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The American University in Cairo

School of Science and Engineering

The Impact of Nutrition and Feeding Time on the Regulation of Gene Expression in the
Central Circadian Clock of *Drosophila Melanogaster*

A Thesis Submitted to

The Department of Chemistry

in Partial Fulfilment of the Requirements for
the Degree of Master of Chemistry with Concentration in Food

by Arwa Mohamed-Rabie Diab

under the supervision of Dr. Hassan Azzazy and Dr. Frank Weber

SPRING/2014

DEDICATION

I dedicate my dissertation work to my family. A special gratefulness to my parents, Dr. and Mrs. Diab, whose help, support and encouragement were always the key for me to continue. Many thanks to my sisters, Asmaa, Amany and Aya, for being helpful and by my side. I will always appreciate all what they have done for me.

I dedicate this work to my wonderful daughters Haya and Hala for tolerating and understanding my circumstances during the entire master program.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Hassan Azzazy for his consistent support, his willingness to offer encouraging chances and for his motivation to let me find wide fields of study. Without his keenness, I wouldn't have been able to carry out my research.

I would also like to thank my co-advisor Dr. Frank Weber for accepting me to carry out my research in his laboratory at Biochemiezentrum der Universität Heidelberg (BZH) in Ruprecht Karl University of Heidelberg and for his planning, consistent follow up, patience and support.

I show my gratitude to Weber's research group at BZH, especially to the post doctorate researcher Hsiu-cheng Hung for her continuous assistance and support and also to the PhD student Daniela Zorn for her good company.

Last but not least, I would like to thank the Chemistry Department in the American University in Cairo for their educational support.

ABSTRACT

The American University in Cairo

The Impact of Nutrition and Feeding Time on the Regulation of Gene Expression in the Central Circadian Clock of *Drosophila Melanogaster*

Arwa Mohamed-Rabie Diab

Under the supervision of Professor Dr. Hassan Azzazy and Professor Dr. Frank Weber

The circadian clock plays an essential role in most organisms by allowing their adaptation to the daily environmental changes mainly light/dark cycles and also temperature. Circadian rhythms persist in constant conditions indicating an auto regulatory molecular circadian clock. Here, we investigated the possibility that the circadian clock of *Drosophila* can also be entrained by scheduled feeding. Cycles of 12 hours feeding and 12 hours starvation could entrain the molecular circadian oscillation in key pacemaker neurons of *Drosophila*. Clock proteins CLOCK (CLK) and PERIOD (PER) showed a 24 hour oscillation in the small and large ventral lateral neurons after entrainment by scheduled feeding of a balanced diet, carbohydrate-only diet, as well as protein only diet. Lipid diet did not prove its effectiveness in the clock's entrainment. The neuropeptide Pigment Dispersing Factor (PDF) plays an important role in entrainment of the circadian clock by light-dark cycles, but it appears to be not essential for entrainment of the circadian clock in response to cyclic feeding. Scheduled feeding resulted in 24 hour oscillations of CLK and PER in small and large ventral lateral neurons of *pigment dispersing factor* mutant flies (*pdf⁰¹*). Time of feeding appears to be a strong entrainment cue for the circadian clock as it affected the phase of amplitude of molecular oscillations even in light dark cycles. These results establish an entrainment of the *Drosophila* circadian clock by scheduled feeding, indicating a regulation of the circadian oscillator by metabolism in *Drosophila*.

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List of Abbreviations

Arnt1	Aryl hydrocarbon receptor nuclear translocator
ASPD	Advanced Sleep Phase Disorder
BMAL1	Brain and Muscle Arnt-1
CAFÉ	Capillary Feeder
CaMK II	Calcium/Calmodulin-dependent Kinase II
CCGs	Circadian Clock Genes
CKI	Casein Kinase I
CK-I-ε	Casein Kinase-I-Epsilon
CKII	Casein Kinase II
CLK	CLOCK protein
<i>clk</i>	<i>clock</i> gene
CNS	Central Nervous System
CRSD	Circadian Rhythm Sleep Disorders
CRY	Cryptochrome
<i>cwo</i>	<i>clock work orange</i> gene
<i>cyc</i>	<i>cycle</i> gene
DBT	Double Time
DD	Constant Darkness
DMH	Dorso-Medial Hypothalamus
DNs	Dorsal Neurons
DR	Dietary Restriction
<i>Drosophila</i> CK-I-ε/DBT	<i>Drosophila</i> Casein Kinase I-ε DOUBLE-TIME
DSPD	Delayed Sleep Phase Disorder
FAA	Food Anticipatory Activity
FRD	Free Running Disorder
Gp	Guinea pig
GSK3	Glycogen Synthase Kinase 3
ICSD	International Classification of Sleep Disorder
ISWPD	Irregular Sleep-Wake Phase Disorder
JLD	Jet Lag Disorder
<i>Kai</i>	means cycle in Japanese
LD	Light Dark cycles
LEA	Light Entrainable Activity
ILNvs	large ventral Lateral Neurons
LNds	dorsal Latel Neurons
LNps	lateral posterior Neurons
LNs	Lateral Neurons
(LNvs)	ventral Lateral Neurons
MAPK	Mitogen Activated Protein Kinase
Mo	Mouse
NGS	Normal Goat Serum

PDF	Pigment Dispersing Factor
<i>(pdf⁰¹)</i> flies	<i>pigment dispersing factor</i> mutant flies
<i>pdp1</i>	<i>par domain protein 1 gene</i>
PDP1	PAR Domain Protein 1
PER	PERIOD protein
<i>per</i>	<i>period gene</i>
PTO	Post Translational Modification Derived Oscillatory Process
Rb	Rabbit
RF	Restricted Feeding
<i>RORA</i>	RAR-Related Orphan Receptor A
SCN	Supra Chiasmatic Nucleus
(sLNvs)	small ventral Lateral Neurons
SWD	Shift Work Disorder
TGF- α	Transforming Growth Factor alpha
TIM	TIMELESS protein
<i>tim</i>	<i>timeless gene</i>
TTO	Transcriptional-Translational Derived Oscillatory Process
VRI	VRILLE Protein
W1118	White type <i>Drosophila</i>
WF	Whole Food
<i>vri</i>	<i>vrille gene</i>
ZT	Zeitgeber Time

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1. Introduction

1.1 Basic functions of the circadian clock

Most living organisms are able to synchronize their physical activities with the environmental changes. Rhythmic environmental changes, such as day and night, are received by the inner molecular clock which thereby coordinates the overall physical and metabolic activities. As a result, activities such as the sleep-wake cycle, hormonal secretion, liver functions, blood pressure, body temperature, neuronal and cognitive functions, immune response and many other functions oscillate rhythmically. Molecular clocks can be entrained by the environmental cues such as light-dark cycles, temperature, pressure, food and many others [1]. Such molecular oscillators can be found in organisms ranging from prokaryote as cyanobacteria to mammals [1, 2]. The molecular oscillator can be entrained by the aforementioned external stimuli and is distinguished from other spontaneous responses by its ability to ‘memorize’, maintain the oscillations after removal of the stimulus (as free-running). The capability to maintain rhythms in free running condition (in the absence of environmental cues) is the key feature of the circadian oscillator. As a result the output circadian rhythms have the following characteristics: 1. they are free-running under constant condition with a periodicity around 24hours; 2. they can be entrained by environmental cues, mainly light and temperature cycles; and 3. they can compensate a broad range of temperature changes and execute constant periodicity. Although the observations of biological rhythms have been long documented, and endogenous rhythms have been distinguished from mere responses to daily stimuli, the existence of the endogenous oscillators was long postulated before it was uncovered. In the 70’s, the first clock gene, *period*, was isolated from a *Drosophila* mutant [3] which initiated the discovery of the core genetic components of the molecular clock [4-7].

After vigorous investigations following the discovery of *period* and many other clock genes in different organisms, a general scheme for the assembly of a circadian oscillator was identified, which includes a central transcriptional component with two interlocked transcriptional-translational feedback loops. Similar core clock components have emerged in bacteria, parasites, fungi, *Drosophila* and mammals. High gene homology between *Drosophila* and mammals has contributed to the discovery of the core clock genes in both organisms: mammalian *period* gene was cloned after its isolation in *Drosophila*, and the *Drosophila clock* gene was cloned after its isolation in mice [8]. In addition to the high genome homology, the well-developed tools for genetic studies make *Drosophila* a highly valuable model organism for circadian research [1, 2, 9-11].

The core molecular clock controls the transcription of key regulatory factors in an oscillating manner and therefore affects physiology and behaviour of an organism in accordance to the external environmental cycles of day and night. Thereby, the circadian clock temporally orchestrates most homeostatic activities with one another [12].

Two fundamental mechanisms regulate the circadian time keeping system. One is the transcriptional-translational derived oscillatory process (TTO) and the other is the post translational modification derived oscillatory process (PTO), which is important for timing, regulation and constitution of the molecular clock [1, 2, 10, 11, 13-16]. The clock proteins are regulated by a group phosphatases and a group of kinases such as casein kinase I (CKI) [17], glycogen synthase kinase 3 (GSK3) [18], casein kinase II (CKII) [19, 20], calcium/calmodulin-dependent kinase II (CaMK II), and mitogen-activated protein kinase (MAPK) [21]

1.2 Cyanobacteria as the simplest organism representing circadian oscillation

Cyanobacteria are the simplest organisms that exhibit circadian rhythm. The genome of *Cyanobacterium Synechococcus elongatus* encodes a gene cluster called *kai* (means cycle in

Japanese) which includes three clock genes: *kaiA*, *kaiB* and *kaiC* [22, 23]. The protein products, KaiA, KaiB and KaiC together form the time-keeping apparatus in this organism. The three proteins interact with each other; KaiA enhances *kaiB* and *C* expression. KaiC is a negative repressor which represses its own expression (negative feedback) and the expression of *kaiB* [24]. However, in cyanobacteria, TTO is not the key regulatory mechanism in timing. Current data suggest that a post translational-derived oscillatory process (PTO) in prokaryotes is instead the main regulatory mechanism [25]. In this scheme, the phosphorylation dynamics of KaiC is essential for time-keeping [26, 27]. KaiC exhibits both auto-kinase and auto-phosphatase activities. When complexed with KaiC, KaiA promotes the phosphorylation of KaiC. The hyperphosphorylated KaiC then binds to KaiB, which brings KaiB and KaiA in contact thereby releasing KaiC. Free KaiC starts a auto-catalytic dephosphorylation. Thereby the phosphorylation of KaiC shows self-sustained circadian rhythmicity; mutations which affect KaiC modification leads to arrhythmicity [26]. Most importantly, robust cycling of KaiC phosphorylation was demonstrated while transcription and translation were inhibited [23]. It also features temperature-compensation. Together with other findings it rules out TTO as the primary time keeping mechanism in these organisms [28].

1.3 Evolution of the circadian clock

Cyclic biological processes and behavioral changes through day-night intervals are found in both prokaryotes and eukaryotes, in single cells and complex organisms. Circadian clocks control behaviors such as the sleep wake cycles and the migration of birds [6]. During evolution, circadian clocks have emerged in most organisms in order to allow them to prepare for cyclic changes in the environment even before these changes happen. For example digestive enzymes are expressed prior to feeding time and the expression of DNA repair genes is up-regulated during late night prior to UV-exposure during daytime. A molecular

circadian clock I found in most cells of multi-cellular organisms. However, to coordinate complex tasks in the whole body, molecular oscillators in higher eukaryotes were further evolved and the tasks were split between the one in central nervous system (CNS) and the ones in peripheral organs, such as liver, heart, pineal gland and adipose tissue [29, 30]. The central oscillator is present in the supra-chiasmatic nucleus in the hypothalamus, while peripheral oscillators are in peripheral organs such as liver, heart, pineal gland and adipose tissue [27, 28].

Following the cloning of the first clock gene, *per*, in *Drosophila*, components of the core molecular clock were also identified and studied in mammals, cyanobacteria and plants [29]. Insects and mammals share similar TTOs and their clock genes show high sequence homology. However, there is little similarity between animals and plants [6]. In prokaryotes, genes with sequences homologous to *kaiB* and *kaiC* were found but they do not exhibit circadian function. The lack of rhythmic KaiC places archaea before cyanobacteria in the evolution origin. It is proposed that cyanobacterial KaiC is drawn from the archaea [23]. The clock system in plants may have evolved from a different origin.

1.4 The circadian clock's dysfunction and diseases related

The integrity of the circadian clock is vital for the safety of all the biological systems in mammals. Any dysfunction of the clock will result in many diseases such as sleeping disorders, psychological disturbances, increased tumour formation, decreased fertility, increased susceptibility to toxicity by chemicals and radiation, hormonal imbalance, stress intolerance, bone deformities, weakened memory [30-35] and metabolic syndrome [36]. The most susceptible people to clock dysfunction are the shift workers and night-time eaters as their probability to develop cancer is higher and they are also more prone to develop metabolic syndrome [36]. Cognitive functions can be affected by Huntington's disease due to its adverse effect on the circadian clock [37]. Also, mice with disturbed clocks showed

decreased stress tolerance and damaged DNA due to exposure to radiation as well as metabolic syndrome, decreased life span and decreased fertility [35].

1.5 Food and molecular clock

For animals, food is an issue of life or death. Especially for small animals, adjusting to the food availability is as essential as to the day-night cycles. Researches on mice indicate the existence of a food-related, food-entrainable clock, and compiling data suggest that food-related clock is the same as the light-related clock [38]. Further studies found that food can mainly entrain the expression of clock genes in peripheral tissues, but not in the SCN where the central circadian clock resides in mammals.

At the molecular levels, metabolism and circadian clock are tightly interconnected. Although the central clock is not affected by feeding cycles, it was found to be affected by nutritional cues, such as caloric restriction which was reported to affect the SCN activities and its responses to light. Several studies indicate that scheduled feeding alleviates a disrupted circadian clock and decreases inflammatory and disease markers. It was also reported that caloric restriction delays the onset of several diseases [39, 40]. Feeding regimens may have practical applications in treating acute and chronic illnesses caused by circadian disorders.

1.6 Aims of the present study

The goal of this study is to investigate in *Drosophila* the impact of food regimens on the central molecular clock. The first aim is to examine the food entrainment on the oscillations of the central molecular clock in otherwise constant conditions, especially to compare different nutrient cues (whole food, carbohydrates, proteins and lipids). The second aim of this study is to investigate if feeding time (day time feeding or night time feeding) can alternate central molecular oscillations. The third aim is to see if the signals from the feeding cue adopt the same neuronal transmission network which is used by light. As light signals

reach the central circadian clock via the Pigment Dispersing Factor (PDF). So, we will investigate whether PDF is also responsible for the transmission of metabolic cues.

2. Literature Review

2.1 Circadian clocks in mammals

In mammals, a second group of circadian oscillators are found in many peripheral tissues and organs. To generate coherent physical and behaviour activities, the phases of these peripheral clocks are orchestrated by the central circadian pacemaker. The central oscillator is located in the suprachiasmatic nucleus (SCN) in the hypothalamus, while peripheral oscillators are found in other regions of the brains such as pineal gland which is situated between the two cerebral hemispheres and is attached to the third ventricle. Other peripheral oscillators are located in the heart, liver, adipose tissues, intestine and retina [41, 42] (figure 1).

The circadian network which resides in the SCN can be dissected into three functional components: a sensor system to receive environmental inputs, an information process centre which is the molecular clock, and the output pathways which pass the central information to the peripheral tissues and organs and coordinate the physical and metabolic rhythm (see figure 2) [43]. In mammals, light stimulus enters through the photosensitive retina of the eye, causing electric signals then passes through the retinohypothalamic tract to reach the SCN. The neuronal signals from SCN are transmitted to the pineal gland (one of the peripheral oscillators) and finally to other peripheral oscillators all over the body so that each will take its physiological action accordingly. In reverse, the tissue signals representing the internal environment may return information to the central pacemaker. A positive or negative feedback stimulus from peripheral oscillators is passed through the pineal gland to the SCN which then sends more stimuli or reduces its stimuli accordingly (figure 1) [44].

The full scheme of the interplay between central and peripheral clocks is not known yet. Previously a ‘master-slave’ model was adopted, which gives the full synchronization power to the central clock. Recently, compiling data show that peripheral clocks can act

independently and it was shown that the peripheral oscillators can act separated from the SCN when the entrainment stimulus is food [45]. A second model is developed to address the ‘orchestrating’ communications between central and peripheral pacemakers. Each peripheral clock can adapt to its own environmental cues, such as feeding cues from liver, but it synchronizes with the light-dark cues sensed by the central clock.

Until now, the signalling output pathways between the central clock and peripheral clocks are not elucidated yet, but many data point out the hormonal factors as a part of the communication network [46]. Transforming growth factor alpha (TGF- α) is one of the signal candidates. It is rhythmically expressed in the SCN and its administration inhibits the locomotor activity. Locomotor activity is one of the behaviors synchronized by the circadian clock via the secreted TGF- α , therefore both TGF- α and Pro-kineticin, (a protein responsible for angiogenesis that potently contracts gastrointestinal smooth muscles) are considered as two of the signal molecules in communicating circadian information.

2.2 The mechanism of the molecular clock in mammals

In mammals, circadian clock is mainly regulated through two transcriptional-translational feedback loops which have positive or negative effects. In mammals, there are nine genes known in this feedback regime: *per1*, *per2*, *per3*, *cry1*, *cry2*, *bmal1*, *clk*, *rev-erb- α* and *ck1- ϵ* (will be outlined below) [47]. These genes encode transcriptional regulators centered on BMAL1 and CLK. BMAL1 (brain and muscle Arnt-1) and CLK forming a heterodimer which binds to the E-box promoter elements (CACGTG) present in the clock and clock-controlled genes, thereby activates the transcription of *per* gene. High accumulation of BMAL1 is to take place in the beginning of a day. BMAL1 binds to CLK to form BMAL1-CLK heterodimer which activates the transcription of *cry* genes to produce CRY1 and CRY2 and on *per* genes to produce PER1, PER2 and PER3. The high amounts of BMAL1 during the day

also result in accumulation of PER proteins in the cytoplasm. Newly synthesized PER is soon phosphorylated by CK-I- ϵ . These phosphorylated PER proteins are highly unstable as they are degraded easily by ubiquitylation and hence PER transcription continues during the day. Until the end of the day when CRY protein accumulates in the cytoplasm and forms CK-I- ϵ /PER/CRY complex, a stable undegradable complex, which by the beginning of night time, enters the nucleus and disrupts the formation of BMAL1-CLK complex leading to reduced transcription of *per*, *cry* and *rev-erb- α* while increased transcription of *bmal1*. This cyclic regulation is referred to as the first feedback loop [43-45, 48] (figure 3).

The second feedback loop is composed of *rev-erb- α* (a nuclear hormone receptor responsible for adipogenesis), RAR-Related Orphan Receptor A (*rora*), and BMAL1. BMAL1-CLK heterodimer binds to the E-box elements of the *rev-erb- α* , and *rora* and activates their transcriptions. Both Rev-Erb- α and Rora binds to the RRE elements in the *bmal1* promoter; Rev-Erb- α protein inhibits the transcription of BMAL1 and CRY whereas Rora enhances it. The competition between these two proteins is driving a daily rhythm of *bmal1* transcription closing the second feedback loop.

2.3 *Drosophila melanogaster* as a model for circadian clock studies and the similarities between its clock and mammalian clock

2.3.1 Why is the *Drosophila* considered a model organism for circadian clock studies?

For decades, scientists have been using *Drosophila melanogaster* for genetic studies to take advantages of its physical size, short life cycle, easy breeding and small genomes (compared to mammals). The body size of *Drosophila* is small, around 3-4 mm long, and therefore it doesn't take a huge place (one vial is enough to breed tens of *Drosophila* flies). The life cycle of *Drosophila* is relatively short; it takes about 10-14 days to breed a new generation. One day after the eggs are laid, the first instar larvae are hatched. Instar is a Latin word means "likeness" which is a developmental stage of arthropods from the beginning till sexual maturity is reached. In three days, the larvae developed into the second and third instar

larvae. Two days later, pre-pupa is formed and turns into pupa and after five more days an adult *Drosophila* ecloses. They can be easily fitted into specific incubators where temperature and Zeitgeber are adjusted easily. Most importantly, their compact genome encodes genes with high homology to their mammalian counterparts. In total, *Drosophila* has four pairs of chromosomes including X-Y chromosome, 2nd, 3rd and 4th chromosomes. The X-Y chromosome is associated with the sex phenotype of *Drosophila*, and the 4th chromosome is too tiny, therefore often ignored. About 75% of known human disease-related genes have a significant match in the *Drosophila* genomes. 50% of the fly proteins can track back to their mammalian homologs. Because of little redundancy in the genome, the biological functions of a gene can be easily identified.

The complete and available genome sequencing of *Drosophila* plus the easy access of a large number of genetically defined mutants that cover almost all the biological aspects of a fly made the *Drosophila* the organism of choice in genetic studies and continues to serve as a powerful tool in finding the genetic roots of the biological and behavioral activities [49].

2.3.2. *Drosophila* circadian clock

In *Drosophila*, there are seven major clock genes encoding the central clock components: *clk*, *per*, *cycle (cyc)*, *timeless (tim)*, *vri*, *par domain protein 1 (pdp1)*, and *clock work orange (cwo)* [50]. The main feature of the central time-keeping is the two interlocked transcriptional/translational feedback loops. By the beginning of the night, CLK binds to CYC protein, the mammalian homolog of BMAL1, giving CLK-CYC complex. This complex activates the *per*, *tim*, *vri* and *pdp1* genes in the nucleus via E-box elements in their promoters. The mRNAs of *per* and *tim* and *vri* migrate into the cytoplasm where they are translated into PER, TIM and VRI proteins. In the cytoplasm, PER protein alone is subjected to phosphorylation by *Drosophila* CK-I- ϵ /DBT (the homolog of the mammalian CK-I- ϵ) which accelerate its degradation. However, later during the night, PER and TIM proteins

form a heterodimer which protects PER from degradation caused by DBT. In the middle of the night, PER protein level reaches maximum and enters the nucleus as a stable dimer with TIM. Inside the nucleus, the PER/TIM heterodimer then binds to the CLK/CYC and blocks the CLK/CYC-mediated transcription, including their own transcription. This is referred to as the first negative feedback loop. TIM is very sensitive to light and degrades rapidly as the sun rises, leaving phosphorylated PER by DBT and targeted for degradation. Thereby the suppression is lifted, the CLK/CYC is free to start a new cycle. Therefore, *per* and *tim* transcription starts during the day but PER and TIM proteins are unable to accumulate. During the night, the CLK-CYC dimer stops the transcription of *per* and *tim* genes with formation of PER-TIM heterodimer complex. In addition to the PER/TIM feedback regime, a second feedback loop is composed of VRI and PDP1, whose gene expression are directly activated by CLK/CYC. VRI and PDP1 proteins feedback and directly regulate *clk* gene expression [51]. PDP1 activates *clk* expression while VRI represses it. PDP1 and VRI proteins accumulate with a phase delay, which regulates the cyclic activation and repression of *clk* transcription. VIR, PDP1 and CLK form the second feedback loop responsible for rhythmic expression of CLK and CYC [12, 47].

2.4 Food entrainable circadian oscillator

Light is the most potent stimulus that can entrain the central clock and the physiological behaviours in response to light-dark stimuli [52]. Food is another kind of stimulus which some studies showed its ability to entrain the physiological behaviours but failed to show its effect on the central clock in the SCN [53]. Compiled data suggest that entrainment to food is mediated by a food entrainable circadian oscillator independent from the SCN-based, light-entrainable oscillator. In mice, some researches were done in studying their behaviour in response to food. Food was introduced during their inactive time of the day (daytime) and deprived during their active time (night-time). As a result, two kinds of activities were

observed in each 24-hour period. One activity happened during the day right before the food was supplied, which is referred as food anticipatory activity (FAA). FAA is thought to be controlled by a SCN-independent food-entrainable clock. The other activity happened during the subjective night (their natural active time), referred to as a light entrainable activity (LEA) controlled by the SCN. Some species of rodents show LEA even in scheduled feeding in constant darkness (DD) and this is thought to be a species dependent SCN entrainment [54]. Previous studies on rodents' behaviour in response to timed feeding showed that hamsters can be behaviourally entrained by scheduled feeding, while mice showed moderate response to entrainment and rats showed no response. Mice could only be entrained when the feeding time was in phase with the free running LEA. The nutrition of the food, normocaloric or hypocaloric, were also found to affect the entrainment. Other studies showed that when mice and rats were fed in the daytime, the oscillations of the clock proteins PER1 and PER2 in SCN were still driven by the light-dark cycles, while the oscillation in the peripheral tissues shifted to synchronize with the feeding timing. But in contrast to these studies in 2004, scientists in Bult-Ito's laboratory, demonstrated that scheduled feeding with nutrition restriction could entrain central clock in SCN by showing that the entrained mice PER2 oscillation in the SCN was in phase with the LEA while the LEA gradually emerged with the FAA [53]. However, the entrainment efficiency showed great discrepancy among the different mice groups.

More controversial data were provided later by two groups of scientists, Mistlberger group and Saper group. In 2007, Landry and Mistlberger first published that food is a dominant Zeitgeber to nearly all organs including brain region [55]. On the other hand, Saper group claimed that instead of entrainment, behaviours as wheel running and other activities are mainly regulated by hourglass processes, which have to be reset everyday by the Zeitgeber as feeding [56]. Another contradicting point between these two parties is whether the removal of

the dorsomedial hypothalamus (DMH) would prevent rats to be behaviourally food entrained, as according to Saper's opinion in 2006. To this point, Mistlberger group demonstrated that nocturnal rats which were served one meal only per day in the middle of day time become more active 1-3 hours before feeding time in their wheel running behaviour, cage activity, food bin activity, infrared motion sensors and also proved when intraperitoneal transmitter's strength is changed. This FAA is established in 1-3 weeks when everyday food is given at the same time and rats continued to show FAA after these 1-3 weeks of entrainment at the regular feeding time even when food was not served at the accustomed feeding time. This FAA plateaued for a while and then declines to low phases until night came. This FAA was observed at the same time for 3-5 days without food served at the regular time. This denotes that rats can be food entrained with ignorance to the hourglass processes. The second contradiction was about the inability to food entrain rats with removed DMH and that rats show FAA in accordance to their hourglass before its removal and this is justified by Landry and Mistlberger as follows: Rats with removed DMH [57] showed reduced nocturnal activity without food entrainment. But when they were food entrained, they showed FAA which becomes prominent day after day [55].

2.5 The art of entrainment (Entrainment versus Synchronization)

Circadian clock's main character is its ability to auto-oscillate in constant conditions, but sometimes it can deviate from the 24hrs rhythm. Hence, entrainment is needed to correct the clock and the circadian clock has evolved to fine tune biological functions to specific times within the day or night, and, when put into constant conditions, it free runs close to 24 h [52]

Although entrainment was a very appealing field for the researchers, most attention is centered on few aspects of light entrainment. These aspects are: (i) the light receiving apparatus such as photoreceptors (ii) the signal pathway between the photoreceptors and the

molecular clock in SCN, and (iii) synchronization of the circadian clock and the signal output [52]. Therefore, little is known when feeding is the entrainment cue as for where the signal inputs are received, how the molecular clock is synchronized and through what are the signal output pathways. There is a difference between the term "entrainment" and the term "synchronization". Synchronization refers to the passive and fast response of a system to an environmental stimulus, such as light or temperature and when this stimulus stops, no further response is observed. On the contrary, entrainment refers to the active response of a system to an environmental stimulus, a Zeitgeber. Once the action is established, it can be and will be continued even after the Zeitgeber is removed. So, entrainment is an active and slow response because oscillators usually require several days until they are synchronized with the Zeitgeber.

In circadian research, a 24-hour cycle has two phases, one with the environmental cue (the Zeitgeber), such as light or food availability, the other one without. Driven by the environment phases, an observed activity, such as a protein accumulation at the molecular level or sleep-wake at the physical level, peaks or troughs rhythmically. Thereby, the observed activities also show two phases in a 24-hour cycle. For example, the Zeitgeber is food availability in this study, and a 24-hour cycle is composed of the feeding and the starvation phases. The observed activities are the clock protein oscillations in the SCN neurons. The clock protein accumulations peak in the feeding phase and trough in the dark phase. Changes of the feeding time will result in the phase advance or delay of the protein accumulation in order to adapt to the new environmental phases given by the Zeitgeber, which is referred as phase shift in the circadian study [52].

2.6 Optimization of dietary restriction

In 2007, Bass and Grandison studied the effect of optimized dietary restriction (DR) on the life span of *Drosophila*. They defined DR as: "*a moderate reduction of food intake that leads*

to extension of life span beyond that of normal, healthy individuals" [58]. In Rats, It is found that DR of whole food (WF-DR) leads to 40% increase in the life span of rats [59]. Also, specific reduction of certain food components leads to increase of their life span while the reduction of other components has no effect. While the reduction of proteins in the food caused a minor increase of their life span [60], the selective reduction of methionine [61] and/or tryptophan [62] however led to 40% increase exactly as with WF-DR. Also, the reduction of lipids, vitamins or minerals components in WF did not cause any change in the life span. In *Caenorhabditis elegans* , a free living nematode in soil, the life span is increased when the bacterial content of its food is reduced , completely removed or changed with another strain [63]. In *Drosophila*, diet has an effect on the life span and the fecundity of the flies. In natural environment, fruit flies feed on the fermenting fruits, while in the lab food is prepared. The sucrose concentration in a rotting banana does not exceed 4.5 g/L. Diet with high sucrose concentration was reported to have a negative effect on their fecundity and reduce their life span as well. Therefore, *Drosophila's* food should have low sucrose concentration as it is in natural bananas. Yeast concentration in *Drosophila's* food has an effect on their life span and fecundity as well. Yeast extract is the resource of proteins and vitamins. When yeast concentration is increased, on one hand fecundity is increased but on the other hand, its life span is decreased. This effect on the life span is thought to be due to the toxic effect of some of the yeast components. It is also possible that these components adversely affect the fecundity of the flies. The increased life span of the flies from DR may not be due to the reduction in richness of the diet, instead, it benefits from less uptake of the toxic components in the food [58].

2.7 Circadian regulation of metabolism and feeding behaviour in *Drosophila*

Circadian clocks, central and peripheral, regulate the feeding behaviour and metabolism of *Drosophila* via its 24 hour-cycles. Timing in the metabolic tissues is an orchestrated action

between its own clock (peripheral clock) and the central clock. It was long reported that metabolic genes are rhythmically oscillated in a daily cycle [64]. Mutations in clock genes also affect metabolic activities. For example, it was reported that the mutations in *bmall* (the mammalian homolog of the *Drosophila Cyc*) affected the diurnal expression of glucose and triglycerides in the circulating blood of *Bmall* deficient mutant mice [65]. Also, the mutations of *bmall* and *clk* affect lipid metabolism, gluconeogenesis and adipogenesis. In human, many evidences suggest a strong relationship between timing disturbance and the metabolic illnesses). For example, the insulin secretion of the diabetic people is not only reduced but also arrhythmic less insulin [66, 67]. Shift workers and night time eaters are more susceptible to metabolic syndromes [36]. In rodents, the mice with clock mutations have also shown signs and symptoms of metabolic syndrome [68]. In diabetic and obese mice, the circadian synthesis of leptin, adiponectin and adipokinase is also adversely affected [69].

In *Drosophila* each organ has a specific function different from the other and this is revealed by the microarray analyses as it proved that each organ has specific circadian clock genes (CCGs) different from the other. In the wild and in the incubator, *Drosophila* tends to eat in light phase (daytime). This is because of the light which drives the flies to eat during daytime. And because of the auto-regulation of the circadian clock that even in constant darkness; flies tend to eat at the same time of the day.

2.8 Regulation of locomotor activity by central clock in *Drosophila*

Drosophila movement in nature or in lab is called locomotor activity. There is a connection between the locomotor activity and the circadian clock which was first recognized in 1971 by Konopka and Benzer [3]. They studied flies with altered rhythms in locomotor activity and pupa eclosion. One fly line became totally arrhythmic, the second has a period of 19 hours

and the third has a period of 28 hrs. These three phenotypes were genetically mapped to the same position on the X-chromosome where the gene *per* is located. The gene was cloned and further studied. Soon it was found that both mRNA and protein products of *per* were expressed in rhythmic manners in a day. PER protein expression is rhythmic and the rhythmicity can be synchronized with the environmental Zeitgeber. This rhythmic expression is essential for the physiological activities adapt to the Zeitgeber [70, 71]. Following the identification of *per*, 7 other clock genes were found as the components of the central molecular oscillator. As the mutations in *per*, the mutations on any of the clock genes resulted in various changes in the locomotor activities. During daytime, the flies become active while during night time, the flies become relatively inactive. Notably, *Drosophila* is one of the many animals showing ‘bimodal’ activity rhythms with two activity bouts: one activity maximum, the “lights-on” peak (also referred as morning peak), is synchronized with the onset, while the other, the “lights-off” peak (referred as evening peak), occurs shortly after the offset of light. If the animals become active only once in a day with one maximal bout, the activity pattern is referred as ‘unimodal’ [72]. One long-standing model proposed that underlie these two activity bouts there are two mutually coupled oscillators with different sensitivities to light.

There are about 150 clock-protein expressing neurons in SCNs of *Drosophila*, they can be divided into groups based on the location in the brain. Each group of neurons has its own distinct function in circadian regulation and may be responsible for a specific locomotor activity [73]. According to their locations, the neurons are named as follows: lateral neurons (LNs), dorsal neurons (DNs), and lateral posterior neurons (LNps). Lateral neurons are further split into ventral lateral (LNvs) and dorsal lateral neurons (LNds). The LNvs include 5 large neurons (ILNvs) and 5 small (sLNvs). DNs are classified into 3 groups, named as DN1, DN2 and DN3 [74]. Although the model of two oscillators for the bimodal activity was long

proposed, it was not until 2004 that scientists were able to identify the neurons with function as the ‘morning’ oscillator and the ones with the ‘evening’ oscillator functions. In 2004, two groups of scientist, Rouyer and Rosbash, published simultaneously their findings, that for the first time defined regulatory function was assigned to specific group of neurons. Both groups claimed that the two distinct components of locomotor activity, are regulated by different neurons; sLNvs regulate the morning activity bout while LNds are in charge of the evening one [75, 76]. While 4 of the PDF-expressing sLNv are responsible for morning activity, the 5th sLNv (non-PDF expressing) along with the LNds are responsible for the evening activity. They also provided sufficient data showing that sLNv are the main oscillator which alone can drive circadian rhythm in constant darkness (auto-regulation) [73].

2.9 Pigment dispersing factor; a neuropeptide responsible for signal transduction

Pigment dispersing factor (PDF) is a neuropeptide which is named after its role in the diurnal movement of pigment in the crustacean retinal cells [77]. The gene of this neuropeptide was first isolated in 1998 at Brandeis University by Halls, J. Lab [78]. In *Drosophila*, PDF is the only found neuropeptide engaged with the signal output pathways; it transmits the chronological signals among other clock neurons distributed within the brain. Hence, it is important to rhythmic locomotor activity [79].

PDF is expressed in lLNv and in 4 of the sLNvs while the 5th sLNv does not express PDF [80]. In nerve endings, the expression of PDF is found to be cyclically which peaks during the sunrise and troughs during the sunset. This feature made it a promising candidate in maintaining the rhythmic circadian activities. Some studies showed that the locomotor activity rhythm was lost in *pdf⁰¹* flies [81, 82], while the others reported a rhythmic behavior in the light-dark entrainment phase and a fast lose of rhythmicity in the free running condition [80]. In 2004, Lin et al carried out a time series immunohistochemistry staining of PER protein in *Drosophila*. They entrained the wild type flies and the *pdf⁰¹* flies then released

the flies in DD for 9 days. In both groups, the oscillations of PER protein were observed; but the *pdf⁰¹* flies showed phase shifting and oscillation amplitude was reduced. Since PDF is not required to maintain clock protein oscillation, but it is required for the communication among the diverse pacemakers in coordinating their phases and amplitudes, therefore, PDF is placed downstream of the central oscillator in the hierarchy of circadian pacemaker circuitry [83].

