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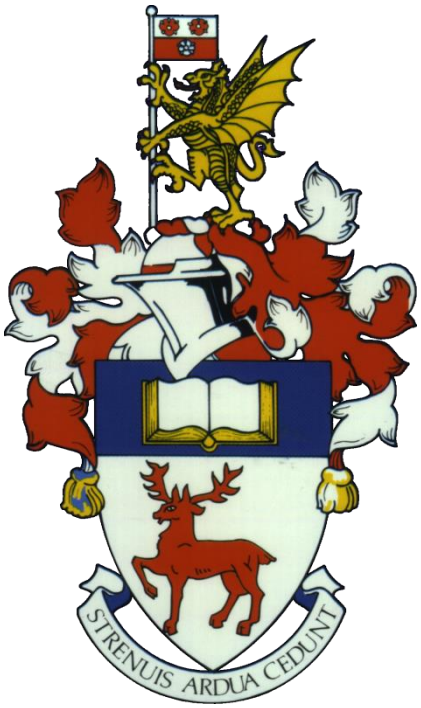

Developmental requirement for adult behavioural rhythmicity in *Drosophila melanogaster*

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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DEVELOPMENTAL REQUIREMENTS FOR ADULT BEHAVIOURAL RHYTHMICITY IN *DROSOPHILA MELANOGASTER*

Karolina Wiktoria Mirowska

Endogenous circadian clocks are a consequence of the periodic nature of the environment we live in. They allow organisms to anticipate daily environmental changes and organize a wide array of biological functions, such as daily activity, sleep and feeding. The molecular clock circuits driving rhythmic locomotor behaviour in *Drosophila melanogaster* consist of two interlocked negative-feedback transcription loops, with a conserved central role for the heterodimeric transcription factor CLOCK/CYCLE (CLK/CYC) and its inhibitor PERIOD (PER). Research presented in this thesis aims to describe underlying developmental requirements for clock function in adult *D. melanogaster*. It has been demonstrated previously that major oscillator components CLK, CYC and PER are present in larvae and some circadian modulation to behavioural is demonstrated in larvae themselves. Moreover, a light pulse administered early in development is enough to establish a phase of the locomotor rhythm of adult flies, pointing out to the connection between a function of the circadian clock during development and adulthood.

This research reveals that adult circadian behaviour does not require either a functioning clock or the expression of *per* during prior development (Chapter 3). However, inhibition of CLK/CYC activity during metamorphosis (post-pupal formation), either by depletion of CYC or over-expression of its inhibitor PER, irreversibly affects clock-controlled locomotor activity in adult flies (Chapter 4 and 5). Even when PER over-expression is restricted to 18 ventral lateral clock neurons (LN_vs) expressing the neuropeptide PIGMENT DISPERSING FACTOR (PDF), subsequent adult circadian behaviour is disrupted. A subset of small ventral lateral neurons (s-LN_vs) was implicated as particularly sensitive to CLK/CYC inhibition through constitutive PER over-expression, suggesting that these cells require CLK/CYC function during development (Chapter 5).

Circadian oscillations in the peripheral tissue of adult flies were less dependent on developmental CLK/CYC activity than locomotor behaviour (Chapter 5). Thus, the newly discovered developmental function for CLK/CYC appears to be specific to the neural clock circuits. Analysis of the daily rhythms of TIMELESS protein localisation within clock neurons revealed that molecular oscillator is severely disrupted in small ventral lateral neurons (s-LN_vs). Therefore my research suggests CLK/CYC is necessary during metamorphosis to establish a proper function of the molecular oscillator in s-LN_vs (Chapter 5). I hypothesized that genes downstream from CLK/CYC are involved in this process, with *Pdp1*ε and *Mef2* as the strongest candidates, however this has not been confirmed (Chapter 6). Moreover, it appears that chromatin modifications are not involved in mediating the phenotype observed as result of developmental CLK/CYC inhibition (Chapter 6).

In summary, my work presents data confirming that CLK/CYC, but not PER activity, is required in PDF-expressing ventrolateral neurons during metamorphosis for establishing adult locomotor rhythmicity.

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DECLARATION OF AUTHORSHIP

I, Karolina Wiktoria Mirowska declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Developmental requirements for adult behavioural rhythmicity in *Drosophila melanogaster*

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

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Signed:.....

Date:.....

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Definitions and abbreviations

When referring to a gene, the name is written in italics. When referring to protein, name is written using capital letters. For example, *Pdf* or *cyc* denotes a gene while PDF or CYC is a translated protein. For simplicity, only one explanation of each abbreviation is used below.

0 – null mutant

A- Adenine

aanat - Arylalkylamine-(Serotonin) N-Acetyltransferase

adj - adjusted

aMe - accessory Medulla

AMPH - Ampiphysin

AOT - Anterior Optic Tract

ar – arrhythmic

ARID - AT-Rich Interaction Domain

ATP – Adenosine Triphosphate

AVP - Vasopressin

bHLH - Basic Helix-Loop-Helix

BMAL1 - Brain And Muscle Arnt-Like Protein

BO - Bolwig's Organs

bZip – basic Leucine Zipper

C – Cytosine

cAMP - Cyclic Adenosine Monophosphate

CBD - CLOCK Binding Domain

CBP - CREB-Binding Protein

CCID - CLK/CYC Inhibitory Domain

cDNA - complementary DNA

C.elegans - Caenorhabditis elegans

CHINMO – Chronologically Inappropriate Morphogenesis

CK – Casein Kinase

CLK – Clock, Circadian Locomotor Output Cycles Kaput

corr - correction

CRF - Corticotropin-Releasing Factor

CRY - Cryptochrome

CSN - COP9 Signalosome

CT - Circadian Time, time in free-running conditions

CtBP - C-terminal Binding Protein

CUL – Cullin

CWO – Clockwork Orange

CYC – Cycle

CyO^{1} - Curly

D – Dark portion of the cycle

DBP - D element Binding Protein

DBT – Doubletime

DCR - Dicer

DD – Constant Darkness

DESAT1 – Desaturase 1

df- degrees of freedom

DFC – Dorsal Fusion Commissure

DILP2 – Insulin-Like Peptide

DLG1 – Discs, Large Homologue 1

D. melanogaster – Drosophila melanogaster

DN – Dorsal Neurons

DNA – Deoxyribonucleic Acid

Dpf - Days Postfertilization

dsRNA – double-stranded RNA

DvGluT - Vesicular Glutamate Transporter

E - Evening (peak, oscillator or neurons)

E – Ebony

E – Embryonic Day

E-box – Enhancer box

ELAV – Embryonic Lethal, Abnormal Vision

EMS - Ethyl Methane Sulfonate

FAS-2 – Fasciclin-2

FASPS - Familial Advanced Phase Syndrome

FER2 - 48-Related 2

FFT-NLLS - Fast Fourier Transform – Non-Linear Last Squares

FRET - Fluorescence Resonance Energy Transfer

G – Guanine

GABAA - γ–Aminobutyric Acid A

GFP – Green Fluorescent Protein

GOI – Gene of Interest

GPRK2 - G Protein-Coupled Receptor Kinase 2

GRNs - Gustatory Receptor Neurons

GSK3β - Glycogen Synthase Kinase-3β

GT - Giant

GV - Germinal Vesicle

H – Histone

H - Hairy

hr(s) – hour(s)

HAT - Histone Acetyltransferases

HDAC – Histone Deacetylase

Hid – Head Involution Defective

HNF4 – Hepatocyte Nuclear Factor 4

Hpf – Hours Postfertillization

HR38 – Hormone Receptor – Like in 38

hs - heat-shock promoter

HSP-70 - Heat-Shock Protein 70

If[1] – Irregular Facet

IMD – Immune Deficiency

ILP3 – Insulin-Like Peptide 3

ipRGC - intrinsically photoreceptive Retinal Ganglion Cells

IR – Inward Rectifier

ITP – Ion Transport Peptide

JAK – Janus Kinase

JET – Jetlag

JMJC - JumonjiC

JRK – Jerk

kDa - kilodalton

KIS - Kismet

l – long

L – Light portion of the cycle

L1 – first instar

L2 – second instar

L3 – third instar

LD – Light/Dark cycle

LED – Light-Emitting Diode

LID – Little Imaginal Discs

LL – Constant Light

l-LN_vs – large ventrolateral Neurons

LN_ds – dorsolateral Neurons

LNs - Lateral Neurons

LOLA - Longitudinals Lacking

LPN - Lateral Posterior Neurons

LUC - Luciferase

M - Morning (peak, oscillator or neurons)

