DIAPAUSE AND THE CIRCADIAN CLOCK IN *DROSOPHILA MELANOGASTER*

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by

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ABSTRACT

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As a strategy to survive to the upcoming winter, many insects enter diapause (a typical overwintering response that results on their developmental arrest). *Drosophila melanogaster* undergoes an adult or reproductive diapause that can be easily spotted by looking at the stage of development of the females' ovaries. The possibility of the circadian clock influencing this phenotype was proposed to explain photoperiodic differences in induction levels. Nevertheless, to the date the debate is still on.

In this thesis, I looked at several canonical clock mutants and assessed their impact on diapause, finding that 1) depending on the temperature in which they were reared the effects on the adult flies varied enormously 2) most of the clock mutants gave a strong effect in one or other growing conditions. In particular, Pdf^{0} and Clk^{Jrk} mutants behave in completely opposite ways.

A second part of the project consisted on looking at the effects of *period* temperature-sensitive splicing in diapause. Using splicing locked transgenic flies provided by Isaac Edery, I found that expression of the summer isoform impaired the ability of the flies to undergo diapause. Hence, I cloned the different splicing variants into a pUAST vector and generated UAS lines to perform a neuroanatomical dissection of the phenotype.

Also, related with the previous project, I decided to look if any miRNA could be regulating diapause by affecting any of the splicing variants. I found several possible miRNAs that could target the summer (intron-containing) non-splicing isoform. I found that one particular*, miRNA-276b*, was having a huge effect on diapause. Using a sponge particularly against this miRNA (which would result in its downregulation) diapause levels halved compared to all the controls that were performed in parallel.

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As part of the PhD, some of the work has been published in peer-reviewed journals. The publications and the contributions to each publication are summarised below.

Chapter 3

Anduaga, A. M., D. Nagy, R. Costa & C. P. Kyriacou (2018) Diapause in *Drosophila melanogaster* - Photoperiodicity, cold tolerance and metabolites. *J Insect Physiol*, 105, 46-53.

Contributions: AMA: Experiments, analysis, guided project, wrote and revised manuscript. DN: Revised manuscript. RC: Revised manuscript. CPK: guided project, wrote and revised manuscript.

ABBREVIATIONS

aa: Amino acid
AKH: adipokinetic hormone
ANOVA: Analysis of variance
Bp: base pairs
CA: *corpora allata*
CC: *corpora cardiaca*
CLK: CLOCK protein (or *clk,* gene and mRNA)
CNS: central nervous system
CRY: CRYPTOCHROME protein (or *cry,* gene and mRNA)
Crz: corazonin
CYC: CYCLE protein, (or *cyc,* gene and mRNA)
dmpi8: *D. melanogaster per intron 8*
DD: constant darkness
DILP1-8: *Drosophila melanogaster* insulin-like protein 1-8
DN1/2/3: Cluster 1, 2 or 3 of dorsal clock neurons
DNA: deoxyribonucleic acid
Ecd: Ecdysone
EDTA: Ethylenediaminetetraacetic acid
EtOH: Ethanol
GFP: green fluorescent protein
gr: grams
h: hour
IPC: insuling producing cell
JH: juvenile hormone
Kb: Kilo base
L: Litre
LD cycle: light-dark cycle
l-LNvs: large ventrolateral neurons
LNds: dorsal lateral neurons
LS-TIM: long (l) and short (s) forms of TIMELESS protein (or *ls-tim*, gene and mRNA)
min: minute
miRNA: micro RNA
mRNA: messanger RNA
N.A.: Not available
ns: not significant
PBS: Phosphate-Buffered Saline
PCR: Polymerase Chain Reaction
PDF: PIGMENT DISPERSING FACTOR protein (or *Pdf,* gene and mRNA)
PDFR: pigment dispersing factor receptor (also known as han)
PDF-Tri: PDF neurons in the *tritocerebrum*
PDP1ε: PAR DOMAIN PROTEIN 1ε protein (or *Pdp1e*, gene and mRNA)
PER: PERIOD protein (or *per,* gene and mRNA)
PG: prothoracic gland
PI: *pars intercerebralis*
PL: *pars lateralis*
RNA: ribonucleic acid
SEM: Standard error of the mean
s-LNvs: small ventrolateral neurons
SNP: Single nucleotide polymorphism
TG: Thoracic Gland
TIM: TIMELESS protein (or *tim*, gene and mRNA)
WT: Wild type
ZT: Zeitgeber Time

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1. INTRODUCTION

1.1 Introduction

The conditions to which animals are exposed are not constant throughout the year; temperature and photoperiod cycles create predictable rhythms (Ben-Shlomo and Kyriacou, 2002). The 24h rotation of the Earth (generating day and night) and its 23.5° tilt as it orbits around the Sun generates the seasons (Koupelis, 2014). As shown in Figure 1-1, there is a direct correlation between latitude and the fluctuation of photoperiod and temperature throughout the year. Major differences during the year can be found at the Poles, whereas variations in these two aspects are less pronounced in the equatorial areas (Wilczek et al., 2010). Even so, within this zone there are factors, such as humidity levels, which fluctuate during the year, leading to seasonal variations.

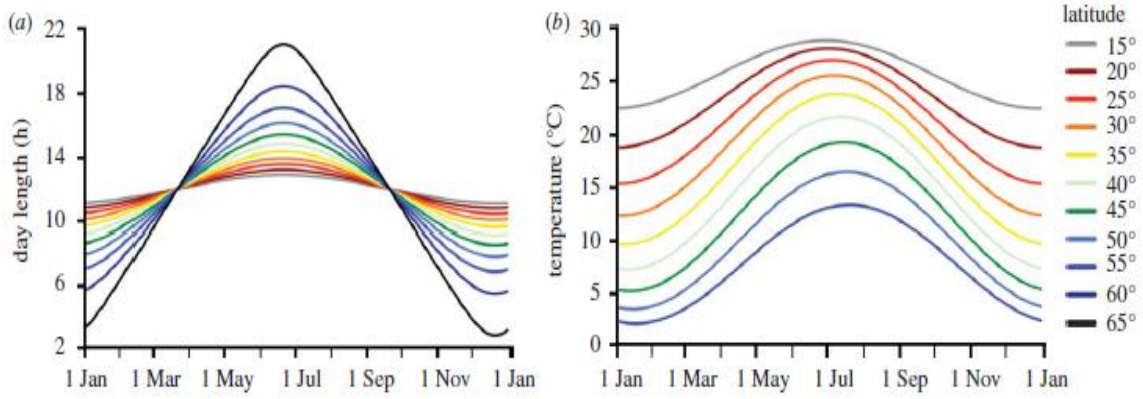


Figure 1-1. Changes in photoperiod (a) and daily average temperature (b) throughout the year that correlate with an increase in latitude. From Wilczek et al. (2010).

Therefore, animals must not only adapt to the specific climate of the region they inhabit, but also to all the putative changes that it may be exposed to during the

course of the year. Thus, the coordination of development and reproduction with seasonal timing is essential to assure the survival of the individual, and the ability to predict seasonal changes and to adapt to them becomes a matter of life or death (Powell and Logan, 2005).

1.2 Adaptations to seasonality

The optimal conditions for reproduction and/or survival are extraordinarily diverse among the animal kingdom. Although many organisms show a stress response when confronting low temperatures or in a winter-like condition, there are others which will have to deal with excessively high temperatures, for example in the deserts or tropics. As well as the circumstances that trigger them, the survival strategies vary among the different animal classes and even among species within the same class (Danks, 1987). **Migration** to places with better environmental conditions is a widespread mechanism in the animal kingdom. Birds as well as some fish, such as *Oncorhynchus gorbuscha* (pink salmon), travel thousands of km every year to escape from unfavourable environments (Winger et al., 2014). There are even some documented Drosophilids that migrate, although their abilities are much more limited (Coyne et al., 1982). Others will enter into a state of inactivity, low body temperature and slow metabolism, which will allow them to sleep during the inhospitable months. Depending on the time of the year in which it occurs, inactivity can be differentiated between **hibernation** (in winter) and **aestivation** (in summer). Many mammals, such as squirrels, and cold-blooded animals, such as lizards, hibernate. However, **dormancy** is the strategy of choice for many insects, the main difference with hibernation being that the animals' development is arrested. It can be subdivided into quiescence and diapause. ***Quiescence*** would be an immediate response to any environmental factor, whereas ***diapause*** is an endogenously and centrally mediated interruption of development, usually in anticipation to an adverse condition (Table 1-1) (Danks, 1987; Kostal, 2006).

Table 1-1 Main differences between quiescence and diapause. Modified from Danks (1987).

Quiescence	Diapause
Immediate response	Programmed (delayed) response – endogenous and centrally mediated.
Environmental factors directly limit development	Environmental factors indirectly modify developmental pathways
Arrested development concurrent with adverse conditions	Start of diapause usually precedes the adverse conditions. Suppressed development lasts longer than adverse conditions

1.3 Diapause

1.3.1 Classification

There are many possible ways to classify diapause (Danks, 1987). However, due to the huge diversity of developmental programmes and their control, it is complicated to catalogue them (Tauber and Tauber, 1981).

According to the developmental stage in which it occurs it several categories have been outlined (See Figure 1-2):

- **Embryonic diapause:** in the case of *Bombyx mori*, diapause is under maternal control. The adult female senses the long summer days and anticipates the arrival of winter by producing diapausing eggs (Sato et al., 2014).
- **Larval diapause:** *Nasonia vitripennis*, a hymenopteran that has a maternally-controlled larval diapause (Wolschin and Gadau, 2009).
- **Pupal diapause:** *Helicoverpa armigera,* the Cotton Bollworm which is a important insect pest, has a pupal diapause for overwintering (Lu et al., 2014).
- **Adult diapause:** in contrast does not entail a failure to moult to the next stage of development, but a delay in reproduction. This is the reason why it is also known as **reproductive diapause** (Danks, 1987). *Pyrrhocoris apterus* (linden bug) and *Drosophila melanogaster* have this type of diapause (Dolezel, 2015; Saunders et al., 1989).

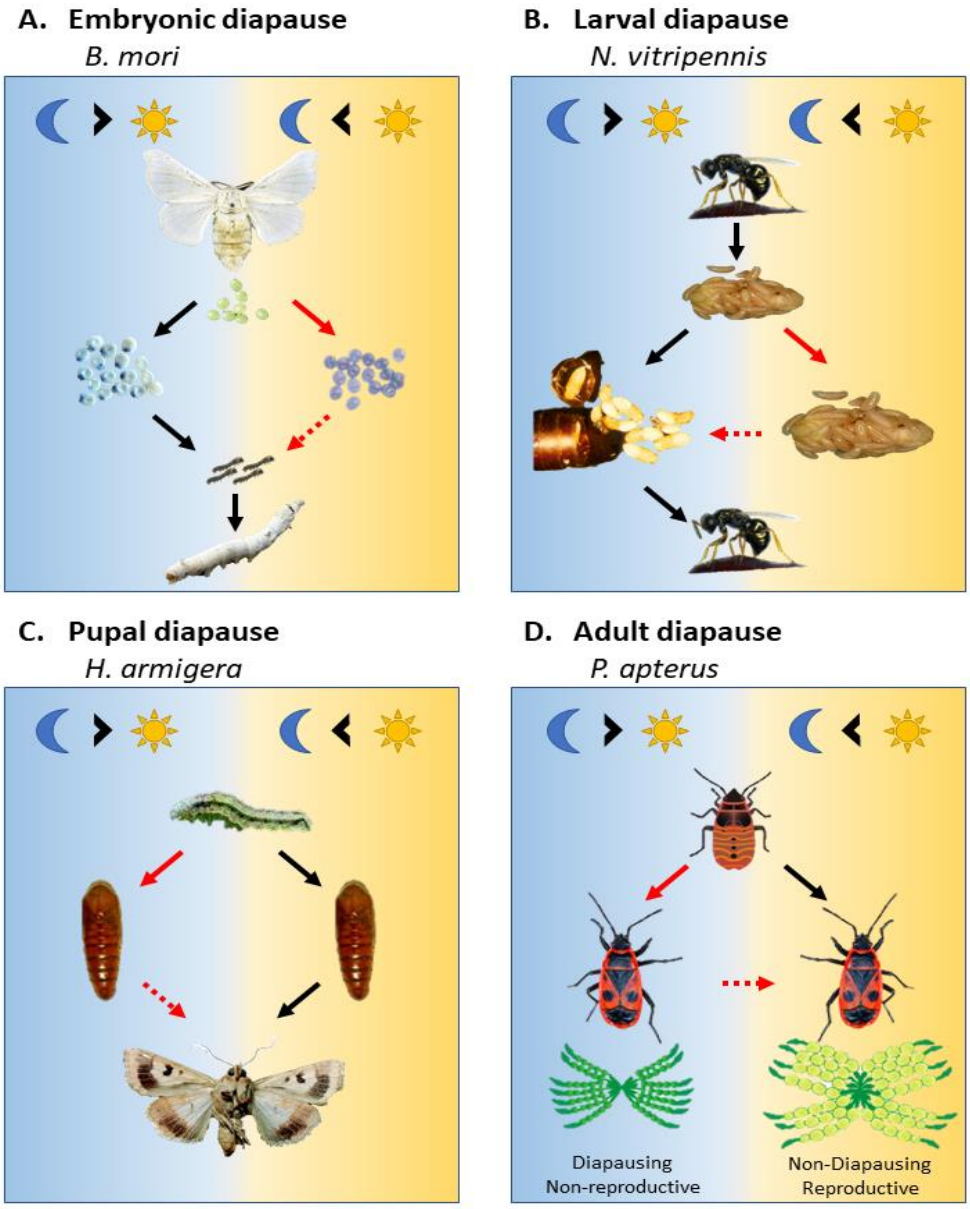


Figure 1-2 Classification of diapause according to the developmental stage in which it occurs. (A) Embryonic diapause in *Bombyx mori* is maternally controlled. Long summer days and high temperature are sensed and used as a cue to produce diapausing eggs, which turn from yellow to brown and finally grey as opposed to the non-diapausing eggs that remain yellow. (B) *Nasonia vitripennis*, the parasitic wasp, undergoes a larval diapause. It is also maternally controlled and triggered by long days, entailing a failure to moult to the pupal stage. (C) *H. armigera* has a pupal diapause induced by exposure of larvae to short days and low temperatures. (D) *P. apterus* has a well characterised adult or reproductive diapause induced by exposure to short days of the young animal. Short and long days are represented by blue and yellow backgrounds, respectively. Black arrows represent non-diapausing development while red ones, the diapausing pathway. Continuation of the usual developmental program after diapause termination is illustrated by the discontinuous red arrows. Adapted from Dolezel (2015).

Taking into account the compulsoriness to undergo diapause, compulsory and facultative diapauses have been described. Animals with compulsory diapause will undergo developmental arrest regardless of the environmental conditions. For example, the gypsy moth *Lymantria dispar* undergoes obligatory diapause as first instar larvae (Atay-Kadiri and Benhsain, 2005). On the other hand, environmental cues such as those described below in Section 1.3.3 will affect the fate of facultative diapausing animals. This type is more common than the compulsory one; indeed, all the examples from above for the different developmental diapauses are facultative.

1.3.2 Phases of diapause

Diapause is a process rather than a state. Kostal (2006) described three eco-physiological phases (pre-diapause, diapause and post-diapause) that are regulated by both endogenous and exogenous factors. **Pre-diapause** is a phase in which development is not yet interrupted, but the individual gets predetermined to enter diapause (Danks, 1987). It can be further sub-divided into an *induction* (perception of diapause-triggering cues during the sensitive period of the animal) and a *preparation* phase (in which the animal is still developing but changes that will lead to the entrance to diapause might occur, for example accumulation of several metabolites) (Kostal, 2006). **Diapause** corresponds to the moment in which the development *per se* of the animal has been interrupted (Denlinger, 2002). During *initiation* development is stopped and, in many cases, it entails metabolic suppression. *Maintenance* is a critical part as this arrest of development must continue for as long as the adverse environmental conditions persist (or until favourable ones appear). Finally, in the *termination* of diapause different changes in the environment stimulate its end (Danks, 1987; Denlinger, 2002). The animal continues being in developmental arrest, but it reached a physiological state in which development might resume. Last but not least, development re-starts during the **post-diapause** phase (Kostal, 2006). However, end of diapause and resumption of development might be triggered by different conditions. This has led to the description of yet another phase: *post-diapause quiescence*, which happens after the termination of diapause if the environmental conditions are not favourable for the resumption of normal development (Kostal, 2006).

1.3.3 Diapause triggers

The stimuli that initiate diapause during the *induction phase* are very diverse. However, as previously shown in Figure 1-1, each time of the year is characterised by different temperature and photoperiod combinations making these two the most obvious cues (Wilczek et al., 2010). Between them, light is a more powerful cue although it is usually modulated by temperature (Nylin, 2013). Moreover, depending on the geographical region, there are many other modulators of diapause such as the humidity levels in the tropics, the CO_2 content or food availability (Kostal, 2006).

1.3.4 Photoperiodic diapause

The role of photoperiod on diapause was first proposed by Kogure (1933) for the silkworm. Since then, photoperiodic diapause has been confirmed in many different animal models including *D. melanogaster* (Pegoraro et al., 2017; Saunders et al., 1989; Tauber et al., 2007; Zonato et al., 2017).

1.3.4.1 Photoperiodic response curve and Critical photoperiod

The photoperiodic response curve (PPRC) is the graphic representation of the variation of diapause (or any other photoperiodic response) according to the photoperiod to which they are exposed. The shape it takes differs widely among different organisms and allows the classification of their responses in two major groups: long-day and short-day. The ones with long-day response, will react mostly when exposed to long hours of light; while the ones with short-day response will have a preference for less hours of light (Figure 1-3) (Vaz Nunes and Saunders, 1999).

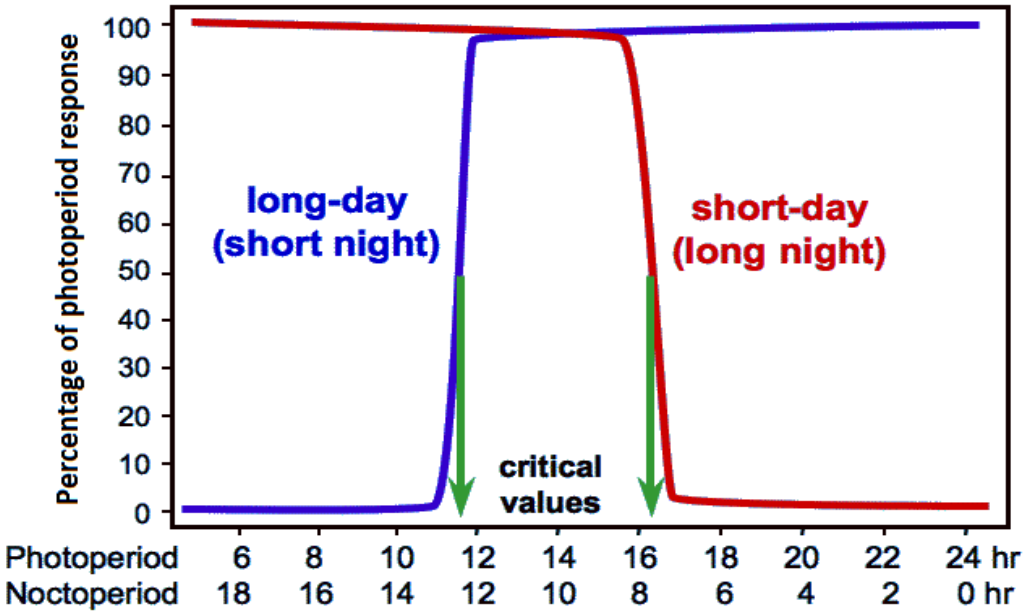


Figure 1-3 Hypothetical photoperiodic response curves to short- or long-days represented in red and blue, respectively. The critical values for each curve are represented by the green arrows. From Koning (1994).

Nevertheless, the PPRC is usually more complex. In the case of *D. melanogaster,* diapause incidence increases in short photoperiods or LL conditions, whereas it typically decreases in long photoperiod or DD conditions (Figure 1-4) (Saunders et al., 1989). Ambient temperature also can affect the PPRCs. For example, in *P. apterus* an increase in temperature results in a shift of the PPRC towards the left (shorter photoperiods, Figure 1-5_A). However, in *S. argyrostoma* increasing the temperature leads to a reduction in pupal diapause levels (Figure 1-5_B) (Dolezel, 2015).

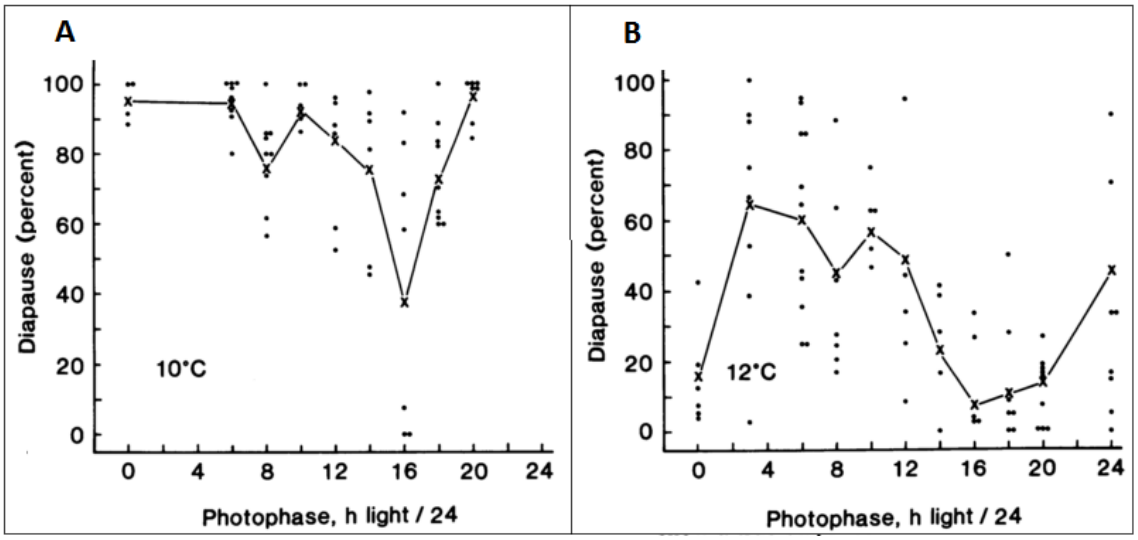


Figure 1-4 Photoperiodic response curve of diapause in *Drosophila* at 10°C (A) or 12°C (B). From Saunders et al. (1989).

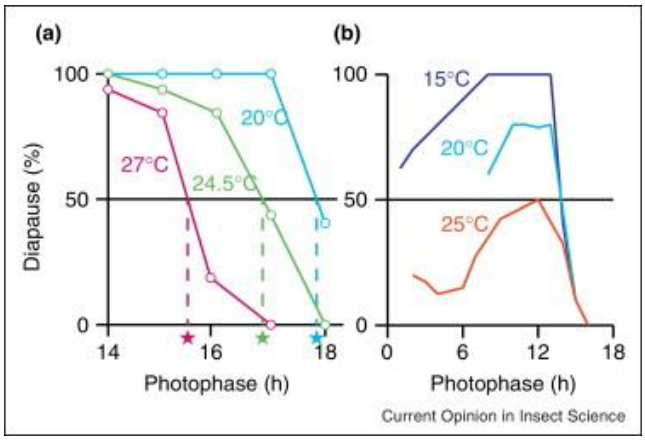


Figure 1-5 Photoperiodic response curve of diapause in *P. apterus* (A) and *S. argyrostoma* (B). From Dolezel (2015).

The PPRC is used to calculate the critical photoperiod, which is the length of the day that leads to half of the population entering diapause while the rest continues normal development (Emerson et al., 2009). As the photoperiodic response curve, the critical photoperiod is organism-specific and is modified by several external factors, such as temperature (Figure 1-4) and latitude (Saunders et al., 1989).

1.3.4.2 Photoperiod sensing models

Since the hypothesis of Kogure (1933) that photoperiod regulates diapause, several models have tried to explain how photoperiod may be measured. Some of these speculate that the circadian clock (the endogenous and autonomous 24h oscillator) could be implicated. This hypothesis has been confirmed in plants: in *Arabidopsis Thaliana* the CONSTANS protein, has a crucial function in the integration of circadian and light signals to measure day-length (Hayama and Coupland, 2004; Suarez-Lopez et al., 2001). However, depending on the species chosen as a model, a wide range of explanations have been put forward. A summary of some of them can be found in Table 1-2.

Table 1-2 Summary of the main photoperiodic measurement. Adapted from Vaz Nunes and Saunders (1999).

Model	First mention
A clock role for the circadian system	
External coincidence See Figure 1-6_A and text.	(Pittendrigh, 1966)

Internal coincidence See Figure 1-6_B and text.	(Tyshchenko, 1966)
The amplitude model The clock is a circadian oscillator whose amplitude is temperature dependent.	(Pittendrigh et al., 1991)
Quantitative clock models	
Clock-commander model The clock measures night length quantitatively, while the commander (similar to a photoperiodic counter) determines if the night is short or long.	(Zaslavski, 1988)
The double circadian oscillator model The clock consists of two damping circadian oscillators that measure night length assigning a quantitative value (≥0). The counter determines critical night length.	(Vaz Nunes, 1998)
Non-clock role for the circadian system	
The resonance effect Magnitude of the photoperiodic response depends on the circadian system's proximity to resonance (i.e.: when T [period of light cycle] is close to t [period of circadian system]). The clock can be an hourglass or a circadian oscillator.	(Pittendrigh, 1972)
The hourglass time-oscillator counter model The clock is an hourglass, but the circadian system affects the counter.	(Vaz Nunes and Veerman, 1982)
Hourglass clock The circadian clock has no influence.	(Lees, 1973)

Bünning (1936) proposed the external coincidence model, in which light acts as the circadian rhythm entrainer and as a photoinductor (Saunders, 2005). It implies the existence of a circadian rhythm of photoperiodic photosensitivity. In this model, the circadian clock-determined subjective day-phase would be photo-insensitive, while the photosensitive phase would be during the subjective night. This way, the lengthening of the day during spring and summer would impose illumination of the photosensitive subjective night-phase, leading to photoperiodic changes (Figure 1-6A). Many variations of this mechanism have been proposed since it was first elucidated by Bünning, such as the possibility of an external coincidence model with one, or more, damped circadian oscillator(s) (Vaz Nunes and Saunders, 1999).

Another option would be an internal coincidence and multioscillator model. This was proposed by Pittendrigh in 1960 (Saunders and Bertossa, 2011) and several variations of it have been described. The “dawn”-“dusk” model of Tyshchenko in 1966, contemplates two pacemakers entrained by dawn or dusk respectively and whose internal phase relationship varies according to the duration of the photophase (Vaz Nunes and Saunders, 1999) (Figure 1-6B). This time, the photoperiodic changes (resulting in the induction of diapause or not) will be triggered depending on the overlap between the two pacemakers. In contrast with previous model, light only plays a role in entrainment (Vaz Nunes and Saunders, 1999).

There are many other circadian clock based models, such as the amplitude model, double circadian oscillator model and plenty more that suggest a non-clock role for the circadian system (Vaz Nunes and Saunders, 1999). However, there is also the possibility of a model without the involvement of the circadian clock. The hourglass model assumes the gradual accumulation of some element that once reaching a specific threshold leads to the physiological response (Figure 1-6C). This model argues against the involvement of the circadian clock in the photoperiodic response, however it does not exclude it as a possible modulator (Saunders, 2005).

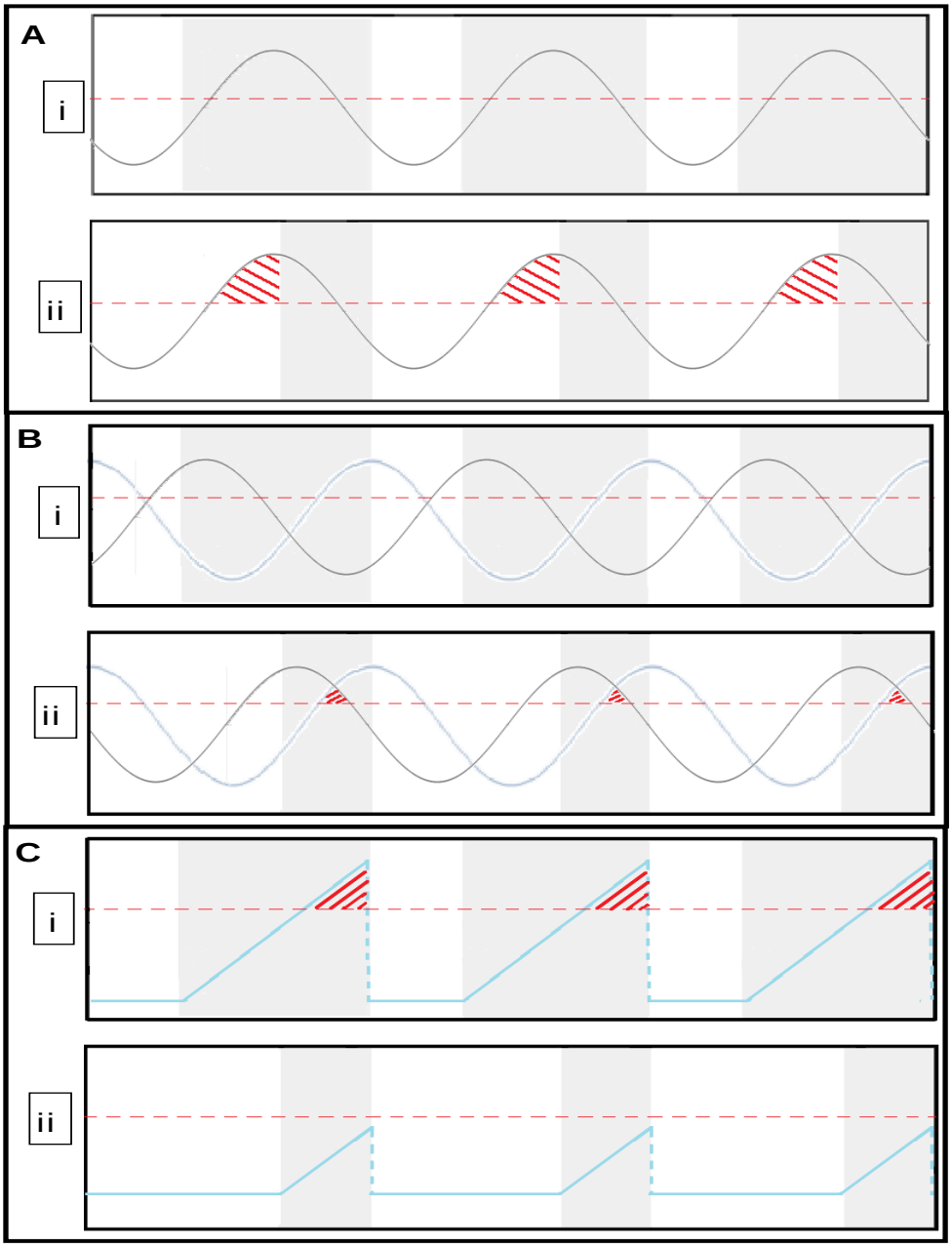


Figure 1-6 Models of photoperiod measurement. (A) In the external coincidence model, during the short days of winter (i) the photosensitive phase will be during the night whereas in the long days of summer (ii), there will be light during the photosensitive phase, thus triggering the photoperiodic response. (B) According to the internal coincidence model, the photoperiodic response will be triggered depending on the overlap between two independent circadian oscillators, one of which could be entrained by dawn while the other by dusk. (C) As for the hourglass model, it speculates that the photoperiodic response is triggered when a specific molecule accumulates over the threshold. In the picture, hours of light and dark are represented by a white or grey background, respectively. The red dotted line represents the threshold over which the photoperiodic response will be triggered, which will be represented by the red areas.

1.3.5 Hormonal control

Development towards adulthood is regulated by several hormones that will control and modify it according to environmental conditions (Rewitz et al., 2013). How these environmental cues lead to changes in hormonal signalling that result in developmental arrest is still unknown (Allen, 2007). In *D. melanogaster,* the involvement of neurons in the *pars intercerebralis* (PI) and *pars lateralis* (PL), which are located in the dorsal protocerebrum, in the transduction of these environmental triggers has been suggested. These generate and release numerous growth factors and neuropeptides, hence exerting a regulatory role in insect metabolism, growth and development (Shiga and Numata, 2007). The ring-gland, which is composed of the prothoracic gland (PG), *corpora allata* (CA) and *corpora cardiaca* (CC), is the main hormonal centre in larvae (Dubrovsky, 2005). It is heavily innervated by neurosecretory cells and it generates and releases hormones in the haemolymph, resulting in a tight regulation of insect growth (Richard et al., 2001; Richard et al., 1998). After metamorphosis, the larval ring-gland is completely reorganised: the role of the PG is taken over by the ovarian follicles or the nurse cells; while CA and CC complex maintains a similar function as in the larvae (Schiesari et al., 2011). There are three main classes of hormones with significant effect in the developmental timing of insects: **Ecdysteroids** (which stimulate developmental transitions), **prothoracicotropic hormone** (**PPTH**, which triggers the synthesis of ecdysteroids) and **juvenile hormone** (**JH**, which inhibits developmental transitions) (Di Cara and King-Jones, 2013).

In *D. melanogaster*, during larval stages, **ecdysone** is generated by the PG (Huang et al., 2008). In the haemolymph, P450 monooxygenase converts ecdysone to its active form (20-hydroxyecdysone), which then will be able to reach its target tissues (Petryk et al., 2003). As previously mentioned this hormone stimulates developmental transitions, such as larval moulting and metamorphosis (Truman and Riddiford, 2002). In adult females, ovarian follicles and/or nurse cells take over the role of the PG (Buszczak et al., 1999). Ecdysone is controlled by **JH**, which is generated in the CA. During development, the interaction between these hormones is critical: in the presence of high JH, ecdysone and 20-hydroxyecdysone, new larval cuticle will be

produced (promoting a larva-to-larva moult); however, low levels of JH are necessary for the larva-to-pupa transition (metamorphosis) (Di Cara and King-Jones, 2013).

Similarly, these hormones play an important role in the generation of diapause. Depending on the stage at which the organisms enters, the hormonal control and the relative importance of each of those hormones will vary (Schiesari and O'Connor, 2013). JH has been described to be key in several other processes, amongst them ovarian growth and diapause in adult flies (Dubrovsky, 2005; Riddiford, 1994; Saunders et al., 1990). In the fat body and ovarian follicle cells, JH has been reported to promote vitellogenesis (process of generation, transport and accumulation of yolk protein in the oocytes) as it induces production of yolk protein (Richard et al., 1998). Indeed, reduced levels of both ecdysone and JH have been described in diapausing females, which is characterised by previtellogenic oocytes (in which the yolk deposition has been arrested). In this situation (low JH → diapause), high levels of yolk protein have been reported circulating in the haemolymph, however the deposition of it in the ovaries was minimal (Saunders et al., 1990). Saunders et al. (1990) observed that complementing JH deficient flies with JH resulted in accumulation of yolk protein in the ovaries, resuming reproductive state. Similar to *Drosophila*, Hodkova (1976) reported that JH triggers yolk protein synthesis in fat bodies and ovarian follicle cells in *Pyrrhocoris apterus*, which also undergoes adult or reproductive diapause.

Ecdysone, which in adult flies is produced by the ovaries upon JH signalling, stimulates yolk protein production by the fat body. Richard et al. (1998) showed that ecdysone is responsible for the initiation of vitellogenesis and that its application in diapausing flies results in ovarian maturation. However, this hormonal regulation of diapause will be different in animals that have a non-reproductive type of diapause (Di Cara and King-Jones, 2013). Most of the animals entering larval or pupal diapauses fail to generate ecdysteroids, either because the PPTH is not being released or because the PG is non-responsive to it, and some will retain high JH levels (Denlinger, 2002). In the case of *Bombyx mori*, Diapause Hormone (DH) is essential for the determination of the fate of the animal and induction of embryonic diapause (Yamashita, 1996).

On the other hand, ***Insulin-like peptides* (*ilps*)** have also been shown to regulate ovarian development in many species (Kimura et al., 1997; Kubrak et al., 2014; Schiesari et al., 2011; Sim and Denlinger, 2008). Insulin signalling regulates many processes such as growth, lifespan, reproduction and stress resistance (Tatar et al., 2003). The main components of the insulin signalling pathway are outlined below (1.3.5.1). Recently, the involvement of this pathway in the regulation of diapause in *D. melanogaster* (Schiesari et al., 2016), as well as in other animals with reproductive diapause such as the mosquito *Culex pipiens* (Sim and Denlinger, 2008), has been described. In *D. melanogaster* several of the DILPs are expressed and secreted by a subset of cells located in the *pars intercerebralis* known as Insulin Producing Cells (IPCs) which have been hypothesised to be involved in the transduction of environmental cues. Indeed, insulin signalling seems to regulate ovarian maturation via induction of JH and ECD synthesis by the *corpora allata* or ovary, respectively (Flatt et al., 2005) (see Figure 1-7).

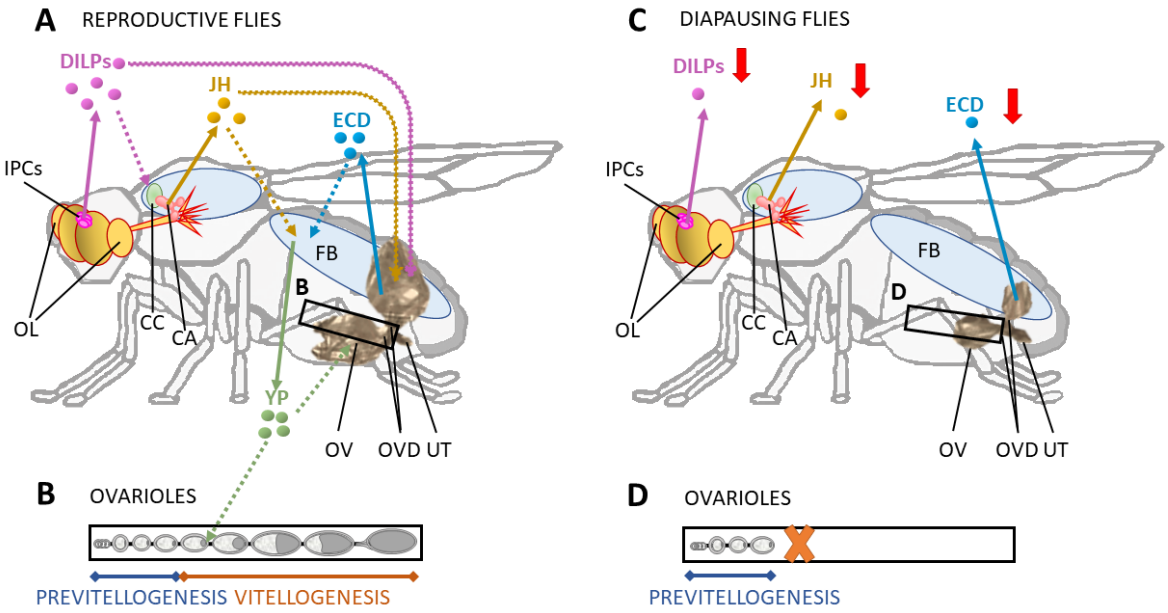


Figure 1-7 Neuroendocrine signalling in *D. melanogaster* under the control of the shift between reproductive or diapausing flies. (A) In reproductive flies, environmental cues will lead to the secretion of DILPs by the IPCs. These will trigger JH secretion by the *corpora allata* and induce egg chamber development growth and vitellogenesis in the ovaries. Moreover, JH signals ovarian follicle cells and fat body to generate YP. JH signalling in the ovaries will also result in them generating ECD, which enhances YP uptake by the ovaries and ovarian maturation. (C) diapausing flies have

the insulin-signalling pathway downregulated, which results in a reduction of JH and ECD and leads to an impairment in maturation of the ovaries. (B) and (D) represent amplifications of the ovariole marked by the rectangles in the ovaries, showing the ability and impairment, respectively, of the flies to enter the vitellogenic stages of egg-chamber development. IPCs, insulin producing cells; OL, optic lobe; CC, *corpora cardiaca*; CA, *corpora allata*; FB, fat body; OV, ovary; OVD, oviduct; UT, uterus; DILPs, Drosophila insulin-like peptides; JH, juvenile hormone; ECD, ecdysone; YP, yolk protein (vitellogenin). Adapted from Schiesari et al. (2011).

1.3.5.1 Insulin signalling pathway

The insulin signalling pathway is involved in the regulation of several physiological processes such as development, growth and metabolic homeostasis. Hence, it is not surprising that it is a highly conserved pathway among different species (insects, mammals etc). In *D. melanogaster*, environmental factors can trigger expression of several insulin-like peptides (dILP) in different cell types and tissues. These dILPs will then be released to the haemolymph, from which they will arrive to their target tissues (such as ovary, *corpora allata* or fat body). Until quite recently, a single *Drosophila* insulin receptor (dInR) of the tyrosine kinase (RTK) family had been described. Colombani et al. (2015) described a second Leucin-rich repeat-containing G protein-coupled receptor (Lgr3) that binds to DILP8. Binding of ILP to their corresponding receptor will start a cascade of (mainly) phosphorylation events that will end with the phosphorylation of FOXO transcriptional factor. This renders the transcription factor inactive, preventing its entrance to the nucleus (where it activates the transcription of several stress and anti-growth genes and plays a key role in transcription and cell-cycle progression, apoptosis and regulation of metabolic genes) and promoting its ubiquitination and consequent degradation (Huang and Tindall, 2011) (See Figure 1-8).

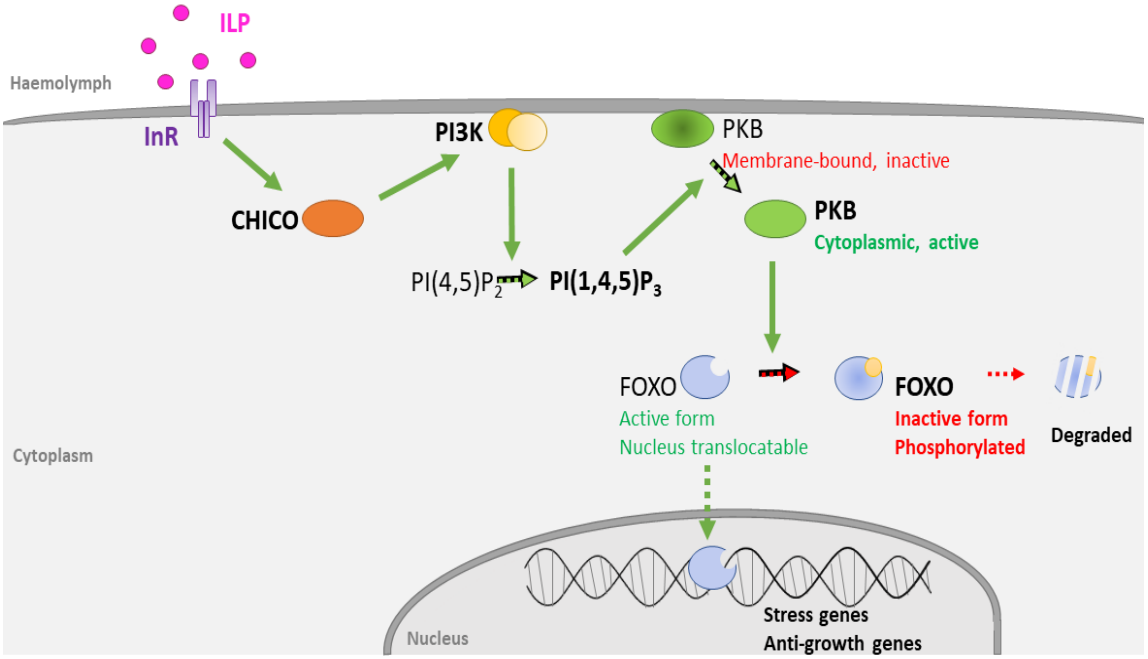


Figure 1-8 Canonical insulin-like peptides pathway in insects. Binding of circulating ILPs to their target receptor (InR) lead to the phosphorylation and activation of CHICO and subsequent activation of PI3K. This enzyme which contains two subunits (dp110, catalytic; and dp60, regulatory) promotes the phosphorylation and conversion of $PI(4,5)P_2$ into $PI(1,4,5)P_3$, which is necessary for the activation and release from the plasma membrane to the cytoplasm of PKB. Finally, PKB will phosphorylate and inactivate FOXO, as this phosphorylation prevents the translocation of the transcription factor to the nucleus as well as promotes its ubiquitination and degradation. On the contrary, if PKB does not phosphorylate FOXO, it is translocated to the nucleus where it activates the transcription of several stress- and anti-growth-related genes. *ILP, insulin-like peptide. InR, insulin receptor. CHICO, insulin receptor substrate in* Drosophila melanogaster*. PI3K, phosphatidylinositol 3-kinase. $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate. $PI(1,4,5)P_3$, phosphatidylinositol-3,4,5-trisphosphate. PKB, protein kinase B (or "Akt"). FOXO, Forkhead box-O transcription factor.*

1.3.6 Diapause in D. melanogaster

1.3.6.1 Reproductive system in *D. melanogaster*

The adult ovary consists of 16–20 ovarioles that contain a series of growing egg chambers. Each ovariole opens into a lateral oviduct and these two lateral oviducts merge into a common oviduct that is connected to the uterus, where the eggs will be fertilised, and, finally, to the vagina. Three regions can be identified inside each ovariole: a terminal filament, a germarium and a vitellarium (Figure 1-9) (Ogienko et

al., 2007). Egg-chamber development in the vitellarium is usually divided into 14 stages by morphological criteria. Until stage 7, the egg-chamber is in the previtellogenic stage. Stage 8 is the earliest yolky stage, and from this point on the egg chambers will be in their vitellogenic stages. At stage 11, more than 2/3 of the egg will be yolk, nurse cells start to degenerate and the inner endochorion is formed (Figure 1-10) (Cummings and King, 1969).

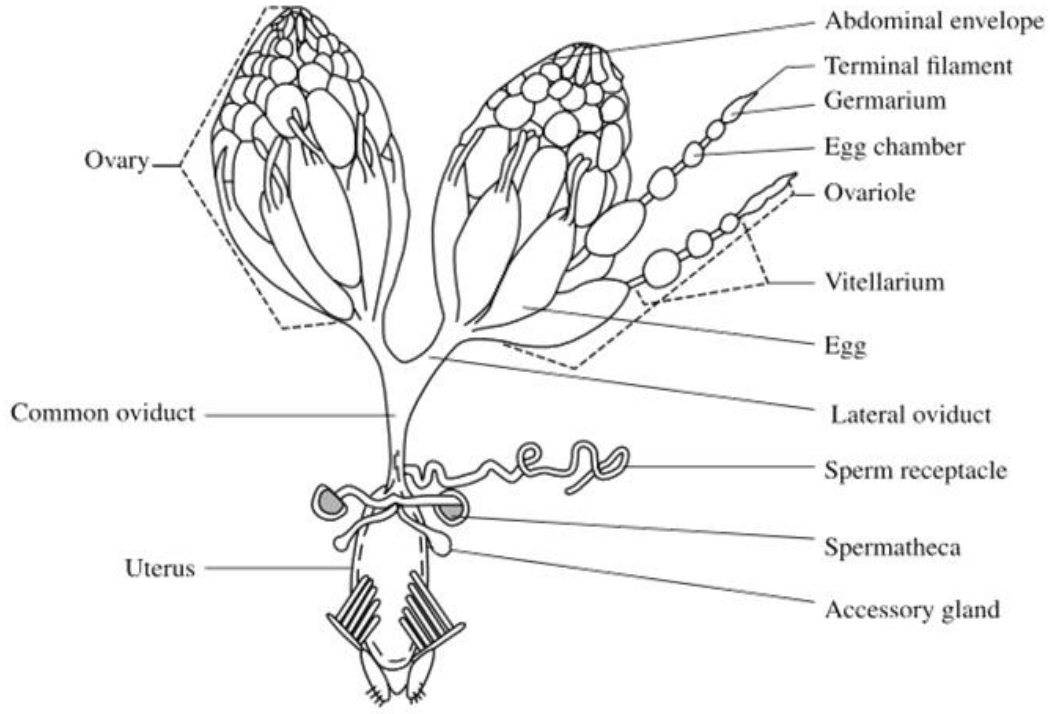


Figure 1-9 Schematic representation of the adult reproductive system of *Drosophila melanogaster*. See text for further information. From Ogienko et al. (2007).

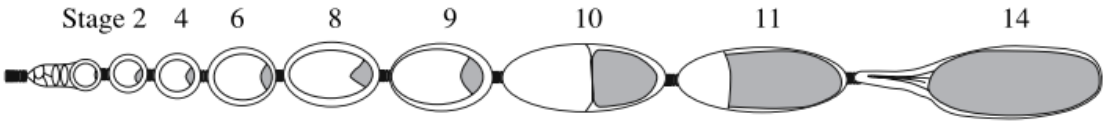


Figure 1-10 Stages of egg-chamber development. From Ogienko et al. (2007).

1.3.6.2 Diapause phenotype in *D. melanogaster*

As previously stated (1.3.1), *D. melanogaster* has a reproductive diapause. Commonly, diapause is scored when both ovaries are in the previtallogenic stage (before stage 8) (Saunders et al., 1989). Contrary to other *Drosophila* species such as *D. montana* or *ezoana* that present a very clear and strong photoperiodic diapause (Salminen and Hoikkala, 2013), in *D. melanogaster* the phenotype is shallower, with a marked temperature effect and higher variability among replicates (Zonato et al.,

2017). Recently, Schiesari (2016) showed a strong control over diapause levels from several *dilps*, reflecting a strong neurohormonal control.

Whether diapause (due to the anticipatory element and strong hormonal control) or quiescence (because warm temperatures terminate it) is more appropriate to describe this phenomenon is more accurate is still disputed (Tatar et al., 2001). The best term in this situation would probably be dormancy (as it englobes both phenomena) (Kostal, 2006); nevertheless, I shall use the term 'diapause' simply for continuity with most *D. melanogaster* literature. Being *D. melanogaster* such an important model for genetic manipulations, research in this organism's ovarian arrest is informative and useful and provides a good molecular model for diapause.

1.4 Circadian clock

The circadian clock is an endogenous time-keeping mechanism with a period of ≈24h. Indeed, it gets its name from the Latin "*circa"*=about and *"diem*"=day. The rotation of the Earth around its axis results in predictable changes (in temperature, light…) throughout the day and having biological clocks might be expected to give a fitness advantage due to their synchronising and coordinating properties. Their importance is highlighted by the fact that most species have one: from prokaryotes to eukaryotes, from cyanobacteria to mammals passing through insects and plants. In the past years considerable effort has been focused towards understanding the different clocks, their mechanisms and regulation. Circadian clocks share three properties: they are "self-sustainable" (this endogenous 24h period must persist under constant conditions, in the absence of environmental cues), "entrainable" (they must be flexible to respond to environmental changes) and show "temperature compensation" (maintain their periodicity within a temperature range).

Insects, in particular *D. melanogaster*, have been key to broadening our knowledge on the clock as it was in the fruitfly that the first clock gene, *period*, was identified (Konopka and Benzer 1971). This fascinating discovery led to the subsequent dissection of the molecular mechanism underlying the clock in *Drosophila* and opened

up the field to many other organisms (Hardin et al., 1990; Zehring et al., 1984). This past year, 2017, has been particularly thrilling for the field as Jeffrey C. Hall, Michael Rosbash and Michael W. Young have been awarded the Nobel Prize in Physiology or Medicine for their genetic and molecular analysis of fly circadian behaviour.

1.4.1 Circadian clock in *Drosophila*

D. melanogaster's circadian clock is composed by two interlocked negative transcription/translation feedback loops (TTFL model): *per/tim* loop and *Clk/cyc* loop (Hardin, 2005). On the one hand, *per/tim* transcription is promoted by the **CLK/CYC** heterodimer with PER/TIM accumulating in the cytoplasm during the night. Once phosphorylated, they enter the nucleus, where they will regulate the transcription of several genes. Additionally, they will promote the phosphorylation of CLK and consequently inhibit their own transcription. In the morning, **TIM** will suffer light-induced degradation, which is followed by **PER** degradation, and the cycle is restarted by CLK/CYC mediating *per/tim* transcription (Hardin, 2011). On the other hand, CLK/CYC heterodimers promote the transcription of several genes during the day, such as the already mentioned *per* and *tim*, but also others: ***vri*** and ***cwo*** (which will inhibit *Clk* transcription) or ***Pdp1e*** (which enhances *Clk* transcription) (Hardin, 2011). The importance of their role for the circadian rhythm is not equal: *per/tim* loop is necessary for the function of the *Clk* loop, but not the other way around. This is the reason why the *per/tim* loop is known also as the core feedback loop and the *Clk* loop, as the interlocked feedback loop (Brown et al., 2012; Hardin, 2011). A schematic representation of the TTFL model can be found in Figure 1-11.

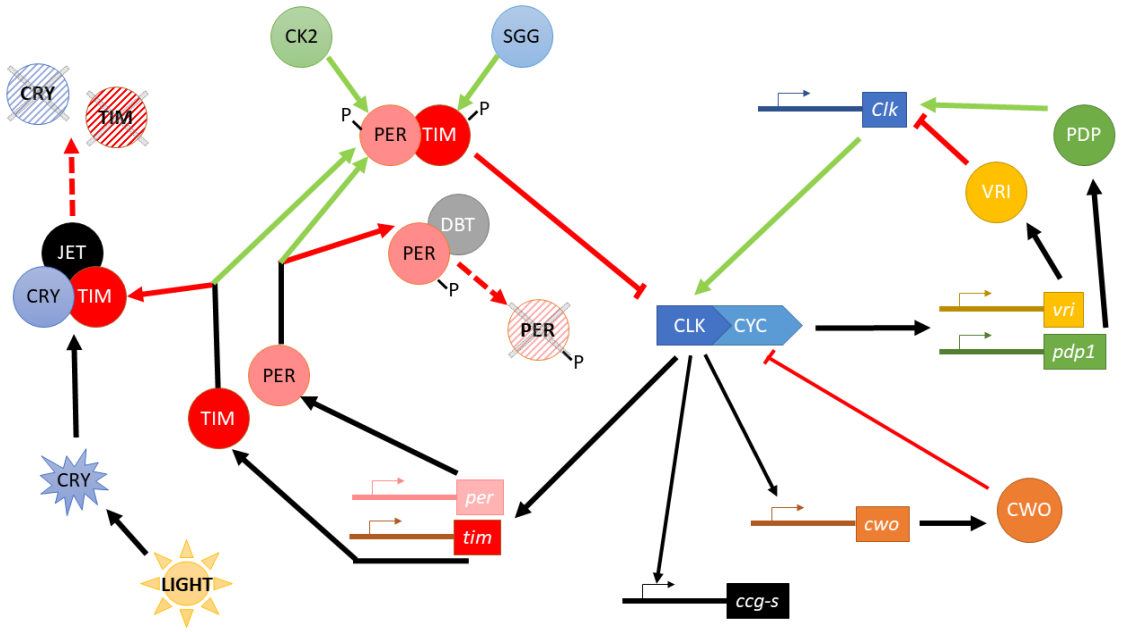


Figure 1-11 Molecular mechanism of the circadian clock in *D. melanogaster*. CLK/CYC activate the transcription of *vri* and *pdp1,* which regulate the expression level of *Clk,* and of several clock-controlled genes (*ccg-s*, for example *Pdf*). On the other hand, the heterodimer also activates the transcription of *per* and *tim*. During the day, light activates the blue photoreceptor CRY, which will bind to TIM, and this complex is recognised by JET leading to their degradation. Without TIM to stabilise it, PER is phosphorylated by DBT and degraded. However, during the night, TIM and PER levels increase, and once they bind together, the kinases SGG and CK2 phosphorylate them, respectively. Finally, they will enter the nucleus where they will repress CLK/CYC actions. Hence, this way the internal clock gets synchronised with the environmental cycles of day/night. CWO is expressed under the control of CLK/CYC and acts as a negative regulator. *ccg-s, clock-controlled genes; cwo, clockwork orange; pdp1, PAS domain protein 1ε; vri, vrille; Clk, Clock; per, period; tim, timeless*; CYC, CYCLE; CRY, CRYPTOCHROME; JET, JETLAG; DBT, DOUBLETIME; CK2, CASEIN KINASE 2; SGG, SHAGGY. Adapted from Gesto (2011).

Drosophila **CRYPTOCHROME** protein **(CRY)** functions as a blue-light photoreceptor that interacts with TIM promoting its degradation in a light-dependent manner and, additionally, modulates transcriptional activity like mammalian CRYs in peripheral tissues (Ceriani et al., 1999; Collins et al., 2006). Being directly influenced by light signals it is the main input signal to the circadian clock (Ozkaya and Rosato, 2012).

PIGMENT-DISPERSING FACTOR (PDF) is the main output signal of *D. melanogasters'* circadian clock, being crucial for the synchronization of the clock neurons (Rosato et al., 2006). However, it is not the only one. Recently, Damulewicz et

al. (2013) suggested that **ION TRANSPORT PEPTIDE (ITP)** is also important for coupling clock cells together.

1.4.1.1 Anatomy of the *Drosophila* pacemaker

The main circadian clock pacemakers in the brain were discovered by immunohistochemical staining of PER and TIM (Rosato et al., 2006). The 150 clock are classified into 6 main clusters: three dorsal neurons (DN_1, DN_2 and DN_3) and three lateral ones (LN) (Damulewicz et al., 2013). The lateral neurons are divided into a dorsal group (LN_ds), and 2 ventral PDF-expressing groups that are classified according to their relative sizes as large (l-LN_vs) or small (s-LN_vs). Exceptionally, the fifth s-LNv neuron does not express PDF. PDF is the main output signal from the circadian clock, so these lateral ventral neurons are crucial for the synchronization of the network (Liang et al., 2016; Rosato et al., 2006; Seluzicki et al., 2014; Shafer et al., 2008). Finally, a seventh cluster known as the lateral posterior neurons (LPNs) has been identified (Peschel and Helfrich-Forster, 2011; Shafer et al., 2006) (See Figure 1-12).

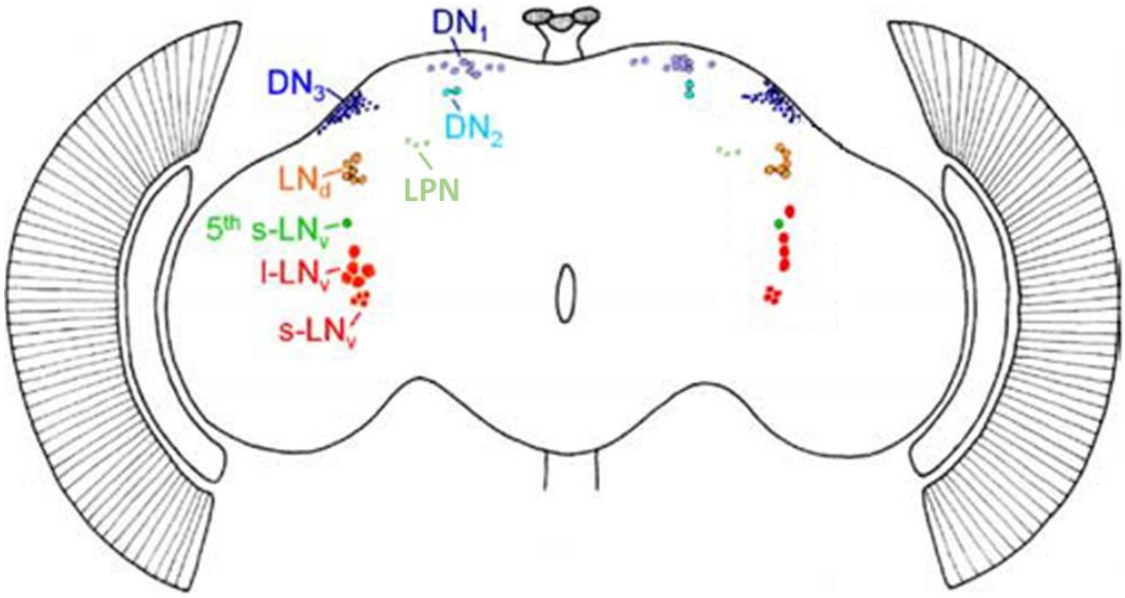


Figure 1-12 Circadian pacemaker neurons in *Drosophila melanogaster*'s brain. The three dorsal clusters are represented in blue while red or orange is used to for PDF expressing s-LN_v and LN_d, respectively. The green dot represents the PDF-negative fifth s-LN_v. The lateral posterior neurons are coloured in a pale-green. Adapted from Rieger et al. (2006).

1.4.1.2 Peripheral clock

Circadian rhythms can also be found in the peripheral tissues such as the fat body. These can be synchronized by the core clock through several factors, but can also entrain their own independent rhythm (Di Cara and King-Jones, 2013).

1.4.2 Inputs of the clock

The circadian clock gets information about the environment via various Zeitgebers (ZT, which means "time-giver" in German). Although the clock is self-sustained, external information can entrain the clock. The most obvious cue is **light**, which resets the clock every day via CRY (and its consequent light-induced degradation of TIM) and the visual system (Ashmore and Sehgal, 2003). **Temperature** also cycles throughout the day, however much less is known about the processing of this signal and Sehadova et al. (2009) showed that, in *D. melanogaster*, the circadian clock requires signals from the peripheral tissues (chordotonal organs) to process temperature information. Finally, other factors such as **social cues** or **feeding** can also entrain the clock. Levine et al. (2004) found that social interactions could change period and phase, whereas Fuller et al. (2008) described an increase in the locomotor activity in anticipation of food availability in temporal restricted feeding mammals.

1.4.3 Outputs of the clock

Due to the high periodicity of the environment we inhabit, it is not surprising that organisms developed a 24h circadian pattern to anticipate these changes (Ben-Shlomo and Kyriacou, 2002). Hence, many behavioural outputs are under circadian control (See Figure 1-13). In the following sections some of these will be mentioned.

1.4.3.1 Locomotor activity

In the case of *Drosophila*, they have a crepuscular activity pattern, which means that they tend to be more active during dawn (morning) and dusk (evening), with a period of inactivity in the middle of the day that is known as "siesta". In LD 12:12 conditions (with 12h of light and 12h of darkness), these bouts of activity anticipate light-on and light-off transitions in WT flies (Rosato and Kyriacou, 2006). This characteristic is lost in clock gene mutants. Different clock cells are responsible for the

morning and evening anticipation: s-LN_vs are known as morning ("M") cells whereas LN_ds and DN_1s are evening ("E") cells (Grima et al., 2004; Stoleru et al., 2007; Stoleru et al., 2004; Stoleru et al., 2005). However, this coupled dual-oscillator model might be too simplistic and more recent studies suggest that the network is more complex and probably composed of multiple oscillators (Nitabach and Taghert, 2008; Sheeba et al., 2008). As it has been mentioned, a key feature of the circadian clock is that it is self-sustainable. Indeed, when keeping WT flies in DD (constant darkness), they retain a period of ≈24h. Nevertheless, mutations in clock genes render the flies arrhythmic. Recently, it has been suggested that this rhythmicity might be the result of a more complex network formed by multiple oscillators, in which different clusters of circadian cells might play different roles in the regulation of this free-running period (Beckwith and Ceriani, 2015; Dissel et al., 2014; Yao and Shafer, 2014).

A phenotype that is usually studied separately, but is closely related to locomotor activity, is sleep. Considerable effort is being placed into the understanding of its control as it is important for human health. Alterations in circadian neuronal clusters have been shown to affect sleep whilst many clock mutants show sleep pattern alterations (Barber et al., 2016; Chen et al., 2013; Rosato and Kyriacou, 2008).

1.4.3.2 Eclosion

Eclosion (emergence of the adult fly from their pupal case) pattern in *Drosophila* has one peak around dawn (in natural conditions) or lights-on transition (in rectangular laboratory conditions), followed by a gradual decrease in the eclosion rate with few flies emerging at night. This eclosion rhythm is controlled by the circadian clock and mutations on its core genes lead to its disruption (Konopka and Benzer, 1971; Myers et al., 2003).