2.10 Peripheral circadian clocks

Circadian oscillators are also found in the peripheral tissues as retina, gut and proboscis [84] with oscillations of mRNA and protein similarly to its oscillation in the central circadian clock [85]. In mammals, peripheral clocks can free-run and maintain rhythmicity in an ‘orchestra’ relationship with the central clock in the SCN. For example, circadian timekeepers in liver and lung explants could maintain daily cycles of *Per2*-luciferase expression for more than 20 days [86]

In *Drosophila*, rhythmic PER protein expression was found in wings, legs, antenna and proboscis. PER protein was measured using bioluminescence assay [87]. This result denoted that there are molecular oscillators in many peripheral tissues and organs, which can adapt to the local environmental cues under the conduction of the central oscillator. For example, cuticular hydrocarbons' synthesis is regulated by the circadian clock [88] in the peripheral tissues. Also in flies, peripheral clocks were found in the smell and taste organs, in the neurons of the antennae and proboscis, which drives the rhythmicity in taste sensitivity, appetite and feeding. [84, 89].

2.11 Circadian feeding pattern in flies

It was reported in 2010 by Shekhar and Levine that feeding of *Drosophila* is circadian-regulated. Using a modified capillary feeder (CAFE), developed by Ja et al in 2007 [90], they

measured real-time food consumed by each individual fly [91]. CAFE is composed of micro capillaries and each capillary is filled with liquid food. Each fly has an access to its own capillary and consumes its food. Fly were entrained with light-dark cycles while hourly photos of the capillaries were taken to measure the reduction of food volum. The results showed that flies ate all day long but they tended to consume more food specifically at transition of lighting. So, in the beginning of light-on (ZT0) and shortly before the light-off (ZT12), flies consumed much more food. Also, there was difference in food consumption between the light phase and the dark phase. Flies tend to consume more food during the light phase than in the dark. These results demonstrated that feeding of the flies is circadian regulated [91]. To investigate whether this feeding behaviour is regulated by the circadian clock or it is just an arouse response to the light, the same CAFE measurement was done with the flies released in DD after light-dark entrainment. The result showed that feeding stayed rhythmic in constant darkness although the overall food consumption was reduced. The locomotor activity in constant darkness was also studied which showed similar rhythmicity as the feeding. This denotes that there is a relationship between the mechanism of moving and the one of feeding [91].

2.12 The effect of poor nutrition on the circadian clock

The circadian clock can be deregulated when the mice are subjected to poor and imbalanced diet. When mice were subjected to high fat diet, this resulted in two negative consequences. The first is a disruption of the clock gene *rev-erba* expression in the beta cells in the pancreas. The second consequence is the impairment of the rhythmic secretion of insulin [92]. Another study on mice exposed to high fat diet also which also showed attenuated clock gene expression in liver, adipose tissues and hypothalamus along with altered behavioral rhythms. These mice developed eventually metabolic pathogenesis as hyper-lipidemia, hyper-leptinemia, hyper-insulinemia, hyper-glycemia, fatty liver and obesity [65, 68]. It suggested

that an imbalanced diet might disturb the orchestration between the central and the downstream peripheral clocks.

2.13 Circadian rhythm and sleep disorders

In clinical practice, many studies have been conducted to understand the biology of the circadian rhythm and to classify circadian rhythm sleep disorders (CRSD). CRSD classification is made based on the symptoms of individuals. CRSD is classified into six types according to the International Classification of Sleep Disorder (ICSD) by the American Academy of Sleep Medicine [93]. They are classified as followed: 1) advanced sleep phase disorder ASPD, 2) delayed sleep phase type disorder DSPD, 3) irregular sleep-wake phase disorder ISWPD, 4) free running disorder FRD, 5) jet lag disorder JLD and 6) shift work disorder SWD. CRSD has some important features. The essential feature is the recurrence or persistence of sleep disorder caused by endogenous or exogenous factors, and in most cases, it is the result of both endogenous and exogenous elements together. Hence this will hinder the jet lag disorder JLD patient to adjust his/her sleep-wake cycle according to his/her needs or his/her routine demands. Many factors contribute to the occurrence of CRSD which include endogenous factors such as gender, familial predisposition (genetic factor) and exogenous factors as inappropriate light exposure whereas excessive, insufficient or at a wrong time [94].

2.14 Concluding Summary

Since the cloning of first clock gene, *per*, many studies have advanced our knowledge regarding the circuits of the central clock, from the signal input pathways, central clock components to output signal network. There is still a great deal of information needed to complete the regulatory scheme, especially when other environmental cues such as feeding

and temperature are taken into concern. For a research with inter-crossed topics, *Drosophila* serves as an ideal model organism based on all the aforementioned advantages. Hence we chose *Drosophila* as our model in our studying of the nutrient effect on the circadian pacemaker, in search for more scientific explanation for the nutrition-caused circadian disorders.

3. Materials and Methods

3.1 Materials

3.1.1 Fly lines

Drosophila melanogaster w1118 was used in this study as wild type flies. The line was originally obtained from Bloomington Drosophila Stock Center (stock number 3605). The fly line, *pdf⁰¹*, with a knockout mutation in the PDF gene was also obtained from Stock Center (Bloomington, IN, USA) (stock number 26654).

3.1.2 Fly food

Whole food, a rich source of sugar, amino acids and lipids, was prepared according to Weber's lab's recipe [95] : 4 liters of deionized water were boiled with 64g of agar until agar is completely dissolved. In another container, 2 liters of deionized water were added and mixed with 0.64g of corn flour, 80g of soy flour and 200g of ground Brewer's yeast. Then they were mixed very well and poured into the boiling pot with continuous and vigorous mixing. The same method was used to mix 1 liter of deionized water with 0.64kg malt and 175g treacle syrup and it was added into the boiling mixture. A glass pipette was used to mark the liquid level in the pot. The whole mixture was cooked for another 30 minutes with continuous stirring. After that, the mixture was left to cool with half-closed lids to 55-60 °C with continuous stirring. To make up the water loss during cooking, water was added. After the fly food was cooled to 60 °C or less, preservatives were added (preservatives cannot be added while the food is hot). 12g of Nipagin (methyl 4-hydroxybenzoate) were dissolved in ethanol and then were added to the pot. In a fume hood 75ml of propionic acid were added to 1 liter of water and then were added to the cooled food mixture with vigorous stirring to ensure the consistency [95].

3.1.3 Food with special nutritional components

Carbohydrates-only meal was taken from Good and Tatar with 11% g w/v of sugar to 1.1% w/v of agar [96]. Protein enriched meal was prepared on the aforementioned sucrose meal with the same concentration of carbohydrates with the addition of 1% casein protein (casein hydrolysate, standard for molecular biology, Roth) [13]. Lipid enriched meal was also prepared on the sucrose meal with the same concentration of carbohydrates but with the addition of 10% oil (olive oil, sunflower oil, canola oil with equal concentration of each 1:1:1) + 10% ethanol to allow oil to dissolve. Starvation vials were prepared with a 1 cm thick piece of cotton soaked in distilled water.

3.1.4 Antibodies

PER antibody PER 21-A (referred as anti-PER) raised in rabbits was purchased from Alpha-Diagnostic(San Antonio, Texas). CLK antibody (referred as anti-CLK) against a peptide in c-terminus of CLK was raised in guinea pig and affinity-purified against the peptide [21]. PDF monoclonal antibody PDF C7 (referred as anti-PDF) was purchased from Developmental Studies Hybridoma Bank (DSHB) (Iowa City, Iowa, USA). Fluorescence dye-conjugated secondary antibodies, Cy2 conjugated anti-mouse (Cy2-anti Mo), Cy3-conjugated anti-rabbit (Cy3-anti Rb) and Cy5-conjugated anti-guinea pig (C5-anti Gp) were purchased from Jackson ImmunoResearch (Newmarket, Suffolk CB8 7SY, England).

3.2 Experimental Design:

3.2.1 Experiment 1

The aim of Exp.1 is to answer the following questions: will the circadian clock proteins, CLK and PER, oscillate in response to timed feeding in the absence of light-dark stimuli? Will carbohydrate only diet affect the clock proteins similarly as the whole food diet?

3.2.1.a Entrainment

Six experiments were held in this research. Exp. 1 was done on arrhythmic W1118 flies emerged in LL conditions as mentioned above (see 3.1). The flies were entrained in three different conditions for 5 days. Their vials were labelled A, B and C. Flies in vials A are the control flies which are exposed to Light-Dark cycles and have whole food 24 hours a day (LD-WF-24hrs). Flies in vials B were exposed to constant darkness and shifted between Whole Food and starvation every 12 hours (DD- WF: Starve- 12:12hrs). Flies in vials C were exposed to constant darkness with shifting between Carbohydrates meal and starvation every 12 hours (DD- CHO: Starve- 12:12hrs). The temperature was 20 (+/-2) °C. Shifting between feeding-starvation vials was done every 12 hours in dark room under infra-red light type (Kindermann, dukalux x-tronic). In normal *Drosophila* breeding conditions, Light exposure is from ZT 0 - ZT 12 & Dark exposure is from ZT 12 - ZT 24. But here the work is on the timed feeding with different nutrients and its effect on the circadian proteins in brains in constant darkness (DD) and the flies are fed during the light phase of the control flies (Vials A). So there isn't any stimulus other than food.

3.2.1.b Harvesting, fixation & dissection

After 5 days, entrained flies were harvested from vials A, B and C at ZT2, ZT10, ZT 14 and ZT22 in the dark room under infra-red light. Tubes were prepared to harvest the flies in and tubes for vials B, C and ZT14 and ZT22 harvest of A vials were covered with aluminium foil and labelled with the letter of the group harvested A, B or C and the ZT this group was harvested on ZT2, 10, 14 or 22. So, only ZT2 and ZT10 harvest of A vials were put in non-covered tubes. All flies were harvested into fixing solution [1x PBS + 0.1% T_{x-100} + 4% Formaldehyde] which was prepared on the day of the harvest. Each group was put into 10ml fixing solution in the dark labelled 10 ml tube for 2.5 hours with continuous rolling on a rolling machine (CAT- RM10W-30V) at 25 roll/min speed. After fixation, flies were washed

3x15 minutes with 1xPBS with continuous rolling. Then, the flies' brains were dissected under microscopy (Zeiss) with light source using two forceps (Dumont #5 Forceps, 11252-20) in small black plates. Dissected brains of each group were put in their relevant plate labelled with the group name and the harvest Zeitgeber. 8 whole brains were dissected of each group of flies.

3.2.1.c Immunohistochemistry(IHC) (blocking, staining& mounting)

The brains were put in a blocking buffer [1xPBS + 5%NGS + 0.5% T_{x-100}] and left overnight (O/N) in the cool room at 4°C on a see-saw rocker (Stuart) at 10 osc. /min speed. On the second day, each group of blocked brains were put in 200µl Primary antibodies [*guinea pig* anti-CLK 1:100, *rabbit* anti-PER 1:200 and *mouse* PDF 1:100] in freshly prepared blocking buffer for 2 hours on the see-saw rocker in room temperature. After 2 hours, brains were washed 5x10 minutes with 1xPBST, 0.5% T_{x-100}. After this the brains were left overnight in Secondary antibodies [*mouse* Cy2 1:400, *rabbit* Cy3 1:400 and *guinea pig* Cy5 1:400] in blocking buffer in the cool room at 4°C on the see-saw rocker at 10 osc. /min speed. The brains were put in dark from this step on to prevent stain bleaching. They were put in a closed box. On the following day, the brains were washed 5x10 minutes with 1xPBST, 0.5% T_{x-100} and then with 1xPBST, 0.1 % T_{x-100} once. Then, the stained brains were mounted on slides using whatman paper for absorbing the washing buffer, adding 20 ul of Glycerol and covering the brains with a cover glass and applying transparent nail polish to the edges of the cover glass for proper sticking.

3.2.1.d Confocal microscopy LSM

In the confocal microscopy room, the slides were scanned by laser. 4 whole brains (8 halves) were scanned in each slide. In each half, three channels were scanned; the CLK stain in red, the PER stain in blue and the PDF stain in green. The ventral lateral neurons (LN_v) were scanned. Anti CLK and anti PER stained the nuclei of the LN_v small and large (sLN_v&lLN_v) while PDF stained the cytoplasm. LSM scans the slides giving images for each scanned layer. Later on, these images were quantified using Image J software, analysed in excel and interpreted into graphs.

3.2.2 Experiment 2

It is the repetition of Exp.1 with some changes; it had no control flies and the primary antibody, *guinea pig anti-CLK*, concentration changed into 1:400. All other conditions were exactly the same.

3.2.3 Experiment 3

The results of this experiment will answer the following: will the circadian clock proteins, CLK and PER, oscillate in response to timed feeding in the absence of light-dark stimuli? (Confirming the data given in Ex.1 and 2) How will carbohydrate only diet, protein enriched diet and lipid enriched diet affect the clock proteins?

The protocol used in Exp.1 was followed in Exp.3 as well. The flies were entrained in five different conditions for 5 days. Their vials were labelled A, B, C, D and E. Flies in vials A are the control flies LD- WF- 24hrs. Flies in vials B were exposed to DD- WF:Starve- 12:12hrs. Flies in vials C were exposed to DD- CHO:Starve- 12:12hrs. Flies in vials D were exposed to DD- Protein:Starve- 12:12hrs. Flies in vials E were exposed to DD- Lipids:Starve- 12:12hrs. The temperature was 20 (+/-2) °C. All the following steps will be done as mentioned in Exp.1.

3.2.4 Experiment4

How will clock proteins oscillate when the W1118 flies are fed during light phase and when another group is fed during dark phase? Which has stronger effect on the circadian oscillation, the light-dark stimulus or the food-starvation stimulus.

In this experiment, two groups of flies were entrained in light dark cycles. Each group was fed on the opposite time. One group was fed during light and the other group was fed during dark. The entrainment of the two groups started together and ended together for five days. The first group was fed from ZT0 till ZT12 (WF 12-12 light) and the other group was fed from ZT12 till ZT24 (WF 12-12 dark). Both groups were fed with WF. For whole food recipe see materials and methods.

3.2.5 Experiment5

Exp. 5 was done on arrhythmic pdf0 flies emerged in LL conditions as mentioned above. The aim of it is to prove whether the effect of food on the nuclei of ventral lateral neurons is transmitted to them through the PDF or through another way.

Two groups of flies were entrained for 5 days; group A , the control group, exposed to LD-WF-24hrs and group B exposed to DD- WF: Starve- 12:12hrs. All other procedures were followed as in Exp.1

3.2.6 Experiment6

This experiment is done to confirm the results of experiments 1,2 and 3. The flies were entrained in four different conditions for 5 days. Their vials were labelled B, C, D and F. Flies in vials B were exposed to DD- WF:Starve- 12:12hrs. Flies in vials C were exposed to DD- CHO:Starve- 12:12hrs. Flies in vials D were exposed to DD- Protein:Starve- 12:12hrs. Flies in vials F were exposed to DD-WF- 24hrs. The temperature was 20 (+/-2) °C. All the following steps were done as mentioned in Exp.1. F vials were subjected to a new kind of entrainment in total darkness with whole food for the whole 5 days, which means there aren't

any kind of stimuli whether light or timed feeding. So, by this we will see if clock proteins oscillate in response to timed feeding in the absence of light stimulus as in other experiments or do they simultaneously oscillate in the absence of light or timed feeding?

Locomotor activity was measured also for these four groups of flies

3.3 Methods

3.3.1 Light entrainment versus food entrainment

Adult flies were removed from the bottle with whole food, leaving only the larvae and pupa in the vial. Such bottles were incubated in constant light at constant temperature ($20 \pm 1^\circ\text{C}$) and freshly eclosed flies were collected after 5 days in order to obtain one to five days old flies. Each flies group was exposed a different condition for 5 days. one group served as the control. They were continuously supplied with whole food and incubated with 12 hour light-12 hours dark cycles (LD entrainment). Another group was exposed to constant darkness and shifted between 12 hours of whole food availability and 12 hours of starvation (WF entrainment). Another group was exposed to constant darkness with shifting between 12 hours of carbohydrate rich meal and 12 hours of starvation (CHO entrainment). The fourth group was subjected to protein enriched diet entrainment and the fifth group was subjected to lipids enriched diet entrainment. The temperature was kept constant at $20 \pm 1^\circ\text{C}$. Shifting between feeding-starvation vials was done every 12 hours in the dark room under infra-red light type (Kindermann, dukalux x-tronic). A Zeitgeber is any external cue (e.g. light or temperature and potentially food) that entrains, or synchronizes, an organism's biological rhythms to the environment daily 24-hour cycle. Day time during entrainment is given as Zeitgeber Time (ZT). In Control flies, the Zeitgeber was light with a 12 hour-light on and 12 hour-light off schedule. ZT0 is defined as the time when light is switched on and ZT12 is the time when the light is switched off and flies go into darkness. WF entrained flies and other nutrient

entrainment groups, the Zeitgeber is food availability. ZT0 is the time flies were transferred to food and ZT12 was the time they were removed from food, marking the start of starvation.

3.3.2 Immunohistochemistry

After 5 days, entrained flies were harvested from vials A, B and C at ZT2, ZT10, ZT14 and ZT22 in the dark room under infra-red light. Flies incubated in constant darkness, namely, flies in Group B and C, together with Group A flies harvested at ZT14 and ZT22 (in the dark cycle) were harvested and fixed in darkness to prevent light-induced protein degradation. Flies harvest in the light cycle, at ZT2 and ZT10 proceed to fixation in light. All flies were harvested into freshly-prepared fixing solution [1xPhosphate buffered saline (PBS) + 0.1% Triton X-100 + 4% formaldehyde]. PBS is isotonic because its osmolarity and ion concentration is equal to that in the human body [97]. Each group was put into 10ml fixing solution for 2.5 hours at room temperature with continuous rotation on a rolling machine (CAT- RM10W-30V) at 25 roll/min speed. After fixation, flies were washed 3 times with 10ml of 1xPBS, each time with rotation for 15 minutes. Then, the flies' brains were dissected to remove fat tissues and eye discs.

After dissection, brains were incubated overnight with blocking buffer [1xPBS + 5% NGS + 0.5% TritonX-100] in the cooling room (at 4°C) on a see-saw rocker (Stuart) at 10 osc /min speed. On the second day, the brains were transferred into 200µl freshly prepared blocking buffer with primary antibodies (a mixture of antibodies with guinea pig anti-CLK 1:100, rabbit anti-PER 1:200 and mouse PDF 1:100 for 2 hours at room temperature with gentle agitation. After 2 hours, brains were washed 5 times with 1 ml of 1xPBST, 0.5% TritonX-100. Each wash lasted for 10 minutes with gentle agitation. The brains were then incubated with secondary antibodies (200 ml blocking solution with Cy2-anti Mo 1:400, Cy3-anti Rb 1:400 and Cy5-anti Gp 1:400) at 4°C on a see-saw rocker at 10 osc. /min speed. On the

following day, the brains were washed 5 times with 1 ml of 1xPBST, 0.5% Triton X-100 and then with 1xPBST, 0.1 % Triton X-100 once. Each wash lasted for 10 minutes at room temperature with mild agitation. Then, the stained brains were mounted with 20 μ l of the glycerol-based mounting solution (Dabco in 95% glycerol in 1 M Tris, pH7).

3.3.3 Confocal microscopy

Once mounted, the samples were processed to confocal microscopy right away and all samples, including controls, were scanned in one day in order to be quantitatively compared. Zeiss confocal laser scanning microscope 510 (Carl Zeiss, Jena, Germany) equipped with three lasers was used for all the experiments. The scanning parameters for each laser were fixed for all the experiments, however, the stepwise sections were individually adjusted to obtain the best images and the most complete information. For each experimental group, 4 whole brains (8 hemispheres) were scanned in each slide. In each brain hemisphere, the section depth and number were selected to cover most if not all ILNvs and sLNvs. Both neuronal groups were identified by Cy2-anti Mo-anti PDF staining which can be excited by the argon laser (488 nm line) and fluorescence emitted at 510 nm in the green region of the visible spectrum. PER expression in these neurons was identified by Cy3-anti Rb-anti, which was excited by the helium laser (543 nm line) and emitted maximal fluorescence at 550 nm in the red region of the visible spectrum. CLK expression was detected by Cy5-anti Gp-anti CLK, which was excited by helium laser (633 nm) and emitted maximal blue fluorescence at 670 nm. All samples were scanned through with three laser lines, the emitted fluorescence signals were digitally recorded and stored as images files. In both sLNvs and ILNvs, PDF was present only in the cytoplasm, which emitted green fluorescence after immune-staining upon laser scanning. PER could be detected in the nuclei or cytoplasm, which emitted red fluorescence after immune-staining upon laser scanning. CLK was only found in the nuclei,

which emitted blue fluorescence after immune-staining upon laser scanning. Later on, these images were quantified using Image J software, statistically analysed with MS Excel and the results were presented as column or linear graphs.

3.3.4 Image analysis

ImageJ is an image processing program developed at the National Institutes of health (Bethesda, Maryland, USA). It is equipped with Java plugins and recordable macros. Image J plugins allows its user to analyse and process the images. In this study ImageJ program was used to quantitate the levels of PER and CLK proteins in the sLNvs and lLNvs. Since PER protein could be found both in cytoplasm (at ZT22) and in nucleus (at ZT2), the signal collection area should be set to include both regions. An analytic procedure was set as following: 1. to split the image file into 3 individual files, each image with information only from one fluorescence. 2. to find the neurons and select the whole neurons based on the PDF staining, taking measurement of the whole neurons on the CLK-only or PER-only image. 3. to take measurement of the non-staining region of the image with the same area setting as PDF, this measurement serves as a noise control and later was subtracted from measure obtained from step two. 4. to select the nucleus also based on image with PDF staining, which was blank center, and take measurement on the other images. 5. to take measurement of the non-staining region of the image with the same area setting as PDF, this measurement serves as a noise control and later was subtracted from measure obtained from step 4. This procedures was applied first to analyze lLNvs in one group of brains (6-12 hemispheres, each with 3-5 neurons), the measurements were saved in a file to be statistically analyzed later using MS Excel. Same procedure was repeated to sLNv's.

3.3.5 Statistic analysis

The measurements taken from ImageJ analysis were further analysed using statistic programs in Excel. The measurements were taken in a fixed sequence so that they could be easily adopted into an analytic format in Excel. Each file contained measurement from one type of neurons (either only sLNvs or lonely ILNvs) from entrainment group (about 24-32 neurons in total). The net signal of a clock protein (CLK or PER) was obtained by subtracting the noise. Such noise subtraction was formatted to apply to individual neurons to obtain the net value of the examined protein. Thereafter the net values from 24-32 neurons were pooled together to obtain the average value and standard deviation. The signals from whole neurons and signals from nuclei were analyzed separately.

As addressed before, each entrainment group (light or food) had 4 time points (ZT2, Z10, ZT14 and ZT22). From these 4 time points, the lowest average value was set to 1, and all the measurements were adjusted accordingly. As a result, the clock protein (PER or CLK) oscillation in a day in response to one environmental cue (light or food) was statistically evaluated and compared. To get conclusive results, each entrainment experiment was repeated at least twice and data from these two experiments were pooled together and submitted to the analysis as addressed above. Using Excel, the final data were plotted and graphically presented for easy assessment.

4. Results

4.1 Whole Food entrainment of the central circadian clock

To study the effect of whole food entrainment on the circadian clock in *Drosophila*, flies were entrained with 12-hour feeding with whole food (WF)/12 hrs starvation cycles in constant darkness for 5 days (WF entrained flies) after freshly eclosed (1-5 days old) flies were exposed to constant light to become arrhythmic. During the starvation phase, WF entrained flies were not supplied with food at all but with cotton soaked with water to prevent dehydration. There are no cyclic environmental stimuli other than food. Temperature was kept constant in the incubator [20 (+/-1) °C]. WF entrained flies is compared with Light-Dark entrained flies which were entrained in 12-hour light/12-hour darkness cycles with constant whole food for 5 days (LD entrained flies). The oscillations of the two clock components, PER and CLK, were used in this study as indicators of a functional molecular clock. At the end of the entrainment, flies were fixed with formaldehyde and the brains were harvested and stained with three antibodies: anti-PDF to stain the cytoplasm of the neurons and hence, the whole neuron can be detected, anti-PER and anti-CLK. Among 150 circadian neurons in the SCN, we focused on the sLN_vs and ILN_vs, which express PDF, for their easy identification and core pacemaker function among the neuronal circadian network.

4.1.1 Effect of whole food entrainment on the CLK in ILN_v

Molecular oscillations of the circadian clock were compared between LD entrained flies (figure 4) and WF entrained flies (figure 5). We first examined the oscillation of CLK protein in ILN_vs. It has been shown that CLK protein accumulation peaks in LN_vs at ZT 2 and a trough has been observed at ZT 10. At each time point 3 to 6 flies were harvested. From those 6 to 12 brain hemispheres, CLK protein levels in ILN_vs were quantified. The average protein level at the trough was set to 1 and the levels from other time points were adjusted accordingly. Figure 4 shows the oscillation of CLK in ILN_vs in LD entrained flies. Figure 5

represents the average of the results of 4 independent experiments in WF entrained flies. As shown in figure 4, LD entrained flies show a strong oscillation of the CLK in ILNvs with a 2-fold amplitude. The peak is at ZT 2 and the trough is at ZT 10. In figure 5, WF entrained flies show a low 1.3-fold amplitude oscillation of the CLK protein with a peak at ZT 2 and a trough at ZT 14. The CLK levels seem to be driven in a 24-hour cycle by feeding. It starts to decrease after ZT 2 till ZT 14 (the trough), then it increases again at ZT 22 until it peaks at ZT 2. To control the condition in constant darkness, we also examined flies kept in constant darkness without feeding cycles (arrhythmic flies). The results are shown in Figure 6. There is no oscillation observed at ZT 10 and ZT 22. For confocal microscopy images (figure 7).

This data give the first indication that in *Drosophila*, whole food entrainment could also drive the circadian molecular oscillator. In constant darkness, cyclic CLK protein accumulations is observed in the ILNvs according to the cyclic food availability.

4.1.1.a Comparison of total CLK protein concentrations in ILNv between LD, WF and arrhythmic entrainments

As shown in figure 8, total CLK concentrations in large ventral lateral neurons are shown in LD entrained flies, WF entrained flies and arrhythmic flies. It shows the following: The peak at ZT 2 in LD entrained flies is 3.6 times higher than the peak at ZT 2 in WF entrained flies and the trough at ZT 10 in LD entrained flies is 2.4 times higher than the trough at ZT 14 in WF entrained flies and almost 1.5 times higher than CLK expression at ZT 10 and ZT 22 in arrhythmic control flies.

4.1.2 Effect of whole food entrainment on the CLK in sLNv:

In sLNv, molecular oscillations were compared between LD entrained flies (Figure 9) and WF entrained (figure 10). LD entrained flies show 24-hour oscillation of CLK protein in

sLN_v. Its amplitude is 2.2-fold high with the peak at ZT 2 and the trough at ZT 22. Whole food entrained flies show a 24-hour oscillation. The peak appears at ZT 2 whose amplitude is 1.3-fold high. The trough appears at ZT 14. The third group of flies is the arrhythmic group that was kept in constant darkness with whole food available at all times (figure 11). As expected, there is no significant oscillation of CLK because there were no light or food stimuli. For confocal microscopy images see figure 12.

4.1.2.a Comparison of total CLK protein concentrations in sLN_v between LD, WF and arrhythmic entrainments

As shown in figure 13, total CLK concentrations in large ventral lateral neurons are shown in LD entrained flies, WF entrained flies and arrhythmic flies. The peak at ZT 2 in LD entrained flies is 2.8 times higher than the peak at ZT 2 in WF entrained flies. The trough at ZT 22 in LD entrained flies is 1.7 times higher than the trough at ZT 14 in WF entrained flies and is almost 1.6 times higher than CLK expression at ZT 10 and ZT 22 in arrhythmic control flies.

4.1.3 Effect of whole food entrainment on the PER protein in ILN_v:

Molecular oscillations of the circadian clock were analyzed by determining PERIOD (PER) protein concentrations between LD entrained flies (Figure 14) and WF entrained flies (Figure 15). PER in LD entrained flies shows 24-hour oscillations with a peak at ZT 2 that is 2.7-fold than the trough at ZT 10. In WF entrained flies, there is also a 24-hour oscillation where PER shows its peak at ZT22 which is 1.6 fold higher than the trough at ZT14. After ZT22 PER concentration keeps decreasing passing by ZT2 and then ZT10 till it reaches its trough at ZT 14 and then it starts to rise again till its peak at ZT22 in the next day (figure 15). Figure 16 shows the third group of flies kept in constant darkness with constant feeding with whole food (arrhythmic flies) shows no significant oscillation due to the absence of any of the entrainment cues (light or food). For confocal microscopy images, see figure 17.

4.1.3.a Comparison of total PER protein concentrations in ILNv between LD, WF and arrhythmic entrainments

Figure 18 shows the difference in PER concentrations between the three fly-groups kept under different conditions described in the previous paragraph. At ZT 2, the peak of PER expression in control flies is 2 times higher than the peak at ZT 22 in WF entrained flies. At ZT 10, the trough of PER expression in LD entrained flies is 1.2 times higher than the trough in WF entrained flies. At ZT 10 and ZT 22, PER expression in arrhythmic flies is 1.2 times higher than PER expression at ZT 10 (the trough) of LD entrained flies and WF entrained flies. Therefore, rhythmic entrainment of the circadian clock in WF entrained flies results in high levels of PER at peak time, while arrhythmic flies express low levels of PER at all times that are almost similar to the trough in the food entrainment conditions.

4.1.4 Effect of whole food entrainment on the PER protein in sLNv:

Molecular oscillations of the circadian clock were analysed by measuring PER in the small ventral lateral neurons (sLNv). PER was measured in LD entrained flies (figure 19), WF entrained flies (figure 20) and in arrhythmic flies (figure 21). LD entrained flies show very strong 24-hour molecular oscillations of PER with a peak at ZT2 and a trough at ZT10. The peak is 10.76-fold high. In WF entrained flies, there is also a 24-hour oscillation where PER shows its peak at ZT22 which is 2-fold higher than the trough at ZT 14. Analysis of PER in the arrhythmic flies does not show any significant oscillation. For confocal microscopy images, see figure 22.

4.1.4.a Comparison of total PER protein concentrations in sLNv between LD, WF and arrhythmic entrainments

Figure 23 shows the difference in PER concentrations between the three fly-groups kept under different conditions as described in the previous paragraph. At ZT 2, the peak of PER expression in LD entrained flies is 2.1 times higher than the peak of PER expression in WF

entrained flies. The trough at ZT 14 in WF entrained flies is 2.3 times higher than the trough at ZT 10 in LD entrained flies.

4.2 Carbohydrate entrainment of the central circadian clock and comparison with the WF entrainment

In this part, *Drosophila* flies were entrained for 5 days in constant darkness with carbohydrate only diet for 12 hours followed by 12 hours of starvation. Carbohydrate meal recipe was taken from Good and Tatar materials and methods [96]. There are no cyclic environmental stimuli other than food. Temperature was kept constant in the incubator [20 (+/-1) °C]

4.2.1 Effect of carbohydrate entrainment on the CLK protein in ILNv:

Carbohydrates entrainment of *Drosophila* shows noticeable impact on the CLK in ILNv (figure 24). It shows 24-hour oscillation. The peak appears at ZT 22 with 1.3-fold high amplitude than the trough at ZT 14. Comparing this graph with the CLK expression in ILNv in WF entrained flies (figure 25), it also shows a 24-hour oscillation with its peak at ZT 2 and an amplitude of 1.4-fold higher than the trough. Both graphs show troughs at ZT14. So the magnitude of oscillation in the two conditions is almost the same (1.3-fold and 1.4-fold respectively) with a 4-hour phase advance for CHO entrained flies. This concludes that carbohydrate entrain molecular oscillations of the circadian clock as efficient as whole food. For confocal microscopy images, see figure 26.

4.2.1.a Comparison of total CLK protein concentrations in ILNv between LD, WF and carbohydrate entrainments

Figure 27 shows the difference in CLK concentrations between the three fly-groups kept under different conditions described in the previous paragraph. At ZT 2, the peak of CLK

expression in LD entrained flies is 3.5 times higher than the peak in WF entrained flies and 3 times higher than the peak of CLK expression in CHO entrained flies at ZT 22. At ZT 10, the trough of CLK expression in LD entrained flies is 2 times higher than the trough of WF entrained flies at ZT 14 and 2.4 times higher than the trough of CHO entrained flies at ZT 14.

4.2.2 Effect of carbohydrate entrainment on the CLK protein in sLNv:

Molecular oscillations of the circadian clock in sLNv were analysed by measuring CLK concentrations. CHO entrained flies show 24 hour oscillation of CLK level in the sLNv (figure 28). The peak amplitude at ZT2 is 1.3-fold higher than the trough level at ZT14. Comparing carbohydrate entrainment with the whole food entrainment (figure 29) shows parallel graphs. Both entrainments show 24-hour oscillation with peaks at ZT2 with the same amplitude (1.3-fold) and troughs at ZT14. Interestingly s-LNv do not show a phase advance for carbohydrates entrained flies compared to the whole food entrainment as observed in ILNv. For confocal microscopy images, see figure 30.

4.2.2.a Comparison of total CLK protein concentrations in sLNv between LD, WF and carbohydrate entrainments

Figure 31 shows total CLK concentrations in sLNv for the three entrainment conditions mentioned in the previous paragraph. At ZT 2, the peaks of CLK expression in the three kinds of entrainments appear. LD entrained flies' peak of CLK expression is 2.5 times higher than the peak in CHO entrainment and 2.8 times higher than the peak in WF entrained flies. The trough of LD entrainment at ZT 14 is 1.5 times higher than the trough of CHO entrainment at both ZT 10 and ZT 14 and 1.7 times higher than the trough of WF entrainment at ZT 14.

4.2.3 Effect of carbohydrate entrainment on the PER protein in ILNv:

Molecular oscillations of the circadian clock in ILNv were analysed by measuring PER concentration. Figure 32 shows a 24-hour oscillation in response to carbohydrate entrainment. The peak is at ZT22. Its amplitude is 1.5-fold higher than the trough at ZT2. Comparing this oscillation with the oscillation of PER in WF entrained flies shows that both have the same peak at ZT22 but the troughs are different. In WF entrainment, the trough is at ZT14. Both graphs show a 24-hour oscillation and have similar amplitudes (figure 33). For confocal microscopy images, see figure 34.

4.2.3.a Comparison of total PER protein concentrations in ILNv between LD, WF and carbohydrate entrainments

Figure 35 shows the total PER concentrations in ILNv in the three conditions mentioned in the previous paragraph. At ZT 2, LD entrained flies show their peak of PER expression which is 2.2 times higher than the peak of CHO entrainment at ZT 22 and 1.8 times higher than the peak of WF entrained flies. At ZT 10, PER expression in LD entrained flies show their trough of PER expression which is 1.2 times higher than the trough in CHO entrainment at ZT 2 and 1.2 times higher than the trough of WF entrainment at ZT 14.

4.2.4 Effect of carbohydrate entrainment on the PER protein in sLNv:

The molecular oscillations of the circadian clock in the sLNv is analysed by measuring PER concentration of period protein in small ventral lateral neurons of flies subjected to carbohydrate entrainment in constant darkness for 5 days (figure 36). This treatment revealed an entrainment of the circadian clock. At ZT22 to ZT 2 PER levels peak reaching the trough at ZT10. Comparing this graph with PER expression in sLNv in WF entrained flies (figure 37) shows that they both have peaks at ZT22 with almost the same amplitude (2.1-fold). For confocal microscopy images, see figure 38.