MAPK - Mitogen-Activated Protein Kinase

MEF2 – Myocyte Enhancer Factor 2

miRNA - microRNA

mGluRA - metabotropic Glutamate Receptors

mRNA – messenger RNA

n - number

NaBu – Sodium Butyrate

NaChBac - Bacterial Sodium Channel

NAD - Nicotinamide Adenine Dinucleotide

NF1 - Neurofibromatosis-1

NGS - Normal Goat Serum

NLS - Nuclear Location Signal

NOC – Nocturnin

NOCTE - No Circadian Temperature Entrainment

NORPA - No Receptor Potential A

NPAS2 - Neuronal PAS Domain-Containing Protein 2

NPF – Neuropeptide F

NPY – Neuropeptide Y

NSAIDs - Non-Steroidal Anti-Inflammatory Drugs

Or47b – Odorant Receptor 47b

org - original

OSNs - Olfactory Sensory Neurons

P - Promoter

φ – Phase

PAL - Protocerebral Anterior Lateral

PAM - Protocerebral Anterior Medial

PAR – Proline and Amino acid Rich Domain

PAS - Per-Arnt-Sim

PBT - Phosphate Buffered Solution/Tween

PCR - Polymerase Chain Reaction

PDBD - Period Doubletime Binding Domain

PDF – Pigment Dispersing Factor

PDFR – Pigment Dispersing Factor Receptor

PDH – Pigment Dispersing Hormone

PDK1 - Phosphoinositide-Dependent Kinase 1

PDP1 - PAR-Domain Protein 1

PER – Period

PHR - Photolyase Homology Region

PI - Pars Intercerebralis

PKA – Protein Kinase A

PP – Protein Phosphatase

PRC - Phase Response Curve

PRMT5 – Protein Arginine Methyl Transferase 5

P-TRCP – P-Transducin Repeat-Containing Protein

PUC – Puckered

qPCR – quantitative PCR

qRT-PCR - quantitative reverse transcriptase Polymerase Chain Reaction

QSM – Quasimodo

r - rhythmic

RAE - Relative Rhythmic Error

RH - Rhodopsin

RISC – RNA-Induced Silencing Complex

RNA – Ribonucleic Acid

RNAi – RNA interference

RRP – Relative Rhythmic Power

RT – Room Temperature

s – short

S2 – Schneider 2

S6KII - p90 Ribosomal S6 Kinase

Sb^{1} - Stubby

SCF - SKP1/CUL1/F-box

SCN - Suprachiasmatic Nuclei

SEM – Standard Error of Mean

Ser^{1} - Serrate

Ser - Serine

SGG – Shaggy

SIR/SIRT - Sirtuin

SKP1 - S-Phase Kinase-Associated Protein 1

SLIMB – Supernumerary limbs

s-LN_vs – small ventrolateral Neurons

SLOB – Slowpoke Binding Protein

SMI35A – Smell Impaired 35A

sNPF - Short Neuropeptide F

spp - species

STAT - Signal Transducer and Activator of Transcription or Signal Transduction And transcription

SUMO - Small Ubiquitin-Related Modifier

τ – tau, period length

T – Thymine

TARGET - Temporal And Regional Gene Expression Targeting

Tb^1 – Tubby

T_{dev} – developmental temperature

TH – Tyrosine Hydroxylase

Thr – Threonine

TIM - Timeless

Trp – Tryptophan

TRX - Trithorax

trxG - TAC1 trithorax Group

ts – temperature-sensitive

TUB – Tubulin

TUG – tim(UAS)Gal4

UAS - Upstream Activation Sequence

UPD - Unpaired

UTR – Untranslated Region

UV – Ultraviolet

W – White

WDS – Will Die Slowly

wr – weakly rhythmic

V/P box – VRI/PDP1 box

VRI – Vrille

Y - Yellow

zfaanat - arylalkylamine-(serotonin) N-acetyltransferase in zebrafish

ZT - Zeitgeber Time, time according to LD cycle

Chapter 1: General introduction

1.1 Structure of the introduction

The main aim of this project was to understand the development of circadian clock function in adult *Drosophila melanogaster*. To properly introduce this topic, I will start by explaining more general information first and progress to information specific to the development of the clocks.

In the first part of the introduction, I will introduce the field of chronobiology and rhythmical properties in nature, then move on to the description of the circadian rhythms, including the information on their conservation and connection to health (1.2). I will progress to describe the mechanisms regulating circadian rhythms in flies both at the molecular (1.3) and neuroanatomical level (1.4). Finally, I will summarise the existing information on the development of the circadian clocks in flies, mammals and zebrafish (1.5). Lastly, I will describe aims of the project (1.6).

1.2 Rhythms in nature

The rotation of the Earth around its axis causes periodic changes in conditions on earth. We experience a 24-hour cycle of solar radiation (light and darkness), which is also accompanied by changes in temperature and humidity. In addition, interaction of the Earth with the Moon and the rotation around the Sun on an elliptical orbit produces rhythms in nature with other periodicities, of 29.5 and 365 days respectively. Organisms inhabiting the Earth are capable of synchronizing their behaviour to these changes so that they can increase their chances for survival or reproduction. Most of these represent not simply a direct response to the periodicities in nature but are manifestations of endogenous rhythms in organisms, meaning that organisms not only respond to these changes but also actively anticipate them. Endogenous origin of the rhythms can be especially observed when organisms are removed from the periodic environment and introduced to constant conditions, where they display free-running period (Dunlap, 1999; McClung, 2006).

1.2.1 Introduction to circadian rhythms

Chronobiology is a formal name given to the field of studying periodic phenomena that occur in nature, in some prokaryotes and all eukaryotes. The term was first introduced by Halberg and uses the Greek word *chronos* (meaning “time”). It studies infradian rhythms (longer than a day), ultradian (shorter than a day, such as REM cycle, respiration, heartbeat, blinking or growth hormone production) and circadian rhythms (Dunlap *et al.*, 2004). A special kind of ultradian rhythm called circatidal is associated with the cycles of high and low tide and consequently have period of around 12.4 hours. They mainly affect marine organisms, such as diatom *Hantzschia,* for example influencing their vertical migration (Binkley, 1998). Infradian rhythms can be further divided into circalunal (synchronised to the lunar month with period of 29.5 days, for example breeding of Atlantic fireworms *Odontosyllis* spp., pit building by antlions *Myrmeleontidae* or human menstrual cycles), circannual (with a period of a year, for example changes in gonad size and body weight in some organisms, antler shedding, nest building, reproduction of certain organisms, such as Palolo worms *Eunice viridis* and *Eunice fucata*, flowering of

some plants) (Cloudsley-Thompson, 1980; Goodenough, 1993; Binkley, 1998; Dunlap *et al*., 2004). There are also rhythms with periods longer than a year. For example some species of bamboo flower with large regular breaks (up to 120 years), and some species of cicada emerge every 13 or 17 years (Thomas *et al*., 1979; Simon *et al*., 2000). Circadian rhythms are the most ubiquitous of those and have a period of about 24 hrs (Cloudsley-Thompson, 1980; Dunlap *et al*., 2004).

Every rhythm can be characterized by the same properties, such as period (tau, τ), which is the time necessary to complete each cycle; amplitude, which is the difference between the highest and lowest value and phase, which is the time of an arbitrarily selected point in the cycle. Time of the increased activity is called acrophase with peak or maximum referring to the highest value. Contrary, bathyphase or through is the time of the lowest activity with nadir used to describe the lowest point (Refinetti, 2005).

The term *circadian* was coined by Franz Halberg in 1959 to unify the existing nomenclature. It uses the combination of Latin words *circa*, meaning "around" and *diem*, meaning "day" to describe the biological phenomena with a period of around 24 hours (20-28 hours) that can be either synchronised or desynchronised from the environment (Halberg, 1959). Before that, such rhythms were called "diurnal" (by the English), "daily" (by Americans), "endodiurnals" (by Germans) or "diel" (by Canadians). There was also no consensus on using the term "rhythms", as "cycles", "periods" and "periodicities" were all used (Chandrashekaran, 1998).

Circadian rhythms are also known as biological clocks. This term was inspired by research on the time compensation of the sun navigation in birds performed by Gustav Kramer and later was used as a name for the seminal 1960 Cold Spring Harbour Symposium of Quantitative Biology (Chandrashekaran, 1998; Kuhlman *et al*., 2007). It gained widespread use since it was emphasising the innate nature of the circadian rhythms and their main function of measuring time (Pittendrigh, 1960; Moore-Ede *et al*., 1982).