1.4.3.3 Others

In *D. melanogaster,* many other rhythmic outputs have been studied for example mating, oviposition and olfaction (Allemand and David, 1984; Krishnan et al., 1999; Tauber et al., 2003). The circadian clock also controls developmental timing of insects in different points through the release of several hormones such as ecdysteroids (ECD),

prothoracicotropic hormone (PTTH) or juvenile hormone (JH) (Di Cara and King-Jones, 2013).

Finally, the circadian clock has also been implicated in the control of diapause. In some *Drosophila* species, mutations in different circadian genes lead to a disruption in normal diapause induction (Ikeno et al., 2010; Kauranen et al., 2013; Meuti et al., 2015; Saunders, 1990; Shiga and Numata, 2007; Yamada and Yamamoto, 2011). However, whether this is a direct effect of the clock over diapause or a pleiotropic effect (as the circadian clock controls physiology at so many levels that is difficult to find any biological function that is not somehow altered by it) is still under debate (Emerson et al., 2009). This hypothesis (and the arguments for and against it) will be expanded in the following chapters (in particular, in Chapter 4).

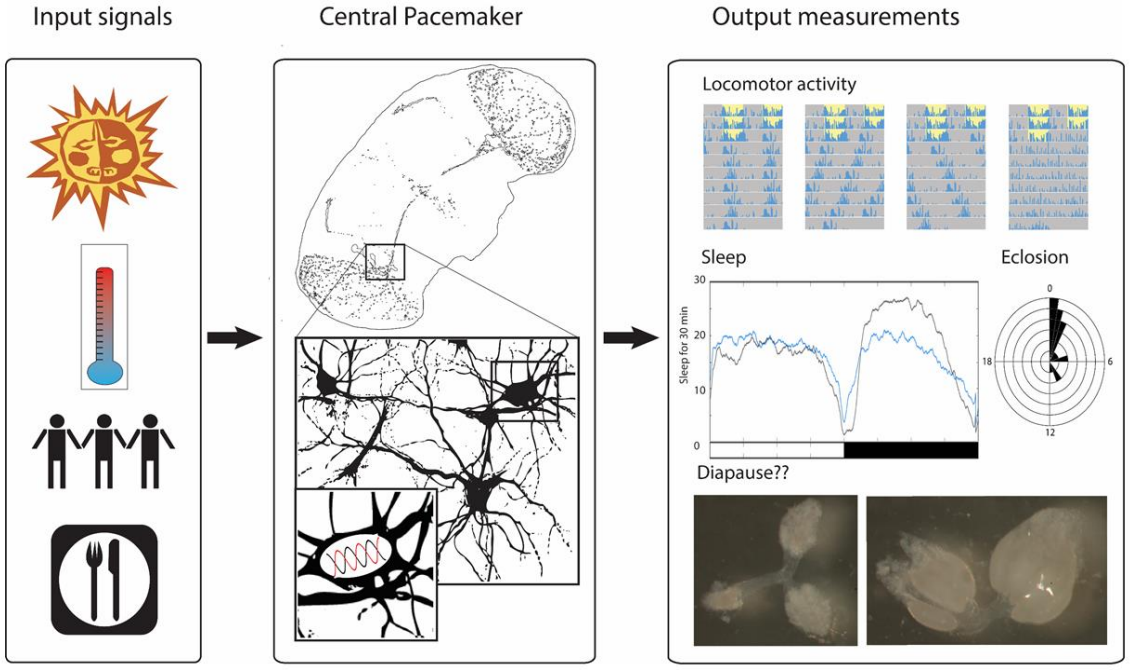


Figure 1-13 Summary of the inputs, pacemaker and outputs in *Drosophila melanogaster*. Schematic representation of different input signals (mostly light and temperature, but also social cues and food intake) that synchronise the circadian clock. In turn, this will affect several features and behaviours that can be studied (outputs). Examples of locomotor activity (represented in actograms) of normal, short and long period and arrhythmic flies are shown, respectively. A closely related phenotype, sleep, is also regulated by the central pacemaker. Eclosion rhythms are known to be circadian, with higher number of flies emerging after dawn. The ability to undergo diapause (lack of development of the egg-chambers in the ovaries in anticipation to winter) has been shown to be impaired when the circadian clock is genetically altered, nevertheless its implication in diapause regulation is disputed.

1.5 Motivation and Aims

Understanding diapause and its regulation is important for several reasons. In the past, its study led to the development of a new group of insecticides targeting JH (Staal, 1975). Furthermore, it could be useful in order to anticipate the impact of climate change and global warming on different ecosystems (Bradshaw and Holzapfel, 2001). Additionally, and taking into account the strong link between diapause and hormonal control, it could lead to many other potentially meaningful discoveries. Many human diseases such as metabolic diseases (Maury et al., 2010) or even several cancers (Savvidis and Koutsilieris, 2012) have been linked to disruptions of the circadian clock, shift work and so on, but how this imbalance in the clock can result in metabolic and/or developmental deregulations in humans is not understood. The link between diapause (an arrest of development with strong metabolic alterations) and the circadian clock in insects has been under strong debate for decades. Nevertheless, one could imagine that if, indeed, a connection between the clock and diapause is confirmed, it could have serious implications for humans, identifying possible pathways to approach and prevent these diseases.

Knowing that the photoperiodic diapause phenotype in *D. melanogaster* is weak compared to other insects, such as the pitcher plant mosquitos or the Linden bug (Bradshaw et al., 2012; Dolezel, 2015) and that low temperatures are required for its induction, the choice of this organism might seem questionable. Nevertheless, the extraordinarily wide range of molecular and genetic tools available for this model organism, as well as the short generation time and cheap maintenance, allow a genetic dissection that is unthinkable in any other insect.

Having stated the evident lack of consensus in whether the circadian clock is implicated in the induction of photoperiodic diapause or ovarian development arrest in *D. melanogaster*, my main objective is to broaden our understanding of this phenomenon and analyse any role that the clock might play. The following chapters:

- Analyse the role of temperature and light as cues for diapause induction (Chapter 3).

- Investigate the effect on diapause, and some other related traits, of rearing the flies in a more natural environment (Chapter 3).
- Use several canonical clock mutants (Chapters 4 and 5) and *period* 3' UTR splicing locked transgenic strains (Chapter 6) to elucidate whether the circadian clock is acting as a module to regulate diapause. Additionally, the role of miRNA in *per* splicing and diapause is investigated (Chapter 7).
- Dissect the role of different cell clusters in diapause regulation (Chapters 5 and 6).

2. MATERIALS AND METHODS

2.1 Fly stocks

2.1.1 Stock maintenance

Flies were maintained in a standard maize-based medium (Table 2-1). Stocks were kept at 18°C in LD 12:12 and the flies used for experiments were raised at 25°C LD 12:12, unless otherwise stated.

Table 2-1 Recipe of the fly-food. [1]: Those components were added after boiling.

Ingredient	Quantity
Water	7.5 l
Maize meal	504 g
Brewer's yeast	350 g
Agar	59.5 g
Glucose	555 g
20% in ethanol Nipagen[1]	94.5 ml
Propionic acid[1]	21 ml

2.1.2 Fly lines

The specific fly strains used will be described in the "Material and Methods" section of the respective chapters.

2.2 Diapause

2.2.1 General diapause induction protocol

Fly lines were expanded at 25 °C prior to any diapause experiment. The lines had different rearing conditions that will be expanded on in each chapter. Nevertheless, diapause-induction methodology remained the same for most the experiments (unless otherwise stated): 60-70 flies that had hatched within 0-5h were pushed into a fresh food vial and placed in an LMS^{TM} 201 cooled incubator (11853410, ThermoFisher Scientific) at 12 °C in LD 8:16 for 12 days.

2.2.2 Dissection and scoring of diapause

After 12 days in diapause inducing conditions, flies were anesthetised by CO_2 exposure and dissected in 1X Phosphate-Buffered Saline (PBS), which was prepared as described by (Wulbeck and Helfrich-Forster, 2007) (Table 2-2).

Table 2-2 Recipe for the preparation of 1 L (10X) PBS stock.

Compound	Amount
NaCl	200 g
KCl	5 g
KH_2PO_4	5 g
$Na_2HPO_4 \cdot 2H_2O$	27.8 g

The classical criteria described by Saunders et al. (1989) was used to score diapause: the most advanced developed egg chamber in both ovaries needed to be below stage 8, and hence previtellogenic, for the fly to be considered as diapausing. See Figure 2-1 for some examples of diapausing and non-diapausing ovaries.

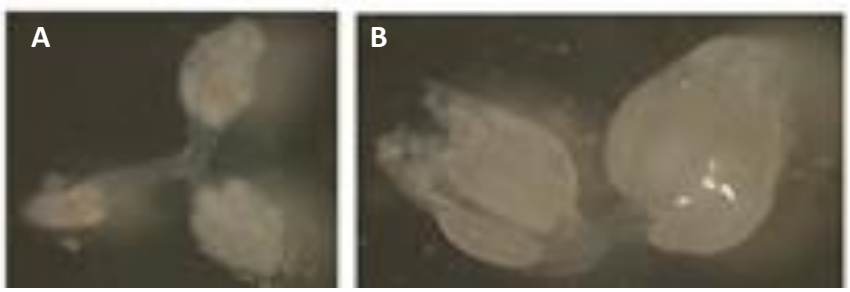


Figure 2-1 Examples of diapausing (A) and non-diapausing (B) ovaries in *D. melanogaster*.

An arcsine transformation of the diapause results was applied before any further analysis by ANOVA, which was followed by Tukey's multiple comparisons test.

2.3 Locomotor activity

Experimental set-ups vary between the chapters, hence will be described in the respective "Material and Methods" sections.

Trikinetics *Drosophila* Activity Monitoring Systems (DAM) and software (Trikinetiks Inc, Waltham, USA) were used to assess locomotor activity of single flies as described in Rosato and Kyriacou (2006). Flies were placed inside a small glass tube containing: on one side, the same food used to rear the flies sealed with a plastic cap and, on the other side, a cotton plug to prevent the flies from escaping. These vials were arranged inside a monitor with an infra-red beam which is connected to a computer through a Power Supply Interface Unit (Trikinetiks Inc). Using the DAMSystem 2.1.3 software (Trikinetiks Inc) the number of times each fly crosses, and hence blocks, the beam was recorded in 30 min intervals (or bins).

Flies were kept at LD12:12 for five days and then released into free-running conditions (DD) for the following seven days. Only flies that survived through all the experiment were analysed. Average activity histograms of the last three days in LD were plotted using Microsoft Excel® 2016. Spectral analysis and autocorrelation, integrated in BeFly! (Allebrandt et al., 2013) were used to assess the rhythmicity of the flies and their period as described in Rosato and Kyriacou (2006). In the **spectral analysis**, the activity is broken down into sine and cosine waves, with the frequencies giving the closest matches to the data being displayed as a spectrogram. Finally, Monte Carlo simulations are used to generate 100 randomisations on the data in order to calculate the 95% and 99% confidence limits (Peixoto et al., 1998). Flies were considered rhythmic when the significant peak (over 99% confidence limits) observed in the spectral analysis was confirmed in the autocorrelogram (see Figure 2-2 for examples of rhythmic and arrhythmic flies).

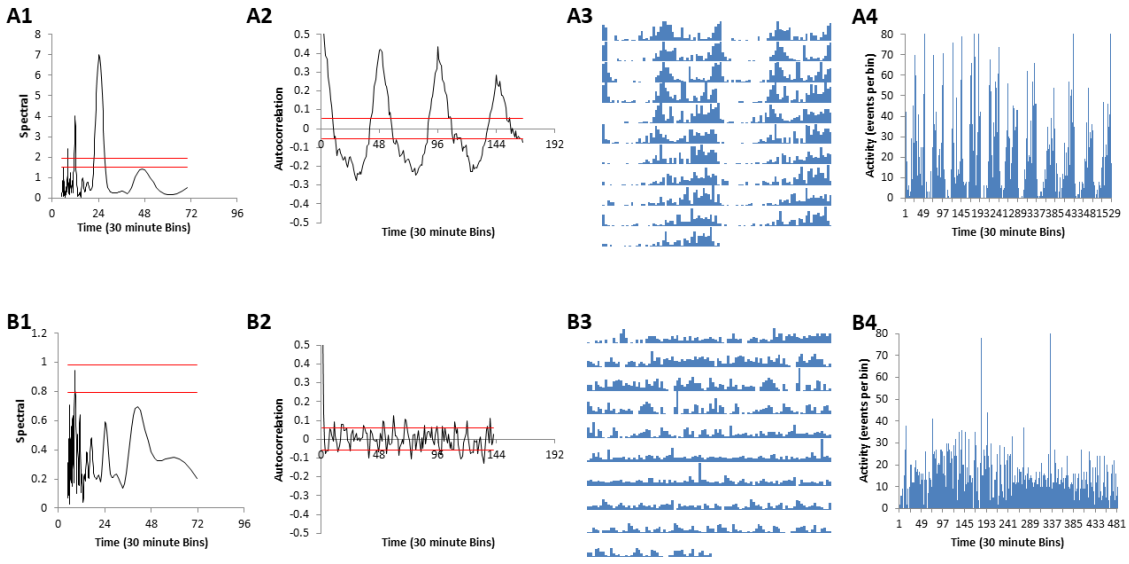


Figure 2-2 Examples of rhythmic (A) and arrhythmic (B) flies. 1) Spectral analysis; 2) Autocorrelation; 3) Actogram; and 4) Overall activity pattern of the fly.

2.4 Genomic DNA extraction

An individual fly was placed in a 0.5 ml tube and smashed in 50 µl of squishing buffer, which contains TrisHCl, EDTA, NaCl and Proteinase K in the proportions shown in Table 2-3. The Proteinase K was added fresh every time. 5 µl of 40 µg/ml Proteinase K stock were added for 1ml of Squishing buffer. Afterwards, the samples were subject to the DNA extraction programme (Table 2-4).

Table 2-3 Components and proportions necessary to prepare Squishing buffer.

Amount	Component	Final concentration of component
80 µl	EDTA (0.5M = 500 mM)	2mM
200 µl	Tris HCl pH 8.2 (1 M)	10mM
110 µl	NaCl (4.5 M)	25 mM
19610 µl	ddH_2O (autoclaved)	

Table 2-4 DNA extraction program.

Temperature	Time
37°C	45 min
95°C	3 min
10 °C	Forever

2.5 RNA extraction

To avoid RNA degradation, flies were snap-frozen in liquid nitrogen. The flies were subjected to three rounds of vigorous vortexing for 15s followed by freezing them in liquid nitrogen. The heads were separated from the bodies using a metal sieve kept on dry ice.

2.5.1 Trizol RNA extraction

25-30 heads were homogeneised with a plastic pestle in 1000 µl of TRIzol® Reagent (15596026, ThermoFisher Scientific) and centrifuged at 4 °C for 10 min at 12.000 xg. The supernatant was incubated for 5 min at room temperature. Afterwards, 200 µl of chloroform were added and the mixture was vortexed for 15 s. After 3 min incubation at room temperature, samples were centrifuged for 15 min at 4°C. This results in a phase separation of the sample into three layers: a red phenol-chloroform phase, an interphase and the colourless upper aqueous phase. The upper layer containing the RNA was moved to a fresh RNase-free tube. RNA was precipitated with 250 µl of isopropanol followed by a 10 min incubation at room temperature and a 10 min centrifugation at 4°C. The supernatant was discarded and the RNA pellet was washed three times with 500 µl of 75% ethanol by inverting the tube. Finally, the supernatant was discarded and the pellet was air dried for 5 minutes, after which it was resuspended in 20 µl of RNase free water.

2.5.2 RNA extraction with Maxwell instrument

Maxwell® 16 LEV simplyRNA Cells Kit (Promega, AS1270) was used to perform RNA extraction of 25-30 *Drosophila* heads with the Maxwell Promega machine from NUCLEUS Genomics (University of Leicester). The protocol provided by the manufacturer was followed with a couple of exceptions: (1) homogenisation of the heads was performed in ice using an electric pestle; (2) 10µl of DNase were added to the cartridge. After filling the cartridges, the instrument was turned on and the 1h long automatic protocol started. After this, you ended up with 50 µl of high quality RNA ready to use.

2.6 DNA and RNA quantification

NanoDrop 8000 Spectrophotometer from NUCLEUS Genomics (University of Leicester) was used to check DNA/RNA quality and quantity.

2.7 DNAse treatment

Any trace of genomic DNA was removed from Trizol-extracted RNA using the TURBO DNAse kit (AM1907, ThermoFisher Scientific). 0.1 µl of 10x TURBO DNase Buffer together with 1 µl of TURBO DNase were added per 1 µg of RNA (final volume of 10 µl). The samples were incubated at 37°C for 30 min. 0.1 volume of DNase Inactivation Reagent was added to the sample and the samples were incubated for 5 min at room temperature in a shaker. After a 1'5 min centrifugation at 10.000xg, the supernatant was transferred to a fresh RNase-free tube.

2.8 cDNA synthesis

cDNA was generated using the two different systems that are described in the following paragraphs.

2.8.1 GoScript™ Reverse Transcription System

GoScript™ Reverse Transcription Kit (A5001, Promega) was used for cDNA synthesis from Trizol-extracted RNA, after the TURBO DNase treatment. A mixture containing 1:6 oligo-dT:random primers was used to enhance poly-A transcripts but avoiding the 3' bias of the transcripts. Up to 5 µg of RNA together with 1 µl of the primer mix were mixed in 5 µl of total volume. The samples were heated to 70 °C for 5 min and immediately chilled on ice for 5 min. After a brief centrifugation to collect the contents of the tube 4 µl of 5x GoScript Reaction Buffer, 2 µl of $MgCl_2$, 1 µl of dNTPs, 0.5 µl of Recombinant RNasin Ribonuclease inhibitor, 1 µl of GoScript Reverse Transcriptase topped up to a total of 15 µl with nuclease-free water were added to the sample (no-transcriptase controls were run in parallel). Afterwards, the sample was

incubated at 25 °C for 5 min (annealing) and at 42 °C for 1 h (elongation). Finally, it was kept at 70 °C for 15 min to inactivate the Reverse Transcriptase.

2.8.2 QuantiTect® Reverse Transcription kit

QuantiTect® reverse transcription kit (205313, Qiagen) was used for cDNA synthesis following RNA extraction with Maxwell instrument. 0.25µg of template RNA was mixed with 2µl of 7x gDNA Wipeout Buffer in a 14µl reaction. Then the samples were incubated at 42°C for 2 min and immediately placed on ice. Finally, 6µl of the reverse-transcription mastermix containing the retrotrascriptase, its buffer and primers were added to the samples. Finally, the additional 15 min of incubation at 42°C were followed by a shorter 3 min incubation at 95°C to inactivate the retrotranscriptase.

2.9 Polymerase Chain Reaction (PCR)

DNA (or cDNA) amplification was achieved via PCR after setting up the reaction mix described in Table 2-5. The primers used had been previously described or designed using Primer3web, version 4.1.0 (Koressaar and Remm, 2007; Untergasser et al., 2012). Table 2-6 shows a general PCR amplification program that will be modified to optimize the reaction. PCRs were performed using a GS4 Multi Block Thermal Cycler (G-Storm).

Table 2-5 Set-up for a 10 µl PCR reaction. [1]: 5x Buffer already contains magnesium as well as the dNTPs.

Component	Quantity
5x Buffer[1]	2 µl
Forward and Reverse Primers	0.25+0.25 µl
Taq	0.2 µl
DNA sample	1 µl
water	6.3 µl

Table 2-6 PCR amplification program. An initial denaturalization of 2 min at 92°C will be followed by 25-30 cycles of denaturation (92°C), annealing and elongation (72°C). [1]: Optimal temperature of annealing will depend on the primers. [2]: Elongation time depends on the length of the amplified fragment.

Cycles		Temperature	Time
Initial Denaturation			
1		92°C	2 min
Amplification			
25-30	Denaturation	92°C	45 s
	Annealing	50-65°C[1]	30-60 s
	Elongation	72°C	45 s -2 min[2]
Final elongation			
1		72°C	10 min
Keeping		10°C	Forever

2.9.1 Genotyping

A PCR-based approach was used to confirm the flies' genotype. Table 2-7 contains a list of primers along with their specific temperature of melting (Tm) and elongation time as well as whether the amplicon had to be digested or not.

Table 2-7 List of primers and PCR conditions for the confirmation of the mutant lines. Tm, temperature of melting; Elong, elongation time.

Genotype	Primers	PCR conditions	Digestion	Length of the amplicon
Pdf^{0}	F: TGCTGCCAGTGGGGATAA R: CTTACTTGCCCGCATCGT	Tm: 66°C Elong: 30s	NO	265bp
Clk^{Jrk}	F: GATGGGATTCGCACCTGG R: TGCCTCCATTGTAGCTTTGATT	Tm: 60°C Elong: 30s	YES BfaI	530bp *325+205*
cry^{b}	F: GCATGGAGGGCAATGACAT R: GACCGACCAATCCGCATT	Tm: 66°C Elong: 30s	NO	257bp
cyc^{01}	F: AGCGGCAGCTTACATCCA R: CCTTAACCTTGCCTATGTCCTA	Tm: 66 Elong: 30s	NO	241bp
per^{01}	F: ACGGTAATGAAGAAGGGTCAGA R: GGGTCCTGGAAGGTGAAATG	Tm: 60 Elong: 30s	YES BfaI/XbaI	236bp *170+66*
tim^{01}	F: GCTCATCGCTTTTCATATGTT R: AGGATGTGATTGGTAACCAC	Tm: 57 Elong: 45s	NO	WT: 602bp Mut: 538bp

2.9.2 *timeless* haplotype

A PCR-based strategy was used to determine the *tim* haplotype of each line. The primers (Table 2-8) and PCR conditions have previously been described in Tauber et al. (2007). Two individual reactions were performed per fly. Each reaction will have a specific forward primer, either s-timF (for *s-tim*) or ls-timF (*ls-tim*), with the common reverse primer (timR). A parallel amplification of a different region of the gene will be performed in both reactions by a pair of control primers (timCF and timCR) to verify the integrity of the reaction.

Table 2-8 List of primers necessary for the *timeless* haplotyping.

Genotype	Primers	PCR conditions	Length of the amplicon
s-timF	TGGAATAATCAGAACTTTAT	Tm: 55°C Elong: 1 min	s-tim: 692 bp ls-tim: 693 bp Control: 487 bp
ls-timF	TGGAATAATCAGAACTTTGA		
timR	AGATTCCACAAGATCGTGTT		
timCF	CATTCATTCCAAGCAGTATC		
timCR	TATTCATGAACTTGTGAATC		

2.9.3 High fidelity PCR

For cloning, Q5® Hot Start High-Fidelity 2x Master Mix (M0494S) was used. As it is a mastermix, containing dNTPs and polymerase, only the primers and DNA need to be added to the reaction (Table 2-9). This polymerase is quicker than the usual Taq polymerase, hence the amplification program is different to the one previously described (Table 2-10).

Table 2-9 Components of High-Fidelity PCR with Q5 polymerase.

Component	Quantity
2x Buffer	12.5 µl
Forward and Reverse Primers	1.25+1.25 µl
DNA sample	4 µl
water	6 µl

Table 2-10 Q5 PCR amplification program. [1]: Optimal temperature of annealing will depend on the primers. [2]: Elongation time depends on the length of the amplified fragment.

Cycles		Temperature	Time
Initial Denaturation			
1		98 °C	30 s
Amplification			
30	Denaturation	98 °C	10 s
	Annealing	50-72 °C[1]	15 s
	Elongation	72 °C	20–30 s/kb[2]
Final elongation			
1		72 °C	2 min
Keeping		10 °C	Forever

2.9.4 Quantitative PCR (qPCR)

qPCR was performed using Maxima SYBR Green qPCR Master Mix (2X), with separate ROX vial (K0251, Fisher Scientific UK LTD). The primer mix was prepared adding 7.5µl from the mother solution (100µM) of the forward and reverse primers which were designed to amplify short ≈100 bp sequences using Primer3web (Koressaar and Remm, 2007; Untergasser et al., 2012). See Table 2-11 for a list of the primers. cDNA obtained using the QuantiTect Reverse Transcription kit was 2x diluted by adding 20µl of RNase free water. The qPCR reaction, with a final volume of 5µl, was prepared as described in Table 2-12. Finally, the samples were run using the LightCycler 480 system (Roche) from Nucleus Genomics (University of Leicester) using the amplification program and melting curve analysis described in Table 2-13. Four technical replicates and a no-reverse-transcription control were performed per sample.

LightCycler 480 Software (Roche) was used to calculate the crossing points (Cp) by the second derivative method. For quantification of relative expression, raw fluorescence data of technical replicates with Cp values within 0.5 cycles of each other were averaged. Finally, the “pcrbatch” and “ratiobatch” functions in “qpcR” R-

package[1] were used to calculate amplification efficiency and expression-ratio compared to controls, respectively.

Table 2-11 List of primers used for qPCR amplification of *perA/perB/rlp32*.

Primer name	Sequence	Target
perAB_F1	AGGAGGACCAGACACAGCAC	*perA* and *perB*
perA_R1	AGGCAATTGCTCACTCGTTT	*perA*
perB_R1	CGAAGAATCGTTTCCAGGAC	*perB*
rlp32_F	ATCGGTTACGGATCGAACAA	*rlp32*
rlp32_R	ACAATCTCCTTGCGCTTCT	*rlp32*

Table 2-12 Components of qPCR with SYBR-Green.

Component	Quantity
2x SYBR-Green Buffer	2.5 µl
Forward and Reverse Primers mix	1 µl
cDNA sample	1 µl
water	0.5 µl

Table 2-13 qPCR amplification and melting curve program.

Cycles		Temperature	Time
Initial Denaturation			
1		95 °C	5 min
Amplification			
45	Denaturation	95 °C	15 s
	Annealing	62 °C	30 s
	Elongation	72 °C	30 s
Melting curve		65-97 °C	~30 min

2.9.5 Electrophoresis

The amplified DNA fragments, were run in agarose gels (0.5-2% according to the size of the DNA fragment) containing ethidium bromide (0.5µg/ml). 2µl of 6x Loading dye (Table 2-14) were mixed with 10µl of PCR reaction before they were run on individual gel lanes under constant voltage (100V) in a tank with 1X TBE (Table 2-15). ΦX-174 HaeIII Digest (New England Biolabs) and/or Hyperladder I (Bioline) DNA markers were run parallelly to the samples to verify their molecular weight. Finally, a

[1] https://CRAN.R-project.org/package=qpcR

GeneGenius bio-imaging system (Syngene) with GeneSnap 6.00.23 (Synoptics) software was used to visualize the gels.

Table 2-14 Composition of 6x Loading dye.

Component	Quantity
Bromophenol blue	0.25 g
Ficoll	31.25 g
5x TBE	250 ml

Table 2-15 Recipe for of 10 L (10X) TBE.

Component	Quantity
Trisma Base	1090 g
Boric Acid	550 g
EDTA	93 g
Distilled Water	To 10 l

2.10 DNA purification

2.10.1 Plasmid DNA

Plasmid DNA was extracted using E.Z.N.A® Plasmid Mini Kit I (D6942, Omega Bio-Tek). The protocol recommended by the manufacturer was followed, including the heating of the Elution Buffer to 70°C and the "Column Equilibration" and "second DNA Wash" that were listed as optional steps. To increase the total Plasmid DNA yield, two steps of elution using 30 µl of Elution Buffer in each were performed.

2.10.2 After enzymatic reaction

Some of the components of enzymatic reactions (such as the buffer or polymerase from the PCR) may interfere with the activity of future enzymes (for example, restriction enzymes). In order to avoid this, DNA was cleaned before proceeding with any downstream application. This was accomplished using E.Z.N.A® MicroElute DNA Clean Up Kit (D6296, Omega Bio-Tek) following manufacturers protocol and eluting the sample with 10µl of nuclease-free water.

2.10.3Gel purification

Plasmid DNA was extracted using E.Z.N.A® Gel Extraction Kit (D2500, Omega Bio-Tek) following manufacturers protocol and eluting the sample with 30µl of nuclease-free water.

2.11 Restriction enzyme reaction

Some of the PCR reactions were followed by the digestion of the amplificated product either for genotyping purposes (See chapter 4) or for inserting the product in a secondary vector. The digestion was performed following manufacturer instructions (New England Biolabs) with a final volume of 20µl or 50µl for PCR or cloning, respectively.

2.12 DNA sequencing

5 µl DNA mixed with 5 µl of primer (5µM) were sent to GATC Biotech for Lightrun Sanger sequencing (SKU#B50200200, GATC) to be run on an ABI 3730xl DNA Analyzer system. The sequence was analysed using Staden Package (http://staden.sourceforge.net/).

2.13 Protein Extraction

Protein extraction was performed as described in Emery (2007). 25-30 *Drosophila* heads were homogenised in 30 µl of Extraction buffer (Table 2-16) and centrifuged at 13.000 rpm 4 °C for 10 min. The supernatant was carefully transferred to a fresh tube and quantified by mixing 1 µl of the sample with 200 µl of 1:5 dilution of Bradford's Reagent (B6916, Sigma-Aldrich) on a 96 well plate and measuring the optical density (OD) at 595 nm on FLUOstar Omega Microplate Reader using Omega 5.10 R2 software (BMG LABTECH). Finally, the samples were equalised to the less concentrated sample using extraction buffer.

Table 2-16 Composition of Extraction Buffer.

Component	Proportion
HEPES, pH=7.5	20 mM
KCl	100 mM
Glycerol	5%
EDTA	10 mM
Triton	0.1%
Dithiothreitol	1 mM
Phenylmethylsulfonyl fluoride	0.5 mM
Proteinase inhibitor (88666, ThermoFisher Scientific)	1 tablet
Water	Up to 10 ml

2.14 Western Blot

10 µl of sample were mixed with 2 µl of 6x Loading Buffer (Table 2-17). After a 5 min incubation at 98 °C for denaturation of the proteins, the samples were briefly centrifuged and loaded in Novex™ 4-20% Tris-Glycine Mini Gels (XP04205BOX, ThermoFisher Scientific). Additionally, a Novex™ Sharp Pre-Stained Protein Marker (LC5800, ThermoFisher Scientific) and a Standard Sample, which was created by mixing 5 µl of each sample, was added in all the gels to verify their molecular weight and to allow comparison between different gels, respectively.

The gels were run at 225 V constant voltage for 40 min in a XCell SureLock Mini-Cell (EI0001, ThermoFisher Scientific) tank full of Running Buffer (Table 2-18) to allow separation of the proteins. The proteins were afterwards transferred onto an Amersham Protran 0.45 µm nitrocellulose membrane (10600002, GE Healthcare Bio-Sciences) using a Wet/Tank Blotting System (Bio-Rad) filled with Transfer Buffer (Table 2-19). The transfer was performed at 400 mA for 1’5 h at 4 °C to prevent overheating. The nitrocellulose membrane was blocked for 1 h in 5% milk buffer (adding 5 g of milk powder in 100ml of TBST (Table 2-20)) and probed with specific antibodies diluted as described in Table 2-21. Primary antibody was probed overnight at 4 °C in a shaker. Afterwards, the membrane was washed three times for 15 min in TBST. The secondary antibody was probed at room temperature for 2 h and was followed by another round of three 15 min washes in TBST. Finally, SuperSignal™ West Dura Extended Duration Substrate (34075, ThermoFisher Scientific) was added to allow the visualisation of the

antibody bound to the protein of interest using GeneGnome (Syngene) with GeneSys version 1.5.0.0 (Synoptics) software.

ImageJ was used to analyse protein levels using the Gels submenu from Analysis as described in a tutorial by Luke Miller (2010)[2]. PER expression levels were compared to the mean of the two internal controls, TUB and HSP70.

Table 2-17 Recipe for 10 ml of 4x Loading buffer.

Component	Quantity	Final concentration
Tris HCl pH=6.8	2.4 ml	300mM
SDS	0.8 g	10%
100% Glycerol	4 ml	40%
β-mercaptoethanol	0.5 ml	5%
Bromophenol Blue	0.004 g	0.01%
Water	3.1 ml	

Table 2-18 Recipe for 1 l of 10x Running Buffer. Dilute 100 ml of 10X Running buffer with 900 ml of water for the working solution.

Component	Quantity	Final concentration (in x1)
Tris Base	30.3 g	25 mM
Glycine	144 g	190 mM
SDS	10 g	0.1%
Water	Up to 1l	

Table 2-19 Recipe for Transfer Buffer.

Component	Quantity
10x Running Buffer	100 ml
Methanol	200 ml
Water	700 ml

Table 2-20 Recipe for 1l of TBST.

Component	Quantity
1M Tris HCl pH=7.5	20 ml
NaCl	8.18 g
10% Tween 20	5 ml
Water	Up to 1 l

[2] http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/

Table 2-21 Primary and secondary antibodies for WB.

	Antibody	Product n° Company	Dilution (in Blocking solution)
Primary	Mouse α-HSP70	MA3-008 ThermoFisher Scientific	1:10.000
	Mouse α-TUB	AA43 DSHB	1:10.000
	Rabbit α-PER1 (H-120)	Sc-25362 Santa Cruz	1:2.500
Secondary	Goat α-mouse IgG (H+L) conjugated to WestVision™ Peroxidase Polymer	WB-2000 Vector Laboratories	1:10.000
	Goat α-Rabbit (H+L) conjugated to WestVision™ Peroxidase Polymer	WB-1000 Vector Laboratories	1:10.000

2.15 Statistical analysis

GraphPad Prism 7 software was used for preparing the images and performing the statistical tests, except for three (or more)- way ANOVAs which were analysed using R or otherwise stated.

3. EFFECTS OF DEVELOPMENTAL CONDITIONS IN ADULT *D. melanogaster*

Part of this work has been published in Anduaga et al. (2018).

3.1 Introduction

3.1.1 Seasonal adaptation and diapause

Temperature and day-length vary during the year in a predictable way. This has led to the development of different mechanisms to enhance survival and reproduction, from migration to hibernation. Nevertheless, dormancies and in particular diapause is the mechanism of choice for many insects, among them, *D. melanogaster* (Danks, 1987).

3.1.2 Insects at low temperatures

3.1.2.1 Cold-induced injuries

Environmental temperatures restrict organisms particularly in temperate or polar regions where temperatures can drop below freezing. This is partially due to the water-ice transition, which can lead to critical **freezing injury** of the organism, although **indirect and direct chilling injurie**s (which are, respectively, chronic or acute exposures to mild or severe cold temperatures that lead to disturbances in metabolic pathways, protein denaturation, oxidative stress…) can also be extremely damaging (Kostal et al., 2011; Sinclair et al., 2003). In such habitats, the survival of the individual will depend on their physiological response to cold exposure (Sinclair et al., 2015).

3.1.2.2 Cold tolerance strategy

Strategies to avoid freeze-induced injuries are variable and organism- and situation-dependant. Chill susceptibility reflects a situation in which the insects die when exposed to cold, but not due to the formation of internal ice (Bale, 1996). For example, Boardman et al. (2012) describe this phenomenon in *Thaumatotobia leucotreta* or the false codling moth larvae, which die after an acute exposure to -8 °C to -12 °C, but do not freeze unless the temperature goes below -13 °C. However, two main strategies (freeze avoidance and freeze tolerance) have been described in insects (Table 3-1) (Sinclair et al., 2003).

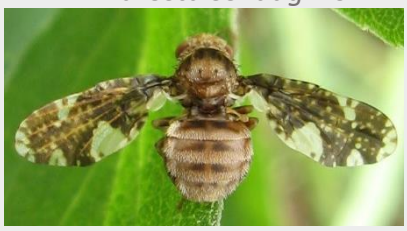
Freeze avoidance consists of maintaining body fluids in a liquid state below their usual melting point in a supercooled state (Sinclair et al., 2003). Species are able to tolerate cold as long as there is no internal ice formation (Sinclair et al., 2015). They manage to do so by several physiological changes such as removing ice nucleators (initiators of ice formation), accumulation of polyols and sugars (for example trehalose, which will contribute to lowering the crystallization temperature and stabilization of the membranes) or generation of antifreeze proteins (Ramlov, 2000; Zachariassen, 1985). Prepupae of *Agrilus planipennis*, the emerald ash borer, withstand long-term exposures to subzero temperatures, as long as they do not freeze (Crosthwaite et al., 2011). Some organisms undergo **cryoprotective dehydration**, a special freeze-avoidance strategy for which a dynamic animal-environment interaction is vital to avoid generation of ice. It consists on an extreme gradual loss of water content as well as increase in trehalose and/or other cryoprotectants, all of which leads to an increase in their melting point. It was discovered in *Onychiurus arcticus*, the Arctic springtail, which is able to avoid freezing, and hence survive, after exposure to temperatures as low as -30 °C (Holmstrup et al., 2002).

In contrast, **freeze tolerance** involves survival to ice formation where in most cases, ice will be formed in the extracellular compartments rather than inside the cells, hence protecting them from damage (Ramlov, 2000; Sinclair et al., 2003). The biochemical components facilitating this mechanism are shared with freeze avoidance, however their function is slightly different. Ice nucleators start freezing in the

haemolymph or gut, while polyols and carbohydrates have the dual effect of protecting proteins and membranes and controlling ice fraction size. Antifreeze proteins will play a role in this mechanism by controlling and preventing growth and redistribution of the formed ice (Ramlov, 2000; Zachariassen, 1985). For instance, Morrissey and Baust (1976) reported that in *Eurosta solidaginis*, the golden rod gall fly, pre-pupae freeze at -8 °C; however, they are able to recover and death only occurs if the temperature drops below -25 °C.

Interestingly, the strategy of choice of a species to enhance cold tolerance can be modified. Some insects will have different strategies depending on the season or even among populations located in different regions (Sinclair et al., 2015). For example, *Pyrrhocoris apterus* is a freeze-avoiding insect during winter, but chill-susceptible in summer (Kostal and Simek, 2000).

Table 3-1 Summary of cold tolerance strategies in insects according to the final result: insects' survival (or not) and the formation (or the lack of) ice. Adapted from Sinclair et al. (2015).

	Internal ice formed	No internal ice formed
Alive	**Freeze-tolerant** Ex. *Eurosta solidaginis* 	**Chill-susceptible or freeze-avoidant** Ex. *Agrilus planipennis* 
Dead	**Chill-susceptible or freeze-avoidant** Ex. *Agrilus planipennis* 	**Chill-susceptible** Ex. *Thaumatotobia leucotreta* 

3.1.2.3 Measuring Cold tolerance

Methods to measure cold tolerance are very diverse and difficult to compare. In *D. melanogaster*, some of the most common assays consist on assessing:

- **Cold shock:** survival following an acute exposure to low temperatures followed by a recovery time. For example, Overgaard et al. (2007) exposed adult females to -5 °C for 1h.
- **Chill-coma recovery time:** flies are kept at 0-4 °C for several hours, hence inducing a reversible coma; afterwards, the time the flies start to regain mobility is recorded (Colinet et al., 2013a; Pegoraro et al., 2014).
- **Critical minimum temperature (CT_{min}):** the temperature to which the flies are exposed is decreased from 25 °C at a slow rate and the time in which the flies stop moving is recorded (Colinet et al., 2013b; Sinclair et al., 2015).
- **Lethal time (LT_{50}):** the time of exposure to cold temperatures in which half of the flies die (Kostal et al., 2011).

3.1.2.4 Enhancing cold tolerance

Several insects can increase their cold tolerance depending on the environment to which they are exposed. Lee and Denlinger (1991) showed the importance of pre-winter acclimation to enhance survival in subsequent extreme environments (Hazel, 1991). Cold tolerance can be achieved by several mechanisms such as cold hardening (exposure to sub-lethal temperature for a short period of time) or cold acclimation (long-term exposure) (Kostal et al., 2011; Rako and Hoffmann, 2006; Vesala and Hoikkala, 2011). Even the temperature at which the flies are reared has been shown to play a role (Ayrinhac et al., 2004).

Cold acclimation (for days or weeks) to challenging (but within viable limits) conditions results in changes in metabolism, cell structure and so on that anticipate gradual changes in the environment (Colinet et al., 2013a). These changes are reversible; however, they will endure for as long as the new challenging condition persists. For example, *D. melanogaster* larvae were found to have enhanced cold tolerance (having lethal time, LT_{50}, as the output) to a 0 °C exposure when acclimated

to 15 °C followed by 2 days at 6 °C compared to the controls that were kept at 25 °C (Kostal et al., 2011).

Rapid cold hardening is a quick, although short-term, response to an acute condition that enhances later survival to more extreme temperatures (Bowler, 2005; Sinclair et al., 2003). Lee et al. (1987) reported in several species, amongst them *Sarcophaga crassipalpis* and *S. bullata*, two flesh fly species, an increase from 5-30% to 90% survival after a two hour exposure to -10 °C after pre-treating the flies with a 0 °C exposure also for two hours. This rapid cold hardening has also been described in *D. melanogaster* (Kelty and Lee, 1999).

3.1.3 Developmental environment effect on adults

The environmental conditions during early stages of life have an effect on the adult. Just as in humans there are several studies documenting an increased risk of mental disorders (such as schizophrenia) depending on the conditions to which the foetus was exposed during early stages of gestation (Opler and Susser, 2005), there are also several studies with insects showing the importance of developmental conditions. Aboagye-Antwi and Tripet (2010) limited the nutritional resources at the larval stage of the malarial mosquito, *An. gambiae*. This resulted in significantly smaller adult female body size, with poorer phenotypic quality that affected the ability to survive dessication. This is in line with the "**silver-spoon**" effect: favourable conditions during early stages results in enhanced fitness for the adult (Nylin and Gotthard, 1998). However, not all organisms behave in the same way and the opposite scenario is also possible. The **environment-matching hypothesis** states that only if the conditions are also favourable during adulthood will the silver-spoon effect be observed (Monaghan, 2008). Chill-coma recovery experiments with *D. melanogaster* found that the rearing temperature of the flies was a major factor for recovery time; the colder they were reared, the less time it took them to recover (Ayrinhac et al., 2004). A related study observed that female flies raised in long summer photoperiods were less resistant to chill coma as adults than when raised in short, winter photoperiods and this effect had both a photo- and thermo-periodic component (Pegoraro et al., 2014).

3.1.4 Triggers for diapause and cold-tolerance

Depending on the organism and diapause strategy, the relevance of environmental triggers for diapause could be different, including humidity levels in the tropics, the CO_2 content or food availability (Kostal, 2006). However, each season is characterised by different temperature and photoperiod combinations making these two the most obvious cues for diapause and cold-tolerance (Nylin, 2013). Lanciani (1992) demonstrated that photoperiod can influence cold tolerance in *Drosophila.* On the other hand, (Kogure, 1933) was the first to report photoperiodic diapause in the silkworm and, from then, it has been confirmed in several species, *D. melanogaster* amongst them (Saunders et al., 1989). Nevertheless, recently the involvement of day length in *D. melanogaster*'s diapause induction is been challenged (Emerson et al., 2009b; Tatar et al., 2001).

It is important to bear in mind that, even if diapause and cold-tolerance share the common goal of increasing the chances of survival to cold or adverse conditions, they may work through very distinct mechanisms (Loeschcke and Sørensen, 2005; Teets and Denlinger, 2013).

3.1.5 Metabolic state and diapause

In Drosophila insulin-like peptides (dilps) are known to influence diapause (Schiesari et al., 2011; Schiesari and O'Connor, 2013). Moreover, sugar levels in the haemolymph are regulated by dilps (Williams et al., 2006), and perhaps not surprisingly, diapausing organisms show variations in carbohydrate levels (Kubrak et al., 2014; Rako and Hoffmann, 2006). Changes in metabolites such as glycerol, glycogen, sorbitol and trehalose have been thoroughly studied in the diapause field. In *H. armigera*, diapausing pupae have increased sorbitol and trehalose levels, which play a role as energetic and cryoprotectant (or antifreeze) molecules (Xu et al., 2009). In *Bombyx mori*, during their embryonic diapause, sorbitol and glycerol are generated from glycogen (Chino, 1957). Similarly, decrease of glycogen and increase of glycerol have been reported during pupal diapause of *Sarcophaga crassipalpis* and the larval diapause of *Chilo suppressalis*. Using metabolomics to understand the metabolic state of the animals during diapause has shown that there are many additional key

metabolites (Michaud and Denlinger, 2007). For example, Lu et al. (2011) described 55 metabolites in the haemolymph and 52 in the brain of *Helicoverpa armigera* that are involved in diapause.

However, in this chapter, I will focus my attention on three carbohydrates of special interest: glucose, glycogen and trehalose (Figure 3-1). **Glucose** is a monosaccharide that is the main energy source for many organisms and it can be stored as **glycogen** (Arrese and Soulages, 2010). Different patterns of stored glycogen have been reported in diapausing flies (Kubrak et al., 2014). **Trehalose** is a disaccharide that is synthesized from glucose. As in most insects, it is the predominant sugar in *D. melanogaster's* haemolymph and has protective properties against cold, desiccation and several other stresses as well as being a source of energy (Reyes-DelaTorre et al., 2012). Experiments performed with trehalose-deficient flies suggest a role for trehalose under starvation conditions and consequently it is crucial for the adaptation to changes in nutrition (Matsuda et al., 2015). See Figure 3-1 for seeing the relationship between these metabolites.

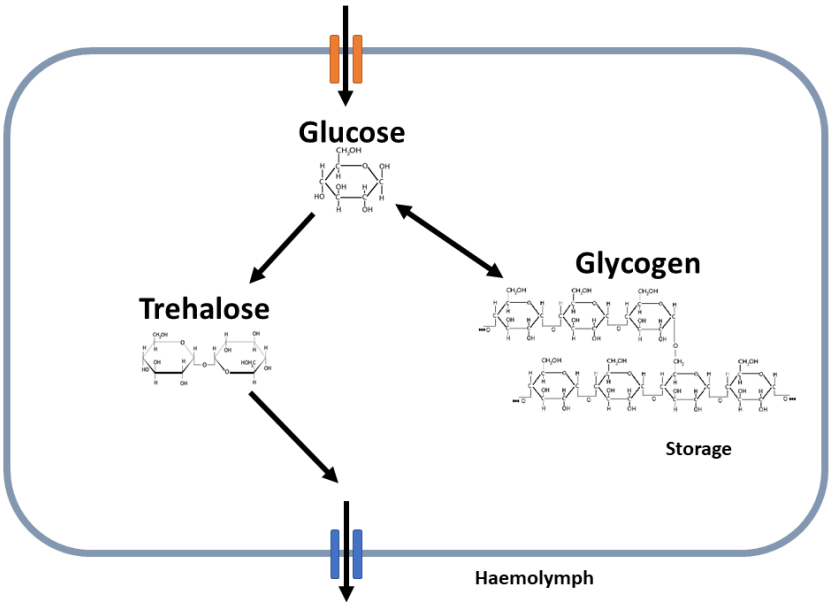


Figure 3-1 Glucose, glycogen and trehalose association in metabolism. Glucose obtained through the diet can be stored as glycogen in fat body cells or used for synthesis of trehalose and released into the haemolymph.

3.2 Aims

Knowing the effects that early stage environment may have on adult performance (Dmitriew and Rowe, 2011), in this chapter I inspect the effects of larval photoperiod on fly development and diapause in wild-type flies. Furthermore, I test a more natural protocol for inducing diapause, raising the flies at 15 °C or 18 °C from late embryonic-early larval stages under short (8:16 LD) or long (16:8 LD) photoperiods before placing them at 12 °C and comparing them with the older protocol of growing them at 25 °C. I also record their survival to a chill shock as well as measure their total glucose, glycogen and trehalose.

3.3 Methods

3.3.1 *Drosophila* Stocks

Fly lines:

- w^{1118}: *white*-null fly line commonly used for generation of transgenic lines.
- *Natural wild isolates*: Isofemale lines collected from different parts of Europe (Figure 3-2) and generously donated by Dr M. Pegoraro.
    - **Rende:** Italy 2006
    - **Houten:** Netherlands 2000
    - **Korpilahti:** Finland 2008

Figure 3-2 Map of Europe highlighting the provenance of the different populations used in the chapter. Orange, Rende (Italy); Green, Houten (Netherlands); Blue, Korpilahti (Finland).

3.3.2 Developmental timing assay

Adult flies were left to lay eggs for two hours at 25 °C. The following day first instar larvae were collected and placed in vials on humid food. The larvae were grown at 15 °C either under short (8:16 LD) or long (16:8 LD) photoperiods. The number of these larvae that reached the pupal (or adult) stage was recorded. 5-10 replicates (vials) with 30 larvae each were examined with another 5-10 replicates containing 20 larvae each placed in constant darkness (DD) but under the same conditions as the flies exposed to the photoperiods. These served as thermal controls. Two-way ANOVA of the day in which 50% of the larvae reached pupal stage was performed to assess the statistical differences between the lines.

3.3.3 General experimental workflow

Vials containing eggs were placed at 15 °C, 18 °C or 25 °C under either short (8:16 LD) or long (16:8 LD) photoperiods until the flies hatched, after which they were placed at 12 °C for 12 days, maintaining the same photoperiod in which they had been grown. After this time, the corresponding experiment was performed (dissection of the ovaries, exposure to -20 °C or quantification of several metabolites) (Figure 3-3). The three experiments were performed in parallel, as well as constant dark (DD) thermal controls of each condition.

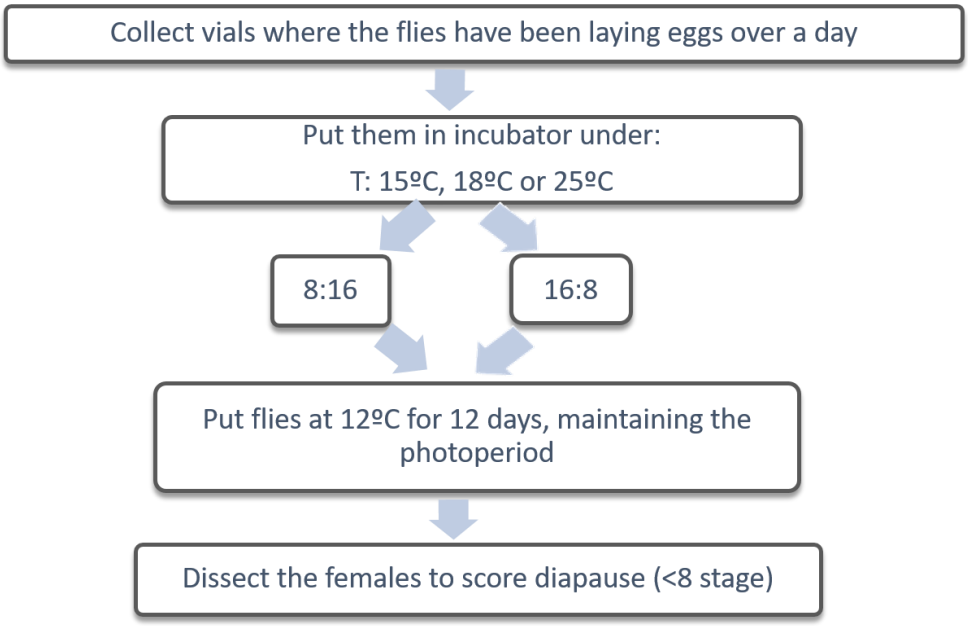


Figure 3-3 General experiment workflow. Read text for further explanation.

3.3.3.1 Scoring diapause

Diapause was scored as described in Material and Methods 2.2.2.

3.3.3.2 Chill-Survivorship

Flies were exposed to -20 °C for different lengths of time (5 min, 10 min, 15 min or 20 min) and transferred to new vials with fresh food. After 24h the number of flies that survived was scored by sex. 6 replicates of 50-75 flies were performed per genotype and condition, along with another 3 DD replicas.

3.3.3.3 Levels of carbohydrate and cryoprotective agents

10 flies were weighed with a precision balance (Precisa 180A) and rinsed with cold PBS several times before being homogenised in 200µl of cold PBS and span down for 1 min at 5,000 RPM. 20 µl of the supernatant were set aside for total protein quantification. The supernatant was heated at 70 °C for 5 min prior to a maximum speed centrifugation for 3 min at 4 °C. Remaining supernatant was used to measure glucose, glycogen and trehalose levels of the samples. 3-4 replicates of 10 flies with their respective 3 DD controls were analysed in triplicate.

The quantification of the metabolites was performed using the protocol described in Zonato et al. (2017). Glucose (GO) Assay Kit (GAGO20-1KT) from Sigma Aldrich was used to score glucose levels of the samples. Standards at 0.01, 0.02, 0.04, 0.08 and 0.16µg/µl were used to generate the standard curve and get the total amount of the different metabolites. Glycogen and trehalose levels were obtained indirectly, by adding amylglucosidase from Aspergillus niger (A1602-25MG, Sigma-Aldrich) or trehalase from porcine kidney (T8778-1UN, Sigma-Aldrich), respectively, to convert them into glucose. Total protein levels were obtained using Bradford assay reagent (B6916, Sigma-Aldrich) using dilutions of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5µg/µl of bovine serum albumin as standards. Samples were quantified spectrophotometrically at 540nm (for glucose, glycogen or trehalose) or at 595nm (for protein).

3.3.4 Statistics

For chill-survival analysis, an arcsine transformation was applied before analysis by three-way ANOVA followed by Tukey's multiple comparisons test. GraphPad Prism 7 was used to generate the correlations between diapause and chill-survivorship or several different metabolites, as well as for the developmental timing analysis.

3.3.5 Temperature control

The temperature inside the incubator was measured using a Tinytag Datalogger from Gemini Dataloggers (product number: SWCD-0040) and HOBO Pendant® Temperature/Light 8K Data Logger (Part # UA-002-08). A recording example of the temperature is shown in Figure 3-4_A.

3.3.5.1 Constant temperature

The temperature difference between lights on and off was recorded for 2-3 days and compensated manually to keep it at 12 °C and avoid overcompensation (See Figure 3-4_B).

3.3.5.2 Temperature-photoperiod cycles inversion

Incubators with long photoperiod and short temperature cycle, and *vice versa,* were generated with the addition of aluminium-foil-covered lights, which were switched on during the night – hence equalising the temperature generated by the lights during day-time but without producing light – as well as by manipulation of the two fans inside the incubator (See Figure 3-5 for schematic representation of the protocol and Figure 3-4_C for an example).

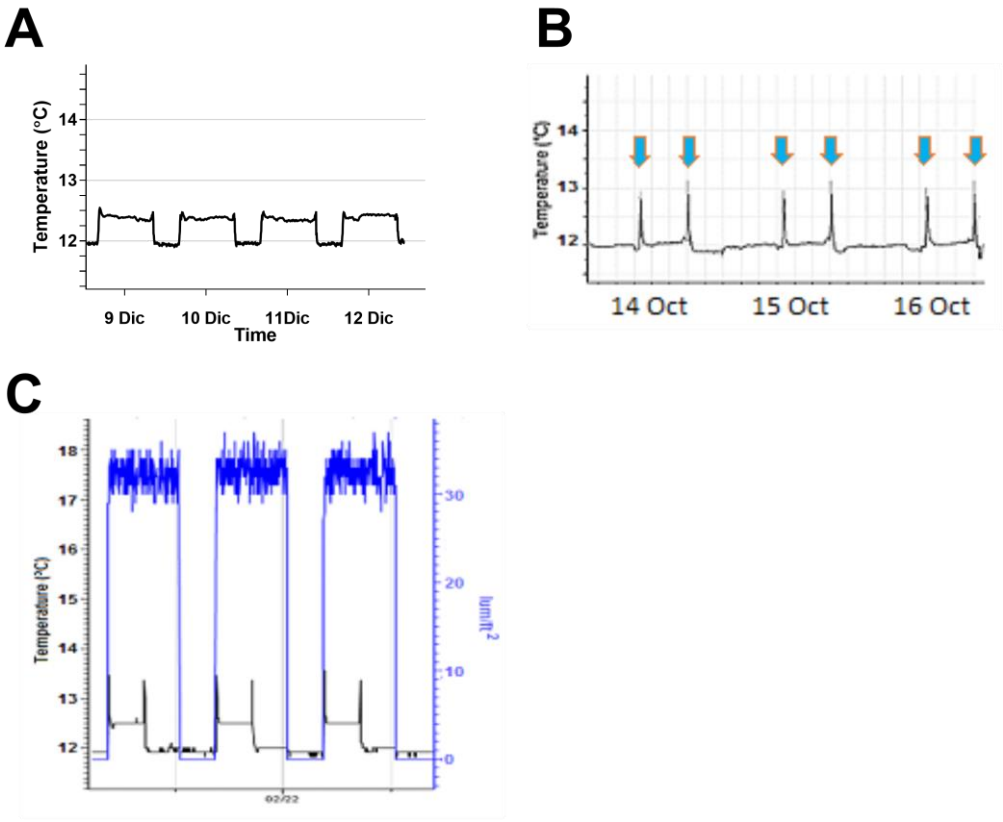


Figure 3-4 Temperature recording of the 12 °C incubator under long photoperiod over several days. A) Temperature is 12 °C when the lights are off (1am to 9am), however it rises up to 12.4 °C when they are on, creating a temperature oscillation of 0.4 °C between lights on and off. B) Temperature is maintained at 12 °C throughout the day. The peaks of temperature (marked by the arrows) are caused by the change from using one fan to the other in the incubator. C) Long photoperiod with short temperature cycle. Black lines represent the temperature cycle inside the incubator: 12.4 °C for 8h and 12.0 °C for 16h. Blue lines represent light intensity, with a high lux/ft^2 for 16h and none for the 8h of night.

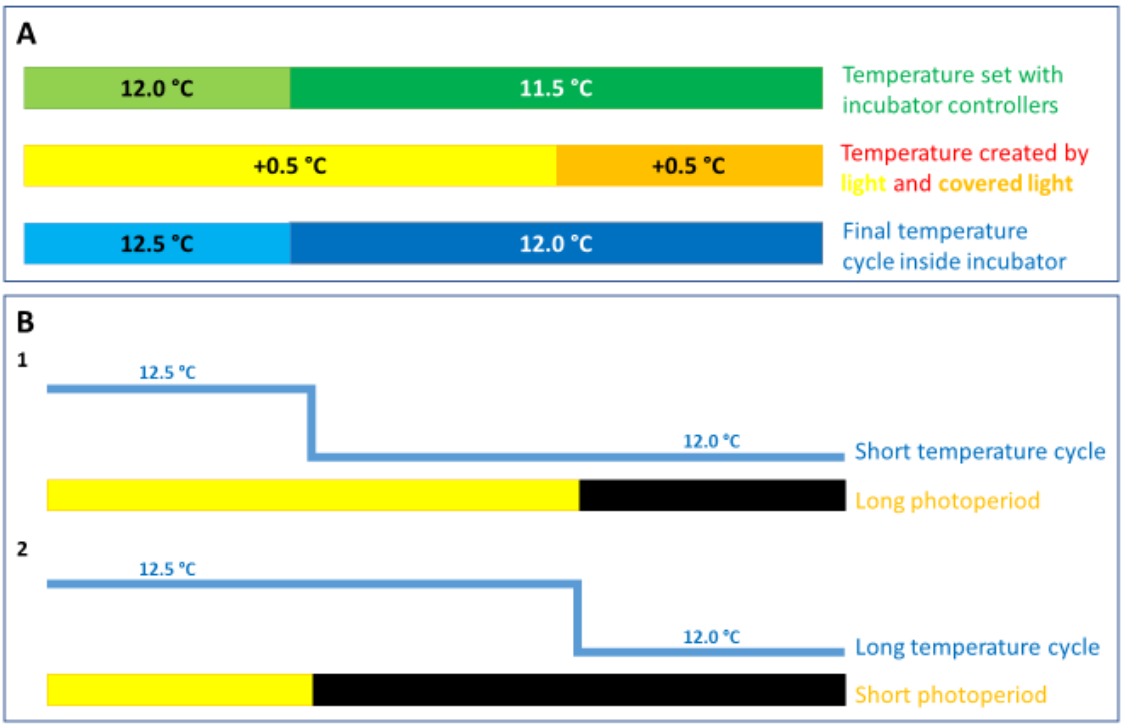


Figure 3-5 Generation of inverse light and temperature cycles inside the incubators. A) Schematic representation of the temperature components inside the incubator. Light sources inside the incubator (cool fluorescent) produce 0.5 °C. This increase in temperature during the light phase is balanced by switching on an additional fluorescent light during the night that will generate the same 0.5 °C, but is covered in aluminium foil and hence the flies will be in complete darkness. To generate a low amplitude temperature cycle, the incubator's temperature controllers can be used (for example, by setting up the temperature to 12.5 °C during the first 8 h and 12 °C for the rest of the day, you get a short-day 0.5 °C oscillation). B) Schematic representation of the final output of light (ON, yellow, and OFF, black) and temperature (blue) inside the incubator. (1) Short temperature cycle with a long photoperiod. (2) Long temperature cycle with a short photoperiod.

3.4 Results

3.4.1 Temperature cycles have a strong effect on developmental timing and diapause in *w*^{1118}

3.4.1.1 Developmental timing

The developmental timing of *w*^{1118} flies under short or long photoperiods at 15 °C was recorded by daily checking the number of larvae that reached pupal-stage from first instar larvae. DD controls were performed to estimate the relative importance of photoperiod and the mild 0.4 °C temperature cycles inside the incubator, as the incubator in the longer photoperiod, by providing 8h more of light per day, will be exposed to a slightly warmer temperature for those 8h (see Methods 3.3.5).

w^{1118} flies kept at 15 °C in the long photoperiod and in the corresponding DD control arrive at the pupal stage on day 12. In contrast those raised under short photoperiods start to pupate at day 18, five days later than their DD controls ($p<0.001$). Clearly the photoperiod itself seems to be having a large delaying effect on development although the slightly lower temperature of the short photoperiod may also be contributing (Figure 3-6_A).

A repetition of the experiment under balanced-temperature conditions (see Methods 3.3.5.1) revealed a major difference with the previous results. The interval in which larvae start arriving at the pupal stage is narrower between the conditions. Even if it is clear that the balancing of the temperature is far from perfect (DD controls are still different from each other, $p=0.0024$), a large difference is observed between the results with balancing versus not balancing the temperature (Figure 3-6_B).