4.2.4.a Comparison of total PER protein concentrations in sLNv between LD, WF and carbohydrate entrainments

Figure 39 shows the total PER concentrations in sLNv in the three mentioned entrainment conditions. At ZT2, LD entrained flies show their peak of PER expression which is 2 times higher than the peak in CHO entrainment at ZT 2 and ZT 22 and 2.1 times higher than in WF entrained flies. At ZT10, PER expression in LD entrained flies shows its trough. The trough of PER expression in CHO entrainment is 2.4 times more than the trough in LD entrained flies both at ZT 10 and it is the same as the trough in WF entrained flies.a

4.3 Protein enriched entrainment of the central circadian clock and comparing it with carbohydrates entrainment.

In this part *Drosophila* flies were entrained with protein enriched diet for 12 hours followed by 12 hours of starvation in constant darkness for 5 days. In starvation phase, flies were bred on cotton soaked with water only. Protein enriched meal was prepared on the aforementioned sucrose. meal with addition of 1% casein protein (casein hydrolysate, standard for molecular biology, Roth) [13]. Starvation vials were prepared with a 1 cm thick piece of cotton soaked with distilled water. There are no environmental stimuli other than food. Temperature was kept constant in the incubator [20 (+/-1) °C]

4.3.1 Effect of protein enriched diet entrainment on the CLK protein in ILNv:

Figure 40 shows the oscillation of CLK in ILNv in protein entrained flies entrained in constant darkness for 5 days. It shows 24-hour oscillation. The peak appears at ZT22 with 1.7-fold amplitude of oscillation. This graph shows two time points with low levels of CLK concentration. These two troughs appear at ZT 2 and ZT 14. After ZT14 it rises again to reach its peak at ZT2.

4.3.1.a Comparison of total CLK concentrations in ILNv between LD, Carbohydrate and protein entrainments

As shown in figure 41, CLK concentrations in large ventral lateral neurons are shown in control flies, flies entrained with carbohydrate diet and flies entrained with protein enriched diet. It shows the following: At ZT2, the CLK expression in LD entrained flies shows its peak that is 2.8 higher than the peak in protein entrained flies at ZT 22 and 3 times higher than the peak CHO entrainment at ZT 22. The trough in LD entrained at ZT 10 is 2.4 times higher than the trough of protein entrainment at ZT 2 and 2.4 times higher than the trough of CHO entrainment at ZT 14.

4.3.2 Effect of protein enriched diet entrainment on the CLK protein in sLNv:

CLK in small ventral lateral neurons oscillates in response to protein enriched entrainment. It shows 24-hour oscillation. The peak appears at ZT 10 with 1.5-fold amplitude while the trough appears at ZT 2. After the peak at ZT 10 CLK expression decreases passing by a ZT 14 and ZT 22 reaching the trough at ZT 2 (figure 42).

4.3.2.a Comparison of total CLK concentrations in sLNv between LD, Carbohydrate and protein entrainments

Figure 43 shows the average of CLK expression in sLNv of the three previously mentioned fly-groups. The peak of CLK expression in LD entrained flies at ZT 2 is 3 times higher than the peak in protein entrainment at ZT22 and 2.5 times higher than the CHO entrainment at ZT 2. The trough of CLK expression in LD entrainment at ZT 22 is 1.6 times higher than the trough in protein entrainment at ZT 14 and 1.5 times higher than the trough in CHO entrainment.

4.3.3 Effect of protein enriched diet entrainment on the PERIOD protein in ILNv:

In protein enriched entrainment, PER in the large ventral lateral neurons oscillates showing the peak at ZT 10 with 1.8-fold amplitude (figure 44). There are two time points showing drop in CLK expression at ZT 2 and ZT 14.

4.3.3.a Comparison of total PERIOD protein concentrations in ILNv between LD, Carbohydrate and protein entrainments

As shown in figure 45, the values of PER concentrations in large ventral lateral neurons are shown in LD entrained flies, CHO entrained flies and protein entrained flies. It shows the following: At ZT 2, the peak of PER concentration in LD entrained flies is 3 times higher than the peak of protein entrainment at ZT 10 and almost 2 times higher than the peak in carbohydrate entrainment at ZT 22. The trough of LD entrained flies at ZT 10 is 2 times higher than the trough in protein entrained flies at ZT 2 and 1.2 times higher than in CHO entrained flies at ZT 2.

4.3.4 Effect of protein enriched diet entrainment on the PERIOD protein in sLNv:

PER in small ventral lateral neurons responds to the protein enriched diet entrainment. It shows 24-hour entrainment. PER shows a 1.3-fold amplitude oscillation with a peak at ZT 10 and two troughs at ZT 14 and ZT 22 (figure 46).

4.3.4.a Comparison of total PERIOD protein concentrations in sLNv between LD, Carbohydrate and protein entrainments

At ZT 2, LD entrained flies show their peak of PER expression in sLNv which is 2 times higher than the peak in protein entrainment at ZT 10 and 2 times higher than the peak in carbohydrate entrainment at ZT 2. CHO entrainment show its trough at ZT 10 which is 1.5 times higher than the trough in protein entrainment at ZT 14 and 2.4 times higher than the trough in LD entrained flies at ZT 10 (figure 47).

4.4 lipid enriched entrainment of the central circadian clock and a comparison with carbohydrate entrainment

In this part *Drosophila* flies were entrained with lipids enriched diet supplied for 12 hours followed by 12 hours of starvation in constant darkness for 5 days. In starvation phase, flies were bred on cotton soaked with water only. Lipid enriched meal was prepared on the sucrose meal with the addition of 10% oil (olive oil, sunflower oil, canola oil with equal concentration of each 1:1:1) + 10% ethanol to allow oil to dissolve. Starvation vials were prepared with a 1 cm thick piece of cotton soaked with distilled water. There are no environmental stimuli other than food. Temperature was kept constant in the incubator [20 (+/-1) °C]

4.4.1 Effect of lipids enriched diet entrainment on the CLK protein in ILN_v:

Molecular oscillations of the circadian clock are analysed by measuring CLK in the large ventral lateral neurons. Figure 48 shows a 24-hour oscillation in CLK expression. At ZT2, CLK shows its lowest concentration (the trough) while at ZT10, it shows its highest concentrations with a 2-fold amplitude oscillation. Comparing this figure with the oscillation after carbohydrate entrainment (figure 49) shows that both treatments entrain 24-hour oscillations, but carbohydrates entrainment shows an opposite phase of oscillation with a peak at ZT22 and a trough at ZT14.

4.4.1.a Comparison of total CLK protein concentrations in ILN_v between LD, Carbohydrate and lipid entrainments

In this graph (figure 50), CLK protein expression in ILN_v in lipids entrained flies is compared with LD entrainment and carbohydrate entrainment. At ZT2, the CLK expression in LD entrained flies shows its peak that is 4.3 times higher than the peak in lipid entrained flies at ZT 10 and 3 times higher than the peak CHO entrainment at ZT 22. The trough in LD

entrained at ZT 10 is 4 times higher than the trough of lipid entrainment at ZT 2 and 2.4 times higher than the trough of CHO entrainment at ZT 14.

4.4.2 Effect of lipids enriched diet entrainment on the CLK protein in sLNv:

Molecular oscillations of the circadian clock in response to feeding with lipids enriched diet for 12 hours followed by starvation for 12 hours in constant darkness for 5 days was analysed by measuring CLK in the sLNv. At ZT2, ZT10 and ZT14 CLK expression is almost the same. The trough appears at ZT22. The whole cycle shows no prominent oscillation with no CLK prominent changes in its expression (figure 51). Comparing this oscillation with the CLK expression in sLNv in carbohydrates entrainment (figure 52) shows the peak at ZT2 with 1.31-fold amplitude and the trough is at ZT14. In carbohydrates entrainment there is 24-hour oscillation while in lipids entrainment, the oscillation is very weak.

4.4.2.a Comparison of total CLK protein concentrations in sLNv between LD, Carbohydrate and lipid entrainments

By comparing the lipids entrainment with the LD entrained flies and carbohydrate entrainment. Figure 53 shows the average of CLK expression in sLNv of the three previously mentioned fly-groups. The peak of CLK expression in LD entrained flies at ZT 2 is almost 3.5 times higher than the CLK expression in lipid entrainment at ZT 2, ZT 10 and ZT 14 and 2.5 times higher than the CHO entrainment at ZT 2. The trough of CLK expression in LD entrainment at ZT 22 is 2 times higher than the trough in lipid entrainment at ZT 22 and 1.5 times higher than the trough in CHO entrainment at ZT 14.

4.4.3 Effect of lipids enriched diet entrainment on the PERIOD protein in ILNv

PERIOD protein shows oscillation in the small ventral lateral neurons. Its expression varies between different time points. It has 2 peaks: one at ZT10 and the other at ZT22 with very close amplitudes (1.8 and 1.9 respectively). The trough appears at ZT2. Between ZT22 and

ZT2, there is a sharp reduction in PER expression. Between ZT2 and ZT10, there is a sharp increase in PER expression (figure 54). Because the normal 24 hour rhythm should have one peak, this kind of oscillation denotes an error and hence no reliable information can be concluded.

4.4.3.a Comparison of total PER protein concentrations in ILNv between LD, Carbohydrate and lipid entrainments

figure 55 shows the difference in the total concentrations of PER in lipid entrainment, carbohydrates entrainments and the control flies. Since lipid entrainment did not show successful entrainment, no reliable comparison can be done.

4.4.4 Effect of lipids enriched diet entrainment on the PERIOD protein in sLNv

The small ventral lateral neurons show a low amplitude of oscillation of PER expression with a trough at ZT14 and a broad peak during feeding time (figure 56). Comparing this graph with the carbohydrate entrainment shows different patterns of oscillation (figure 57). They both show 24 hour oscillation. In carbohydrate entrainment, the peak appears at ZT2 and the trough appears at ZT10 while in lipid entrainment, the peak is broad involving 3 time points and the trough is at ZT14.

4.4.4.a Comparison of total CLK protein concentrations in sLNv between LD, Carbohydrate and lipid entrainments

At ZT 2, LD entrained flies show their peak of PER expression in sLNv which is almost 4 times higher than the amplitude of oscillation in lipid entrainment at 2 times higher than the peak in carbohydrate entrainment at ZT2. CHO entrainment show its trough at ZT10 which is 2.4 times higher than the trough in LD entrained flies at ZT 10 and 2 times higher than the trough in lipid entrained flies (figure 58).

Data from lipid entrainment have to be interpreted with caution since this experiment has been done only once and needs to be repeated.

4.5 Entrainment of *pdf*⁰ flies

Entrainment of *pdf*⁰ flies in two different conditions using the same procedure used for *w1118* flies (see materials and methods). The first condition is the control where flies were entrained in light dark cycles with constant feeding with whole food for 5 days. In the second condition, the flies were entrained with cycles of 12 hours feeding/ 12 hours starvation in constant darkness for 5 days. The recipe of whole food is mentioned in the materials and methods section. Temperature was kept constant in the incubator [20 (+/-1) °C]. In this analysis CLK and PER proteins were measured only in the nucleus because the cytoplasm couldn't be measured due to the lack of its identification protein which is the pigment dispersing factor (PDF).

4.5.1 Effect of whole food entrainment on the CLOCK protein in ILNv in *pdf*⁰ flies

Molecular oscillation of the circadian clock is analyzed by measuring CLK concentration in the ILNv of *pdf*⁰ flies. LD entrained *pdf*⁰ flies show inconsistent CLK expression with an amplitude of 2.2-fold. WF entrained *pdf*⁰ flies show 24-hour oscillation. The peak is at ZT14 with an amplitude 2.9-fold higher than the trough at ZT2 (figure 59). In this graph, *pdf*⁰ flies appear to entrain better to feeding cycles than to light-dark cycles. For confocal microscopy images, see figure 60.

4.5.1.a Comparison of total CLK in ILNv between LD and WF entrained *pdf*⁰ flies

Total concentrations of CLK has been compared between the previously mentioned two conditions, the LD entrained *pdf*⁰ flies and the WF entrained *pdf*⁰ flies. The amplitude of both entrainments is almost equal and the trough of LD entrainment at ZT10 is 1.3 times higher than the trough of WF entrainment at ZT2 (figure 61).

4.5.2 Effect of whole food entrainment on the CLOCK protein in sLNv in *pdf*⁰ flies

Molecular oscillation of circadian clock was analysed by measuring CLK in the sLNv of LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies. LD entrainment showed an error in their oscillation as the CLK expression is opposite at every time point. The amplitude of oscillation is 2-fold. CLK expression in WF entrainment shows 24-hour oscillation. The peak appears at ZT14 with 2-fold amplitude more than the trough at ZT2. At ZT22, CLK expression was not measured because no small ventral lateral neurons could be detected in any of the scanned brains at this time point (figure 62). This may be due to the very low expression of CLK at this time point. *pdf*⁰ flies appear to entrain to feeding cycles while it does not entrain in response to light-dark cycles, indicating that PDF is more important for light signalling than for metabolic signaling into the circadian clock. For confocal microscopy images, see figure 63.

4.5.2.a Comparison of total CLK in sLNv between LD and WF entrained *pdf*⁰ flies

CLK expression is compared between light and whole food entrainments of *pdf*⁰ flies. The peak of CLK expression in sLNv in WF entrained flies at ZT 14 is 1.2 times higher than the amplitude of CLK expression in LD entrainment, while the trough of CLK expression in LD at ZT 10 is 1.8 times higher than the trough in WF entrainment at ZT 2 (figure 64).

4.5.3 Effect of whole food entrainment on the PERIOD protein in lLNv in *pdf*⁰ flies

Molecular oscillation of the circadian clock was analysed by measuring PER in the lLNv of LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies. PER expression in LD entrainment shows 24-hour oscillation. LD entrained *pdf*⁰ flies show their peak at ZT 22 with 24-fold amplitude. Their trough is at ZT 14. WF entrained flies also show 24-hour oscillation. The peak is at ZT 14 and its amplitude is 4-fold. The trough is at ZT10 (figure 65). This graph shows that PER oscillations entrain better to light dark entrainment than to whole food entrainment. For confocal microscopy images, see figure 66.

4.5.3.a Comparison of total PER in ILNv between LD and WF entrained *pdf*⁰ flies

Comparing total PER expression in LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies showed a peak of PER expression in LD entrainment at ZT 22 which is 3 times higher than the peak in WF entrainment at ZT 14. The trough of PER expression in WF entrainment at ZT 10 is 2 times higher than the trough in LD entrainment at ZT 14. On one hand, this denotes that PDF is important in modulation of PER expression in LD entrainment but not in metabolic entrainment, while on the other hand, PER protein in both entrainment conditions showed 24-hour oscillation in absence of PDF (figure 67).

4.5.4 Effect of whole food entrainment on the PERIOD protein in sLNv in *pdf*⁰ flies

PER expression in sLNv of LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies was analyzed to study the molecular oscillation of the circadian clock. PER expression in LD entrainment shows 24-hour oscillations unlike CLK expression. The peak is at ZT22 and its amplitude is 48-fold more than the trough at ZT14. PER expression in WF entrainment also shows 24-hour oscillation. The peak is at ZT14. Its amplitude is 3.5-fold. The trough is at ZT2. At ZT22, PER expression was not measured because no small ventral lateral neurons could be detected in any of the scanned brains at this time point (figure 68). For confocal microscopy images, see figure 69.

4.5.4.a Comparison of total PER in sLNv between LD and WF entrained *pdf*⁰ flies

PER expression is compared between LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies. The peak of PER expression in LD entrainment at ZT 22 is almost equal to the peak in WF entrainment at ZT 14, while the trough of PER expression in WF entrainment at ZT 2 is 13 times higher than the peak in LD entrainment. On one hand, this denotes that PDF is important in modulation of PER expression in LD entrainment but not in metabolic entrainment, while on the other hand, PER protein in both entrainment conditions showed 24-hour oscillation in absence of PDF (figure 70).

4.6 The effect of feeding time on the circadian clock oscillation of *w1118* flies

This experiment is done to find out whether light entrainment or feeding entrainment is the more powerful entrainment cues. Therefore, both entrainments were given together in the same phase in one fly-group and in the opposite phase in the other fly-group. In this experiment, the two fly-groups were entrained in light dark cycles. One group was fed during light and the other group was fed during dark. The entrainment of the two groups started together and ended together for five days. The first group was fed from ZT0 till ZT12 and the other group was fed from ZT12 till ZT24. Both groups were fed with whole food. For whole food recipe see materials and methods.

4.6.1 Effect of feeding time on the CLK protein in ILNv in *w1118* flies

Molecular oscillation of the circadian clock in *w1118* flies fed during dark is measured versus *w1118* flies fed during light. CLK concentration is measured in ILNv. WF in light entrainment shows 24-hour oscillation. The peak is at ZT 2 and its amplitude is 1.8-fold. The trough is at ZT 14. WF in dark entrainment also shows 24-hour oscillation. The peak is at ZT22 and its amplitude is 1.8-fold higher than the trough at ZT 14. Both conditions show almost the same oscillation with close amplitude but the peak is shifted from ZT 22 in WF in dark entrainment to ZT2 in WF in light entrainment (figure 71). Since feeding during the night causes a 4-hour phase advance of molecular oscillations, this demonstrates that feeding is a potent Zeitgeber for the ILNv. For confocal microscopy images, see figure 72.

4.6.1.a Comparison of total CLK in ILNv between WF in light and WF in dark entrained *w1118* flies.

Total concentration of CLK protein is measured at every time point. The peak of CLK expression in WF in dark entrainment at ZT 22 is 1.5 times higher than the peak of CLK expression in WF in light entrainment at ZT 2. The trough of CLK expression in WF in dark

entrainment at ZT 14 is 1.4 times higher than the peak of CLK expression in WF in light entrainment at ZT 14 (figure 73).

4.6.2 Effect of feeding time on the CLOCK protein in sLNv in *w1118* flies

CLK protein expression in sLNv shows 24-hour oscillation in both WF in light flies and WF in dark flies. WF in light flies show their peak at ZT2 with 2.9-fold amplitude. The trough is at ZT10. CLK expression in WF in dark flies shows its peak at ZT22 and its amplitude is 1.4-fold and the trough is at ZT14 (figure 74). Feeding during the dark phase almost abolishes the molecular oscillation in sLNv again demonstrating that feeding is a very potent Zeitgeber. For confocal microscopy images, see figure 75.

4.6.2.a Comparison of total CLK in sLNv between WF in light and WF in dark entrained *w1118* flies.

In WF in light entrained flies, the peak of CLK expression at ZT 2 is 1.7 times higher than the peak in WF in dark entrained flies at ZT 22 and ZT 2. The trough of CLK expression in WF in dark entrainment at ZT 14 is 1.2 times higher than the trough in WF in light entrained flies at ZT 10 (figure 76).

4.6.3 Effect of feeding time on the PERIOD protein in lLNv in *w1118* flies

PER expression in WF in light entrainment and WF in dark entrainment is measured. In WF in light flies there is a 24-hour oscillation. The peak is at ZT2 and its amplitude is 4.5-fold and the trough is at ZT10. WF in dark flies show also 24-hour oscillation and the peak is at ZT22 and its amplitude is 3.6-fold and the trough is at ZT14 (figure 77). PER expression is affected less than CLK expression indicating that PER as expected is controlled stronger by light than by metabolism. However, CLK appears to be important for metabolic regulation of the circadian clock. For confocal microscopy images, see figure 78.

4.6.3.a Comparison of total PER in sLN_v between WF in light and WF in dark entrained *w¹¹¹⁸* flies.

Total expression of PER at every time point is compared between the two mentioned conditions. The peak of PER expression in WF in dark entrainment at ZT 22 is 1.2 times higher than the peak of PER expression in WF in light entrainment at ZT 2. The trough of PER expression in WF in dark entrainment at ZT 14 is 1.5 times higher than the trough of PER expression in WF in light entrainment at ZT 10 (figure 79).

4.6.4 Effect of feeding time on the PERIOD protein in sLN_v in *w¹¹¹⁸* flies

In WF in light flies, PER expression in sLN_v shows 24-hour oscillation. The peak of PER expression is at ZT 2 and its amplitude is 7.3-fold. The trough is at ZT 10. On the other hand, PER expression in WF in dark flies also shows 24-hour oscillation and its peak is at ZT 22 and its amplitude is 6.2-fold. The trough is at ZT14 (figure 80). Feeding during the dark phase causes a phase advance of PER expression in sLN_v, indicating that feeding is an important Zeitgeber. For confocal microscopy images, see figure 81.

4.6.4.a Comparison of total PER in sLN_v between WF in light and WF in dark entrained *w¹¹¹⁸* flies.

Total expression of PER at every time point is compared between the two mentioned conditions. The peak of PER expression in WF in light entrainment at ZT 2 is almost as same as the peak of PER expression in WF in dark entrainment at ZT 22. The trough of PER expression in WF in dark entrainment at ZT 14 is 1.1 times higher than the trough of PER expression in WF in light entrainment at ZT 10 (figure 82).

5. Discussion

This research was done to investigate the impact of feeding on the molecular circadian clock. First, we aimed to investigate the ability of food to entrain the circadian clock. Our results reveal that scheduled feeding can entrain molecular oscillations of the circadian clock. Different nutrition shows differences in the amplitude and phase of molecular oscillations. The second aim was to test the effect of feeding time on light entrainment of the circadian clock. These experiments showed that feeding is a strong entrainment factor that alters the phase of light-entrained molecular oscillations. Finally, the impact of pigment dispersing factor, which is important for synchronization of the circadian neuronal network by light-dark cycles, on the circadian clock proteins in response to food entrainment has been investigated. These results indicate that PDF is more important for light-entrainment than for entrainment by scheduled feeding, suggesting independent signalling pathways that mediate a light- and food- response of the circadian clock.

5.1 Response of circadian clock to WF entrainment

5.1.1 Response of CLK protein to WF entrainment

Whole food restricted feeding schedules in *Drosophila* could successfully entrain the central circadian clock. It shows 24-hour oscillation and hence it shows that circadian clock can be entrained centrally by complete balanced diet. These findings are consistent with previous studies in rodents that showed that the SCN in rodents is entrainable by scheduled feeding in the absence of photic stimuli [53]. Similar entrainment of circadian oscillations has not been shown to date in *Drosophila*. Importantly, our data show an entrainment of molecular oscillations in neuronal pacemaker cells, which has not been observed in mammals, where feeding schedules is a key entrainment factor for the digestive organs, but not the SCN.

CLK protein shows gradual oscillation along the 24-hours cycle with the peak at ZT2 which is two hours after food is supplied and the trough at ZT14 which is two hours after food is stopped (starvation). This is seen in the oscillation of CLK in ILNV and in sLNv as well. Both large and small ventral lateral neurons responds positively to a well balanced diet and negatively to starvation. The concentration of CLK protein in ILNV and sLNv of flies entrained in constant darkness with constant food is nearly the same with no oscillation since there are no changes in any of the environmental factors whether food, light or temperature. This in turn proves that restricted feeding RF can entrain oscillations in CLK protein levels.

5.1.2 Response of PER protein to WF entrainment

PER protein response to RF is not different from CLK response. PER shows 24 hour oscillation with the peak at ZT22, two hours before the introduction of food and its trough at ZT14, two hours after the beginning of starvation. The appearance of the peak at ZT22 is considered as food anticipatory activity (FAA) of PER protein. This response appears similarly in the oscillation of PER in the small and large ventral lateral neurons. It also denotes that PER expression responds positively to a well balanced diet and negatively to starvation. Comparing this oscillation with PER oscillation in LD entrained flies, both show 24 hour oscillation but the magnitude of oscillation in control flies is more than that in RF. Arrhythmic flies showed no difference in PER expression since there is no change in the surrounding environmental entrainment factors as food or light. This outcome of the ability of whole food to entrain PER protein as well as CLK protein is giving a clue that in mammals, when a well balanced diet is maintained at specific times, it can affect the mammalian circadian clock proteins. Consequently, when circadian clock proteins can respond towards feeding, this can be used in treatment of specific diseases related to circadian clock impairment.

5.2 Response of circadian clock to carbohydrates entrainment

5.2.1 Response of CLK protein to carbohydrates entrainment

Our results show that the circadian clock can be entrained by carbohydrates. CLK protein showed 24-hour oscillation in small and large ventral lateral neurons. Both neuron types expressed more CLK around the onset of carbohydrates feeding time. CLK expression is high at ZT2 and ZT22 which is two hours before and two hours after the beginning of feeding time. This can be interpreted as FAA. In ILNv the peak is at ZT22 and in sLNv, it is at ZT2. This pattern of CLK response is comparable with its response towards starvation at ZT10 and ZT14. CLK expression is markedly decreased at ZT10 and ZT14 which is two hours before and two hours after the onset of starvation. So, CLK in ILNv and sLNv responds positively towards carbohydrates feeding and negatively towards starvation. This change in CLK levels before the change in feeding onset or offset indicates an autonomous oscillation of the circadian clock. This kind of response is the same as that in whole food entrainment.

5.2.2 Response of PER protein to carbohydrates entrainment

PER expression in response to carbohydrate entrainment shows 24-hour oscillation. Its expression in ILNv is different from that in sLNv. PER in ILNv peaks at ZT22 which is two hours before the onset of carbohydrates feeding and drops showing its trough two hours after the onset of feeding. sLNv shows phase shift of its peak as the peak is shifted to ZT2 and the trough shifted to ZT10. PER expression in sLNv is high during carbohydrate feeding and low during starvation. This cannot be also applied in ILNV because of the appeared trough at ZT2. But overall, carbohydrates restricted feeding can entrain PER protein in small and large ventral lateral neurons.

5.3 Response of circadian clock to proteins entrainment

CLK and PER expression in protein entrainment showed 24- hour oscillation in both sLNv and ILNv but with low amplitude. Since the protein was added to the same carbohydrate meal discussed above, proteins may have a suppressive effect on the circadian proteins. However, protein entrainment was done only twice and hence, it should be further studied and repeated to confirm the results.

5.4 Response of circadian clock to lipids entrainment

Lipids entrainment of the circadian clock did not give any solid results. Clock proteins did not show 24-hour oscillation in all cases except in CLK expression in ILNv which showed 24-hour oscillation with low amplitude. Although lipids meal was prepared on carbohydrate media using the same carbohydrate concentration used in the carbohydrate entrainment, there was no oscillation. It may be because oil floated over the meal surface although alcohol was used to dissolve the lipids composition and hence the flies couldn't feed from the sugar composition. Another reason may be suggested, is that alcohol may be toxic. This experiment was done only once and hence, it should be repeated for confirmation.

5.5 Role of pigment dispersing factor in controlling the expression of circadian clock proteins.

Pigment dispersing factor PDF is a neuropeptide responsible for modulation of the circadian clock. It coordinates fly circadian behaviour as it is the primary output of oscillation within the lateral ventral neurons [80].

We further studied its role in the circadian clock proteins expression in response to restricted feeding in constant darkness in *pdf⁰* flies and comparing it to the LD entrained *pdf⁰* flies.

5.5.1 Response of CLK protein in ILNv of *pdf*⁰ flies to whole food restricted feeding

*pdf*⁰ flies under RF shows 24-hour oscillation with its peak two hours after the onset of starvation at ZT14 and its trough is two hours after the onset of feeding. This kind of oscillation proves that PDF does not play important role in controlling CLK expression in ILNv. While in LD entrained *pdf*⁰ flies, they don't show 24-hour oscillation but instead, CLK expression is inconsistent and this is due to the lack of PDF. This denotes that PDF is more important for light signalling than for metabolic signalling into the circadian clock.

5.5.2 Response of CLK protein in sLNv of *pdf*⁰ flies to whole food restricted feeding

sLNv show similar molecular oscillation of CLK as in ILNv in response to restricted feeding. It shows 24-hour oscillation which peaks 2 hours after the onset of starvation and the trough appears at ZT2 which means 2 hours after the onset of feeding. At ZT22, no sLNv was detected at all and this is most probably due to the very low expression of CLK that is not detected even under very high laser intensities during the scanning process under confocal microscopy. This oscillation is almost the same as in ILNv. So as mentioned in the large ventral lateral neurons, this kind of oscillation in small ventral lateral neurons proves that PDF does not play important role in controlling CLK expression in response to restricted feeding. While in LD entrained *pdf*⁰ flies, they don't show 24-hour oscillation but instead, CLK expression is arrhythmic. This oscillation is considered as an error and it may be due to the lack of PDF. So hereby, restricted feeding can entrain CLK expression in large and small ventral lateral neurons in *pdf*⁰ flies.

5.5.3 Response of PER protein in ILNv of *pdf*⁰ flies to whole food restricted feeding

PER expression in ILNv in WF entrained *pdf*⁰ flies shows 24-hour oscillation which peaks 2 hours after the onset of starvation and the expression drops at ZT10 during the starvation phase. LD entrained *pdf*⁰ flies also show 24-hour oscillation and its amplitude is much higher than that in WF entrainment. PER response to light dark entrainment is different than CLK

response. PER seems to be independent of PDF and hence showed proper 24-hour oscillation. While in RF, PER response is the same as *CLK*. As a result, this kind of oscillation in large ventral lateral neurons proves that PDF does not play a role in controlling PER expression whether in light dark entrainment or food entrainment.

5.5.4 Response of PER protein in sLNv of *pdf*⁰ flies to whole food restricted feeding

PER expression in sLNv in LD entrained *pdf*⁰ flies and RF *pdf*⁰ flies is the same as in ILNv mentioned in the previous paragraph. They both show 24-hour oscillation and the *magnitude* of oscillation in LD entrainment is much more than the magnitude in WF entrainment. In WF entrainment, PER response is the same as CLK. As a result, this kind of oscillation in large ventral lateral neurons proves that PDF does not play a role in controlling PER expression whether in light dark entrainment or food entrainment unlike the CLK response in control *pdf*⁰ flies.

5.6 Changes in the molecular oscillation of circadian clock in response to feeding at the light phase in comparison to feeding at dark phase

5.6.1 Response of CLK in ILNv to feeding at the light phase and feeding at dark phase

The two entrainments, WF in light and WF in dark entrainments, show similar oscillatory pattern of CLK expression in ILNv with 4-hour phase advance of WF in dark entrainment.

This demonstrates that feeding is a potent Zeitgeber for the ILNv.

5.6.2 Response of CLK in sLN_v to feeding at the light phase and feeding at the dark phase

CLK expression in sLN_v is affected by WF in light versus WF in dark entrainments. CLK expression in WF in dark is very low in comparison with WF in light entrainment. Therefore, WF in dark almost abolishes the molecular oscillation in sLN_v again demonstrating that feeding is a very potent Zeitgeber.

5.6.3 Response of PER in ILN_v to feeding at the light phase and feeding at the dark phase

PER expression in the ILN_v shows similar pattern between the two entrainment conditions, WF in light and WF in dark entrainments. As mentioned in 5.5.1 and 5.5.2, both entrainments yielded very close oscillation with a phase advance in WF in dark entrained flies. For that reason, feeding is considered as a very potent Zeitgeber.

5.6.4 Response of PER in sLN_v to feeding at the light phase and feeding at the dark phase

Molecular oscillation of PER in sLN_v of WF in light and WF in dark entrainments showed that WF in light entrainment shows 24-hour oscillation with a very high amplitude, while in WF in dark entrainment the amplitude is reduced with a phase advance. So, as mentioned before, food can be considered as a very potent Zeitgeber.

6. Conclusions and future prospects

Scheduled feeding can entrain molecular oscillation in circadian pacemaker neurons in *Drosophila*. A whole food diet (a well balanced diet) can drive the expression of circadian clock proteins centrally. This also applies in carbohydrate only diet and protein enriched diet. Circadian clock proteins, CLOCK and PERIOD, show proper oscillation with proper 24-hour oscillation, but carbohydrates are more efficient in synchronizing molecular oscillation than proteins. Lipid enriched diets did not show proper driving effect on the central circadian clock. Lipid entrainment experiment may be repeated for further confirmation.

Pigment dispersing factor (PDF) shows its importance to drive clock proteins in light dark cycles. Meanwhile, it appeared to be insignificant in entrainment by scheduled feeding. This can be justified that light perception is transmitted to the central circadian clock via PDF while food entrainment drive is transmitted to the central clock via different ways, indicating that PDF is more important for light signalling than for metabolic signalling into the circadian clock. Time of feeding during day or during night had an effect on the molecular oscillation of the central clock except by a phase shift of CLK and PER in sLN_v and lLN_v. This data suggest that eating habits impact circadian synchronization.

The above data establishes *Drosophila* as a model for the investigation of metabolic regulation of the circadian clock which can be further applied in mammals.

This study emphasizes the importance of nutrition and nutrients types and their effect on the circadian clock. They can positively or negatively affect it. Further study should focus on the treatment of certain diseases in people related to circadian clock dysfunctions using a certain dietary regimes with specific nutrients.

Figures

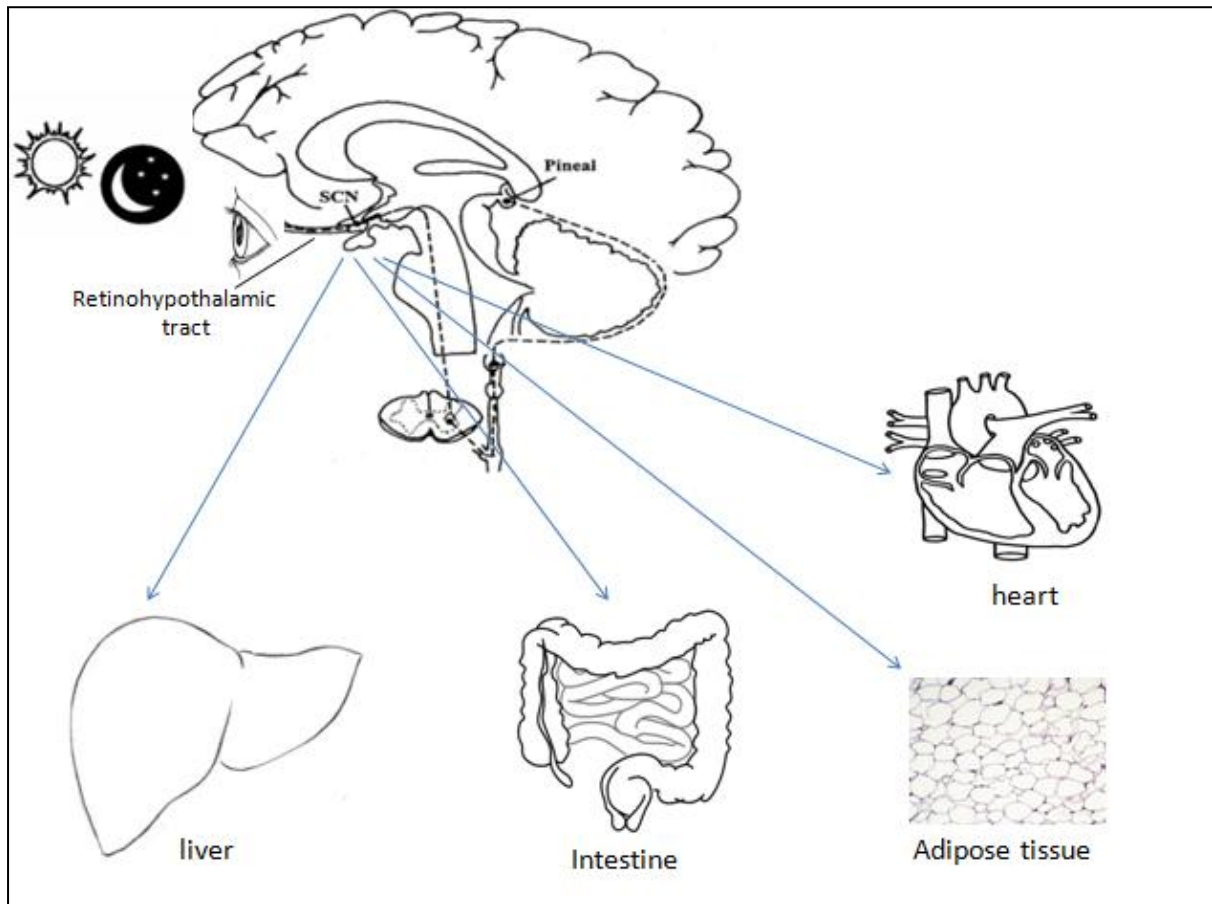


Figure 1. Central and peripheral circadian clock distribution in human body.