It is important not to confuse the biological clocks with biorhythms. The latter belongs to pseudoscience and describes an idea that there exists a set rhythm to our intellectual abilities (with period of 33 days), self-awareness (48-day period) and spirituality (53-day period), among others (Gittelson, 1983).

Biorhythms are even classified as mythology by a National Institute of Mental Health (USA) (Gittelson, 1983).

1.2.2 Properties of circadian rhythms

Circadian rhythms can be defined by three general characteristics (Pittendrigh, 1954; Pittendrigh, 1960; Dunlap *et al.*, 2004):

- Their free-running period, which is the time necessary to complete a full cycle in the absence of environmental cues, lasts about 24 hours (± 4 hours). The rhythms have endogenous origin and are self-sustaining, meaning they can manifest themselves and persist in the aperiodic environment. This property distinguishes true circadian rhythms from rhythms that are just a response to environmental cycles.
- They are entrainable, meaning that even though clocks sustain their activity in the absence of external cues, they can be reset by the exposure to certain external stimulus. In fact, it is quite unusual to find aperiodic environment outside of the laboratory and most of organisms have their rhythms constantly synchronised to environmental rhythms thanks to Zeitgebers (synchronizing cues, Aschoff, 1960).
- They are temperature-compensated, so their period is relatively stable over a range of ambient temperatures. This is because many organisms experience a wide range of temperatures, and despite the fact that temperature changes affect the kinetics of molecular processes, the period of circadian rhythms has to stay relatively stable (Sweeney and Hastings, 1960). This does not mean that the rhythms are completely independent of temperature but they are less dependent than expected, as the Q10 temperature coefficient (ratio of the rate of any process at any temperature to the ratio of the same process at temperature 10˚C lower) remains close to 1, as opposed to other biochemical processes that have Q10 between 2 and 3 (Gibbs, 1981). The only way this could be achieved is if even when the individual reactions are affected by temperature, the whole system is buffered so that the output is not variable (Johnson, 2004; Kuhlman *et al.*, 2007). The precise mechanism depends on the organism (Majercak *et al.*, 1999; Gould *et al.*, 2006).

1.2.3 History of the research on circadian rhythms

The first recorded account of the rhythmical events in living organisms comes from around 400 BC. Androsthenes of Thasos, an admiral, geographer and scribe travelling with the Alexander the Great during his expeditions in Asia, described movements in leaves of the tamarind tree (*Tamarindus indicus*) on the island of Tylos in the Persian Gulf (McClung, 2006). To him, it appeared that the rhythmical movements of leaves were caused by the presence of light. It took over 2000 years to realize that the rhythms were in fact endogenous, internal to the organisms, and not merely caused by the external factors. Before that, unknown to western world, the first observation of a rhythmic (circadian or diurnal) process in humans appeared in Chinese medical texts around the 13th century, including the "Noon and Midnight Manual" and the "Mnemonic Rhyme to Aid in the Selection of Acu-points According to the Diurnal Cycle", "The Day of the Month and the Season of the Year" (Gwei-Djen lu, 2002).

In 1729, Jacques d'Ortous de Mairan, French astronomer, noticed rhythms in the leaves of sensitive heliotrope plant (probably *Mimosa pudica*), which were showing daily pattern of opening and closing (de Mairan, 1729). de Mairan went further than Androsthenes of Thasos and tested whether these rhythms were just a response to presence of light and he observed behaviour of the plant in constant darkness. He discovered, that even in the absence of light, the plant was opening its leaves during the daylight hours and closing them at night time. Even though de Mairan did not conclude that these observations confirmed the existence of the endogenous biological clock (and he even did not publish his work, it was his colleague M. Marchand who did), these observations were the first recorded scientific proof of the presence of rhythms even in the absence of light/dark cycle.

Following 30 years were a time of further refinement of de Mairan's work. Taking the idea of plants showing remarkable correlation of behaviour with a time of the day, in 1751 Carl Linnaeus described a concept for a *Horologium Florae* (flower clock). It involved using several different plants that open and close their petals at different times to indicate a time of the day (Linnaeus,

1751). Three independent researchers, Hill, Duhamel du Monceau and Zinn also excluded fluctuations in the environmental temperature as the factor influencing the leaf movement, confirming that the biological clock is endogenous (Hill, 1757; Duhamel du Monceau, 1759; Zinn, 1759; reviewed in McClung, 2006).

Many years later, in 1832 De Candolle demonstrated that the period of leaf movements of sensitive plants in constant light is close to but not equal 24 hours (De Candolle, 1832; McClung, 2006). Thus, the first demonstration of the free-running (clock drifting away from the 24 hour period as it is not entrained to the external cues) was provided. Another interesting observation provided by De Candolle was that by altering the light/dark cycle, the biological rhythm of plants could be reverted. Both of these findings were later confirmed by Bose and Kleinhoonte, who formally concluded that the movement of plants can entrain to light/dark cycles and recorded free-running rhythms both in constant light and constant darkness (Bose, 1919; Kleinhoonte, 1929).

As evident from previous description, all of the early research on circadian rhythms was focusing on using plants, as circadian rhythms in other organisms were unknown. Circadian research on animals started late in 19th century and became more popular in 20th century. Notable examples of first descriptions of the rhythms of animals include the description of pigment rhythms in arthropods (Kiesel, 1894) and research demonstrating that goldfish show a 24 hour rhythms in swimming, even when placed in constant darkness (Szymanski, 1914). Circadian rhythmicity in primates was uncovered rather early (Simpson and Gaibraith, 1906) but it took over half a century for similar observations in human (Aschoff and Weaver, 1962). Over time, circadian rhythms were discovered in rodents (Richter, 1922), insects (Beling, 1929) and birds (Kramer, 1952). Slowly evidence for the presence of endogenous circadian rhythms in other organisms started appearing, including single-cell eukaryotes (Sweeney and Hastings, 1957) and bacteria (Mitsui *et al.*, 1986).

In late 19th century it was suggested that circadian rhythms are heritable (Darwin and Darwin, 1880). In 1932 first evidence for the genetic basis of circadian rhythms was presented by showing that period length is heritable in plants (Bünning, 1932). It was later confirmed in 1971 when Seymour Benzer

and Ron Konopka identified the first gene involved in the generation of circadian rhythms in *D. melanogaster* and called it *period* (Konopka and Benzer, 1971). Two additional lines of evidence were uncovered confirming this. First, even after multiple generations in aperiodic environment, rhythms still persisted (Paranjape *et al.*, 2003; Imafuku and Haramura, 2011). Second, organisms were able to entrain to non-24 hour cycles of Zeigebers, but upon release to constant conditions were displaying the period of behaviour dictated by their internal timekeeping mechanism (Bünning, 1973).

More discoveries of genes involved in generation of circadian rhythms followed, with identification of such candidates in green algae *Chlamydomonas reinhardtii* (Bruce, 1972), fungi *Neurospora crassa*, where identified gene was called *Frequency* (Feldman and Coyle, 1973) and hamsters (Ralph and Menaker, 1988). 1990s were a time of cloning and molecular characterization of the genes found (Dunlap, 1999). Later, a first mammalian circadian gene, *Clock* was found by Joe Takahashi's laboratory group (Vitaterna *et al.*, 1994; King *et al.*, 1997). It also became possible to create a completely artificial circadian oscillator in a test tube using circadian proteins from *Cyanobacteria* and ATP (Nakajima *et al.*, 2005).

History of the research on human circadian rhythms is especially fascinating. To study the free-running rhythms, you need to remove test subjects from periodic environment, which is not an easy task, or subject them to a different period length. In 1938 Nathaniel Kleitman decided to confine himself and his assistant, Bruce Richardson, to a Mammoth Cave in Kentucky. Away from the external 24-hour cycle, they were planning to live in an environment which had a period of 28 hours, both in terms of light/dark cycle and meal schedule. However, their core temperature still oscillated with a period of 24 hours. This was further evidence for the endogenous nature of circadian rhythms that persist not only in the absence of cues but also in non-circadian environments (Kleitman, 1963). Jürgen Aschoff was also studying circadian behaviour of people by locking them in underground bunkers and measuring their physiological functions and locomotor activity during both 24 hour light/dark cycle and constant darkness. He uncovered that social cues (such as meal time) were acting as a strong Zeitgeber and synchronizing test subjects' rhythms, and that in light/dark cycles, internal rhythms were slightly longer than 24hours (Aschoff *et al.*, 1971).