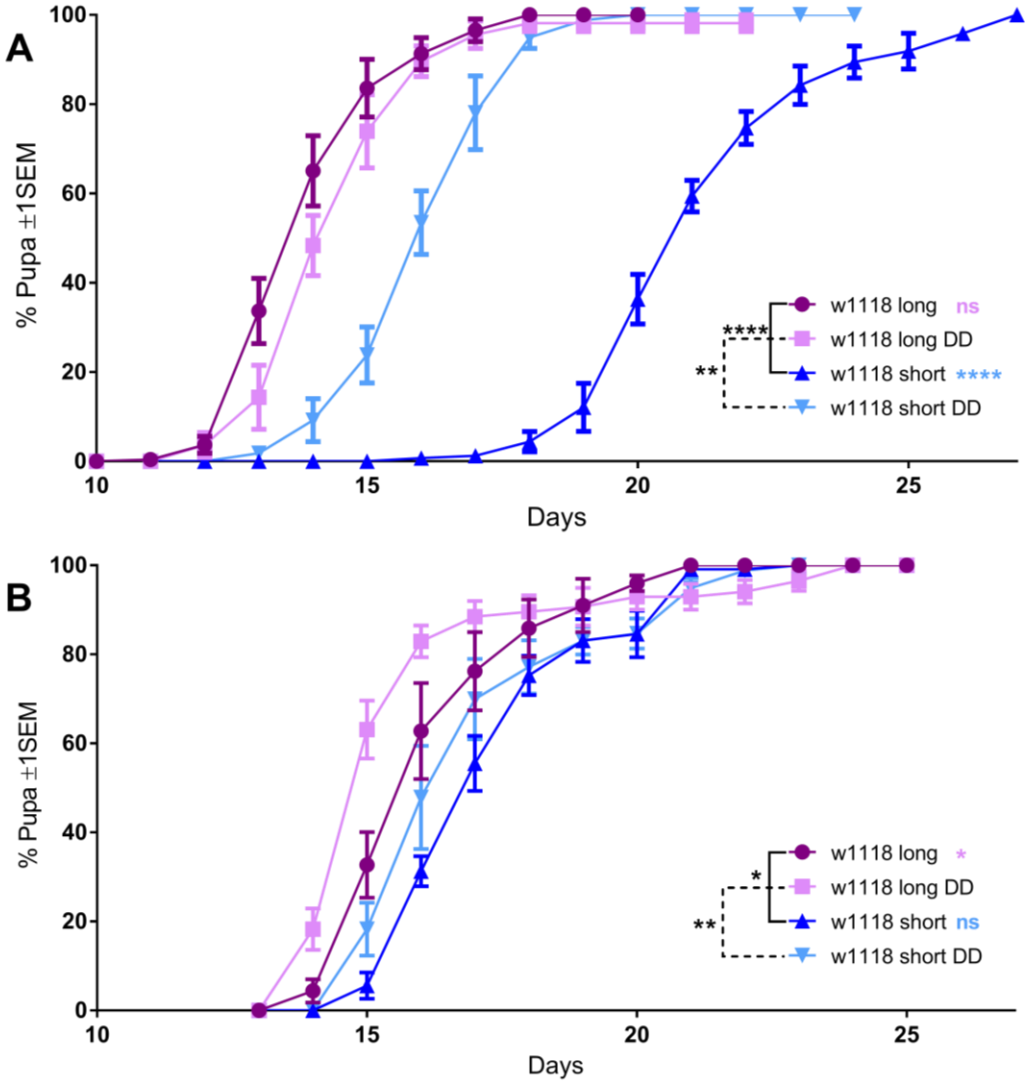


Figure 3-6 Developmental timing at 15 °C of *w*^{1118}. Long (purple) and short (blue) photoperiod are plotted. A) Developmental timing with 0.5 °C temperature cycle inside the incubator. B) Developmental timing "without" the temperature cycle.Dark and light lines correspond to experimental and its corresponding DD controls, respectively. % arriving to pupa-stage ±SEM. Replicates = 10 (A) and 5 (B). ***** ≤ 0.0001; ** ≤ 0.01; * ≤ 0.05; ns = non significant*

3.4.1.2 Diapause

From the previous developmental timing assay, it is clear that the 0.4 °C temperature cycle previously thought to be negligible, may be responsible for significant differences in development. Using *w*^{1118} again, I therefore studied their diapause responses under different light and temperature regimes after growing them at 18 °C.

Two-way ANOVA results (Table 3-2) indicate that depending on the conditions of growth or maintenance, diapause responds differently to photoperiod. Figure 3-7 shows that there are significantly different levels of diapause of w^{1118} raised in a short or long photoperiod (*post hoc,* p<0.0001). This dramatic photoperiodic difference is almost completely abolished if the temperature inside the incubator is maintained at 12 °C (*post hoc,* p=0.57). The flies that were placed under inverse temperature and light cycles also display strong differences amongst photoperiods. However, in this case they have the opposite direction: significantly higher levels of diapause are reported when the flies are placed in a long photoperiod but under short temperature cycle (*post hoc,* p=0.01). These results suggest that low temperature cycles can have considerable impact on photoperiodic diapause, whilst the inversion of photo- and thermo-period reveals the dominant effect of thermoperiod for diapause induction in this strain. Nonetheless, a detailed inspection of the figure shows that flies kept in short photoperiod with long temperature cycle have significantly higher diapause levels than the ones kept on the same thermoperiod but with a long photoperiod (*post hoc,* p<0.0001). This suggests that, although sometimes it might be masked by the temperature, photoperiod still plays a role in diapause induction (See Table 9-1 in the Appendix).

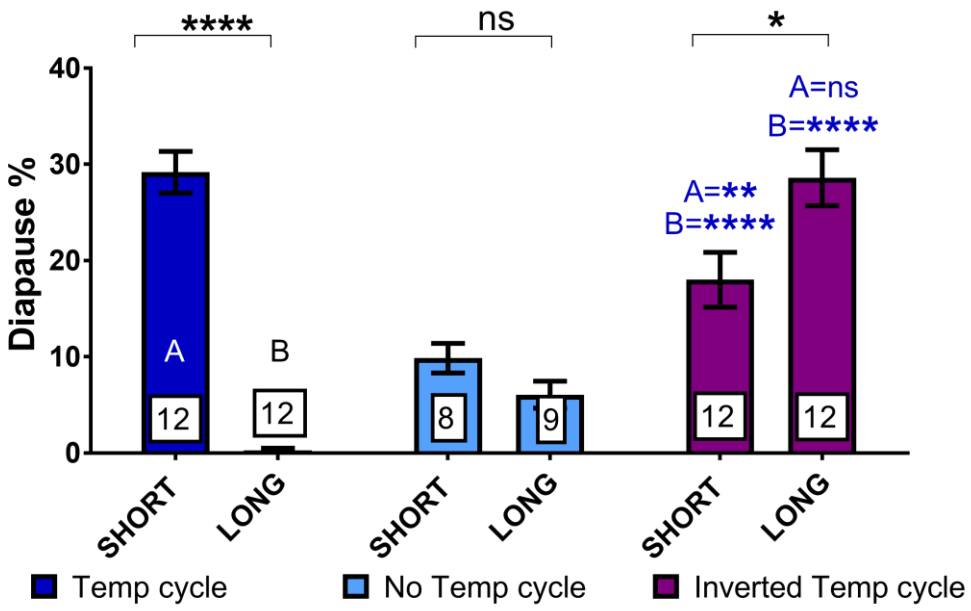


Figure 3-7 Diapause levels of w^{1118} grown at 18 °C in short or long photoperiod from larval stage either with the temperature cycle (dark blue), without it (light blue) or with an inverted temperature-light cycle (purple). The comparison between the

short or long photoperiod with the natural temperature cycle (A or B, respectively) with the inverted temperature-light cycles is represented in dark blue. Mean ± SEM are shown. The numbers inside the squares reflect the number of replicates. *ns = non significant. * ≤ 0.05. **≤ 0.01. ****≤ 0.0001.*

Table 3-2 Summary of Two-way ANOVA results for w^{1118} diapause under different rearing and keeping conditions mentioned in Figure 3-7. Significant components are highlighted in bold.

	Df	Sum Sq	F value	Pr(>F)
Condition	**2**	**0.67**	**33.70**	**<0.0001**
Photoperiod	**1**	**0.49**	**49.81**	**<0.0001**
Condition:Photoperiod	**2**	**1.43**	**71.98**	**<0.0001**
Residuals	80	0.83		

3.4.2 Testing a more realistic protocol to induce diapause

In most diapause experiments with *D. melanogaster*, flies are raised at 25 °C, females are collected 0-5h post eclosion and placed with males at 12 °C for two weeks to one month before dissection to score ovarian development (Schmidt et al., 2005; Tauber et al., 2007). Here I studied the effect on diapause of raising different European isofemale lines at a more realistic temperature of 18 °C or 15 °C under short (8:16 LD) or long (16:8 LD) photoperiod and compare them with the already established protocol of growing the flies at 25 °C. I also looked at the survival levels of the flies to a cold stress and measured several metabolites to corroborate diapause results.

3.4.2.1 Diapause

Table 3-3 summarises the ANOVA results which show significant strain and temperature effects ($F_{2,173}$=4.85, p=0.009 and $F_{2,173}$=24.89, p<0.0001, respectively). Examination of Figure 3-8 shows that when rearing the flies at 18 °C all lines tested display around 40% diapause, resulting in a significant increase in total diapause compared to growing them at any of the other two temperatures (p<0.0001 for 15 °C and p=0.037 for 25 °C). Korpilathi strain shows no significant temperature-of-growth effect whilst rearing Houten or Rende at 15 °C or 25 °C leads to clear (p<0.0001 and p<0.0002) or marginal (p=0.05 and p=0.21) reduction in diapause, respectively. None of the lines show photoperiodic differences ($F_{1,173}$=0.026, p=0.87).

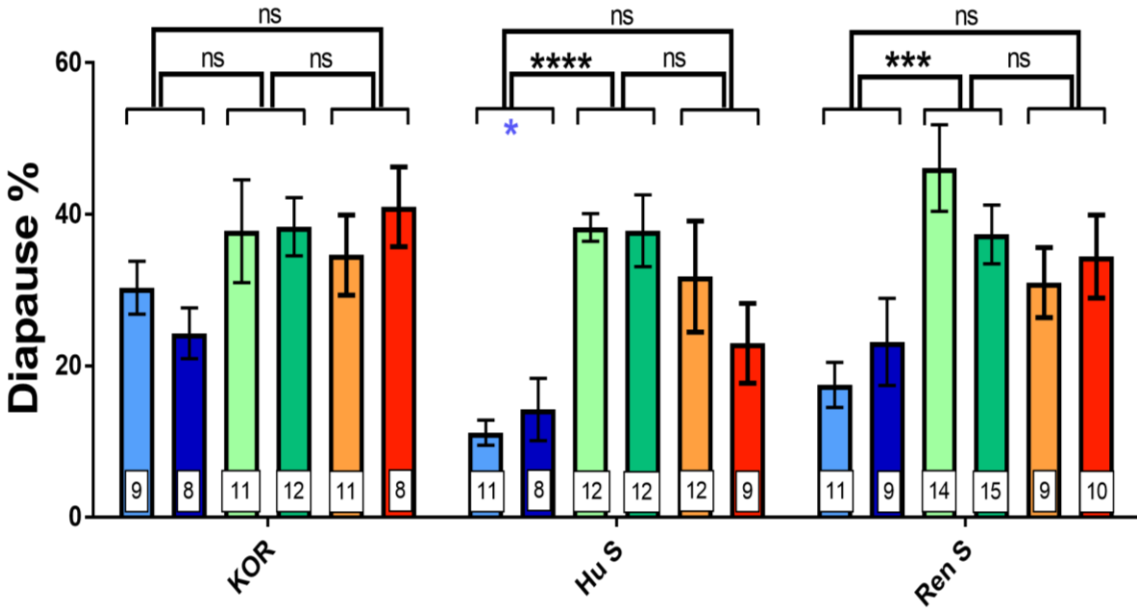


Figure 3-8 Diapause levels of flies raised at 15 °C (blue), 18 °C (green) or 25 °C (red) and short (light colours) or long (dark colours) photoperiod. Mean ± SEM are shown. The number inside the squares reflects the number of replicates. The blue star represents significance levels compared to the Korpilahti population reared at 15 °C. *ns = non significant. * ≤ 0.05. ***≤ 0.001. ****≤ 0.0001.*

Table 3-3 Three-way ANOVA of diapause results of the natural strains represented in Figure 3-8. The significant values are highlighted in bold.

	Df	SumSq	Fvalue	Pr(>F)
Photoperiod	1	0.001	0.03	0.87
Strain	**2**	**0.32**	**4.85**	**0.01**
Temperature	**2**	**1.66**	**24.89**	**<0.0001**
Photoperiod:Strain	2	0.001	0.009	0.99
Photoperiod:Temperature	2	0.02	0.34	0.71
Strain:Temperature	4	0.22	1.65	0.16
Photoperiod:Strain:Temperature	4	0.13	0.96	0.43
Residuals	173	5.76		

3.4.2.2 Chill-Survivorship

For this experiment, the flies were reared at 15 °C, 18 °C or 25 °C in short or long photoperiods and placed at 12 °C after eclosion. Following 12 days in diapause inducing conditions, the survivorship of the flies 24h after an acute stress at -20 °C for 5, 10, 15 or 20 min was assessed separately for females and males. The main components driving the survival to the acute cold exposure were the time of exposure as well as genotype and the temperature in which the flies had been reared, whereas

other factors such as sex had no effect on this phenotype (see Table 9-2 in Appendix). After placing the flies at -20 °C for 5 min most of the flies survive, whereas after 20 min approximately all the flies die. Exposure for 10 or 15 min give intermediate survivorships. Raw results on the survivorship of the different populations as well as the distribution of chill-survival rates after each exposure time can be found in the Appendix (Figure 9-1 to Figure 9-4).

Focusing on the results obtained after a 10 min exposure (which were normally distributed) for females, photoperiod and temperature in which the flies were raised ($F_{1,77}$=4.40, p=0.039 and $F_{2,77}$=43.6, p<0.0001, respectively) have significant effects (Table 3-4). As previously seen in diapause results, rearing the flies at 18 °C significantly increases the chances of survival compared to the other two conditions (p<0.0001 for both 15 and 25 °C) (Figure 3-9_A). Afterwards, the survival to 10 min exposure at -20 °C of each population was plotted against the diapause levels of the strain and condition reported on the previous subchapter 3.4.2.1. Examination of Figure 3-9_B indicate that the two phenomena are positively associated (R^2=0.30, p of the slope being different to 0 being <0.0001)(See Figure 3-9_B).

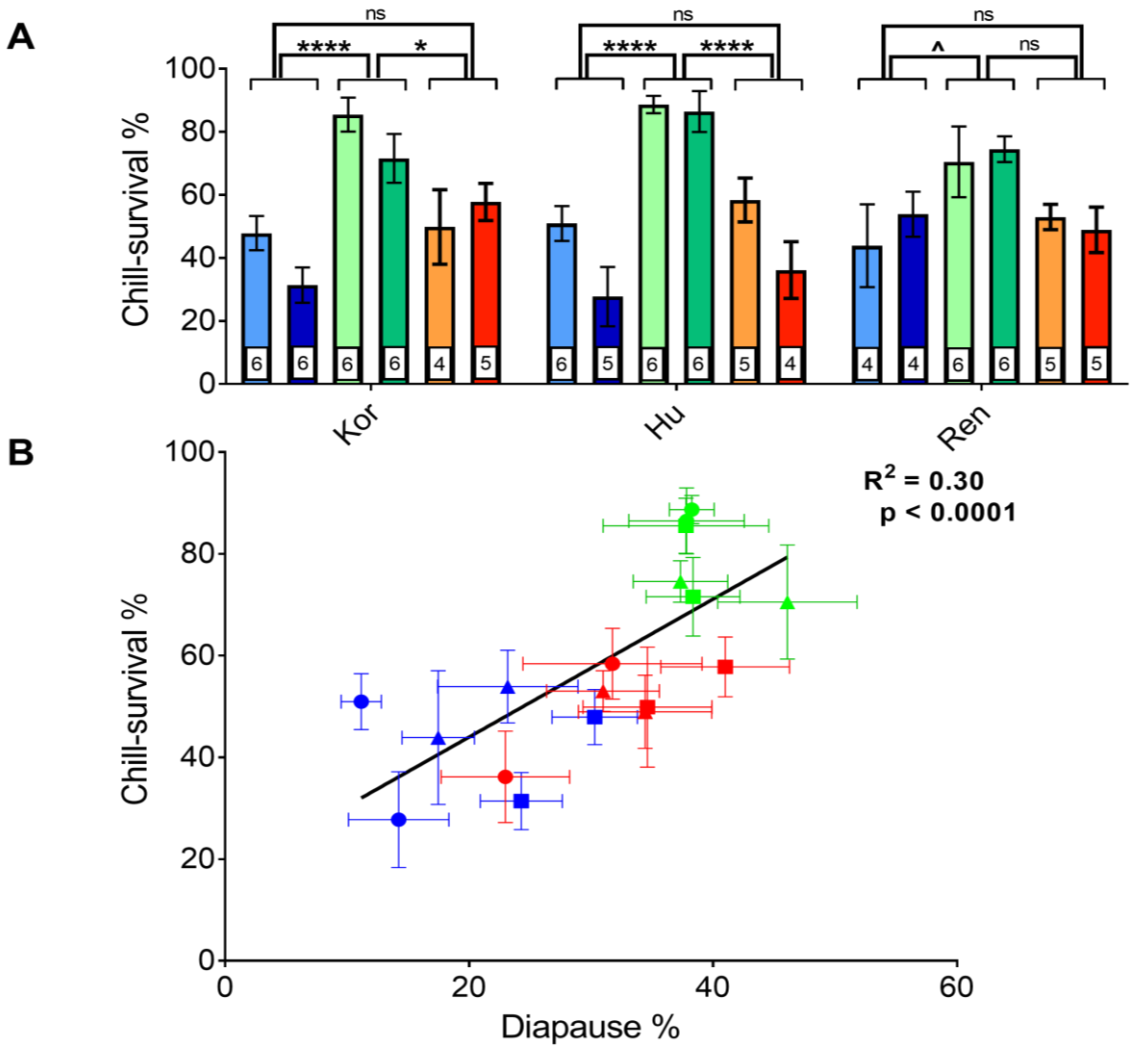


Figure 3-9 Survivorship to -20 °C exposure for 10 minutes of several European strains. A) Raw survivorship results of three natural strains grown at 25 °C (red), 18 °C (green) or 15 °C (blue) and short or long photoperiod (light vs dark colours, after 10 min exposure to -20 °C. Mean ± SEM are shown. The number inside the squares reflects the number of replicate vials. *ns = non-significant. ^=0.06. * ≤ 0.05. ****≤ 0.0001.* B) Correlation between survivorship and diapause. Survivorship levels of natural lines (Rende, triangle; Houten, circle and Korpilahti, square) grown at 25 °C (red), 18 °C (green) or 15 °C (blue) after 10 min exposure to -20 °C plotted against the diapause levels for the corresponding strain under the same conditions. Mean ± SEM are shown. Linear regression between diapause and survival levels is represented in black, with its respective the coefficient of determination (R^2) and p-value to test whether the slope is significantly different from 0.

Table 3-4 Three-way ANOVA of data in Figure 3-9. Survivorship to -20 °C exposure for 10 minutes when raising the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. The significant values are highlighted in bold.

	Df	SumSq	Fvalue	Pr(>F)
Photoperiod	**1**	**0.20**	**4.40**	**0.04**
Strain	2	0	0.003	>0.99
Temperature	**2**	**3.89**	**43.60**	**<0.0001**
Photoperiod:Strain	2	0.13	1.42	0.25
Photoperiod:Temperature	2	0.02	0.27	0.77
Strain:Temperature	4	0.31	1.75	0.15
Photoperiod:Strain:Temperature	4	0.30	1.68	0.16
Residuals	77	3.43		

3.4.2.3 Cryoprotective agents

The levels of glucose, glycogen and trehalose in all the different conditions were also determined. Most of them vary according to sex, but I will focus in the females' results (Appendix Table 9-3 to Table 9-7).

Several of the metabolites measured after growing the flies at 15 or 25 °C correlate with the diapause levels reported in section 3.4.2.1 for the strain and condition (at these temperatures diapause levels were the most variable). Since total weight correlates positively with diapause (Figure 3-10_A R^2=0.17, p=0.01), the different metabolites are normalised against weight. Correlation between diapause and protein is lost after the normalisation (Figure 3-10_B R^2=0.01, p=0.5), supporting the effectivity of this method. Hence, glucose, glycogen and trehalose were adjusted against total weight prior to performing the correlations. As expected, both glucose and trehalose show a positive association with diapause whereas no correlation was found between glycogen and diapause (Figure 3-10_C-E R^2=0.29, p=0.0006; R^2=0.30, p=0.0005 and R^2=0.005, p=0.68, respectively). Nevertheless, the ratio between them might be of greater interest than the total amounts of the different saccharides, since it gives an overall idea of the metabolic state of the flies. As diapause levels increase so do the trehalose:glucose and trehalose:glycogen (Figure 3-10_F-G R^2=0.43, p<0.0001 and R^2=0.19, p=0.0064) ratios while glycogen:glucose levels have a negative association with diapause (Figure 3-10_H R^2=0.18, p=0.009).

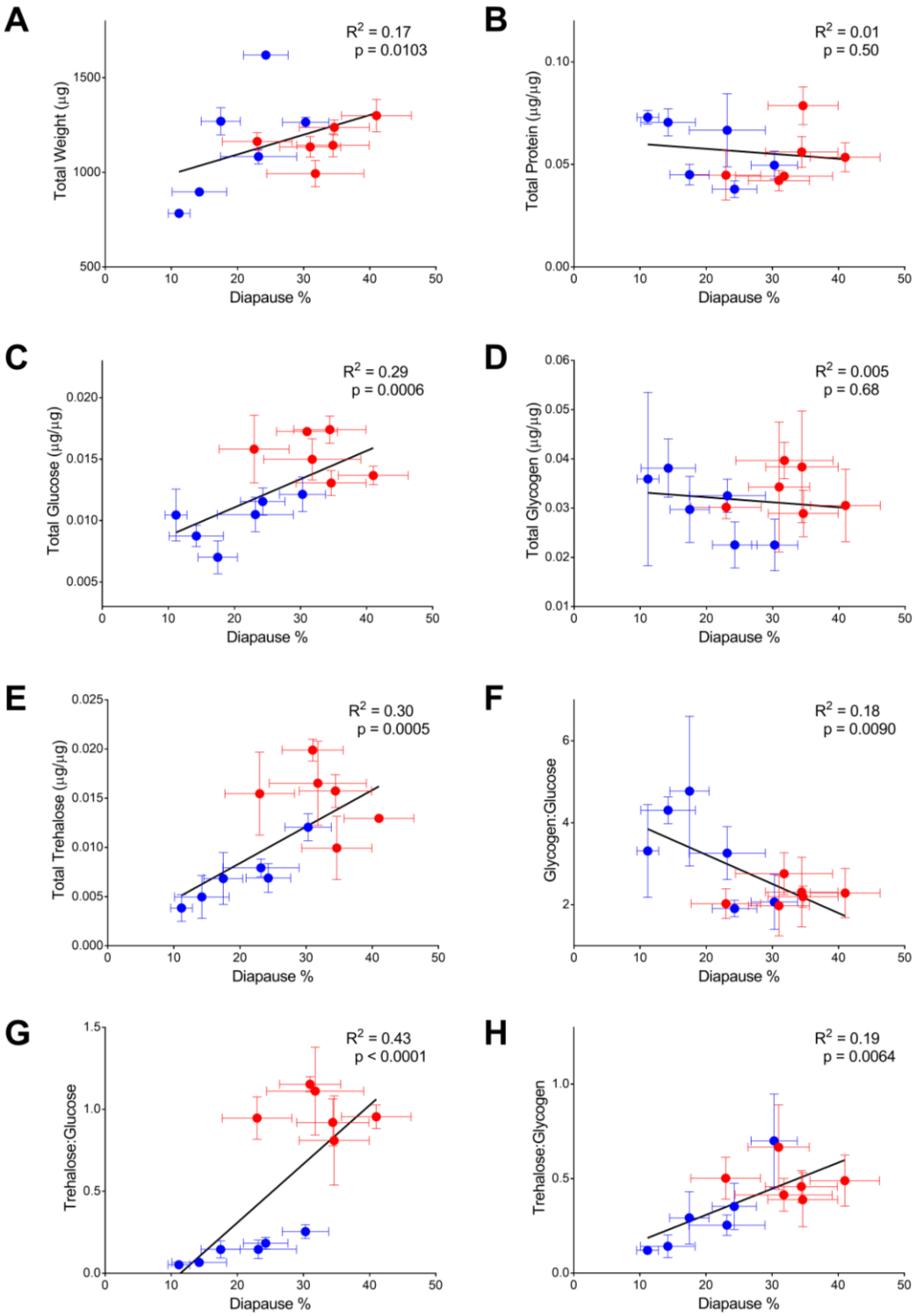


Figure 3-10 Different metabolite levels of the natural strains grown at 15 °C (blue) or 25 °C (red). Total weight plotted against diapause levels (A). Total protein (B), glucose (C), glycogen (D) and trehalose (E) were normalised against weight. Correlation between diapause and the ratios those carbohydrates are plotted in F (Glycogen:Glucose), G (Trehalose:Glucose) and H (Trehalose:Glycogen). Linear regression between diapause and the metabolite is represented in black, with its respective the coefficient of determination (R^2) and p-value to test whether the slope is significantly different from 0. Mean ± SEM.

3.5 Discussion

As seen with both w^{1118} and the natural isolates, the conditions in which the flies are reared have a significant effect on the adult phenotypes and their ability to cope with different stresses.

The difference of over 7 days in development time between raising flies at 15 °C in short versus long photoperiod is reduced when the temperature inside the incubator is stabilised (Figure 3-6). The DD controls are still slightly different between the long and short photoperiod conditions so the balancing of temperature within the incubator is not perfect. Faster development was observed when the flies were kept in the long photoperiod (for both the experimental and the DD controls) suggesting some temperature fluctuation inside the incubator that is responsible for the developmental differences. Flies are ectothermic and their growth rate is known to be affected by the temperature (Angilletta et al., 2004). Shortest growth rate is achieved at 28 °C, where it just takes 7 days to reach adulthood, and it becomes longer as the temperature decreases (10 days at 25 °C and 20 days at 18 °C) (Ashburner et al., 2004). However, it is surprising that such enormous differences in developmental time can be caused by a minimal (0.4 °C) temperature variation. Perhaps the time required to reach maturity increases non-linearly as the temperature deviates from 28 °C (AlSaffar et al., 1995). These results suggest that the initial effect on developmental timing between long and short photoperiods when exposed to light is determined solely, or at least mostly, by temperature differences.

Diapause of w^{1118} revealed that small temperature changes of less than 0.4 °C inside the incubator can lead to major effects (See Figure 3-7). When scoring diapause after keeping the flies in diapause-inducing conditions, moderate levels of diapause (~30%) were found in the vials that had been kept in short photoperiods against nearly zero levels in the long photoperiod. When this minimal temperature cycle inside the incubator was removed, these differences were almost completely abolished. As *white* gene encodes for an ABP transporter (Borycz et al., 2008), w^{1118} flies have metabolic differences that could lead to this effect. However, several natural lines from different parts of Europe have been tested, none of which have shown any photoperiodic effect

under the stabilised temperature for diapause (Figure 3-8). Furthermore, an inversion of temperature and photoperiod revealed that the levels of diapause reversed, with significantly higher levels of diapause found under long photoperiods (~30%) as compared to the short photoperiod (~20%). This suggests a major role for the low-amplitude temperature cycle in diapause induction in comparison with the photoperiod.

There is some debate in the field about whether *D. melanogaster* diapause is photoperiodic or not (Emerson et al., 2009a). I show that minimal temperature oscillations could account for the discrepancies between laboratories that do or do not find photoperiodic differences in diapause in this species (Emerson et al., 2009a; Saunders et al., 1989; Tauber et al., 2007). Clearly, there is a difference in outcome between stabilising the temperature in the incubator between long and short photoperiods compared to covering flies to create corresponding DD conditions, which is an otherwise reasonable thermal control (Pegoraro et al., 2017; Tauber et al., 2007; Zonato et al., 2017). It is possible that a "greenhouse" effect occurs when flies in transparent plastic/glass vials are exposed to incubators lights, which will not exist when the vials are wrapped in aluminium foil. , Nevertheless, the fact that the flies are still able to enter diapause in the short photoperiod with long temperature cycle (contrary to the previous results where the flies kept in long photoperiod did not diapause, see Figure 3-7), suggests that photoperiod plays some marginal role as it does in chill coma studies (Pegoraro et al., 2014). The interaction between temperature and photoperiod might be the real key for triggering the response, even if temperature cycle is the main driver. It is important to note that temperature fluctuations follow the light in natural conditions so it is not unusual to find that both temperature and photoperiod need to act in synchrony (Pegoraro et al., 2014).

Contrary to what was expected, diapause levels of Rende and Houten lines significantly fall when they are reared at 15 °C. This could be due to the temperature step down from 15 to 12 °C not being large enough to induce diapause. Nevertheless, the Korpilahti strain maintains high diapause levels (Figure 3-8). The daily average temperatures are extremely different depending on latitude. Korpilahti originates from Finland (latitude 60°), where the average temperatures throughout the year are 5-10°C

(Wilczek et al., 2010), which could explain the higher diapause induction in the strain compared to the more southern populations. Might Korpilahti produce similar diapause levels if the diapause-inducing temperature is reduced to 9 °C, preserving the 6 °C fall from 15 °C that is also generated from a developmental temperature of 18 °C falling subsequently to 12 °C? Is absolute fall in temperature the key here?

Acclimation to colder temperatures is known to improve cold-tolerance (Rako and Hoffmann, 2006), so the unexpected fall in diapause levels when Rende and Houten flies were raised at 15 °C could be explained by an association between diapause and cold-hardening (Kostal et al., 2012) (See Figure 3-8). The survivorship after a strong and acute cold stress was measured to assess cold-tolerance of the lines. Rearing the flies at the different temperatures has a dramatic effect in that lower temperature (15 °C) has a prejudicial effect on their survival, so they seem to be less cold-adapted. On the contrary, growing the flies at 18 °C seems to improve the chances of survival to the cold stress in all tested lines. Korpilahti shows photoperiodic difference in survivorship when raised at 15 °C. When grown in a long (summer) photoperiod it presents a significantly lower survivorship rate after stress. However, there is no difference between rearing Korpilahti at 15 °C in a short photoperiod or at 18 °C at any of the photoperiods (See Figure 3-9).

All three strains reared at these colder temperatures (15 °C) are disadvantaged in terms of survival to the particular cold stress tested in this chapter, which appears rather paradoxical but has been reported before. In fact, similar experiments in which the Mediterranean flour moth (*Anagasta kühmiella*) was exposed to -15 °C found that acclimation to colder temperatures improved the chances of survival but just up to a limit - acclimation to less than 5 °C resulted in the moth performing poorly (Salt, 1961). In my case, something similar might be happening. Survival to the -20 °C exposure after growing the flies at 25 °C resulted in survival levels that were similar or nearly equivalent to when the flies were reared at 15 °C (Figure 3-9). Rearing the flies at 18 °C had a positive effect on the survival levels. Even if both are measuring chances of survival against cold-adverse conditions, the mechanisms of induction of diapause and cold-tolerance are very different so they do not necessarily need to correlate (Sinclair and Roberts, 2005). For example, in previous work on *Drosophila* cold-tolerance

focused on chill-comma recovery time (CCRt), Pegoraro et al. (2014) suggested that these two phenomena could be triggered by different environmental cues. However, Higuchi and Kimura (1985) reported that diapausing adult *D. triauraria* survive cold shock better than non-diapausing flies. More recently, Vesala and Hoikkala (2011) showed in *D. montana* that diapause can increase cold tolerance in the female. Additionally in my case, the fall in survivorship to -20 °C is correlated with a decreased ability to enter diapause (even if the photoperiodic difference of Korpilahti at 15 °C is not as clear) (Figure 3-9).

Another plausible explanation for the fall of diapause at 15 °C is that, as they develop more slowly, larvae are storing more reserves and cryoprotective agents, hence increasing the flies' resistance to cold and preventing them from entering diapause at 12 °C. There is evidence of a connection between rearing temperature, growth rate and body size (Angilletta et al., 2004). My data corroborate this result, as weight of the fly seems to increase in parallel to its ability to enter diapause (Figure 3-10_A). Carbohydrate homeostasis has been shown to be especially important for diapause and its maintenance (Hahn and Denlinger, 2011; Williams et al., 2006). In this report, I focused my attention in three carbohydrates that have been related to diapause in several insects: glucose, glycogen and trehalose (Colinet et al., 2012; Kubrak et al., 2014; Overgaard et al., 2007). Accumulation of stored nutrients is vital for animals undergoing developmental diapause. During the preparatory phase they acquire the nutrients that will be consumed while in diapause (Kubrak et al., 2014). Due to the large number of conditions and lines examined, low numbers of biological replicates were performed, which is not ideal for picking modest differences in the levels of metabolites (Kubrak et al., 2014). As a result, very little variation in overall levels of the chosen metabolites was observed. However, I found a strong correlation between some of them and diapause levels. I observed positive correlations between diapause and total trehalose and glucose, but none with total glycogen. However, our results do not differentiate between stored and circulating levels, which might explain the lack of correlation for this last carbohydrate. The Glycogen:Glucose ratio had a negative correlation with diapause levels, hinting that glycogen is broken down to produce glucose and/or trehalose. This is in line with observations in diapausing flies in

which they halve their levels of glycogen compared to control flies (Kubrak et al., 2014). On the other hand, both Trehalose:Glucose and Trehalose:Glycogen ratios correlate directly with the diapause levels (Watanabe et al., 2002). So overall, in diapause all metabolism is shifted towards the generation of trehalose and mobilization of the stored carbohydrates.

The levels of carbohydrates and of their ratios correlate with the proportion of flies that enter diapause. However, not many differences between the growing conditions in the raw levels were observed. For this purpose, it might have been better to measure the levels of each carbohydrate just after eclosion, since after keeping them under the same diapause inducing conditions for 12 days any treatment differences might have faded. It could be useful to see the "starting" metabolic situation to compare with the final point after 12 days at 12 °C. This would allow to discern if growing the flies at different conditions has an effect on the storage strategy of the adult fly, which could explain phenotypical differences on the adult as suggested by Shreve et al. (2004). The fact that no striking differences in metabolites between the rearing conditions were found does not mean there are not any. I focused on some of the most common diapause-related carbohydrates but there are many other metabolites of interest that I could have investigated. For example, triglycerides are known to correlate with cold-tolerance and diapause as do free amino acids and many other metabolites (Arrese and Soulages, 2010; Rako and Hoffmann, 2006).

Different laboratories studying diapause in *D. melanogaster* measure diapause after maintaining flies for 12 or 28 days under diapause-promoting conditions (Emerson et al., 2009a; Saunders et al., 1989; Schmidt and Paaby, 2008; Tauber et al., 2007). This raises the question that at constant 12 °C there may not be a real diapause after 12 days, but simply a developmental slowdown, which after 28 days, has largely run its course. However, the chill shock analysis revealed a correlation between diapause at 12 days and survival, which would be expected if the flies were in a "real" diapause and, consequently, more resistant to stress. The metabolic analysis also suggests that after 12 days flies are showing profiles that resemble those for diapause, so it would seem that maintaining flies at 12 °C for 12 days is not simply reflecting reproductive quiescence but a more dynamic phenomenon. Indeed, Zonato et al.

(2016), demonstrated that when more natural conditions were employed to mimic the approach of winter by weekly reducing temperature and photoperiod , diapause levels were maintained for many weeks (Rosato et al., 1997; Sandrelli et al., 2007; Tauber et al., 2007). These results suggested that keeping flies at constant 12 °C, and hence, without any feedback from falling temperatures/reducing photoperiod, females may switch back to a reproductive state after 28 days. In addition, genetic ablation of the Drosophila insulin-like peptides (dILPs) has been shown to mediate ovarian diapause even when the flies were kept at temperatures as high as 19 °C, clearly implicating a dynamic hormonally mediated process underlying the phenotype (Schiesari et al., 2016).

Overall, this chapter suggests that:

- Temperature seems to be the main cue for developmental speed as well as for diapause induction in *D. melanogaster,* although photoperiod might still play a marginal role in the latter. In line with this, a collaborator has found that when using a more natural light profile, strains that were not photoperiodic using a rectangular light profile had strong photoperiodic diapause (See Appendix Figure 9-5 from Nagy et al. (2018)).
- Conditions in which the flies are reared have a major effect on the adult in all the observed phenotypes. Indeed, raising the flies at 18 °C leads to higher diapause induction that is correlated with increased survival to an acute stress compared to the other two temperatures. However, it should be borne in mind that “correlation does not imply causation”.
- Correlation between chill shock and several of the metabolites with diapause suggests that we are seeing a diapause rather than a simple reproductive quiescence.

4. CANONICAL CLOCK MUTANTS AND DIAPAUSE

4.1 Introduction

4.1.1 Drosophila clock components

In *D. melanogaster*, the circadian clock consists of interlocked transcription/translation feedback loops. *Clk/cyc* are the positive components whereas *per/tim* are the negative ones.

4.1.1.1 Negative components: period (*per*) and timeless (*tim*)

period was the first clock gene to be discovered by Konopka and Benzer (1971) and is located on the X chromosome. Using chemical mutagenesis, they identified three mutants with alterations in free-running rhythms: per^s (≈19 h); per^l (≈28h) and per^0, which was arrhythmic. It took many years before the second clock component, *timeless*, was identified (Sehgal et al. 1994, Myers et al. 1995). This gene is located on the second chromosome and the initial *tim* mutant was arrhythmic in DD. In tim^0, *per* mRNA rhythms are disrupted (Sehgal et al. 1994) and after *tim* was identified it was observed that *tim* mRNA rhythms are also disrupted in per^0 mutants (Price et al. 1995). Consequently, the *per* and *tim* genes require each other for mRNA cycling, suggesting that they are partners. Both their mRNAs accumulate through the day with a peak around dusk, whereas their corresponding proteins peaks late at night (Ozkaya and Rosato 2012). These observations whereby the mRNA and proteins are out-of-phase by ~6 h suggested a negative feedback loop, in which PER/TIM accumulation resulted in a reduction of their own transcription (Hardin, Hall and Rosbash 1990, Hardin 2011).

Furthermore, Zeng et al. (1996) found that TIM was light-sensitive, which explains the lower levels of TIM during the day. Moreover, in *tim*^{0} mutants, PER is retained in the cytoplasm: it does not accumulate and cannot be translocated to the nucleus (Sehgal et al. 1995, Shafer, Rosbash and Truman 2002). This implied that TIM plays a role in PER stability (Vosshall et al. 1994) and that these two proteins need to heterodimerize to be translocated to the nucleus, where they repress their own transcription. Nevertheless, none of these two proteins have any DNA binding domain highlighting the implication of some additional players (Huang, Edery and Rosbash 1993).

4.1.1.2 Positive loop: Clock (*Clk*) and cycle (*cyc*)

As opposed to the negative regulators of the *Drosophila* clock, the positive components (CLOCK and BMAL1) were first discovered in mouse (King et al. 1997, Vitaterna et al. 1994). *Clock* and *cycle* (homologous to mouse *Bmal1*) belong to the basic helix-loop-helix (bHLH)/PAS transcription factors family (Allada et al. 1998). They form heterodimers that bind to E-box elements on promoter sequences of several genes and activate their expression. This way, they control the expression of a wide range of proteins, among them *per* and *tim* which ultimately inhibit CLK/CYC (Allada et al. 1998, Rutila et al. 1998, Kyriacou and Rosato 2000, Hardin 2005, Ozkaya and Rosato 2012). Furthermore, in *Drosophila*, *Clk* and *cyc* mutants are arrhythmic in DD and fail to activate *per* and *tim* transcription (Allada et al. 1998, Rutila et al. 1998).

CYC levels are maintained through the day (Rutila et al. 1998), while CLK peaks in the morning (ZT23-ZT4) (Allada et al. 1998). The molecular mechanism behind this rhythmic expression of *Clk* transcript involves VRILLE and PDP1ε which compete for the same regulatory elements (V/P boxes) in the *Clk* promoter and have opposing roles. *vri* encodes a basic leucine zipper (bZIP) transcription factor which represses *Clk* transcription (Glossop et al. 2003) while *PAR domain protein 1* activates *Clk* (Cyran et al. 2003). *vri/Pdp1e* expression is activated by the CLK/CYC heterodimer, with a faster accumulation of VRI which peaks at ZT15 as opposed to PDP1ε at ZT18 (Cyran et al. 2003, Hardin 2011). As *vri* is involved in development, its mutants are lethal as homozygotes. However, *vri* heterozygotes display a shortened DD rhythm (Blau and Young 1999). On the other hand, *PDP1ε* is thought to be involved in the output

signalling pathway of the clock as its mutation results in behavioural deficits while it does not affect expression levels or patterns of output mRNAs such as *takeout* (Benito, Zheng and Hardin 2007).

Another transcriptional regulator of *Clk* was also identified: *clockwork orange* (*cwo*) repress CLK/CYC activity and acts in a similar way as the PER/TIM heterodimer (Kadener et al. 2007, Matsumoto et al. 2007, Richier et al. 2008). This is considered to be the third negative feedback loop of the circadian clock in *D. melanogaster*.

4.1.1.3 Others: Cryptochrome (*cry*) and Pigment dispersing factor (*Pdf*)

CRYPTOCHROME (CRY) is a blue-light photoreceptor and the main circadian photoreceptor in *Drosophila cry* is expressed cyclically, with a peak around ZT1-5 (Hao et al. 2008). During the day, CRY is activated and mediates TIM degradation by the ubiquitin-proteasome pathway with JETLAG (JET) (Ceriani et al. 1999, Koh, Zheng and Sehgal 2006). Hence, it prevents the PER/TIM dimer from being formed and translocated into the nucleus. JET binds to CRY and promotes its degradation. However, it does this with a weaker affinity than to TIM, which results on the generation of this dimers only during the day, when TIM levels are low (Peschel et al. 2009). cry^b mutants fail to respond to a light pulse but are still entrainable to the light-dark regime and maintain rhythmicity in constant conditions, even in constant light, LL, that render WT flies arrhythmic (Stanewsky et al. 1998, Stoleru et al. 2007).

PIGMENT DISPERSING FACTOR (PDF) is the main output molecule from the clock and it is indispensable for the synchronization of different pacemaker cells. Indeed, Pdf^0 lines display abnormal clock-controlled behaviours: most flies were arrhythmic in DD and the few that remained rhythmic had a shortened period (Renn et al. 1999). Its transcription is initiated indirectly by CLK/CYC and mutations in genes regulating CLK/CYC such as *vri*, *per* and *tim* alter the final protein levels without altering *Pdf* transcription (Blau and Young 1999, Park et al. 2000, Rosato, Tauber and Kyriacou 2006).

4.1.2 Temperature and the clock

One of the main properties of the clock is the "temperature compensation". However, temperature can also act as a zeitgeber and flies can entrain to daily temperature cycles when kept in total darkness (Roessingh, Wolfgang and Stanewsky 2015, Kidd, Young and Siggia 2015, Roessingh and Stanewsky 2017). Indeed, Yoshii et al. (2002) found that temperature cycles can even restore rhythmicity in *per*, *tim*, *Clk* and *cyc* mutants. Majercak et al. (1999) kept WT flies at 18 °C, 25 °C and 29 °C in for four days in LD 12:12 followed by several days in DD and found differences among temperature conditions. During the LD cycles, they describe an increased activity during the day when the flies were kept in the lower temperature while the flies in the two higher temperatures displayed an increased siesta (inactivity) in the middle of the day. Several differences were also found in DD: in higher temperatures the vast majority of the flies kept a bimodal activity with the same peak of activity as in LD, while at 18 °C they had a single peak of activity reaching a steady-phase 4h before than the peak of activity in LD by the third day in DD (Figure 4-1).

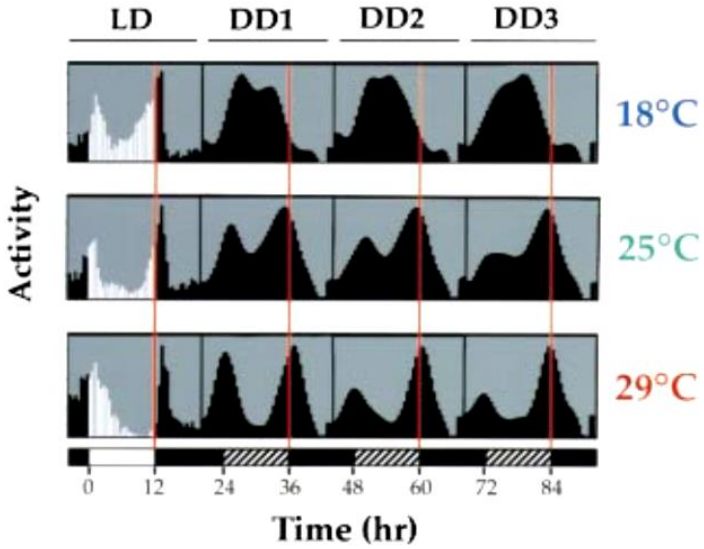


Figure 4-1 Effects of temperature in the locomotor behaviour of *D. melanogaster*. From Majercak et al. (1999).

4.1.3 Circadian vs Seasonal clock

Being able to anticipate and respond to a changing environment on a tilted, rotating planet is advantageous for fitness, and this has resulted in the evolution of a seasonal as well as a circadian rhythm. The four main differences between these two types of cycles are summarised in Table 4-1. Erwin Bünning (1936) initially suggested a role for the circadian system in seasonal photoperiodic measurement but how or whether these two processes cooperate or are independent from each other has generated considerable debate (Emerson, Bradshaw and Holzapfel 2009).

Table 4-1 Main differences between the circadian and the seasonal clock.

	Circadian clock	Seasonal clock
Period of oscillation	24h	12 months
Free Run	No need for environmental cues to maintain rhythmicity	Needs appropriate environmental cues
Temperature compensation	Period stays constant in different temperatures	Strong dependence on temperature of seasonal output
Molecular components and Mechanism	Much known (read above)	Not much known

4.1.4 The clock and diapause

Saunders (1990) initially excluded the possibility that core clock genes were involved in photoperiodicity after examining the classic *per* mutants for their diapause response. However, there were some caveats to his study, including the fact that Saunders had not taken into account that per^L and per^s mutants have more similar periods at colder temperatures (Matsumoto et al. 1998). However, *tim* haplotypes have been shown to modify the occurrence of diapause. A new natural variant, *ls-tim*, is under natural selection in Europe because it enhances diapause in temperate and seasonal environments (Tauber et al. 2007, Sandrelli et al. 2008). In addition, two SNPs in *couch potato* (*cpo*) (Ala/Val347- SNP and 48034[A/T]) have been reported to correlate with diapause clines in USA (Schmidt et al. 2008, Cogni et al. 2014). In Europe, only a cline in Ala/Val347-SNP has been described (Zonato, Fedele and Kyriacou 2016), while in Australia no correlation between diapause clines and *cpo* haplotypes has been found (Lee et al. 2011). Similarly, the circadian clock genes *tim*

and *cry* have been implicated in diapause induction in *D. triauraria* (Yamada and Yamamoto 2011). In a different northern fly species (*D. montana*) the possibility of their photoperiodic diapause response being partly based on the circadian oscillator has also been suggested (Kauranen, Tyukmaeva and Hoikkala 2013).

In some species, such as *Riptortus pedestris*, the involvement of *per*, *cry2* and *cyc* in the initial phases of diapause induction has been demonstrated (Ikeno et al. 2010, Ikeno, Numata and Goto 2011a, Ikeno, Numata and Goto 2011b, Ikeno et al. 2014). Nonetheless, in *D. melanogaster* it is still unknown how the *tim* variants affect diapause levels as they do not affect the photoperiodic response (Tauber et al. 2007). Is it a direct control of diapause by the circadian gene or is it due to indirect pleiotropic effects, as the circadian clock controls daily timing at many different levels in the organisms. Thus, it is difficult to find any physiological function that is not affected in some way by the clock (Emerson et al. 2009, Bradshaw and Holzapfel 2010).

4.2 Aims

As seen in Chapter 3, the conditions in which the flies are reared alter the adult's ability to undergo diapause. A previous PhD student in the laboratory, Joao Gesto, had performed a systematic study of the effect of different clock mutants on diapause in the HU genetic background (Chapter 3) following the established protocol of growing the flies at 25 °C. It therefore seemed reasonable to see if these clock mutants behaved in the same way following my protocol of growing them at 18 °C.

4.3 Methods

4.3.1 *Drosophila* Stocks

In this chapter, I used several canonical clock mutants that were backcrossed for eight generations into the natural Houten *s-tim* (Holland) genetic background by Gesto (2011) (See Table 4-2 for a complete list on the mutants).

Table 4-2 List of canonical clock mutants used in the chapter. The generation of the mutant (most of them by chemical mutagenesis, EMS) as well as the nature of the mutation are reported.

Mutant	Origin of the mutation	Mutation	References
per^{01}	EMS	Nucleotide substitution resulting in a STOP codon (Q464STOP)	(Konopka and Benzer 1971, Yu et al. 1987)
tim^{01}	P-element mutagenesis	64bp deletion that results in a frame shift	(Sehgal et al. 1994, Myers et al. 1995)
Clk^{Jrk}	EMS	Nucleotide substitution resulting in a STOP codon (Q776STOP)	(Allada et al. 1998)
cyc^{01}	EMS	Nucleotide substitution resulting in a STOP codon (K159STOP)	(Rutila et al. 1998)
cry^{b}	EMS	Nucleotide substitution resulting in amino acid replacement (D410N)	(Stanewsky et al. 1998)
Pdf^{01}	EMS	Nucleotide substitution resulting in a STOP codon (Y21STOP)	(Renn et al. 1999)

4.3.2 Scoring diapause

Flies were reared at 18 °C or 25 °C under short (LD 8:16) photoperiod until the flies hatched, after which they were placed at 12 °C for 12 days, maintaining the same photoperiod. After this time, females were dissected to score ovarian diapause as described in Material and Methods 2.2.2. 10 replicas per genotype and condition were performed, with the exception of *per^{01}; tim^{01}* grown at 18 °C which has 5 replicas.

4.3.3 Locomotor activity

Flies were reared at 18 or 25 °C in LD 12:12. 1-2 day-old males were put inside the Trikinetics activity system. Flies were kept at either 18 or 25 °C for 5 days in a LD 12-12 regime followed by 7 days in DD conditions (See Figure 4-2. Only the last three days of LD were used to analyse the LD behaviour to avoid confounding effects due to the temperature changes. Similarly, the first day in DD was skipped from the DD analysis. Between 16 and 32 individual males were analysed per genotype in condition as described in Material and Methods 2.3.

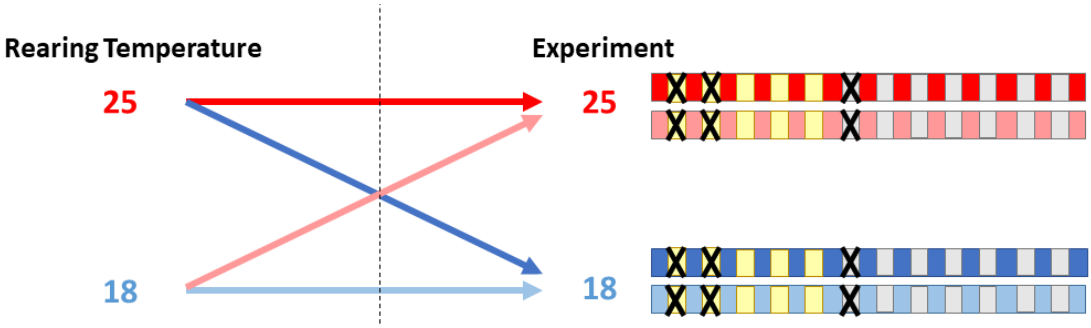


Figure 4-2 Locomotor activity experimental set-up. The different clock mutants were reared at 25 °C (darker) or 18 °C (lighter) and 1-2 days old males were placed either at 25 °C (red) or 18 °C (blue) for five days in LD (lights-on represented by the yellow rectangles) and seven days in DD (subjective day represented in grey). The first two days of LD and first one of DD were not used for the analysis (black crosses).

4.4 Results

4.4.1 Diapause

4.4.1.1 Diapause levels of the different canonical mutants differ according to rearing temperature

Inspection of Figure 4-3 reveals that the temperature in which the flies are reared has a significant effect on the ability of several of the lines to undergo diapause. ANOVA reports a clear Genotype and Temperature effect ($p<0.0001$ and $p=0.003$, respectively), but also a significant Genotype:Temperature interaction ($p<0.0001$) (See Table 4-3). When the flies are reared following the standard protocol (25 °C) the results are consistent with those reported by Gesto (2011) in the *ls-tim* background (See Figure 9-6 in the Appendix): Clk^{Jrk}, cyc^{0} and per^{0} mutants show a significant reduction of diapause levels ($p=0.0001$, 0.0001 and 0.0002), whilst cry^{b} flies have significantly higher diapause compared to wild-type ($p=0.0001$). Surprisingly, diapause levels under the lower rearing temperature are quite different. Only cry^{b} seems to be indifferent to rearing temperature with higher levels than wild type ($p=0.0001$) and increased diapause compared to when the mutants were developed at 25 °C ($p=0.024$). On the other hand, Pdf^{0} flies show a significant reduction of diapause under colder rearing ($p=0.0012$, Figure 4-3) in contrast to *Clk* and *cyc* which have the opposite effect ($p=0.0001$ and $p<0.0001$).

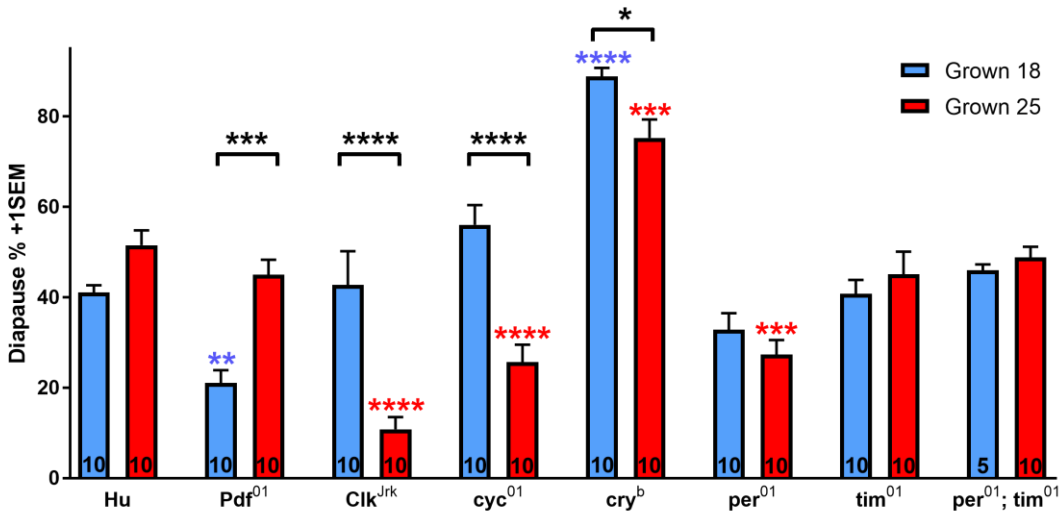


Figure 4-3 Diapause level of clock mutants reared at 18 °C or 25 °C. Blue and red asterisks represent significant differences compared to Hu control. Black asterisks represent differences between temperatures. The numbers inside the columns represent replicas. Means + SEM. ** p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.*

Table 4-3 ANOVA results of data presented in Figure 4-3.

	Df	SumSq	F value	Pr(>F)
Genotype	7	17858	43.67	<0.0001
RearingT	1	518.4	8.873	0.003
Genotype:RearingT	7	5710	13.96	<0.0001
Residuals	139	8121		

4.4.1.2 Hu *s-* and *ls-tim* backgrounds show similar diapause trends

When the mutants are reared at 25 °C and placed at 12 °C for 12 days (using the traditional diapause-induction protocol), ANOVA reports a strong genotype (p<0.0001) and a significant *s-/ls-tim* (p=0.01) effect (Table 4-4), which is most dramatic for Pdf^{0} (p=0.02). Comparison of diapause levels of the clock mutants in Houten *s-* and *ls-tim* background show similar trends in both genetic backgrounds compared to Hu controls. Both Clk^{Jrk} (p<0.0001) and per^{0} (p=0.002 and p<0.0001) have significantly reduced diapause compared to their respective controls. The only exception happens with cry^{b} mutant, that has significantly increased diapause level in *s-tim* (p=0.001), matching the levels of the same mutant in the *ls-tim* background (Figure 4-4).

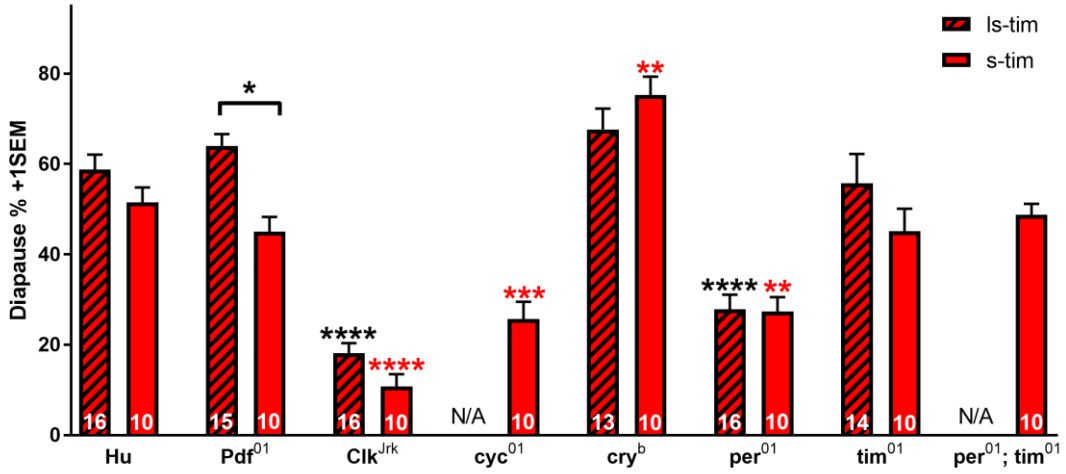


Figure 4-4 Differences on diapause levels of the canonical clock mutants between *s-* and *ls-tim* background grown at 25 °C. *ls-tim* data (with diagonal stripes) from Gesto (2011) and *s-tim* data (in blank) from the previous Figure 4-3. N/A: no available data for that genotype. Black and red stars represent significant differences compared to corresponding Hu controls. The numbers inside the columns represent replicas. Means + SEM. ** p<0.05; ** p<0.01; ***<0.001; ****<0.0001.*

Table 4-4 ANOVA results of data presented in Figure 4-4.

	Df	SumSq	Fvalue	Pr(>F)
Genotype	5	6.67	53.08	<0.0001
s/ls-tim	1	0.16	6.50	0.012
Genotype:s/ls-tim	5	0.28	2.27	0.051
Residuals	138	3.47		

4.4.2 Locomotor activity

Flies reared at 18 °C or 25 °C were placed for five days at either 18 °C or 25 °C in LD 12:12 followed by 7 days of DD.

4.4.2.1 LD 12:12 display experimental-temperature dependent changes

The first two days served to acclimate the flies to the experimental temperature and were not included in the analysis of the LD data. Hence, the last three days in LD were averaged and used to analyse the ability of the different lines to entrain to light.

Figure 4-5 shows that Houten *s-tim* control lines entrain well to LD cycles regardless of the temperature of rearing and maintenance, although the flies that had been constantly maintained at 25 °C display higher activity with typical bimodal

patterns, and higher activity anticipating light-dark or dark-light transitions (morning and evening peaks). Nevertheless, the phase of the evening peak seems advanced when the flies are kept as adults at 18 °C.

As previously reported, mutations in the core clock genes (*per*, *tim*, *Clk* and *cyc*) severely impair the ability to anticipate light-dark transitions. However, in most cases an increase of activity after a transition is still present (startle response), so they are still able to react. Two clear exceptions are Clk^{Jrk} and cyc^{01}, in which no startle response is observed in the lights-off-to-on transition. As in the WT flies, the mutants that were kept at 25 °C show higher activity counts than their 18 °C counterparts, regardless of developmental temperature. In most cases, the profiles on the different temperatures are similar, but there are a couple of exceptions in per^{01}; tim^{01} double-mutants and in cry^{b}. In the former, the activity count is extremely low when the flies are kept at 18 °C and also if the flies are grown at 18 °C and placed at 25 °C for the experiment. Astonishingly, if the flies had developed and been kept at 25 °C they displayed a more WT-like pattern with anticipation to the lights-on transition. In cry^{b} flies, the strong startle response after lights-off that is observed when the flies were at 25 °C is diminished when the experiment was performed at 18 °C; and the same happens with the morning anticipation, which is present at 25 °C, but not at 18 °C. Finally, Pdf^{01} mutants show no morning anticipation, but maintain a strong evening anticipation. When the flies had been kept at 18 °C, the phase of the evening peak seems to be advanced (ZT9-10, followed by a decrease or maintenance of the activity levels until the startle response to lights off). Also, when the flies were raised and kept at 25 °C the evening activity started earlier than when the flies had been reared at 18 °C (ZT6 *vs* ZT9).

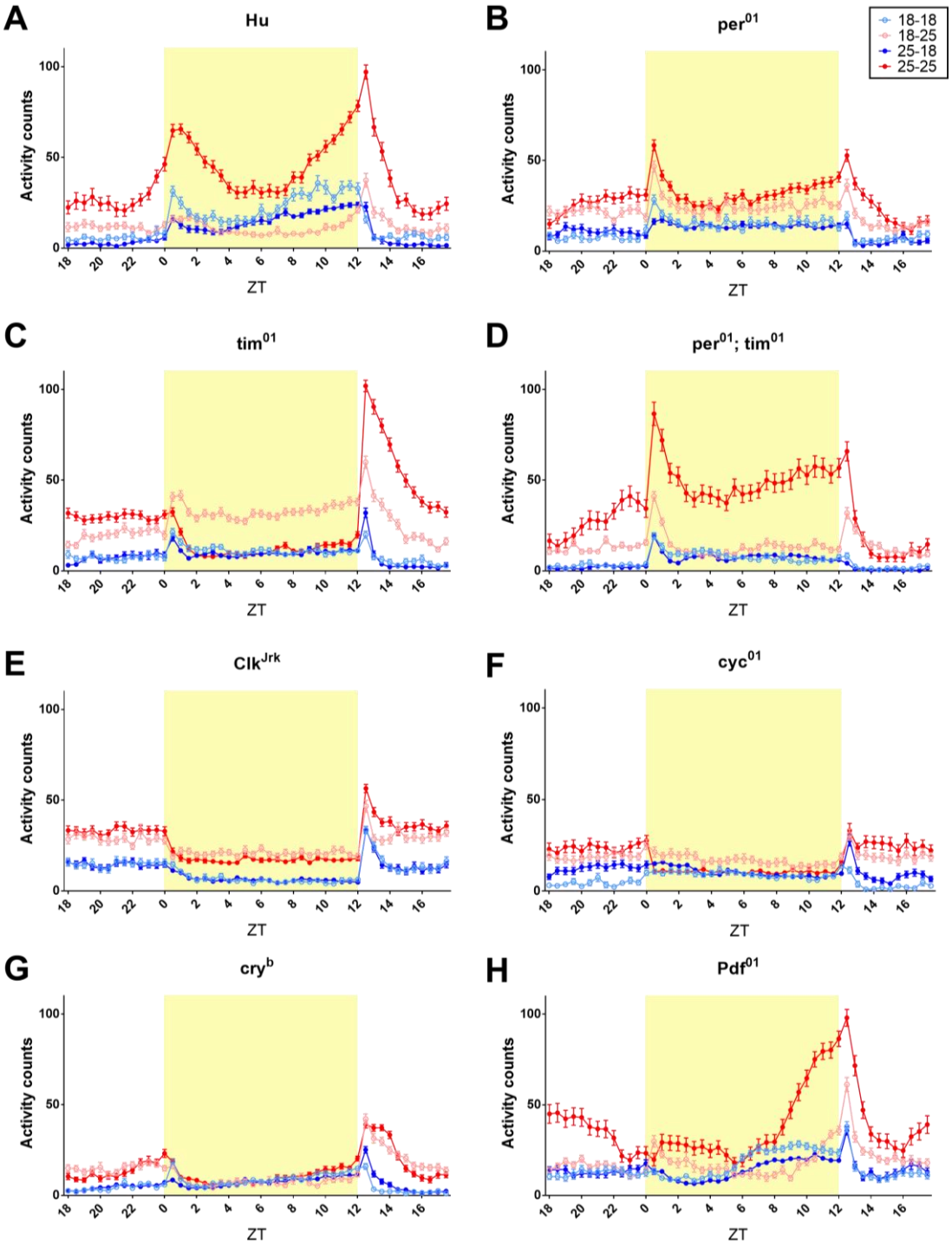


Figure 4-5 Locomotor activity in LD 12:12. (A) Hu-S. (B) *per*$^{01}_{HU}$. (C) *tim*$^{01}_{HU}$. (D) *per*^{01}*; tim*$^{01}_{HU}$. (E) *Clk*$^{Jrk}_{HU}$. (F) *cyc*$^{01}_{HU}$. (G) *cry*$^{b}_{HU}$. (H) *Pdf*$^{01}_{HU}$. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Flies reared at 18 °C (lighter) or 25 °C (darker). The yellow bars in the graphs represent lights-on during the LD regime, with lights on at ZT0 and lights off at ZT12. Mean ±SEM. *If the SEM bars are shorter than the height of the symbol, they are not represented.*

4.4.2.2 DD

Figure 4-6 summarises the behaviour in DD of the strains under different developmental and/or experimental temperatures. The first day in DD was eliminated from the analysis and the last six days were used to assess rhythmicity and period length using spectral analysis and autocorrelation. As expected, HuS (Houten *s-tim*) controls display high rhythmicity (>80%) in all conditions except when grown and kept at 18 °C, but still over 70% of the flies remain rhythmic. Even if some of the clock mutants had 1-5 flies which exhibited some rhythmicity, most of these strains (per^{01}, tim^{01}, per^{01}; tim^{01}, Clk^{Jrk} and cyc^{01}) are arrhythmic. The free-running period of all these clock mutants tend to have much larger standard errors, which is due to ultradian and circadian periodicities being present. However, there are some exceptions: tim^{01} and per^{01} flies that have been reared at 18 °C but the experiment was performed at 25 °C, display a slight improvement in rhythmicity (although it is in the ultradian range and not circadian). Moreover, per^{01}; tim^{01} double mutants are rhythmic when grown at 18 °C and kept at 25 °C during the experiment - **59%** of the flies are rhythmic (7:10 circadian *vs* ultradian rhythms; See Figure 4-7 for examples and Figure 9-7 in the Appendix for additional examples).

cry^{b} mutants are highly rhythmic under all conditions (>90% when grown at 25 °C and >70% at 18 °C). Finally, Pdf^{0} mutants are surprisingly rhythmic but their behaviour changes dramatically depending on rearing and experimental conditions. When reared at 18 °C they display ~50% rhythmicity independently of experimental temperature. Nevertheless, when reared and kept at 25 °C, over 70% of the flies are rhythmic; and when reared at 25 °C and kept at 18 °C throughout the experiment, only 25% are rhythmic.