Circadian clock has central oscillator and peripheral oscillators. The central oscillator is located in the Suprachiasmatic nucleus (SCN) in the hypothalamus while Peripheral oscillators are located in several are which are; pineal gland which is situated between the two cerebral hemispheres and is attached to the third ventricle. Other peripheral oscillators are located in the heart, liver, adipose tissues, intestine and retina [42].

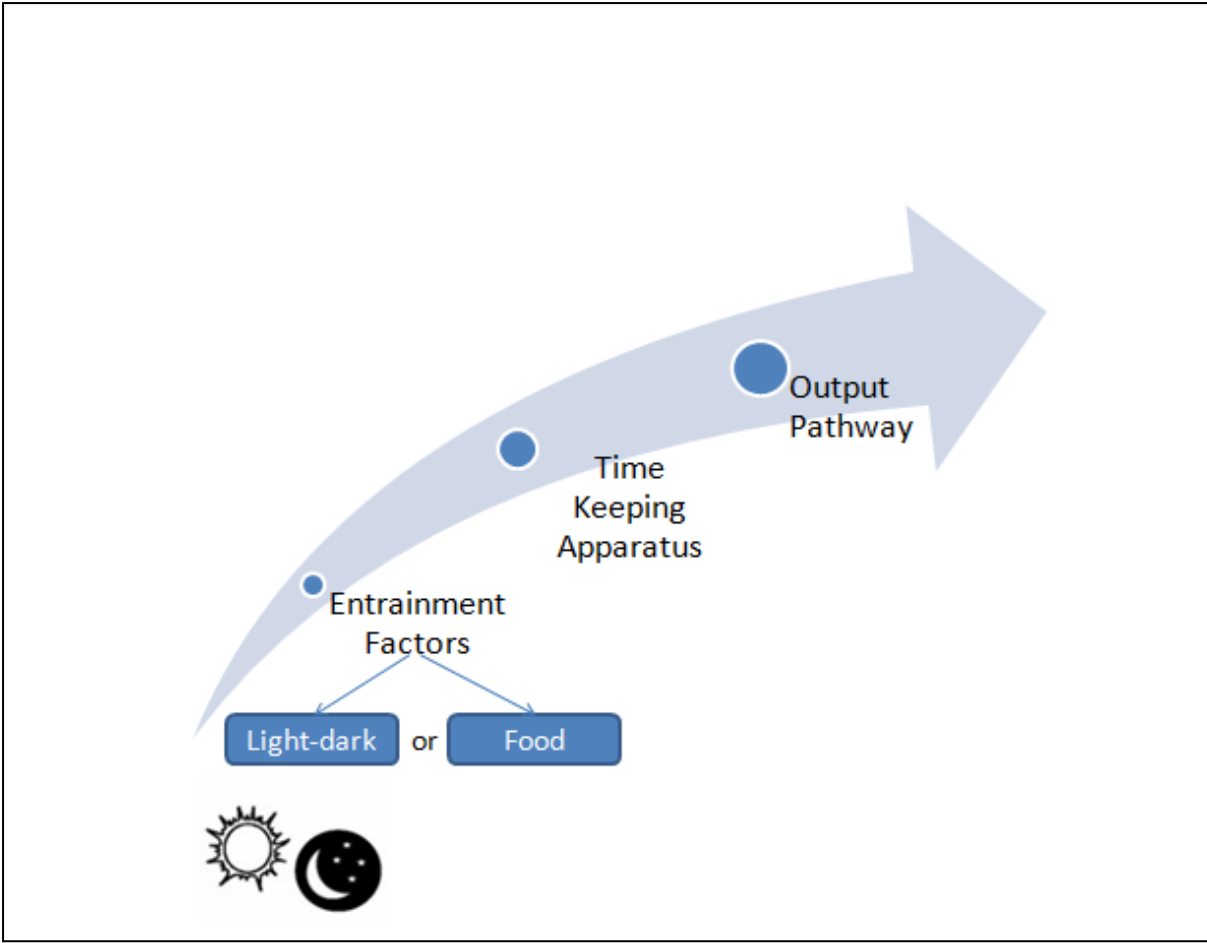


Figure 2. The circadian clock pathway is composed of three successive steps

An entrainment pathway which receives the environmental stimulus (usually light) and transmits it to the next step, timekeeping apparatus which keeps the entrainment history and does its function even in the absence of the external stimulus and the output pathway which results in the physiological behaviour at certain times of the day [43]

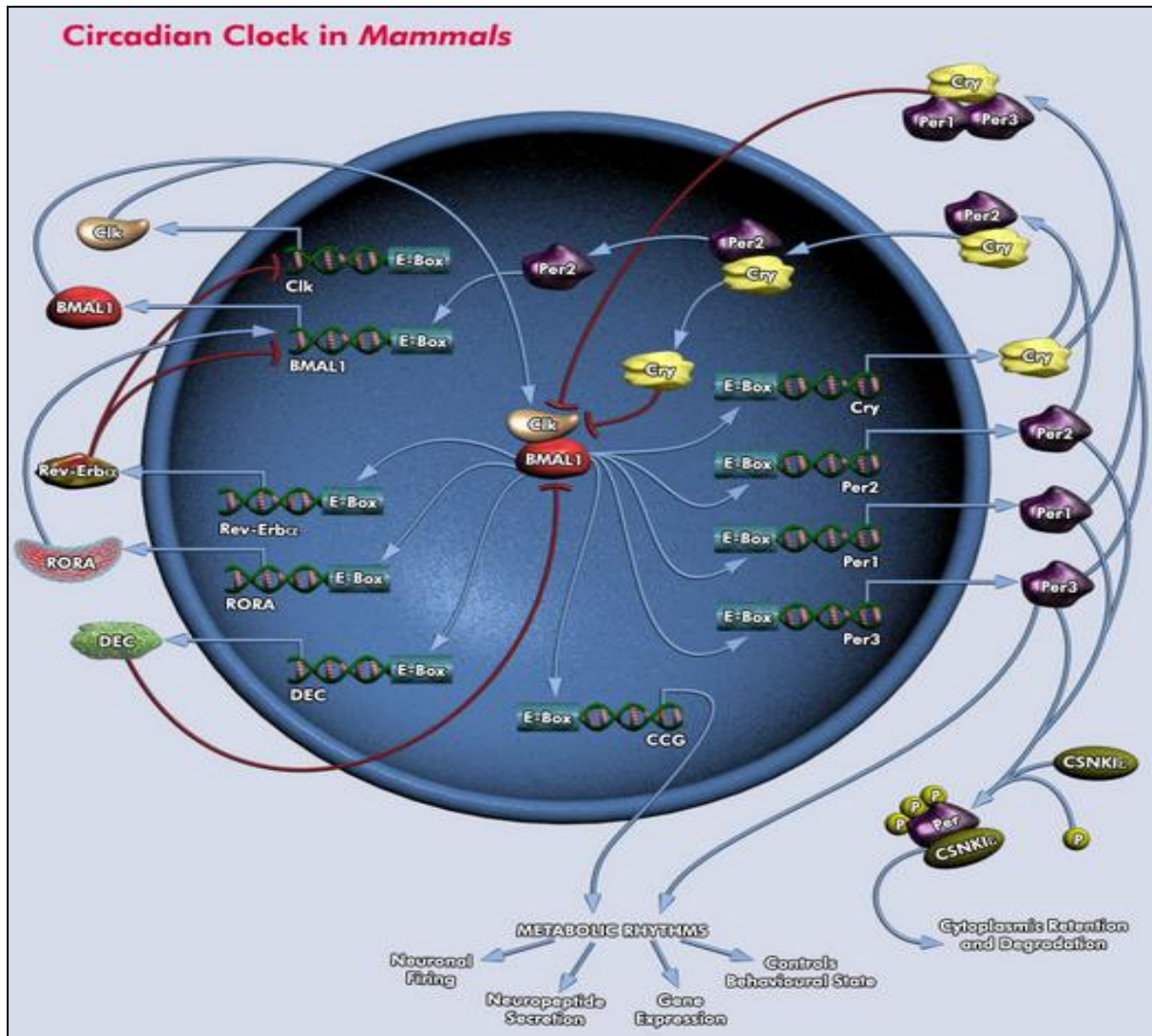


Figure 3. The mechanism of Circadian Clock in Mammals

BMAL1 binds to CLK to form BMAL1-CLK heterodimer which activates the transcription of CRY proteins giving CRY1 and CRY2 and on PER proteins giving PER1, PER2 and PER3. The high amounts of BMAL1 on the day also result in accumulation of PER proteins in the cytoplasm. Newly synthesized PER is soon phosphorylated by CK-I-ε. These phosphorylated PER proteins are highly unstable as they are degraded easily by ubiquitylation and hence PER transcription continue during the day. Until the end of the day when CRY protein accumulates in the cytoplasm and forms CK-I-ε /PER/CRY complex, a stable undegradable complex, which by the beginning of night time enters the nucleus disrupting the transcription of BMAL1-CLK complex leading to reduced transcription of per, cry and rev-erb- α while increased transcription of bmal1. This cyclic regulation is referred as the first feedback loop. The second feedback loop is composed of rev-erb- α (a nuclear hormone receptor responsible for adipogenesis), RAR-Related Orphan Receptor A (rora), and BMAL1. BMAL1-CLK heterodimer binds to the E-box elements of the rev-erb- α , and rora, activate their transcriptions. Both Rev-Erb- α and Rora binds to the RRE elements in the bmal1 promoter; Rev-Erb- α protein inhibits the transcription of BMAL1 and CRY whereas Rora enhances it [48].

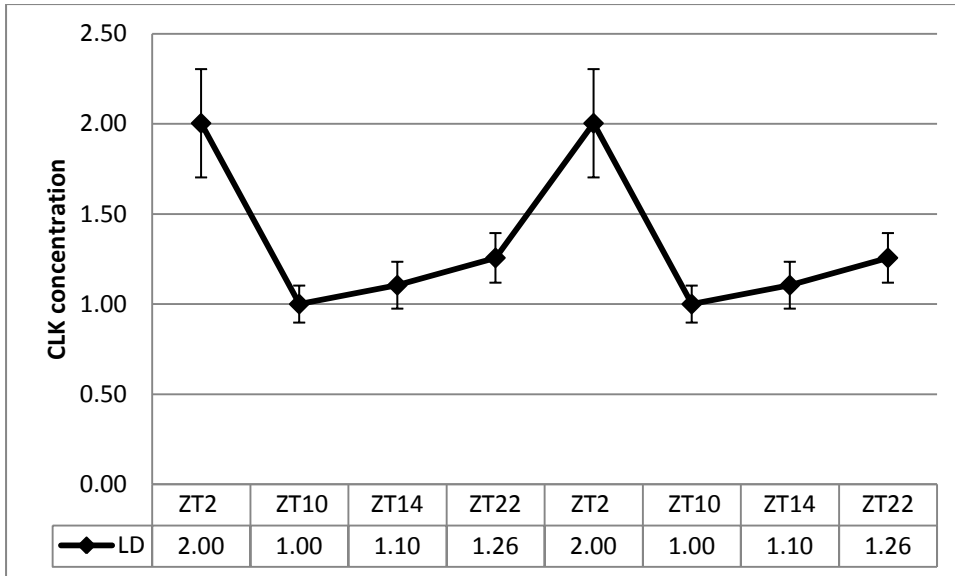


Figure 4. Oscillation of CLK protein in the ILNv in response to exposure to light-dark cycles with constant whole food diet for 5 days (Control flies).

The expression level of CLK at ZT10 was set to 1 and the levels from other time points are shown relative to ZT 10. Data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light; ZT14: 2 hours in darkness; ILNv: large ventral lateral neurons.

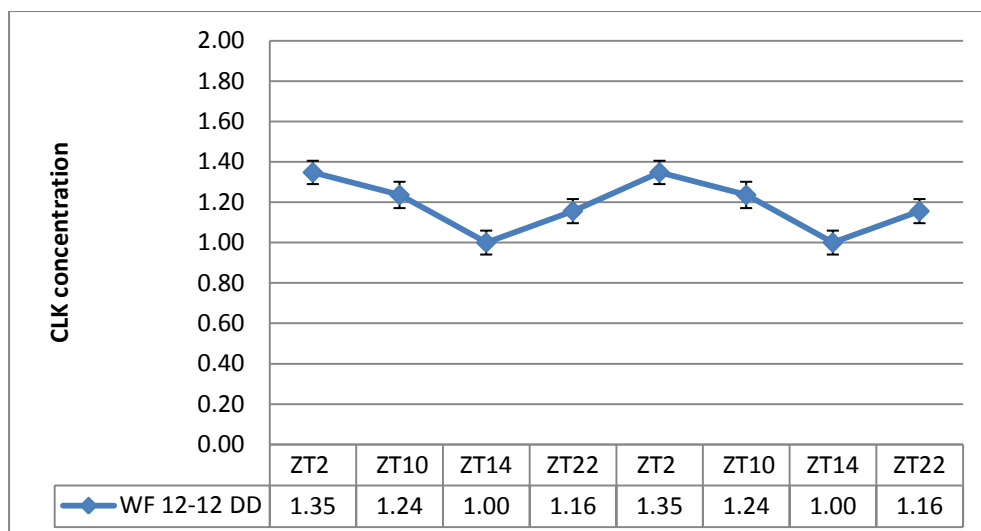


Figure 5. Oscillation of CLK protein in the large ventral lateral neurons in response to exposure to 12 hours of whole food diet followed by 12 hours starvation in constant darkness for 5 days.

The expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

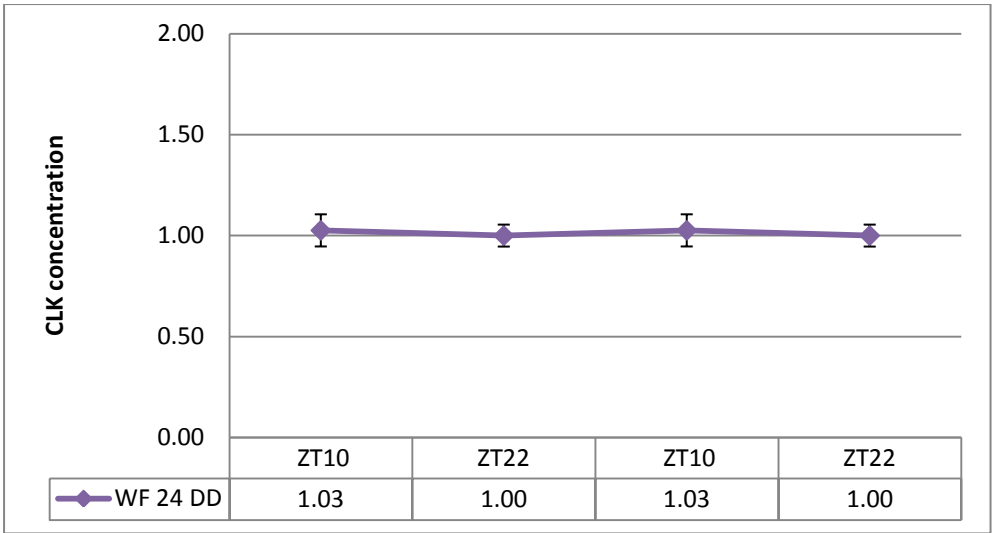


Figure 6. CLK protein levels in the ILNv in the absence of environmental cycles (flies kept on whole food diet all times in constant darkness for 5 days).

Data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

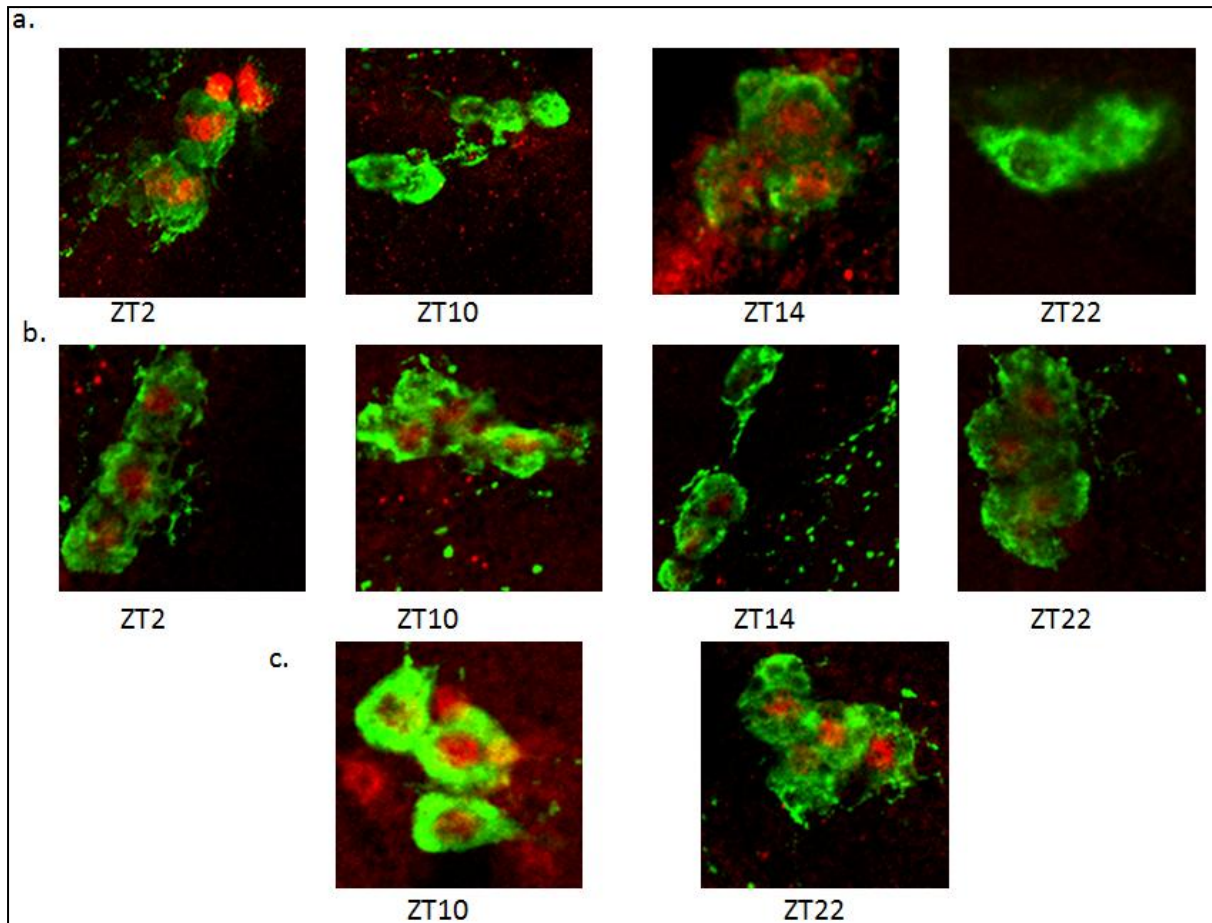


Figure 7. Oscillation of CLK in the ILNv in response to: a. LD entrainment, b. WF entrainment c. arrhythmic conditions. CLK in red and PDF in green.

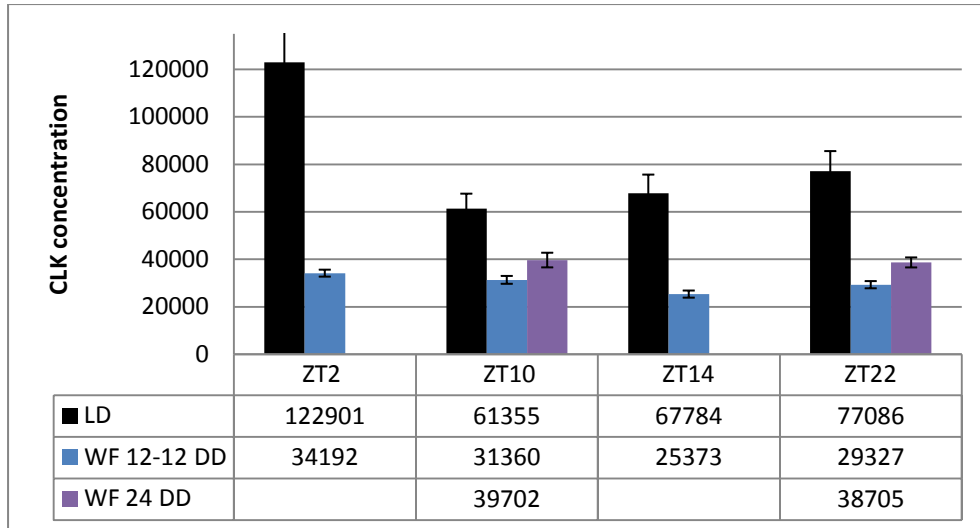


Figure 8. Comparison of total CLK concentrations in ILNv between LD, WF and arrhythmic entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 24 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; CLK: CLOCK protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

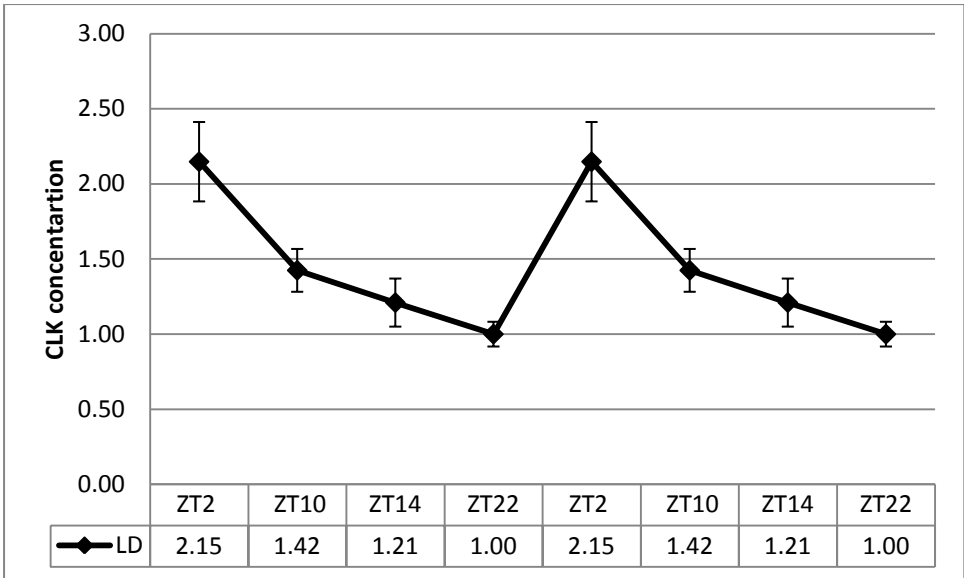


Figure 9. Oscillation of CLK protein in the sLNv in response to exposure to light-dark cycles with constant whole food diet in for 5 days.

The expression level of CLK at ZT 22 was set to 1 and the levels from other time points are shown relative to ZT 22. Data shown at each time point are the average of at least 4 flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light; ZT14: 2 hours in darkness; sLNv: small ventral lateral neurons.

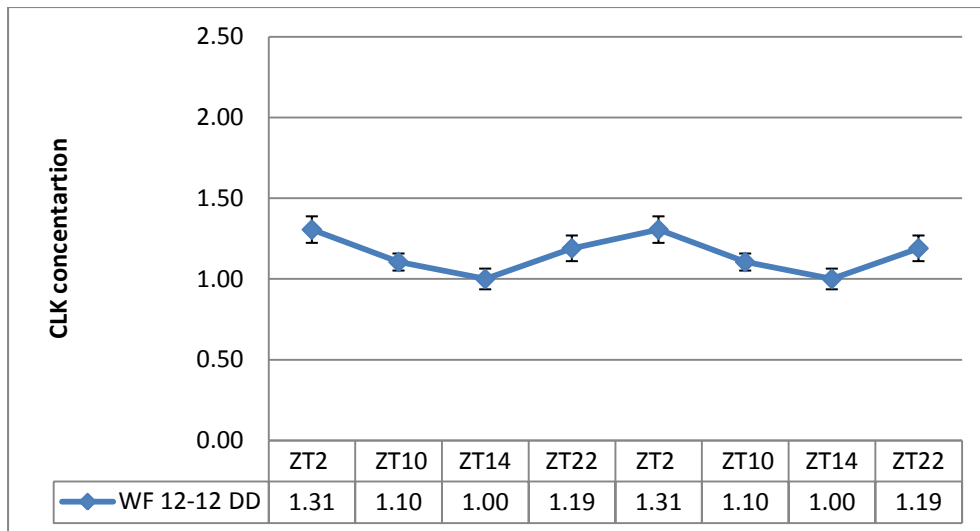


Figure 10. Oscillation of CLK protein in the sLNv in response to WF entrainment.

The expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are of at least 16 flies/ 32 hemispheres from 4 experiments.

Abbreviations: WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

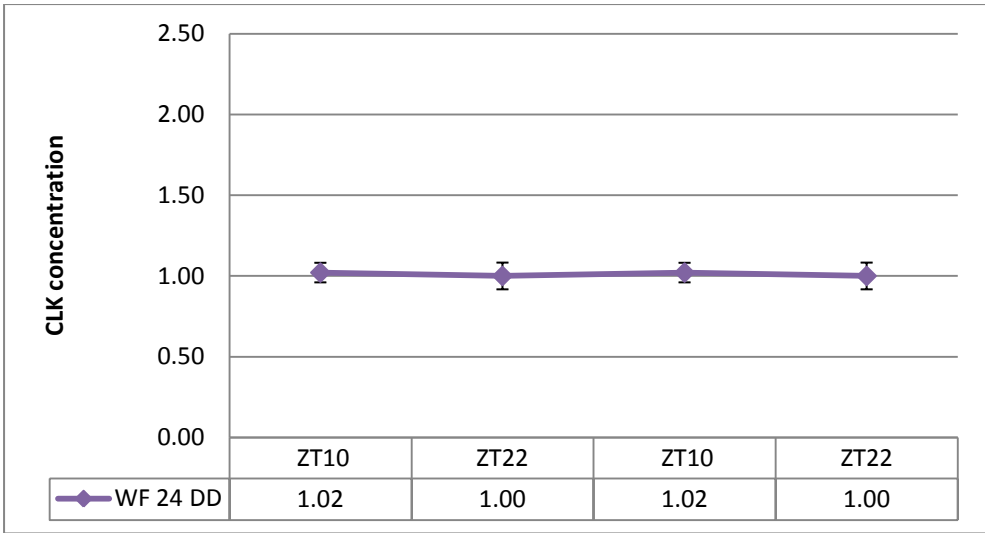


Figure 11. CLK protein levels in the sLNv in the absence of environmental cycles

(flies kept on whole food diet all times in constant darkness for 5 days). Data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

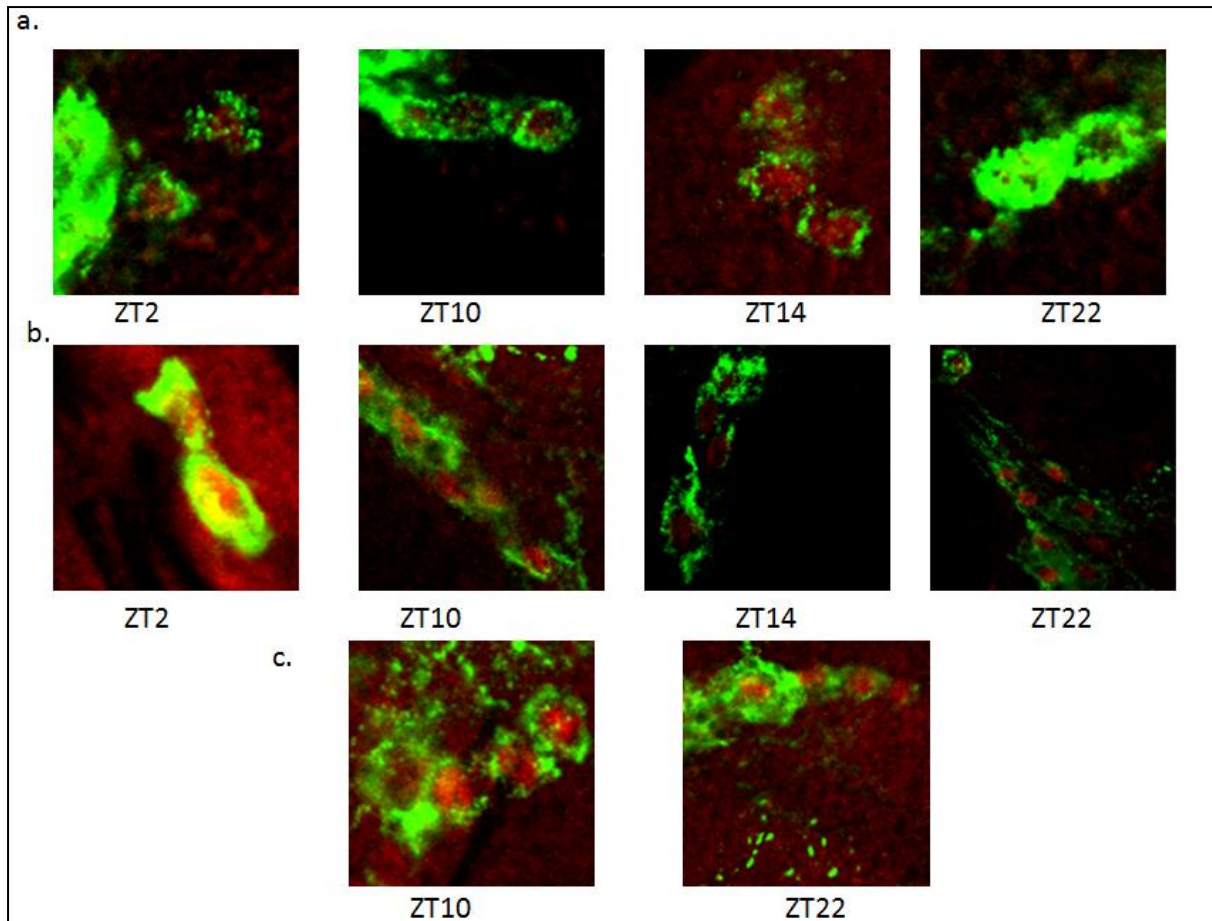


Figure 12. Oscillation of CLK protein in the sLNv in response to: a. LD entrainment, b. WF entrainment c. arrhythmic conditions. CLK in red and PDF in green.

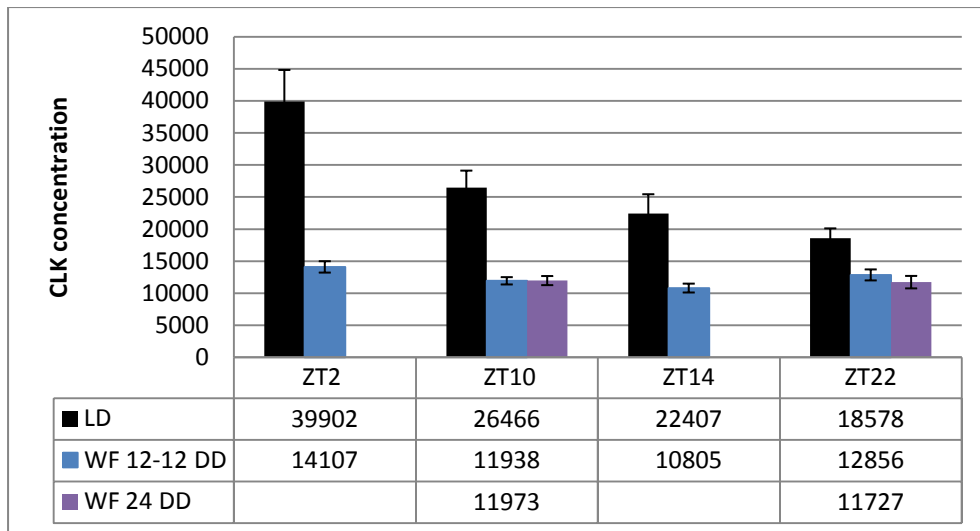


Figure 13. Comparison of total CLK concentrations in sLNv between LD, WF and arrhythmic entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 24 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons

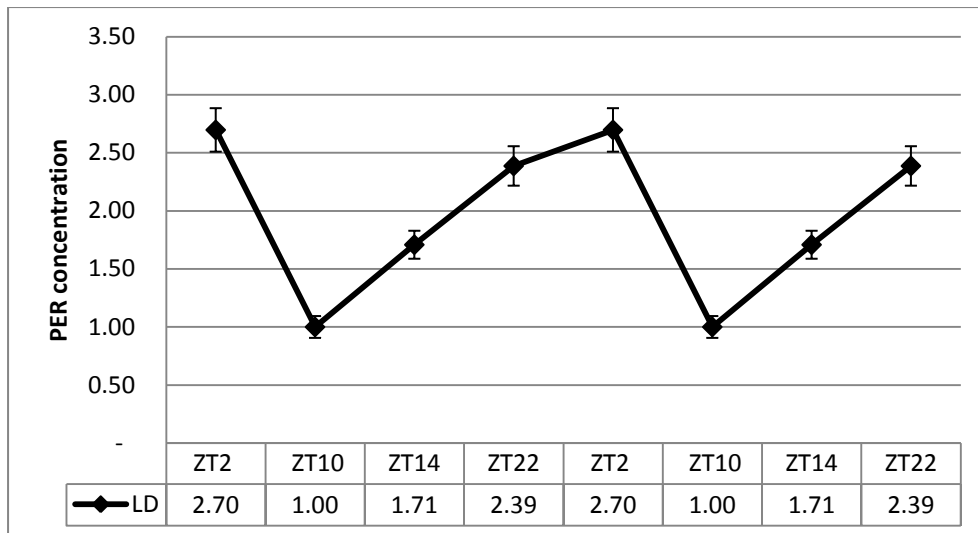


Figure 14. Oscillation of PER in the ILNv in response to exposure LD entrainment.

The expression level of PER at ZT 10 was set to 1 and the levels from other time points are shown relative to ZT 10. Data shown at each time point are the average of at least 8 flies/16 hemispheres from 2 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light; ZT14: 2 hours in darkness; ILNv: large ventral lateral neurons.

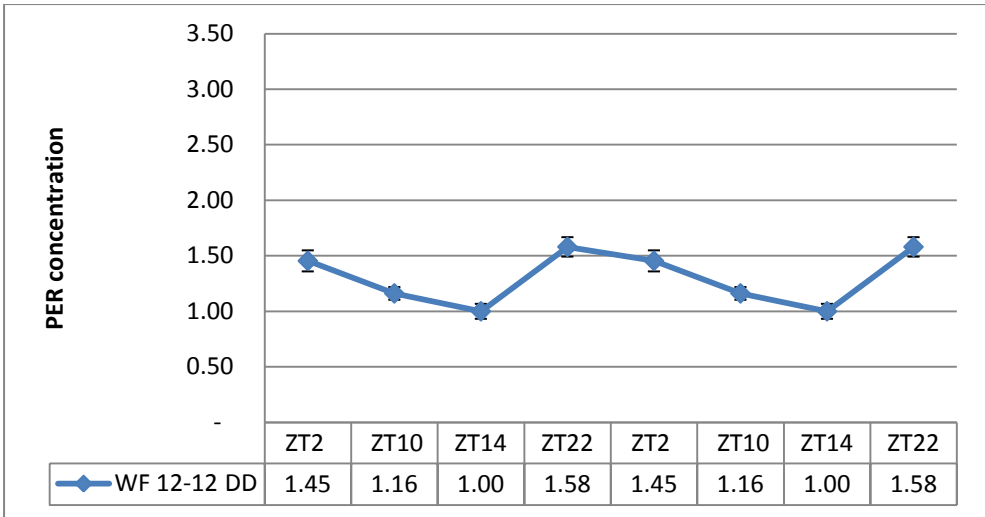


Figure 15. Oscillation of PER in the ILNv in response WF entrainment.

The expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are of at least 16 flies/ 32 hemispheres from 4 experiments.