Many years after experiments suggested the endogenous nature of the biological rhythms; some scientists still believed that the rhythms occur in response to either light leaking into darkrooms or caves (Pfeffer, 1873) or some mysterious exogenous factors (Brown, 1960). Brown postulated the existence of 'subtle geophysical factors' (like electrostatic and magnetic field variations), which would be providing synchronizing cues to organisms even at apparently constant conditions. However, it was the endogenous clock theory that became widely accepted, not because of the overwhelming evidence but since it was "(…) simply chosen as the preferred hypothesis" (Brown, 1974). One of the most convincing evidence to the endogenous nature of the circadian clocks (other than discovery of clock genes) came from the experiments on the fungus (*Neurospora crassa*) conducted in space, where the rhythms persisted despite removal of any periodic changes or mysterious geophysical cues (Sulzman *et al*., 1984). Interestingly, some researchers were still not convinced (Brady, 1987).

1.2.4 Emergence and conservation of circadian rhythms

Scientists used to believe that circadian rhythms are a property of only eukaryotes, as prokaryotes were thought to be too simple (Dunlap, 1999). However, researchers demonstrated existence of rhythms in many prokaryotes, including cyanobacteria and archaebacteria (Golden *et al*., 1997; Dvornyk *et al*., 2003; Whitehead *et al*., 2009). These findings might prove the adaptive value of the circadian rhythms since these prokaryotes represent the oldest organisms on the Earth, meaning that circadian clocks were adding some advantage to even simple organisms and since circadian rhythms are a common phenomenon in organisms, it suggests evolutionary conservation of rhythms (Dvornyk *et al*., 2003).

It is currently believed that circadian rhythms accompanied emergence of photosensitive proteins and processes in early cells to protect the sensitive process (such as DNA replication) from ultraviolet radiation that could introduce mutations. This way, circadian rhythms could restrict this process to dark portion of day (Pittendrigh, 1993). This mechanism is still present in the fungus *Neurospora crassa* (Heintzen and Liu, 2007).

It was always assumed that circadian clocks conferred some fitness advantage. After all, it seems logical that organisms that can anticipate environmental events would be better prepared that those who simply respond to changes (Sharma, 2003). For example, by knowing which time of day your prey is the most vulnerable gives you time to position yourself in their vicinity. However, it was fairly difficult to come up with a definite proof for this, since laboratory conditions create a kind of 'perfect' environment, where existence of circadian rhythms might be less crucial than in real life.

Early experiments managed to suggest that circadian clocks confer fitness advantage by demonstrating that plants grow best when exposed to 24 hours light cycle as compared to short or long cycles or constant light (Withrow and Withrow, 1949; Highkin and Hanson, 1954; Hilman, 1956). More convincing proof for this came later. For example, when grown under a short cycle of 20 hours, *Arabidopsis thaliana* clock mutants with a short internal period have larger biomass and better rates of chlorophyll production and carbon fixation than plants with a long internal period (Green *et al.*, 2002; Dodd *et al.*, 2005). In addition, for both cyanobacteria and *Arabidopsis* in experimental settings, organisms that had a period closest to an environmental period were outcompeting or growing faster than those with a mismatched period (Ouyang *et al.*, 1998; Dodd *et al.*, 2005; Johnson, 2005). Similarly, *D. melanogaster* with a functional clock has greater reproductive fitness than flies with clock mutations (Beaver *et al.*, 2002). Another line of evidence for the importance of circadian rhythms comes from the fact that organisms retain them even after many generations in aperiodic environment (Sheeba *et al.*, 1999).

Most of organisms employ the same regulatory mechanism governing their biological rhythmicity, which is based on a negative feedback loop (Figure 1.1). Oscillations are generated at a level of transcription and translation and are a result of the delays in those processes. The period of those oscillations match a period of environmental events important for an organism. However, while it is fairly easy to find homology between clock components in eukaryotes, no such homology exists between circadian genes in prokaryotes and eukaryotes (Hut and Beersma, 2011).

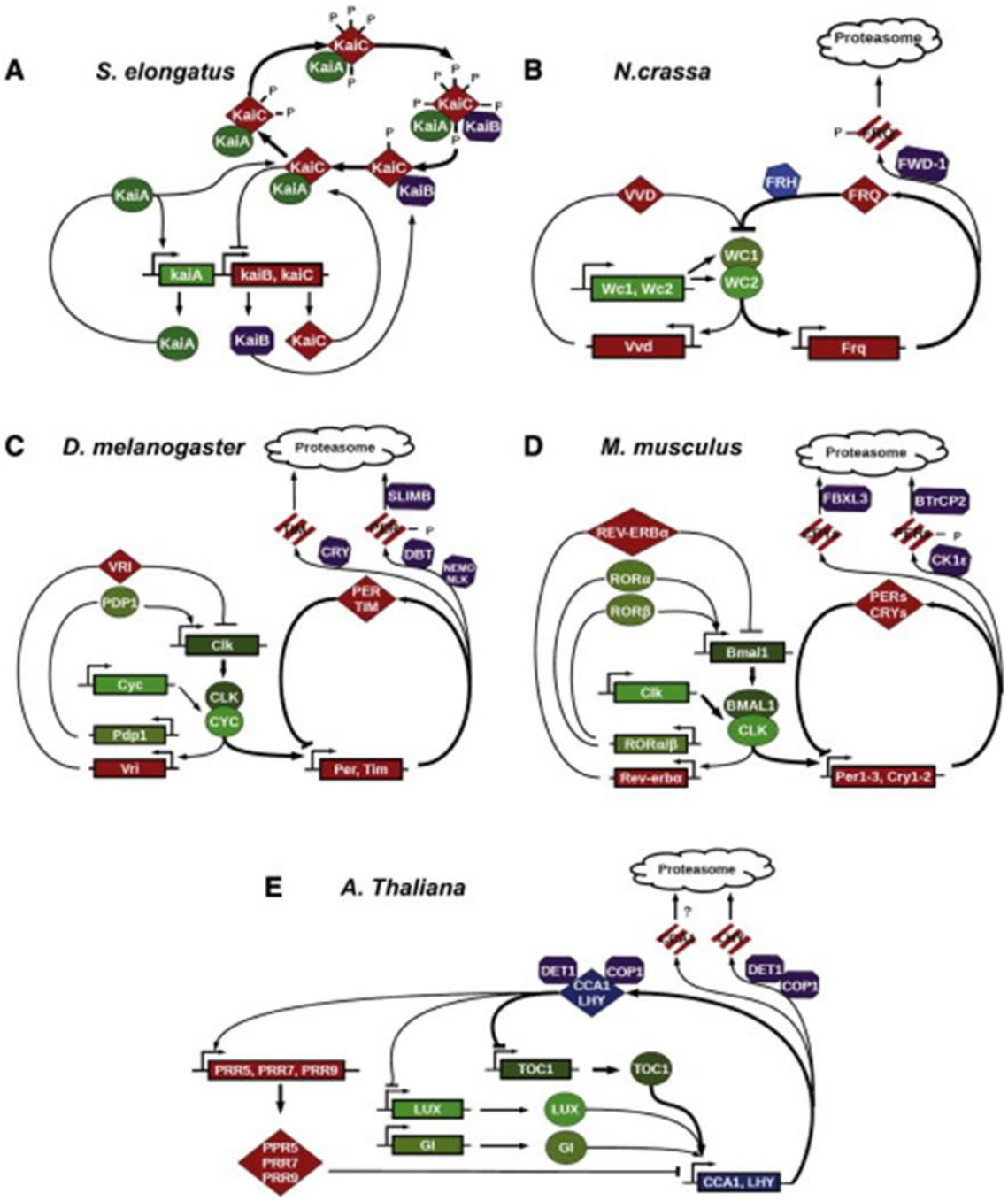


Figure 1.1. Comparison of molecular oscillators driving circadian rhythms in different organisms (reproduced from Brown *et al.*, 2012).

Schematic representation of canonical circadian feedback loops in cyanobacteria *Synechococcus elongates* (A), fungus *Neurospora crassa* (B), fly *Drosophila melanogaster* (C), mouse *Mus musculus* (D), and plant *Arabidopsis thaliana* (E). A large degree of similarity can be observed, as in all cases a principal loop can be identified (marked with bold lines) which is accompanied with additional loops (marked with thinner lines). These loops share

components that can act as activators (green) or repressors (red). A large degree of homology can be particularly observed for molecular oscillator in flies and mammals.