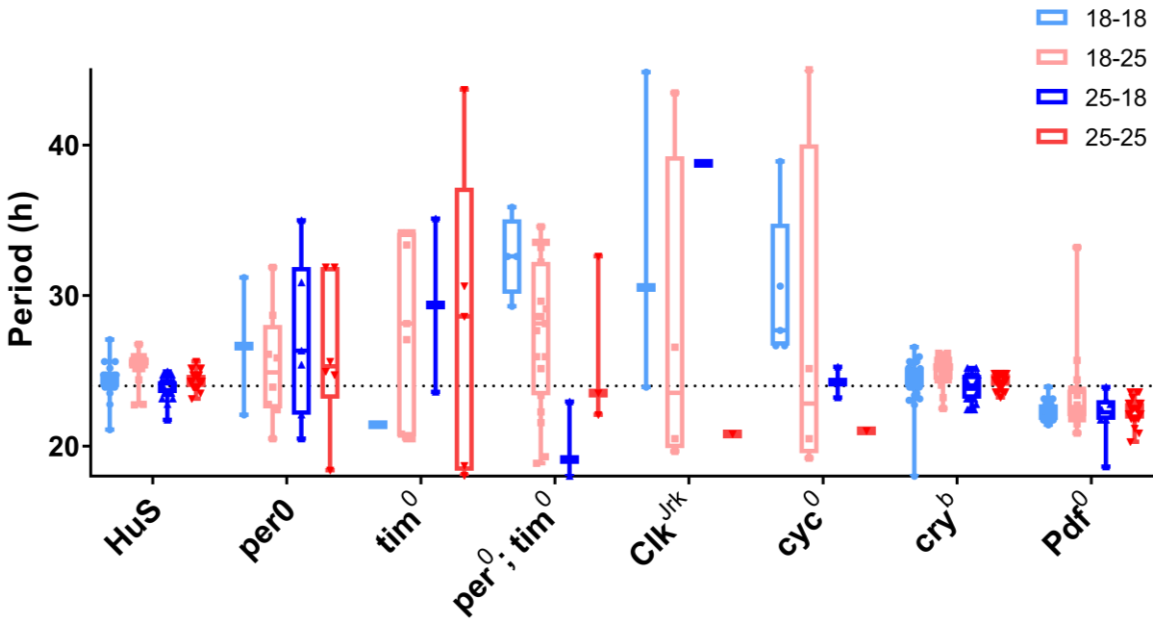


Figure 4-6 Min to max box-and-whiskers plot of free-running behaviour in clock mutants reared and/or maintained at 18 or 25 °C. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Each point represents a rhythmic fly. Flies reared at 18 °C (lighter) or 25 °C (darker). The horizontal dotted line marks a 24h period. *Several strains had only a single rhythmic fly per condition and, hence, have no whiskers.*

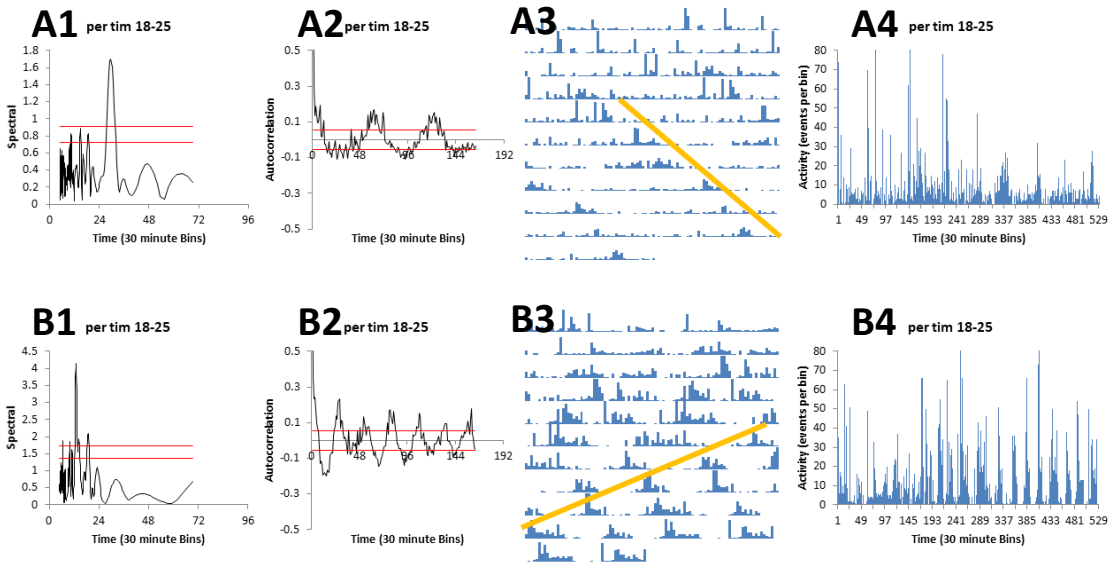


Figure 4-7 Examples of rhythmic *per*^{01}*; tim*^{01} flies at 25 °C reared at 18 °C. (A) displays a long 27h free-running period, while (B) has a 14h ultradian rhythm. 1) Spectral analysis; 2) Autocorrelation; 3) Actogram; and 4) Histogram of activity pattern of the fly.

When focusing on the period length of the rhythmic strains (HuS control, *cry*^{b} and *Pdf*^{0}), a clear genotype, rearing temperature and experimental temperature effect (p<0.0001, <0.0001 and =0.0001, respectively) is observed, with no interactions (Table 4-5). Rearing the flies at 18 °C lengthens free-running period, as well as performing the experiment at 25 °C (See summary data in Appendix Table 9-8 and Table 9-9). Analysis of the free-running period of different strains show that *cry*^{b} mutants behave as WT controls, while period is significantly reduced in *Pdf*^{0} mutants (See summary data in Appendix Table 9-10). This is also obvious when inspecting Figure 4-8, as both control and *cry*^{b} mutants display periods slightly longer than 24h, whilst *Pdf*^{0} flies clearly have a much shorter period (p<0.0001, post hoc).

Table 4-5 ANOVA table of the rhythmic strains: HuS control, *cry*^{b} and *Pdf*^{0}. 'Exp_T', 'Experimental temperature' and 'Grown_T', 'Grown temperature'. Significant results are highlighted in bold.

	Df	SumSq	Fvalue	Pr(>F)
Exp_T	**1**	**26.13**	**17.36**	**<0.0001**
Genotype	**2**	**164.18**	**54.54**	**<0.0001**
Grown_T	**1**	**23.47**	**15.59**	**0.0001**
Exp_T:Genotype	2	0.72	0.24	0.79
Exp_T:Grown_T	1	2.1	1.39	0.24
Genotype:Grown_T	2	0.98	0.33	0.72
Exp_T:Genotype:Grown_T	2	0.6	0.20	0.82
Residuals	248	373.29		

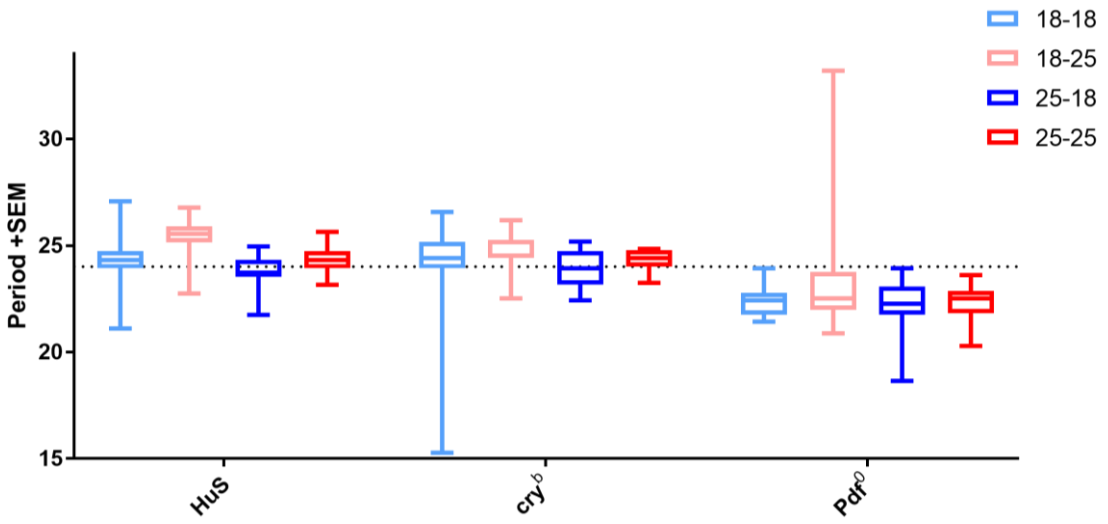


Figure 4-8 2.5 to 97.5 percentile box-and-whiskers plot of period length of HuS, *cry*^{b} and *Pdf*^{0}. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Flies reared at 18 °C (lighter) or 25 °C (darker). The horizontal dotted line marks a 24h period.

4.5 Discussion

In this chapter, I repeated the systematic diapause study of the canonical clock mutants previously performed by Gesto (2011) using a different *timeless* background and two different diapause-inducing protocols (the standard protocol and a more natural one). The main difference between these two protocols resides in their developmental conditions: in the standard protocol, the flies are reared at 25 °C in LD 8:16; while in the natural protocol, at 18 °C in LD 8:16. However, the diapause induction stage (12 °C in LD 8:16 for 12 days) and scoring remains the same for both of them. Hence, for the first time I looked at the effect of developmental conditions in adult diapause for different clock mutants.

Most of the clock mutants tested showed some diapause phenotype. Interestingly, this diapause phenotype differed significantly depending on the protocol used (hence, on the rearing conditions). The most remarkable changes occurred when the positive components of the circadian clock were manipulated (Clk^{Jrk} and cyc^{0}) as well as when the main output was absent (Pdf^{0}). However, the disruption of the negative components of the clock in per^{0}, tim^{0} and per^{0}; tim^{0} double-mutant had little or no effect. Only the disruption of *per* in standard rearing conditions showed a significant reduction in diapause levels. Finally, cry^{b} mutants display very high diapause levels regardless of the protocol. Although we cannot exclude pleiotropic effects (Bradshaw and Holzapfel 2010, Emerson et al. 2009) we are inclined towards the clock network as a module regulating diapause, as all the clock mutants show some differences in diapause levels, either using one or other developmental condition. These results permit a very simple but speculative model to be developed to explain how diapause could be regulated by the circadian clock, which is summarised in Figure 4-9. How Clk^{Jrk}, cyc^{01} and Pdf^{01} mutants could be inverting their roles in diapause induction depending on the rearing conditions is not known.

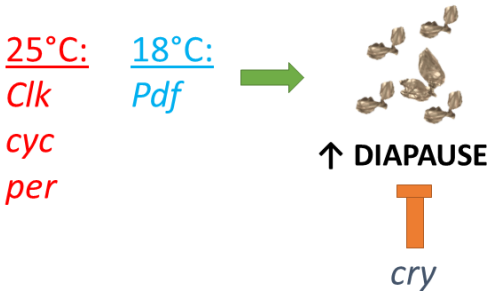


Figure 4-9 Summary of diapause control by the core clock genes. *cry* has an inhibitory role independent of rearing conditions. *Clk*, *cyc* and *per* promote diapause when the flies are developed at 25 °C while *Pdf* promotes diapause after rearing the flies at 18 °C.

When the flies were reared a 25 °C, disruption of *Clk*, *cyc* and *per* resulted in a reduction of diapause levels. However, after rearing the flies at the colder temperatures the lines displayed control diapause levels, which were significantly higher than when following the standard protocol for the two positive components of the clock. CLK/CYC could regulate diapause via expression of one (or several) of their target genes. For example, a direct target of CLK (*Pdp1ε*) activates *takeout* expression (Benito et al. 2010), which is involved in the regulation of lifespan (Bauer et al. 2010) and might be a carrier protein for JH (Meunier, Belgacem and Martin 2007) which, as mentioned in the Introduction (1.3.5), modulates diapause in *D. melanogaster*. This role inversion was milder in the case of *period*, where independently of the developmental conditions ~30% of the females were in diapause. These results are in contrast to those obtained by Ikeno et al. (2010) in which opposite roles for *period* and *cycle/Clock* are described: the former reduces diapause whilst the latter increase it in *Riptortus pedestris*. Nevertheless, in the case of *D. melanogaster*, per^{01} mutant flies are known to have low levels of *Clk* (Glossop, Lyons and Hardin 1999). This would explain both the positive and negative clock components exerting the same effect on diapause induction.

The involvement of PDF in diapause induction is controversial. Pdf^{0} mutants have been reported to have no effect on diapause or to promote it in *D. melanogaster* (Gesto 2011, Nagy 2017). On the other hand, knocking down *Pdf* with RNAi in *R. pedestris* had no effect in diapause (Ikeno et al. 2014), yet this same downregulation in *C. pipiens* prevented development even under favourable conditions (Meuti et al.

2015) and, recently, in a specific larval stage in *B. mori,* PDF was found to stimulate ecdysone (Iga et al. 2014). My results suggest a temperature-dependent role for *Pdf*, which seems to enhance diapause when the flies are reared in more natural-like conditions (lower temperature and shorter photoperiod, this has been independently replicated by Nagy (2017), our collaborator in Padova). The importance of PDF may reside in its role as a signalling molecule. In *D. melanogaster*, PDF expressing cells send axonal projections to the dorsal brain in close proximity to the *pars lateralis* and *pars intercerebralis*, which have been associated with diapause (Schiesari et al. 2016, Kubrak et al. 2014). Ablation of PDF-expressing cells promoted development in *R. pedestris* (Ikeno et al. 2014) and resulted in intermediate numbers of reproductive animals under long and short photoperiods in *Protophormia terraenovae* (Shiga and Numata 2009). Genetic manipulations of the PDF-expressing cells in *D. melanogaster* showed that PDF^{+} neurons regulate diapause: their overexcitement resulted in a reduction of diapause; while silencing or ablating them, generated an increase (Nagy 2017).

cry^{b} mutants have significantly increased diapause in both experimental set-ups. This is in agreement with the previous results from Gesto (2011), where cry^{b} was found to promote diapause and disrupt photoperiodic diapause. Since I have performed the experiments under a short photoperiod, I cannot confirm its involvement in photoperiod-sensing. Nevertheless, cry^{b} has been reported to disrupt CRY mediated circadian photoreception and photic entrainment (Stanewsky et al. 1998), which suggests at least a role in the transduction of environmental light signals in diapause.

Surprisingly, the tim^{01} mutant does not have a clear diapause phenotype, in contrast to the increased diapause reported in Gesto (2011) and Tauber et al. (2007) for *D. melanogaster* or in *C. costata* by Stehlik et al. (2008). The dramatic differences that have been previously reported in diapause with the natural polymorphism in *timeless* (Tauber et al. 2007, Sandrelli et al. 2007) suggested the null mutant should have a large effect on diapause. Moreover, my results, in *s-tim*, are startlingly similar to those from Gesto (2011) whose experiments were performed in the *ls-tim* background. Although an overall higher diapause level is observed in his flies, only *s-tim;* Pdf^{01} and *ls-tim;* Pdf^{01} are significantly different from each other when comparing

within individual genes (contrary to what is reported in Gesto's thesis, in which carrying *s-tim* significantly lowered diapause levels in all the mutants).

There might be many reasons for this variability including that I was collecting **0-5 h** old flies, with **30-40 females** per replica, and keeping them in diapause-inducing conditions for **12 days**, while he was collecting ***0-10h*** old flies, with ***20-30 individuals*** (males and females) and keeping them in diapause-inducing conditions for ***15 days*** prior to their dissection. Finally, I kept the flies at 12 °C with no light-induced temperature increase during the day-time. On the contrary, Gesto et al. (2011) performed the experiments using lightboxes, in which the temperature can be up to 1 °C higher during lights-on. Hence, he probably performed the experiments in a slightly higher absolute temperature and, as negligible as that may seem, I have already shown in Chapter 3 the significant effect that even a 0.5 °C fluctuation during 8 h can have in diapause. Overall, putting both our datasets together serves to confirm that clock mutants have significant effects on diapause inducibility.

Finally, I assessed whether rearing conditions affect circadian locomotor phenotypes of WT and mutant flies. In LD 12:12, performing the experiment at higher temperature seemed to enhance activity in all the lines. Counter-intuitively, the mid-day siesta at 25 °C in WT flies that had been reared at 18 °C is enhanced compared to when the same line was grown and maintained at 25 °C. Similarly, the phase of the evening peak seems to be advanced when the flies are kept at 18 °C, regardless of the developmental conditions, which has already been described (Majercak et al. 1999). As expected, mutations in the core clock genes (*per*, *tim*, *Clk* and *cyc*) severely impairs the ability to anticipate to light-dark transitions (Konopka and Benzer 1971, Sehgal et al. 1994, Allada et al. 1998, Grima et al. 2004). In most cases, the profile of the different temperatures is relatively similar, but there are a couple of exceptions in per^{01}; tim^{01} double-mutant and in cry^{b}. Pdf^{01} mutants behave as WT flies but without the morning anticipation and with an advance in the evening peak (Stoleru et al. 2004).

Rearing the flies at 18 °C tends to increase rhythmicity in DD of the clock mutants. In agreement with the literature, most of them remain arrhythmic (Konopka and Benzer 1971, Sehgal et al. 1994, Allada et al. 1998, Grima et al. 2004), with an

exceptional rhythmicity observed in per^{01}; tim^{01} flies when they were reared at 18 °C and placed at 25 °C. They mainly display a shorter ultradian period, which has been previously described in per^{01} mutants (Dowse, Hall and Ringo 1987) with high variability between the individuals. A partial rescue of per^{01} locomotor behaviour by another mutant, cry^{b}, has been reported (Collins et al. 2005). Nevertheless, in this former case a PER-independent role of TIM was suggested to be involved. Clearly, this is not my case and additionally it is extremely curious that the rescue only occurs under this particular rearing environment. Is it possible that the lack of a PER/TIM negative feedback loop is allowing some previously masked rhythm gene to be expressed?

Locomotor activity rhythms at lower temperatures tends to have shorter periods (Majercak et al. 1999). Indeed, statistical comparison of the rhythmic strains (Hu *s-tim*, cry^{b} and Pdf^{01}) shows an experimental temperature effect, with more rhythmic flies and longer periods at 25 °C compared to 18 °C. Unexpectedly, a strong developmental temperature effect is also present: rearing the flies at lower temperature significantly lengthens the period and, at the same time, weakens rhythmicity. As previously described, Pdf^{01} mutants have significantly shorter periods than the controls (Renn et al. 1999). Rhythmicity levels are higher than expected for this line (Renn et al. 1999), but this might be because the experiments were performed on an *s-tim* background as Gesto (2011) found a an increase in rhythmicity on this *timeless* background. Finally, cry^{b} mutants maintain WT-comparable rhythmicity and free-running period, which are evidence of a functional clock (Stanewsky et al. 1998). In *cry* mutants light input signalling to the clock is impaired: these mutants are rhythmic in LD and DD; and they stay rhythmic in LL, when WT flies would be otherwise arrhythmic (Emery et al. 1998). As described in Gesto (2011), the evening anticipation is not present in the *s-tim;* cry^{b} line. The strong startle response after lights-off that is observed when the flies were at 25 °C is diminished when the experiment was performed at 18 °C; and the same happens with the morning anticipation, which is present at 25 °C, but not at 18 °C. The evening cell oscillator synchronises to LD cycles by two mechanisms: CRY expression and visual system inputs via PDF (Cusumano et al. 2009). Hence, it is not surprising that these two mutants resulted in altered evening peak activity.

Overall this chapter suggests that:

- In diapause, developmental conditions shape the role of the different circadian genes in diapause regulation. When following the standard protocol, Clk, cyc and per promote diapause. Similarly, after developing the flies with the more natural protocol, *Pdf* also seems to be promoting diapause.
- Regardless of the rearing conditions, *cry* always inhibits diapause induction. Might cry^b be temperature insensitive?
- Experimental conditions and not rearing conditions seem to be important for the locomotor behaviour of WT and mutant strains. Nevertheless, in the rhythmic strains, rearing the flies at 18 °C lengthens tau but decreases rhythmicity; while performing the experiment at 25 °C, lengthens tau and increases rhythmicity.

5. STRUCTURAL CHANGES IN DIAPAUSING FLY BRAINS

5.1 Introduction

5.1.1 Neurobiology of the circadian pacemaker

Seven clusters of clock neurons, named after their anatomical locations have been described (Figure 5-1). They create a complex network in which the different clusters communicate with each other either directly (synaptic connections) or indirectly (through neuropeptide release). The best studied are those of the small and large ventral lateral neurons. The **s-LN$_v$**s drive free-running locomotor behaviour in DD (Park et al., 2000; Stoleru et al., 2005). These cells send projections towards the dorsal area of the brain, near the DN1 and DN2s with the s-LN$_v$s directly connected with the DN1 (Seluzicki et al., 2014). The arborisation of these projections is under a tight circadian control, resulting in changes in their length and in their ability to communicate with their synaptic partners (Fernandez et al., 2008; Gorostiza et al., 2014; Petsakou et al., 2015). There are four PDF expressing s-LN$_v$s in each brain hemisphere as well as a 5th non-PDF expressing cell, whose arborization pattern is likely to be similar to their PDF-expressing counterparts (Helfrich-Forster et al., 2007). On the other hand, the axonal projections from the **l-LN$_v$**s enter Cucatti's bundle and interconnect the two medullae, where they arborize, via the posterior optic tract (POT) (Helfrich-Forster and Homberg, 1993). These large cells have been connected with sleep and arousal as an alteration of their excitability promotes higher activity at night (Sheeba et al., 2008). Additionally, they may be important in signal transduction to

and/or from the optic lobe or eye (Helfrich-Forster et al., 2007). Ablation of **LN_d**s presents an arrhythmic phenotype suggesting a role as a critical pacemaker (Renn et al., 1999). Additionally, these cells are thought to play a role in the control of the evening locomotor peak in LD (Grima et al., 2004). Their axons innervate the dorsal protocerebrum, overlapping with the dorsal cells (Helfrich-Forster et al., 2007). The last set of lateral neurons, the **lateral posterior neurons (LPNs)**, have been more recently characterised (Shafer et al., 2006). These cells, together with the DN2s have been linked to temperature entrainment (Miyasako et al., 2007).

Three main dorsal neuron clusters have been defined: DN1, DN2 and DN3, from more medial to more lateral location. There are 14-16 **DN1**s in each hemisphere and they can be further divided into two anterior (aDN1) and posterior (pDN1) cells. The former express neuropeptide IPNamide (IPNa) and have been related with oscillating behaviours in LL conditions (Stoleru et al., 2007). In contrast, the GLASS-positive pDN1s are associated with light sensitivity of the clock in a CRY independent manner (Rieger et al., 2003). TIM and PER oscillation in the two **DN2**s is in antiphase with most of the other clock cells (Stoleru et al., 2005) and as mentioned before they have been associated with temperature entrainment. Finally, there are approximately 40 **DN3**s in each of the brain hemispheres and they have been related with evening activity (Veleri et al., 2003). Some of the DN1 and DN3s send projections towards the accessory medulla, where the s- and l-LN_vs are located, but most of the DNs project towards the dorsal protocerebrum.

Indeed, apart from the l-LN_vs, all clock neurons show projections towards the dorsal protocerebrum, a region for the integration of sensory and circadian stimuli from all the pacemaker cells. This information might by subsequently passed to the *pars intercerebralis* and *lateralis*, resulting in an indirect regulation of various physiological effects (Helfrich-Forster et al., 2007).

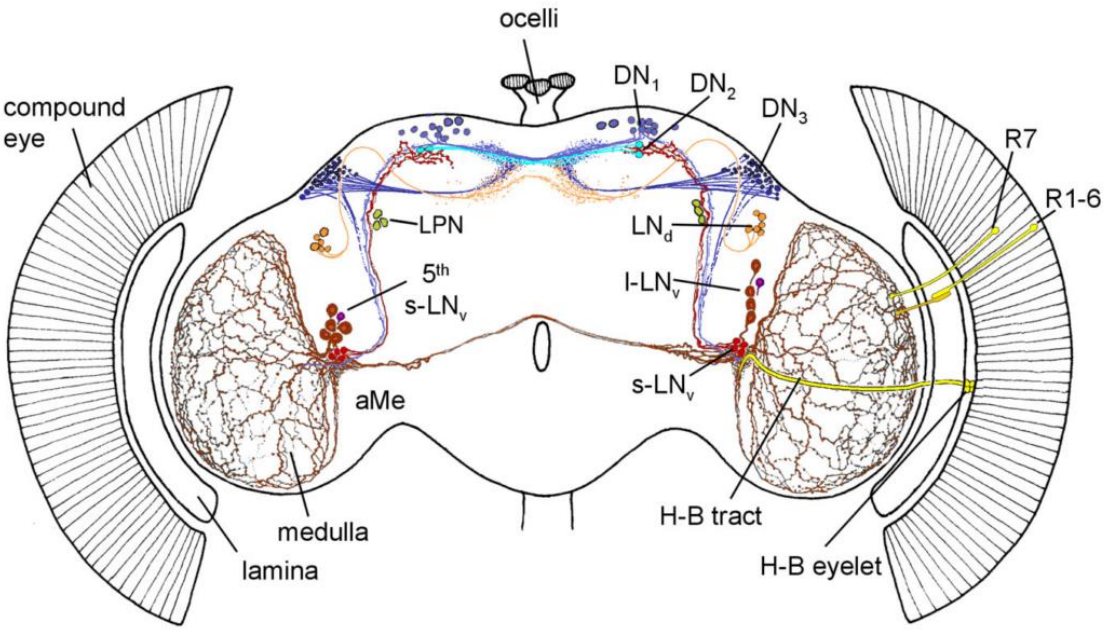


Figure 5-1 Schematic representation of the known pacemaker cells and their putative connections in *Drosophila*. From Helfrich-Forster (2007).

5.1.2 IPCs

Four out of the eight identified *dilp*s (DILP1, 2, 3 and 5) are expressed by ~14 cells located in the *pars intercerebralis*, the Insulin Producing Cells (IPCs) (Broughton et al., 2008; Liu et al., 2016; Slaidina et al., 2009). These send axons towards the *tritocerebrum*, *corpora cardiaca,* proventriculus and the crop (Nassel et al., 2013). Rulifson et al. (2002) suggested an analogous role of the IPCs to human pancreatic islet B cells, as flies missing the IPCs showed some similarities will *diabetes mellitus* patients (for example fasting glucose levels in the haemolymph). However, even after considerable effort we still do not know which conditions induce their activation nor how their activity is regulated. IPCs express a wide range of receptors that specifically recognise Adipokinetic hormone (AkhR), Adiponectin (AdipoR), Corazonin (CrzR) and sNPF (sNPFR1), but also octopamine, serotonin and GABA (Kapan et al., 2012; Kwak et al., 2013; Luo et al., 2012; Luo et al., 2014).

Apart from the well-characterised role of IPCs in growth, development and metabolic control, they may be involved in regulation of several other phenotypes. For example, they have been hypothesised to regulate sleep via the octopamine receptor (Cong et al., 2015; Crocker et al., 2010). Indeed, Cong et al. (2015) reported a decrease

in total sleep in *InR* and *dILP* mutants (except for *dILP4*). Belgacem and Martin (2006) described a possible regulatory effect of these cells in locomotor activity while Zheng et al. (2007) found that they might be implicated in the regulation of responsiveness of the circadian clock to oxidative stress. Similarly, Barber et al. (2016) recently showed a neuronal connection between the IPCs and the circadian central pacemaker as they confirmed that the DN1 innervated the IPCs.

5.1.3 Neuropeptides and diapause in *D. melanogaster*

5.1.3.1 Drosophila insulin-like proteins (dilps)

In *D. melanogaster,* eight *dilp* genes have been described. They are expressed in different tissues and at different developmental times (See Table 5-1 for a summary). Some level of redundancy and compensation among this DILPs has been reported, for example Broughton et al. (2008) recorded an upregulation in *dilp3* and *dilp5* mRNA levels when knocking down *dilp2*. In this chapter, I will focus in DILP2 which is mainly expressed in the IPCs, although also in imaginal discs, salivary glands and some glial cells of the CNS during the larval stage.

The involvement of this pathway in regulation of several trait such as stress resistance, metabolism, fecundity and longevity is well characterised. Some of these characters are also modulated in diapausing animals, hence the possibility that this pathway is involved in diapause induction. Williams et al. (2006) reported significant changes in diapause levels associated with a mutation in the catalytic subunit of PI3K, *dp110*. Mutations in *dilp2* and *dilp5* promote diapause, (Kubrak et al., 2014; Schiesari et al., 2016). Additionally, Schiesari et al. (2016) showed that manipulation of the excitability of the IPCs (the main DILP generators in *Drosophila*) also resulted in changes in diapause levels (more DILP release, less diapause; less DILP release, more diapause). *InR* hypomorphic mutations cause sterility in *Drosophila* females which is accompanied by reduced levels of JH (Tatar et al., 2001). Interestingly, mutations in the insulin receptor substrate *chico* showed low vitellogenesis and increase diapause incidence accompanied by an extended lifespan, even if levels of JH and ecdysone are normal (Clancy et al., 2001; Richard et al., 2005). Finally, significant increase in diapause induction has been reported in flies overexpressing FOXO (Hwangbo et al.,

2004). Similarly, mutations in *dilp*s in other organisms such as *Culex pipiens*, also result in alterations of diapause levels (Sim and Denlinger, 2008; Sim et al., 2015). Also, *daf-2* (*InR* homologue) mutants in *C. elegans*, stop their development at the dauer larval (diapause-like) stage. Hence, insulin signalling pathway regulates both longevity and diapause in this species (Kimura et al., 1997). In summary, mutations in different components of the Insulin signalling pathway have been linked to diapause-like phenomena beyond *Drosophila*.

Table 5-1 Expression and axon terminations of the eight DILPs in *D. melanogaster*. Adapted from Nassel et al. (2013).

DILPs	Location		Axon terminations	References
	Larvae	Adult		
DILP1	IPCs	-	-	(Rulifson et al., 2002)
DILP2	IPCs Imaginal discs Salivary glands Glial cells of CNS	IPCs	Brain neuropil Corpora cardiaca Anterior aorta Proventriculus Crop	(Brogiolo et al., 2001; Cao and Brown, 2001; Rulifson et al., 2002)
DILP3	IPCs	IPCs Muscle cells of midgut	Corpora cardiaca Anterior aorta Proventriculus Crop	(Brogiolo et al., 2001; Rulifson et al., 2002; Veenstra et al., 2008)
DILP4	Anterior midgut	-	-	(Brogiolo et al., 2001)
DILP5	IPCs Principal cells in renal tubules	IPCs Follicle cells of ovary Principal cells in renal tubules	Corpora cardiaca Anterior aorta Proventriulus Crop	(Brogiolo et al., 2001; Rulifson et al., 2002; Soderberg et al., 2011)
DILP6	Adipose cells Salivary glands Heart Glial cells of CNS	Adipose cells	-	(Okamoto et al., 2009; Slaidina et al., 2009)
DILP7	Abdominal neuromeres	Abdominal neuromeres	Brain neuropil Hindgut Reproductive tract	(Miguel-Aliaga et al., 2008; Yang et al., 2008)
DILP8	Imaginal discs	Ovary	-	(Colombani et al., 2012; Garelli et al., 2012)

5.1.3.2 Pigment dispersing factor (PDF)

PDF expressed by several neurons in the lateral ventral region of *D. melanogaster* pacemaker: all four l-LN_vs and four of the s-LN_vs (the fifth one does not express PDF, although it contains PDFR) (See Table 5-1). Its expression has also been reported in two other non-circadian clock neuron clusters: PDF-Ab and PDF-Tri (Helfrich-Forster, 1997; Park et al., 2000; Renn et al., 1999). The latter were reported as developmentally-transient cells that undergo apoptosis early after hatching, not being detectable in 1-2 day old adults.

Table 5-2 PDF expressing cells in adult D. melanogaster. Adapted from Helfrich-Förster (1997).

PDF expressing cells	N in adults	Location of somata	Axon terminations	References
s-LNvs	4	Ventral lateral protocerebrum Near accessory medulla	Dorsal protocerebrum	(Helfrich-Forster and Homberg, 1993)
l-LNvs	4-6	Ventral lateral protocerebrum	Ipsi- and contralateral medulla cortex, accessory medulla	(Helfrich-Forster and Homberg, 1993)
PDF-Tri	2-4	Anterior-ventral Tritocerebrum	Ventrally and laterally in the esophageal foramen, median bundle, pars intercerebralis and lateralis	(Helfrich-Forster, 1997)
Eighth abdominal neuromere	4	Ventrally in the fused abdominal ganglia	Dorsal area of fused abdominal ganglia	(Helfrich-Forster, 1997)

The connection between PDF and diapause is not straightforward. *Pdf^0* mutants have been reported to have no effect on diapause or to promote it in *D. melanogaster* (Gesto, 2011; Nagy, 2017; Chapter 4). *Pdf* knock down with RNAi in *R. pedestris* had no effect on diapause (Ikeno et al., 2014), while in *C. pipiens,* RNAi prevented development even under favourable conditions (Meuti et al., 2015). Perhaps PDF regulates key hormones, such as ecdysone and juvenile hormone, which are known to

be implicated in diapause control (Allen, 2007; Denlinger, 2002; Richard et al., 2001). In fact, in *B. mori,* PDF has been shown to promote synthesis of ecdysone (Iga et al., 2014).

5.1.3.3 Others

Apart from DILPs and PDF, there are several other neuropeptides that could be involved in triggering or avoiding diapause. A subset of dorsal-lateral peptidergic (DLPs) corazonin-expressing neurons are located in the *pars lateralis* and could also be involved in the signalling of environmental conditions to the IPCs. As corazonin is a hormone, receptors for it have been found on peripheral tissues such as the fat body (Sha et al., 2014). It has been linked to metabolism, desiccation and resistance to starvation and oxidative stress in addition to food ingestion (Kapan et al., 2012; Kubrak et al., 2016; Sha et al., 2014). These cells also express **short neuropeptide F (sNPF)**, which is also expressed in the s-LN$_v$s. Importantly, IPCs have receptors for both of these neuropeptides. Both sNPF and corazonin have been implicated in insect diapause in several studies (Huybrechts et al., 2004; Nassel et al., 2013; Siga, 2003).

5.2 Aims

In this chapter, PDF and DILP2 expression is studied in the brains of diapausing or reproductive females. A model in which PDF control over diapause and the DILP2 will be presented and a preliminary diapause experiment testing the model will be described. Additionally, after rearing the flies using the standard and more natural protocols described in the previous chapters, I studied the expression of PDF and DILP2 in several of the canonical clock mutants.

5.3 Methods

5.3.1 *Drosophila* Stocks

per^0, *Clk^{Jrk}* and *Pdf^0* mutants in the Houten background described in Chapter 4 (4.3.1) as well as the WT Hu control were used in this chapter. Additionally, GRASP

(described in 5.3.4) and diapause experiments were performed using the flies described in Table 5-3.

Table 5-3 List of flies used for GRASP. [1] Dorsal-lateral peptidergic neurons.

Stock	Information	Reference
w; Pdf-LexA,LexAop-CD4::GFP11/CyO; UAS-CD4::GFP1-10/TM6b	Expresses half of GFP in the PDF+ cells using the LexA system, while the rest of the GFP is under a UAS construct.	Gift from F. Rouyer
Crz1-Gal4	Expresses GAL4 in the corazonin cells (DLPs1)	(Zhao et al., 2010)
dilp2-Gal4	Expresses GAL4 in the IPC from 2nd instar larvae	(Rulifson et al., 2002)
Gal1118	Expresses GAL4 in the PDF+ cells, but not in the PDF-Tri	(Blanchardon et al., 2001)
Pdf-Gal4	Expresses GAL4 in the PDF+cells, including PDF-Tri	(Park et al., 2000; Renn et al., 1999)
UAS-Pdf-RNAi	Expresses Pdf-RNAi under UAS control	Bloomington 25802
UAS-rpr-RNAi	Expresses rpr-RNAi under UAS control	Bloomington 51846

5.3.2 Diapause experiment

Flies were reared 25°C under short (LD 8:16) photoperiod and placed at 11 °C for 12 days after hatching. After this time, females were dissected to score ovarian diapause as described in Material and Methods 2.2.2. 4-7 replicas per genotype and condition were performed.

5.3.3 Immunohistochemistry (ICC)

5.3.3.1 General protocol

Flies were fixed in 4% PFA (Paraformaldehyde P6148-500g, Sigma) at ZT1 and a modified version of the protocol described in Dissel et al. (2004): primary antibodies were incubated at 4°C for 4 days and secondary antibodies at 4°C overnight. See Table 5-4 for the list of antibodies used and their working dilutions. Once the brains were

labelled against the protein of interest, the brains were mounted onto microscope slides using “antifade” (Table 5-5). The Olympus FV1000 CLSM confocal microscope from the Advanced Imaging Facility (AIF) of the University of Leicester was used for the imaging of the brains. The step size between the sections forming a Z-series was 1.16 µm (with the 20x objective) and 0.5µm (with 40x and 60x).

Table 5-4 Primary and secondary antibodies

	Antibody	Source	Dilution
Primary	Mouse α-PDF C7-s	DBSH	1:50.000
	Rabbit α-DILP2	Jan A. Veenstra (University of Bordeaux)	1:2.000
	Mouse α-GFP	Sigma G6539	1:100
Secondary	Goat α-mouse IgG (H+L) Alexa Fluor®488	Thermo Sientific A-11001	1:100
	Goat α-Rabbit (H+L) Cy3	Thermo Scientific A-10520	1:100

Table 5-5 Antifade composition. * Was added after the propyl gallate had been completely dissolved in glycerol.

Component	Amount
Propyl gallate (P3130, Sigma)	1.5 g
Glycerol	40 ml
dH2O*	10 ml

5.3.3.2 Specific experiments

Two extensive sets of ICC experiments were performed. In the first, HuS control flies were reared at 25 °C and placed under diapause-inducing conditions for 12 days after hatching. After fixing the flies at ZT1, the females were dissected to assess the egg-chamber maturation and the brains of clearly diapausing or reproductive (egg-chamber development beyond stage 11) flies were harvested separately. Finally, the brains were stained against PDF and DILP2 (Figure 5-2).

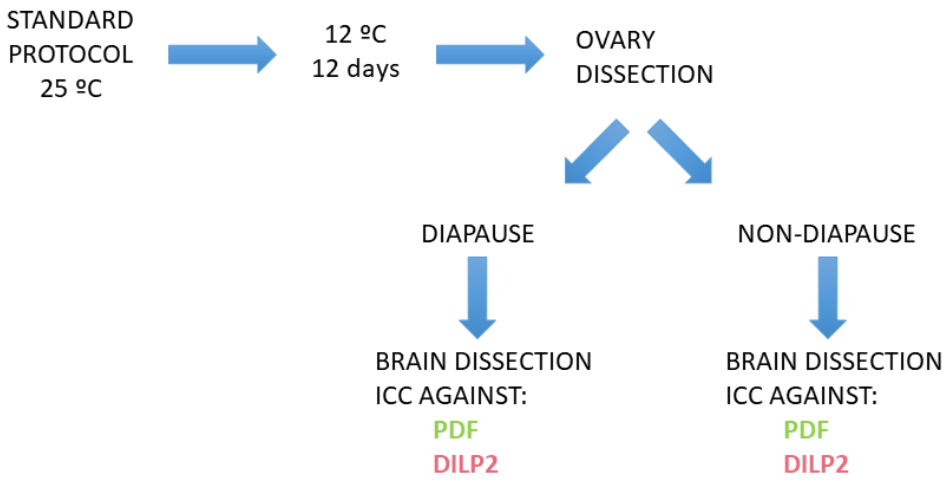


Figure 5-2 Experimental workflow for Diapause *vs* Non-diapause ICC.

For the second set of experiments, HuS control flies as well as *Clk*^{Jrk}, *per*^{0}, and *Pdf*^{0} flies were grown at either 25 °C or 18 °C and placed for 12 days at 12 °C or 25 °C. Finally, flies were fixed at ZT1 and dissected.

5.3.3.3 Analysis of the images

ImageJ software was used for quantification of different structures in the *Drosophila* brain (Figure 5-3). Due to the large scale of the experiments several macros were written to analyse PDF-Tri, the different axonal projections or to remove the background signal from the pictures. For quantification of the axonal projections, the macro used an ImageJ plugin named "Simple Neurite Tracker" which allowed tracking the axonal projections along the Z stack, giving information on the volume of the axon as well as the PDF or DILP2 intensity of it. For the quantification of the PDF-Tri, a Z-stack containing the 15 slides in which the PDF-Tri were present was generated. Afterwards, a circle was placed on the images directly under the posterior tract, and using the threshold feature the PDF-Tri were selected. The average of the intensity outside the selection was taken as the background intensity for the image. Finally, as the l-LN$_v$ (and IPCs) were in many occasions overlapping, it was not possible to generate an automated way to quantify PDF (or DILP2) staining in the cells. Hence, the individual cells were outlined manually.

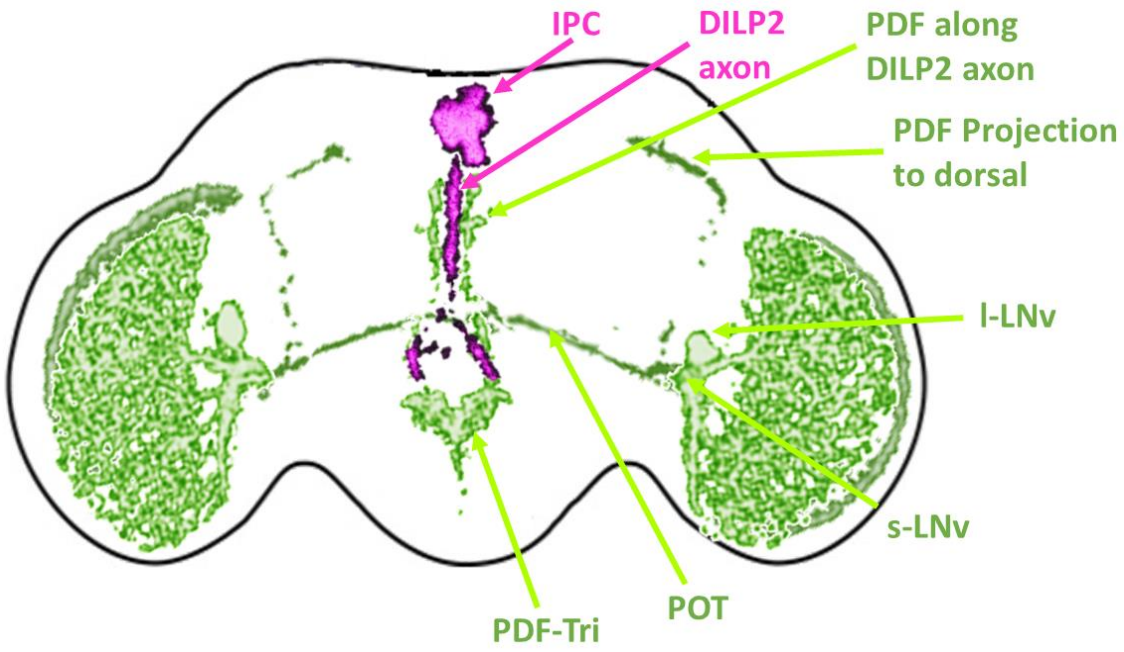


Figure 5-3 Schematic representation of the different regions of interest for PDF (green) and DILP2 (magenta) quantification.

The imaging settings were maintained when possible. However, some of the mutants and/or conditions had significantly different intensities of the protein of interest, and the PMT voltage of the laser had to be adjusted. In order to be able to compare and merge images taken with different PMT voltage, PS-Speck™ Microscope Point Source kit P-7220 (Fisher) was used to normalise the intensity values. These fluorescent beads were placed in a slide and images of them were taken using different PMT voltage. Finally, the intensity of a specific area was measured in all the images using ImageJ and plotted against the PMT voltage to obtain a linear curve (Appendix Figure 9-10). The intensity of the samples was finally corrected against this controls and the data was analysed.

5.3.4 GFP Reconstitution Across Synaptic Partners (GRASP)

Feinberg (2008) described this system which allows the identification of direct interactions between different cell types *in vivo*. GFP is divided into two split-GFP fragments: a longer GFP_{1-10} and a short GFP_{11}. Each fragment is fused to the mammalian membrane protein CD4; therefore, it is directed to the plasma membrane

and it becomes exposed to the extracellular space. Each fragment is expressed on a different type of cell between which we want to test physical interactions. If the two cells are in contact, a functional GFP is reconstituted and its signal is visible at the points of contact. In this chapter, I use this technology to study the putative interaction between PDF^+ and $DILP2^+$ or CRZ^+ cells. The crosses were performed at 18 °C and placed at 12 °C or 25 °C for 12 days after hatching. On the last day, flies were fixed at ZT1, which is when the projections from the s-LN_vs are more expanded towards the dorsal protocerebrum (Fernandez et al., 2008; Gorostiza et al., 2014). In order to amplify the GFP signal, an IHC against GFP was performed as described in 5.3.3.1. This antibody recognises specifically the reconstituted GFP. Only the GFP_{1-10} fragment is recognised at very low levels (Frank et al., 2015).

5.3.5 Statistics

The intensity and size results of the different brain structures from the diapause *vs* non-diapause brains were analysis using pairwise t-tests or Mann-Whitney U ranking test, depending on whether the data was normally distributed or not. PDF signalling data from the ICC of the clock mutants was normalised using reciprocal, logarithmic or square-root transformation prior to a three-way ANOVA using R. The rest of the analysis was performed as described in Material and Methods 2.1.15.

5.4 Results

5.4.1 PDF and DILP2 staining reveals brain changes between diapausing and reproductive flies

In this section, PDF and DILP2 were quantified in diapausing and developing adult females' brains to assess their putative involvement in this phenotype. In order to do so, the standard diapause-inducing protocol was used: WT flies were grown at 25 °C and placed at 12 °C for 12 days after hatching. Figure 5-4 displays representative examples of diapause *vs* reproductive brains.

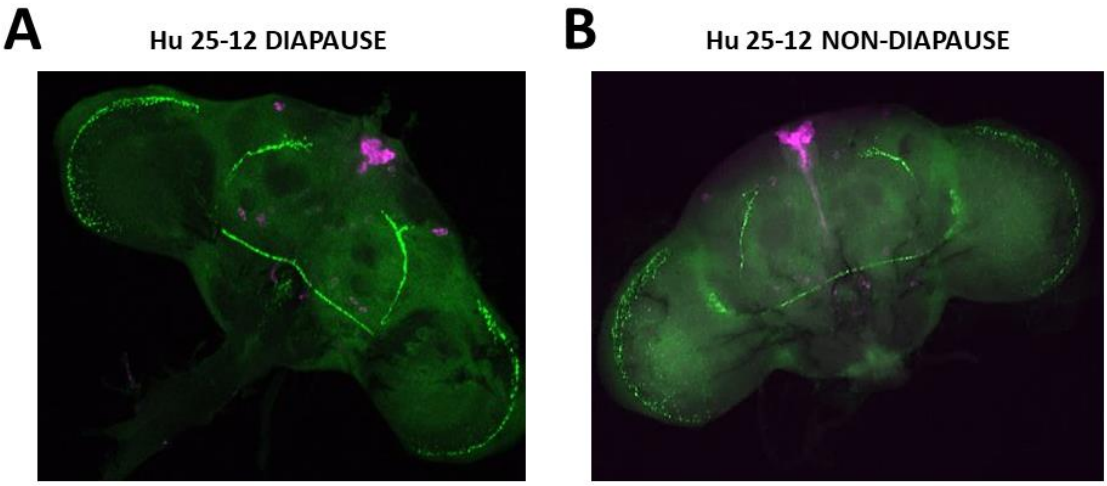


Figure 5-4 Representative examples of PDF (green) and DILP2 (magenta) staining of diapausing (A) or non-diapausing (B) *Drosophila* brains.

Many different features were quantified (See Figure 5-5 and Appendix Figure 9-11 and Figure 9-12). The differences in the brains between the two conditions were subtle and are summarised in Table 5-6. As seen in Figure 5-5, the brains of diapausing females tend to be smaller ($p=0.02$, $p=0.0002$ and $p<0.0001$, for POT, l-LN_v and IPC, respectively). No significant differences in PDF expression levels in POT, nor DILP2 in IPC were observed ($p=0.18$ and $p=0.09$, respectively). On the other hand, PDF-Tri and l-LN_v displayed higher levels of total PDF in the diapausing flies ($p=0.046$ and $p<0.0001$, respectively for mean and total intensity). Additionally, the axonal projections from the s-LN_vs towards the dorsal area were significantly smaller, resulting in a significantly higher PDF mean staining (Appendix Figure 9-11). Surprisingly, the DILP2 quantification along the axonal projections of the IPC showed a significant increase in DILP2 levels in diapausing flies. Finally, the area of the PDF-Tri axons along the DILP2 projections tended to be lower in reproductive flies, although the differences were not significant.

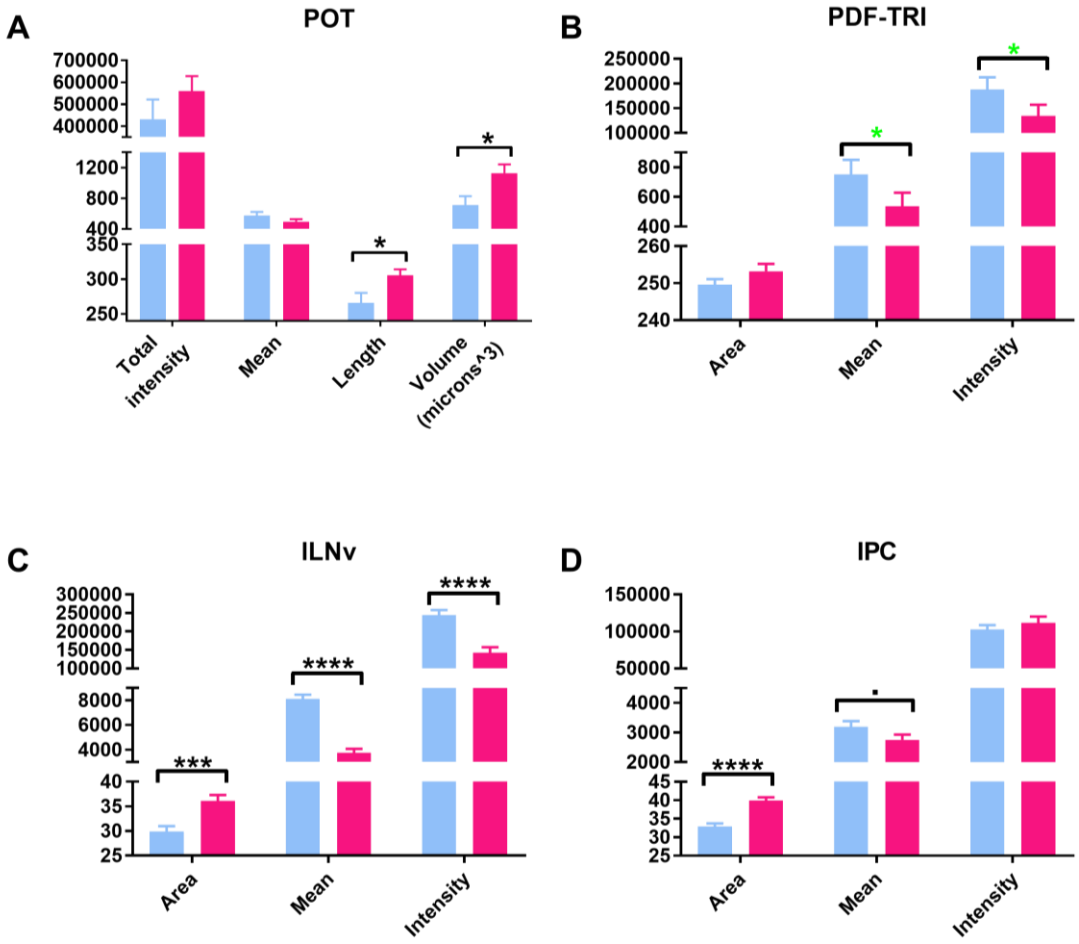


Figure 5-5 PDF quantification in the (A) posterior optic tract (POT); (B) Tritocerebrum and (C) l-LN$_v$s. (D) DILP2 quantification in the IPC. Comparison between the diapause (blue) and reproductive (magenta) was analysed using t-tests (black stars) or Mann-Whitney U test (green stars). Mean + SEM ·, *p<0.1; *, p<0.05; ***, p<0.001; ****, p<0.0001.*

Table 5-6 Summary of effects on diapause, compared to the brains of reproductive females. Mean intensity corresponds to the ratio of total intensity divided by volume (for the axons) or area (cells and PDF-Tri).

LOCATION	EFFECTS OF DIAPAUSE on brains
POT	Projection shorter, smaller volume. Similar mean intensity.
PDF DORSAL axons	No change total intensity. Smaller area and volume. Higher mean intensity.
l-LNv	Smaller area. Higher mean and total intensity.
PDF-TRI	Higher mean and total intensity.
IPC	Smaller area.
DILP2 axon	Higher mean and total intensity.
PDF along DILP2 axon	Non-significant increase in area.

5.4.2 Clock mutants' brains display structural differences

In this section, I tried to correlate the changes in brain structure observed for clock mutants with the dramatic differences in diapause described in Chapter 4. For this, I grew *Clk*^{Jrk}, *per*^{0} and *Pdf*^{0} mutants at high (25 °C) or low (18 °C) temperature and placed the newly hatched flies at 25 °C or at 12 °C for 12 days. Contrary to what I did in the previous section, here I did not select between diapausing and non-diapausing individuals, but I pooled together all the brains from each separate experimental condition (ie: grown at 25 °C and kept at 12 °C for 12 days). Then I focused on the analysis of several features that were found to be altered between diapausing and reproductive individuals. I measured PDF immunostaining in the Tritocerebrum and along the POT and then I compared the intensity of the staining with levels of diapause. For this comparison, I used the observations reported in Chapter 4, where I have described dramatic differences in diapause levels among several clock mutants depending on their rearing conditions. Although I performed the experiment on *Clk*^{Jrk}, *per*^{0} and *Pdf*^{0} mutants, only the first two will be discussed here. *Pdf*^{0} flies do not have PDF preventing the measurements described above. It would have been important to quantify the immunostaining of PDF in the l-LN$_v$ and of DILP2 in IPCs (this last quantification could be extended to the *Pdf*^{0} mutants additionally) as they displayed the strongest effects in the previous section. However, due to lack of time, these analyses were not included.

The POT volume and its PDF intensity as well as the area of the PDF-Tri and their PDF intensity were analysed using separate 3-way ANOVAs that would evaluate the effects of genotype, rearing temperature and experimental temperature for each feature. There were a large number of main effects and interactions which can be found in Appendix (9.3.2, Table 9-11 to Table 9-15). From these, we can distil the following results: *Clk*^{Jrk} brains had significantly higher staining than WT and *per*^{0} flies in the PDF-Tri area, independently of the experimental temperature ($p<0.0001$, for both comparisons). This area showed a significant increase in flies that had been reared at 25 °C and kept at 12 °C compared to those that were grown and maintained at 25 °C ($p=0.0001$). Both *Clk*^{Jrk} and *per*^{0} mutants displayed a significantly higher PDF-Tri signal

than control flies (p<0.0001 and p=0.01 for *Clk*^{Jrk} and *per*^{0}, respectively). Surprisingly, for the *per*^{0} mutants that had been grown at 18 °C, the flies that were placed at 25 °C had significantly higher levels of PDF in the Tritocerebrum than those kept in diapause-inducing conditions (p<0.0001). PDF levels in the POT were significantly higher when the flies were kept at 25 °C or when they were reared at 25 °C (p<0.0001 and p=0.006, respectively). In POT, *per*^{0} displayed significantly higher levels of PDF than HuS controls (p=0.0005).

Overall, PDF-Tri levels are enhanced when the adult flies are kept at colder temperatures while, in the POT, PDF exhibit the opposite trend and is significantly reduced at 12 °C (Figure 5-6).

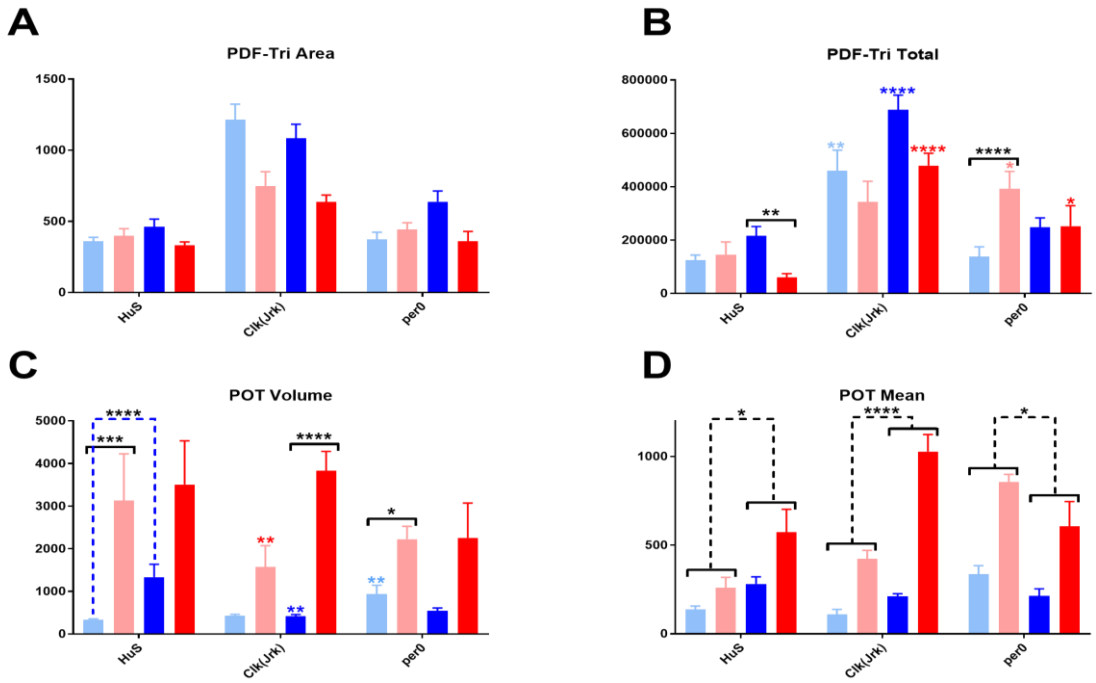


Figure 5-6 Analysis of PDF staining in PDF-Tri (A) area and (B) total intensity and in POT (C) volume and (D) mean intensity in HuS and *Clk*^{Jrk} and *per*^{0} mutants. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Flies reared at 18 °C (lighter) or 25°C (darker). Coloured stars represent significant differences compared to its respective HuS control (ie: dark red depicts differences compared to the controls reared and kept at 25 °C). Mean + SEM. *<0.05; **<0.01; ***<0.001; ****<0.0001.

Finally, the data described above were correlated with the diapause level obtained for each of the strains. Figure 5-7 shows that the general trend of larger PDF-Tri area and POT volume when diapause is higher, which is accompanied by higher PDF levels in both of these locations, is disturbed in both of the clock mutants.

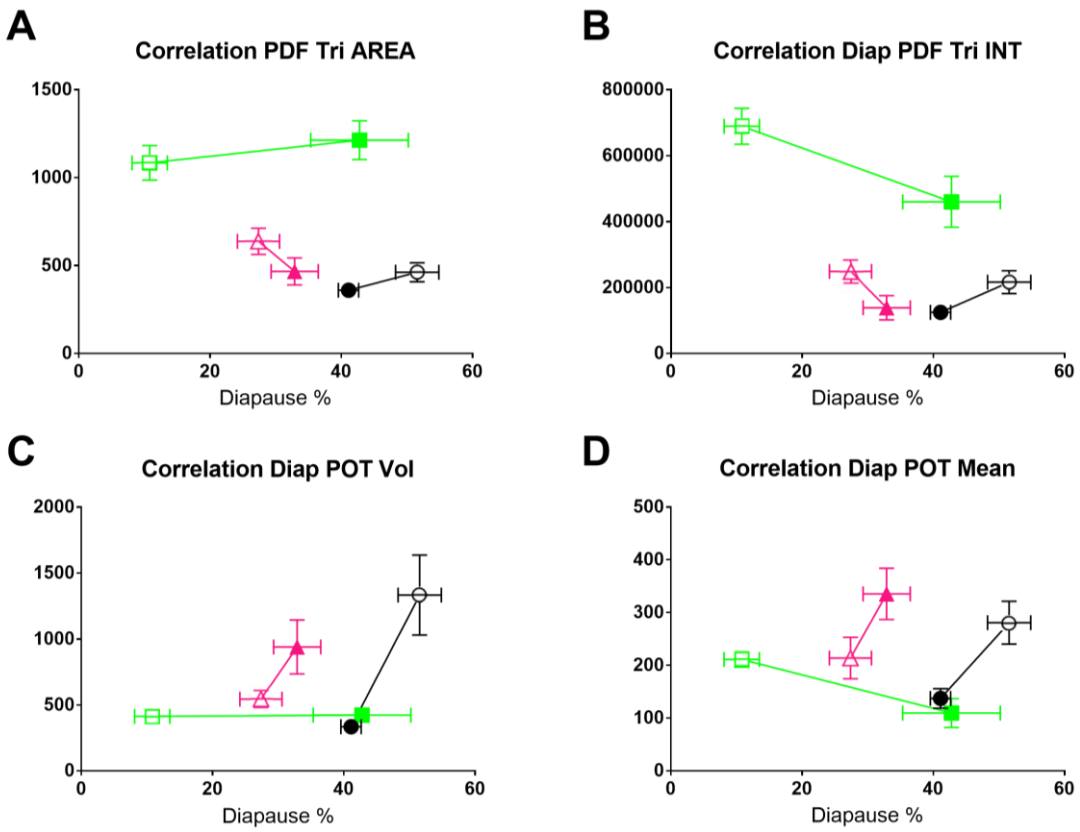


Figure 5-7 Correlation between diapause levels and PDF quantification for (A) PDF-Tri area, (B) PDF-Tri total intensity, (C) Volume of POT and (D) Mean intensity of POT. Results from HuS control flies, *Clk*^{Jrk} and *per*^{0} are represented in black, green and magenta, respectively. Data from 25 °C (open) *vs* 18 °C (closed). Mean ± SEM.