Abbreviations: WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

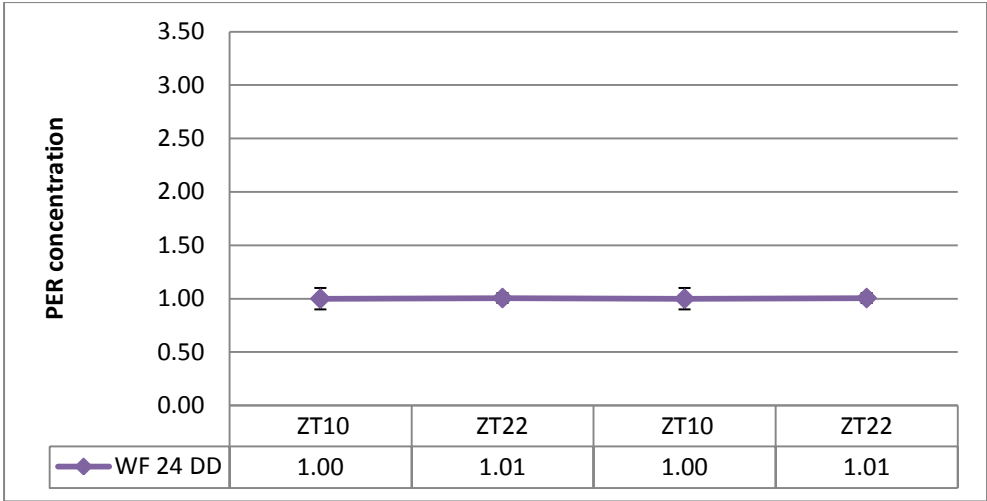


Figure 16. PER levels in the ILNv in the absence of environmental cycles (flies kept on whole food diet all times in constant darkness for 5 days).

Data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF 24 DD: 24h of whole food diet in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

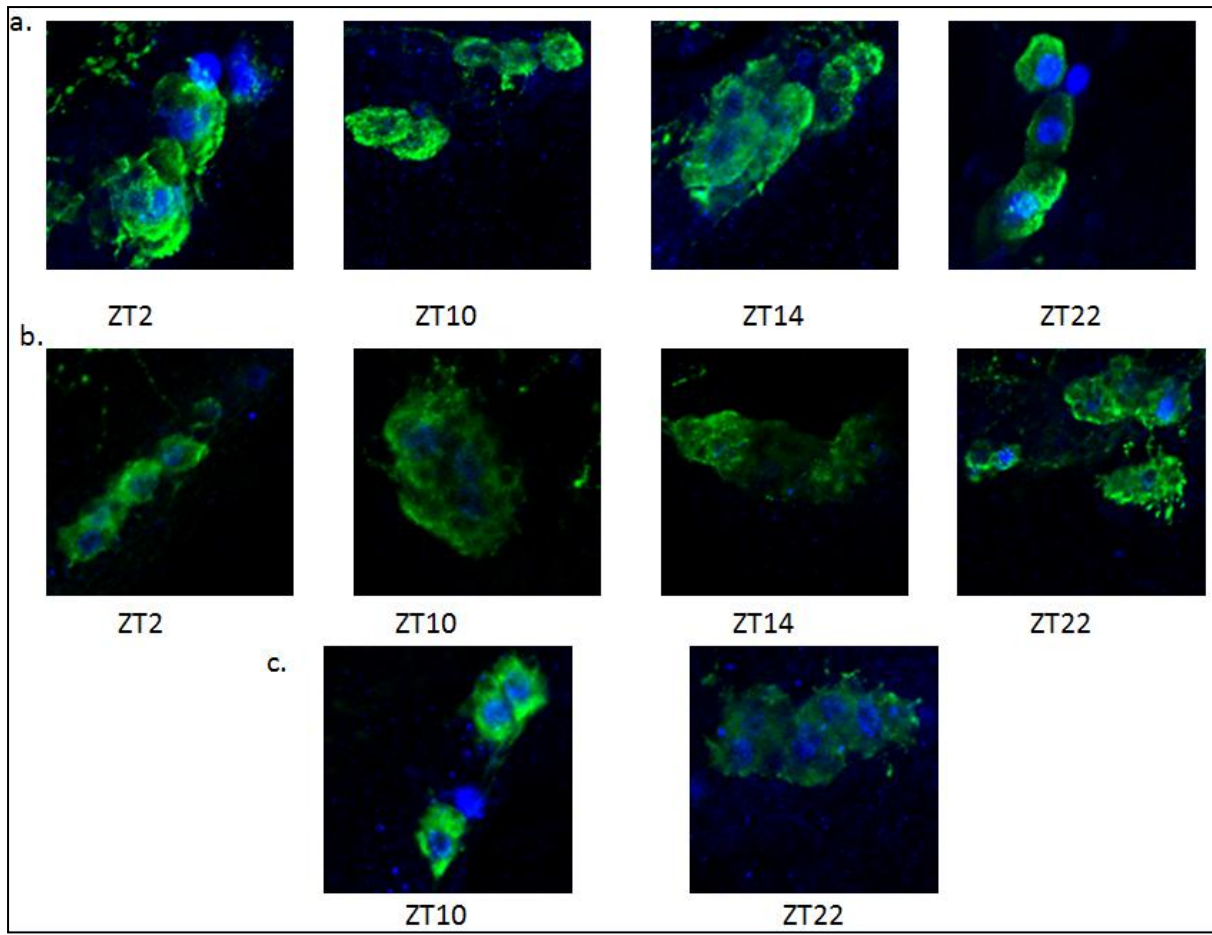


Figure 17. Real images scanned of PER protein in ILNv in response to: Oscillation of CLK protein in the sLNv in response to: a. LD entrainment, b. WF entrainment c. arrhythmic conditions. PER in blue and PDF in green.

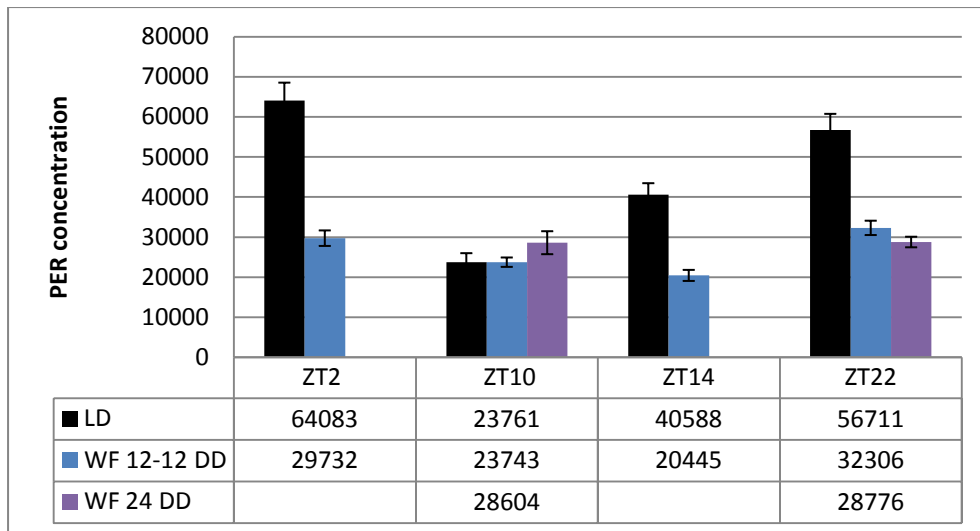


Figure 18. Comparison of total PER concentrations in ILNv between LD, WF and arrhythmic entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 8 Flies/16 hemispheres from two representative experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 24 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons

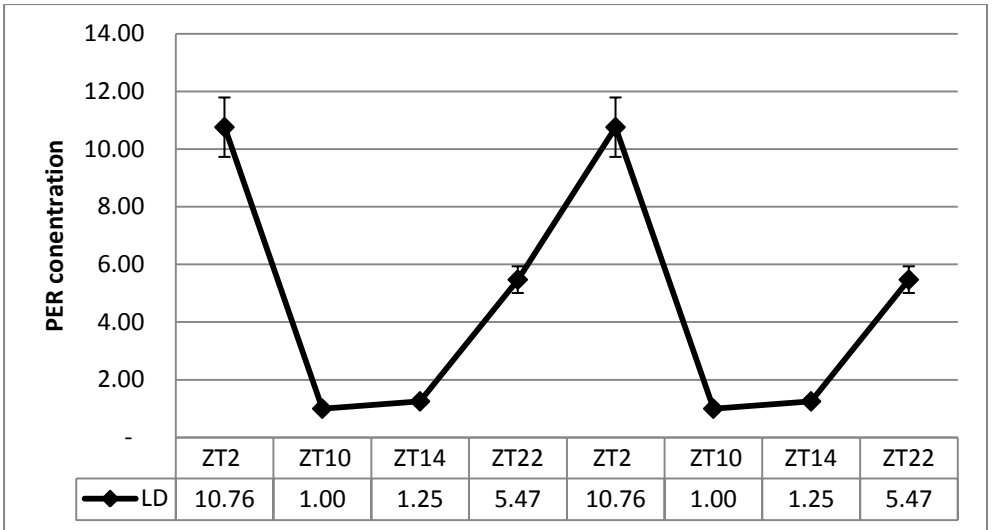


Figure 19. Oscillation of PER protein in the sLNv in response to LD entrainment.

The expression level of PER at ZT 10 was set to 1 and the levels from other time points are shown relative to ZT 10. Data shown at each time point are the average of at least 8 flies/16 hemispheres from 2 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light; ZT14: 2 hours in darkness; sLNv: small ventral lateral neurons.

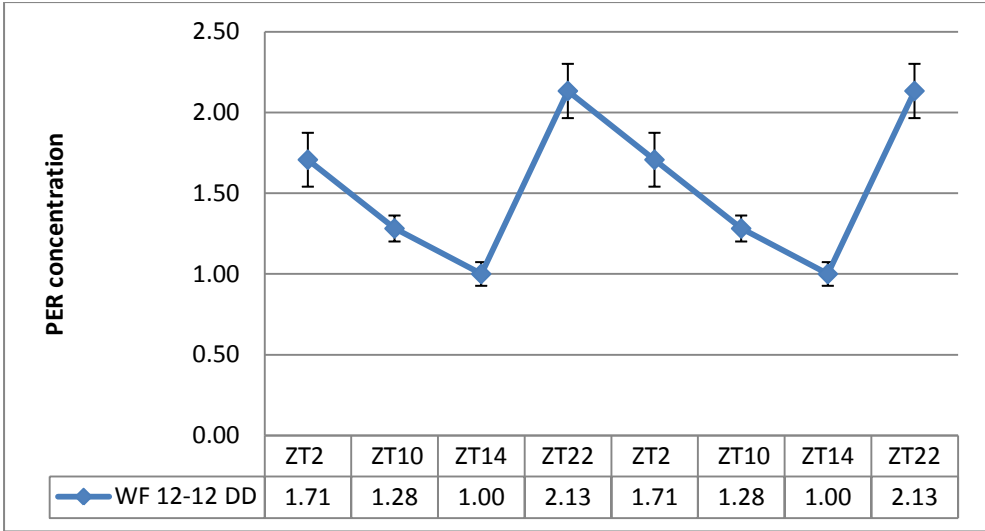


Figure 20. Oscillation of PER protein in the sLNv in response to WF entrainment.

The expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are of at least 16 flies/ 32 hemispheres from 4 experiments.

Abbreviations: WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

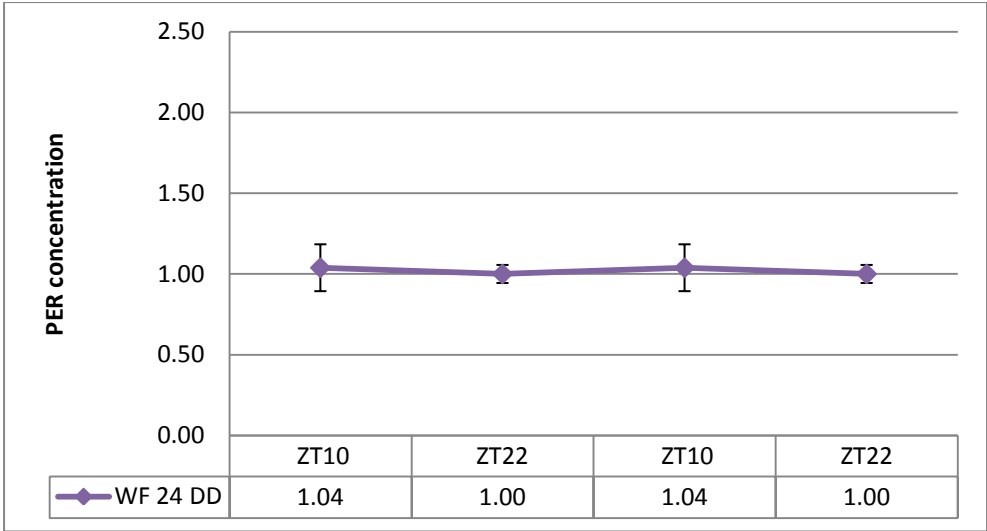


Figure 21. PER protein levels in the sLNv in the absence of environmental cycles (flies kept on whole food diet all times in constant darkness for 5 days).

Data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF 24 DD: 24h of whole food diet in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

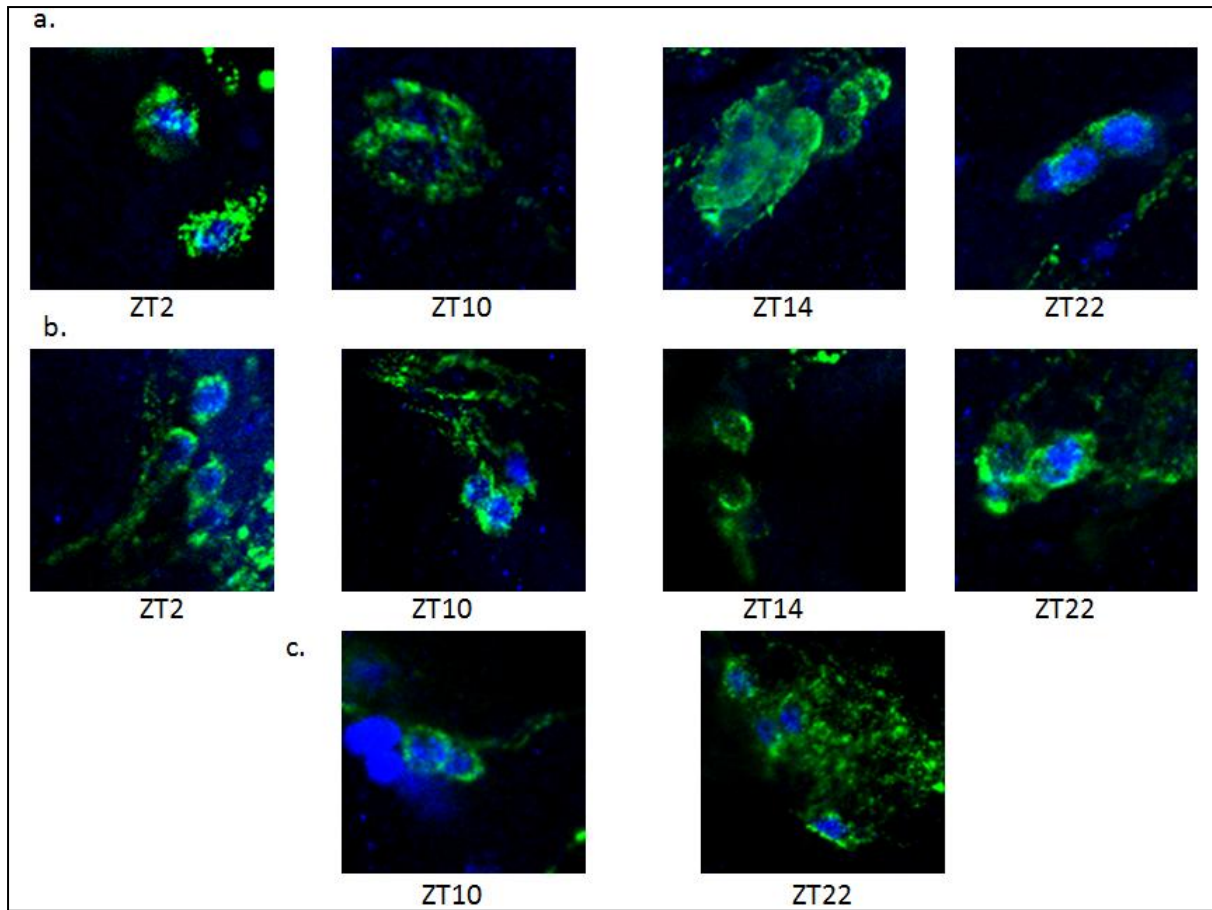


Figure 22. Real images scanned of PER protein in sLNv in response to: a. LD entrainment, b. WF entrainment c. arrhythmic conditions. PER in blue and PDF in green.

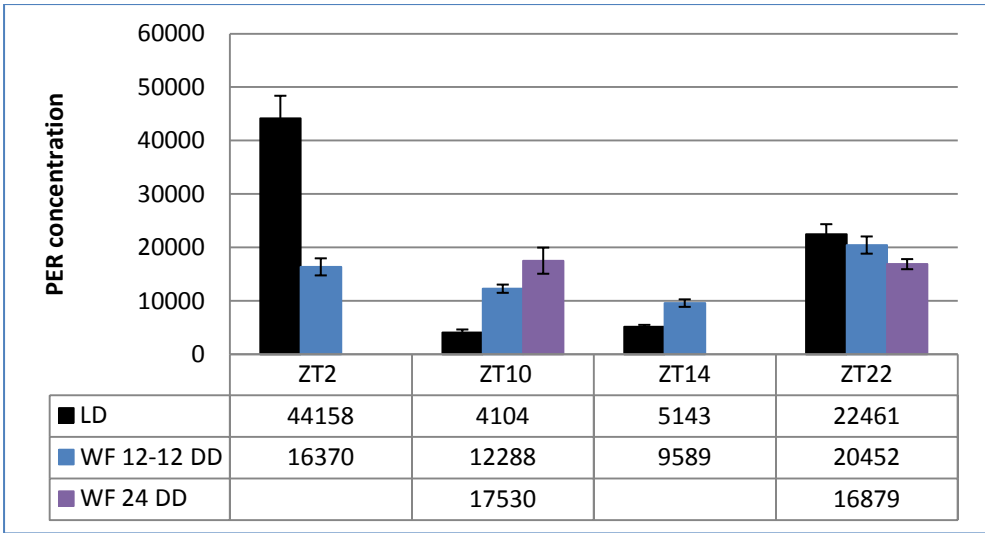


Figure 23. Comparison of total PER concentrations in sLNv between LD, WF and arrhythmic entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 8 Flies/16 hemispheres from two representative experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 24 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; WF 24 DD: 24h of whole food diet in constant darkness; CLK: CLOCK protein; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

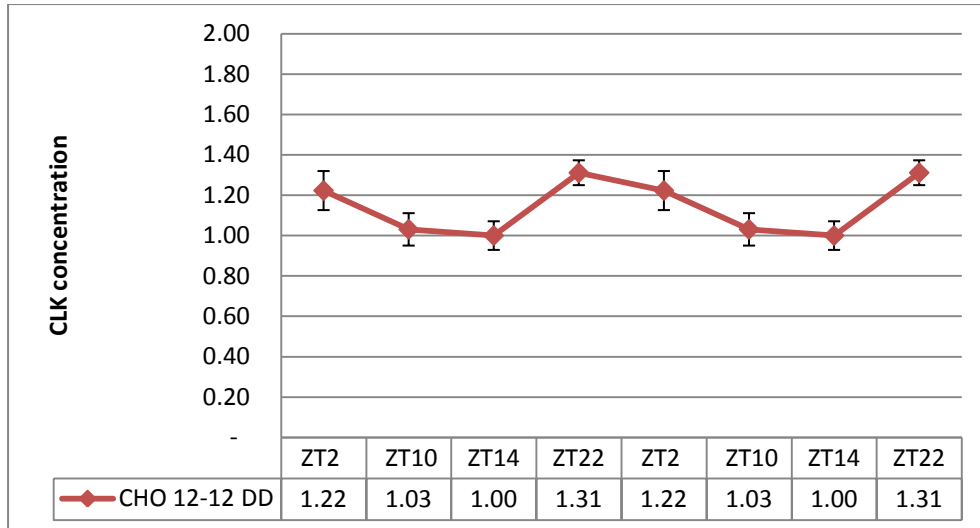


Figure 24. Oscillation of CLK in the ILNv in carbohydrate entrainment.

The expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

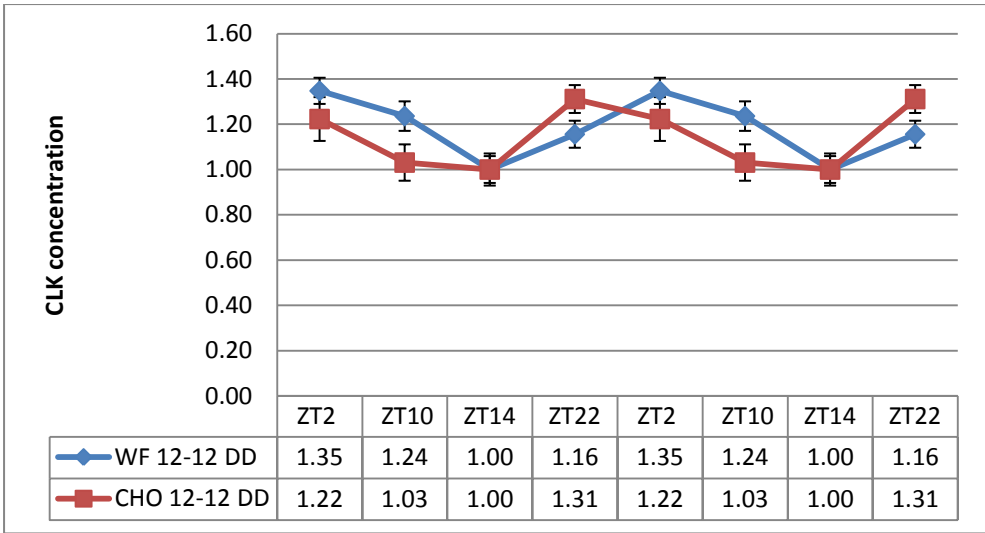


Figure 25. Oscillation of CLK in the ILNv in carbohydrate and WF entrainments.

In both fly-groups the expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

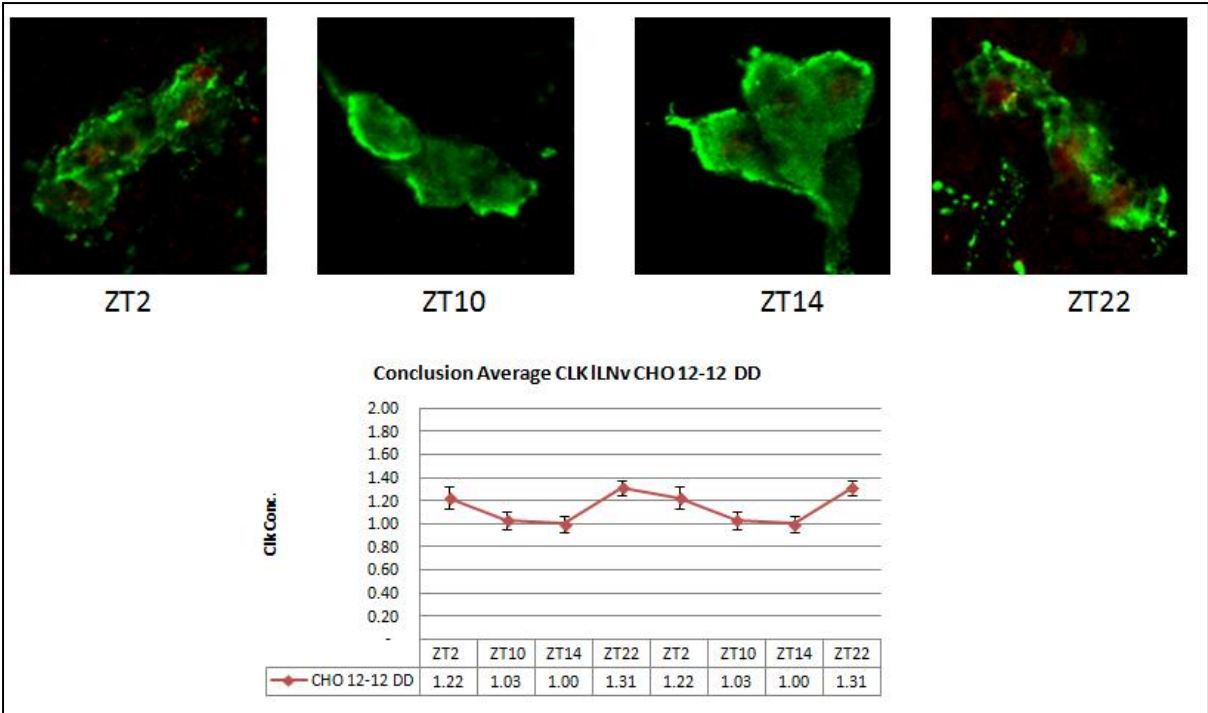


Figure 26. Real images scanned of CLK protein in the ILNv in response to carbohydrate entrainment. CLK in red and PDF in green.

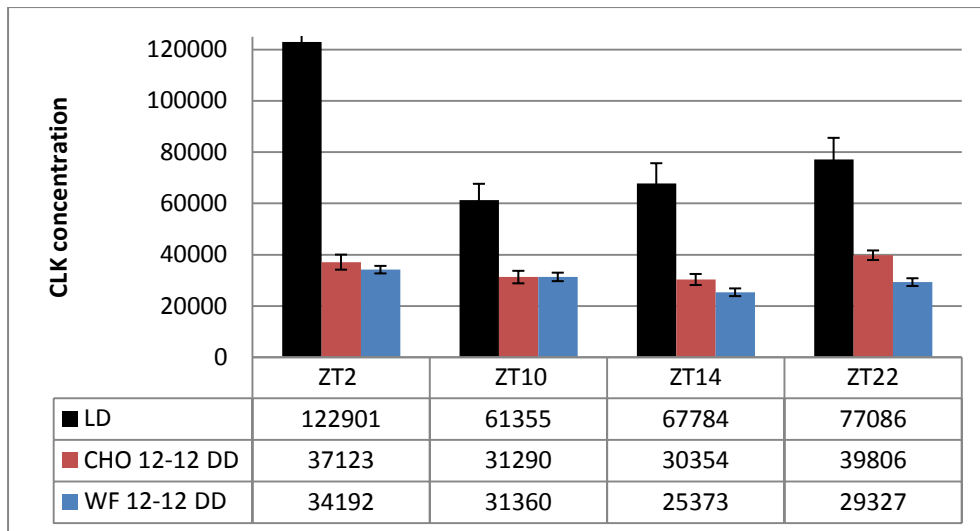


Figure 27. Comparison of total CLK concentrations in ILNv between LD, WF and carbohydrate entrained *w¹¹¹⁸* flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; ; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

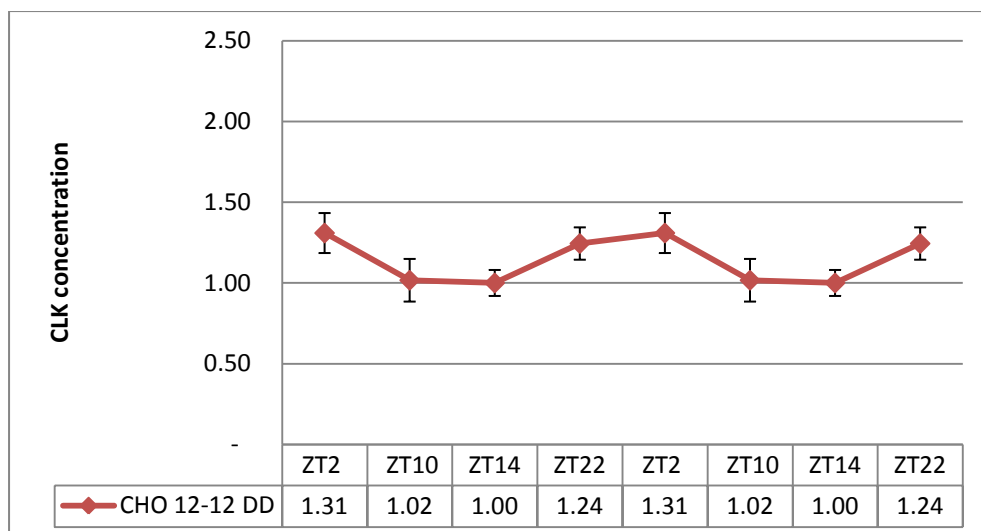


Figure 28. Oscillation of CLK in the sLNv in response to carbohydrates entrainment.

The expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

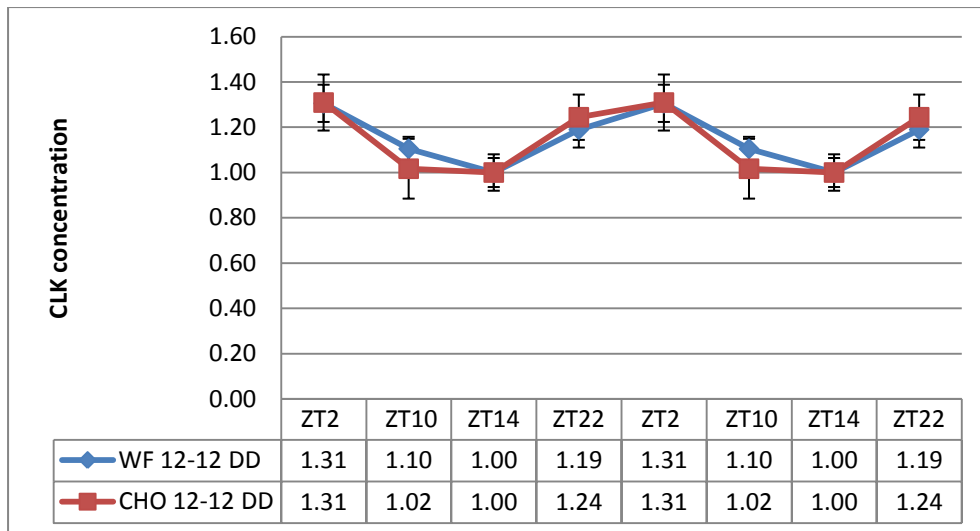


Figure 29. Oscillation of CLK in the sLNv in carbohydrate and WF entrained flies.

In both fly-groups the expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

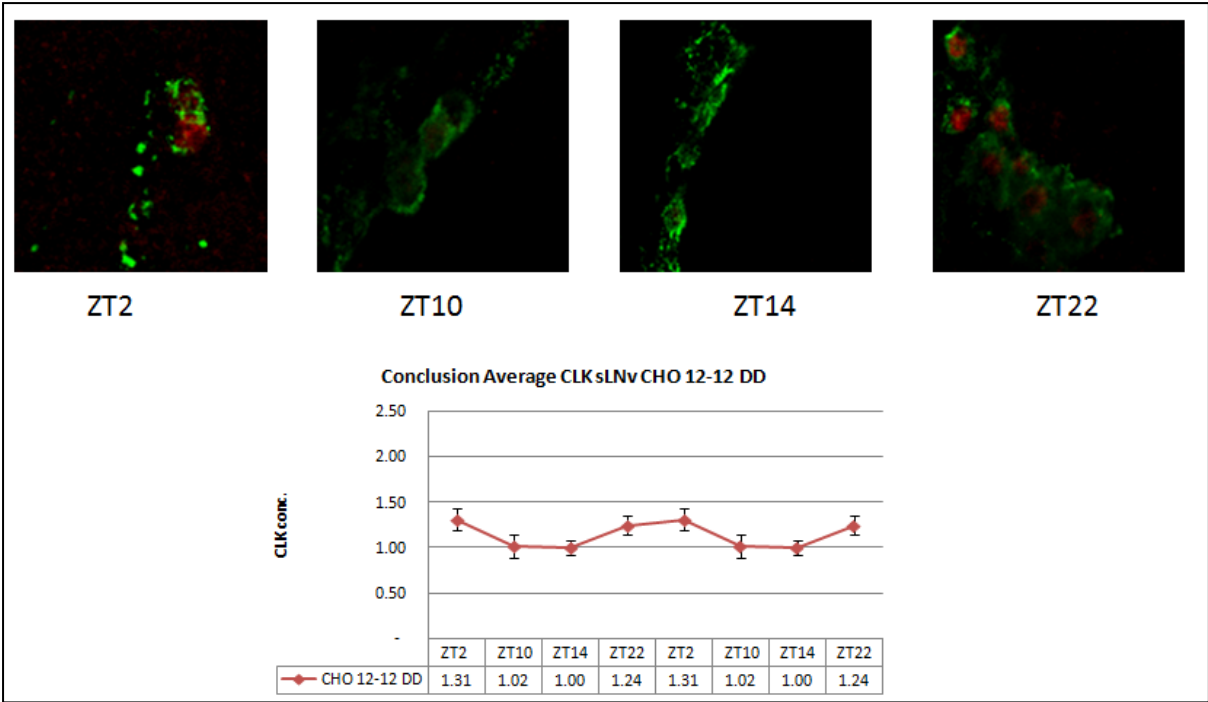


Figure 30. Real images scanned of CLOCK protein in the sLNv in response to carbohydrate entrainment. CLK in red and PDF in green.

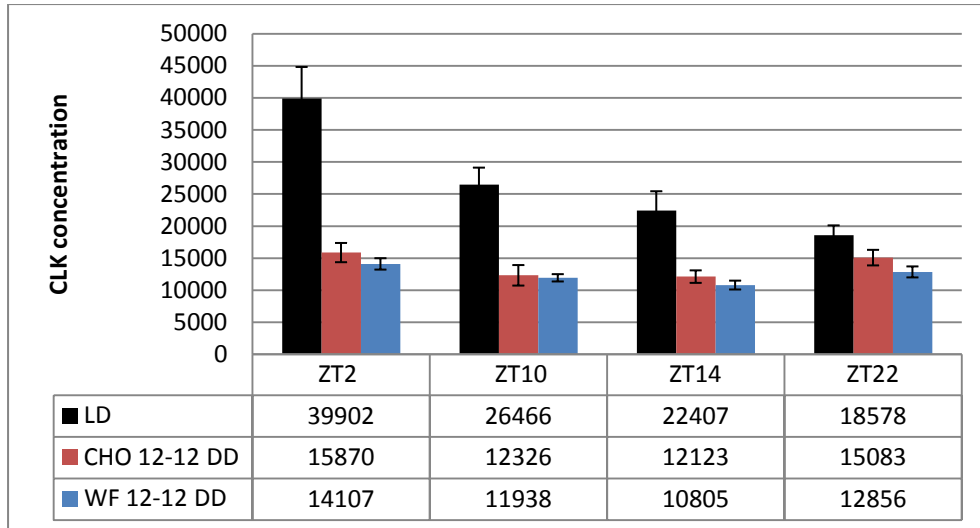


Figure 31. Comparison of total CLK concentrations in sLNv between LD, WF and carbohydrate entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

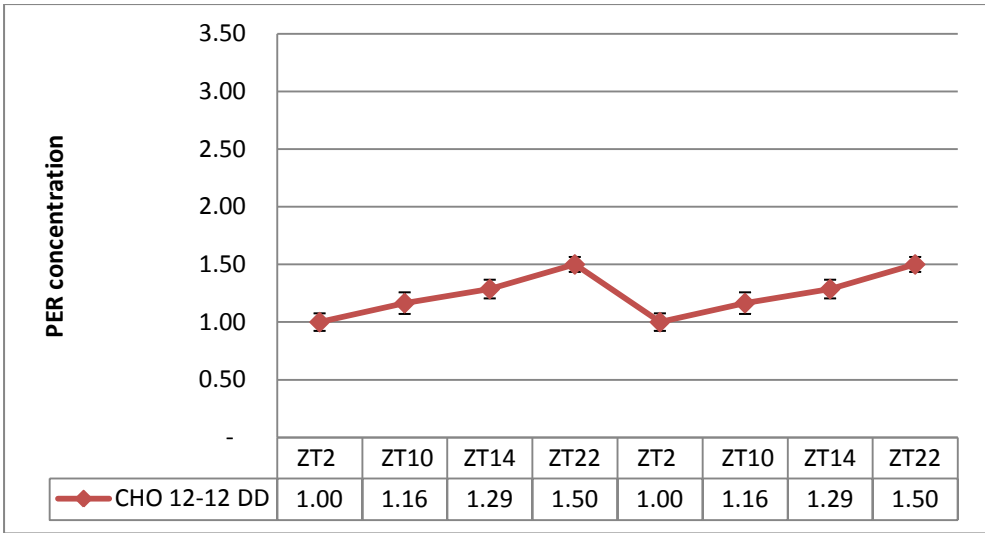


Figure 32. Oscillation of PER in the ILNv in response to exposure to carbohydrate entrainment.