1.2.5 Circadian clocks, health and well-being

Circadian clocks contribute to the health of organisms. The incidences of some diseases are correlated with a time of the day, which might represent the state of a molecular oscillator. For example, morning is shown to be particularly dangerous for people suffering from cardiovascular diseases (Goldberg *et al.*, 1990). There is a 40% greater chance of an initiation of acute myocardial infarction (AMI) in the morning (Cohen *et al.*, 1997). Also there is a 30% increased risk of sudden cardiac death in the morning, contributing to about 7% of overall cases (Cohen *et al.*, 1997). Both of these might be connected to an increased blood pressure that occurs in the morning (Lemmer, 1996). Also, a risk of blood clots forming and their resistance to degradation is increased in the morning as the efficacy of the thrombolytic agents that break down blood clots, such as tissue plasminogen activator, is the lowest in the morning (Decousus *et al.*, 1985; Krulder *et al.*, 1994; Braunwald, 1995; Kurnik, 1995).

In humans, prolonged desynchronization from external environmental cycle, such as shift work, has been shown to be linked to a variety of diseases, such as cardiovascular diseases (Kivimaki *et al.*, 2006), metabolic disorders (Rudic *et al.*, 2004; Turek *et al.*, 2005; Staels, 2006); diabetes (Morikava *et al.*, 2005), cancer (Fu *et al.*, 2002; Konlon *et al.*, 2007; Reddy and O'Neill, 2010; Hansen and Stevens, 2012), miscarriage (Knutsson, 2003), pain and inflammation (Narasimamurthy *et al.*, 2012; Tong *et al.*, 2012; Zhang *et al.*, 2012). Moreover, shift work also increases incidence of psychological disorders and depression (Bildt and Michelsen, 2002).

Since the circadian clock also regulates metabolism, it comes as no surprise that various drugs, especially non-steroidal anti-inflammatory drugs (NSAIDs) and statins have different efficiency and even different side effects depending on a time they are administered (Lévi, 2003; Mück *et al.*, 2000; Lévi and Schibler, 2007; Baraldo, 2008). For example, statins and NSAIDs administered in the evening have higher efficacy and lower toxicity (Mück *et al.*, 2000; Lévi

and Schibler, 2007). Moreover, the therapeutic outcomes and survival after chemotherapy in case of patients with metastatic colorectal cancer was impacted by circadian rhythmicity of patients (Mormont *et al*., 2000). Maintaining a circadian sleep-wake cycle after chemotherapy could also improve patients' wellbeing (Ortiz-Tudela *et al*., 2014).

Finally, dominant mutations in components of the oscillator were shown to link to a disease affecting sleep-wake cycle, causing patients to wake up and fall asleep unusually early (around 4:30 am and 7:30 pm, respectively) (Jones *et al*., 1999). A hyperphosphorylation of PER2, which can occur either due to the mutation of CK1ε, CK1δ or the mutation of phosphorylation site in the PER2, causes 2-4 hours period shortening in the patients suffering from Familial Advanced Phase Syndrome (FASPS). This is in turn causing advancement of waking and sleep onset, a trough in core body temperature and onset of melatonin secretion (Toh *et al*., 2001; Xu *et al*., 2005).

1.3 Circadian rhythms in *Drosophila melanogaster*

1.3.1 Use of *Drosophila melanogaster* in circadian research

Drosophila melanogaster, also called the fruit fly, has been used as a model organism in laboratories across the globe for over 100 years and still continues to be one of the most popular study subjects, contributing to major breakthroughs in almost every area of biological sciences, from ecology to toxicology, from evolutionary studies to neuroscience. It can also be credited for the major advancement of the circadian research. This section will provide a brief explanation for popularity and appropriateness of *D. melanogaster* use in research on circadian rhythms.

1.3.1.1 Advantages of *D. melanogaster* as a model organism

Drosophila melanogaster fulfils the requirements for model organism listed by Bolker in 1995. These are:

- Ease of cultivation – it is relatively simple to store large numbers of flies even in a small laboratory. In addition, the reagents necessary for the flies' husbandry are relatively inexpensive;
- Fast development and short life cycle – even when it is necessary to create new combinations of genetic elements it is possible to achieve that in just couple months. New offspring can be obtained in under two weeks at a standard temperature;
- Large generation size – it is possible to obtain hundreds of offspring from a single female (female flies can lay on average 700-1000 eggs, at the rate of about 100 eggs per day (Shapiro, 1932; Gowen and Johnson, 1946)). Therefore it is relatively easy to obtain the necessary amount of flies for an experiment;
- Optimal size – flies are small enough to allow for easy cultivation but large enough to allow a variety of experiments, such as brain extractions;
- Simple genetics – not only is the entire genome of *D. melanogaster* known (Adams *et al.*, 2000), it is easy to manipulate a genotype of flies. With only two copies of each of four chromosomes, balancer chromosomes marked with easily recognizable dominant mutations and recessive lethal mutations, it is quite easy to assign and track the genotype. Moreover, males do not have chromosomal recombination, keeping their chromosomes stable;
- Availability of stocks – scientists working on flies are always willing to share their stocks. In addition to that, commercial stock centres have a large number of genetic constructs which can be readily purchased and shipped with relative ease. When there is no genetic construct available, thanks to other properties of flies, it is relatively simple and fast to create new transgenic flies.

(Bolker, 1995)

An additional advantage of flies for a use in circadian rhythms research is the relative simplicity of conducting experiments with them. Starting from eclosion

experiments, which look at the presence of rhythms in development of a population of flies and going to locomotor activity experiments, where an automated recording system facilitates data collection, information on rhythmicity of the fly can be obtained within couple weeks. Moreover, flies allow for relatively straightforward neuroanatomical studies. Therefore flies are great for use in circadian biology – they allow easy manipulation of clock function and simple determination of phenotype associated with it.

1.3.1.2 History of *Drosophila melanogaster* research

Drosophila melanogaster was first introduced to a laboratory probably by Charles W Woodworth, an entomologist. Studies involving *Drosophila melanogaster* were the foundation of modern genetics and molecular biology, mainly due to work of Thomas Hunt Morgan and his students: Alfred Sturtevant, Calvin Bridges and Herman Muller. Even though at the very beginning of twentieth century William W Castle started conducting the inbreeding studies with *D. melanogaster*, it was not until 1909 that Thomas Hunt Morgan introduced flies into "fly room", his laboratory at the Columbia University, when the potential of flies was recognized.

In 1910, for the first time Morgan managed to demonstrate sex-linkage of a particular trait, white eyes (Morgan, 1910). Discovery of more traits followed, leading to the theory of recombination (Morgan, 1911 a and b) and Sturtevant publishing the first linkage map of genetic traits (Sturtevant, 1913). These discoveries, described later in seminal work "The Mechanism of Mendelian Heredity" (Morgan *et al.*, 1915) served as a beautiful confirmation of genetic theory of inheritance, suggested by Gregor Mendel and later described by Sutton and Boveri and known as the Boveri-Sutton chromosome theory (Sutton, 1902; Sutton, 1903). In subsequent years, many more laboratories recognized the potential of flies and started using them as primary model organism.

Drosophila melanogaster played a very important role for improvement of chronobiology. In the early 1950s, it was noted that adult eclosion can be studied to further the understanding of circadian clocks, especially their entrainment, free-running, and temperature compensation (Pittendrigh, 1960). Over ten years later, in the early 1970s, Ron Konopka and Seymour Benzer

started looking for single gene mutants in flies that affect the eclosion time. They were hoping that it would lead to a better understanding of molecular mechanism behind the rhythms.

Ron Konopka and Seymour Benzer exposed flies to mutagenic substance, ethyl methane sulfonate (EMS). When they analysed activity of 2000 offspring, they found three whose progeny had abnormal period of eclosion. Unlike most flies that showed 24 hour circadian rhythmicity, one fly had a shorter 19 hour period, one had longer 28 hour period and one did not display any rhythmicity. The descendants of mutant lines also showed similar impairment of locomotor rhythmicity rhythms. Surprisingly, all three mutations appeared to be on the same gene located on X chromosome (Konopka and Benzer, 1971). This gene was later named *period* and the point mutations found were per^{S} (from short), per^{L} (from long) and per^{0} (null), respectively. After these results were published, two things became obvious. First, it appeared that *period* is a key element driving many circadian rhythms in flies, since both eclosion and locomotor rhythmicity were affected. Second, locomotor activity could replace eclosion as experimental readout for screening experiments, as it was faster and involved measuring rhythms in individual flies rather than in population. Thanks to this discovery, circadian clock in *Drosophila melanogaster* remains one of the most studied and most well understood molecular oscillator (Hall, 2003).