Additionally, I realised that the *Clk*^{Jrk} flies that had been reared at 18 °C displayed a certain improvement in their brain architecture (Figure 5-8). These mutants have extremely aberrant s-LN$_v$ axonal projections but I noted that the flies that had been reared at lower temperature had visible (although still not normal) axonal projections (compare Figure 5-8 A, in which the LUT settings have been highly increased, with Figure 5-8 B), even when the flies were placed at 25 °C after hatching (Figure 5-8 C and D). Indeed, these axonal projections were visible in 11/16 and 8/12 brains of flies reared at 18 °C and placed at 12 or 25 °C, as opposed to the 7/18 and 2/10 times that is detectable in the flies that were reared at 25 °C (Fisher's exact test reported $p>0.99$, $p=0.42$, $p=0.10$ and $p=0.04$ for reared at 18 °C or 25 °C and maintained at 12 °C or 25 °C, respectively). This may suggest an improvement of this aberrant phenotype after rearing them at colder temperatures, especially when the flies are maintained at 25 °C. Nevertheless, this would need to be repeated and confirmed with a larger sample size as a correction for multiple comparison would render the results non-significant.

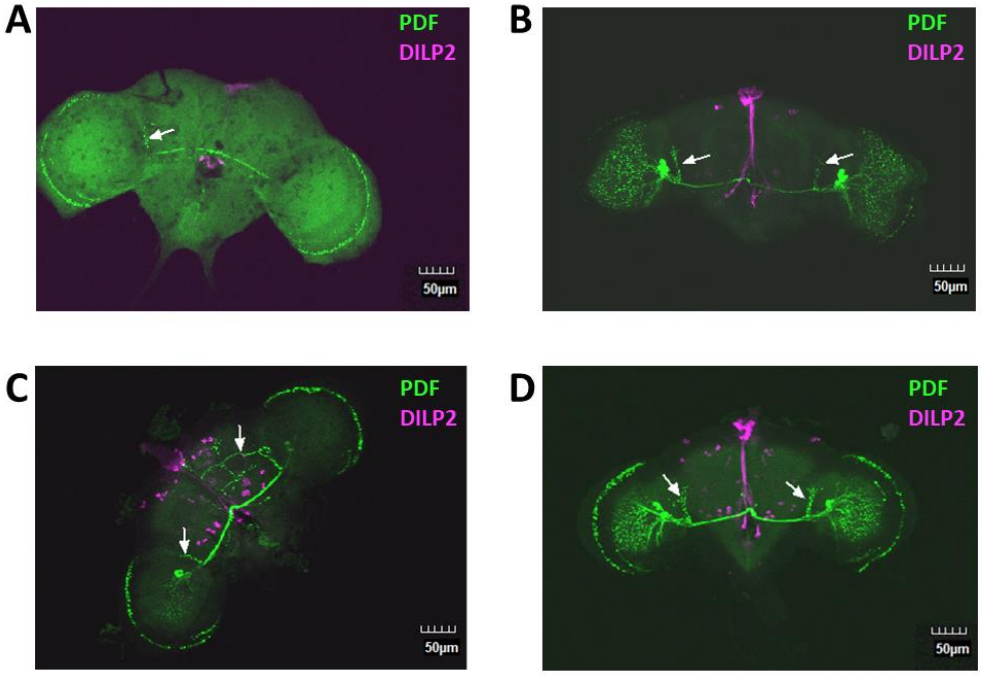


Figure 5-8 PDF (green) and DILP2 (magenta) staining in *Clk$^{Jrk}_{Hu}$* flies (A) reared at 25 °C and placed at 12 °C, (B) 18 °C → 12 °C; (C and D) 18 °C → 25 °C. Arrows indicate the axonal projection from s-LN$_v$.

5.4.3 GRASP

5.4.3.1 GRASP signal appears along the axonal projection from the IPCs in cold conditions

Using the GFP Reconstitution Across Synaptic Partners, the putative interaction between PDF and DILP2 was studied. Expression of Pdf-LEXA>CD4-GFP$_{11}$ and dilp2(P)-GAL4>CD4-GFP$_{1-10}$ in flies reared at 18 °C showed a GRASP signal under diapause-inducing conditions in 9:17 female brains (Figure 5-9). However, when the flies were kept at 25 °C, only 4:16 brains presented a GRASP signal (Fisher's exact test, p=0.15).

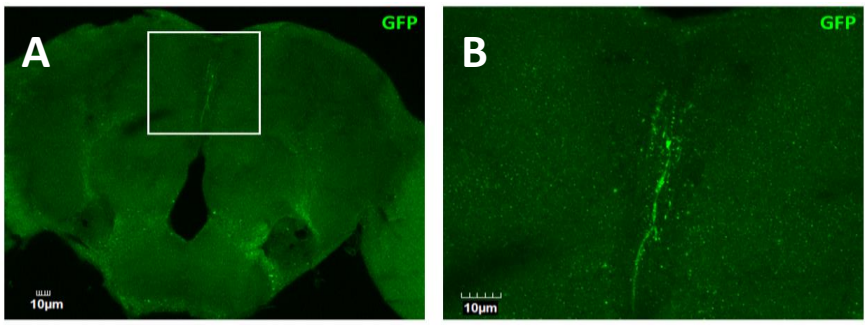


Figure 5-9 GRASP signal (green) between PDF and DILP2 expressing cells using 40x objective (A) and an amplification of the area inside the white square using 60x objective (B).

5.4.3.2 GRASP signals are present in the Tritocerebrum and DLP area when GFP_{1-10} expression is driven by a Corazonin driver

Similarly, the possibility of PDF cells interacting with dorsal-lateral peptidergic neurons was assessed. Expression of GFP_{1-10} in the corazonin cells showed a very distinctive pattern. When the flies were kept at low temperatures (12 °C), a GRASP signal was detected in the anterior aspect of the brain (in the tritocerebrum and along its axonal projection towards the dorsal area), as well as in the medial-posterior aspect (where the DLPs are located) (Figure 5-10). The fluorescent signal near the DLPs is probably an artefact due to the use of an anti-GFP antibody to increase the GRASP signal. However, the axonal projections towards the IPC area and along the posterior lateral tract are clearly marked. When the flies were placed under diapause-inducing temperatures, 12/17 brains displayed strong GRASP signals in the posterior and anterior sections. On the other hand, when the new-born adults were placed at 25 °C, none of the brains showed any GRASP signal in the tritocerebrum (Fisher's exact test reports p=0.002) while 3/8 brains showed the non-specific staining in the posterior sections of the brain.

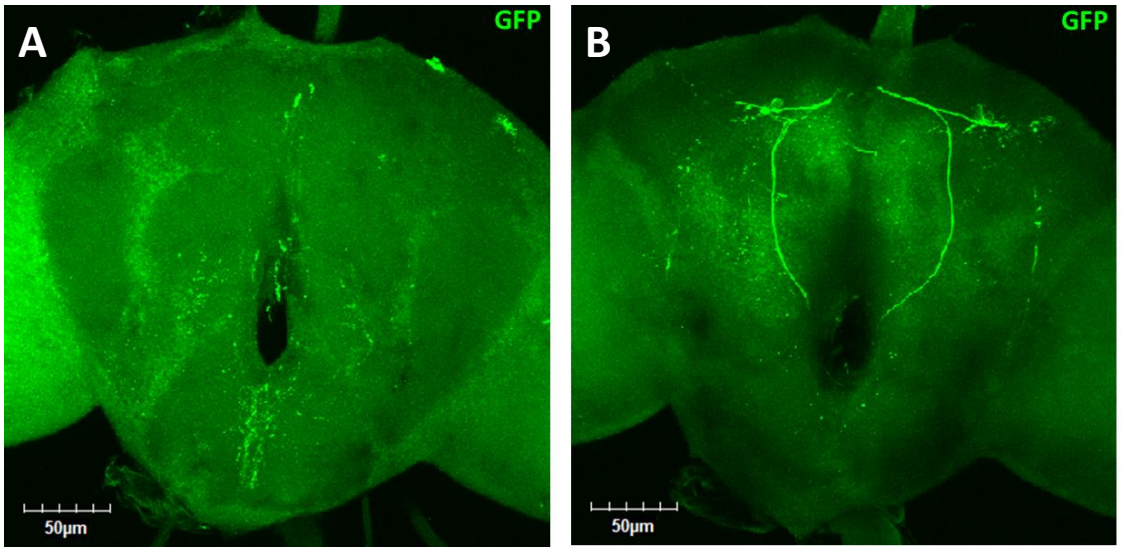


Figure 5-10 GRASP signal (green) between PDF and Corazonin expressing cells. (A) Stack pictures of the anterior (A) and posterior-medial (B) aspects of the same brain using 40x objective.

5.4.4 Downregulation of *rpr* using *Pdf-Gal4* driver inhibits diapause

In order to test the role of PDF-Tri cells in diapause induction, I performed a preliminary experiment in which *Pdf-RNAi* was expressed using *Pdf-Gal4* and *gal1118* (which does not express Gal4 in the PDF-Tri) drivers (Figure 5-11). Additionally, as PDF-Tri have been reported as developmentally transient undergoing apoptosis shortly after metamorphosis, these same drivers were used to express *rpr-RNAi* and promote PDF-Tri maintenance in adult flies. ANOVAs on the *Pdf* and *rpr* downregulation suggest significant differences between the genotypes for both ($F_{(4, 17)}$=9.18, p=0.0004 and $F_{(4, 21)}$=21.04, p<0.0001). However, Tukey's multiple comparison test indicates no significant effect when *Pdf* is downregulated: expression using the *gal1118* driver is significantly different from the Gal4 control, but not from the UAS one. Inspection of Figure 5-11 B shows that expression of *rpr-RNAi* with *Pdf-Gal4*, but not *Gal1118*, downregulates diapause levels significantly in comparison to the driver and UAS controls (p=0.0017 and p=0.0002). On the other hand, *Gal1118>UAS-rpr-RNAi* has lower diapause than the Gal4 control, but it is not different from the UAS control (p=0.0004 and p=0.25).

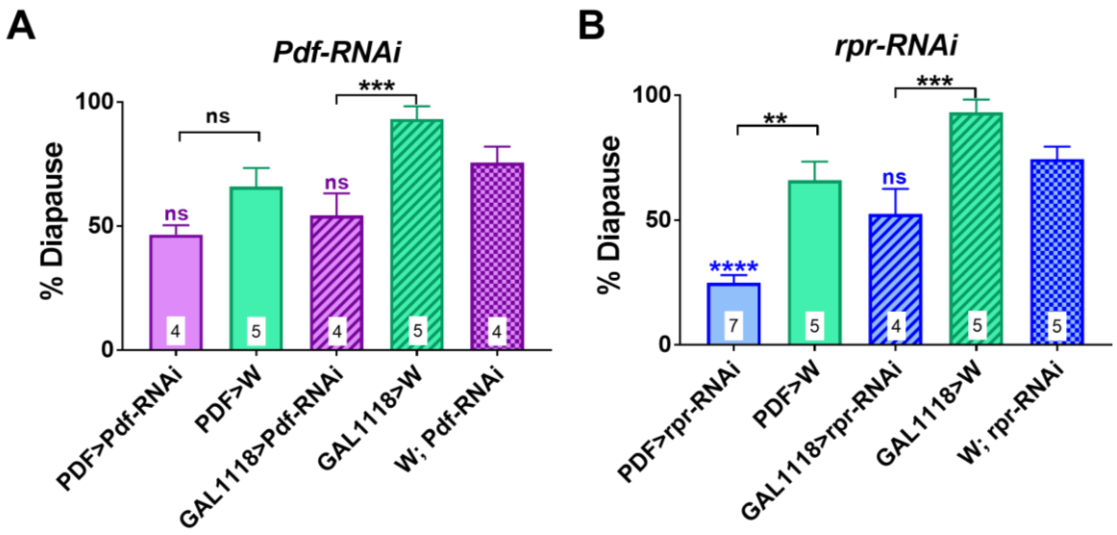


Figure 5-11 Diapause levels at 11 °C for (A) *Pdf-RNAi* (purple) and (B) *rpr-RNAi* (blue) experiments. Data for the driver controls (green) is the same for both graphs. Mean + SEM. Numbers inside the columns represent number of replicas.

5.5 Discussion

PDF and DILP2 staining in adult *D. melanogaster* females detects several structural changes between diapausing and reproductive fly brains. In most of the structures, cells were smaller and axonal projections shorter in the diapausing females, which might suggest that they really are in a dormancy state. Indeed, Kubrak et al. (2014) reported a decreased body size in diapausing flies. The analysis of PDF staining in l-LN_vs reveals a significant increase in both total and mean intensity of PDF in these cells. However, there are no significant changes in the intensity of the posterior optic tract (POT), in which no release of PDF is thought to occur (Helfrich-Forster, 2009). This could indicate a reduced PDF signalling in diapausing flies. Additionally, an increase in the PDF-Tri intensity was observed in the diapausing flies. Since these cells and their arborisations had been reported to undergo apoptosis soon after hatching, it was remarkable finding them in 12 days old adults. It is possible that, with diapause being a state of arrested development, their presence is merely a reflection of this interrupted growth. However, it is also possible that their presence *per se* has an effect over the regulation of this phenotype.

When Cavanaugh et al. (2014) used GRASP to study the possible synaptic connection between the *pars intercerebralis* cells and PDF^+ cells, they concluded that there is no direct contact between them. Nevertheless, when I expressed GFP in the PDF^+ cells and in the IPCs using a *dilp2(P)* driver, I found that 9 out of 17 female brains presented a GRASP signal along the IPCs axonal projections in 12 day old adults that had been kept under diapause inducing temperatures. This means that in approximately half of the WT flies that have been kept at 12 °C there is a direct synaptic connection between PDF^+ and $DILP2^+$ cells, similar to the percentage of flies that undergo diapause in these conditions (see Figure 3.8 in Chapter 3). This signal was temperature-dependent, with more brains with GRASP signal being observed at lower temperature. This may suggest that this interaction is developed via the PDF-Tri as these cells are similarly upregulated in cold temperatures and are known to send projections towards the IPCs. While the axonal projections from the canonical PDF cells have recently been suggested to arrive into the *protocerebrum* and reach the IPCs (Nagy et al., In preparation), they could not generate the signal across the IPC

projections. Although it is also possible that PDF is being released in a paracrine manner until it reaches the IPC axons, this kind of connection would not lead to a GRASP signal, which needs physical interaction between the putative synaptic partners. Nevertheless, such diffusion of PDF has already been reported for activation of PdfR on the LN_ds by the s-LN_vs (Kim et al., 2013), however it seems unlikely as the length of their projections towards the *PI* tend to be smaller in diapausing brains.

The Dorsal lateral peptidergic (DLP) cells are known to regulate the IPCs via two neuropeptides (CRZ and sNPF) that have been linked with stress and reproduction (Kapan, 2012; Kapan et al., 2012; Kubrak et al., 2016; Zhao et al., 2010), so it seemed worthwhile to study whether they could be directly interacting with the PDF^+ cells. Expression of GFP_{1-10} in the corazonin cells produced a GRASP signal in the anterior sections of the brain, near the tritocerebrum and along the IPC axon exclusively in the brains of flies kept at low temperatures. The fact that this signal is only present at 12 °C provides an additional indication that this area in the *tritocerebrum* could be important for cold stress and/or diapause regulation. Nevertheless, after performing several manipulations with *NaChBac* and overexpression of *sNPF* and downregulation of *Crz* experiments, Nagy (2017) concluded that the DLPs were not likely to be involved in diapause regulation in *Drosophila*. However, it should be taken into account that: (1) the use of RNAi has been reported to downregulate *Crz* expression by ~62% at 25 °C (McClure and Heberlein, 2013), however the downregulation might have been significantly lower when the experiment is performed at 12 °C as most of the GAL4 lines contains the 3'UTR of *Hsp70* (and some of the UAS lines contain its 5' UTR), resulting in a rapid degradation of it under non-heat shock conditions (Petersen and Lindquist, 1989; Pfeiffer et al., 2010); and (2) those analysis were performed after growing the flies at 25 °C, while my GRASP experiments were performed after growing the flies at 18 °C. Hence, it follows that a repetition of both the GRASP and diapause experiments under the different protocols might prove fruitful.

DILP2 staining indicated that the IPCs also were significantly smaller in diapausing brains. However, no change in total DILP staining intensity was observed, resulting in a non-significant trend towards higher mean intensity in this condition. Unexpectedly, DILP2 levels seem to be higher in the IPC axonal projections during

diapause. This increase in DILP levels has been reported before: qPCR analysis of *dilp2* and *dilp5* showed an increase of both of these mRNAs in diapause inducing conditions (Kubrak et al., 2014; Schiesari et al., 2016). It is possible that this increase in *dilp2* and DILP2 along the axonal projection is due to a lower release of the neuropeptide to the haemolymph, with its consequent accumulation. It is known that arrival of a neuropeptide to its target tissue can result in negative feedback loop that inhibits the generation and/or release of the neuropeptide. If DILPs are not being released, not only would they accumulate, but the IPCs might receive DILP-production activating signals (or stop receiving production inhibitory ones) which would lead to the increase in production. For example, DILP6, which is produced in the fat body depending on food intake, and is known to inhibit *dilp2* and *dilp5* mRNA and a decrease in circulating DILP2 (Bai et al., 2012; Nassel et al., 2013). It is also possible that these DILPs are sequestered in the haemolymph, for example by Imaginal morphogenesis protein-Late2 (Imp-L2), which can bind to DILP2 and DILP5 and block their interaction with their receptor (Arquier et al., 2008), or secreted decoy of InR (SDR) with a similar effect during development (Okamoto et al., 2013).

There is evidence for PDF^{+} cells being involved in diapause of several insects (Gesto, 2011; Nagy, 2017; Shiga and Numata, 2009). Nagy (2017) observed that hypersensitisation (using *NaChBac*) or overexpression of PDF in PDF^{+} cells led to a significant decrease in diapause. However, a simple model in which PDF inhibits DILP production or release in the IPCs (Figure 5-12_A) could explain the non-significant differences in diapause between the different rearing conditions in the WT flies (Figure 5-12_B) but does not explain the dramatic difference in diapause levels reported for Pdf^{0} mutants grown following the standard or the more natural protocol described in Chapter 4 (Figure 5-12_C).

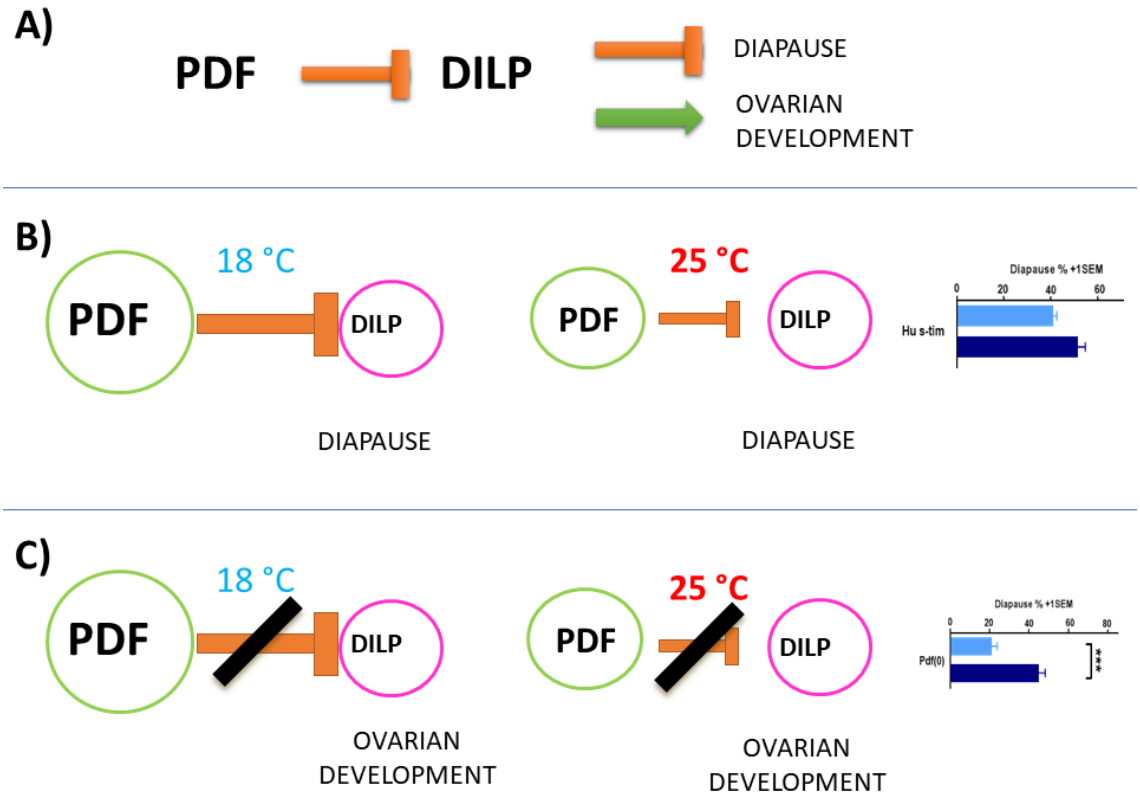


Figure 5-12 Simple model of diapause regulation by PDF. (A) PDF inhibits DILPs, which promote ovarian development and inhibit diapause; overall, enhancing diapause. (B) Testing the model in a WT background, it seems to fit adequately: slight increase in PDF from the Tritocerebrum when the flies are reared at 18 °C results in a non-significant decrease in diapause for the strain. (C) However, when the model is tested in a *Pdf*^{0} background it fails as independently of the rearing temperature, the absence of PDF should result in a decreased inhibition of the IPCs and consequently in ovarian development. However, the *Pdf*^{0} mutant has been reported to have high levels of diapause, comparable to WT or higher. As PDF levels in the Tritocerebrum are increased when the flies have been reared at 18 °C, the PDF circles are slightly bigger in this rearing condition.

This discrepancy in the *Pdf*^{0} flies might suggests the existence of (an) additional regulator(s) of the DILPs of at least equal strength than PDF (Figure 5-13_A), that in WT conditions would balance each other out, leaving a roughly 50:50 diapause induction (Figure 5-13_B), while in the *Pdf*^{0} mutants a higher expression of this DILP activator when the flies are reared at lower temperatures might explain the discrepancy in the diapause levels between the two rearing conditions (Figure 5-13_C).

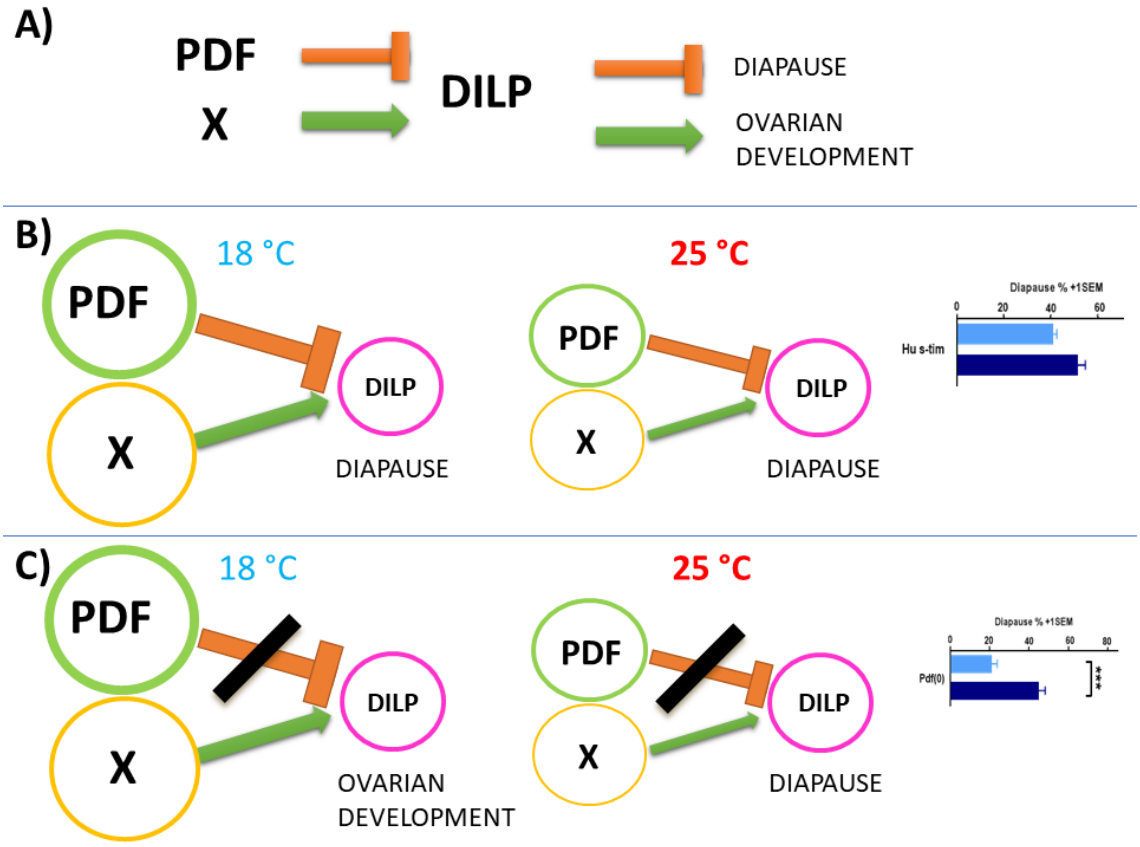


Figure 5-13 Model of diapause regulation by PDF and an additional unidentified factor (X). (A) PDF inhibits DILPs and enhances diapause, while X has the opposing effect promoting DILPs and ovarian development. (B) Testing the model in a WT background, it seems to fit adequately: slight increase in PDF from the Tritocerebrum when the flies are reared at 18 °C is compensated by a similar increase of X which results in similar diapause levels in both rearing conditions. (C) In this model, as both PDF and its antagonist, X, have higher expression levels when the flies are reared in colder temperatures, the mutant will have a stronger pro-development signal at 18 °C than at 25°C, which could account for the higher development reported in the former. The size and thickness of the circles is proportional to the amount of the peptide (PDF, X, or DILPs).

Moreover, PDF has been reported to inhibit diapause in *D. melanogaster* (Nagy, 2017) while the presence of PDF-Tri at cold temperatures seemed to enhance diapause. It has been reported that PDF can have different actions depending on their target. For example, longer period length was observed after increasing PDF levels in the accessory medulla, but it was shortened when this PDF increase was in the dorsal protocerebrum (Wulbeck et al., 2008; Yoshii et al., 2009). It is possible that the cellular responses after PDF binding are different depending on the origin of the neuropeptide, resulting in these diverse effects. Additionally, the PDF expressed in the large and small

LN_vs is different. The expression of *dimmed* in the l-LN_vs results in the amidation of the C-terminus of PDF, while the s-LN_vs generate a non-amidated form (Helfrich-Forster, 2009; Park et al., 2008). These amidated and non-amidated peptides have different stabilities and activity profiles: the amidated PDF has a longer half-life and is more active (Helfrich-Forster, 2009). This ratio between amidated and non-amidated PDF might be temperature-dependant (indeed a significant increase in the amidated PDF produced by l-LN_vs is reported in the diapausing brains) and affects whether the fly enters diapause (Figure 5-5_C). Additionally, as the PDF-Tri had been classified as developmentally transient, it is not known whether these cells express the amidated or non-amidated form of the peptide. However, the fact that a strong PHM (one of the two amidation-catalysing enzymes in *Drosophila*) staining was reported in the neuropil surrounding the esophageal foramen, where the PDF-Tri are found (Taghert et al., 2001), may suggest that PDF-Tri carries the amidated form. However, the specific cells within the esophageal foramen containing the enzyme were not identified.

The neuronal network of the circadian cells has been broadly studied. The different clusters have been shown to communicate with each other either directly, through axonal projections, or indirectly, through peptide release (for example, PDF). Shafer et al. (2008) determined that most of the clock neurons, except for the l-LN_vs, are responsive to PDF as they express a PdfR. It has been determined that the s-LN_v directly interact with the DN1s (Seluzicki et al., 2014). Additionally, PDF secreted from these small cells has been reported to activate PdfR in LN_d, increasing mating time (Kim et al., 2013). PDF released from the l-LN_v has been established, through diffusion, to activate its receptor on the s-LN_vs (Shafer et al., 2008), but also some of the evening cells such as PdfR expressing LN_ds and 5^{th} PDF^- s-LN_v (Schlichting et al., 2016). DNs and LN_ds send projections towards the protocerebrum and *pars intercerebralis*, were the IPCs are located.

A third cluster of (apparently) non-circadian PDF^+ cells are located in the tritocerebrum (PDF-Tri) and are known to extend their projections towards the *pars intercerebralis* near the IPCs, which in turn send axonal projections towards the tritocerebrum. These PDF-Tri cells have been reported to not express PER (Gatto and Broadie, 2011) and their retention does not cause alteration on the rhythmicity of the

flies (Renn et al., 1999). Although PDFR expression in the IPCs has not yet been reported, Lear et al. (2005) described a high expression of its transcripts in the *pars intercerebralis*. Indeed, Nagy (2017) determined that IPCs respond to bath applications of both PDF and sNPF, which appeared to act synergistically. Surprisingly, *han* PDFR mutants not only impaired the response to PDF but also to sNPF, suggesting that cAMP of sNPF was PDF-dependant.

From locomotor activity studies, it is known that some of the DNs contribute more towards the circadian network when there is less PDF (ie. period gets shorter) (Helfrich-Forster, 2009). If at colder temperatures, there is a decrease in PDF signalling (as suggested by the increase in PDF in l-LN$_v$s with no consequent increase in their POT projections in diapausing flies) it is possible that these DNs might be more active and have an inhibitory effect towards the IPCs, promoting diapause. On the other hand, PDF-Tri staining is higher when the fly is in diapause. This allows the generation a preliminary model of diapause regulation in the brain of *D. melanogaster* in which PDF could be having a dual effect over the IPCs: directly (through the PDF-Tri) it could exert an inhibitory role on development, promoting diapause; while indirectly (through inhibition of DN and LN$_d$s) it could function as an IPC activator (Figure 5-14).

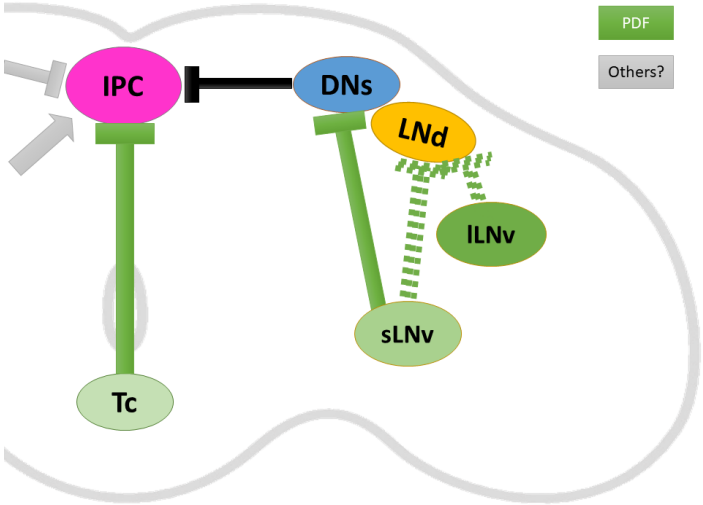


Figure 5-14 Model of PDF regulation of diapause in *D. melanogaster*. s-LN$_v$s and l-LN$_v$s regulate LN$_d$s by diffusion of PDF, while s-LN$_v$s directly interact with DN1s. DN1s directly interact with the IPC and inhibit DILP synthesis and/or release. This way, PDF signal from LN$_v$s have an IPC activating role (promoting ovarian development). On the other hand, PDF from the tritocerebrum area acts directly over the IPCs, inhibiting them and promoting diapause. Additionally, some other IPC activating and inhibitory signals might help to shape the balance between diapause or reproduction.

There are two key hypotheses in this model: first, that the DN inhibit IPCs and, second, that PDF-Tri inhibit IPCs. To gain further insight into the effect exerted by the DNs over the IPCs in a diapause context several diapause experiments could be performed. For example, if the DNs are inhibiting IPCs and promoting diapause, expression of CRYΔ or NaChBac using PDF-Gal4 should result in a reduction of the DNs activity (locomotor period gets longer) and diapause levels should decrease. Indeed, Nagy (2017) reported a reduction on diapause using NaChBac. On the other hand, the effect of expressing CRYΔ or NaChBac in the DNs using *tim-Gal4; cry-Gal80*, should result on higher diapause levels.

To my knowledge, there is no Gal4-driver expressing specifically in the PDF-Tri, so an indirect approach was used in order to test the role of the PDF-Tri in diapause induction. *Pdf-* or *rpr-RNAi* were expressed either under *Pdf-Gal4* driver, which drives expression in all the PDF^+ cells including the PDF-Tri, or under the enhancer trap *gal1118* line. This line drives expression in PDF^+ cells except for the PDF-Tri; however, it has also been shown to drive expression in other clock neurons, medulla, *pars intercerebralis* amongst others (Blanchardon et al., 2001). Furthermore, due to the lack of time, the number of replicas performed is low. Hence, the results obtained should be treated with caution. No significant effects were observed when *Pdf* was downregulated. Inhibition of apoptosis using *rpr-RNAi* seems to significantly reduce diapause levels with *Pdf-Gal4* but not with *gal1118*, which could indicate that the PDF-Tri plays a role in diapause induction. This apparent lack of effect when downregulating PDF, may suggest that the tritocerebrum cells may use a different neuropeptide. A candidate might be sNPF (which is expressed broadly in the *Drosophila* brain, and by s-LN_vs). Nevertheless, the experiment was performed after rearing the flies at 25 °C and even Pdf^0 mutants did not display significant diapause changes using this protocol. It would be useful to assess the effect of these genetic manipulations after rearing the flies at 18 °C.

Finally, clock mutants show dramatic structural differences compared to the WT flies such as increased PDF-Tri staining, even at high temperatures, and aberrant projections. Some of these changes, such as the projection towards the dorsal protocerebrum from the s-LN_vs in Clk^{Jrk} mutants, seem to be temperature-of-growth

dependant. Hence, the differences reported in Chapter 4 between the two rearing conditions for some of these clock mutants might be due to the severity of the brain phenotype rather than to an effect of a disrupted clock. Additionally, it should be taken into account, that the differences observed in the diapause *vs* non-diapause brains were very marginal. If the differences between 100% diapause and 100% reproductive are small, the differences between a line with 40% or 60% diapause will be even smaller. Nevertheless, comparison between the lines kept at 12 °C or at 25 °C show that PDF-Tri levels tend to be enhanced and POT smaller in diapause-inducing conditions.

Overall this chapter suggests that:

- Structural changes in the diapausing brains include a trend towards smaller cells and shorter projections, which could suggest that this phenomenon is indeed a dormancy state.
- PDF signalling from the s-LN_vs seems to be reduced in diapausing flies, although a significant increase in PDF-Tri staining (which had been reported to undergo apoptosis in adult flies) is observed. Unexpectedly, DILP2 levels seem to be increased in diapausing flies. However, it is possible that this increase in DILP2 reported along the axonal projection is due to a lower release of the neuropeptide to the haemolymph.
- A preliminary model in which PDF might have a dual effect in diapause regulation depending on whether they regulate the IPCs directly (PDF from the PDF-Tri inhibit IPCs) or indirectly (PDF from LN_vs through DNs or LN_ds activates IPCs) was presented.
- Clock mutants display significant structural differences compared to WT flies, which might explain their diapause levels alterations.

6. *period* SPLICING

6.1 Introduction

6.1.1 Alternative splicing of *per* 3'UTR

The alternative splicing of the 89bp *dmpi8* located in the 3' UTR of *per* has been associated with seasonal adaptation (Collins et al., 2004; Low et al., 2008; Majercak et al., 1999). At colder temperatures, the spliced variant is more abundant, which results in a faster accumulation of *per* mRNA and an earlier clock phase (with reduced siesta time and an earlier evening peak). On the other hand, at warmer temperature, there is a low level of splicing of *dmpi8*, slower accumulation of *per* (and PER) and, consequently, a later clock phase with an enhanced siesta time and morning and evening activity levels focused around the lights-on/off transitions (Collins et al., 2004; Majercak et al., 2004; Majercak et al., 1999) (See Figure 6-1). Additionally, short photoperiods have also been shown to enhance splicing in the 3'UTR of *per*. Majercak et al. (1999) found that long photoperiods counteracted the effects of cold temperature. This is because long photoperiods delay TIM accumulation, affecting PER stability and accumulation. Hence, *per* splicing seems to be important for seasonal adaptation via PER accumulation related to both temperature (which has a direct effect on *splicing*) and photoperiod (indirectly, by changes in TIM accumulation). Furthermore, Collins et al. (2004) showed that mutants of *norpA* which encodes phospholipase C (PLC), regulates *period* splicing, because the mutants had a higher level of *per* splicing and displayed cold-adapted locomotor behaviour independently of the temperature and time of the day (Collins et al., 2004). Indeed, *norpA* mutants are

unable to entrain to temperature cycles in LL (Glaser and Stanewsky, 2005) possibly because they do not have normal temperature-dependent *per* splicing.

Low et al. (2012) identified two *per* 3'UTR natural variants (known as VT1.1 and VT1.2 and which differ by six polymorphisms, four SNP and two deletions) that significantly affected splicing of *dmpi8*. In particular, they found that the last two SNPs, which are located 3' to the *dmpi8*, enhance splicing. This temperature controlled *dmpi8* splicing may be species-specific as even if similar introns in the 3' UTRs of *D. yakuba* and *M. domestica* have been found, their splicing has been shown to be temperature-independent (Bazalova and Dolezel, 2017; Low et al., 2008). However, the molecular mechanisms that lead to flies expressing different 3' UTR splicing variants, with no change in the protein sequence, is unclear. In this case, the different UTR-splicing events are thought to play a role in mRNA stability and retaining (or not) the intron results in greater (or lower) mRNA stability and hence control the accumulation of the final product.

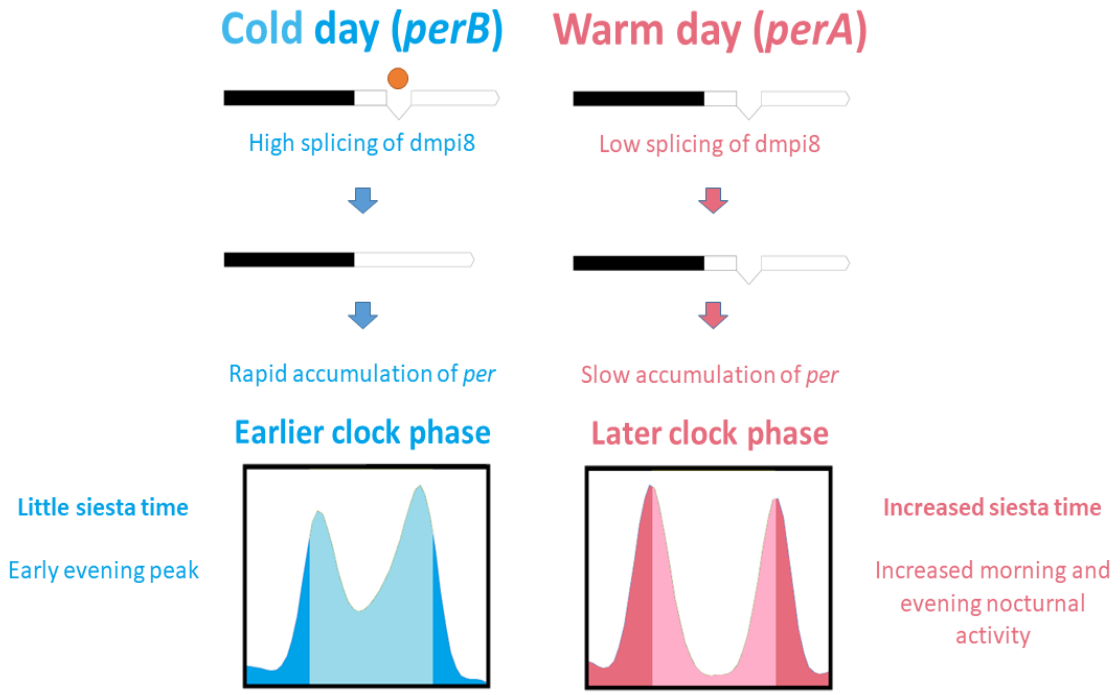


Figure 6-1 Cold days enhance *period* splicing, which results in a faster PER accumulation and an earlier clock phase with reduced siesta time and advance in the evening peak. Adapted from Majercak et al. (1999).

Similarly, *timeless* also displays temperature-sensitive splicing. In this case, the $tim^{unspliced}$ variant predominates at cold temperatures generating a 33 aa shorter TIM

isoform as the intron contains a premature STOP codon (Boothroyd et al., 2007). This isoform has been shown to display increased affinity for CRY over the warmer *tim*$^{spliced=normal}$ form (independently on the *s/ls-tim* polymorphism) and is speculated to control seasonal responses (Montelli et al., 2015).

6.2 Aims

Collins (2014), tested the effect of *dmpi8* splicing on diapause. He used the splicing-locked flies generated by Cheng et al. (1998) and observed that the summer *per* variant (unspliced) showed very low levels of diapause whereas the winter splice-locked variant showed the same levels as the wild-type transgene. In this chapter, I confirm his preliminary results and generate UAS-*per* splicing locked transgenic flies in an attempt to dissect the neuroanatomic areas that regulate diapause using several Gal4 drivers to express *per* isoforms in different cell subsets.

6.3 Methods

6.3.1 Generation of *UAS-per* splicing transgenic flies

6.3.1.1 pUASTattB Plasmid

pUASTattB plasmid described in Bischof et al. (2007) was kindly donated by Prof. Basler. It contains the same *UAS-MCS-SV40* cassette as the original *pUAST* vector by Brand and Perrimon (1993) [which allows transcription of whichever gene is inserted within the multiple cloning site, MCS, adjacent to the *Upstream Activating Sequence*. Additionally, there is a SV40 termination signal]. The *white*$^{+}$ sequence allows easy identification of the transformants when the plasmid is injected in *white*$^{-/-}$ flies. An *attB* [bacterial attachment site, also known as donor sequence] is also present (See Figure 6-2).

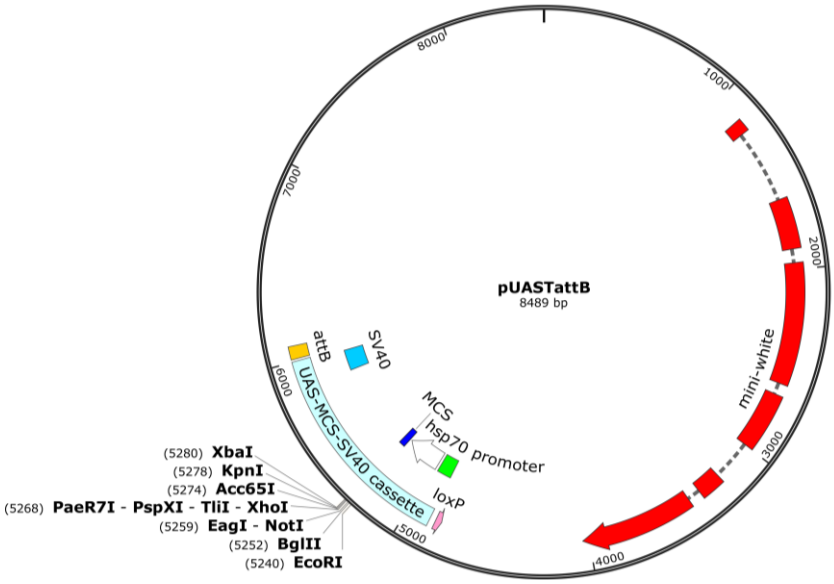


Figure 6-2 pUASTattB map vector. *white*, loxP *UAS-MCS-SV40* and *attB* sequences are reported. Created with SnapGene®.

This plasmid is injected into flies that contain the 221 bp *attP* or phage attachment site and ΦC31 integrase mediates recombination between these two attachment sites and allows the integration of pUASTattB cytospecifically into the landing site. When this happens, two hybrid sites are generated which are no longer recognised by the ΦC31 integrase, hence resulting on an irreversible insertion of the plasmid (Figure 6-3).

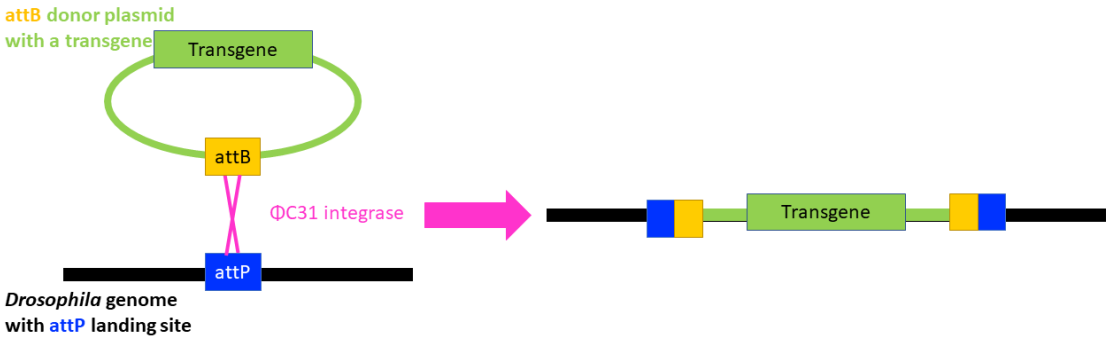


Figure 6-3 Schematic mechanism of ΦC31-mediated integration of *pUASTattB* into a landing-site containing *Drosophila*. Adapted from Fish et al. (2007).

6.3.1.2 Bacterial Transformation

Chemical transformation was carried out by mixing 5 ng of PCR product or DNA for transformation with StrataClone or Stellar™ Competent Cells, respectively. Cells

were incubated on ice for 20-30 min. Afterwards, they were heat shocked for 45 s at 42 °C and placed back on ice for 1-2 min. 250 µl or 500 µl, respectively, of pre-warmed LB medium (Appendix Table 9-16) were added to the mixture and the cells were incubated at 37 °C for 1 h with agitation. After this recovery time, 5 µl and 100 µl of the transformation mixture was spread in LB plates (Appendix Table 9-17) with the correct antibiotic for selection of the plasmid-of-interest containing cells. The plates were incubated overnight at 37 °C and single transformant colonies were selected and tested for *period* via PCR. Several of the colonies were then grown in ~ 5 ml of LB with antibiotic and incubated at 37 °C overnight. These cultures were used for plasmid DNA extraction as explained in Material and Methods 2.10.1 and sequenced.

6.3.1.3 Cloning and injection strategy

Due to the size of the gene and, specially, to the fact that three different 3'UTRs were to be attached to the same gene, a two-step cloning strategy was taken to generate p*UASTattB-perA* (unspliced), p*UASTattB-perB* (default splicing) and p*UASTattB-perG* (WT, with temperature-dependent splicing). cDNA from the *period* splicing-locked transgenic flies from Cheng et al. (1998) was generated as outlined in Material and Methods 2.5-2.8 (using the Trizol method). The first half of the *per* cDNA (1-2418 bp) was amplified and cloned into *pUASTattB,* inserting a *XhoI* site in the 3' of the sequence with a synonym single nucleotide change G→C. The second half (2418-4157 bp) containing the 3'UTRs were cloned into the StrataClone vector and sequenced. The vectors containing the correct 3' UTRs were digested with *XhoI* and *XbaI* to extract the second half of *period* and were inserted into a previously linearized (by digestion with *XhoI* and *XbaI*) *pUASTattB_per5* (Figure 6-4). A list of the primers used for cloning and/or sequencing can be found in Appendix Table 9-18.

The plasmids were subsequently sent to Fly Facility (Genetics Department, University of Cambridge) for injection in *vas-int; attp-86Fb* flies which express PhiC36 integrase under the control of *vasa* and contains an attP site in the 3rd chromosome. For simplicity, the lines will be referred to as *UAS-perA*, *UAS-perB* and *UAS-perG*. The original *vas-int; attp-86Fb* flies (24749, Bloomington) with no vector will be used as an additional control.

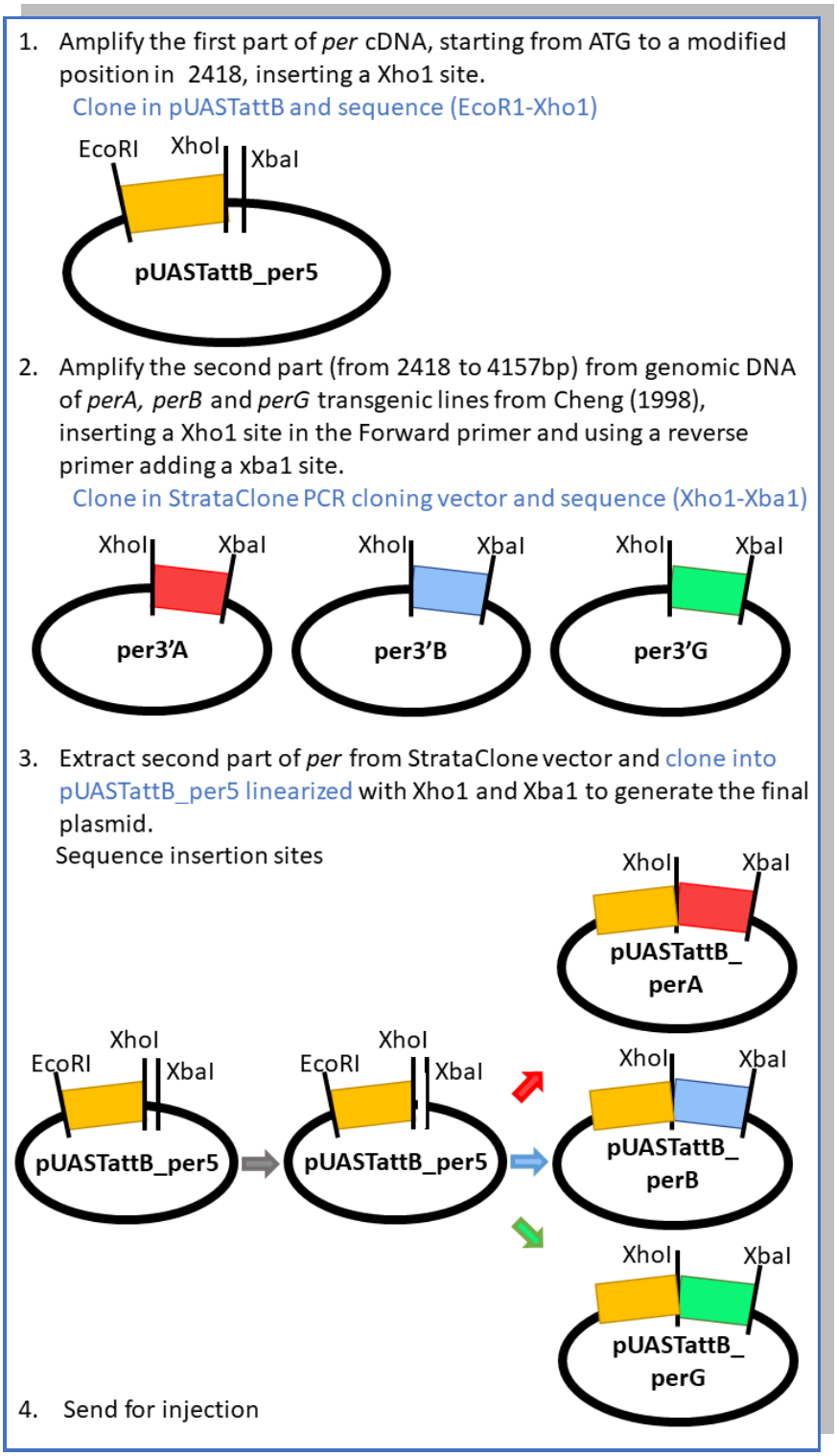


Figure 6-4 Overview of *pUASTattB-per* splicing locked flies' generation.

6.3.2 Drosophila Stocks

per splicing locked transgenic lines in a per^{01} background were obtained from Isaac Edery (Rutgers University, NJ). *perA* expresses the summer-like unspliced variant;

perB, the winter-line default splicing variant (the transgene does not contain the intron) and *perG*, encodes a WT *per* transcript, which undergoes natural temperature-controlled splicing (Cheng et al., 1998).

Additionally, *UAS-perA/B/G/-* generated as described in the previous section 6.3.1 and several Gal4 lines to drive the expression of different splicing variants in specific subsets of cells were used (see Table 6-1). All lines were put in the *w, per^{01}* background following the backcrossing scheme shown in Figure 6-5. Although, it shows the crosses when your favourite gene (YFG) is in the third chromosome, this same method could be used for flies with the YFG in the second chromosome. None of the lines used had the COI in the first chromosome.

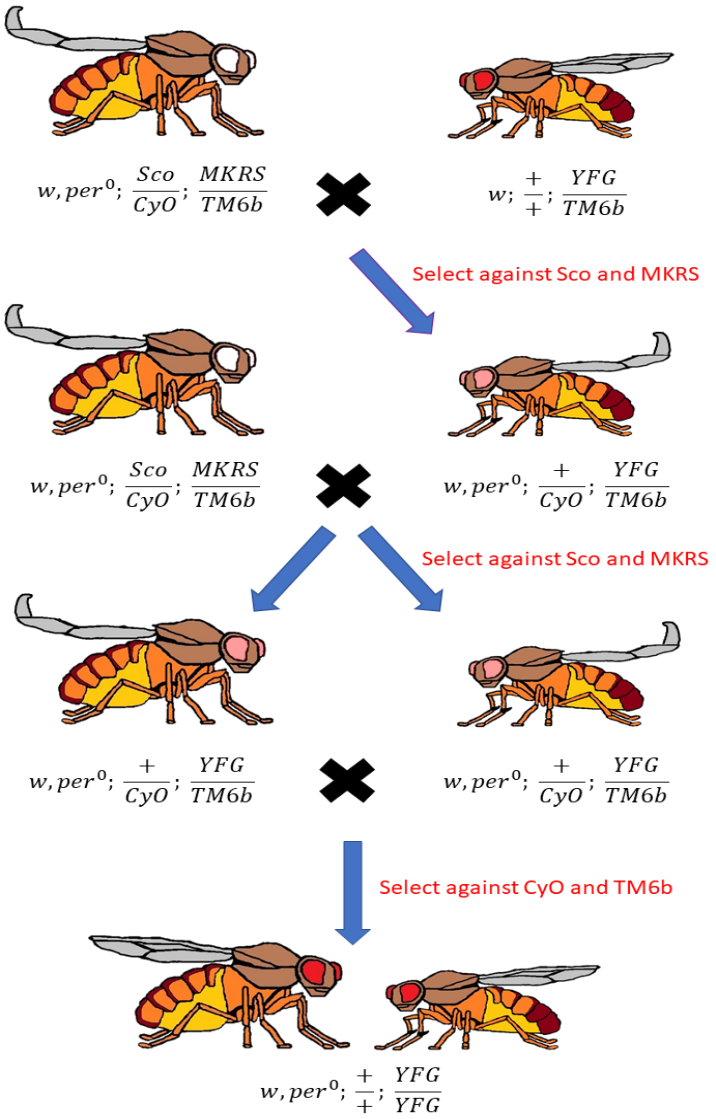


Figure 6-5 Crossing scheme to place the transgenes into *w, per^0*. YFG, your favourite gene (could be a Gal4-driver or a UAS line).

Table 6-1 List of GAL4 drivers, expression pattern and their origin. MB, mushroom bodies; Me, medulla; *PI, pars intercerebralis*.

Fly Stock	Expression in adults (cells per hemisphere)	Reference
tim-Gal4	5 s-LNv + 4 l-LNv 6 LNd + 3 LPN DN1 + DN2 + DN3	(Kaneko et al., 2000; Tataroglu and Emery, 2014)
gmr-Gal4	Eyes, Wing Others	(Freeman, 1996; Li et al., 2012)
Rh6-Gal4	R8 (Yellow ommatidia)	(Sprecher and Desplan, 2008)
Rh5-Gal4	R8 (Pale ommatidia)	(Sprecher and Desplan, 2008)
Pdf-Gal4	4 s-LNv (-5th) + 4 l-LNv PDF-Tri Abdominal ganglion	(Park et al., 2000; Renn et al., 1999)
Gal1118	4 s-LNv (-5th) + 4 l-LNv LNds NO PDF-Tri Few cells ~ MB, Me & PI Subgroup of DN1 + DN2?	(Blanchardon et al., 2001)
R6-Gal4	4 s-LNv (-5th) Tracheal cells?	(Helfrich-Forster et al., 2007)
PdfR-Gal4	5 s-LNv 3 LNd (CRY+) Most DN1 + DN2	(Beckwith and Ceriani, 2015)
cry-Gal4	5 s-LNv + 4 l-LNv 3 LNd 2 DN1	(Klarsfeld et al., 2011)
Mai179-Gal4	5 s-LNv + Some l-LNv 3/6 (PDF-) LNd	(Grima et al., 2004)
Clk4.1M-Gal4	DN1	(Zhang et al., 2010)
Clk9M-Gal4	4 sLNv (-5th) DN2	(Kaneko et al., 2012)
Further manipulations with GAL80		
tim-Gal4; cry-Gal80	3 LNd + 3 LPN Some DN1 + DN2 + DN3	(Tataroglu and Emery, 2014)
tim-Gal4; Pdf-Gal80	5th s-LNv 6 LNd + 3 LPN DN1 + DN2 + DN3	(Stoleru et al., 2004)
Pdf-Gal4; cry-Gal80	PDF-Tri?? Abdominal ganglion??	
cry-Gal4; Pdf-Gal80	5th s-LNv 3 LNd DN1	(Stoleru et al., 2004)

6.3.3 General experimental workflow

Flies were reared using the standard protocol of 25°C in LD 12:12. The diapause induction protocol was slightly altered to test for different maintenance temperatures. Hence, the newly hatched flies were placed for 12 days in LD 8:16 at either 12°C, 11°C or 10°C depending on the experiment. After this time, the corresponding experiment (diapause or Western Blot) was performed.

6.3.3.1 Scoring diapause

Diapause was scored as described in Material and Methods 2.2.2.

6.3.3.2 Western Blot

Protein extraction and subsequent PER/TUB/HSP70 quantification was performed as described in Material and Methods 2.13-2.14 with samples collected every 3 h.

6.3.4 Statistics

CircWave V1.4[1] was used to perform cosinor analysis on the Western Blot data and get the fitted curve, which was then drawn in GraphPad Prism 7 along with the means for each data-point.

6.4 Results

6.4.1 *perA* flies do not enter diapause at 12°C

Inspection of Figure 6-6 shows a significant decrease in diapause levels when the flies only expressed the 'summer-like' unspliced *perA* compared to *perB* ('winter-like' default splicing, $p<0.0001$) and *perG* (WT, with temperature-dependent splicing $p<0.0001$). However, when the experiment is repeated under more restrictive conditions (diapause-induction at 10°C) no differences in diapause were detected.

[1] http://www.rug.nl/fwn/onderzoek/programmas/biologie/chronobiologie/downloads/index

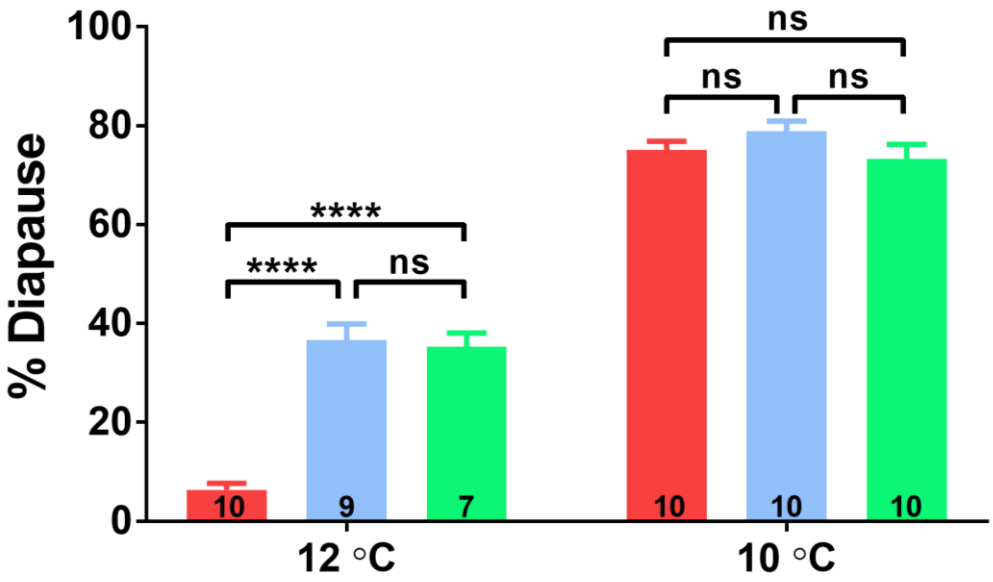


Figure 6-6 Diapause level of *perA* (red), *perB* (blue) and *perG* (green) transgenic flies at 12°C (left) and 10°C (right). The numbers in the bottom of the column represent the number of replicas. Mean + SEM. *****<0.0001; ns=non-significant.*

6.4.2 None of the splicing-locked transgenic flies display rhythmic PER expression at 12 °C

Figure 6-7 displays a one-day time-course of PER levels normalised against the mean of HSP70 and TUB in eight time-points after the splicing-locked transgenic flies had been maintained at 12 °C in LD 8:16 for 12 days. The fitted curves obtained using CircWave V1.4 software were not significantly rhythmic (p=0.53, p=0.46, and p=0.53 for *perA*, *perB* and *perG*). Hence, in these transgenic flies, *period* is not expressed in a cyclic manner at 12 °C. Comparison of the total PER levels, measuring 5 replicates for each transgenic line in which samples of each time point were mixed together, showed no significant difference between the three lines although *perB* displayed a trend to have slightly higher PER levels than the other two (Appendix Figure 9-14).

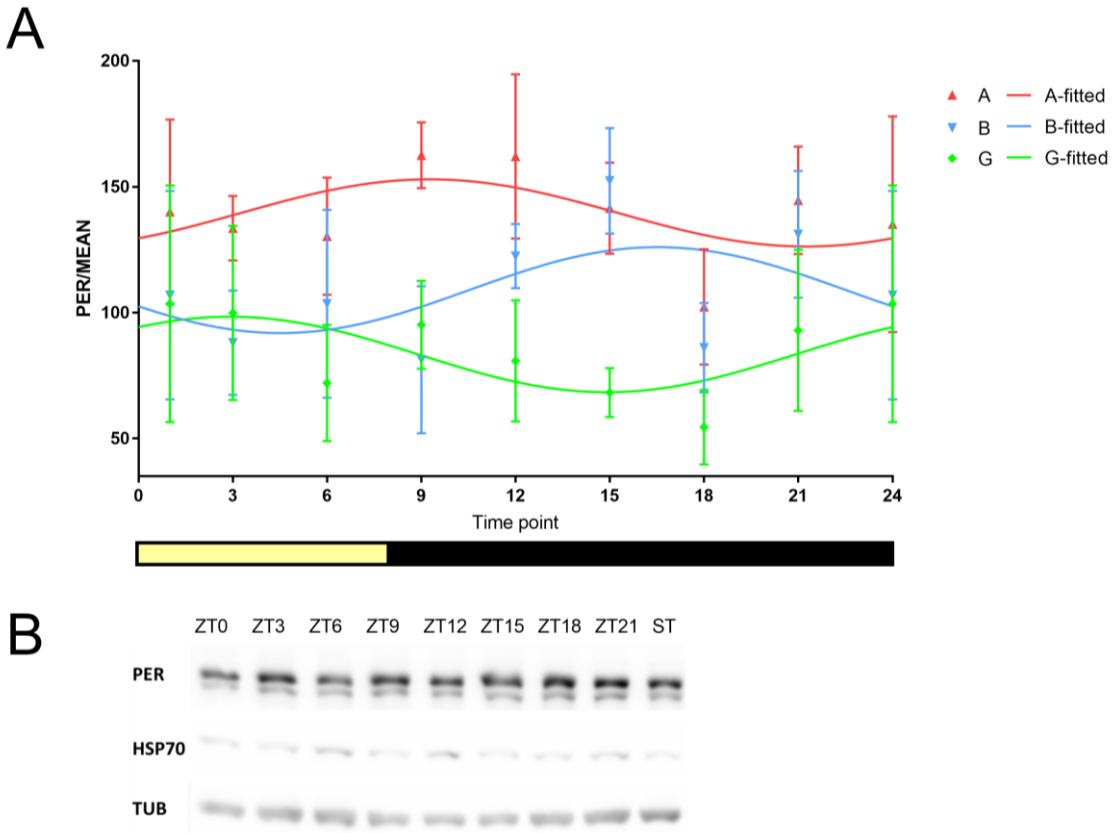


Figure 6-7 (A) Time-course of PER adjusted against two internal controls (HSP70 and TUB) at 12 °C in *perA* (red), *perB* (blue) and *perG* (green) transgenic flies. The '24 h' data-point is the same as ZT 0. Mean ± SEM for each data point and fitted curves are represented. The yellow and black bars underneath represent LD 8:16. (B) Example of a perA WB gel. ST, Standard sample.