The expression level of PER at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

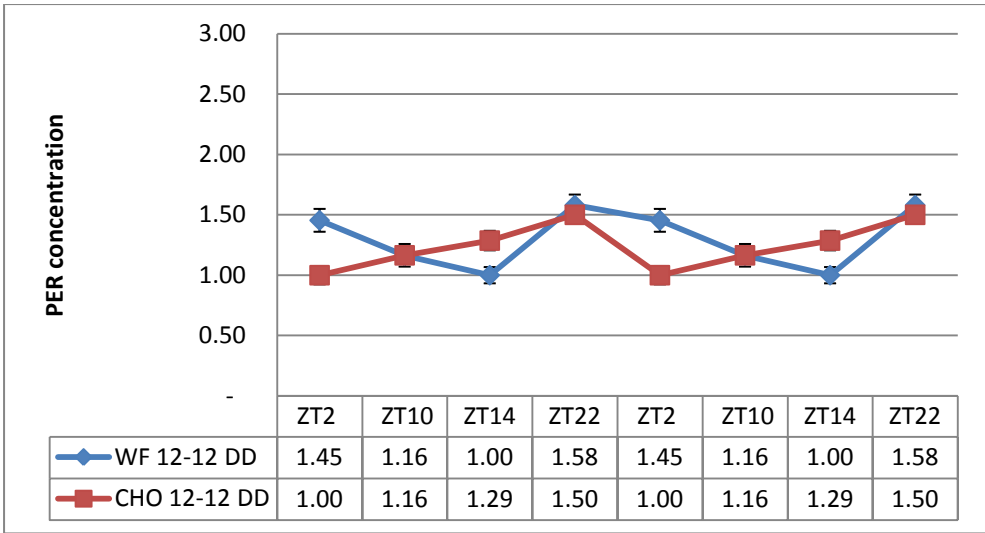


Figure 33. Oscillation of PER in ILNv in carbohydrate entrained flies and WF entrained flies.

In carbohydrates entrained flies, the expression level of PER at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. In whole food entrained flies, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours of in starvation; ILNv: large ventral lateral neurons.

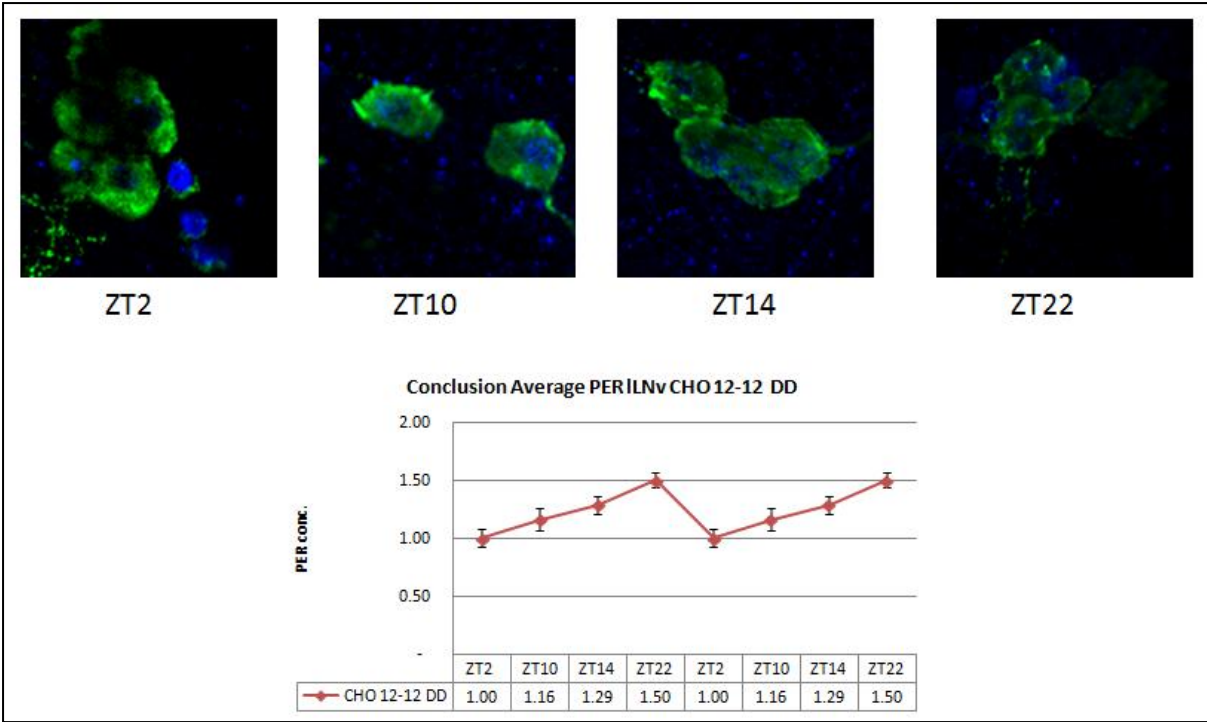


Figure 34. Real images scanned of PER protein in the ILNv in response to exposure to carbohydrate entrainment. PER in blue and PDF in green.

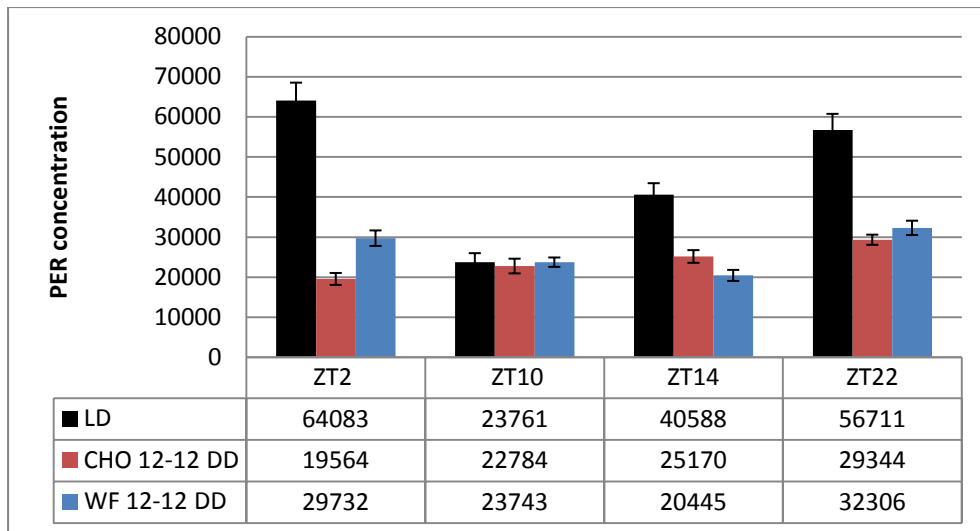


Figure 35. Comparison of total PER concentrations in ILNv between LD, WF and carbohydrate entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

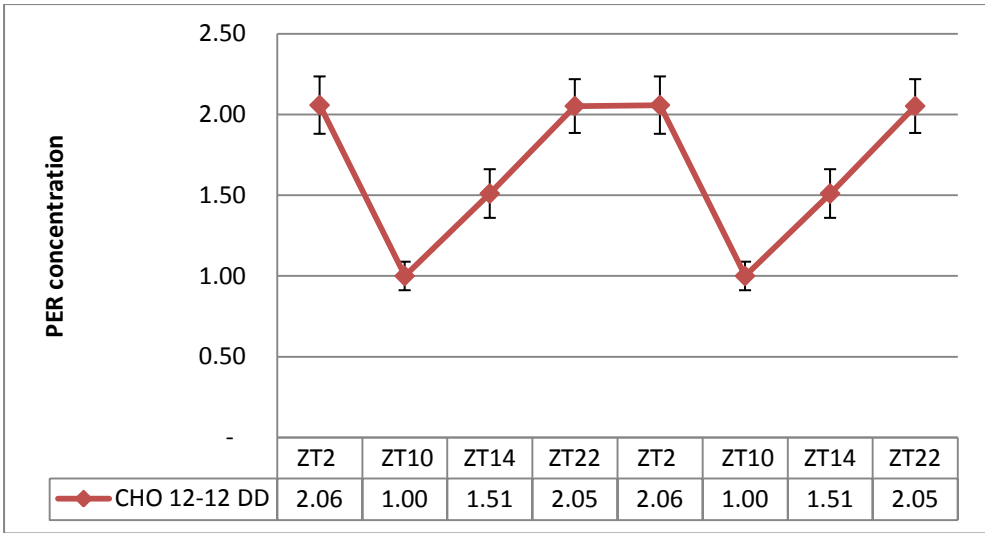


Figure 36. Oscillation of PER in sLNv in response to exposure to carbohydrate entrainment.

The expression level of PER at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

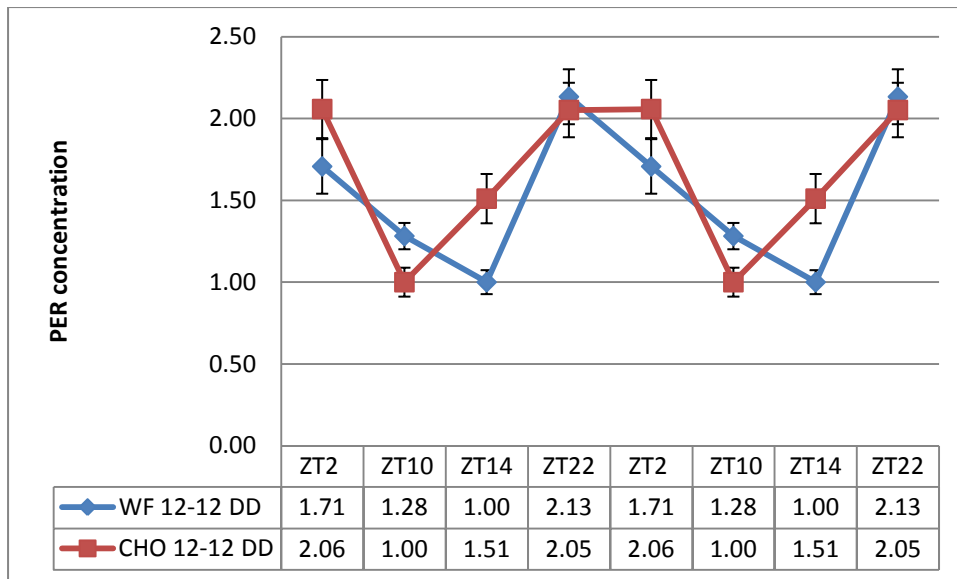


Figure 37. Oscillation of PER in sLNv in carbohydrate and in WF entrained flies.

In carbohydrates entrained flies, the expression level of PER at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In whole food entrained flies, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

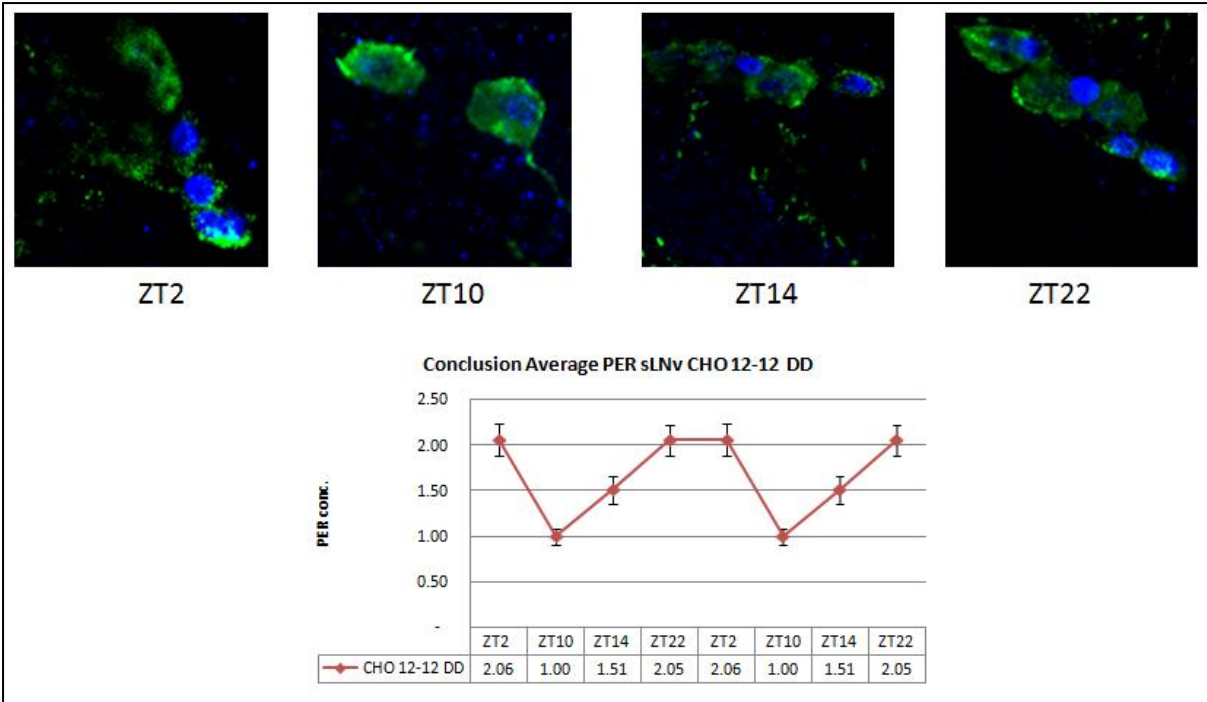


Figure 38. Real images scanned of PER protein in the sLNv in response to carbohydrate entrainment. PER in blue and PDF in green.

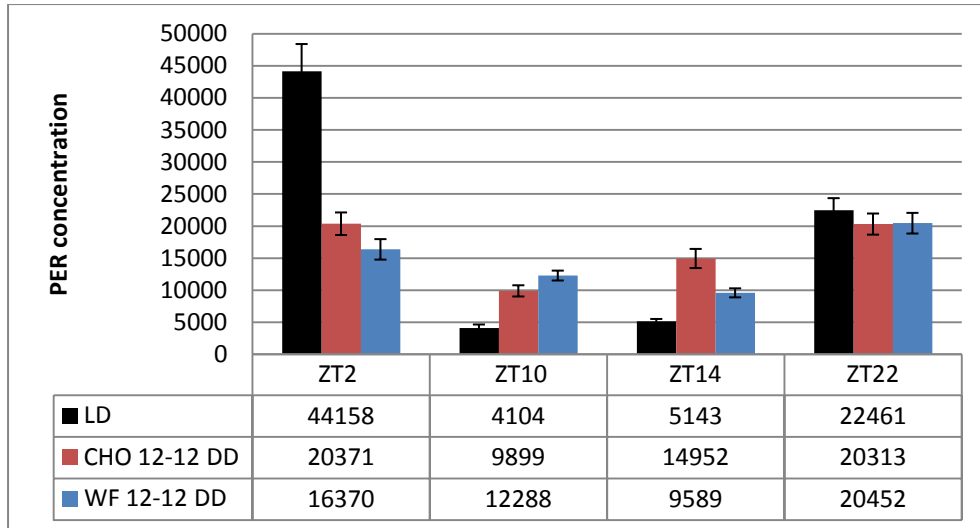


Figure 39. Comparison of total PER concentrations in sLNv between LD, WF and carbohydrate entrained w1118 flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. WF 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; WF 12-12 DD: 12h of whole food diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

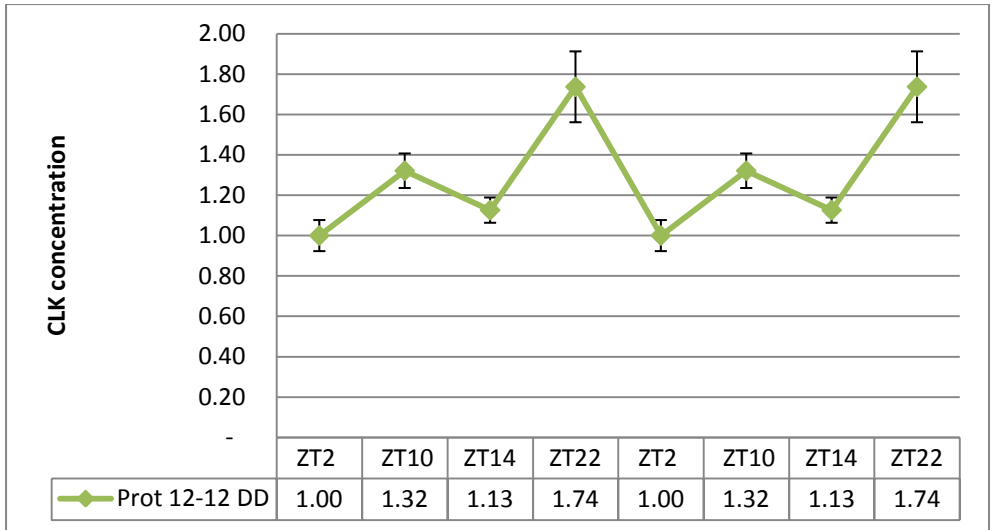


Figure 40. Oscillation of CLK in ILNv in response to exposure to protein entrainment.

The expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are an average of at least 8 flies/16 hemispheres from 2 experiments.

Abbreviations: Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

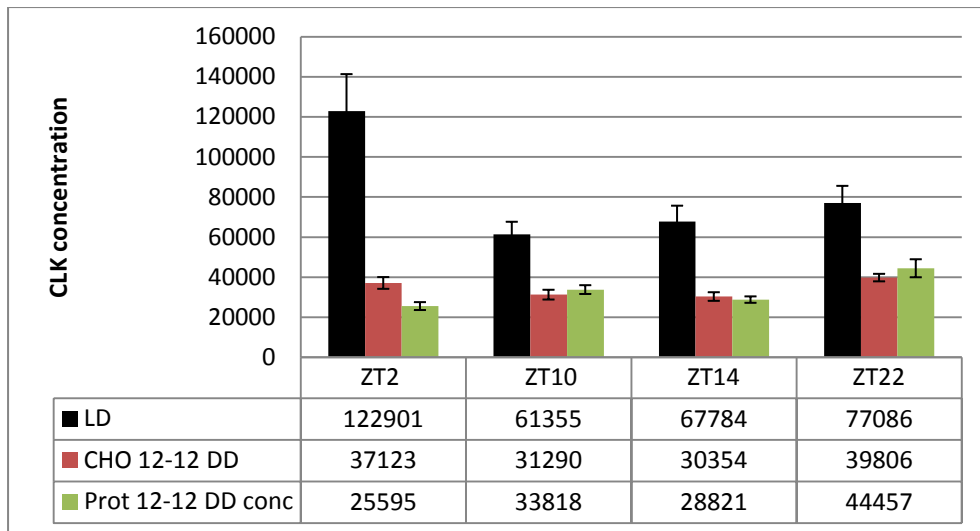


Figure 41. Comparison of total CLK concentrations in ILNv between LD, Carbohydrate and protein entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Prot 12-12 DD data shown at each time point are an average of at least 8 flies/16 hemispheres from 2 experiments.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

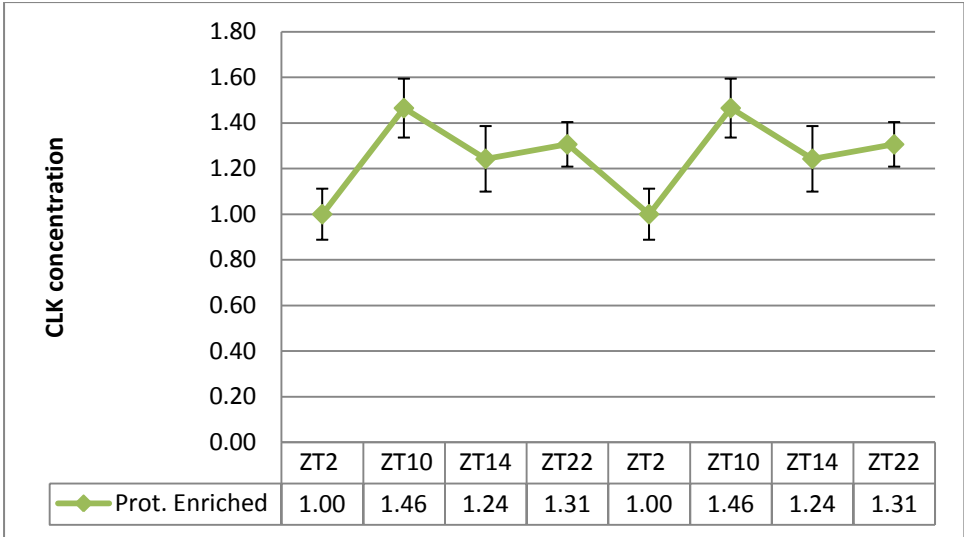


Figure 42. Oscillation of CLK in sLNv in response to exposure to protein entrainment.

The expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

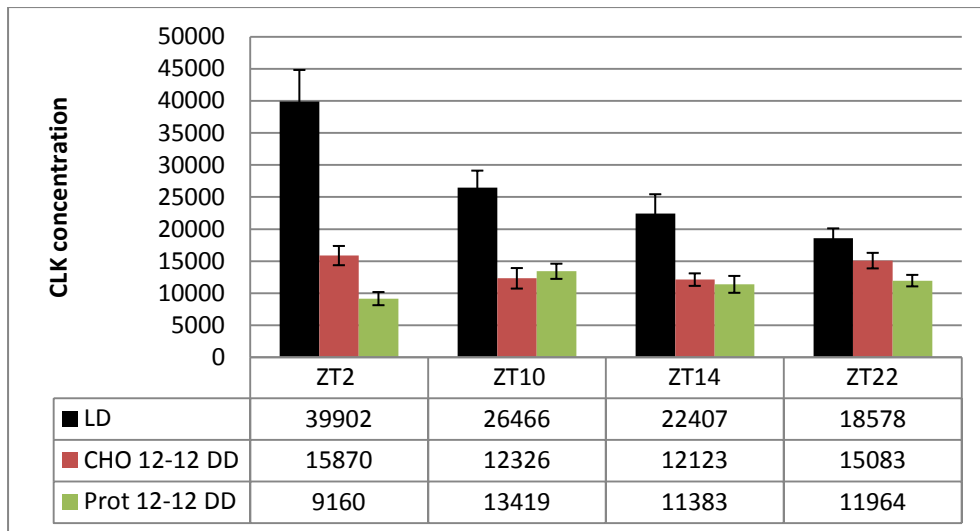


Figure 43. Comparison of total CLK concentrations in sLNv between LD, Carbohydrate, and protein entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Prot 12-12 DD data shown at each time point are an average of at least 4 flies/16 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

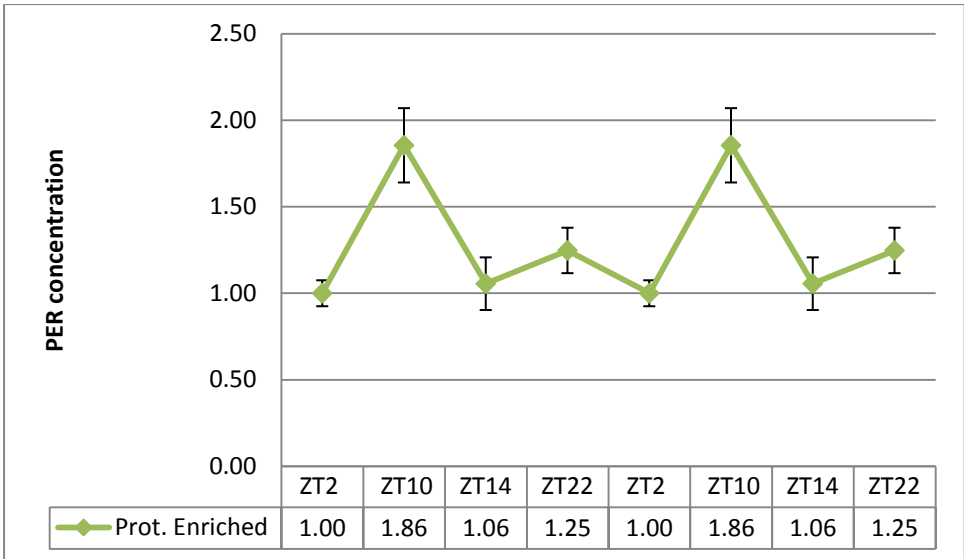


Figure 44. Oscillation of PER in ILNv in response to exposure to protein entrainment.

The expression level of PER at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment out of 2 independent experiments with similar results.

Abbreviations: Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

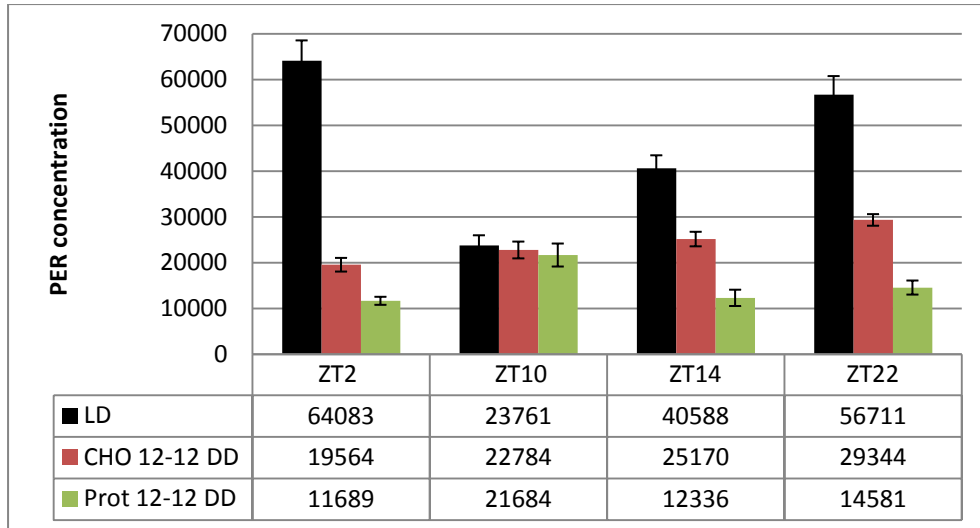


Figure 45. Comparison of total PER concentrations in ILNv between LD, Carbohydrate and protein entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Prot 12-12 DD data shown at each time point are an average of at least 4 flies/16 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

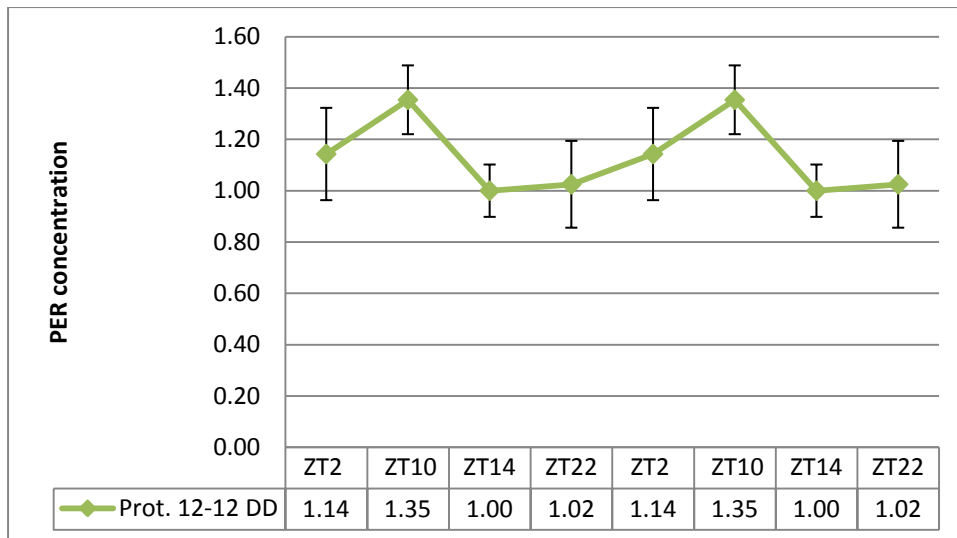


Figure 46. Oscillation of PER in the sLNv in response to exposure to protein entrainment.

The expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment out of 2 independent experiments with similar results.

Abbreviations: Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

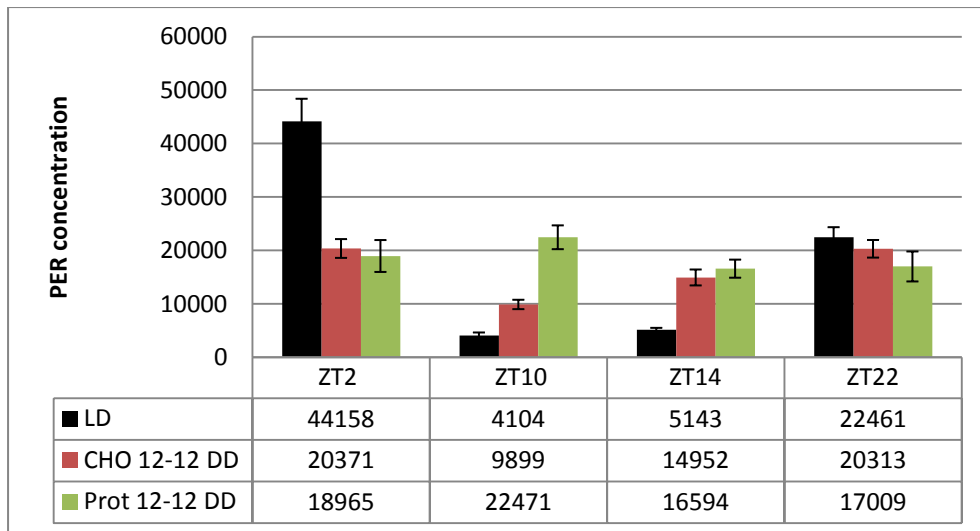


Figure 47. Comparison of total PER concentrations in sLNv between LD, carbohydrate and protein entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Prot 12-12 DD data shown at each time point are an average of at least 4 flies/16 hemispheres from one representative experiment out of 2 independent experiments with similar results.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Prot. 12-12 DD: 12h of protein diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

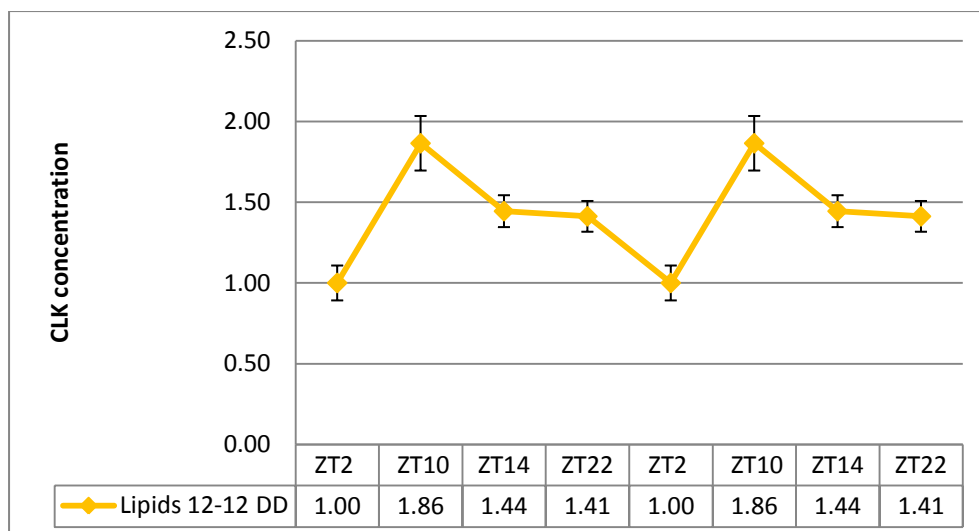


Figure 48. Oscillation of CLK in the ILNv in response to exposure to lipid entrainment.

The expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.



Figure 49. Oscillation of CLK expression in ILNv in lipid and carbohydrate entrained flies.

Abbreviations: Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours of in starvation; ILNv: large ventral lateral neurons.

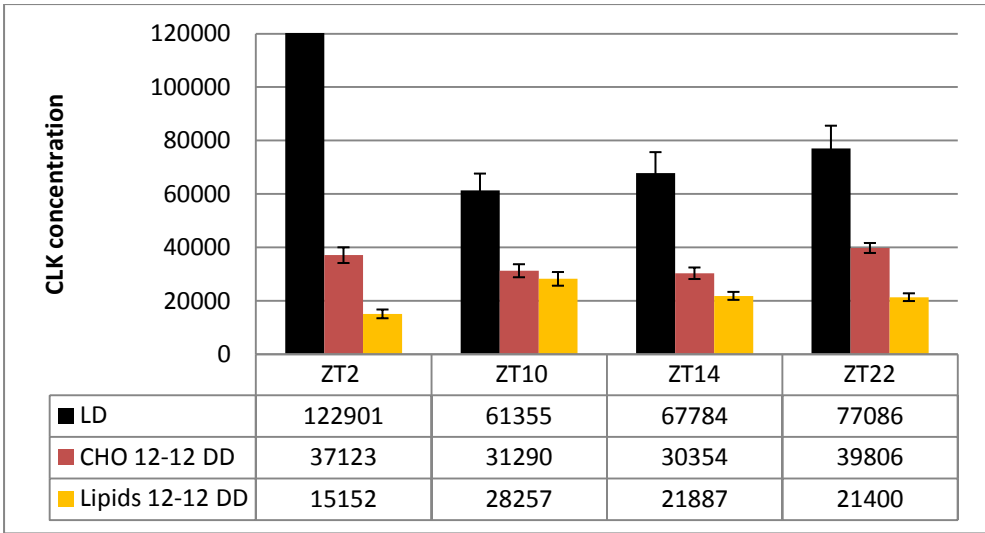


Figure 50. Comparison of total CLK concentrations in ILNv between LD, carbohydrate, and lipids entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Lipid 12-12 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

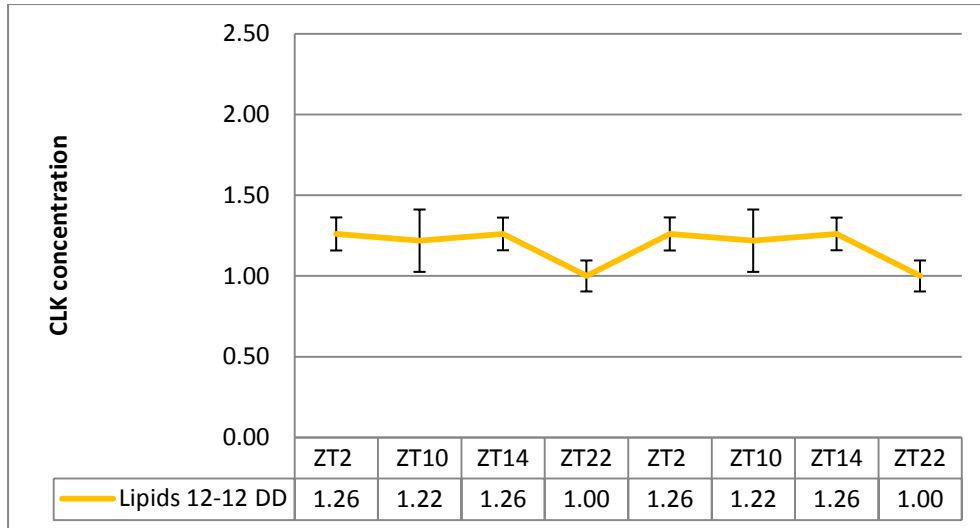


Figure 51. Oscillation of CLK in sLNv in response to lipid entrainment.