1.3.2 Rhythmic properties in *Drosophila melanogaster*

In addition to the molecular rhythms found in *D. melanogaster* that will be discussed later in this chapter, circadian changes in many physiological outputs have been found. I have already mentioned two of these, locomotor activity and eclosion. Eclosion, which is an emergence of an adult fly from a pupal case, was the first discovered property under circadian regulation. This rhythm cannot be observed in a single fly but rather when the population of flies of the same genotype and from the same conditions is studied, except in the case of circadian clock gene mutants, where the rhythms are absent. The eclosion usually happens around dawn (Konopka and Benzer, 1971; Sehgal *et al.*, 1994). Interestingly, these rhythms depend both on a proper function of

the brain pacemaker neurons (especially PDF-positive ventral lateral neurons) and functional peripheral clock in the prothoracic gland (Myers *et al*., 2003).

The most studied circadian rhythm is the locomotor activity (behaviour). Adult *Drosophila melanogaster*, being a crepuscular organism, show two peaks of activity, one around dawn and second around dusk. They are separated by a period of almost complete inactivity during night and reduced activity during the middle portion of the day, called siesta. In free-running conditions, an activity of flies often consolidates over time to one period of activity, with the evening peak of activity roughly staying in place and the morning peak of activity disappearing (Roberts, 1956; Konopka and Benzer, 1971).

Other circadian properties in flies are olfaction (Krishnan *et al*., 1999 and 2005) and feeding (Xu *et al*., 2008). In addition, many processes and behaviours involved in reproduction are under circadian regulation: synthesis of pheromones in oenocytes (Krupp *et al*., 2008), courtship (Roche *et al*., 1998; Fujii *et al*., 2007), mating (Sakai and Ishida, 2001; Becnel *et al*., 2011), oogenesis (Beaver *et al*., 2003) and oviposition (egg-laying, McCabe and Birley, 1998; Paranjpe *et al*., 2004; Howlander and Sharma, 2006; Hari Dass and Sharma, 2008). Finally, it is worth mentioning that circadian modulation of behaviour is not only limited to adult flies. Larvae, until they reach late third instar stage, are photophobic. However, this light sensitivity is also under the clock modulation as larvae show enhanced light avoidance at early morning (Mazzoni *et al*., 2005).

Drosophila melanogaster shows also circadian modulation of immune response and ability to fight pathogenic infections (Lee and Edery, 2008). When *Pseudomonas aeruginosa* or *Staphylococcus aureus* were injected into flies during the day, chances of flies of combating the infection were much lower than if similar injection occurred during the night. This was correlated with stronger induction of antimicrobial peptides through TOLL or IMD pathway (Hoffmann, 2003) that happened at night (Lee and Edery, 2008). This circadian regulation of infection response is similar to circadian modulation of resistance to insecticides found in other insects (Sullivan *et al*., 1970). This is consistent with the fact that genes involved in detoxification are rhythmically expressed, as demonstrated in a whole-genome expression profiling studies (Wijnen and Young, 2006) and confirmed in *D. melanogaster* (Hooven *et al*., 2009).

Unlike other insects, flies do not display the circadian modulation of egg hatching (for examples in other insects see Minis and Pittendrigh, 1968; Sauman *et al*., 1999) or metamorphosis timing. For example, it has been show that ecdysteroid hormones synthesis and release, as well as sensitivity to it, are showing daily oscillations in *Rhodonius prolixus* (Vafopoulou and Steel, 1991, 1996, 1999) and also the release of it is rhythmic in cockroach *Periplaneta Americana* (Richter, 2001). In addition, some insects display a quite intricate mechanism of time-compensation of the sun compass. Circadian clock is used to change the flight direction over time due to changes in the position of the sun during the day. This behaviour is commonly found in insects that are engaging in foraging over long distances or are migratory, for example in Monarch butterflies (Mouritsen and Frost, 2002; Froy *et al*., 2003; Reppert *et al*., 2010) and honey bees (Lindauer, 1960; Bloch, 2010).

1.3.3 Peripheral oscillators

The brain is the site of the main molecular oscillator in *D. melanogaster*, but is not the only organ that contains a molecular oscillator. In fact, circadian clocks were found in a variety of other organs and tissues and these clocks were named peripheral clocks, in contrast to the main oscillator. Peripheral clocks have been observed in eyes (Chen and Stark, 1992; Zeng *et al*., 1994; Cheng and Hardin, 1998; Houl *et al*., 2006), prothoracic glands (Emery *et al*., 1997), Malpighian tubules (Giebultowicz and Hege, 1997; Hege *et al*., 1997; Giebultowicz *et al*., 2000), proboscis, sensory bristles in wings and legs (Plautz *et al*., 1997a and 1997b), antennae (Krishnan *et al*., 1999) and cuticle (Ito *et al*., 2008). Peripheral clock function is independent from the function of the main molecular oscillator and when a tissue is isolated from the brain and kept in a culture, they are capable of sustaining oscillations (Emery *et al*., 1997; Giebultowicz and Hege, 1997, Plautz *et al*., 1997a and 1997b) and can by synchronised by a light stimulus (Plautz *et al.,* 1997a).

Peripheral clocks are involved in the generation of rhythms that affect local physiology, rather than behaviour. For example, as it was mentioned before, flies display robust variation in their sensitivity to odour and taste stimuli, with the strongest response to odours at night (Krishnan *et al*., 1999, 2008; Tanoue

et al., 2004) and to gustatory stimulation at dawn (Chatterjee *et al*., 2010; Chatterjee and Hardin, 2010). When electrophysiological responses were collected from olfactory sensory neurons (OSNs) or labellar gustatory receptor neurons (GRNs), they clearly revealed circadian rhythms in spike amplitude. These oscillations were both necessary and sufficient for the rhythms in olfactory and gustatory responses, respectively. Presence of spike amplitude rhythms was in turn dependent on the presence of the molecular oscillator in the basiconic and trichoid sensillae (for olfaction) or GRNs (for gustation). Both of these circadian rhythms are mediated by the same molecule, G protein-coupled receptor kinase 2 (GPRK2), which regulate localization of olfactory receptors to a dendritic membrane of OSNs (Emery and Francis, 2008; Krishnan *et al*., 2008; Tanoue *et al*., 2008) and possibly works similar in the case of the gustatory receptors.

1.3.4 Molecular oscillator

Each functional circadian pacemaker can be separated into three elements. A core pacemaker element is the molecular oscillator that generates rhythms in transcription and translation. Since it needs to be entrainable, the second element is required to relay environmental signals and synchronize the oscillator. Finally, a mechanism is needed that uses rhythms generated by the molecular oscillator and generates circadian output in the form of biological processes out of them. These three elements are all required for the proper functioning of the circadian clock. They share components and pathways, making them truly interconnected (Menaker *et al*., 1978).

The simplest molecular oscillator was discovered in cyanobacteria *Synechococcus elongatus* (Figure 1.1 A). It comprises of only 3 proteins (KaiA, KaiB and KaiC) and can be recreated in vitro. Upon the addition of ATP and Mg^{2+} to the system, it is possible to sustain 22-hr period for several days (Nakijama *et al*., 2005; Ishiura *et al*., 1998). This molecular oscillator is unique in respect that it does not regulate transcription in circadian manner. KaiC is the main molecular component and the rhythmicity is linked to changes in phosphorylation status. KaiC forms hexamers and has both kinase and phosphatase activities, which allow it to regulate its own phosphorylation state,

with KaiA and KaiB acting as regulators (Nakijama *et al*., 2005; Ishiura *et al*., 1998).

The molecular oscillator in *Drosophila melanogaster* is more complicated than in cyanobacteria. However, since it bears resemblance to a mammalian molecular oscillator both in terms of the main mechanism and homology of many proteins, it is a powerful model for studying the properties of circadian clocks (Dunlap, 1999).