6.4.3 Diapause with *UAS-perA/B/G* lines

6.4.3.1 Best diapause results were obtained when the induction was carried out at 11°C

As previous studies using the UAS-GAL4 system have displayed low diapause levels and *UAS-perA* is expected to lower them even more, an initial assay was performed to choose the experimental temperature. Figure 6-8 displays diapause after crossing the lines with *tim*-GAL4, which drives expression of the different *UAS-per* transgenes in all clock cells. The experiment was performed at 12°C (usual diapause-induction temperature), 11°C and 10°C (temperature in which no difference is observed among the original *perA/B/G* transgenic lines). As *per* splicing is enhanced in cold temperatures, similar diapause induction was expected for the *perB* and *perG*

expressing flies (which would either only or mainly, respectively, contain the spliced variant). On the other hand, the *per*^{0} flies expressing no *per* transgene were expected to have significantly reduced diapause compared to the *perG* controls (Figure 5.2 from Collins (2014)). At 11°C, flies expressing *perA* (red) have similar levels of diapause than the no-transgene *per*^{0} flies (orange) which are significantly reduced compared to both *perB* and *perG* ($p<0.05$). A similar trend is observed when the experiment is performed at slightly higher temperatures, although the differences are significantly reduced. As with the original transgenic lines from Edery's lab, no difference was observed when the experiment was performed at 10°C.

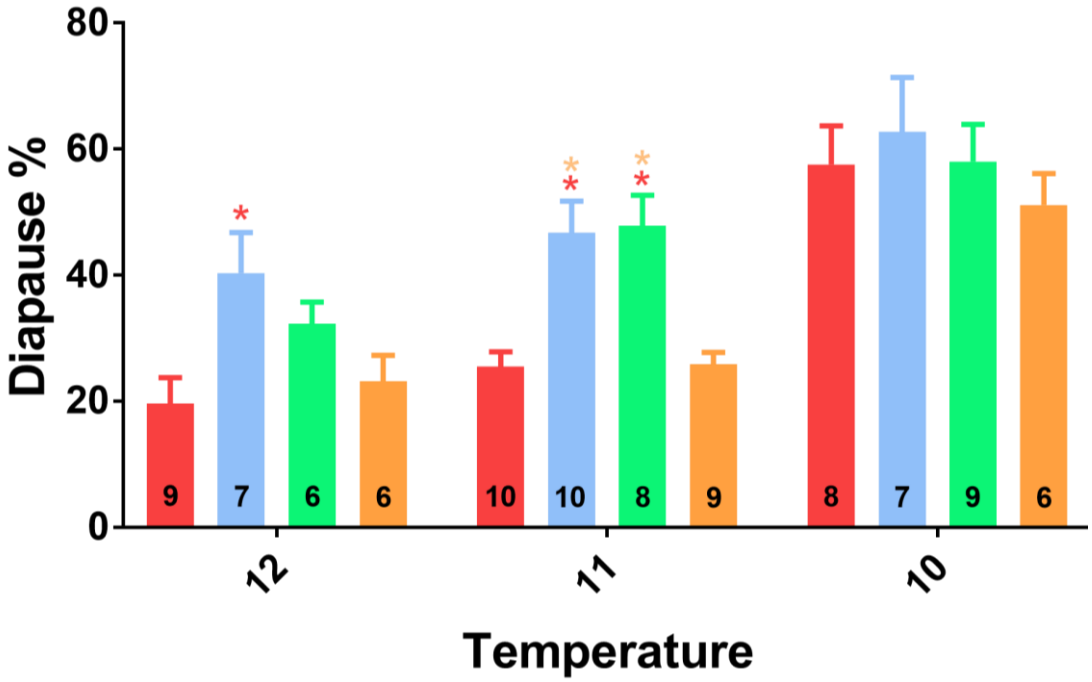


Figure 6-8 Diapause level of *UAS-perA* (red), *-perB* (blue), *-perG* (green) and no-transgene (orange) expressed using *tim-Gal4* driver at 12 °C (left), 11 °C (centre) and 10 °C (right). The numbers in the bottom of the column represent the number of replicas. Mean + SEM. **<0.05.*

6.4.3.2 Genetic dissection of diapause

Given the results of the previous experiment, 11°C appeared to be the optimal temperature at which to carry out further study of the *perA/perB* effects on diapause. Different GAL4 lines were used to drive expression of the *per* splicing variants in specific subsets of cells in an attempt to identify the key neurons.

Figure 6-9 shows the results for various clock drivers. *tim-gal4, cry gal4;Pdf-gal80* and *Clk9M-gal4* as well as the eye drivers *gmr-gal4* and *Rh6-gal4* reveal that the *perB* and *perG* driven constructs generate significantly higher levels of diapause than *perA,* identifying the eyes and several combinations of clock neurons as sufficient for the seasonal difference.

While I was performing these experiments a colleague in the laboratory, observed that the *UAS-perG* line had been contaminated and was no longer in per^0 background. We then discovered that the *w,* per^0*; Cyo/Sco; TM6b/MKRS* double balancer line that was used for placing the lines in the per^0 background had been contaminated with per^+ compromising the whole experiment. Consequently, these results and those with other drivers that were performed before we knew about the contamination (see Appendix Figure 9-15) should be treated with caution.

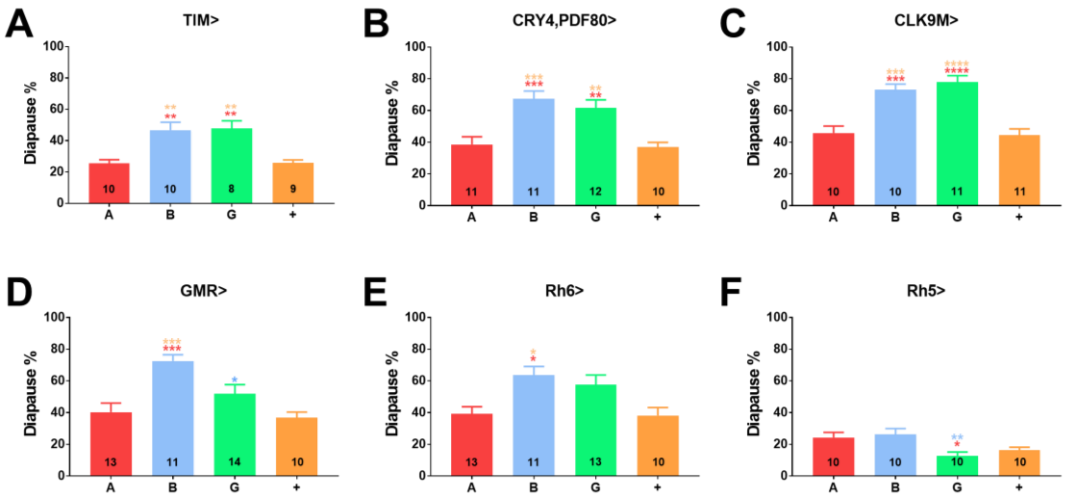


Figure 6-9 Diapause level of UAS-perA (red), -perB (blue), -perG (green) and no-transgene (orange) at 11°C expressed using: (A) tim-Gal4, same data shown as in Figure 6-8; (B) cry-Gal4; Pdf-Gal80; (C) Clk9M-Gal4 (D) GMR-Gal4; (E) Rh6-Gal4; (F) Rh5-Gal4. Mean + SEM. The numbers in the bottom of the column represent the number of replicas. *<0.05; **<0.01; ***<0.001; ****<0.0001.

6.5 Discussion

While any effects of *period* in diapause regulation have been disputed, with Saunders (1990) initially excluding involvement of this gene after an examination of its

classical mutants, my work in this chapter confirms the previous study of Collins (2014): flies expressing solely the *perA* isoform, which retains *dmpi8*, have significantly reduced diapause levels when the experiment is performed at 12 °C. As splicing on the 3' UTR of *period* is known to be temperature and photoperiod dependant, it has been previously associated with seasonal adaptation. Majercak et al. (1999) found that the higher splicing correlated with lower temperatures results in an earlier locomotor phase, with shorter siesta time and an advanced evening peak. My results link the circadian clock with diapause through temperature sensing. Indeed, at 10 °C the difference between the lines is abolished. This might suggest that temperature input was not being processed appropriately at 12 °C in the *perA* transgenic flies. On the other hand, subjecting them to 10 °C might be too strong a stimulus overriding this diminished sensitivity. This could suggest that the low diapause levels at 12 °C are not due to an inability of the flies to trigger diapause induction, but rather to an inefficiency in processing temperature inputs correctly. Additionally, 10 °C might be such a low temperature that is below the threshold in which development can happen.

PER levels had been reported to be lower in colder temperatures of 18 °C (Majercak et al., 1999). However, I went a step further and measured PER levels in diapause-inducing temperatures: at 12 °C. I found that the expression of PER was not rhythmic and that having only one or the other splicing variant did not result in different accumulation levels of the protein. Montelli et al. (2015) studied *per* and *tim* levels under natural conditions and found that the former only displayed a rhythmic pattern in the higher-temperature months of June-July. Additionally, Menegazzi et al. (2013) described that in winter conditions, PER and TIM levels in the clock neurons are significantly reduced, below detectable quantities. All of this suggests that the circadian clock might not be functional at 12 °C and that the results obtained previously on Chapter 4 with the different clock mutants might be due to developmental effects. It also raises the possibility that pleiotropic effects rather than the clock itself are directing diapause induction.

It could be that the transcription of the different clock components is temperature-dependent, suggesting that different components of the clock might play

larger or smaller roles depending on the environmental temperature. If indeed, the clock cells are not expressing PER nor TIM at detectable levels at low temperatures, as seen in Menegazzi et al. (2013), it is understandable why mutating these two genes had little or no effect on a winter phenotype such as diapause. In *Eurydice* tidal timekeeping has been reported to be *Ep-per* and *Ep-tim* independent; nevertheless, pharmacological manipulation of casein kinase 1ε (CK1ε) altered tidal behaviour (Zhang et al., 2013). From these results, the authors suggested that a CK1-dependent pleiotropic effect was mediating the tidal cycle which was independent of circadian-clock control. In diapausing conditions, does the low expression of the canonical negative loop (constituted by PER/TIM) entail a lack of a functional clock? It would be useful to check the levels of other clock proteins that are known to cycle throughout the day, such as CLK, CWO or VRI to confirm whether this lack of rhythmicity at 12 °C is PER-specific or a generalised feature.

My anatomical dissection *Gal4/80-UAS* experiments gave some encouraging results with the *perA* and *perB* transgenes. Expression of the different transgenes in all the circadian cells with a *tim-Gal4* line showed a similar diapause levels to those obtained with the original transgenic lines from Cheng et al. (1998). Comparable patterns were found when expressing the transgenes in different subsets of the clock cells, identifying DN1, DN2, $CRY^{+}LN_{d}$ and s-LN_{v}s as possible regulators of these seasonal phenotype. Additionally, the eyes, and in particular the "yellow" ommatidia (expressing Rh4 and Rh6) seem to play a role for this mechanism. Very unfortunately and belatedly, a contamination of the original *w, per^{0}* double balancer stocks was detected. This contamination must have been at very low levels initially because when I originally checked 6 *per^{0}* **mated females** in the founder stocks I found no evidence for *per^{+}*. This might mean that my initial experiments (illustrated in Figure 6-9), were reasonably free from contamination. Indeed *per^{+}* contamination would minimise the differences between *perA* and *perB*, yet significant effects were observed for the initial set of drivers (apart from *Rh5-gal4).* The results I most suspect are those that were acquired later on and are outlined in Appendix Figure 9-15. As the crosses required to generate a new set of strains, their expansion and testing takes many months, I ran out

of time. However, I suspect that the repetition of the experiment in the correct background will yield similar results to those observed in Figure 6-9.

Overall this chapter suggest that:

- The *perA* splicing variant changes temperature-sensitivity threshold, resulting in no-diapause induction when the adult females are placed at 12 °C but normal diapause at 10 °C.
- The different *per* splicing-locked transgenic flies from Cheng et al. (1998) have similar PER levels and display no significant rhythmic PER expression at 12 °C.
- The optimal temperature for diapause-induction for the systematic dissection of the different *per* splicing-locked isoforms is 11 °C.
- *UAS-perA/B/G* lines that were generated will constitute a useful tool to perform the neuroanatomical dissection of this temperature-dependent –pathway. Although the experiments need to be repeated, the preliminary results obtained in this section implicate DN1, DN2, CRY^+ LN_d and s-LN_vs in processing of temperature information and/or diapause regulation. Additionally, the eyes, and in particular the yellow ommatidia seem to play a role for this mechanism. However, due to an unfortunate contamination by per^+, these results must be treated with caution and the experiment should be repeated.
- The *UAS-perG* line, which undergoes temperature-dependant splicing, might also be used to perform a neuroanatomical dissection of the midday siesta behaviour described by Majercak et al. (1999).

7. microRNA REGULATION OF *period* SPLICING

7.1 Introduction

7.1.1 microRNA

The first evidence indicating the importance of these small (~ 22 nt) non-coding endogenous microRNAs (miRNAs) was discovered in *C. elegans*. Lee et al. (1993) found that *lin-4*, which had been reported to regulate larval developmental timing in the nematode, did not produce a protein, but a pair of small RNAs: one of ~22 nt and its precursor of ~61 nt. It was later discovered that the sequences of these *lin-4* RNAs were complementary to multiple sites in a region of the 3' UTR of *lin-14* which had been suggested to have an inhibitory role. Finally, Wightman et al. (1993) showed that *lin-4* regulated LIN-14 protein levels without altering *lin-14* mRNA. Nevertheless, it was not until seven years later that the second miRNA, *let-7*, was discovered in *C. elegans* (Reinhart et al., 2000). This gene is also important for developmental timing and, for the first time, homologues were found in several animals, amongst them *Drosophila* (Pasquinelli et al., 2000). From here onwards, the biogenesis, mechanisms and regulatory pathways of these tiny but indispensable miRNAs have been broadly documented leading to a Nobel Prize in Medicine or Physiology for Andrew Fire and Craig Mello in 2006.

7.1.2 Biogenesis of miRNA

Primary miRNAs (pri-miRNAs, which can be larger than 1 kb long and form RNA hairpin structures) are transcribed by RNA polymerase II (Qian et al., 2011). Drosha

RNase III endonuclease performs the nuclear cleavage of this pri-miRNA, generating the miRNA precursor (pre-miRNA, ~60-70 nt) (Lee et al., 2002; Zeng et al., 2003). This pre-miRNA is translocated to the cytoplasm, where Dicer processes the other end of the transcript (Lee et al., 2003). As both of these proteins (Drosha and Dicer) are RNase III endonucleases, when they process the transcript each of the strands will end with a 5' phosphate and ~ 2 nt overhang 3' (Lee et al., 2003). The result is a small imperfect duplex formed by the miRNA and its complementary sequence (miRNA*) (Lau et al., 2001). The fact that this miRNA* is present at very low levels in libraries of cloned miRNA suggests that this duplex has a shorter life-time than the miRNA *per se* (Aravin et al., 2003; Lagos-Quintana et al., 2002; Lau et al., 2001). When the miRNA is loaded onto the RNA-induced silencing complex (RISC), the miRNA* is thought to be degraded. Generally, the strand whose 5' is more accessible enters the RISC, suggesting that a helicase might be involved (Schwarz et al., 2003). Nevertheless, in some rare cases in which the ends of both strands had similar stability it has been found that both strands of the miRNA:miRNA* duplex can enter the RISC (the helicase loads one strand *per* duplex, but might choose different strands each time) (Schwarz et al., 2003). Another way of naming the strands would be to use the -5p or -3p nomenclature, which takes into account the position of the strand rather than whether it is the functional strand or not. The first nucleotides on the 5' end of the mature miRNAs (mostly 2-8) constitute the seed region and have been shown to play a key role in target recognition. Nevertheless, this seed region is flexible and regulation of mRNA by the nucleotides 2-7, 2-8 or 3-9 from the 5'-end has been reported (Lewis et al., 2005; Nahvi et al., 2009) (Figure 7-1).

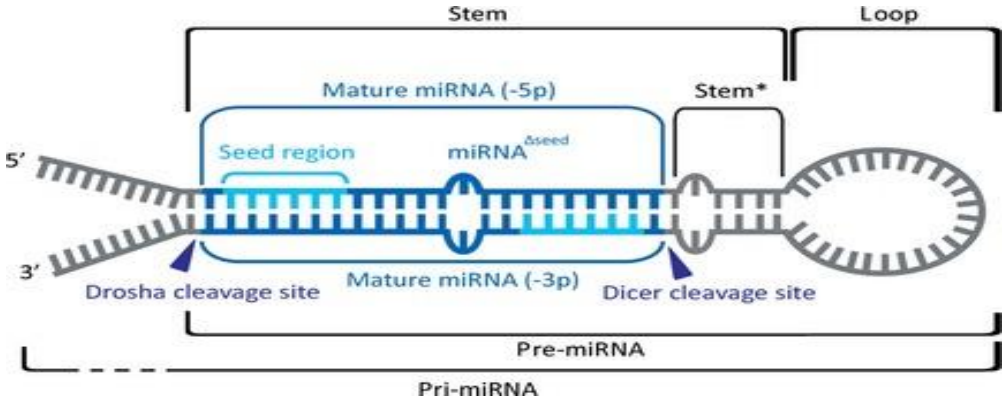


Figure 7-1 miRNA structure. *From Jevsinek Skok et al. (2013) .*

7.1.3 Mechanisms of miRNAs

miRNAs exert their silencing role after binding to RISC (Ameres and Zamore, 2013; Kawamata and Tomari, 2010). In *Drosophila*, this RISC contains the miRNA and an Argonaute protein (AGO1) (Ipsaro and Joshua-Tor, 2015; Wilson and Doudna, 2013). This complex is able to promote degradation of mRNA or repression of its translation, depending on the level of complementarity between the miRNA and its target (Bartel, 2004) (Figure 7-2). When the miRNA is nearly-perfectly complementary to the target mRNA, RISC cleaves it using the RNase H homologous domain in Argonaute proteins (Ameres and Zamore, 2013). Nevertheless, in animals a partial complementarity with the target mRNA is more common, allowing translational repression without directly promoting mRNA cleavage (Bartel, 2004; Iwakawa and Tomari, 2015). Usually, this translational inhibition is followed by mRNA degradation in a cleavage-independent manner and the cases in which translational repression occur without mRNA degradation are rare (Behm-Ansmant et al., 2006; Eulalio et al., 2009; Schratt et al., 2006). The exact mechanism by which miRNA induce translational repression is still not fully understood (Iwakawa and Tomari, 2015). On the other hand, much more is known about the miRNA role in mRNA decay: for example, several deadenylases and decapping factors have been seen to be recruited by RISC (Jager and Dorner, 2010; Nishihara et al., 2013).

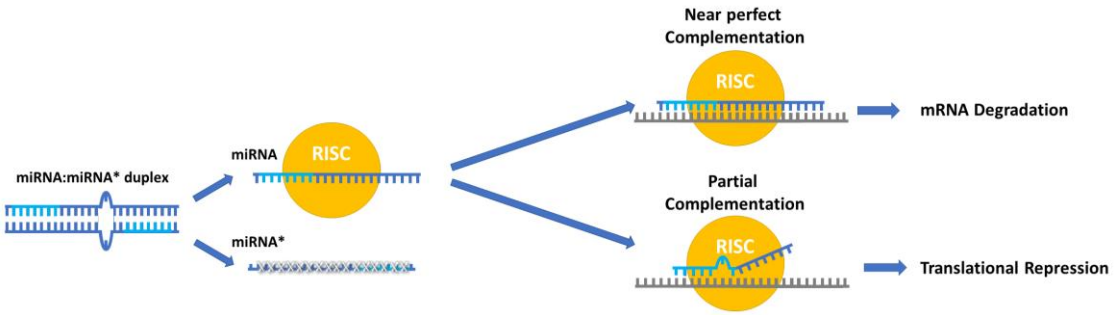


Figure 7-2 miRNA mechanisms of action. Adapted from Ryan et al. (2015).

7.1.4 Regulation of the circadian clock by miRNAs

Many groups are finding that miRNAs are involved in the control of the circadian clock (reviewed by Xue and Zhang (2018)). miRNA *bantam* has been found to target *Clk* and the deletion of its binding sites in the 3' UTR of *Clk* leads to an increased number of PDF-positive cells, which suggests a putative role on circadian neuron development

by this miRNA (Lerner et al., 2015). Additionally, downregulation in the circadian cells of GW182, which is known to interact with RISC and regulate miRNA-induced translational repression, renders the flies arrhythmic (Zhang and Emery, 2013). Some other miRNAs such as *miR-279* and *miR-276a* have been shown to be involved in the regulation of circadian robustness. In particular, *miR-276a* has been reported to target *tim*, and reduce both TIM and PER levels (Chen and Rosbash, 2016). *bantam* and *let-7*, which target *Clk* and *cwo* respectively alter free-running period (Chen et al., 2014a; Kadener et al., 2009). A two hour shortening of period has been reported in mice without *Dicer* as a result of an accelerated PER1 and PER2 translation (Chen et al., 2013). Nevertheless, knocking down of *Dicer1* in *Drosophila* affected amplitude and robustness of the rhythms but did not affect period length at 29 °C (Kadener et al., 2009).

7.2 Aims

As seen in the previous chapter, expressing one or the other of the two *per*-3' UTR splicing variants, which encode the same protein, results in dramatic differences in diapause levels. In this chapter, I will explore the possibility of miRNAs being involved in the regulation and/or degradation of one or the other splicing variant. Several bioinformatic tools will be used to select ~10 miRNA that could potentially target *per* and I will explore their possible effects on both locomotor activity and, in particular, the midday siesta as well as diapause.

7.3 Methods

7.3.1 Bioinformatics

The first step of this project was the identification of putative miRNAs that could be binding to *dmpi8* and regulating *period*. For that, I took advantage of several bioinformatic tools. **TargetScan** uses TargetScanS algorithm to find targets of miRNA within Flybase transcripts searching for conserved (beyond the Sophophora subgenus, for which they align 12 *Drosophila* genomes including *melanogaster*, *simulans* and *yakuba* (Kheradpour et al., 2007)) or non-conserved sites matching the seed region of

individual miRNAs (Lewis et al., 2005; Ruby et al., 2007). **microRNA.org** uses the miRanda algorithm to predict miRNAs targeting the mRNA of interest from their *Drosophila* mRNA and miRNA datasets (Betel et al., 2008; Enright et al., 2003). This algorithm also considers the conservation of the miRNA and mRNA (Betel et al., 2010). **PicTar** algorithm instead predicts ~50 target mRNA from Flybase for each miRNA, hence you can check if your gene of interest has any predicted miRNA (Grun et al., 2005). Finally, **PITA** allows manual input of your 'favourite' sequence(s) and compares them to all miRNAs (Kertesz et al., 2007). Hence, this is the only algorithm that allows one to search for miRNA binding sites in both spliced and unspliced *per* sequences.

7.3.2 *Drosophila* Stocks

7.3.2.1 microRNA sponges

Ebert et al. (2007) developed a new tool in mammalian cells that allowed the inhibition of specific miRNA. It consisted of a reporter construct with several miRNA-seed targets in the 3' UTR. When expressed in the cells, these sponges sequester the miRNA, preventing it from binding to its endogenous target (See Figure 7-3). Later on, this technology was made available for flies. Fulga et al. (2015) generated a series of transgenic flies carrying *mCherry* reporter fused to twenty copies of sequences which are complementary to the miRNA-of-interest seed.

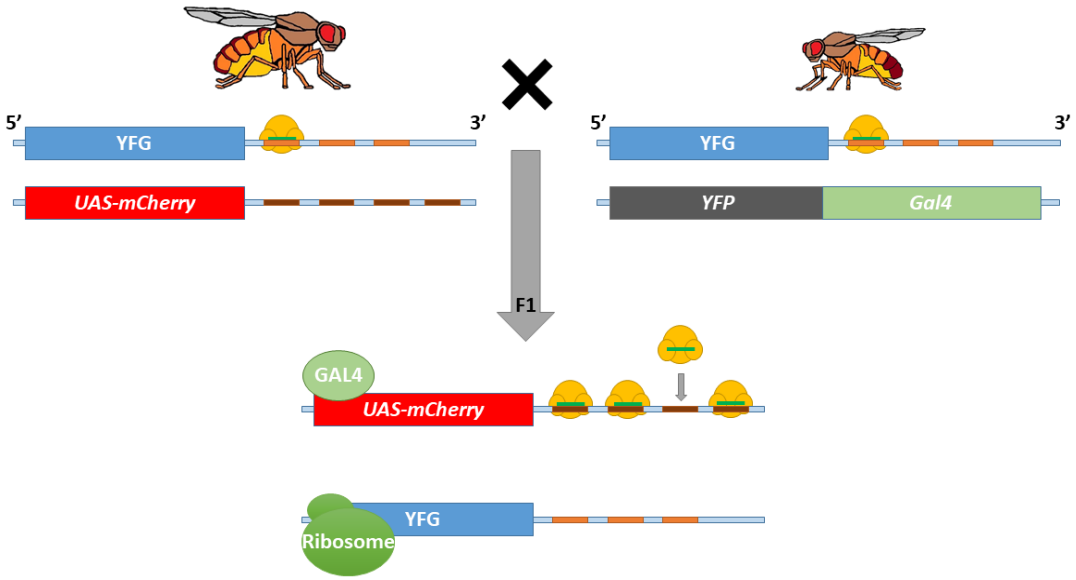


Figure 7-3 Mechanism of action of the microRNA sponges. YFG, your favourite gene targeted by miRNA-RISC complex (yellow). YFP, your favourite promoter, which will drive expression of the GAL4, and consequently the *miRNA-sponge* which is tagged

with *mCherry*. Expression of the *miRNA-sponge* will result on the miRNAs being sequestered and their endogenous targets being released from their regulation, allowing translation. Adapted from Varshney and Subramanian (2015).

7.3.2.2 *Drosophila* stocks

Flies containing miRNA sponges for the predicted *dmpi8*-targetting miRNAs under *UAS* control were obtained from Bloomington Stock Center (NIH P40OD018537, Table 7-1). They were then crosses to *w; tim-Gal4* drivers or to *w* (as a *UAS* control), both of which have been described in the previous chapters.

Table 7-1 List of miRNA sponge lines described in Fulga et al. (2015).

Bloomington Stock number	Target miRNA
61366	mir-2a
61367	mir-2b
61371	mir-5
61398	mir-193
61406	mir-276a
61407	mir-276b
61409	mir-278
61418	mir-286
61423	mir-305
61424	mir-306
61427	mir-310
61428	mir-311
61501	mir-Scramble

7.3.3 Locomotor activity

Flies were crossed and reared at 25 °C in LD 12:12. 1-2 days-old males were put inside the Trikinetics activity system. Flies were kept at either 18 or 29°C for 5 days in a LD 12-12 regime followed by 7 days in DD conditions (Figure 7-4). Only the last three days of LD were used to analyse the LD behaviour to avoid confounding effects due to the temperature changes. Similarly, the first day in DD was skipped from the DD analysis. Between 16 and 32 individual males were analysed per genotype and condition as described in Material and Methods 2.3.

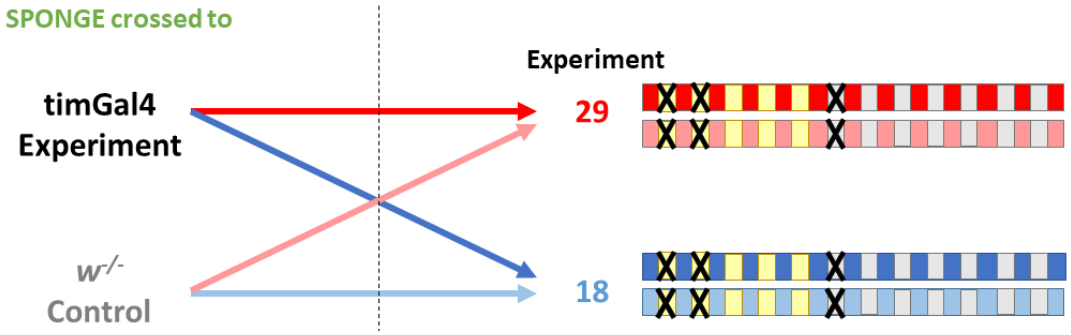


Figure 7-4 Experimental set-up for locomotor activity. *UAS-miRNA_sponge* lines were crossed to *tim-Gal4* (dark) or $w^{-/-}$ (light). The flies were developed at 25 °C and 1-2 days old males were placed either at 29 °C (red) or 18 °C (blue) for five days in LD (lights-on represented by the yellow rectangles) and seven days in DD (subjective day represented in grey). The first two days of LD and first one of DD were not used for the analysis (black crosses).

7.3.4 General experimental workflow

Flies were crossed and reared using the standard protocol of rearing the flies at 25°C in LD 12:12. The newly hatched flies were placed for 12 days in LD 8:16 at either 25 °C or 11°C. After this time, the corresponding experiment was performed.

7.3.4.1 Scoring diapause

Diapause was scored exclusively on the flies that had been maintained at 11°C as described in Material and Methods 2.2.2.

7.3.4.2 qPCR

RNA extraction and subsequent *perA/perB* quantification as well as *rlp32* control was performed as described in Material and Methods 2.5 to 2.9.4 (Maxwell method) with samples collected at ZT15.

7.3.4.3 Western Blot

Protein extraction and subsequent PER/TUB quantification was performed as described in Material and Methods 2.13 and 2.14 with samples collected at ZT21.

7.4 Results

7.4.1 miRNA predictions targeting *dmpi8*

Table 7-2 summarises the list of the miRNA that are predicted to target *dmpi8* using four different bioinformatic tools: microRNA.org, PITA, PicTar and TargetScan Fly. Except for PicTar, which had no predicted miRNA binding to the 3' UTR of *per*, all the other algorithms found several miRNAs. *miRNA-276a* and *miRNA-276b* are the only ones that have been predicted by multiple tools: microRNA.org, PITA and TargetScan (Figure 7-6). However, it is classified as a non-conserved binding site. Indeed, the TargetScan output file in which the 3' UTR of 12 *Drosophila* species are aligned (Figure 7-5) show that the binding site is only present in *D. melanogaster*. *miRNA-278* had the strongest score in PITA, however, none of the other methods confirmed this binding. Finally, as PITA allowed the manual entry of the target sequence, this was the only tool I could use to test if any miRNA could be binding to the junction between the two exons that flank *dpmi8* (targeting specifically the spliced form, *perB*). Indeed, one miRNA, *miRNA-5*, was predicted, although with very low confidence.

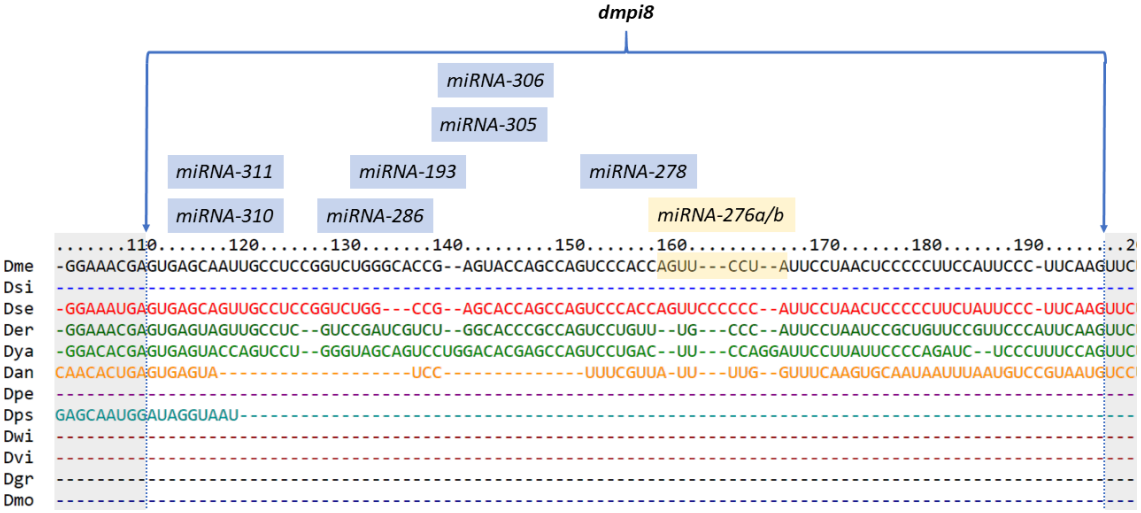


Figure 7-5 TargetScan Fly output for miRNAs targeting *D. melanogaster* 3' UTR (Dme). Highlighted in grey are the exons and the beginning and end of *dmpi8* are marked by the arrows. The putative binding site for the different *miRNAs* is represented in blue and the seed for *miRNA-276a/b* family is highlighted in yellow also in the sequence.

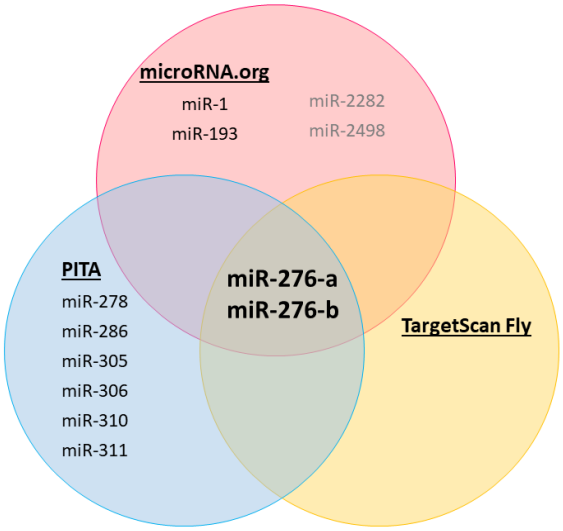


Figure 7-6 Venn diagram of miRNAs targeting *dmpi8* predicted by microRNA.org, PITA and TargetScanFly.

Table 7-2 Summary of miRNA predictions for *dmpi8*. [1] A second miRNA binding site is predicted for them in the 3' of the intron (exactly in 195 from the stop codon) with ddG=-4.3. [2] No conserved miRNAs were found to target the 3'UTR of *per*, the results reported were classified as poorly conserved.

Software	Predicted miRNA	Score	Threshold	Target
microRNA.org	mir-1	mirSVR=-0.038	-0.1	Intron
	mir-193	mirSVR=-0.002		Intron
	mir-276a	mirSVR=-0.427		Intron
	mir-276b	mirSVR=-0.427		Intron
	mir-2282	mirSVR=-0.012		Intron
	mir-2498	mirSVR=-0.003		Intron
PITA	mir-5	ddG=+5.68	-10	NO intron – exons-junction
	mir-276a[1]	ddG=-10.54		Intron
	mir-276b[1]	ddG=-10.54		Intron
	mir-278	ddG=-15.49		Intron
	mir-286	ddG=-6.98		Intron
	mir-305	ddG=-5.26		Intron
	mir-306	ddG=-6.84		Intron
	mir-310	ddG=-7.47		Intron
	mir-311	ddG=-7.17		Intron
PicTar	No predictions	-	-	-
Target Scan Fly2	mir-276a	Branch-Length Score=0.00	3.16 for being classified as conserved	Intron
	mir-276b	Branch-Length Score=0.00		Intron

7.4.2 Locomotor activity screening of putative microRNAs

miRNA sponges (described in Material and Methods 7.3.2.1) for these 10 putative miRNAs as well as two controls that did not appear in any of the tools (*miRNA-2a* and *miRNA-2b*) and the scramble control (which instead of miRNA targets contains random nucleotide sequences) were obtained from Bloomington stock center. An initial screening for locomotor activity behaviour linked with *dmpi8* splicing and the mid-day siesta was performed. When inspecting the behaviour under LD 12:12 entraining conditions, most of the lines displayed decreased morning activity, either when the experiment was performed at higher temperatures or at both high and low temperatures. As even the miRNAs that were not supposed to target *dmpi8* were displaying this phenotype, it was discarded as non-specific. One of the lines displayed higher nocturnality levels than usual (*miRNA-184*). However, the only line that displayed an altered siesta behaviour (Figure 7-7 A) was *miRNA-276b*. When the experiment was repeated, this siesta alteration was not found (Figure 7-7 C). Additionally, the DD behaviour of the sponges was assessed and found that some of them lowered rhythmicity levels of the flies in free-running conditions (*miRNA-5*, *miRNA-276a*, *miRNA-276b*, *miRNA-305* and *miRNA-310*) and two lines significantly lengthened tau (*miRNA-2b* and *miRNA-286*) (Appendix Figure 9-16).

Table 7-3 Summary of the locomotor behaviour of the studied miRNA sponges. [1] The initial experiment (altered siesta and no effect on DD behaviour) was not replicated (see Figure 7-7_A and C).

Phenotype			Strains
LD behaviour	Decreased morning activity	29 °C	mir-2b; mir-193; mir-276a; mir-276b; mir-278; mir-305; mir-306; mir-310; mir-311
		18°C + 29°C	mir-2a; mir-5; mir-286
	Altered siesta behaviour		mir-276b1
	Increased nocturnality		mir-184
	No effect		mir-Scramble
DD behaviour	Lower rhythmicity		mir-5; mir-276a; mir-276b1; mir-305; mir-310
	Lengthen period		mir-2b; mir-286
	No effect		mir-2a; mir-193; mir-276b1; mir-278; mir-306; mir-311; mir-Scramble

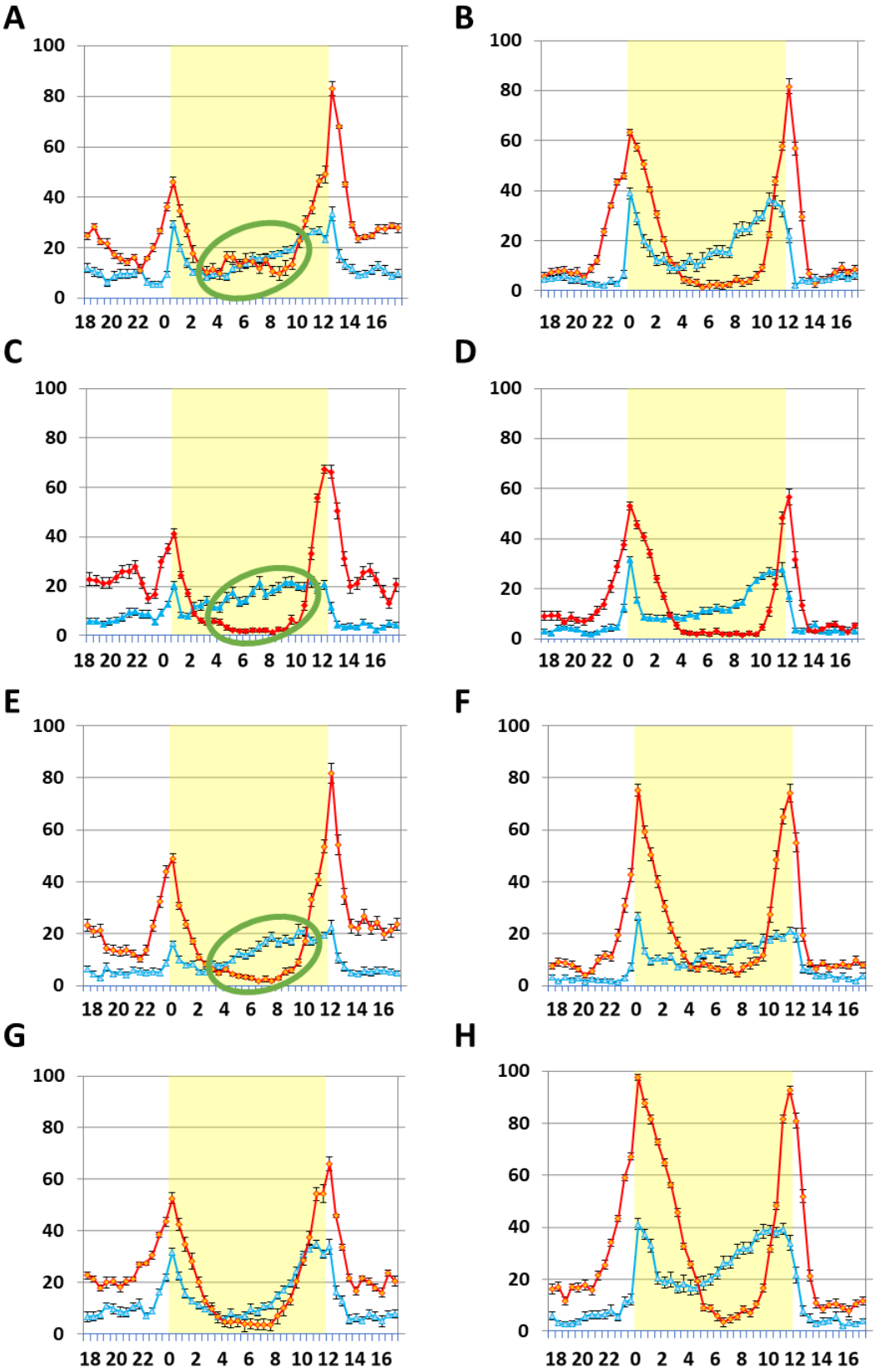


Figure 7-7 Locomotor activity under LD 12:12 at 18 °C (blue) and 29 °C (red). (A,C) *timGal4>miRNA-276b-Sp*. (B,D) *+>miRNA-276b-Sp*. (E) *timGal4>miRNA276a-Sp*. (F) *+>miRNA-276a-Sp*. (G) *timGal4>miRNA-Scramble-Sp*. (H) *timGal4>+*. Circles in A, C and E surround usual mid-day siesta. Sp: Sponge.

7.4.3 qPCR and WB confirmation of lines targeting per

From the results obtained with the locomotor activity screen, the main candidate to be involved in the regulation of *period* 3' UTR was *miRNA-276b*. qPCR (to check *per* mRNA levels of each splicing variant) and PER WB (to look at the final protein levels) were performed on *miRNA-276b-Sponge*, *miRNA-276a*, *miRNA-2b* and *miRNA-Scramble* sponges at ZT15.

ANOVA of *perA/perB* expression ratio for the different sponges showed significant genotype and interaction effects (p=0.035 and p=0.0011) but no temperature effect (p=0.87). Tukey multiple comparison test showed no significant differences in *perA/perB* ratio for any genotype at 12 °C, while astonishingly the scramble control is the only line which has a significantly increased ratio at 25 °C.

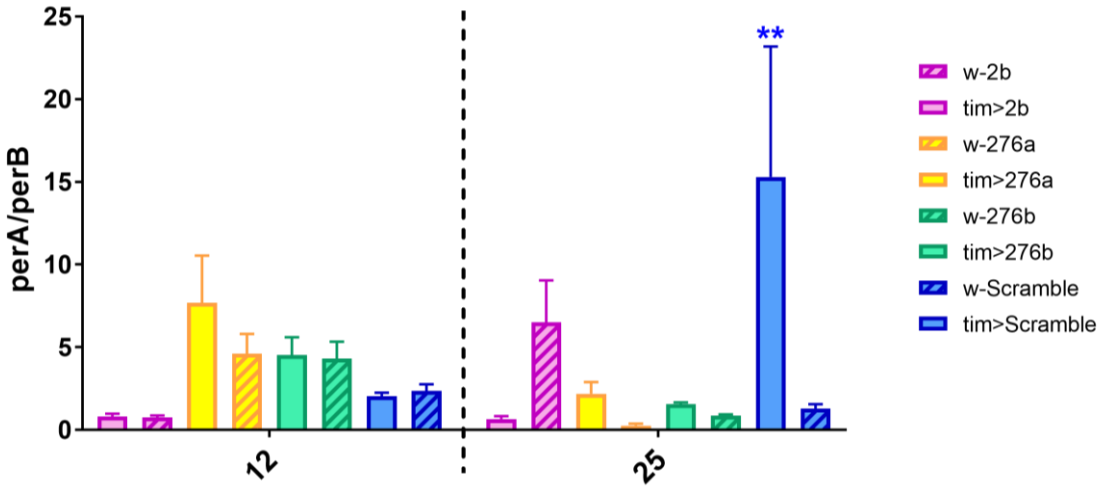


Figure 7-8 *perA* quantification normalised against *perB* levels. Sponges against *miRNA-2b* (purple), *miRNA-276a* (yellow), *miRNA-276b* (green) and *miRNA-Scramble* (blue) crossed with *tim-Gal4* (empty columns) or *w* flies (diagonal bars). Mean + SEM.

On the other hand, ANOVA of PER levels at ZT21 normalised against tubulin show a genotype, temperature and interaction effect (p<0.0001). A non-significant increase in PER levels at 12 °C is observed exclusively when the flies were expressing the *miRNA-276b* sponge (p= 0.081, Figure 7-9_A). Nevertheless, this is mostly due to one lane of the TUB control, which is significantly lower intensity in the experimental flies compared to the controls kept at the same temperature (Figure 7-9_B). At higher temperature, PER levels are, overall, significantly higher (with the exception of *miRNA-276b*).

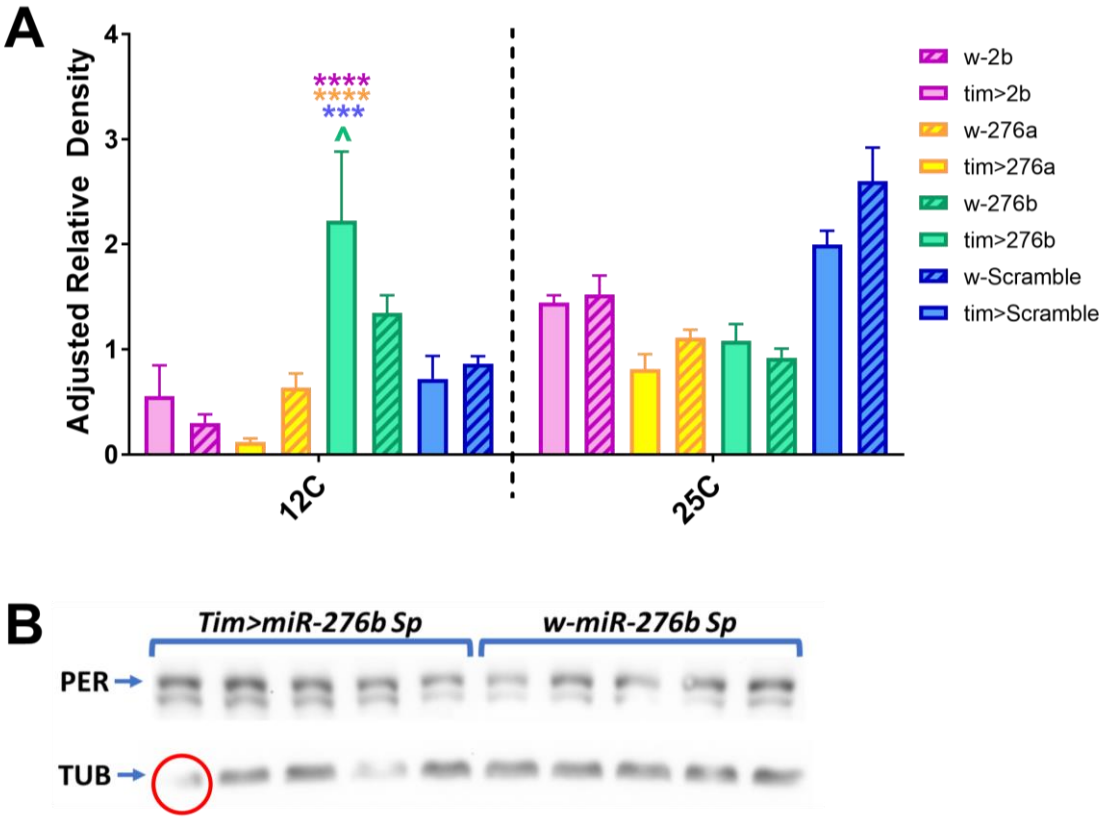


Figure 7-9 PER quantification normalised against TUB levels. (A) Western Blot quantification using sponges against *miRNA-2b* (purple), *miRNA-276a* (yellow), *miRNA-276b* (green) and *miRNA-Scramble* (blue) crossed with *tim-Gal4* (empty columns) or *w* flies (diagonal bars). Mean + SEM. (B) Western blot membrane of *miR-276b* sponge probed against PER and TUB. The first lane that is missing part of the tubulin (circled in red) was not included in the analysis.

7.4.4 Diapause with UAS-sponge lines

Finally, whether *miRNA-276b* had any implications for diapause induction was examined. Again, *miRNA-276a*, *miRNA-2b* and *miRNA-Scramble* sponges were used as controls.

All the lines displayed a high level of diapause when the flies were kept at 11 °C (~70%). Remarkably, when the sponge against *miRNA-276b* was expressed under *tim-Gal4* control the proportion of flies that entered diapause was dramatically reduced compared to all the different controls ($p=0.0001$ and $p<0.0001$, compared to *tim-Gal4>miRNA-2b* and all others, respectively).

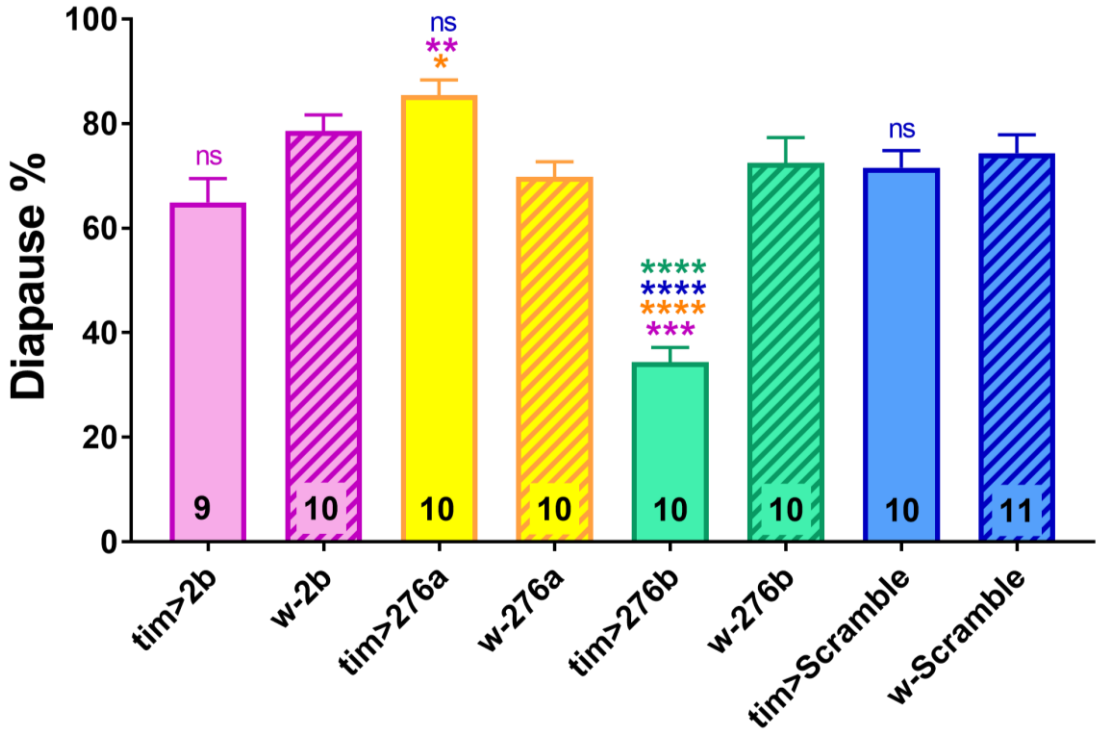


Figure 7-10 Diapause levels of the different miRNA sponge lines. Sponges against *miRNA-2b* (purple), *miRNA-276a* (yellow), *miRNA-276b* (green) and *miRNA-Scramble* (blue) were crossed with *tim-Gal4* (empty columns) or *w* flies (diagonal bars). Mean + SEM.

7.5 Discussion

In this chapter, several putative *dmpi8*-targetting miRNAs were tested. Amongst them, *miRNA-276a* and *miRNA-276b* (both of which were predicted to target *dmpi8* by three different algorithms) and *miRNA-5* (which was the only one predicted to bind specifically to the spliced form of *period*) were of particular interest.

Transgenic flies containing a reporter (*mCherry*) fused to several tandem copies of miRNA targets under *UAS* control (Fulga et al., 2015) were employed to generate knockdowns of the miRNAs of interest in the circadian cells using a *tim-Gal4* driver. When the locomotor behaviour under entraining conditions was studied, most of the lines displayed a decreased morning peak. The most dramatic effect was observed in *miRNA-184*, which had significantly increased nocturnality. Nevertheless, the only lines that displayed a mid-day siesta phenotype were the *miRNA-276b* knock-down flies. The flies had increased mobility in warm conditions, which would be expected if the miRNA was targeting the "summer" unspliced variant and promoting a shift towards a

more winter-like behaviour. Nevertheless, when the experiment was replicated one year later, the results were not comparable and this midday siesta behaviour was not found. It should be taken into account that the main difference between the two experimental results seems to reside in the locomotor behaviour at 29 °C.

Inspection of the sponges under free-running conditions showed that several of the miRNA studied affected the flies' behaviour, either by increasing arrhythmicity level (at one or both experimental temperatures, *mir-5*; *mir-276a*; *mir-276b* [only in the second experiment]; *mir-305*; *mir-310*) or lengthening *tau* (*mir-2b*; *mir-286*). *miRNA-276a* has been reported to target the other negative component of the clock, *tim*, and my results corroborate those presented in Chen and Rosbash (2016). Not much is known about the function of the other miRNA tested, especially in a circadian context. Interestingly, Yang et al. (2008) found several miRNA that could be relevant to the circadian clock in *Drosophila*. Amongst them, *miRNA-276b* displayed significantly increased levels in cyc^0 mutants compared to WT. Additionally, *miRNA-263a* and *miRNA-263b* were the only ones that displayed a strong circadian pattern. Nevertheless, several of the miRNAs tested such as *miRNA-305* and *miRNA-310* displayed weak rhythms. Hence, it would be of interest to check whether any of these circadian-phenotype displaying miRNA could be targeting any other clock related mRNA.

qPCR showed no effect on *per* mRNA levels for any of the miRNA-sponges tested, not even a temperature-effect was found which is surprising as *per* splicing is known to be temperature dependant and higher *perB* levels would be expected for the flies kept at lower temperatures (Collins et al., 2004; Majercak et al., 1999). However, analysis of total PER protein in 12 days old *Drosophila* females shows, as expected, an overall decrease in PER levels when the flies were kept under diapause-inducing conditions as opposed to 25 °C (Menegazzi et al., 2013; Vanin et al., 2012). The main exception resides in the *miRNA-276b* knock-down and control, which display similar PER levels in both temperatures. This experiment should be repeated as the differences between the experimental and control PER levels is minimal and some of the experimental samples have lost part of the TUB band. However, it may suggest a temperature-

dependant miRNA function, which seems to be targeting $per^{unspliced}$ only at colder temperatures.

There still remain a number of questions. Based on the results on AGO1 associated mRNAs, *per* was not expected to be a miRNA target, yet these experiments were performed at high temperatures (25 °C) (Kadener et al., 2009) and, since an effect on PER levels is only observed under cold diapause-inducing temperatures (12 °C) and not at 25 °C, it is understandable that *period* would not be initially identified up as a miRNA target. Hence, the possibility rises that the results obtained by Kadener et al. (2009) are an underestimation of the real involvement of the miRNA-regulated clock genes and it might be of interest to repeat a similar set of experiments under colder temperatures (12 and 18 °C). The quantification of *miRNA-276b* under cold and warm temperatures would be key for understanding the putative changes in PER levels observed uniquely at low temperature. Is it possible that *miRNA-276b* is more abundant at colder temperatures? Additionally, if *per* is found to bind to AGO, and consequently represent a miRNA target, whether *miRNA-276b* specifically targets $per^{unspliced}$ would need to be addressed. For that, the spliced and unspliced 3' UTR of *per* could be fused to a luciferase reporter and co-expressed together with the miRNA in Schneider (S2) cells. If the presence of the unspliced 3' UTR results in a decreased luciferase stability, generation of plasmids with luciferase fused to *per* 3' UTR with the putative *miRNA-276b* target site mutated would need to generated. Finally, this could also be tested *in vivo* either by generating a *UAS-perA** (*perA* 3'UTR with the mutated *miRNA-276b* site) and injecting it as reported in Chapter 6 (6.3.1) or using the CRISPR/Cas9 technology to mutate the site in the endogenous *per*.

Two natural variants of *per* 3' UTR known as VT1.1 and VT1.2, which differ by six polymorphisms (four SNP and two deletions), are known to affect its splicing levels (Low et al., 2012). Collins (2014) investigated the diapause levels of transgenic flies with the different splicing variants finding that the flies with VT1.1, which has higher splicing, overall displayed increased diapause. This is really interesting as *miRNA-276b* is expected to target *dmpi8* recognising the sequence flanking one of the deletions reported in VT1.1 (DEL2, see Figure 7-11). This could explain the decreased $dmpi8^{spliced}/dmpi8^{unspliced}$ ratio for the VT1.2 variant, in which the unspliced variant

might not be efficiently recognised by *mi-276b*, resulting in its accumulation. This is a seductive hypothesis, but when Low et al. (2012) performed a *dmpi8* splicing efficiency test in *Drosophila* S2 cells, they reported little effect on splicing efficiency between the constructs with or without the DEL2. Indeed, the overall splicing levels were very similar between the cells that had been transfected with a plasmid containing VT1.1 and or VT1.1 with the DEL2 polymorphism from VT1.2, although the latter displayed greater variability in splicing levels at 22°C. From their results the main polymorphisms to explain the different splicing levels between these two natural variants reside in both SNP3 and SNP4, located in the 3' of *dmpi8*. Indeed, similar splicing levels were found in cells containing the VT1.2 plasmid and VT1.1 hybrid plasmid with SNP3/4 from VT1.2. Collins (2014) reported an additional SNP (SNP223, also located in the 3' of *dmpi8*) which showed a latitudinal cline, with this SNP less frequent in northern populations of Europe. While speculative, it is possible that these SNP, affect the secondary structure of *per* mRNA making the *miRNA-276b* target site more or less accessible. It could be interesting to test the effect of expressing *miRNA-276b* sponge in flies containing one or other set of SNPs to assess this hypothesis.

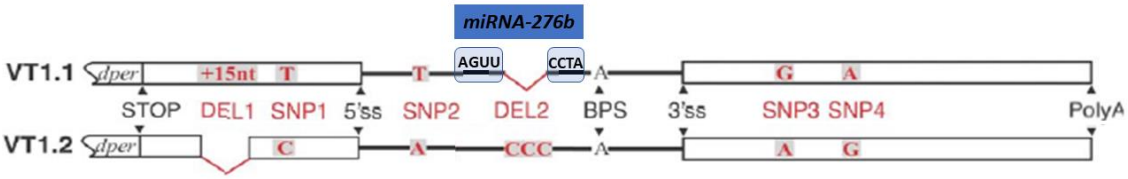


Figure 7-11 Schematic differences of the two natural *per* variants VT1.1 and VT1.2 which influence splicing. The target site for *miRNA-276b* is disrupted in VT1.2 variant which contains a CCC triplet in the middle of the target. *Adapted from Low et al. (2012).*

3' UTR of *per* is not highly conserved among different *Drosophila* species (See Figure 7-5). Studies of *per* splicing in species closely related to *D. melanogaster* such as *D. yakuba* or *D. santomea* (equatorial) showed no thermal regulation, displaying high splicing levels regardless of the temperature. On the other hand, temperature-dependant splicing has been reported in *D. simulans* (which, as *D. melanogaster* is found in temperate regions) (Low et al., 2008). The lack of the putative target site for *mi-276b* in *dmpi8* in *D. yakuba* is consistent with the low thermal regulation of *per* splicing in this species. TargetScan reported no 3' UTR homologous to the *dmpi8* and its flanking regions for *D. simulans* (Figure 7-5). Nevertheless, Low (2008) report a 3'

UTR with an 86 nt-long intron that undergoes temperature-dependant splicing in a *D. melanogaster* manner after sequencing several *D. simulans* strains. Additionally, it would be interesting to analyse the thermal sensitivity of *per* splicing in *D. sechellia*, which, according to TargetScan, contains the target site to *miRNA-276b* with a CCC insertion in the middle of it (just like the VT1.2 natural variants). Is it possible that this species also has two different natural variants and just the one with the triplet has been sequenced and not the one with the deletion?

The levels of diapause in flies expressing *miRNA-276b* sponge are significantly reduced compared to all the controls. It is possible that the ratio between one or other variant is mediating temperature information and the increase in the summer variant misleads the flies into sensing a higher temperature. A simple model would be that *miRNA-276b*, which might be stimulated by cold temperatures, targets the summer unspliced *perA* variant, promoting its degradation or blocking its translation. Consequently, at 11 °C the ratio *perB/perA* is increased, favouring winter behaviour and promoting diapause (Figure 7-12_A). On the other hand, expression of the *miRNA-276b-Sponge* sequesters this *miRNA*, interfering with the natural *perA/perB* ratio and shifting it towards a more summer-like response and inhibiting diapause (Figure 7-12_B). However, the WB results were inconclusive and need to be repeated, so there is no evidence for this molecular mechanism.

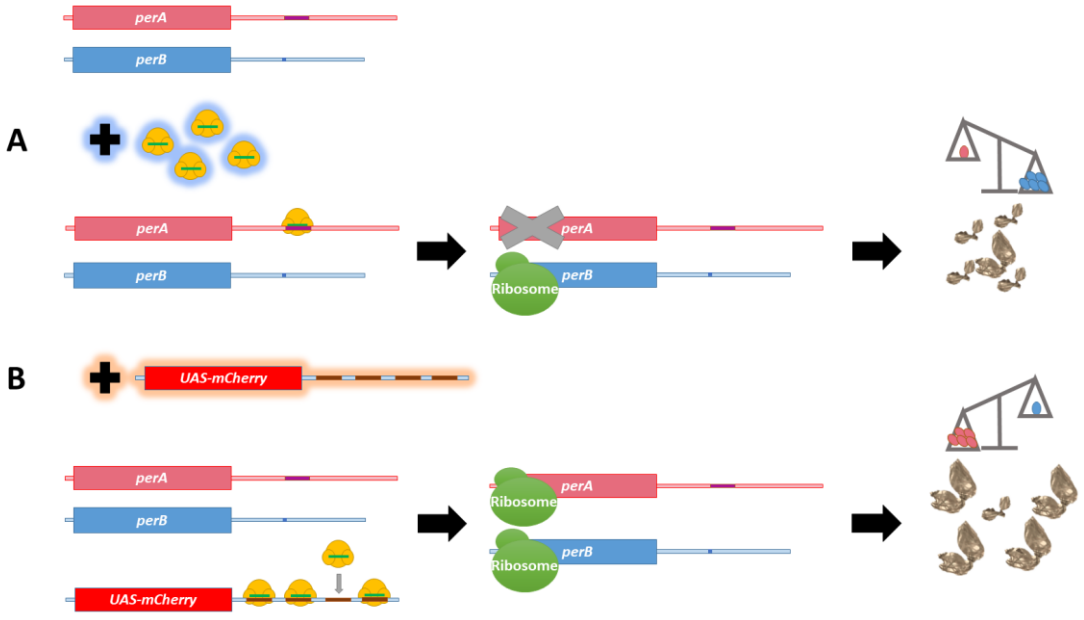


Figure 7-12 Proposed mechanism of *per* and diapause regulation by *miRNA-276b*. See text for further explanations.