The expression level of CLK at ZT 22 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

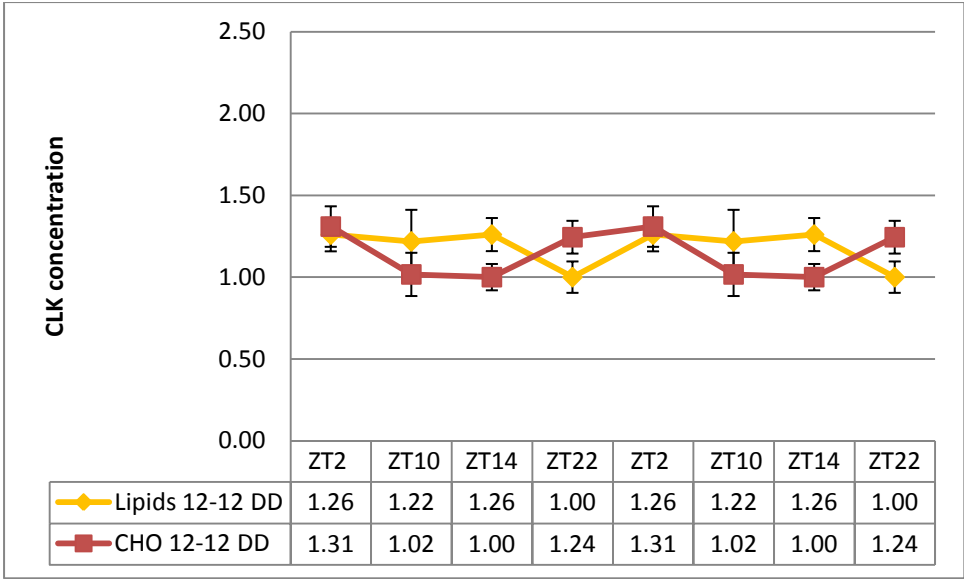


Figure 52. Oscillation of CLK sLNv in lipid and carbohydrate entrained flies.

Abbreviations: Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

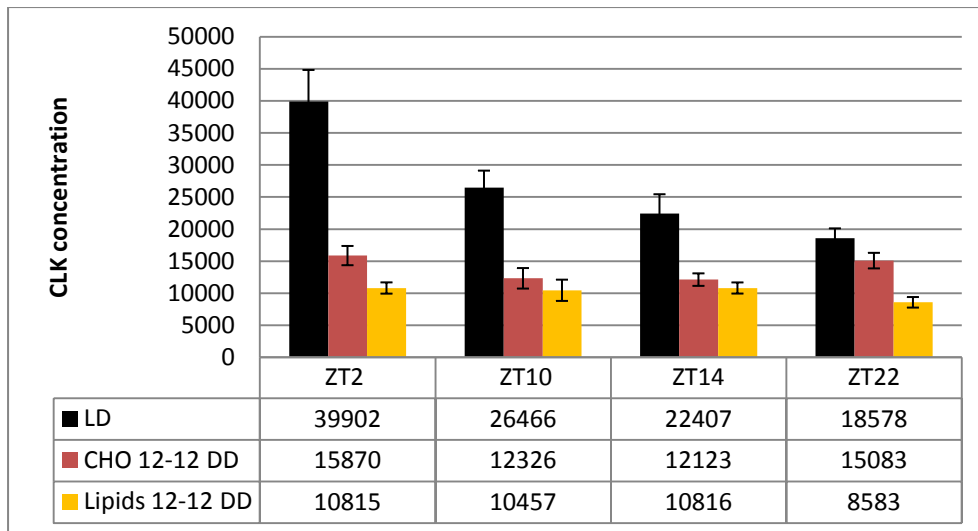


Figure 53. Comparison of total CLK concentrations in sLNv between LD, carbohydrate and lipids entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Lipid 12-12 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

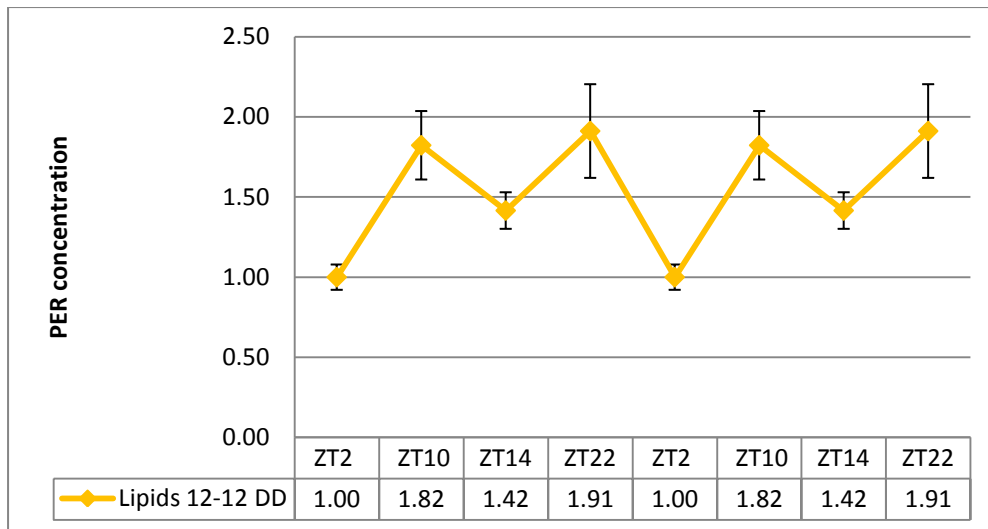


Figure 54. Oscillation of PER in the ILNv neurons in response to exposure to lipid entrainment.

The expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: Lipids12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; ILNv: large ventral lateral neurons.

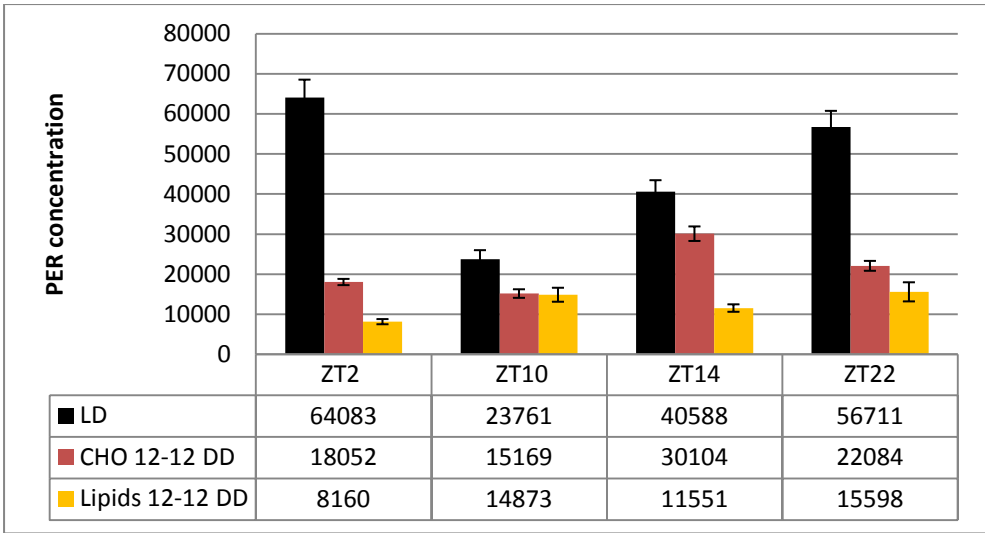


Figure 55. Comparison of total PER concentrations in ILNv between LD, carbohydrate and lipids entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Lipid 12-12 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

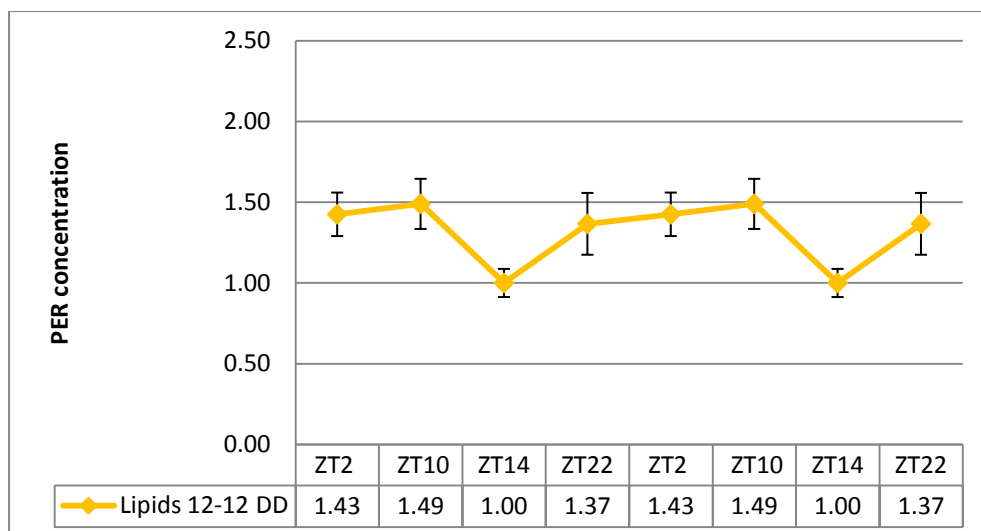


Figure 56. Oscillation of PER in sLNv in response to exposure to lipid entrainment.

The expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: Lipids12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

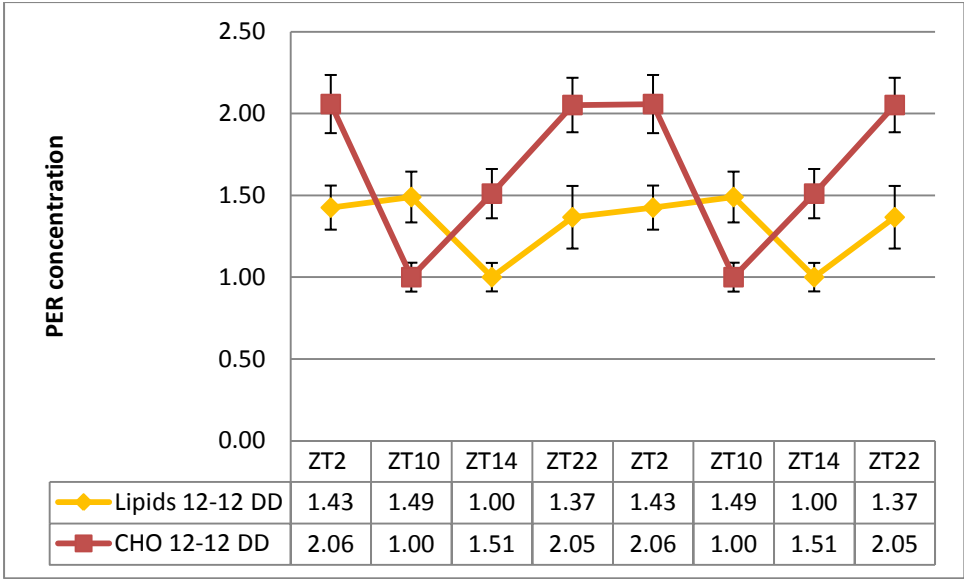


Figure 57. PER expression in sLNv in lipid entrained flies and carbohydrate entrained flies.

Abbreviations: Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in food; ZT14: 2 hours in starvation; sLNv: small ventral lateral neurons.

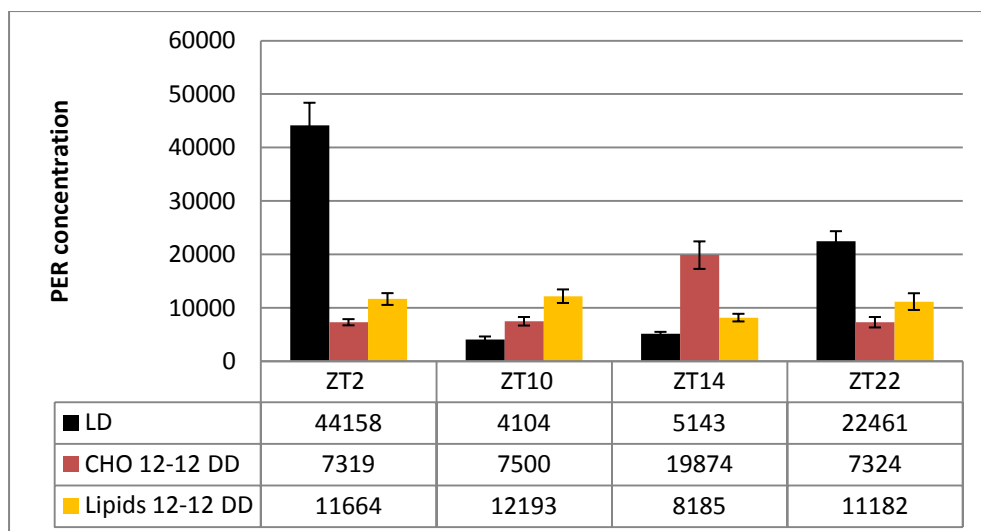


Figure 58. Comparison of total PER concentrations in sLNv between LD, carbohydrate and lipids entrained flies.

WF 24-LD data shown at each time point are the average of at least 4 Flies/8 hemispheres from one representative experiment out of 2 independent experiments with similar results. CHO 12-12 DD data shown at each time point are an average of at least 16 flies/32 hemispheres from 4 experiments. Lipid 12-12 DD data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: LD: 12h light followed by 12h dark with constant whole food diet; CHO 12-12 DD: 12h of carbohydrates diet followed by 12h of starvation in constant darkness; Lipids 12-12 DD: 12h of lipids diet followed by 12h of starvation in constant darkness; PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

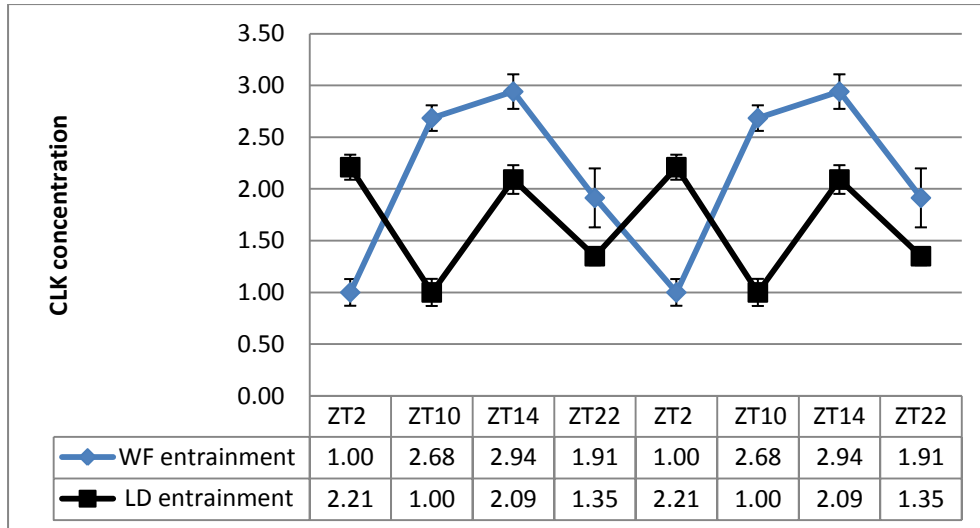


Figure 59. Oscillation of CLK in the ILNv of LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

In LD entrained *pdf⁰* flies, the expression level of CLK at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In WF entrained *pdf⁰* flies, the expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; ILNv: large ventral lateral neurons.

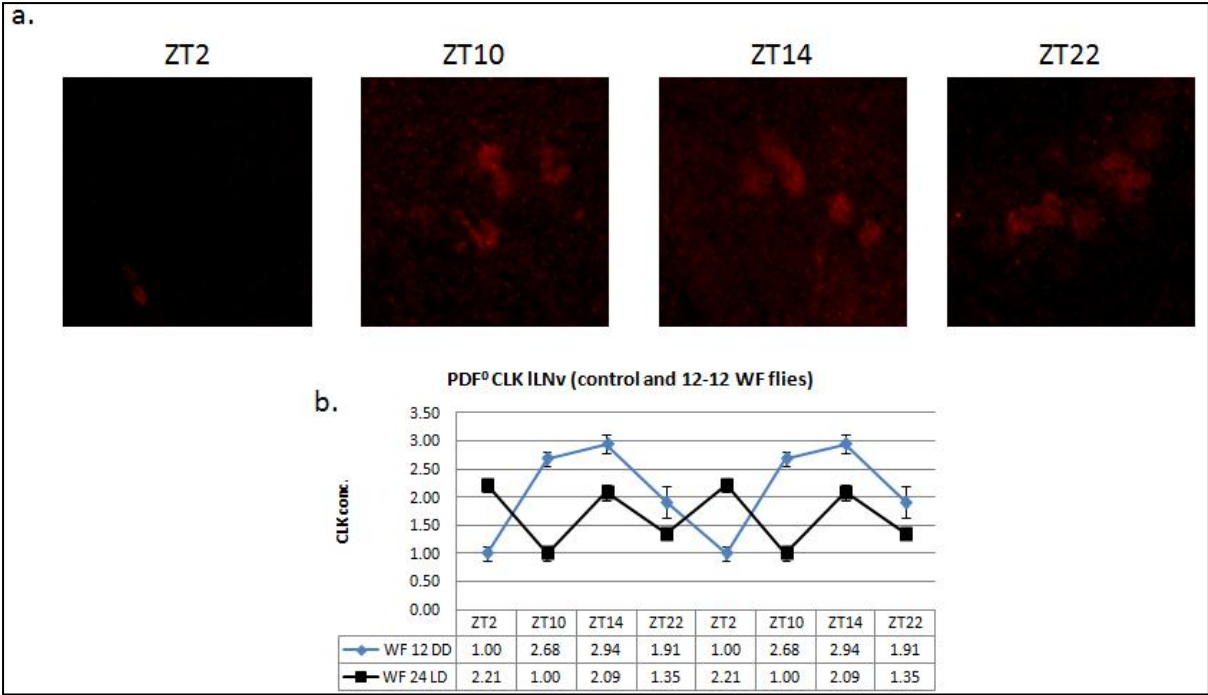


Figure 60. a. Real images scanned of CLK in the ILNv of WF entrained *pdf⁰* flies **b.** the graph representing the oscillation of CLK in ILNv in WF entrained *pdf⁰* flies and LD entrained *pdf⁰* flies. CLK in red.

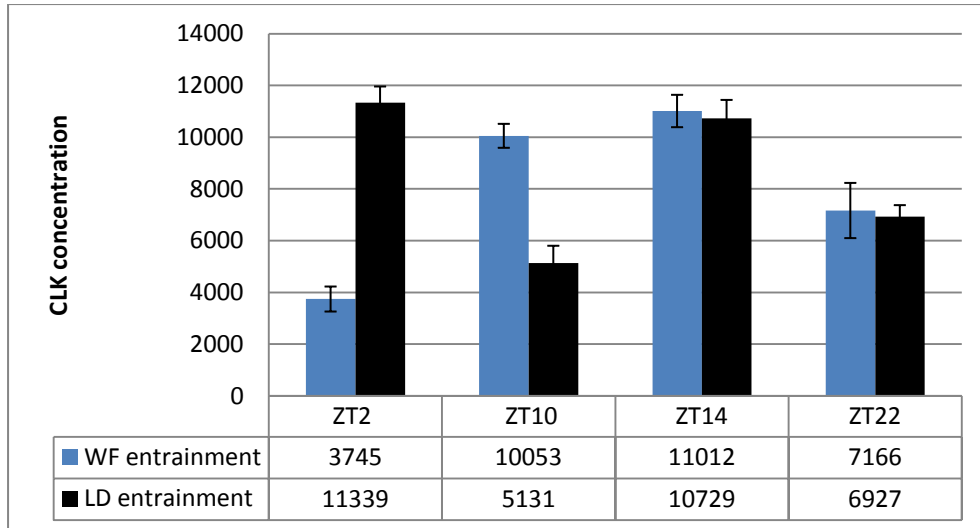


Figure 61. Comparison of total CLK concentrations in ILNv LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; ILNv: large ventral lateral neurons.

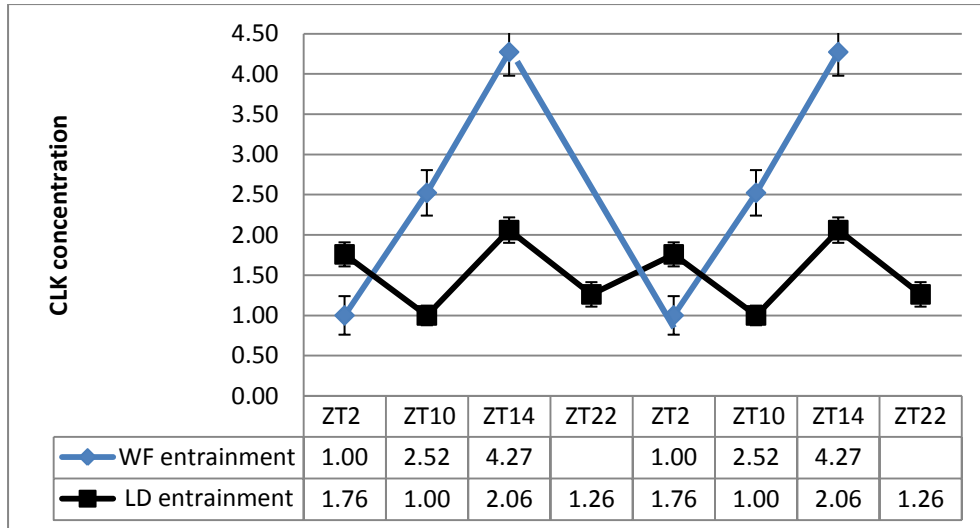


Figure 62. Oscillation of CLK in the sLNv of LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

In LD entrained *pdf⁰* flies, the expression level of CLK at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In WF entrained *pdf⁰* flies, the expression level of CLK at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; sLNv: small ventral lateral neurons.

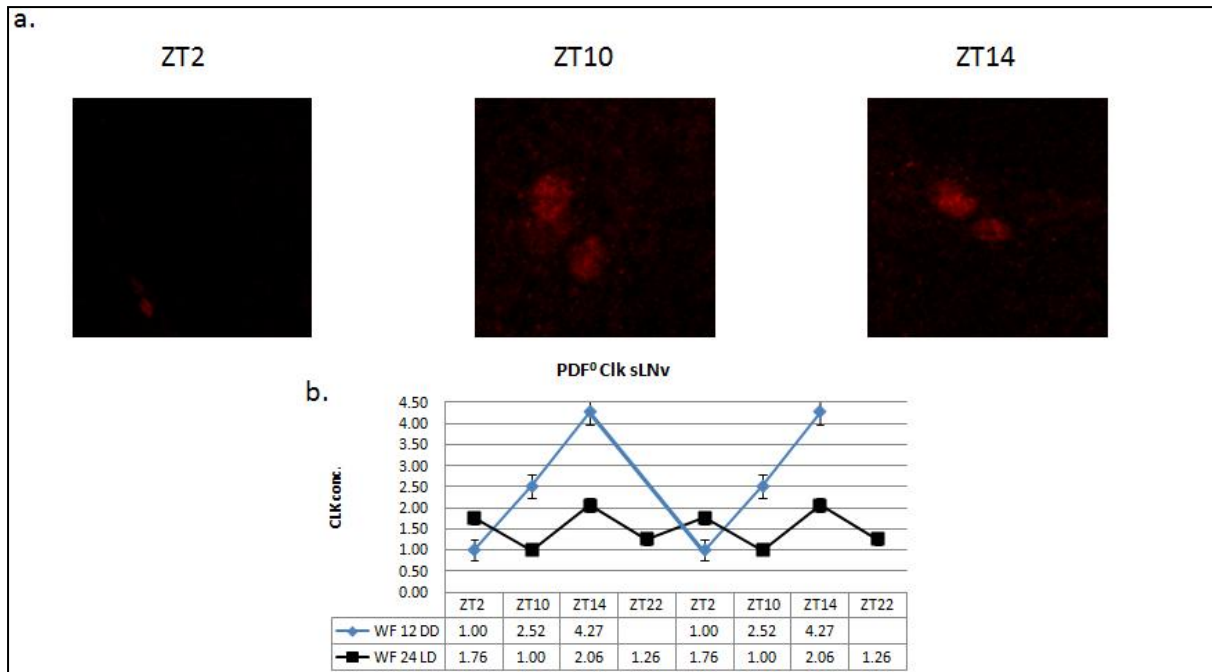


Figure 63. a. Real images scanned of CLK in sLNv of WF entrained *pdf⁰* flies **b.** the graph representing the oscillation of CLK in sLNv in WF entrained *pdf⁰* flies and LD entrained *pdf⁰* flies. CLK in red.

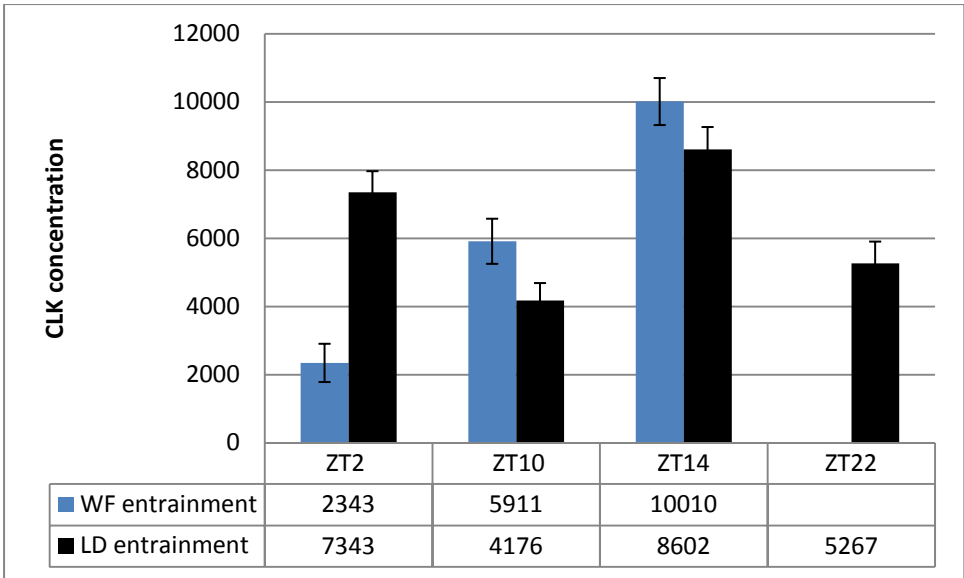


Figure 64. Comparison of total CLK concentrations in sLNv between LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; CLK: CLOCK protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; sLNv: small ventral lateral neurons.

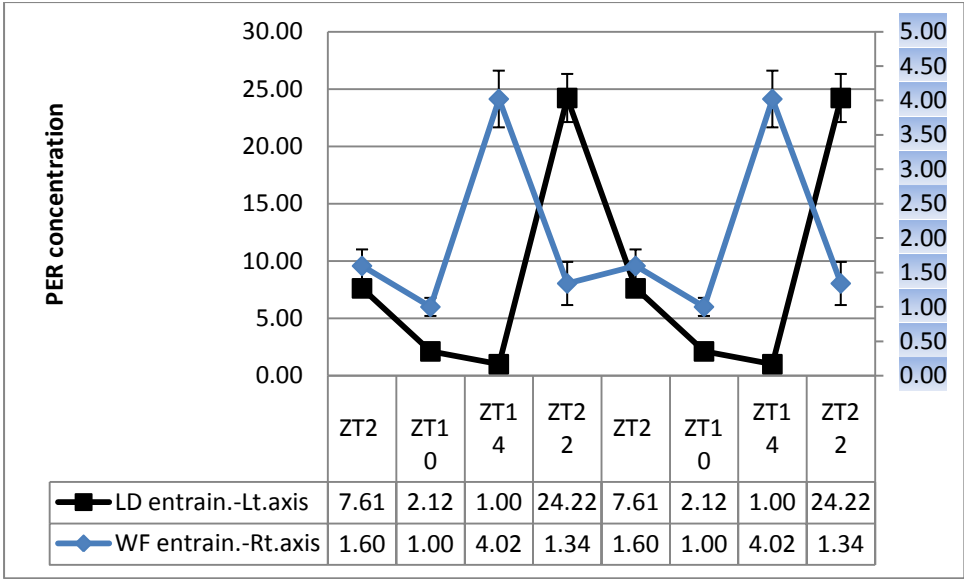


Figure 65. Oscillation of PER in the ILNv of LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

In LD entrained *pdf⁰* flies, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. In WF entrained *pdf⁰* flies, the expression level of PER at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment. The Rt axis is for PER expression in WF entrainment and the Lt axis is for PER expression level in LD entrainment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; ILNv: large ventral lateral neurons.

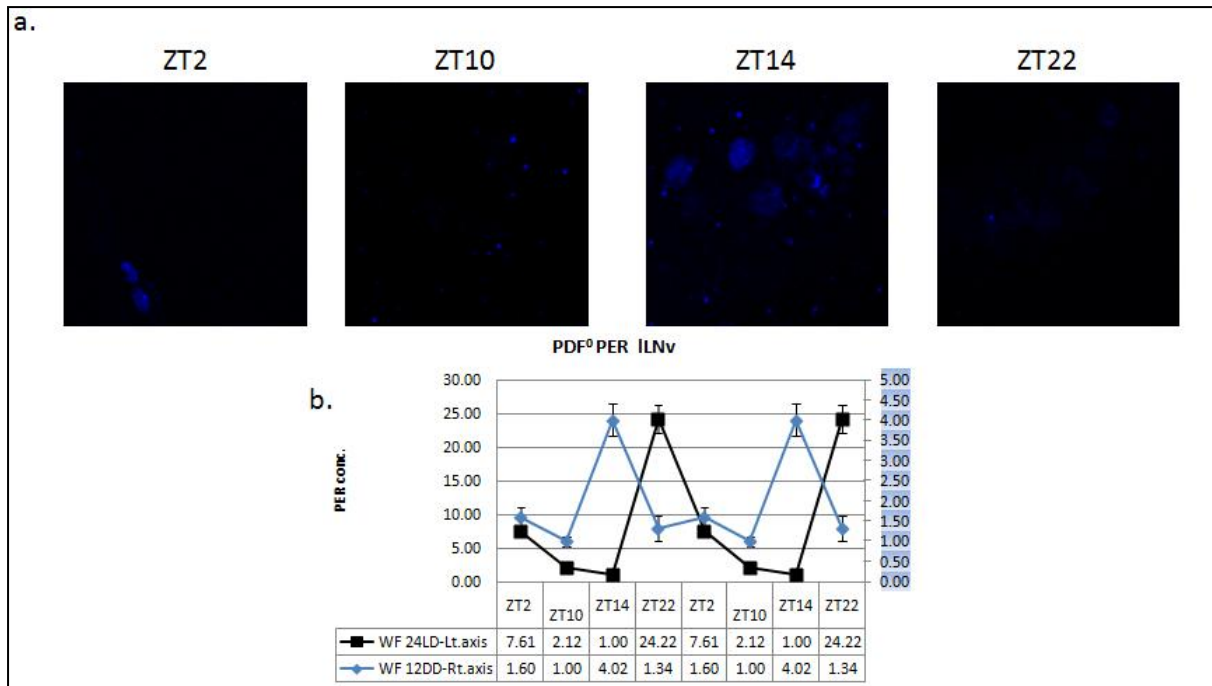


Figure 66. a. Real images scanned of PER ILNv of WF entrained *pdf⁰* flies **b.** the graph representing the oscillation of CLK in ILNv in WF entrained *pdf⁰* flies and LD entrained *pdf⁰* flies. PER in blue.

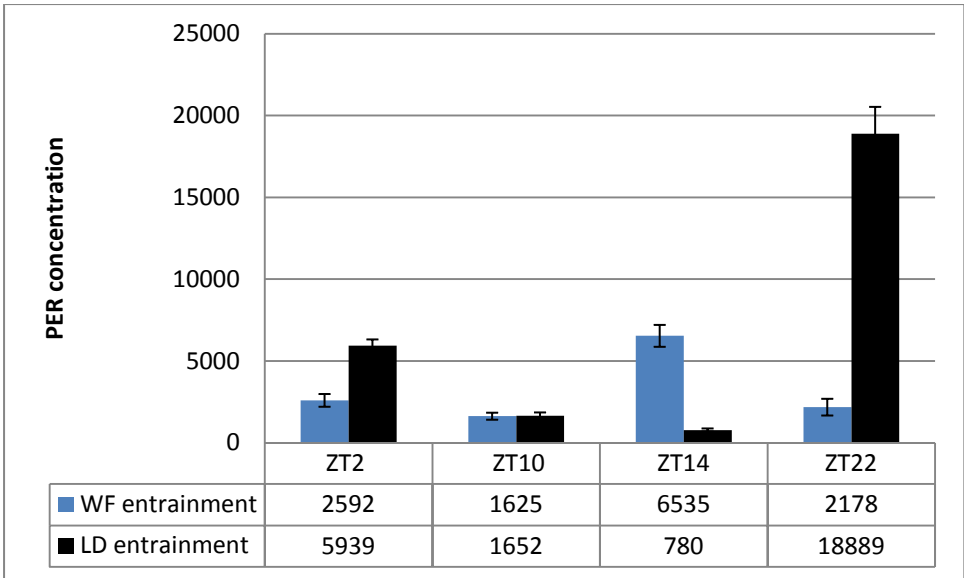


Figure 67. Comparison of total PER concentrations in ILNv between LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; ILNv: large ventral lateral neurons.

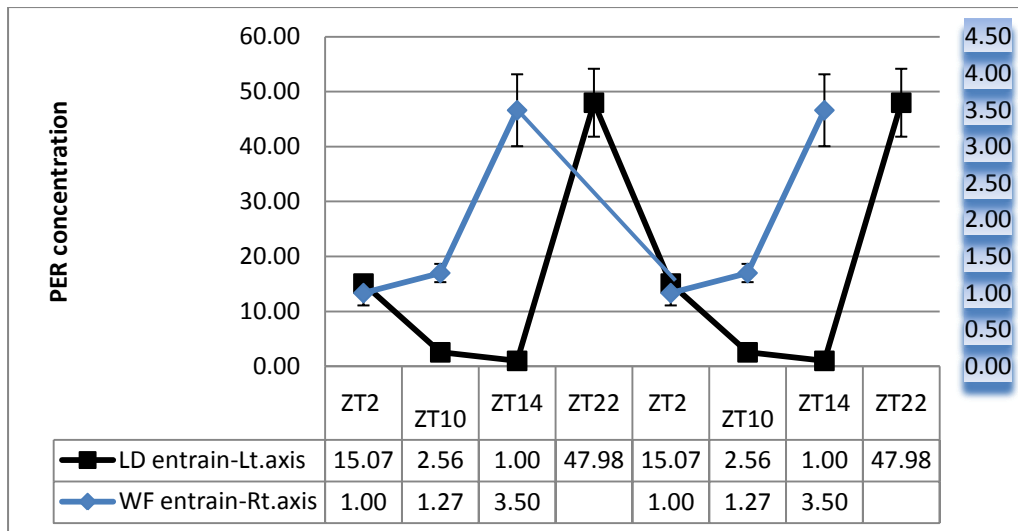


Figure 68. Oscillation of PER protein in the sLNv of LD entrained *pdf*⁰ flies and WF entrained *pdf*⁰ flies.

In LD entrained *pdf*⁰ flies, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. In WF entrained *pdf*⁰ flies, the expression level of PER at ZT 2 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment. The Rt axis is for PER expression in WF entrainment and the Lt axis is for PER expression level in LD entrainment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; sLNv: small ventral lateral neurons.