1.3.4.1 Negative feedback model

The idea of transcription and translation being crucial for generating periodicity came from observation of *period*. Most notably, it was found that *period* mRNA shows daily oscillations in both light: dark (LD) and constant darkness (DD) cycles and the period of those oscillations in DD matches the period (or lack of period) of eclosion and locomotor activity of classical clock mutants (Hardin *et al*., 1990, 1992). Later, the model was expanded and the current understanding is that the molecular oscillator in *D. melanogaster* consists of two interlocked transcription-translation negative feedback loops with complex post-transcriptional modifications, linked by the CLOCK/CYCLE (CLK/CYC) heterodimer (Dunlap, 1999). In the first loop, CLK/CYC regulates the expression of PERIOD (PER) and TIMELESS (TIM), which in turn stop their own transcription by interacting with CLK/CYC. The second loop explains oscillations in *Clock* mRNA. Schematic representation of the interlocked transcription-translation negative feedback loops in *Drosophila melanogaster* is presented in the Figure 1.2 and will be discussed in details in subsequent sections.

1.3.4.2 Major components of the main loop

As described previously, the first gene that was implied to be involved in the molecular oscillator was *period* (Konopka and Benzer, 1971). It took over ten years for *period* to be cloned, but when per^0 mutant arrhythmic flies became rhythmic upon insertion of a fragment of X chromosome, it was a spectacular success (Bargiello *et al*., 1984, Reddy *et al*., 1984; Zehring *et al*., 1984).

PERIOD is a 1224 amino acid protein that contains two Per-Arnt-Sim (PAS) domains, which are a common feature in the basic Helix-Loop-Helix (bHLH) transcription factors, however it lacks a DNA binding domain and thus does not interact with DNA directly (Huang *et al.*, 1993). *per* transcripts show daily patterns of oscillations, with increasing levels during light phase and decreasing levels during night, resulting in a peak in an early evening (Hardin *et al.*, 1990). A similar profile is also observed in the levels of PER protein, with delayed phase resulting in a peak late at night, just before dawn (around ZT0) and trough late in a day, just before dusk (ZT12) (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Zwiebel *et al.*, 1991). In addition to those molecular rhythms, circadian oscillations were also found in the phosphorylation of PER (Edery *et al.*, 1994b) and nuclear transport (Vosshall *et al.*, 1994; Curtin *et al.*, 1995). PER acts as a repressor of its own transcription and levels of *per* are low when PER is over-expressed (Zeng *et al.*, 1994).

The second circadian gene identified in *D. melanogaster* was *timeless* (*tim*), which together with *per* is a crucial part of the molecular oscillator (Sehgal *et al.*, 1994, 1995). It encodes a protein of 1389 amino acids and contains a PAS dimerization domain but no DNA binding domain (Myers *et al.*, 1995). Oscillations of *tim* mRNA have similar phase and amplitude to *per* mRNA (Sehgal *et al.*, 1995). Moreover, mutations to either *tim* or *per* disrupt mRNA cycling of the other one (Sehgal *et al.*, 1994). This suggested that *tim* and *per* work together, with TIM necessary to stabilize PER and contributing to PER nuclear transport (Vosshall *et al.*, 1994; Gekakis *et al.*, 1995). TIM oscillations mimic PER's, however instead of steadily decreasing during light portion, TIM levels dramatically decrease (Hunter-Ensor *et al.*, 1996), suggesting that TIM might be degraded in the presence of light, which would constitute an entrainment mechanism (Zeng *et al.*, 1996).

Study of both *tim* and *per* has revealed that promoter elements driving their expression contain a 69 base pair Circadian Regulatory Sequence that is necessary for driving the rhythmic transcription (in *per*) or ensuring high transcription levels (in *tim*). This promoter sequences contain E-box (Enhancer box) binding sites (CACGTG) (Hao *et al.*, 1997 and 1998; Darlington *et al.*, 1998; McDonald *et al.*, 2001) that are recognized by proteins containing bHLH (Sogawa *et al.*, 1995, Swanson *et al.*, 1995). Neither PER nor TIM has such

domain (Huang *et al*., 1993), so there had to exist an additional member of the molecular oscillator that would be activating their expression.

In fact, soon two members of the bHLH family were found that also contained PAS domain – CLOCK (Circadian Locomotor Output Cycles Kaput, originally called JERK and later renamed to CLOCK after homology to murine CLOCK was discovered) and CYCLE (homologue of mammalian BMAL1) (Allada *et al*., 1998; Darlington *et al*., 1998; Rutila *et al*., 1998). *Clk* mRNA and CLK proteins levels also show daily oscillations in antiphase to *per* and *tim*, with the peak early during a day (Glossop *et al*., 1999). Since *per* and *tim* mRNA levels are severely decreased in both dominant negative Clk^{Jrk} (Allada *et al*., 1998) and null cyc^{0} (Rutila *et al*., 1998) mutant, the role of CLK and CYC was proposed as positive regulators of *per* and *tim* expression. This was later confirmed by showing that CLK and CYC can not only bind to E-boxes (Lee *et al*., 1999) but also directly activate the expression of transgenic elements connected to *per* and *tim* promoter elements in S2 cells, however this ability is greatly reduced in the presence of PER and TIM (Darlington *et al*., 1998). Microarray studies have suggested that almost all genes that show circadian pattern of transcription were either directly or indirectly regulated by CLK, making it the main circadian protein (McDonald and Rosbash, 2001). It has been recently suggested that some proteins, such as C-TERMINAL BINDING PROTEIN (CtBP) might be co-factors necessary for a proper CLK/CYC activity, possibly co-activating it through E-box sequence (Itoh *et al*., 2013).

1.3.4.3 Molecular oscillations in the major oscillator loop

CLK and CYC form a heterodimer and activate daily transcription of target genes, among them core clock genes *per* and *tim*. While *tim* mRNA appears short-lived, the delay between rhythms of *per* transcription and mRNA suggest delay in *per* mRNA accumulation (So and Rosbash, 1997). *per* mRNA was found to be more stable late in the day and less stable during mid and late night. In addition to this, promotorless *per* transgene can produce low amplitude mRNA oscillations even though there are no rhythms to transcription (Frisch *et al*., 1994; So and Rosbash, 1997). The exact mechanism regulating stability and oscillations in *per* mRNA is yet to be discovered, but it was suggested that it involves newly synthesised PER and TIM forming a dimer. Partial evidence for

this comes from the discovery that in the *tim*^{0} background, *per* mRNA can be stabilized by native PER and transgenicaly expressed TIM (Suri *et al*., 1999).

PER and TIM accumulate in a cytoplasm, where TIM stabilizes PER by forming a stable heterodimer through the dimerization Per-Arnt-Sim (PAS) domain (Huang *et al*., 1993; Vosshall *et al*., 1994; Gekakis *et al*., 1995; Myers *et al*., 1995; Saez and Young, 1996; Zeng *et al*., 1996) and protects PER from degradation (Price *et al*., 1995; Kloss *et al*., 2001). The phase delay between PER and TIM oscillations and oscillations of their transcripts is a result of post-translational modifications that are a crucial part of the molecular oscillator for the determination of the pace of the circadian rhythm (Edery *et al*., 1994b; Zeng *et al*., 1996).

The first kinase identified in the genetic screens for circadian modulators was DOUBLETIME (DBT, sometimes also DCO) (Kloss *et al*., 1998; Price *et al*., 1998; Ko *et al*., 2002), which is a homologue of important kinases in mammalian clock – CK1ε (Lowrey *et al*., 2000; Xu *et al*., 2005; Etchegaray *et al*., 2009). DBT binds to PER between the residues 755-809 (in so called Period Doubletime Binding Domain – PDBD, Kim *et al*., 2007) and 762-788 (Nawathean *et al*., 2007) and causes PER phosphorylation at the Serines: 44, 45, 47, 48, 151, 153 and 589 (Kloss *et al*., 2001, Chiu *et al*., 2008; Kivimae *et al*., 2008). The phosphorylation of PER triggers its subsequent degradation that requires presence of SUPERNUMERARY LIMBS (SLIMB), an F-box protein recognizing phosphorylated substrates and a component of the SCF (SKP1/CUL1/F-box) complex that acts as a E3-ubiquitin ligase (Grima *et al*., 2002; Ko *et al*., 2002; Cyran *et al*., 2005). The F-box protein is what determines the specificity of SCF but the CULLIN (CUL1) is responsible for the activation of the complex through neddylation (action of ubiquitin-like peptide NEDD8). Another complex, CSN (COP9 Signalosome), can cause deneddylation of CULLIN and therefore repress the activity of SCF (Cope and Deshaies, 2003). In the absence of functional CSN, the light-dependent TIM degradation is suppressed (Knowles *et al*., 2009).