Overall this chapter suggest that:

- *miRNA-276a* and *miRNA-276b* were the only miRNA that were predicted to target the unspliced summer *per* variant by more than one method.
- Flies expressing *miRNA-276b* sponge under *tim-Gal4* driver displayed a midday siesta behaviour which is linked to *period* splicing, but only on the first set of experiments.
- qPCR and WB quantification of *per* mRNA and PER protein levels were inconclusive.
- Diapause levels are reduced in flies expressing *miRNA-276b sponge*, which results in a knock-down of *miRNA-276a* which is predicted to target the unspliced (*perA*) isoform. This, together with the lower diapause levels in *perA* transgenic flies described in Chapter 6, may suggest that the presence of the summer variant has a negative role in diapause induction.

8. GENERAL DISCUSSION

This thesis has been focused on the study of diapause regulation in *D. melanogaster*. Initially, I established that the conditions in which the flies are reared have a significant impact on the adult flies: in their metabolite levels, ability to undergo diapause and even in their locomotor behaviour. Using several congenic mutants generated by Gesto (2011) as well as other genetic tools, the regulation of diapause by the circadian clock has been assessed in several studies, paying especial attention on the effects of *per* splicing. In this last chapter, I present a summary and discussion of my main findings.

8.1 Temperature as the main cue for diapause induction

There has been a long-standing debate concerning the photoperiodicity of diapause in *D. melanogaster* (Emerson et al. 2009b, Saunders, Henrich and Gilbert 1989, Tauber et al. 2007). Chapter 3 revealed a major effect of temperature in diapause regulation, with a subtler contribution from photoperiod. Removing the minimal temperature oscillations generated by the light inside the incubators abolished the significant differences between the two “photoperiods”. However, temperature and photoperiod inversion experiments revealed that the latter might still be playing a secondary role in diapause regulation, as the flies that are placed within a short photoperiod but with a long-thermocycle maintain high levels of diapause. This could potentially protect the fly from a sudden warm period during “winter”, when exiting diapause would have serious fitness consequences. Similarly to

Pegoraro et al. (2014), I conclude that the interaction between both photoperiod and thermoperiod are important for triggering diapause, although temperature is the main driver. It should be mentioned that the experiments performed were not optimal for an assessment of whether the differences reported in Figure 3-7 are due to changes in the thermoperiod *per se* or on the absolute temperature. The experiments could be planned in a slightly different manner in the future so that the absolute temperature between all the conditions would be the same.

Another group has also performed an assessment of the effect of photoperiod in diapause on different natural variants using a more natural light profile (Nagy et al. 2018). They found that lines that were not photoperiodic using the default rectangular (lights on/off) light profile, displayed strong photoperiodic differences when using the more realistic cycling light profile. On the one hand, it seems that temperature artefacts (Chapter 3) might have contributed to an apparent photoperiodic diapause and limiting the replicability of experiments between laboratories. On the other hand, it is possible that *D. melanogaster* really is more photoperiodic than my results in Chapter 3 might suggest using the more standardised laboratory conditions. Clearly there is a need to investigate this phenotype using more natural protocols.

8.2 Developmental conditions influence adults

Developmental conditions have been shown to influence several phenotypes related to stress resistance such as dessication or cold-shock (Aboagye-Antwi and Tripet 2010, Ayrinhac et al. 2004, Pegoraro et al. 2014). In chapters 3 to 5, I found that the rearing conditions cause significant differences in several adult phenotypes. Growing the flies at 18 °C resulted in an increased ability to undergo diapause, while lowering this temperature to 15 °C gave very low diapause levels. This effect on diapause was shown to correlate with differences in metabolites, such as trehalose or glucose, and survival to cold-shock. Additionally, developmental conditions have been shown to shape the role of the different circadian genes in diapause regulation: developing the flies at 25 °C, I observed that Clk^{Jrk}, cyc^{0} and per^{0} had significantly reduced diapause levels, while if the flies had been reared at 18 °C only Pdf^{0} mutants

showed lower diapause levels. Even in locomotor activity experiments, flies reared at 18 °C tended to have longer periods and lower rhythmicity. Finally, dissection of several WT and clock mutant brains that had been reared at different conditions generated differences between adult brains grown at higher (25 °C) or lower (18 °C) temperatures.

8.3 Involvement of the circadian clock in diapause regulation

8.3.1 *period* splicing

The involvement of *per* in diapause regulation has been controversial. Saunders et al. (1989) initially discarded all regulation of diapause through the circadian clock after testing several *per* mutants and finding no apparent diapause effect. Nevertheless, *per* null mutants have been described to display a reduction in diapause (Collins 2014, Gesto 2011). Temperature-dependent alternative splicing in the *per* 3' UTR has been involved in the control of the seasonal midday siesta (Sidote et al. 1998, Majercak, Chen and Edery 2004) and, recently, it was shown to be involved in the regulation of morning and evening locomotor peaks (Cao and Edery 2017). Given its association with seasonal behaviours, its putative role in diapause regulation was assessed in Chapter 6. Indeed, the alternative splicing of *per*'s 3' UTR proved to be important for diapause, as the transgenic flies only expressing the "summer"-like unspliced variant *(perA)* could not undergo diapause at 12 °C (Chapter 6, Figure 6-6). Additionally, overexpression of a miRNA sponge against *miRNA-276b* (which allegedly targets the unspliced *per* variant, Chapter 7 and theoretically could upregulate the *perA* unspliced variant in all the clock cells) led to a reduction in diapause levels. The expression of the *per*-splicing locked variants in different subsets of neurons suggests that at least some clock cells and the eyes might be involved in the regulation of diapause, although these results must be treated with caution and repeated due to a contamination of the stocks.

Menegazzi et al. (2013) showed that the expression patterns of PER and TIM changed seasonally, resulting in changes in the phases of the two negative components of the clock. They suggested a possible role for the differences between PER and TIM peaks in summer and winter as a molecular mechanism to integrate environmental light and temperature information that may trigger diapause or reproduction (Figure 8-1_A and B). This is reminiscent of the internal coincidence model. Such a mechanism could also explain the differences observed between the unspliced and spliced transgenic flies: the "summer"-like unspliced *perA* isoform results in slower daily PER accumulation and could generate a larger phase difference between the two negative components, effectively mimicking a summer pattern (Figure 8-1_C, disconnected line).

However, when I examined the levels of PER throughout the day in the *per* splicing-locked and wild-type transgenes, they did not show any cycling at 12 °C. The molecular mechanism by which this regulation of diapause is exerted via PER remains obscure. Although the internal coincidence model loses credibility if PER is not cycling, it seems that expressing one or the other *per* variant, or the ratio between them, is translated into a signal of the thermal/photoperiodic conditions and regulates several seasonal outputs, amongst them, siestas and diapause. Indeed, the transgenic *UAS-per* splicing locked transgenics showed that expression of the unspliced summer *perA* variant resulted in decreased diapause levels comparable to per^0 mutants (Figure 6-8).

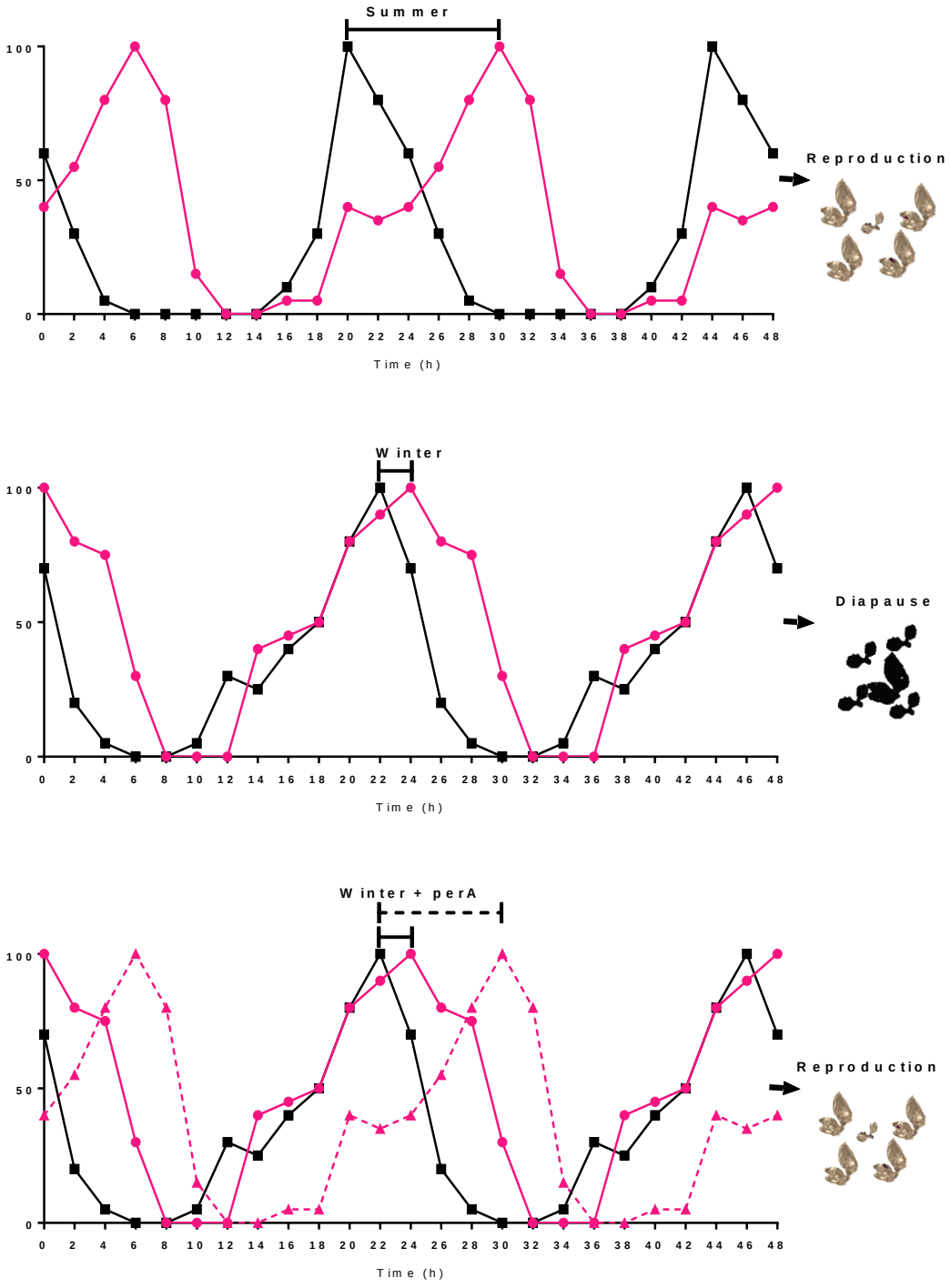


Figure 8-1 Model for PER (magenta) and TIM (black) expression in (A) summer, (B) winter and (C) winter in WT (straight) plus *perA* unspliced (disconnected) flies. The phase differences between the two proteins determine the fate of the flies as diapausing or reproductive adults. *Adapted from Menegazzi et al (2013).*

8.3.2 miRNA control of *per*

In chapter 7, I investigated the possibility of a miRNA regulating *per* and/or PER levels. I found that *miRNA-276b* seemed like a good candidate although the quantifications of *per* mRNA and PER protein by qPCR and WB, respectively, were inconclusive. Indeed, *per* was not found to be bound to AGO1 (protein involved in miRNA target recognition) at 25 °C (Kadener et al. 2009). However, the possibility arises that this miRNA is under temperature control and is expressed at lower levels in high temperatures. If so, could the presence or absence of this miRNA underlie the higher or lower *perA/perB* ratios observed? Clearly the expression of this miRNA at high and low temperatures needs to be studied as well as its expression pattern throughout the day in both conditions.

Use of a sponge specific to this miRNA which would generate a 'knock-down' of this particular *miRNA-276b* and potentially increase *perA* (unspliced 'summer') levels, resulted in a significant reduction of diapause consequent with a putative increase in *perA*. However, it should be remembered that these miRNAs usually have several targets, so this effect could be mediated by other mRNAs. The specificity of this interaction between *miRNA-276b* and *per* 3'UTR could be investigated in S2 cells by fusing the spliced and unspliced UTRs to a luciferase reporter and testing its stability after adding *miRNA-276b*. A decay in luciferase signal specifically in the unspliced *perA* 3'UTR containing cells would support a direct control of *per* via this miRNA. Additionally, this regulation could be further assessed by mutating of the putative miRNA binding site, which should result in no binding of the miRNA and higher stability of the luciferase. This would finally need to be tested *in vivo* by generating a *UAS-perA** line (containing the unspliced *perA* 3'UTR with the mutated *miRNA-276b* binding sites). Overall, this chapter opened up a new approach to studying the regulation of *period* and diapause in *D. melanogaster*.

8.3.3 Direct control by the clock *vs* pleiotropy

Since Kogure (1933) suggested that photoperiod could regulate diapause, photoperiodic diapause has been reported in several organisms, amongst them *D. melanogaster* (Saunders et al. 1989). Nevertheless, how photoperiodic information

might be assimilated is not well understood. I have previously mentioned several models (e.g. external and internal coincidence models), some of which suggest that the circadian clock might be involved on the integration of this photoperiodic cue. Disruption of the clock in some *Drosophila* species, has been shown to lead to changes in the normal diapause levels (Yamada and Yamamoto 2011, Kauranen, Tyukmaeva and Hoikkala 2013). However, whether these effects are due to the circadian clock as a module controlling diapause or to pleiotropic effects is still under dispute (Emerson, Bradshaw and Holzapfel 2009a, Gesto 2011, Bradshaw and Holzapfel 2010). Nevertheless, the circadian clock might be used to measure day-length or also seasonal/daily temperature changes which would be of particular interest in *D. melanogaster* diapause control given the dominance of temperature over light in this phenotype.

Analysis of diapause levels using the congenic canonical clock mutants described by Gesto (2011) show that, apart from tim^{01} and cry^{b}, at one or other rearing temperature, most of them cause alterations to the normal diapause level. The most dramatic changes occurred when the positive components of the circadian clock were manipulated (Clk^{Jrk} and cyc^{0} reared at 25 °C) as well as when the main output was absent (Pdf^{0} reared at 18 °C). Finding that most of the mutants changed diapause levels, it would seem, at least superficially, that these effects are mediated by the circadian clock acting as a module to regulate diapause induction, rather than pleiotropic effects.

However, the most aberrant phenotypes were observed with the Clk^{Jrk} mutants, which have been reported to have the axonal projections originating from the s-LN_vs disrupted (Park et al. 2000). Inspection of the *Clk* mutants confirmed this phenotype. Nevertheless, a slight improvement was observed when the flies were reared at lower temperature with several brains displaying strong (though still aberrant) projections. Interestingly, the levels of diapause in these mutants reverted to WT levels when the flies are reared at these lower temperatures, which suggests that the effects observed in some of the clock mutants could be developmental, implicating a pleiotropic effect.

In chapter 6 the levels of PER were measured across the day in different *per* splicing-locked transgenic flies and I found that PER levels did not cycle at 12 °C. Menegazzi et al. (2013) had already reported non-cyclic PER expression in *Drosophila* heads during the winter months (with temperatures comparable to mine of around 12 °C); indeed, the levels were too low to be quantifiable in the clock cells. This opens up the question to whether the circadian clock is functional at all in these low temperatures. The levels and rhythmic properties of other key clock proteins which are known to cycle throughout the day, such as CLK, PDP1ε, CWO or VRI, should be assessed in order to understand the implications of low PER levels.

There are a number of possible explanations for the results of the clock mutants.

1. **Direct regulation by the clock.** *Clk*, *cyc*, *per* and *Pdf* are directly promoting diapause. Glossop et al. (1999) found that per^{0} flies have lower levels of *Clk*. This might explain why mutating the positive and negative components of the clock results in similar effects on diapause in *D. melanogaster*, instead of having opposite roles as has been reported for *R. pedestris* (Ikeno et al. 2010).

2. Alterations in diapause levels are purely **developmental** as the normal diapause levels on Clk^{Jrk} mutants reared at 18 °C might suggest. Nevertheless, it is counterintuitive that Pdf^{0} mutants would have no effect (or increased diapause (Nagy 2017, Gesto 2011)) when reared at high temperatures while the opposite effect is seen when the rearing condition is lowered to 18 °C, suggesting a role of this peptide in diapause regulation.

3. Diapause effects are purely **pleiotropic**. Benito et al. (2010) showed that a *Clk*-controlled gene, *Pdp1ε*, regulates *takeout*, which has been connected with diapause-relevant JH in *D. melanogaster* (Meunier, Belgacem and Martin 2007). Additionally, differential expression of *Pdp1* in the fat body has been reported between diapausing and non-diapausing *P. apterus* individuals (Dolezel et al. 2008). It is possible that there is a noncircadian involvement of clock genes upon hormonal signalling that ultimately lead to diapause

regulation, as has been suggested for the Linden bug (Bajgar, Dolezel and Hodkova 2013).

Possibly an intermediate situation, in which a mixture of the formerly presented options are regulating the output of the flies, is closest to the real mechanism (Figure 8-2). From the ICCs, I reported differences in the PDF signalling pathway between diapausing and reproductive flies. On the one hand, the normal PDF signalling seemed to be decreased in diapausing brains, which might be linked to the reduced PER levels observed by Menegazzi et al. (2013) as PDF has been reported to enhance PER stability (Li et al. 2014). However, an increase in PDF levels from the developmentally transient PDF-Tri cells was also found. PDF has been suggested to be a link between the circadian pathway and endocrine axis (Schiesari, Kyriacou and Costa 2011, Di Cara and King-Jones 2013). Hence, I propose an involvement of PDF in diapause promotion, although it does not necessarily need to implicate the circadian clock (which may or may not be functional at this temperature).

The dramatic structural changes in the clock mutants, and in particular in Clk^{Jrk}, might suggest that the effects observed on diapause for these lines might be purely developmental: the aberrant structure of the brain connections in these flies avoids normal PDF signalling and results indirectly in changes in diapause levels. An indirect effect due to *Pdp1ε* misregulation (see above) of diapause-related genes in the peripheral tissues cannot be discarded. In order to test this hypothesis, it could be useful to use a temperature sensitive *Gal4/Gal80* system to drive *Clk-RNAi* in the clock cells specifically in the adult flies, so that the brain develops in a normal way and the absence of *Clk* in diapause could be fully assessed. Finally, cry^{b} mutants significantly increase diapause levels regardless of the developmental conditions. These mutants were reported to maintain significantly increased levels of PER and TIM throughout the day (Stanewsky et al. 1998), possibly resulting in alterations in PDF levels as PER and TIM have been suggested to alter PDF levels post-transcriptionally (Park et al. 2000), in the CRY^{+} clock cells which, to my knowledge, do not include the PDF-Tri.

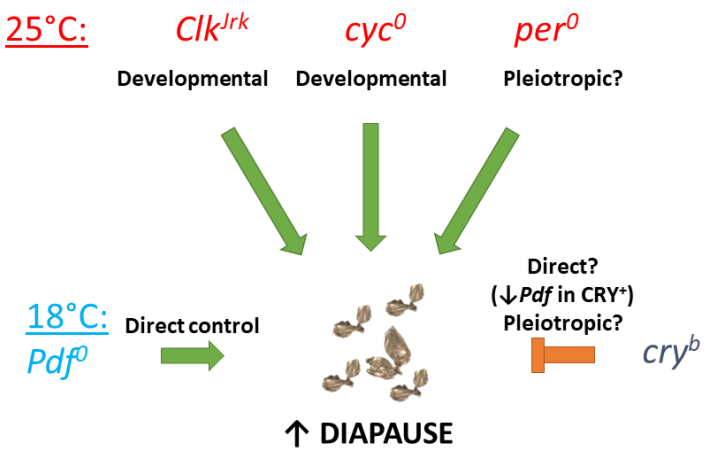


Figure 8-2 Summary of the likely type of regulation of clock genes in diapause. Diapause reduction in *Clk*^{Jrk} and *cyc*^{0} flies that have been reared at 25 °C is probably due to the developmental effects, while *per*^{0} effects might be pleiotropic. *Pdf*^{0} reductions in diapause are a result of a direct regulation of diapause via PDF. Finally, the enhanced diapause in *cry*^{b} could be due to an upregulation of PDF in CRY^{+} cells (LN_vs) or due to pleiotropic effects.

The clock mutants displaying strong developmental defects that might account for most of the alterations in diapause do not exclude a possible role of the circadian clock as one of the modulators of hormones linked with diapause. Indeed, using genetic manipulation, different clock cells have been found to be important for diapause regulation. For example, the seasonal changes in the environment and changes between the phases of expression and/or total levels of different clock proteins could modulate PDF release and regulate the release of DILPs or other diapause-related hormones.

8.4 Which groups of neurons are involved in the regulation of diapause?

Mutations in the insulin pathway have been related with increases in diapause levels (Schiesari et al. 2016, Kubrak et al. 2014). Unexpectedly, in chapter 5, I found a counter-intuitive increase in DILP2 signalling in the axonal projections of this cells at 12 °C. Similar results have been obtained by others who have found increased *dilp2* and *dilp5* levels in the brain in diapausing conditions (Kubrak et al. 2014, Schiesari et al. 2016) and high DILP2 expression in the IPCs, at 12 °C (Nagy 2017). This increase in

DILP2 levels along the IPC axons could be interpreted as an increase in secretion or, most likely, as a decrease in secretion and its consequent within-cell accumulation. Additionally, the lack of release of DILPs might inhibit natural feedback signals, resulting in an increase in DILP-production activating signals or a decrease of DILP-production inhibiting signals.

A role for PDF^+ cells in diapause has been suggested recently in other insects (Shiga and Numata 2009, Meuti et al. 2015). Additionally, Nagy (2017) found that several manipulations that resulted in the genetic ablation, silencing or excitation of these cells resulted in both positive and negative effects on diapause levels in *D. melanogaster*. When I compared diapausing and reproductive fly brains I found significant differences in PDF signalling. On one hand, canonical PDF signalling seemed to be reduced in diapausing flies (in spite of increased PDF in LN_vs but not along the axonal projections, possibly due to a lower release of the peptide). On the other hand, a set of developmentally transient PDF^+ cells (PDF-Tri) was found to be present in 12 day old flies under colder temperatures suggesting a possible role in diapause.

Although the results obtained with the genetic dissection using the different *UAS-per_splicing-locked* flies need to be treated with caution and repeated, the preliminary results suggest that the DN1, DN2, CRY^+ LN_d and some of the LN_vs might be involved in the regulation of diapause. Some of these clusters, such as the DN2s, have been implicated in temperature preference (Kaneko et al. 2012). Additionally, expression of the *per* splicing variants in the eyes (and, in particular, in the yellow ommatidia) seemed to mimic the effects of expression in the clock neurons. It is possible that the input from the eyes is integrated by the l-LNvs, which have been strongly associated with the visual system (Helfrich-Forster et al. 2007).

With this in mind, a preliminary model in which PDF acts as a dual diapause regulator is proposed. Through the PDF^+ clock cells (LN_vs), PDF regulates the IPCs indirectly promoting development: Cavanaugh et al. (2014) found a synaptic connection between LN_vs and DN1 and, more recently, Barber et al. (2016) reported a regulation of the IPCs by the DN1s. Additional regulation of the IPCs via secretion of PDF by l-LN_vs and its diffusion towards other clock cells such as LN_ds is also possible

(Schlichting et al. 2016). Finally, the non-circadian PDF-Tri cells might promote diapause by a direct connection with the IPCs as PDF-Tri send axonal projections towards the dorsal protocerebrum and the IPCs, towards the tritocerebrum area (Chapter 5, Figure 5-14). In this model we are treating each cluster as a homogeneous set of neurons based on their neuroanatomical positions, when it is known that they are heterogeneous in expression of different genes, peptides and/or receptors (Barber et al. 2016). Hence, it is possible that different subsets of these clusters play different roles (or none) in diapause regulation. Additional studies that would allow a more rigorous dissection of the cells would be important in order to complete and/or confirm the proposed model.

8.5 Open questions

As in most PhDs, I have managed to answer a few questions concerning the regulation of diapause in *D. melanogaster*, while many new ones have arisen. Here are some of the most intriguing questions that I consider should be investigated further:

- What is the molecular mechanism behind the differences in diapause levels between the different *per* splicing-locked transgenic lines?
- Is the clock functional at 12 °C? Several natural lines and circadian proteins should be examined Additionally, other cycling proteins such as TIM, CLK, VRI, PDP1ε or CWO should be investigated at low temperatures
- Neurogenetic dissection to assess the cells involved in regulation of diapause using the *UAS-per* splicing locked flies I generated should be continued
- Is *miRNA-276b* really targeting unspliced *per*? How is the expression of this miRNA controlled? Is it temperature dependent? How exactly is *miRNA-276b* regulating diapause?
- The proposed model with PDFs dual function both inhibiting diapause (through indirect activation of the IPCs using DN or LN_ds as intermediaries) and promoting it (through direct inhibition of the IPCs via PDF-Tri) needs to be rigorously tested.

9. APPENDIX

9.1 Appendix Chapter 3

9.1.1 Figures

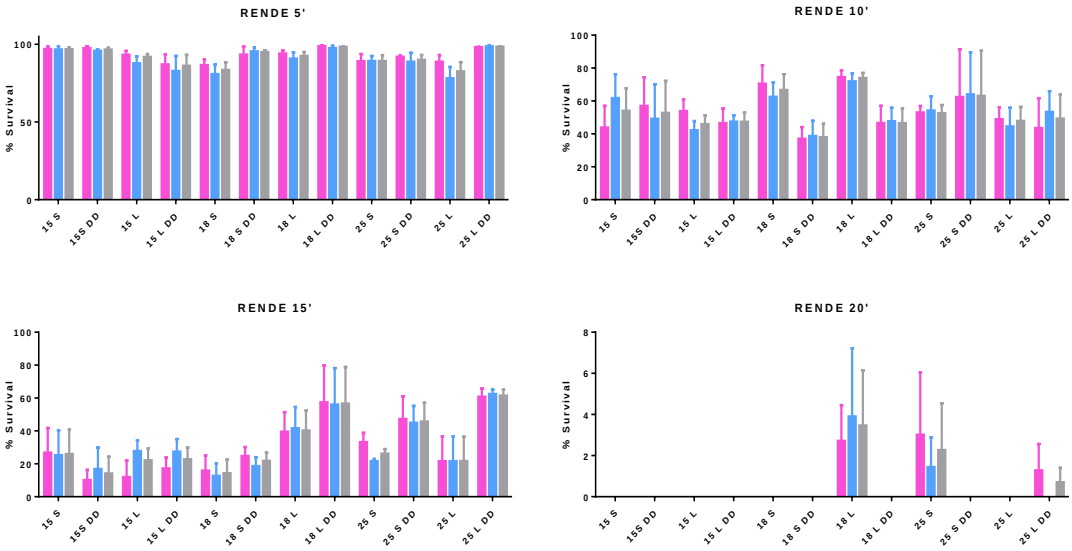


Figure 9-1 Survivorship levels of Rende *s-tim* population 24h after subjecting the flies to -20 °C for 5, 10, 15 or 20 minutes. Mean + SEM are shown. In the graphs, results from the females, males or merged population are represented in pink, blue and grey, respectively.

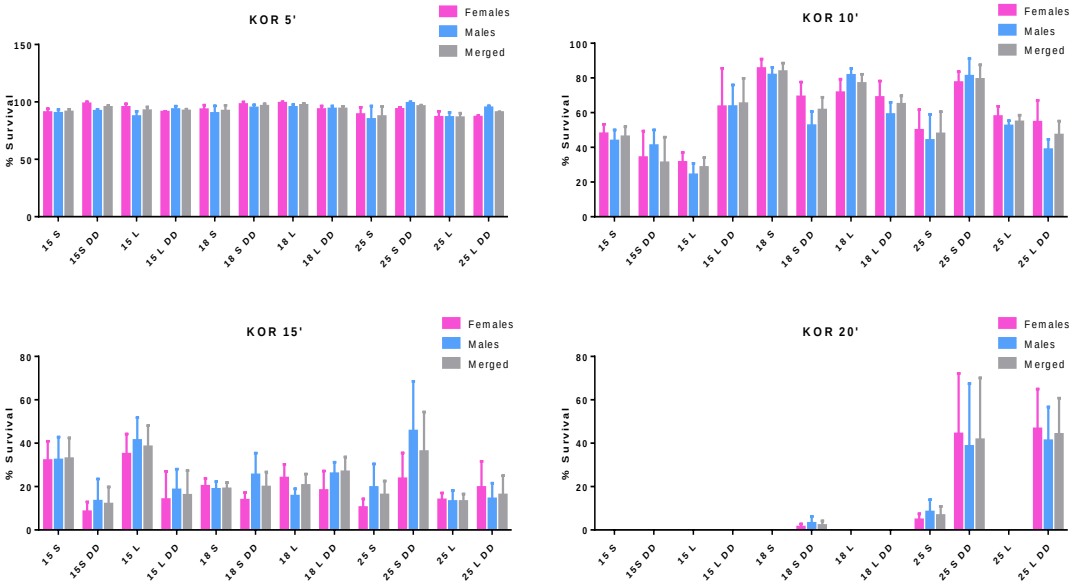


Figure 9-2 Survivorship levels of Korpilahti *s-tim* population 24h after subjecting the flies to -20 °C for 5, 10, 15 or 20 minutes. Mean + SEM are shown. In the graphs, results from the females, males or merged population are represented in pink, blue and grey, respectively.

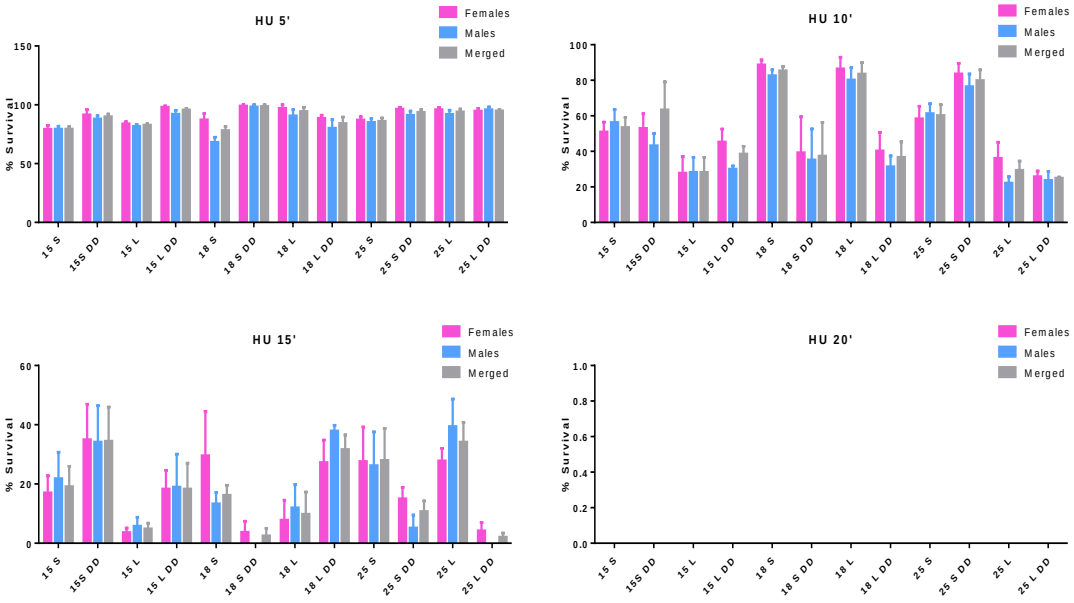


Figure 9-3 Survivorship levels of Houten *s-tim* population 24h after subjecting the flies to -20 °C for 5, 10, 15 or 20 minutes. Mean + SEM are shown. In the graphs, results from the females, males or merged population are represented in pink, blue and grey, respectively.

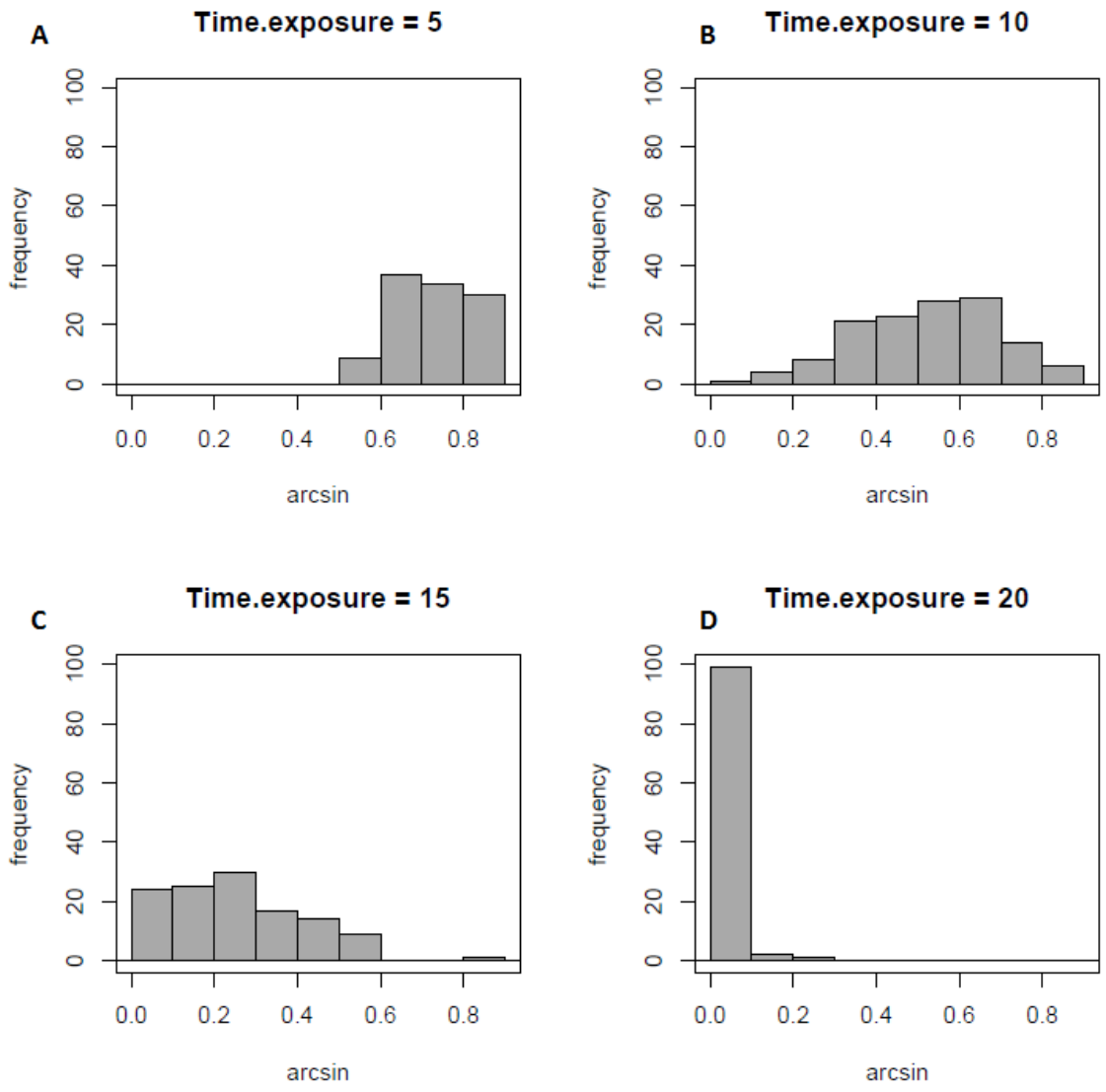


Figure 9-4 Distribution of chill-survival rates after 5, 10, 15 or 20 min of exposure to -20 °C.

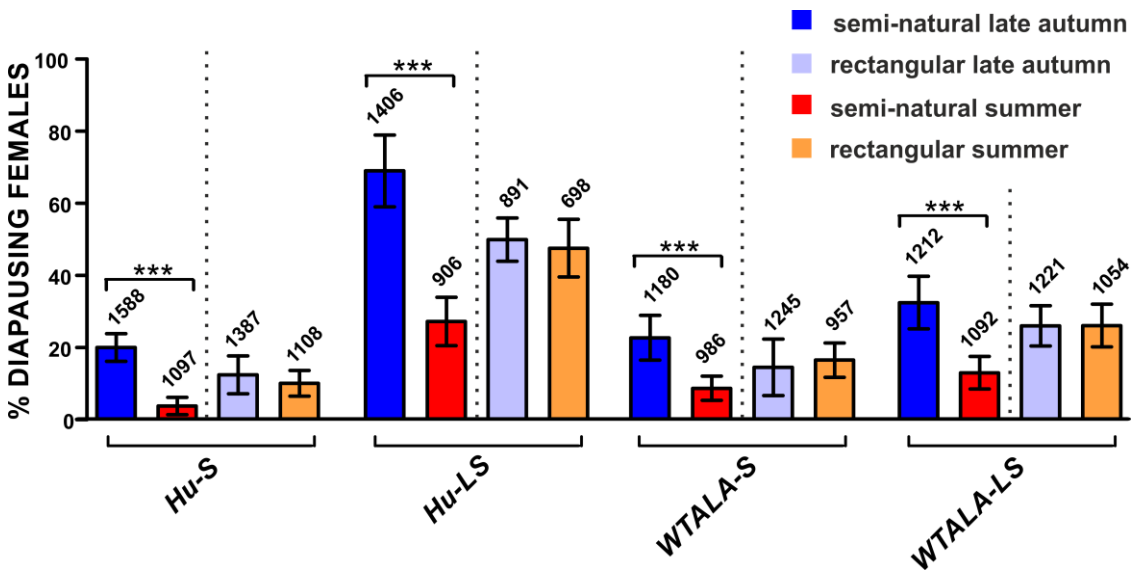


Figure 9-5 Semi-natural light profiles largely affect the incidence of diapause in *Drosophila* field lines. Simulation of consecutive late autumnal days induced a higher proportion of females to enter diapause (dark blue bars) compared to controls kept in short rectangular LD cycles (LD 8:16; light blue bars). Generation of summer light conditions promoted diapause response in a significantly smaller proportion of females (red bars) compared to controls exposed to long rectangular LD cycles (LD 15:9; orange bars). Robust photoperiodic diapause emerged when more realistic light profiles were used, highlighting significantly higher levels of dormancy during late autumnal days (red vs. blue bars). When flies were subjected to rectangular LD cycles, diapause levels did not differ when long and short days were compared (orange vs. light blue bars). Data are presented as mean ± SD. Numbers above each column indicate the number of dissected females. ANOVA after arcsine transformation, followed by post-hoc Tukey HSD test. ***p<0.001. *FIGURE 2 from Nagy et al. (2018).*

9.1.2 Tables

Table 9-1 TukeyHSD *posthoc* multiple comparison test of *w*^{1118}'s diapause levels for the interaction between photoperiod and condition (with or without temperature oscillation or with an inverted temperature and photoperiod cycle). Significant values in bold.

	diff	lwr	upr	P adj
T:LONG- T_inverted :LONG	**-0.536**	**-0.656**	**-0.417**	**<0.0001**
T_inverted:LONG-no_T:LONG	**-0.330**	**-0.460**	**-0.201**	**<0.0001**
T_inverted:SHORT- T_inverted:LONG	**-0.133**	**-0.252**	**-0.013**	**0.02**
T:SHORT- T_inverted:LONG	**0.009**	**-0.110**	**0.129**	**>0.99**
no_T:SHORT- T_inverted:LONG	-0.248	-0.387	-0.108	<0.0001
no_T:LONG-T:LONG	0.206	0.077	0.335	0.0002
T_inverted :SHORT-T:LONG	**0.404**	**0.284**	**0.523**	**<0.0001**
T:SHORT-T:LONG	**0.546**	**0.426**	**0.665**	**<0.0001**
no_T_inverted:SHORT-T:LONG	0.289	0.149	0.428	<0.0001
T_inverted:SHORT-no_T:LONG	0.198	0.068	0.327	0.0005
T:SHORT-no_T:LONG	0.340	0.210	0.469	<0.0001
no_T:SHORT-no_T:LONG	**0.083**	**-0.065**	**0.231**	**0.57**
T:SHORT- T_inverted:SHORT	**0.142**	**0.022**	**0.262**	**0.01**
no_T:SHORT- T_inverted :SHORT	-0.115	-0.255	0.024	0.16
no_T:SHORT-T:SHORT	-0.257	-0.397	-0.118	<0.0001

Table 9-2 Five-way ANOVA of the chill-survivorship after exposure to -20 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	Sum Sq	F value	Pr(>F)
Genotype	**2**	**0.5**	**6.32**	**0.002**
Photoperiod	1	0.04	0.91	0.34
Sex	1	0.11	2.74	0.10
Temperature.raised	**2**	**2.3**	**29.16**	**<0.0001**
Time.exposure	**3**	**139.36**	**1180.27**	**<0.0001**
Genotype:Photoperiod	2	0.17	2.19	0.11
Genotype:Sex	2	0	0.03	0.97
Photoperiod:Sex	1	0	0.01	0.92
Genotype:Temperature.raised	**4**	**0.78**	**4.93**	**0.0007**
Photoperiod:Temperature.raised	**2**	**0.68**	**8.67**	**0.0002**
Sex:Temperature.raised	2	0.11	1.46	0.23
Genotype:Time.exposure	6	0.22	0.92	0.48
Photoperiod:Time.exposure	**3**	**0.59**	**5.01**	**0.002**
Sex:Time.exposure	3	0.19	1.60	0.19
Temperature.raised:Time.exposure	**6**	**4.64**	**1.97**	**<0.0001**
Genotype:Photoperiod:Sex	2	0.01	0.13	0.87
Genotype:Photoperiod:Temperature.raised	**4**	**0.44**	**2.82**	**0.02**
Genotype:Sex:Temperature.raised	4	0.12	0.77	0.55
Photoperiod:Sex:Temperature.raised	2	0.09	1.20	0.30
Genotype:Photoperiod:Time.exposure	**6**	**0.62**	**2.64**	**0.02**
Genotype:Sex:Time.exposure	6	0.01	0.04	>0.99
Photoperiod:Sex:Time.exposure	3	0.08	0.66	0.57
Genotype:Temperature.raised: Time.exposure	**12**	**1.85**	**3.91**	**<0.0001**
Photoperiod:Temperature.raised: Time.exposure	6	0.18	0.77	0.59
Sex:Temperature.raised: Time.exposure	6	0.05	0.21	0.98
Genotype:Photoperiod:Sex: Temperature.raised	4	0.01	0.07	0.99
Genotype:Photoperiod:Sex: Time.exposure	6	0.14	0.57	0.75
Genotype:Photoperiod: Temperature.raised:Time.exposure	**12**	**1.09**	**2.31**	**0.007**
Genotype:Sex:Temperature.raised: Time.exposure	12	0.16	0.33	0.98
Photoperiod:Sex:Temperature.raised: Time.exposure	6	0.07	0.28	0.95
Genotype:Photoperiod:Sex: Temperature.raised:Time.exposure	12	0.26	0.55	0.88
Residuals	528	20.78		

Table 9-3 Four-way ANOVA of weight after 12 day in 12 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	SumSq	F	Pr(>F)
Genotype	**2**	**1639032**	**111.2**	**<0.0001**
Grown.T	1	15816	2.15	0.15
Photop.	1	7872	1.07	0.31
Sex	**1**	**2508993**	**340.44**	**<0.0001**
Genotype:Grown.T	**2**	**395001**	**26.80**	**<0.0001**
Genotype:Photop.	**2**	**855059**	**58.01**	**<0.0001**
Grown.T:Photop.	1	6126	0.83	0.37
Genotype:Sex	2	32042	2.17	0.12
Grown.T:Sex	1	20010	2.72	0.11
Photop.:Sex	1	8596	1.17	0.29
Genotype:Grown.T:Photop.	**2**	**478834**	**32.49**	**<0.0001**
Genotype:Grown.T:Sex	2	15238	1.03	0.36
Genotype:Photop.:Sex	2	37836	2.57	0.09
Grown.T:Photop.:Sex	1	1455	0.20	0.66
Genotype:Grown.T:Photop.:Sex	2	378	0.03	0.97
Residuals	50	368488		

Table 9-4 Four-way ANOVA of total protein levels normalised against total weight after 12 day in 12 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	SumSq	F	Pr(>F)
Genotype	**2**	**0.004**	**4.84**	**0.01**
Grown.T	**1**	**0.002**	**4.25**	**0.04**
Photop.	1	0.0004	0.96	0.33
Sex	1	0.0003	0.75	0.39
Genotype:Grown.T	**2**	**0.005**	**6.30**	**0.004**
Genotype:Photop.	**2**	**0.008**	**10.18**	**0.0002**
Grown.T:Photop.	**1**	**0.004**	**11.71**	**0.001**
Genotype:Sex	2	0.0008	1.03	0.36
Grown.T:Sex	1	0.00005	0.13	0.72
Photop.:Sex	1	0.00003	0.07	0.79
Genotype:Grown.T:Photop.	**2**	**0.004**	**4.99**	**0.01**
Genotype:Grown.T:Sex	2	0.0002	0.26	0.78
Genotype:Photop.:Sex	2	0.0002	0.24	0.79
Grown.T:Photop.:Sex	1	0.000001	0.002	0.96
Genotype:Grown.T:Photop.:Sex	2	0.0004	0.50	0.61
Residuals	50	0.019		

Table 9-5 Four-way ANOVA of total glucose levels normalised against total weight after 12 day in 12 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	SumSq	F	Pr(>F)
Genotype	2	91	0.037	0.96
Grown.T	1	741	0.599	0.44
Photop.	1	27	0.022	0.88
Sex	1	617	0.499	0.48
Genotype:Grown.T	2	60	0.024	0.98
Genotype:Photop.	2	204	0.083	0.92
Grown.T:Photop.	1	1	0.001	0.97
Genotype:Sex	2	1109	0.449	0.64
Grown.T:Sex	1	1059	0.857	0.36
Photop.:Sex	1	1240	1.003	0.32
Genotype:Grown.T:Photop.	2	161	0.065	0.94
Genotype:Grown.T:Sex	2	1469	0.595	0.56
Genotype:Photop.:Sex	2	2241	0.907	0.41
Grown.T:Photop.:Sex	1	1133	0.917	0.34
Genotype:Grown.T:Photop.:Sex	2	1631	0.66	0.52
Residuals	50	61780		

Table 9-6 Four-way ANOVA of total glycogen levels normalised against total weight after 12 day in 12 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	SumSq	F	Pr(>F)
Genotype	2	0.0002	0.69	0.50
Grown.T	1	0.0005	3.73	0.06
Photop.	**1**	**0.0007**	**5.38**	**0.02**
Sex	**1**	**0.005**	**36.18**	**<0.0001**
Genotype:Grown.T	2	0.0001	0.50	0.61
Genotype:Photop.	2	0.0004	1.36	0.27
Grown.T:Photop.	1	0.0003	2.09	0.15
Genotype:Sex	2	0.00002	0.09	0.91
Grown.T:Sex	1	0.00004	0.29	0.59
Photop.:Sex	**1**	**0.001**	**11.37**	**0.001**
Genotype:Grown.T:Photop.	**2**	**0.001**	**4.63**	**0.01**
Genotype:Grown.T:Sex	2	0.00006	0.22	0.80
Genotype:Photop.:Sex	2	0.00006	0.22	0.80
Grown.T:Photop.:Sex	1	0.0002	1.66	0.20
Genotype:Grown.T:Photop.:Sex	2	0.0004	1.39	0.26
Residuals	50	0.006		

Table 9-7 Four-way ANOVA of total trehalose levels normalised against total weight after 12 day in 12 °C when growing the flies at 15 °C, 18 °C or 25 °C under short or long photoperiod. Significant values in bold.

	Df	SumSq	F	Pr(>F)
Genotype	**2**	**0.0004**	**4**	**0.02**
Grown.T	**1**	**0.002**	**35.23**	**<0.0001**
Photop.	**1**	**0.001**	**20.82**	**<0.0001**
Sex	**1**	**0.001**	**18.66**	**<0.0001**
Genotype:Grown.T	2	0.0001	1.19	0.31
Genotype:Photop.	2	0.0003	3.16	0.05
Grown.T:Photop.	1	<0.0001	1.10	0.30
Genotype:Sex	2	0.0001	1.26	0.29
Grown.T:Sex	1	<0.0001	0.001	0.97
Photop.:Sex	**1**	**0.0006**	**11.83**	**0.001**
Genotype:Grown.T:Photop.	2	<0.0001	0.53	0.59
Genotype:Grown.T:Sex	2	0.0001	1.04	0.36
Genotype:Photop.:Sex	2	<0.0001	0.69	0.51
Grown.T:Photop.:Sex	1	0.000007	0.13	0.72
Genotype:Grown.T:Photop.:Sex	2	0.0002	1.96	0.15
Residuals	50	0.003		

9.2 Appendix Chapter 4

9.2.1 Figures

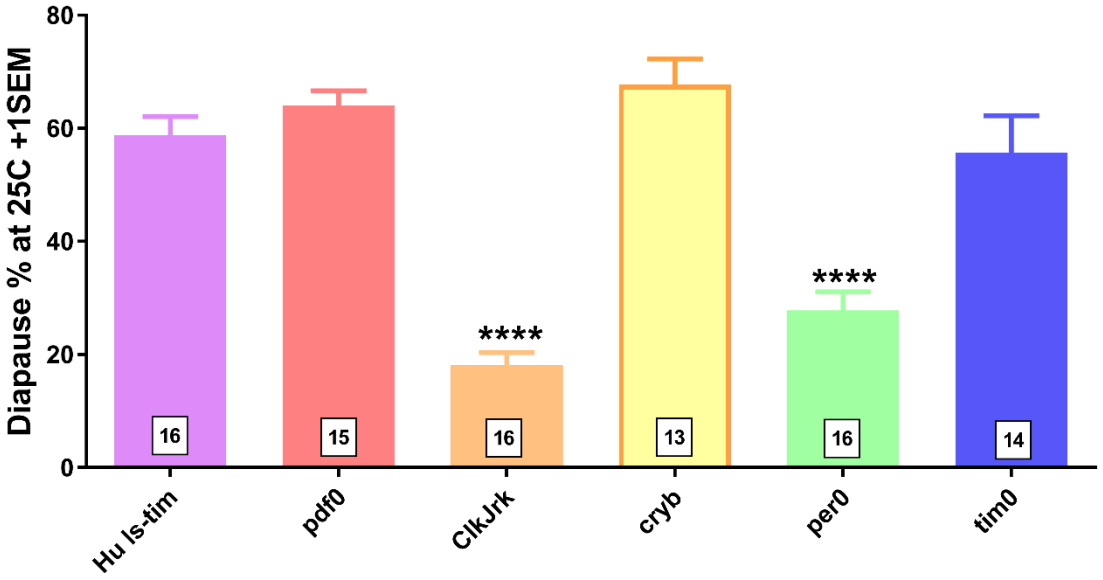


Figure 9-6 Diapause of the canonical clock mutants in Hu *ls-tim* background grown under the conventional 25 °C. *Adapted from Gesto (2011).*

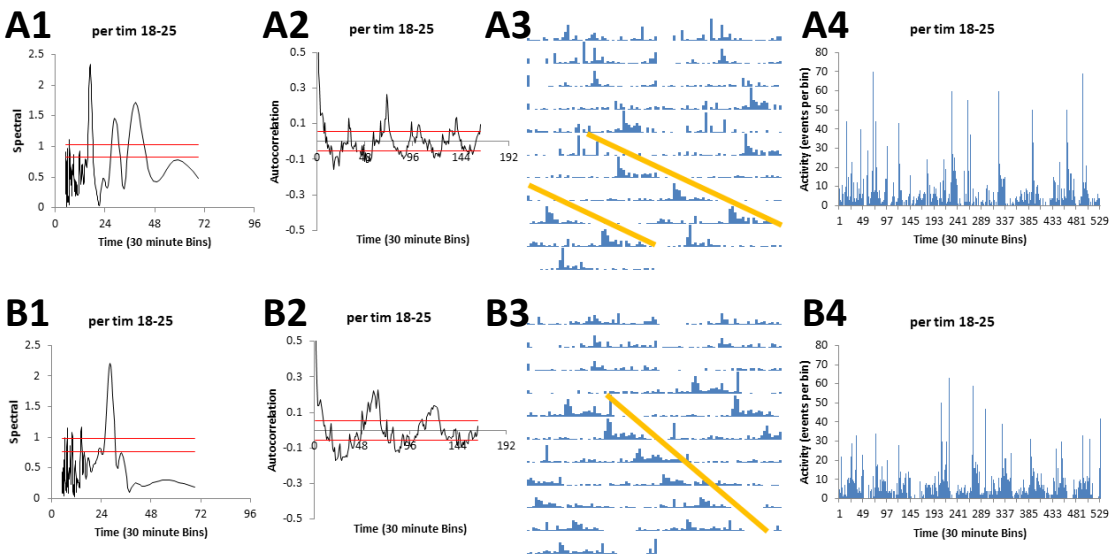


Figure Continues in the following page….

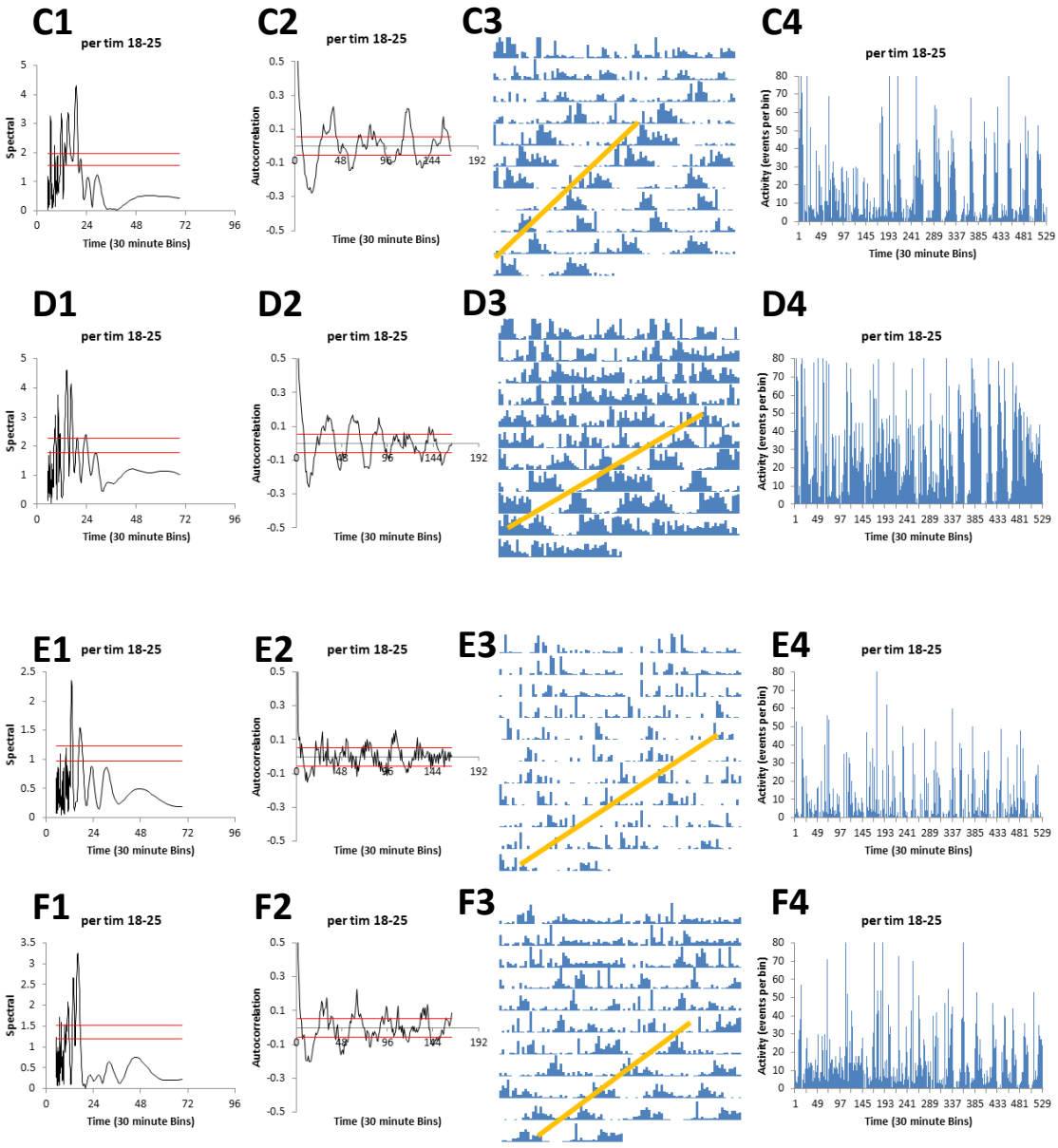


Figure Continues in the following page….

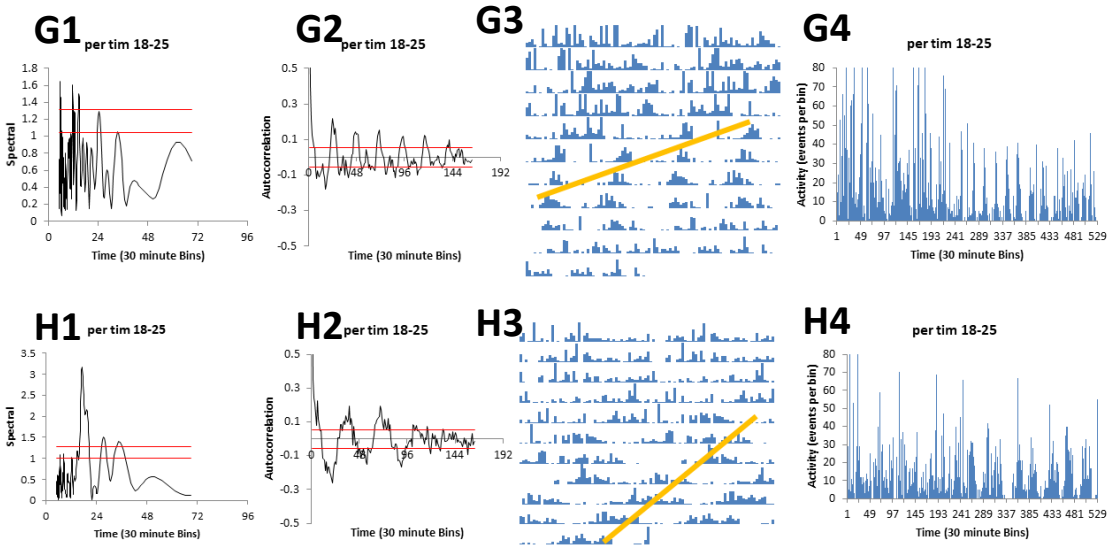


Figure 9-7 More examples of rhythmic *per*^{01}; *tim*^{01} flies at 25 °C reared at 18 °C (A-J). 1) Spectral analysis; 2) Autocorrelation; 3) Actogram; and 4) Overall activity pattern of the fly.

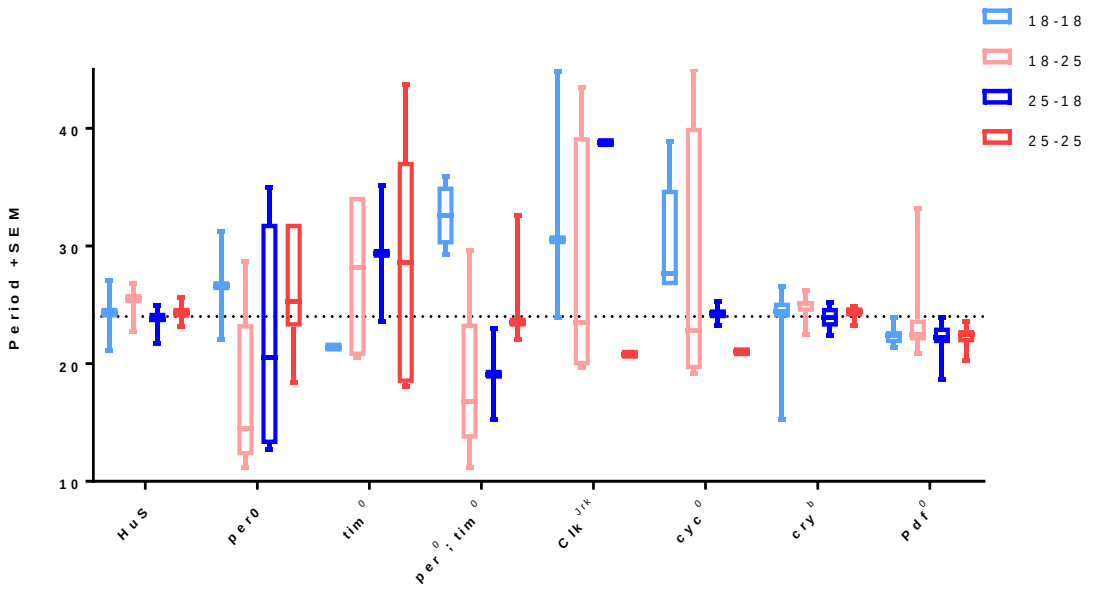


Figure 9-8 2.5 to 97.5 percentile box-and-whiskers plot of period length of the clock mutants and the HuS control. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Flies reared at 18 °C (lighter) or 25 °C (darker). The horizontal dotted line marks a 24h period.

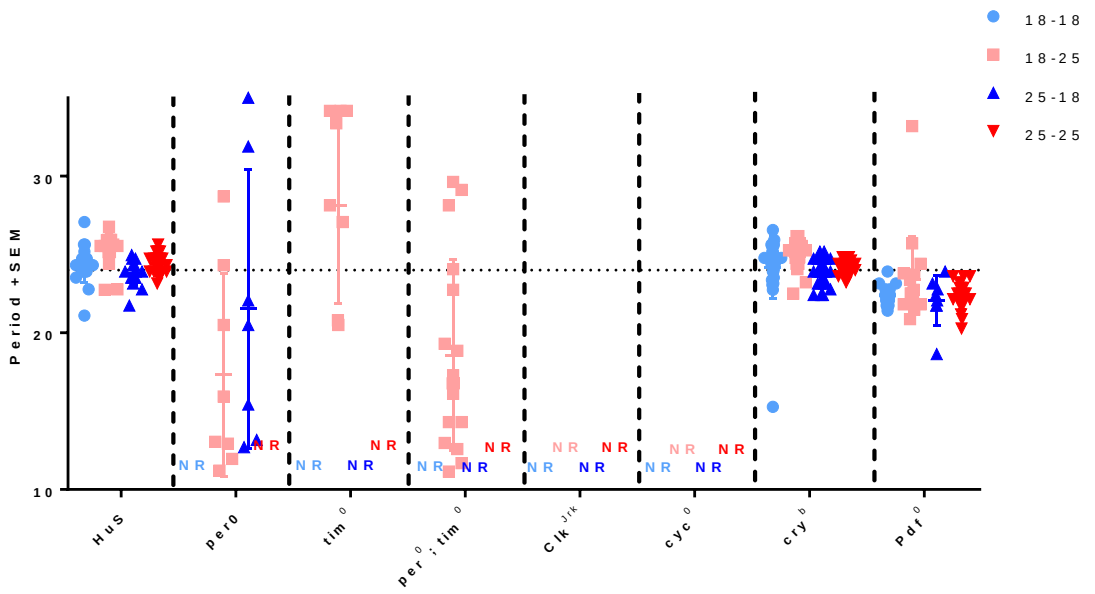


Figure 9-9 Scatter plot with Mean and SEM of the lines with more than 20% rhythmic individuals. Results from the flies kept at 18 °C or 25 °C are represented in blue and red, respectively. Flies reared at 18 °C (lighter) or 25 °C (darker). The horizontal dotted line marks a 24h period. *NR, not rhythmic*.

9.2.2 Tables

Table 9-8 Summary of period length of HuS control, *cry^{b}* and *Pdf^{0}* in the different experimental conditions.

Experimental temperature	18 °C	25 °C
Period (t)	23.70	24.10

Table 9-9 Summary of period length of HuS control, *cry^{b}* and *Pdf^{0}* in the different rearing conditions.