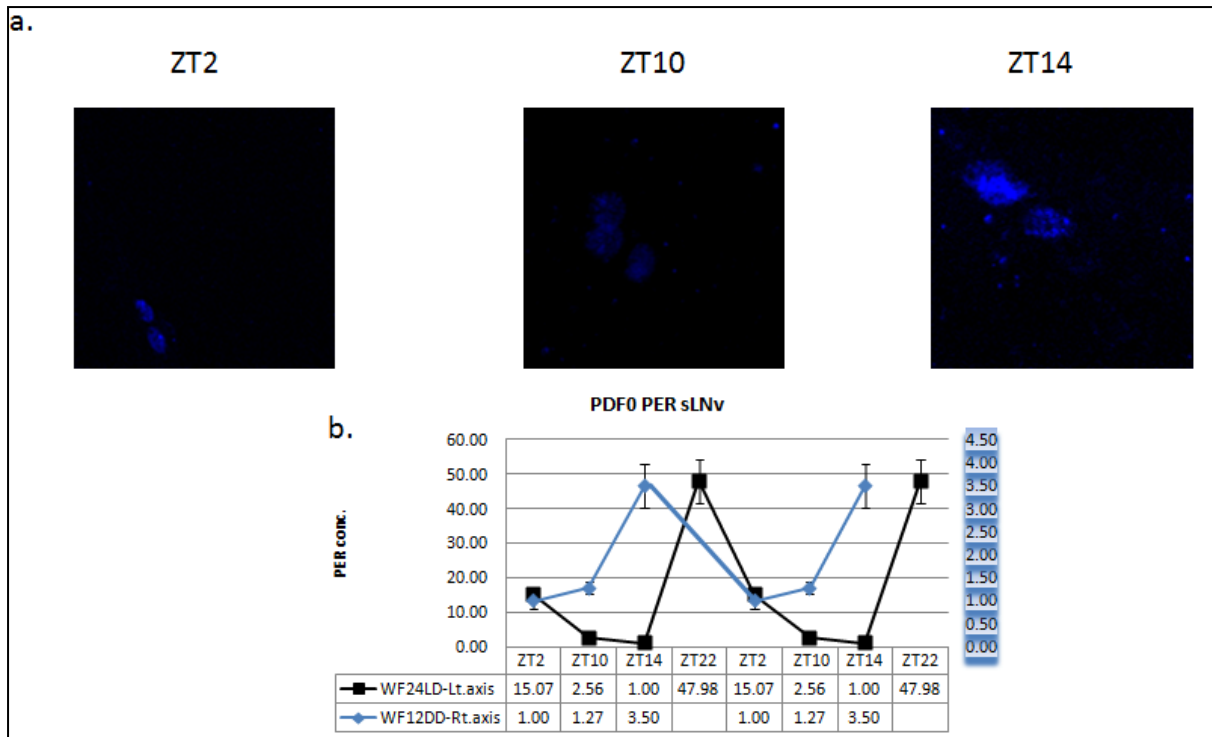


Figure 69. a. Real images scanned of PER in the sLNv WF entrained *pdf⁰* **b.** the graph representing the oscillation of CLK in ILNv in WF entrained *pdf⁰* flies and LD entrained *pdf⁰* flies. PER in blue.

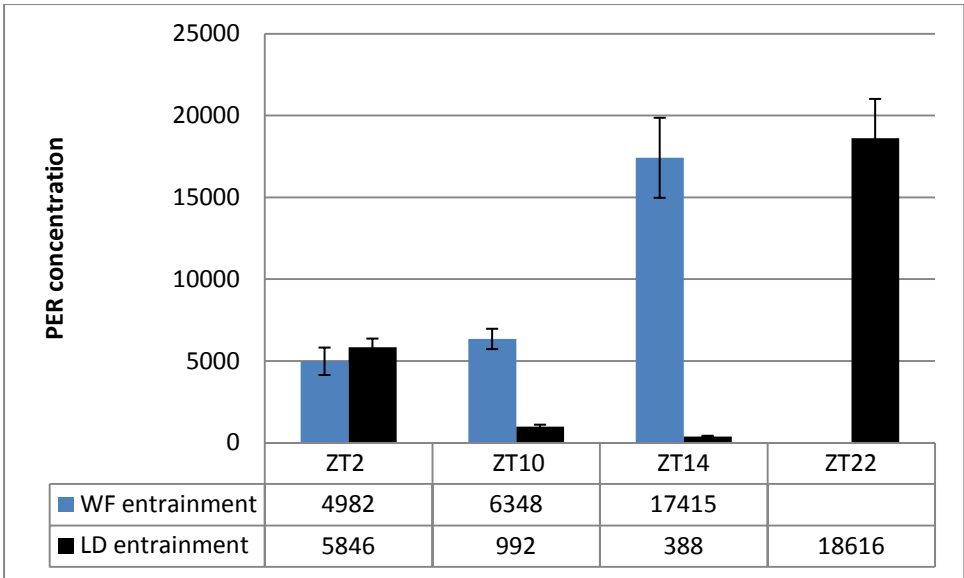


Figure 70. Comparison of total PER protein concentrations in sLNv between LD entrained *pdf⁰* flies and WF entrained *pdf⁰* flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF entrainment: 12h of whole food diet followed by 12h of starvation in constant darkness; LD entrainment: 12h light followed by 12h dark with constant whole food diet; PER: PERIOD protein; ZT: Zeitgeber Time; ZT2: 2 hours in light or food; ZT14: 2 hours in dark or starvation; sLNv: small ventral lateral neurons.

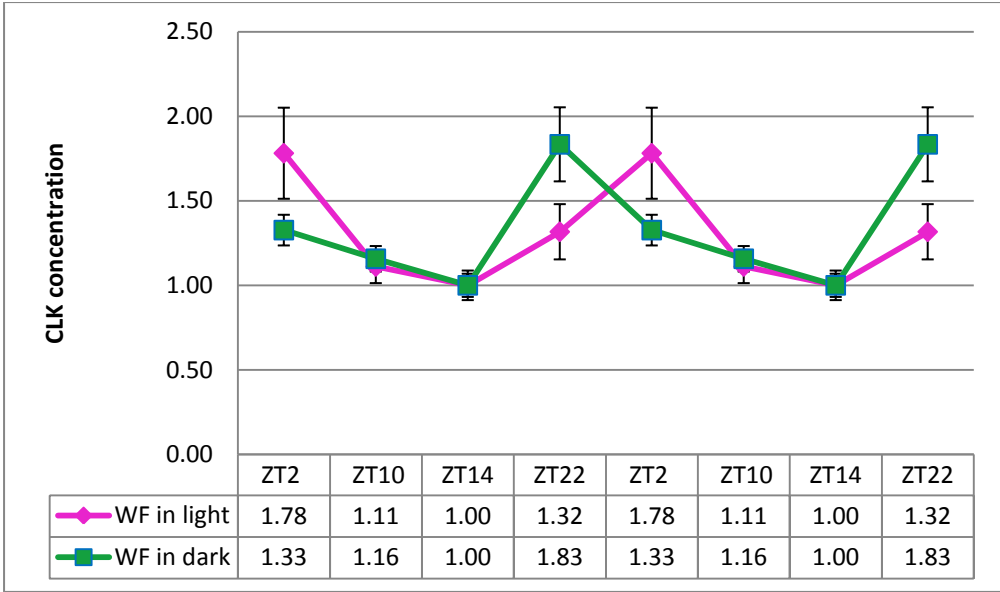


Figure 71. Oscillation of CLK in the ILNv of WF in light and WF in dark entrained flies.

In both entrainments, the expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. CLK: CLOCK protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

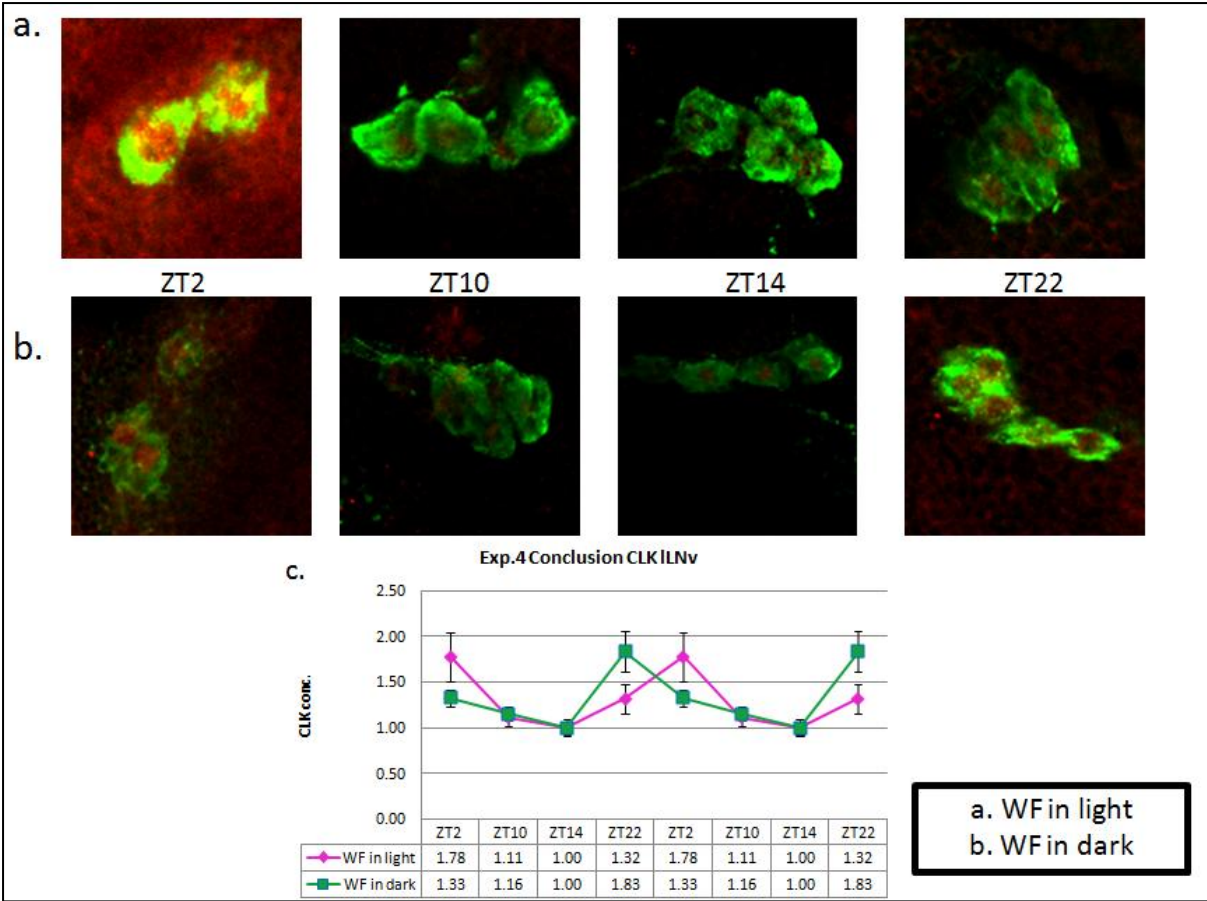


Figure 72. Real images scanned of CLK in the ILNv of **a.** WF in light *w1118* flies, **b.** WF in dark *w1118* flies **c.** graph representing Molecular oscillation of CLK in the ILNv in both entrainments. CLK in red and PDF in green.

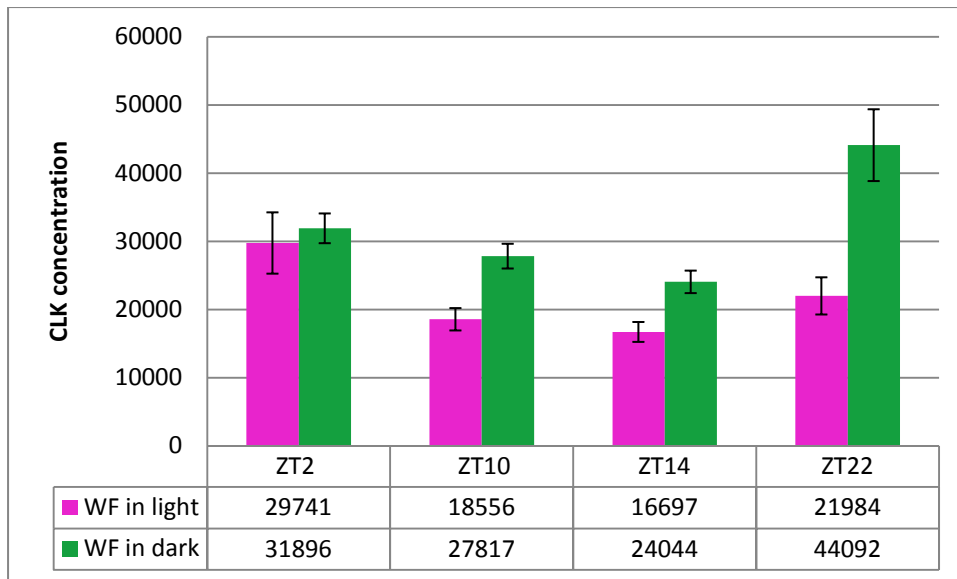


Figure 73. Comparison of total CLK expression in the ILNv of WF in light and WF in dark entrained flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. CLK: CLOCK protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

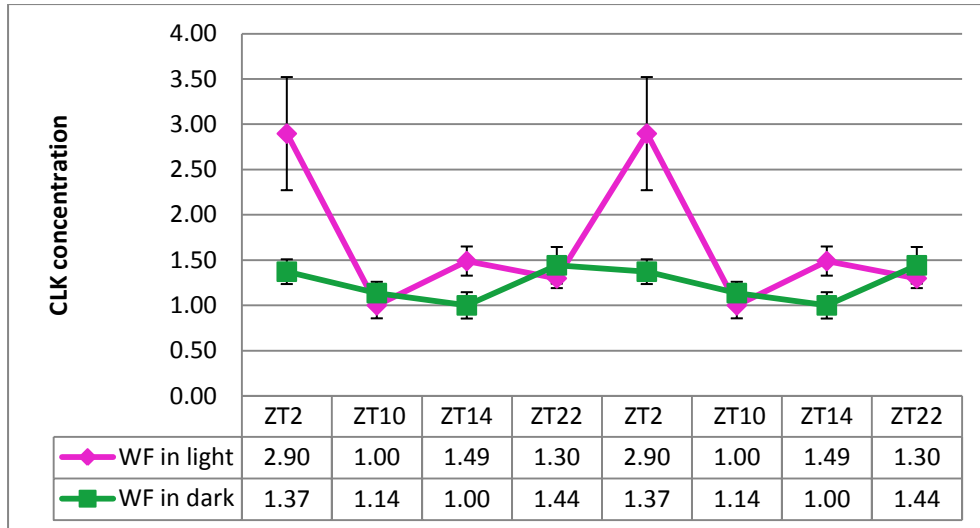


Figure 74. Oscillation of CLK in the sLNv of WF in light and WF in dark entrained flies.

In WF in light entrainment, the expression level of CLK at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In WF in dark entrainment, the expression level of CLK at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

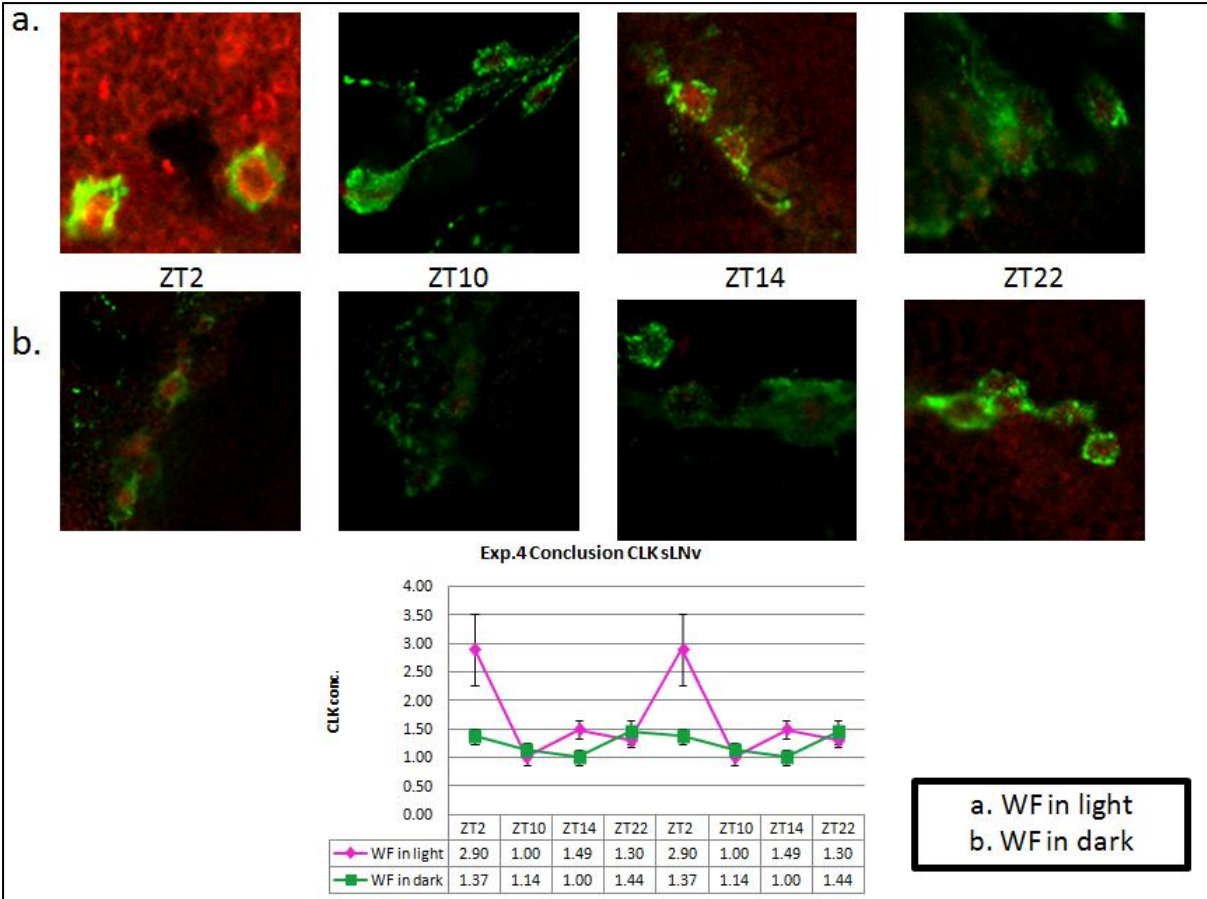


Figure 75. Real images scanned of CLK in the sLNv of a. WF in light *w1118* flies, b. WF in dark *w1118* flies c. graph representing Molecular oscillation of CLK in the ILNv in both entrainments. CLK in red and PDF in green.

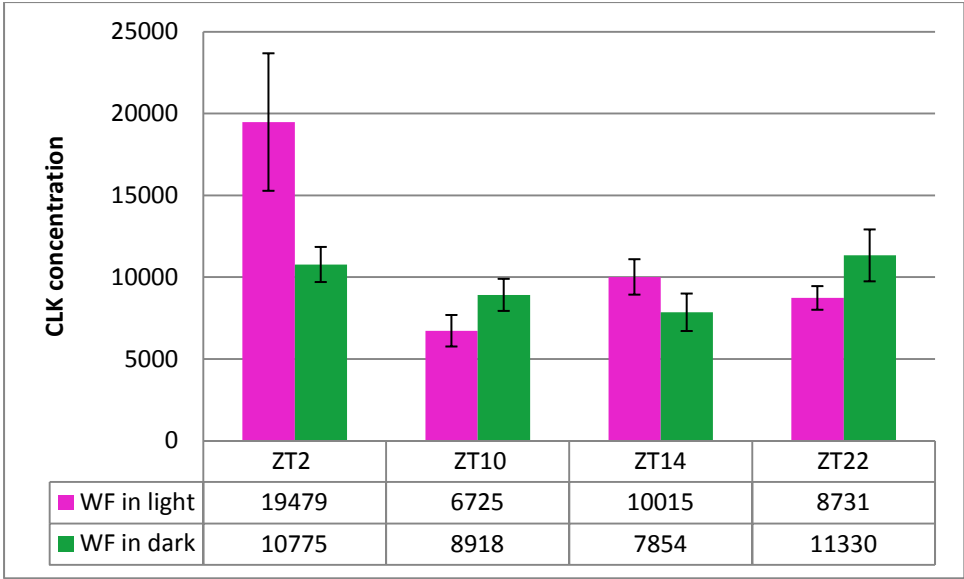


Figure 76. Comparison of total CLK expression in the sLNv of WF in light and WF in dark entrained flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. CLK: CLOCK protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

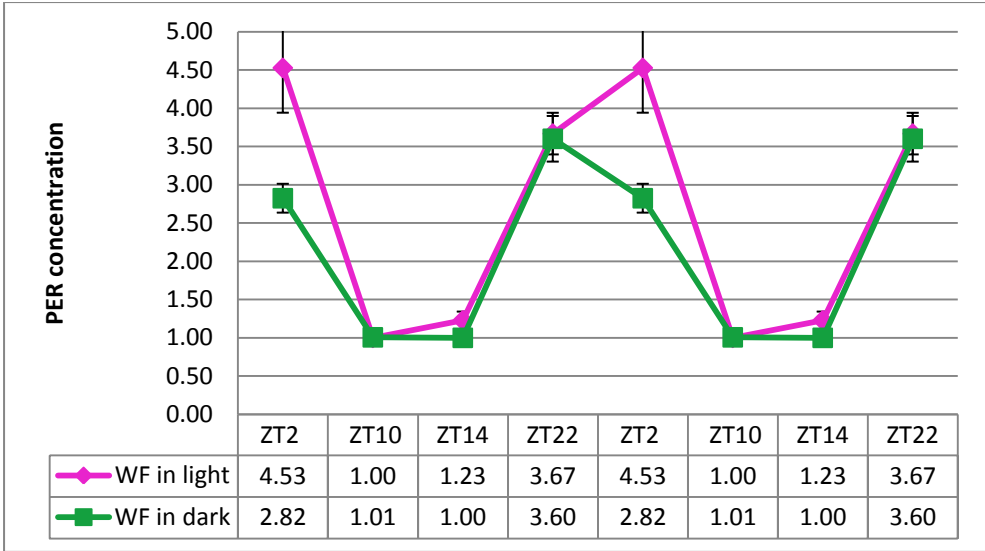


Figure 77. Oscillation of PER in the ILNv of WF in light and WF in dark entrained flies.

In WF in light entrainment, the expression level of PER at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In WF in dark entrainment, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

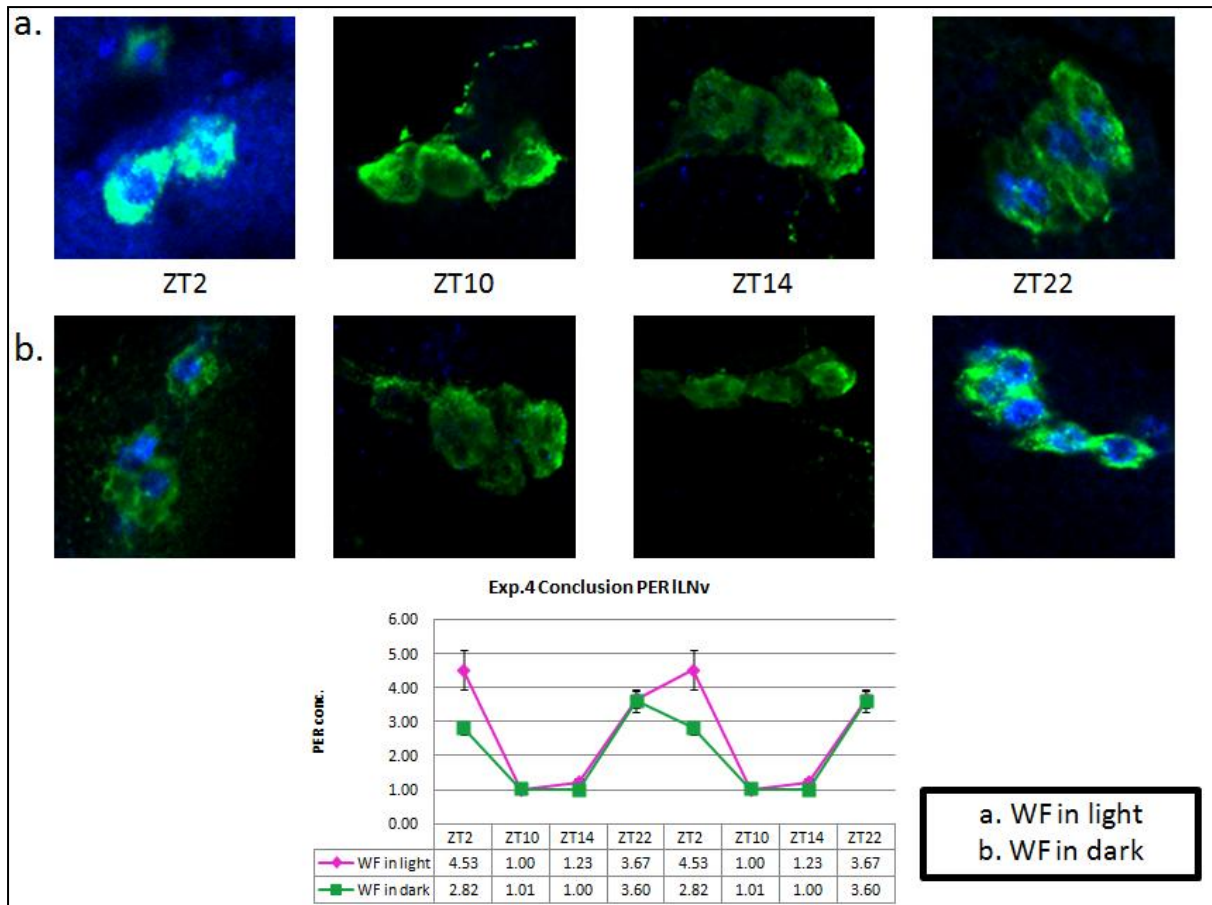


Figure 78. Real images scanned of PER in the ILNv of **a.** WF in light *w1118* flies, **b.** WF in dark *w1118* flies **c.** graph representing Molecular oscillation of CLK in the ILNv in both entrainments. PER in blue and PDF in green.

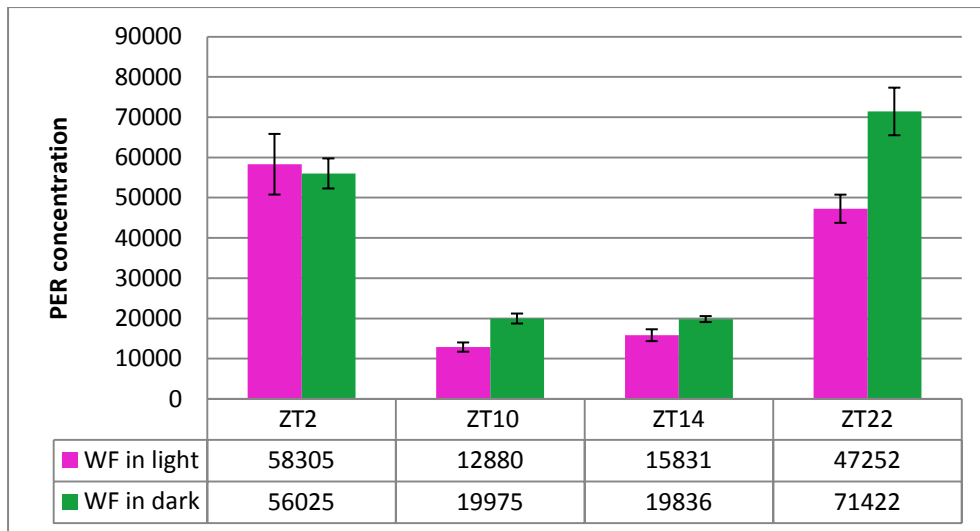


Figure 79. Comparison of total PER expression in the ILNv of WF in light and WF in dark entrained flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. PER: PERIOD protein; ZT: Zeitgeber Time; ILNv: large ventral lateral neurons.

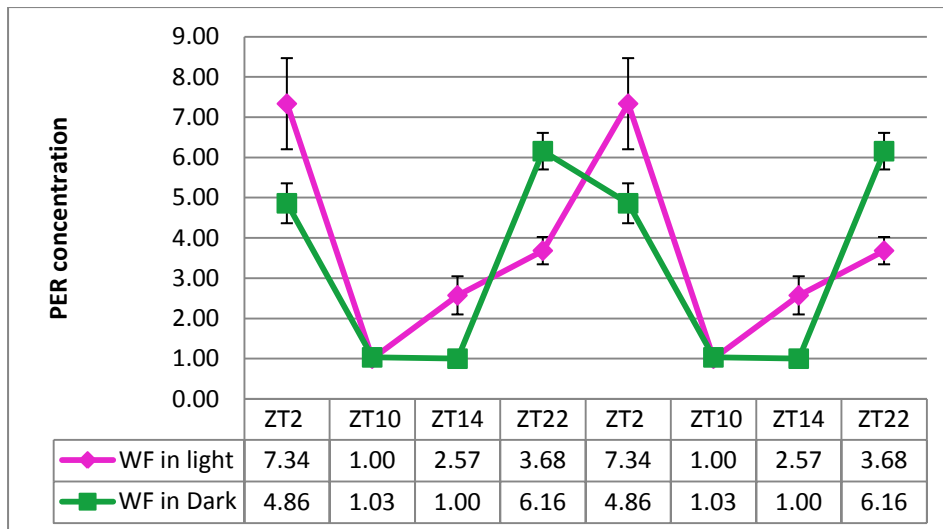


Figure 80. Oscillation of PER protein in the sLNv of WF in light and WF in dark entrained flies.

In WF in light entrainment, the expression level of PER at ZT 10 was set to 1 and the levels from other time points were adjusted accordingly. In WF in dark entrainment, the expression level of PER at ZT 14 was set to 1 and the levels from other time points were adjusted accordingly. Data shown at each time point are the average of at least 4 flies/8 hemispheres from 1 representative experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

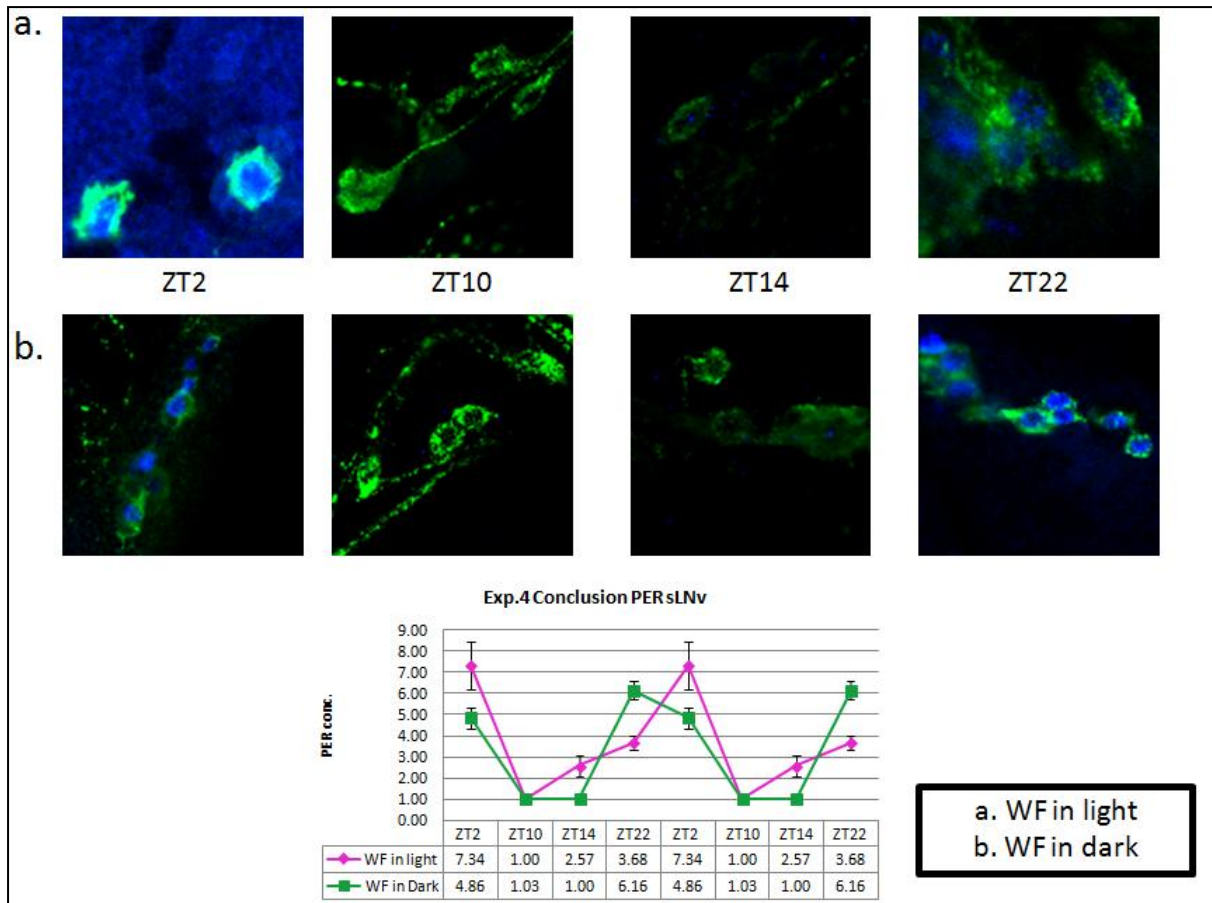


Figure 81. Real images scanned of PER in the sLNv of a. WF in light *w1118* flies, b. WF in dark *w1118* flies c. graph representing Molecular oscillation of CLK in the ILNv in both entrainments. PER in blue and PDF in green.

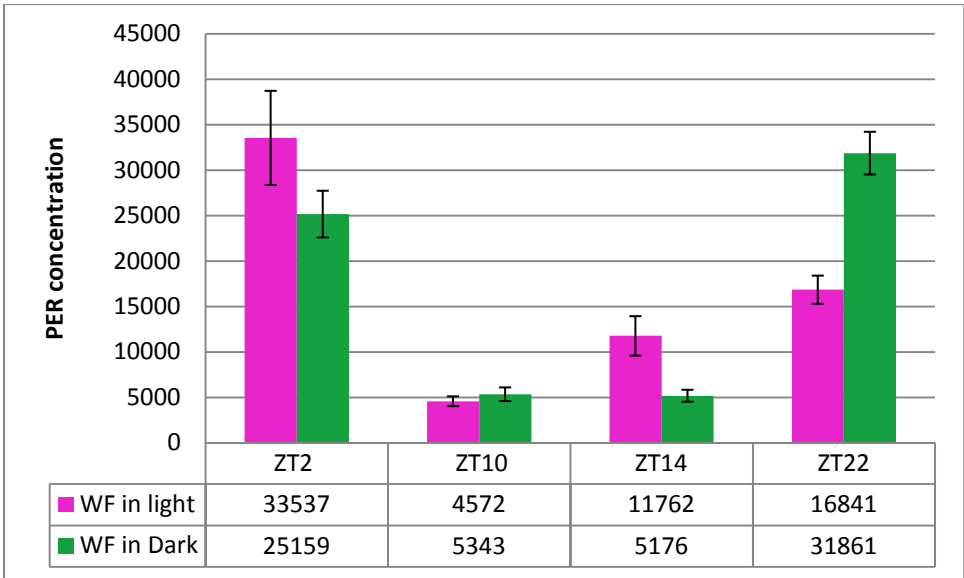


Figure 82. Comparison of total PER expression in the sLNv of WF in light and WF in dark entrained flies.

Both entrainments' data shown at each time point are an average of at least 4 flies/8 hemispheres from 1 experiment.

Abbreviations: WF in Light: cycles of 12h in light with whole food feeding and 12h in dark with starvation; WF in Dark: cycles of 12h in light with starvation and 12h in dark with whole food feeding. PER: PERIOD protein; ZT: Zeitgeber Time; sLNv: small ventral lateral neurons.

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