Rearing temperature	18 °C	25 °C
Period (t)	24.15	23.69

Table 9-10 Summary of period length of HuS control, *cry^{b}* and *Pdf^{0}*.

Genotype	HuS	cry^{b}	Pdf^{0}
Period (t)	24.28	24.41	22.59

9.3 Appendix Chapter 5

9.3.1 Figures

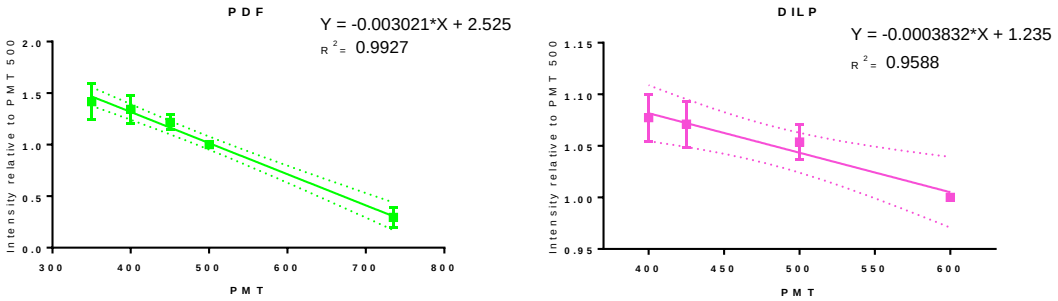


Figure 9-10 PMT vs intensity controls.

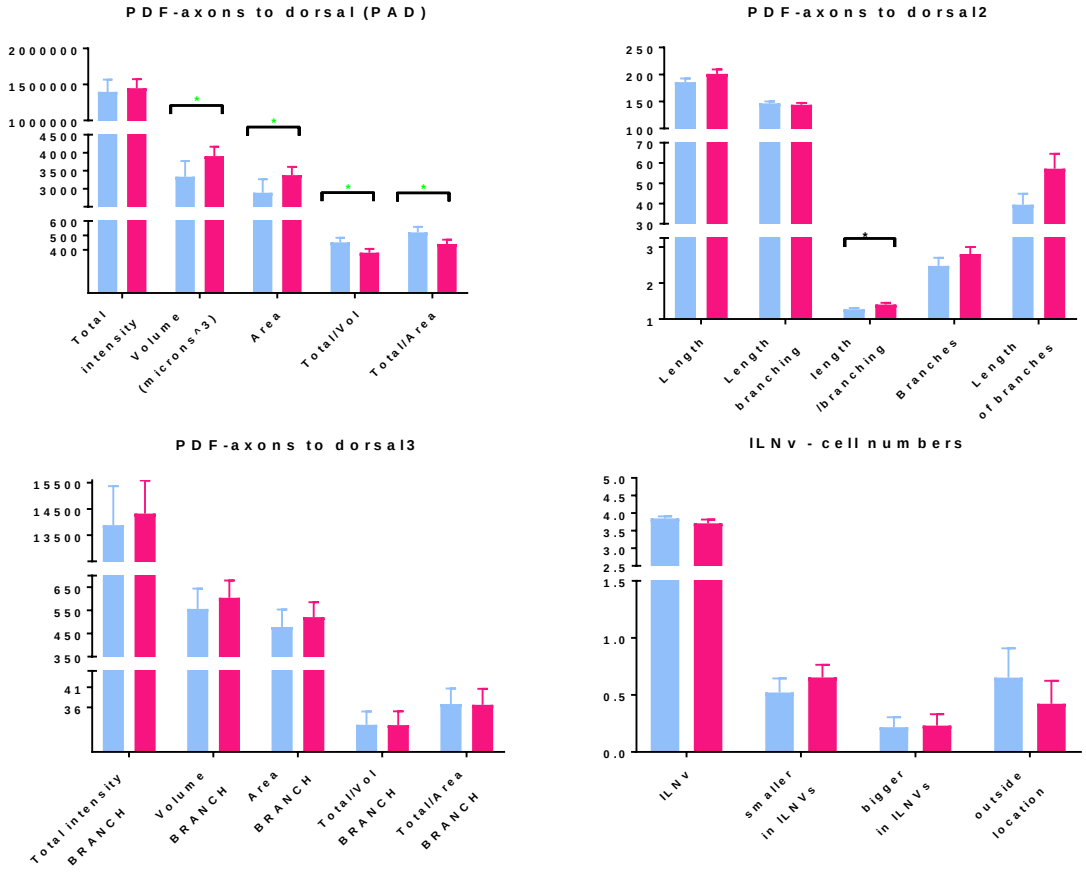


Figure 9-11 Diapause (blue) *vs* reproductive (magenta) values for PDF staining in different structures of *Drosophila* brain.

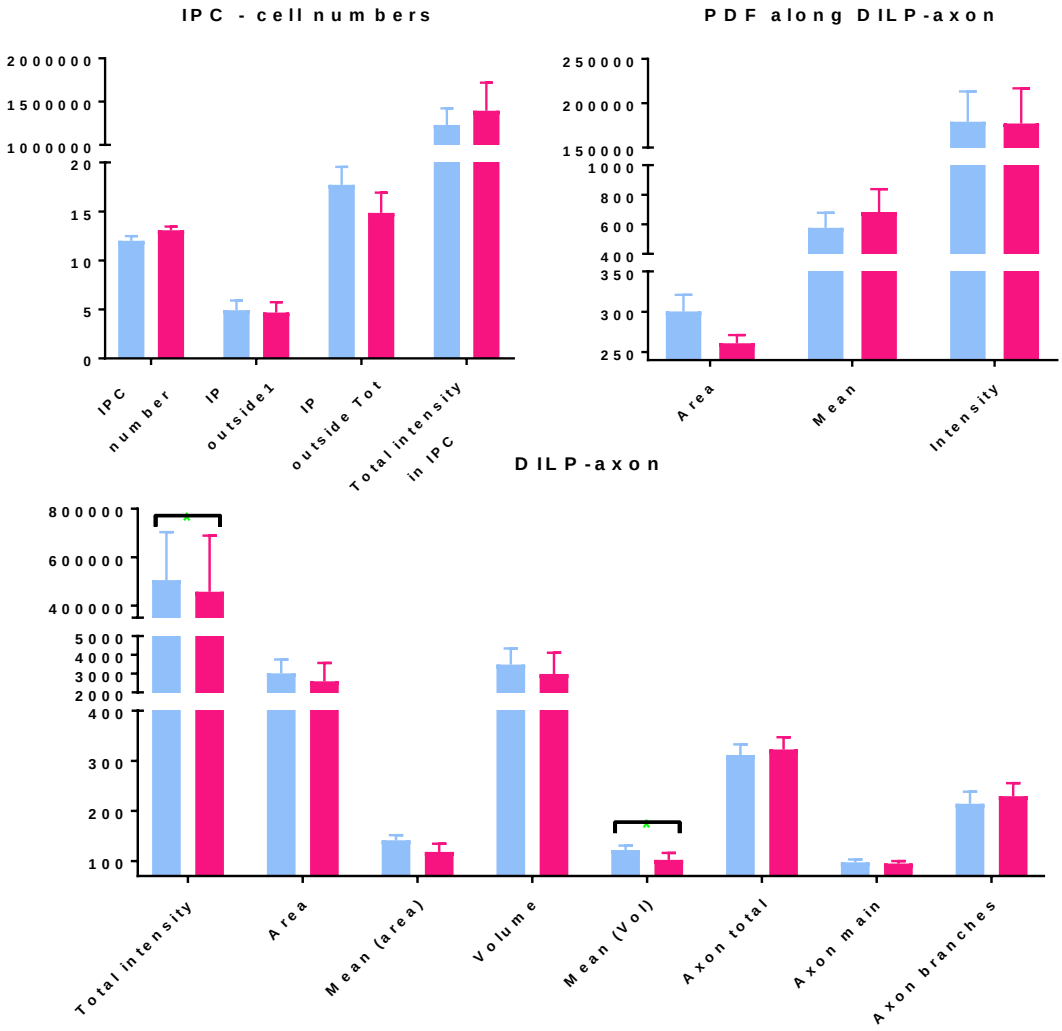


Figure 9-12 Diapause (blue) *vs* reproductive (magenta) values for PDF staining in different structures of *Drosophila* brain.

9.3.2 Tables

3-way ANOVA of the PDF-Tri area (Table 9-11_A) displays a strong Genotype and Experimental temperature effects as well as a Genotype:ExperimentalT and RearingT:ExperimentalT interactions ($p<0.0001$; $p<0.0001$; $p=0.005$ and $p=0.047$, respectively). Indeed, Tukey multiple comparison test shows that Clk^{Jrk} brains have significantly higher staining in the PDF-Tri area, independently of the experimental temperature ($p<0.001$), and the area is significantly increased in flies that have been reared at 25 °C and kept at 12 °C compared to the ones that were grown and maintained at 25 °C ($p=0.0001$). 3-way ANOVA (Table 9-11_B) shows a significant effect of Genotype and RearingT and Genotype:ExperimentalT, RearingT:ExperimentalT and Genotype:RearingT:ExperimentalT interactions ($p<0.0001$; $p=0.003$; $p<0.0001$; $p<0.0002$ and $p=0.018$). Both mutants have significantly higher PDF-Tri signal than the control flies ($p<0.0001$ and $p=0.01$, Clk^{Jrk} and per^{0}). Surprisingly, in per^{0} mutants that had been grown at 18 °C, the flies that were placed at 25 °C had significantly higher levels of PDF in the Tritocerebrum than those kept in diapause-inducing conditions ($p<0.0001$). Analysis of the POT volume reveals a Genotype, RearingT and ExperimentalT as well as Genotype:RearingT and Genotype:RearingT:ExperimentalT interactions ($p=0.003$; $p=0.003$; $p<0.0001$; $p<0.0001$ and $p=0.001$) (Table 9-11_C). The last feature analysed was the mean intensity along the POT (Table 9-11_D) which is influenced by Genotype, RearingT and ExperimentalT as well as Genotype:RearingT and Genotype:ExperimentalT interactions ($p=0.0006$; $p=0.006$; $p<0.0001$; $p<0.0001$ and $p=0.0006$). per^{0} displays significantly higher PDF levels than HuS controls ($p=0.0005$). PDF levels in the POT are significantly higher when the flies were kept at 25 °C ($p<0.0001$, for both mutants and $p=0.04$ for HuS) or when they were reared at 25 °C ($p<0.0001$, $p=0.048$ and $p=0.02$, for Clk^{Jrk}, per^{0} and HuS). Statistics for the Tukey multiple comparison tests performed can be found below (Table 9-12 to Table 9-15).

Table 9-11 3-way ANOVA tables for (A) PDF-Tri Area, (B) PDF-Tri Total intensity, (C) POT Volume and (D) POT Mean intensity.

		AREA TRI	Df	SumSq	Fvalue	Pr(>F)
A	**PDF-Tri Area**	**Genotype**	**2**	**3518**	**68.43**	**<0.0001**
		RearingT	1	28	1.08	0.30
		Exp.T	**1**	**474**	**18.43**	**<0.0001**
		Genotype:RearingT	2	104	2.03	0.14
		Genotype:Exp.T	**2**	**289**	**5.61**	**0.004**
		RearingT:Exp.T	**1**	**104**	**4.03**	**0.047**
		Genotype:RearingT:Exp.T	2	93	1.81	0.17
		Residuals	137	3521		
B	**PDF-Tri Total intensity**	**Genotype**	**2**	**52.49**	**48.17**	**<0.0001**
		RearingT	**1**	**4.84**	**8.89**	**0.003**
		Exp.T	1	0.33	0.60	0.44
		Genotype:RearingT	2	2.96	2.72	0.07
		Genotype:Exp.T	**2**	**16.9**	**15.51**	**<0.0001**
		RearingT:Exp.T	**1**	**7.77**	**14.26**	**0.0002**
		Genotype:RearingT:Exp.T	**2**	**4.54**	**4.16**	**0.018**
		Residuals	136	74.09		
C	**POT Volume**	**Exp.T**	**1**	**<0.0001**	**82.49**	**<0.0001**
		RearingT	**1**	**<0.0001**	**9.13**	**0.003**
		Genotype	**2**	**<0.0001**	**6.14**	**0.003**
		Exp.T:RearingT	1	<0.0001	0.68	0.41
		Exp.T:Genotype	2	<0.0001	0.83	0.44
		RearingT:Genotype	**2**	**<0.0001**	**10.31**	**<0.0001**
		Exp.T:RearingT:Genotype	**2**	**<0.0001**	**7.16**	**0.001**
		Residuals	130	<0.0001		
D	**POT Mean Intensity**	**Exp.T**	**1**	**3131**	**110.44**	**<0.0001**
		RearingT	**1**	**225**	**7.93**	**0.006**
		Genotype	**2**	**446**	**7.87**	**0.0006**
		Exp.T:RearingT	1	48	1.68	0.20
		Exp.T:Genotype	**2**	**446**	**7.86**	**0.0006**
		RearingT:Genotype	**2**	**934**	**16.47**	**<0.0001**
		Exp.T:RearingT:Genotype	2	94	1.66	0.20
		Residuals	130	3685		

Table 9-12 Tukey AREA TRI

Genotype				
	diff	**lwr**	**upr**	**p**
HuS-Clk	**-10.95**	**-13.33**	**-8.56**	**<0.0001**
per0-Clk	**-9.00**	**-11.38**	**-6.59**	**<0.0001**
per0-HuS	1.96	-0.50	4.43	0.15

RearingT				
	diff	**lwr**	**upr**	**p**
25-18	0.86	-0.78	2.50	0.30

Exp.T				
	diff	**lwr**	**upr**	**p**
25-12	**-3.67**	**-5.36**	**-1.97**	**<0.0001**

Genotype:RearingT				
	diff	**lwr**	**upr**	**p**
HuS:18-Clk:18	**-12.13**	**-16.30**	**-7.95**	**<0.0001**
per0:18-Clk:18	**-11.01**	**-15.14**	**-6.89**	**<0.0001**
Clk:25-Clk:18	-1.16	-5.15	2.84	0.96
HuS:25-Clk:18	-10.98	-14.97	-6.98	<0.0001
per0:25-Clk:18	-8.13	-12.20	-4.05	<0.0001
per0:18-HuS:18	1.11	-3.6	5.48	0.98
Clk:25-HuS:18	10.97	6.73	15.22	<0.0001
HuS:25-HuS:18	1.15	-3.09	5.40	0.97
per0:25-HuS:18	4.00	-0.32	8.33	0.09
Clk:25-per0:18	9.86	5.66	14.05	<0.0001
HuS:25-per0:18	0.04	-4.16	4.23	>0.99
per0:25-per0:18	2.89	-1.39	7.17	0.37
HuS:25-Clk:25	**-9.82**	**-13.88**	**-5.75**	**<0.0001**
per0:25-Clk:25	**-6.97**	**-11.12**	**-2.82**	**<0.0001**
per0:25-HuS:25	2.85	-1.30	7.00	0.36

Genotype:Exp.T				
	diff	**lwr**	**upr**	**p**
HuS:12-Clk:12	**-13.30**	**-17.04**	**-9.57**	**<0.0001**
per0:12-Clk:12	**-11.30**	**-14.96**	**-7.63**	**<0.0001**
Clk:25-Clk:12	**-7.42**	**-11.51**	**-3.33**	**<0.0001**
HuS:25-Clk:12	-14.64	-18.86	-10.42	<0.0001
per0:25-Clk:12	-12.97	-17.44	-8.51	<0.0001
per0:12-HuS:12	2.01	-1.78	5.79	0.64
Clk:25-HuS:12	5.89	1.69	10.09	0.001
HuS:25-HuS:12	-1.33	-5.66	2.99	0.95
per0:25-HuS:12	0.33	-4.23	4.89	>0.99
Clk:25-per0:12	3.88	-0.26	8.02	0.08

HuS:25-per0:12	-3.34	-7.61	0.92	0.22
per0:25-per0:12	-1.68	-6.18925	2.832719	0.890384
HuS:25-Clk:25	**-7.22**	**-11.8602**	**-2.58048**	**0.000207**
per0:25-Clk:25	**-5.56**	**-10.4203**	**-0.69452**	**0.015145**
per0:25-HuS:25	1.66	-3.30939	6.635279	0.927651

RearingT:Exp.T				
	diff	**lwr**	**upr**	**p**
25:12-18:12	1.88	-0.86233	4.622481	0.285913
18:25-18:12	-2.02	-5.15916	1.114281	0.33982
25:25-18:12	-3.53	-6.80092	-0.24939	0.029595
18:25-25:12	-3.90	-6.94766	-0.85737	0.006015
25:25-25:12	**-5.41**	**-8.59342**	**-2.21704**	**0.000121**
25:25-18:25	-1.50	-5.03581	2.030386	0.686339

Genotype:RearingT:Exp.T				
	diff	**lwr**	**upr**	**p**
HuS:18:12-Clk:18:12	**-15.37**	**-21.54**	**-9.20**	**<0.0001**
per0:18:12-Clk:18:12	**-15.26**	**-21.56**	**-8.97**	**<0.0001**
Clk:25:12-Clk:18:12	-1.81	-7.69	4.06	>0.99
HuS:25:12-Clk:18:12	-13.16	-19.22	-7.10	<0.0001
per0:25:12-Clk:18:12	-9.62	-15.41	-3.82	<0.0001
Clk:18:25-Clk:18:12	**-7.52**	**-13.96**	**-1.08**	**0.008**
HuS:18:25-Clk:18:12	-14.54	-21.84	-7.24	<0.0001
per0:18:25-Clk:18:12	-13.35	-20.15	-6.56	<0.0001
Clk:25:25-Clk:18:12	-9.09	-16.11	-2.07	0.002
HuS:25:25-Clk:18:12	-16.08	-22.68	-9.47	<0.0001
per0:25:25-Clk:18:12	-15.61	-23.68	-7.54	<0.0001
per0:18:12-HuS:18:12	0.11	-6.38	6.60	>0.99
Clk:25:12-HuS:18:12	13.56	7.48	19.65	<0.0001
HuS:25:12-HuS:18:12	2.22	-4.05	8.48	0.99
per0:25:12-HuS:18:12	5.76	-0.25	11.76	0.07
Clk:18:25-HuS:18:12	7.85	1.22	14.49	0.007
HuS:18:25-HuS:18:12	0.83	-6.64	8.30	>0.99
per0:18:25-HuS:18:12	2.02	-4.96	9.00	>0.99
Clk:25:25-HuS:18:12	6.28	-0.92	13.49	0.15
HuS:25:25-HuS:18:12	-0.71	-7.50	6.09	>0.99
per0:25:25-HuS:18:12	-0.24	-8.47	7.98	>0.99
Clk:25:12-per0:18:12	13.45	7.24	19.66	<0.0001
HuS:25:12-per0:18:12	2.11	-4.28	8.49	0.99
per0:25:12-per0:18:12	5.65	-0.49	11.79	0.10
Clk:18:25-per0:18:12	**7.74**	**0.99**	**14.50**	**0.01**
HuS:18:25-per0:18:12	0.72	-6.86	8.30	>0.99
per0:18:25-per0:18:12	1.91	-5.18	9.00	>0.99
Clk:25:25-per0:18:12	6.17	-1.14	13.48	0.19
HuS:25:25-per0:18:12	-0.82	-7.72	6.09	>0.99

per0:25:25-per0:18:12	-0.35	-8.67	7.97	>0.99
HuS:25:12-Clk:25:12	**-11.35**	**-17.32**	**-5.37**	**<0.0001**
per0:25:12-Clk:25:12	**-7.81**	**-13.51**	**-2.10**	**0.0007**
Clk:18:25-Clk:25:12	-5.71	-12.07	0.65	0.12
HuS:18:25-Clk:25:12	-12.73	-19.96	-5.50	<0.0001
per0:18:25-Clk:25:12	-11.54	-18.26	-4.82	<0.0001
Clk:25:25-Clk:25:12	**-7.28**	**-14.23**	**-0.33**	**0.03**
HuS:25:25-Clk:25:12	-14.27	-20.79	-7.74	<0.0001
per0:25:25-Clk:25:12	-13.80	-21.81	-5.80	<0.0001
per0:25:12-HuS:25:12	3.54	-2.35	9.43	0.69
Clk:18:25-HuS:25:12	5.64	-0.89	12.17	0.16
HuS:18:25-HuS:25:12	-1.39	-8.77	6.00	>0.99
per0:18:25-HuS:25:12	-0.20	-7.08	6.69	>0.99
Clk:25:25-HuS:25:12	4.07	-3.04	11.17	0.76
HuS:25:25-HuS:25:12	-2.92	-9.61	3.77	0.95
per0:25:25-HuS:25:12	-2.46	-10.60	5.69	>0.99
Clk:18:25-per0:25:12	2.10	-4.19	8.38	0.99
HuS:18:25-per0:25:12	-4.93	-12.09	2.24	0.49
per0:18:25-per0:25:12	-3.74	-10.39	2.91	0.78
Clk:25:25-per0:25:12	0.53	-6.356	7.40	>0.99
HuS:25:25-per0:25:12	-6.46	-12.91	-0.01	0.049
per0:25:25-per0:25:12	-6.00	-13.95	1.95	0.34
HuS:18:25-Clk:18:25	-7.02	-14.72	0.67	0.11
per0:18:25-Clk:18:25	-5.83	-13.05	1.38	0.24
Clk:25:25-Clk:18:25	-1.57	-9.01	5.86	>0.99
HuS:25:25-Clk:18:25	**-8.56**	**-15.60**	**-1.52**	**0.005**
per0:25:25-Clk:18:25	-8.10	-16.53	0.33	0.07
per0:18:25-HuS:18:25	1.19	-6.81	9.18	>0.99
Clk:25:25-HuS:18:25	5.45	-2.74	13.64	0.546
HuS:25:25-HuS:18:25	-1.54	-9.37	6.30	>0.99
per0:25:25-HuS:18:25	-1.0	-10.18	8.03	>0.99
Clk:25:25-per0:18:25	4.26	-3.48	12.01	0.80
HuS:25:25-per0:18:25	-2.72	-10.09	4.64	0.99
per0:25:25-per0:18:25	-2.26	-10.97	6.44	>0.99
HuS:25:25-Clk:25:25	-6.99	-14.57	0.59	0.106
per0:25:25-Clk:25:25	-6.52	-15.41	2.36	0.39
per0:25:25-HuS:25:25	0.46	-8.09	9.02	>0.99

Table 9-13 Tukey total Tri

Genotype				
	diff	**lwr**	**upr**	**p**
HuS-Clk	**-1.41**	**-1.75**	**-1.06**	**<0.0001**
per0-Clk	**-0.96**	**-1.31**	**-0.61**	**<0.0001**
per0-HuS	**0.45**	**0.09**	**0.81**	**0.01**

RearingT				
	diff	**lwr**	**upr**	**p**
25-18	**0.36**	**0.12**	**0.60**	**0.003**

Exp.T				
	diff	**lwr**	**upr**	**p**
25-12	-0.10	-0.34	0.15	0.44

Genotype:RearingT				
	diff	**lwr**	**upr**	**p**
HuS:18-Clk:18	**-1.09**	**-1.69**	**-0.48**	**<0.0001**
per0:18-Clk:18	**-0.88**	**-1.49**	**-0.29**	**0.0006**
Clk:25-Clk:18	**0.64**	**0.06**	**1.23**	**0.02**
HuS:25-Clk:18	-1.11	-1.70	-0.53	<0.0001
per0:25-Clk:18	-0.44	-1.03	0.15	0.27
per0:18-HuS:18	0.21	-0.43	0.84	0.94
Clk:25-HuS:18	1.73	1.11	2.35	<0.0001
HuS:25-HuS:18	-0.03	-0.65	0.59	>0.99
per0:25-HuS:18	0.65	0.02	1.28	0.04
Clk:25-per0:18	1.53	0.91	2.14	<0.0001
HuS:25-per0:18	-0.23	-0.84	0.38	0.88
per0:25-per0:18	0.44	-0.18	1.06	0.32
HuS:25-Clk:25	**-1.76**	**-2.36**	**-1.16**	**<0.0001**
per0:25-Clk:25	**-1.08**	**-1.69**	**-0.47**	**<0.0001**
per0:25-HuS:25	**0.68**	**0.07**	**1.28**	**0.02**

Genotype:Exp.T				
	diff	**lwr**	**upr**	**p**
HuS:12-Clk:12	**-1.24**	**-1.78**	**-0.69**	**<0.0001**
per0:12-Clk:12	**-1.40**	**-1.93**	**-0.87**	**<0.0001**
Clk:25-Clk:12	-0.35	-0.95	0.26	0.57
HuS:25-Clk:12	-1.99	-2.61	-1.38	<0.0001
per0:25-Clk:12	-0.49	-1.14	0.16	0.26
per0:12-HuS:12	-0.17	-0.71	0.39	0.96
Clk:25-HuS:12	0.89	0.27	1.51	0.0008
HuS:25-HuS:12	**-0.76**	**-1.39**	**-0.13**	**0.009**
per0:25-HuS:12	0.75	0.08	1.4	0.02
Clk:25-per0:12	1.06	0.44	1.67	<0.0001

HuS:25-per0:12	-0.59	-1.21	0.03	0.07
per0:25-per0:12	**0.92**	**0.26**	**1.57**	**0.001**
HuS:25-Clk:25	**-1.65**	**-2.33**	**-0.96**	**<0.0001**
per0:25-Clk:25	-0.14	-0.86	0.57	0.99
per0:25-HuS:25	**1.50**	**0.78**	**2.23**	**<0.0001**

RearingT:Exp.T				
	diff	**lwr**	**upr**	**p**
25:12-18:12	**0.70**	**0.30**	**1.10**	**<0.0001**
18:25-18:12	0.36	-0.10	0.82	0.18
25:25-18:12	0.12	-0.36	0.60	0.91
18:25-25:12	-0.34	-0.79	0.10	0.19
25:25-25:12	**-0.58**	**-1.05**	**-0.11**	**0.009**
25:25-18:25	-0.24	-0.76	0.28	0.63

Genotype:RearingT:Exp.T				
	diff	**lwr**	**upr**	**p**
HuS:18:12-Clk:18:12	**-1.18**	**-2.08**	**-0.28**	**0.001**
per0:18:12-Clk:18:12	**-1.79**	**-2.71**	**-0.87**	**<0.0001**
Clk:25:12-Clk:18:12	0.63	-0.23	1.48	0.38
HuS:25:12-Clk:18:12	-0.72	-1.60	0.17	0.24
per0:25:12-Clk:18:12	-0.55	-1.40	0.29	0.57
Clk:18:25-Clk:18:12	-0.30	-1.24	0.63	>0.99
HuS:18:25-Clk:18:12	-1.25	-2.32	-0.19	0.007
per0:18:25-Clk:18:12	-0.01	-1.00	0.98	>0.99
Clk:25:25-Clk:18:12	0.28	-0.78	1.34	>0.99
HuS:25:25-Clk:18:12	-1.98	-2.94	-1.02	<0.0001
per0:25:25-Clk:18:12	-0.59	-1.77	0.58	0.88
per0:18:12-HuS:18:12	-0.61	-1.55	0.34	0.59
Clk:25:12-HuS:18:12	1.81	0.92	2.70	<0.0001
HuS:25:12-HuS:18:12	0.47	-0.45	1.38	0.87
per0:25:12-HuS:18:12	0.63	-0.25	1.50	0.42
Clk:18:25-HuS:18:12	0.88	-0.09	1.84	0.11
HuS:18:25-HuS:18:12	-0.07	-1.16	1.01	>0.99
per0:18:25-HuS:18:12	1.17	0.16	2.19	0.01
Clk:25:25-HuS:18:12	1.46	0.37	2.55	0.0009
HuS:25:25-HuS:18:12	-0.80	-1.78	0.19	0.25
per0:25:25-HuS:18:12	0.59	-0.61	1.79	0.89
Clk:25:12-per0:18:12	2.42	1.51	3.32	<0.0001
HuS:25:12-per0:18:12	1.07	0.14	2.00	0.01
per0:25:12-per0:18:12	**1.24**	**0.34**	**2.13**	**0.0006**
Clk:18:25-per0:18:12	1.49	0.50	2.47	<0.0001
HuS:18:25-per0:18:12	0.54	-0.57	1.64	0.90
per0:18:25-per0:18:12	**1.78**	**0.75**	**2.82**	**<0.0001**
Clk:25:25-per0:18:12	2.07	0.97	3.17	<0.0001
HuS:25:25-per0:18:12	-0.19	-1.19	0.82	>0.99

per0:25:25-per0:18:12	1.20	-0.01	2.41	0.06
HuS:25:12-Clk:25:12	**-1.34**	**-2.21**	**-0.48**	**<0.0001**
per0:25:12-Clk:25:12	**-1.18**	**-2.01**	**-0.35**	**0.0003**
Clk:18:25-Clk:25:12	-0.93	-1.86	-0.006	0.047
HuS:18:25-Clk:25:12	-1.88	-2.94	-0.83	<0.0001
per0:18:25-Clk:25:12	-0.64	-1.61	0.34	0.58
Clk:25:25-Clk:25:12	-0.35	-1.40	0.70	0.99
HuS:25:25-Clk:25:12	-2.61	-3.56	-1.66	<0.0001
per0:25:25-Clk:25:12	-1.22	-2.38	-0.05	0.03
per0:25:12-HuS:25:12	0.16	-0.70	1.02	>0.99
Clk:18:25-HuS:25:12	0.41	-0.54	1.36	0.95
HuS:18:25-HuS:25:12	-0.54	-1.61	0.54	0.88
per0:18:25-HuS:25:12	0.71	-0.29	1.71	0.45
Clk:25:25-HuS:25:12	0.99	-0.08	2.07	0.10
HuS:25:25-HuS:25:12	**-1.26**	**-2.24**	**-0.29**	**0.002**
per0:25:25-HuS:25:12	0.12	-1.06	1.31	1
Clk:18:25-per0:25:12	0.25	-0.66	1.16	>0.99
HuS:18:25-per0:25:12	-0.70	-1.74	0.34	0.53
per0:18:25-per0:25:12	0.55	-0.42	1.51	0.77
Clk:25:25-per0:25:12	0.83	-0.21	1.88	0.26
HuS:25:25-per0:25:12	-1.42	-2.36	-0.48	<0.0001
per0:25:25-per0:25:12	-0.04	-1.20	1.12	1
HuS:18:25-Clk:18:25	-0.95	-2.07	0.17	0.18
per0:18:25-Clk:18:25	0.30	-0.76	1.35	>0.99
Clk:25:25-Clk:18:25	0.58	-0.54	1.70	0.85
HuS:25:25-Clk:18:25	-1.67	-2.70	-0.65	<0.0001
per0:25:25-Clk:18:25	-0.29	-1.52	0.94	>0.99
per0:18:25-HuS:18:25	**1.25**	**0.08**	**2.41**	**0.02**
Clk:25:25-HuS:18:25	1.53	0.31	2.76	0.003
HuS:25:25-HuS:18:25	-0.72	-1.86	0.42	0.62
per0:25:25-HuS:18:25	0.66	-0.66	1.99	0.88
Clk:25:25-per0:18:25	0.29	-0.88	1.45	>0.99
HuS:25:25-per0:18:25	-1.97	-3.04	-0.90	<0.0001
per0:25:25-per0:18:25	-0.59	-1.85	0.68	0.93
HuS:25:25-Clk:25:25	**-2.26**	**-3.40**	**-1.12**	**<0.0001**
per0:25:25-Clk:25:25	-0.87	-2.20	0.45	0.56
per0:25:25-HuS:25:25	**1.39**	**0.14**	**2.63**	**0.02**

Table 9-14 Tukey POT Volume

Exp.T				
	diff	**lwr**	**upr**	**p**
25-12	**-0.001**	**-0.002**	**-0.001**	**<0.0001**
RearingT				
	diff	**lwr**	**upr**	**p**
25-18	**-0.0004**	**-0.0007**	**-0.0001**	**0.003**
Genotype				
	diff	**Lwr**	**upr**	**p**
HuS-Clk	-0.0003	-0.0006	0.0001	0.30
per0-Clk	**-0.0006**	**-0.001**	**-0.0002**	**0.002**
per0-HuS	-0.0003	-0.0007	<0.0001	0.14
Exp.T:RearingT				
	diff	**lwr**	**upr**	**p**
25:18-12:18	**-0.001**	**-0.002**	**-0.0006**	**<0.0001**
12:25-12:18	-0.0003	-0.0008	0.0001	0.26
25:25-12:18	-0.002	-0.002	-0.001	<0.0001
12:25-25:18	0.0008	0.0003	0.001	0.0002
25:25-25:18	-0.0006	-0.001	<0.0001	0.06
25:25-12:25	**-0.001**	**-0.002**	**-0.0008**	**<0.0001**
Exp.T:Genotype				
	diff	**lwr**	**upr**	**p**
25:Clk-12:Clk	**<0.0001**	**-0.002**	**-0.0001**	**<0.0001**
12:HuS-12:Clk	<0.0001	-0.001	0.0002	0.38
25:HuS-12:Clk	<0.0001	-0.002	-0.0008	<0.0001
12:per0-12:Clk	**<0.0001**	**-0.001**	**<0.0001**	**0.02**
25:per0-12:Clk	<0.0001	-0.003	-0.001	<0.0001
12:HuS-25:Clk	0.001	0.0004	0.002	0.0004
25:HuS-25:Clk	1.02E-06	-0.0007	0.0007	>0.99
12:per0-25:Clk	0.0008	0.0002	0.002	0.007
25:per0-25:Clk	-0.0004	-0.001	0.0004	0.64
25:HuS-12:HuS	**-0.001**	**-0.002**	**-0.0004**	**0.0004**
12:per0-12:HuS	-0.0002	-0.0009	0.0004	0.88
25:per0-12:HuS	**-0.001**	**-0.002**	**-0.0007**	**<0.0001**
12:per0-25:HuS	**0.0008**	**0.0002**	**0.002**	**0.007**
25:per0-25:HuS	-0.0004	-0.001	0.0004	0.64
25:per0-12:per0	**-0.001**	**-0.002**	**-0.0005**	**<0.0001**
RearingT:Genotype				
	diff	**lwr**	**upr**	**p**
25:Clk-18:Clk	-0.0004	-0.001	0.0002	0.32

18:HuS-18:Clk	0.0002	-0.0005	0.0009	0.98
25:HuS-18:Clk	-0.001	-0.002	-0.0004	0.0002
18:per0-18:Clk	**-0.001**	**-0.002**	**-0.0003**	**0.0004**
25:per0-18:Clk	-0.0006	-0.001	<0.0001	0.11
18:HuS-25:Clk	0.0006	<0.0001	0.001	0.13
25:HuS-25:Clk	-0.0006	-0.001	0.0001	0.17
18:per0-25:Clk	-0.0005	-0.001	0.0002	0.25
25:per0-25:Clk	-0.0001	-0.0008	0.0006	0.99
25:HuS-18:HuS	**-0.001**	**-0.002**	**-0.0005**	**<0.0001**
18:per0-18:HuS	**-0.001**	**-0.002**	**-0.0004**	**0.0002**
25:per0-18:HuS	-0.0008	-0.001	>-0.0001	0.04
18:per0-25:HuS	<0.0001	-0.0006	0.0007	>0.99
25:per0-25:HuS	0.0004	-0.0003	0.001	0.49
25:per0-18:per0	0.0004	-0.0003	0.001	0.61
Exp.T:RearingT:Genotype				
	diff	**lwr**	**upr**	**p**
25:18:Clk-12:18:Clk	-0.0009	-0.002	0.0002	0.18
12:25:Clk-12:18:Clk	<0.0001	-0.0009	0.001	>0.99
25:25:Clk-12:18:Clk	-0.002	-0.003	-0.001	<0.0001
12:18:HuS-12:18:Clk	0.0005	-0.0005	0.002	0.88
25:18:HuS-12:18:Clk	-0.001	-0.002	<0.0001	0.045
12:25:HuS-12:18:Clk	-0.001	-0.002	-0.0002	0.008
25:25:HuS-12:18:Clk	-0.002	-0.003	-0.0007	<0.0001
12:18:per0-12:18:Clk	-0.0008	-0.002	0.0001	0.17
25:18:per0-12:18:Clk	-0.002	-0.003	-0.0009	<0.0001
12:25:per0-12:18:Clk	-0.0004	-0.001	0.0005	0.94
25:25:per0-12:18:Clk	-0.002	-0.003	-0.0004	0.0009
12:25:Clk-25:18:Clk	0.0009	-0.009	0.002	0.11
25:25:Clk-25:18:Clk	**-0.001**	**-0.003**	**-0.0001**	**0.02**
12:18:HuS-25:18:Clk	0.001	0.0003	0.003	0.003
25:18:HuS-25:18:Clk	-0.0003	-0.002	0.0009	>0.99
12:25:HuS-25:18:Clk	-0.0003	-0.001	0.0008	>0.99
25:25:HuS-25:18:Clk	-0.0009	-0.002	0.0002	0.27
12:18:per0-25:18:Clk	<0.0001	-0.001	0.001	>0.99
25:18:per0-25:18:Clk	-0.001	-0.002	<0.0001	0.07
12:25:per0-25:18:Clk	0.0004	-0.0006	0.001	0.96
25:25:per0-25:18:Clk	-0.0008	-0.002	0.0005	0.68
25:25:Clk-12:25:Clk	**-0.002**	**-0.003**	**-0.001**	**<0.0001**
12:18:HuS-12:25:Clk	0.0005	-0.0006	0.002	0.95
25:18:HuS-12:25:Clk	-0.001	-0.002	>-0.0001	0.03
12:25:HuS-12:25:Clk	**-0.001**	**-0.002**	**-0.0002**	**0.004**
25:25:HuS-12:25:Clk	-0.002	-0.003	-0.0008	<0.0001
12:18:per0-12:25:Clk	-0.0009	-0.002	<0.0001	0.1
25:18:per0-12:25:Clk	-0.002	-0.003	-0.001	<0.0001
12:25:per0-12:25:Clk	-0.0005	-0.001	0.0005	0.85

25:25:per0-12:25:Clk	-0.002	-0.003	-0.0005	0.0004
12:18:HuS-25:25:Clk	0.003	0.002	0.004	<0.0001
25:18:HuS-25:25:Clk	0.001	-0.0003	0.002	0.28
12:25:HuS-25:25:Clk	0.001	-0.0001	0.002	0.14
25:25:HuS-25:25:Clk	0.0005	-0.0007	0.002	0.97
12:18:per0-25:25:Clk	0.001	0.0002	0.003	0.006
25:18:per0-25:25:Clk	0.0002	-0.001	0.002	>0.99
12:25:per0-25:25:Clk	0.002	0.0006	0.003	<0.0001
25:25:per0-25:25:Clk	0.0006	-0.0008	0.002	0.96
25:18:HuS-12:18:HuS	**-0.002**	**-0.003**	**-0.0005**	**0.0007**
12:25:HuS-12:18:HuS	**-0.002**	**-0.003**	**-0.0006**	**<0.0001**
25:25:HuS-12:18:HuS	-0.002	-0.003	-0.001	<0.0001
12:18:per0-12:18:HuS	**-0.001**	**-0.002**	**-0.0003**	**0.003**
25:18:per0-12:18:HuS	-0.003	-0.004	-0.001	<0.0001
12:25:per0-12:18:HuS	-0.001	-0.002	0.0001	0.12
25:25:per0-12:18:HuS	-0.002	-0.003	-0.0009	<0.0001
12:25:HuS-25:18:HuS	<0.0001	-0.001	0.001	1
25:25:HuS-25:18:HuS	-0.0005	-0.002	0.0007	0.92
12:18:per0-25:18:HuS	0.0004	-0.0008	0.002	>0.99
25:18:per0-25:18:HuS	-0.0008	-0.002	0.0005	0.61
12:25:per0-25:18:HuS	0.0007	-0.0004	0.002	0.63
25:25:per0-25:18:HuS	-0.0005	-0.002	0.0009	0.99
25:25:HuS-12:25:HuS	-0.0006	-0.002	0.0005	0.83
12:18:per0-12:25:HuS	0.0003	-0.0006	0.001	0.99
25:18:per0-12:25:HuS	-0.0008	-0.002	0.0003	0.43
12:25:per0-12:25:HuS	0.0007	-0.0003	0.002	0.41
25:25:per0-12:25:HuS	-0.0005	-0.002	0.0008	0.98
12:18:per0-25:25:HuS	0.0009	-0.0001	0.002	0.14
25:18:per0-25:25:HuS	-0.0003	-0.001	0.0009	>0.99
12:25:per0-25:25:HuS	**0.001**	**0.0003**	**0.002**	**0.003**
25:25:per0-25:25:HuS	0.0001	-0.001	0.001	>0.99
25:18:per0-12:18:per0	**-0.001**	**-0.002**	**>-0.0001**	**0.03**
12:25:per0-12:18:per0	0.0004	-0.0006	0.001	0.97
25:25:per0-12:18:per0	-0.0008	-0.002	0.0004	0.53
12:25:per0-25:18:per0	0.002	0.0004	0.003	0.0006
25:25:per0-25:18:per0	0.0004	-0.001	0.002	>0.99
25:25:per0-12:25:per0	-0.001	-0.002	<0.0001	0.06

Table 9-15 Tukey POT Mean

Exp.T				
	diff	lwr	upr	p
25-12	**9.61**	**7.80**	**11.42**	**<0.0001**

RearingT				
	diff	lwr	upr	p
25-18	**2.52**	**0.75**	**4.28**	**0.006**

Genotype				
	diff	lwr	upr	p
HuS-Clk	-1.46	-4.05	1.12	0.37
per0-Clk	**2.87**	**0.32**	**5.43**	**0.023**
per0-HuS	**4.34**	**1.67**	**7.00**	**0.0005**

Exp.T:RearingT				
	diff	lwr	upr	p
25:18-12:18	**8.51**	**5.17**	**11.86**	**<0.0001**
12:25-12:18	1.59	-1.40	4.57	0.51
25:25-12:18	**12.47**	**9.05**	**15.88**	**<0.0001**
12:25-25:18	**-6.93**	**-10.24**	**-3.61**	**<0.0001**
25:25-25:18	**3.95**	**0.25**	**7.66**	**0.03**
25:25-12:25	**10.88**	**7.49**	**14.27**	**<0.0001**

Exp.T:Genotype				
	diff	lwr	upr	p
25:Clk-12:Clk	**13.08**	**8.69**	**17.48**	**<0.0001**
12:HuS-12:Clk	2.06	-2.10	6.21	0.71
25:HuS-12:Clk	6.86	2.47	11.25	0.0002
12:per0-12:Clk	3.56	-0.36	7.47	0.10
25:per0-12:Clk	15.04	10.33	19.76	<0.0001
12:HuS-25:Clk	-11.03	-15.69	-6.37	<0.0001
25:HuS-25:Clk	**-6.22**	**-11.09**	**-1.35**	**0.004**
12:per0-25:Clk	-9.53	-13.97	-5.08	<0.0001
25:per0-25:Clk	1.96	-3.21	7.12	0.88
25:HuS-12:HuS	**4.81**	**0.15**	**9.47**	**0.04**
12:per0-12:HuS	1.50	-2.72	5.72	0.91
25:per0-12:HuS	12.99	8.02	17.96	<0.0001
12:per0-25:HuS	-3.31	-7.75	1.14	0.27
25:per0-25:HuS	**8.18**	**3.01**	**13.34**	**0.0002**
25:per0-12:per0	**11.48**	**6.72**	**16.25**	**<0.0001**

RearingT:Genotype				
	diff	lwr	upr	p
25:Clk-18:Clk	**7.14**	**2.86**	**11.42**	**<0.0001**

18:HuS-18:Clk	-0.84	-5.42	3.74	0.99
25:HuS-18:Clk	4.34	0.11	8.58	0.04
18:per0-18:Clk	8.39	4.11	12.68	<0.0001
25:per0-18:Clk	3.83	-0.56	8.21	0.13
18:HuS-25:Clk	-7.98	-12.71	-3.25	<0.0001
25:HuS-25:Clk	-2.80	-7.20	1.61	0.45
18:per0-25:Clk	1.25	-3.19	5.70	0.96
25:per0-25:Clk	-3.31	-7.86	1.23	0.29
25:HuS-18:HuS	**5.18**	**0.49**	**9.87**	**0.02**
18:per0-18:HuS	**9.23**	**4.50**	**13.96**	**<0.0001**
25:per0-18:HuS	4.66	-0.16	9.49	0.06
18:per0-25:HuS	4.05	-0.35	8.45	0.09
25:per0-25:HuS	-0.52	-5.02	3.98	>0.99
25:per0-18:per0	**-4.57**	**-9.11**	**-0.02**	**0.048**
Exp.T:RearingT:Genotype				
	diff	**lwr**	**upr**	**p**
25:18:Clk-12:18:Clk	**10.57**	**3.80**	**17.33**	**<0.0001**
12:25:Clk-12:18:Clk	4.87	-1.40	11.13	0.30
25:25:Clk-12:18:Clk	22.20	14.52	29.87	<0.0001
12:18:HuS-12:18:Clk	1.96	-4.98	8.90	>0.99
25:18:HuS-12:18:Clk	5.61	-2.07	13.28	0.39
12:25:HuS-12:18:Clk	6.57	-0.05	13.18	0.05
25:25:HuS-12:18:Clk	12.41	5.64	19.18	<0.0001
12:18:per0-12:18:Clk	**7.97**	**1.60**	**14.34**	**0.003**
25:18:per0-12:18:Clk	19.67	12.28	27.05	<0.0001
12:25:per0-12:18:Clk	3.97	-2.40	10.34	0.64
25:25:per0-12:18:Clk	14.26	6.23	22.29	<0.0001
12:25:Clk-25:18:Clk	-5.70	-12.46	1.07	0.19
25:25:Clk-25:18:Clk	**11.63**	**3.54**	**19.72**	**0.0003**
12:18:HuS-25:18:Clk	-8.61	-16.00	-1.21	0.01
25:18:HuS-25:18:Clk	-4.96	-13.05	3.13	0.67
12:25:HuS-25:18:Clk	-4.00	-11.09	3.09	0.77
25:25:HuS-25:18:Clk	1.85	-5.39	9.08	>0.99
12:18:per0-25:18:Clk	-2.60	-9.46	4.27	0.98
25:18:per0-25:18:Clk	**9.10**	**1.29**	**16.92**	**0.009**
12:25:per0-25:18:Clk	-6.60	-13.46	0.27	0.07
25:25:per0-25:18:Clk	3.69	-4.74	12.12	0.95
25:25:Clk-12:25:Clk	**17.33**	**9.65**	**25.00**	**<0.0001**
12:18:HuS-12:25:Clk	-2.91	-9.85	4.03	0.96
25:18:HuS-12:25:Clk	0.74	-6.94	8.41	>0.99
12:25:HuS-12:25:Clk	1.70	-4.92	8.31	>0.99
25:25:HuS-12:25:Clk	7.54	0.77	14.31	0.02
12:18:per0-12:25:Clk	3.10	-3.27	9.47	0.90
25:18:per0-12:25:Clk	14.80	7.41	22.18	<0.0001
12:25:per0-12:25:Clk	-0.90	-7.27	5.47	>0.99

25:25:per0-12:25:Clk	9.39	1.36	17.42	0.008
12:18:HuS-25:25:Clk	-20.24	-28.47	-12.00	<0.0001
25:18:HuS-25:25:Clk	-16.59	-25.45	-7.73	<0.0001
12:25:HuS-25:25:Clk	-15.63	-23.60	-7.67	<0.0001
25:25:HuS-25:25:Clk	**-9.79**	**-17.88**	**-1.70**	**0.005**
12:18:per0-25:25:Clk	-14.23	-21.99	-6.47	<0.0001
25:18:per0-25:25:Clk	-2.53	-11.14	6.081	>0.99
12:25:per0-25:25:Clk	-18.23	-25.99	-10.47	<0.0001
25:25:per0-25:25:Clk	-7.94	-17.11	1.23	0.16
25:18:HuS-12:18:HuS	3.65	-4.59	11.88	0.94
12:25:HuS-12:18:HuS	4.61	-2.65	11.87	0.62
25:25:HuS-12:18:HuS	10.45	3.05	17.85	0.0004
12:18:per0-12:18:HuS	6.01	-1.03	13.04	0.17
25:18:per0-12:18:HuS	17.71	9.74	25.67	<0.0001
12:25:per0-12:18:HuS	2.01	-5.02	9.05	>0.99
25:25:per0-12:18:HuS	12.30	3.73	20.87	0.0003
12:25:HuS-25:18:HuS	0.96	-7.005	8.92	>0.99
25:25:HuS-25:18:HuS	6.80	-1.28	14.89	0.19
12:18:per0-25:18:HuS	2.36	-5.40	10.12	>0.99
25:18:per0-25:18:HuS	**14.06**	**5.45**	**22.67**	**<0.0001**
12:25:per0-25:18:HuS	-1.64	-9.40	6.12	>0.99
25:25:per0-25:18:HuS	8.65	-0.52	17.82	0.08
25:25:HuS-12:25:HuS	5.85	-1.25	12.94	0.22
12:18:per0-12:25:HuS	1.40	-5.31	8.12	>0.99
25:18:per0-12:25:HuS	13.10	5.42	20.79	<0.0001
12:25:per0-12:25:HuS	-2.60	-9.31	4.12	0.98
25:25:per0-12:25:HuS	7.69	-0.61	16.00	0.10
12:18:per0-25:25:HuS	-4.44	-11.31	2.42	0.58
25:18:per0-25:25:HuS	7.26	-0.56	15.07	0.10
12:25:per0-25:25:HuS	-8.44	-15.31	-1.58	0.004
25:25:per0-25:25:HuS	1.85	-6.58	10.28	>0.99
25:18:per0-12:18:per0	**11.70**	**4.23**	**19.17**	**<0.0001**
12:25:per0-12:18:per0	-4.00	-10.47	2.47	0.65
25:25:per0-12:18:per0	6.29	-1.82	14.40	0.30
12:25:per0-25:18:per0	-15.70	-23.17	-8.23	<0.0001
25:25:per0-25:18:per0	-5.41	-14.34	3.52	0.68
25:25:per0-12:25:per0	**10.29**	**2.18**	**18.40**	**0.003**

9.4 Appendix Chapter 6

9.4.1 Figures

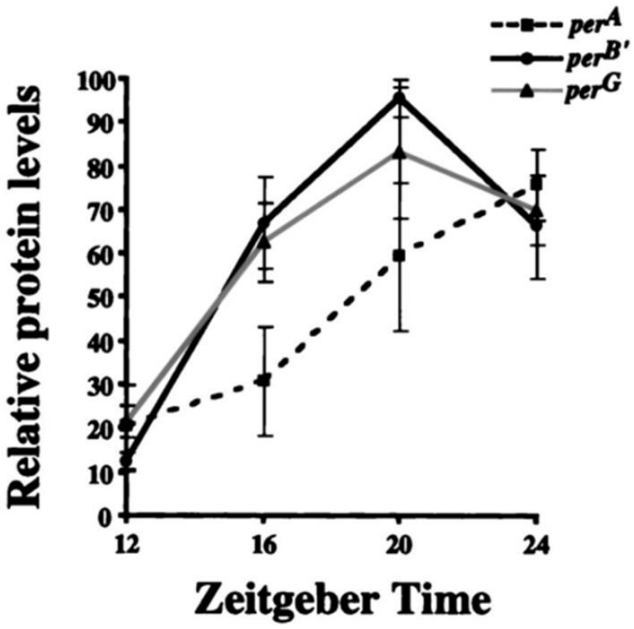


Figure 9-13 WB quantification of PER in the different *perA/B/G* transgenic flies over different time-points at 25 °C. *From Cheng et al. (1998).*

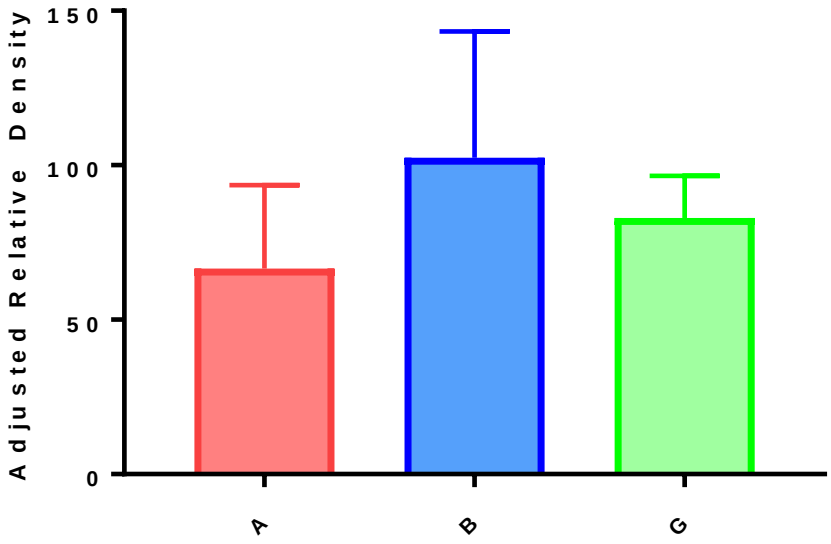


Figure 9-14 Comparison of total PER levels normalised against two internal controls (HSP70 and TUB) at 12 °C.

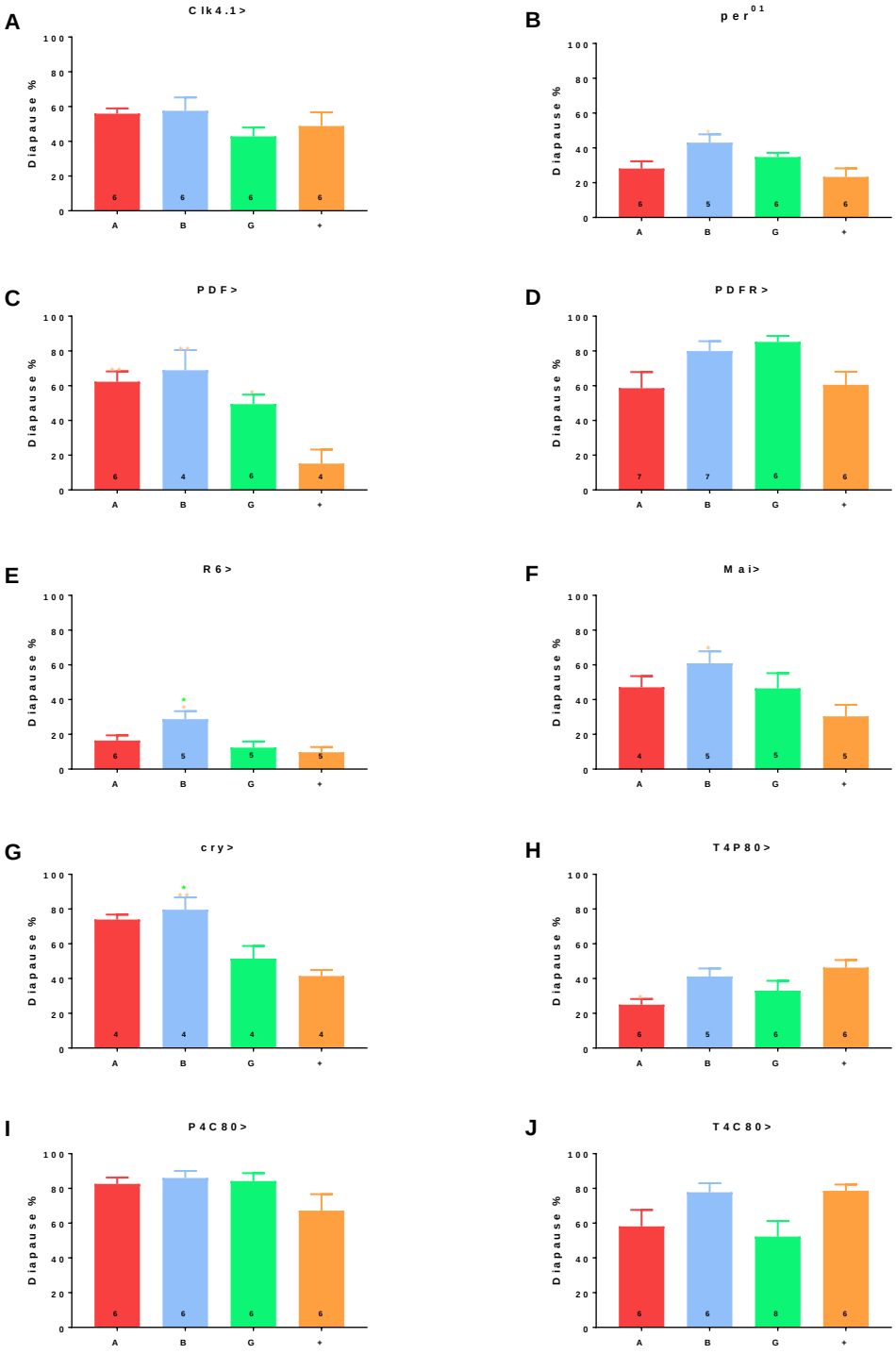


Figure 9-15 Diapause level of UAS-*perA* (red), -*perB* (blue), -*perG* (green) and no-transgene (orange) at 11°C expressed using: (A) Clk4.1-Gal4; and (B) *per*^{0} as a negative control. (C) *Pdf*-Gal4; (D) *PdfR*-Gal4; (E) *R6*-Gal4; (F) *Mai*-Gal4; (G) *cry*-Gal4; (H) tim-Gal4; *Pdf*-Gal80; (I) *Pdf*-Gal4; *cry*-Gal80; and (J) *tim*-Gal4; *cry*-Gal80. The numbers in the bottom of the column represent the number of replicas. **<0.05; **<0.01.*

9.4.2 Tables

Table 9-16 Recipe for 2l of Luria Broth (LB) medium.

Component	Amount
Bactotriptone	20 g
Yeast extract	10 g
NaCl	20 g
Deionized H_20	Until 2 l

Table 9-17 Generation of LB agar for plates

Component	Amount
LB medium	400 ml
Agar	6 g

Table 9-18 Primers used for cloning or sequencing pUASTattB *per* splicing-locked lines. Highlighted in yellow are the binding sites for different restriction enzymes (EcoRI, XbaI or XhoI). The C that is in red is where the XhoI site was generated by changing the original G (no aminoacid change involved). The underlined sections pertain the the pUASTattB vector while the rest are part of *period*. The initial ATG codon in which PER translation starts is highlighted in green.

Primer name	Sequence	Use
pUAS_ perEco_For	CTCTGAATAGGGAATTGGGAATTC ATGGAGGGCGGCGAGTCCACGGAG	Cloning
per_F	ATGGAGGGCGGCGAGTCCACGGAG	Cloning
pUAS_perXho_Rev	CACAAAGATCCTCTAGAGGTACCCTCGAG CATCTTAAGGGTGTCATTGGC	Cloning
perXho_R	CGCTGTACTCGAGCATCTTAAGGG	Cloning
pUAS_per_Xho_For	GCCAATGACACCCTTAAGATGCTCGAG TACAGCGGTCCAGGCCACGGG	Cloning
perXho_F	GATGCTCGAGTACAGCGGTCCAGG	Cloning
pUAS_perXba_Rev	GTAAGGTTCCTTCACAAAGATCCTCTAGA GGCTTTTCGATATTTATTGTAC	Cloning
pUAS_F	CAAATAAACAAGCGCAGCTG	Sequencing
pUAS_R	ACTGCTCCCATTCATCAG	Sequencing
per_1_F	AAGCTGGAGTCCATGACC	Sequencing
per_2_F	CTCTCAGTCATGAAGGAG	Sequencing
per_3_F	ATAACGAGAACCTGCTGC	Sequencing
per_4_F	ATGATGTACCAGCCGATG	Sequencing

9.5 Appendix Chapter 7

9.5.1 Figures

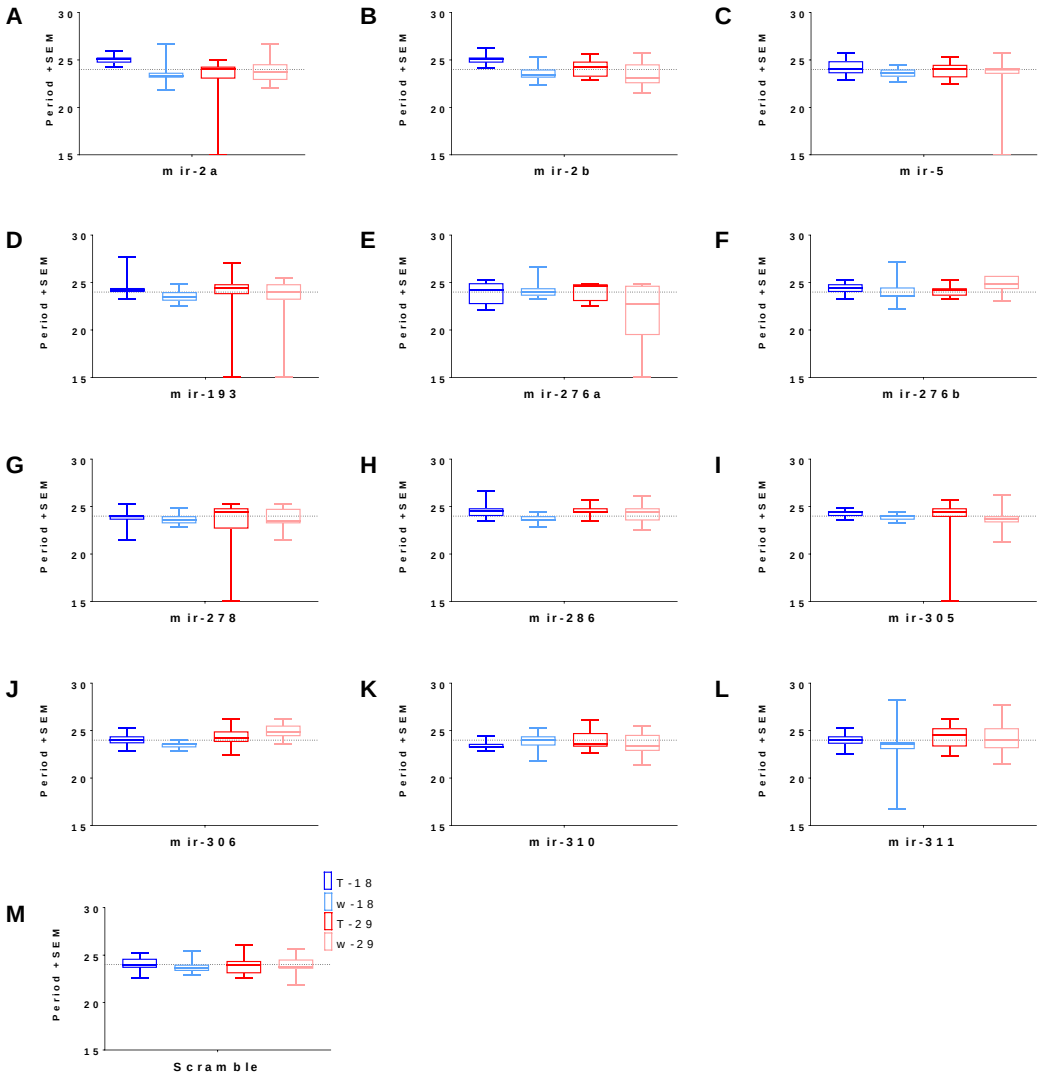


Figure 9-16 2.5 to 97.5 percentile box-and-whiskers plot of period length of the different miRNA sponges. Results from the flies kept at 18 °C or 29 °C are represented in blue and red, respectively. Flies crossed to $w^{-/-}$ controls (lighter) or *tim-Gal4* (darker). (A) *miRNA-2a-SP*. (B) *miRNA-2b-SP*. (C) *miRNA-5-SP*. (D) *miRNA-193-SP*. (E) *miRNA-276a-SP*. (F) *miRNA-276b-SP*. (G) *miRNA-278-SP*. (H) *miRNA-286-SP*. (I) *miRNA-305-SP*. (J) *miRNA-306-SP*. (K) *miRNA-310-SP*. (L) *miRNA-311-SP*. (M) *Scramble-SP*.

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