It was suggested that the phosphorylation of the PER sites occurs after initial phosphorylation by NEMO and is spatially coordinated to create some delay to the phosphorylation of more distal sites, delaying the degradation (Chiu *et al*., 2008). Therefore DBT is crucial for regulating the time of a nuclear entry of PER. Since TIM is protecting PER from the DBT-mediated phosphorylation and

subsequent degradation (Kloss *et al*., 2001), when TIM becomes rapidly degraded upon light exposure, PER becomes vulnerable and its levels slowly drop, which explains the difference between PER and TIM abundance patterns (Hunter-Ensor *et al*., 1996). In the absence of functional DBT, accumulation and nuclear translocation and transcription repression can occur even in the absence of TIM (Price *et al*., 1998; Suri *et al*., 2000; Cyran *et al*., 2005).

Another kinase involved in the phosphorylation of PER and its nuclear entry is CASEIN KINASE II (CKII), a Serine/Threonine kinase which comprises of two catalytic and two modulatory P subunits (Lin *et al*., 2002; Akten *et al*., 2003). CKII phosphorylates PER (on Serine 151 and 153, Lin *et al*., 2005) and also promotes CLK repression (Nawathean and Rosbash, 2004). However, since the same residues can be phosphorylated by DBT (Kivimae *et al*., 2008) and the phenotype observed when CKII is supressed is more severe that the phenotype resulting from mutations at either of those sites, it is possible that CKII phosphorylates also additional targets in the molecular oscillator (Smith *et al*., 2008). One of those targets is probably TIM, since not only can it be phosphorylated in vitro (Zeng *et al*., 1996; Lin *et al*., 2002) but also in CKII mutants TIM nuclear translocation is affected (Lin *et al*., 2002; Akten *et al*., 2003) and TIM has increased stability (Meissner *et al*., 2008). In addition, when a part of TIM containing 4 putative phosphorylation sites is removed, TIM appears hypophosphorylated (Meissner *et al*., 2008) and the free-running period of flies is increased (Ousley *et al*., 1998). CKII can physically interact with another kinase, p90 RIBOSOMAL S6 KINASE (S6KII) (Kusk *et al*., 1999; Akten *et al*., 2009). S6KII has opposite effects on circadian period than CKII –in absence of a functional S6KII period was shortened, *per* mRNA levels lowered while PER levels elevated, which might suggest increased CLK repression by PER (Akten *et al*., 2009). Therefore it has been proposed that S6KII might physically interact with CKII and inhibit its function (Akten *et al*., 2009).

Third kinase important for the determination of circadian pace is SHAGGY (SGG), homologue of the Glycogen Synthase Kinase-3β (GSK3β), discovered in the misexpression studies. Over-expression of SGG severely decreased period length, caused hyperphosphorylation of TIM and advanced nuclear entry of PER and TIM (Matinek *et al*., 2001; Fang *et al*., 2007). SGG is critical during embryonic development but flies with decreased SGG levels have increased period and constantly hypophosphorylated TIM that has impaired oscillations

(Martinek *et al*., 2001). Based on these results it was suggested that TIM is a target of SGG. However, it appears that SGG can also phosphorylate PER on Serine 657 (after phosphorylation of Serine 661 by unidentified kinase) (Ko *et al*., 2010). In fact, it was suggested that several PER phosphorylation events require presence of an earlier phosphorylation, forming a kind of phosphorylation chain (Garbe *et al*., 2013).

In addition to kinases described above, time of nuclear translocation of PER, its phosphorylation and stability is also regulated by phosphatases. PROTEIN PHOSPHATASE 2A (PP2A) stabilizes PER and decreases the period length (as a result of faster nuclear translocation) (Sathyanarayanan *et al*., 2004). It acts antagonistically to DBT on PER phosphorylation but could be also involved in dephosphorylation of CLK (Kim and Edery, 2006). Similarly, PROTEIN PHOSPHATASE 1 (PP1) promotes nuclear translocation, however it removes phosphate groups from TIM rather than PER (Fang *et al*., 2007). It was suggested that PP1 does not simply counterbalance circadian kinases but rather interacts with SGG, either by PP1 dephosphorylating SGG targets on PER or SGG regulating PP1 ability to remove phosphate groups introduced by other kinases, such as CKII (Fang *et al*., 2007).

TIM is crucial for the nuclear entry of PER and DBT (Vosshall *et al*., 1994; Saez and Young, 1996), however, when PER levels are elevated, PER can enter the nucleus even in the absence of TIM (Cyran *et al*., 2005). TIM contains the 14 amino acid sequence called Nuclear Location Signal (NLS) that is not required for nuclear translocation but the lack of it increases period length due to delayed nuclear entry (Saez *et al*., 2011). It is not fully understood if PER enters the nucleus together with TIM, as PER is found in nuclei of circadian neurons before TIM (Shafer *et al*., 2002) and FRET (Fluorescence Resonance Energy Transfer) studies have revealed that PER and TIM enter nucleus separately (Meyer *et al*., 2006). However, since PER and TIM are still present as a heterodimer in perinuclear foci (Meyer *et al*., 2006) and PER mutants with defective homodimerization domain have impaired nuclear localisation (Landskron *et al*., 2009), it was postulated that TIM is important for cytoplasmic catalysis of nuclear entry of the homodimers. The function of TIM inside the nucleus is believed to be to stabilize the interactions between PER and CLK and partially compensate for absence or dysfunction of the CBD (CLOCK Binding Domain) in PER (Sun *et al*., 2010).

PER binds to CLK in nucleus during subjective night and represses transcriptional activity of CLK/CYC complex, leading to decreased transcription of *per* and *tim* (Darlington *et al*., 1998; Lee *et al*., 1998, 1999; Bae *et al*., 2000, Rothenfluh *et al*., 2000). C terminus of PER was shown to contain the CCID (CLK/CYC inhibitory domain) that allows PER to repress CLK/CYC function (Chang and Reppert, 2003). This domain was further characterized and more discrete CBD has been found (Sun *et al*., 2010), which is in fact responsible for binding CLK.

When PER binds to CLK, it displaces it from E-box (Lee *et al*., 1999), which explains a circadian rhythm in the CLK/CYC occupation of *per* promoter with the strongest presence when *per* levels are high and no presence when PER is in nucleus (Yu *et al*., 2006). Recently it was also suggested that PER either coordinates recruitment or activates some corepressors, or inhibits the recruitment or activity of coactivators as PER was found associated with chromatin together with CLK/CYC before they both leave the E-box (Menet *et al*., 2010). It is quite important to realize that TIM is not required for the PER repression of CLK/CYC (Rothenfluh *et al*., 2000; Nawathean and Rosbash, 2004).

One of the proposed mechanisms explaining how PER displaces CLK from E-box is through the DBT-mediated phosphorylation, which is supported by rhythms in a phosphorylation state of CLK (Kim *et al*., 2002 and 2006; Lee *et al*., 1998; Yu *et al*., 2006). The highest phosphorylation of CLK corresponds with the time it is detached from DNA and with its lowest stability (Kim and Edery, 2006; Yu *et al*., 2006; Menet *et al*., 2010).This requires DBT to come in contact with the CLK, which is possible since the DBT domain necessary to initiate repression is also part of the CCID (Chang and Reppert, 2003; Kim *et al*., 2007; Navathean *et al*., 2007; Yu *et al*., 2009). In support of this hypothesis, flies with mutated phosphorylation sites on CLK either had accelerated molecular rhythms, shortened behavioural rhythms or accelerated transcriptional activation, which confirms the impact of CLK phosphorylation on circadian rhythms (Mahesh *et al*., 2014). Interestingly, it is not DBT that causes phosphorylation of CLK but rather it is responsible for recruitment of other kinases (Yu *et al*., 2006, 2009). Therefore, the second role of DBT is to regulate transcriptional ability of CLK. Additionally, CKII was shown to decrease the transcriptional activity of CLK/CYC in a PER-dependent manner (Szabó *et*

al., 2013). Moreover, NEMO promotes CLK degradation and might also be involved in the phosphorylation of CLK (Yu *et al*., 2011).

In nucleus, PER is phosphorylated by DBT following the same phosphorylation cascade as described previously (Kloss *et al*., 1998; Price *et al*., 1998; Chiu *et al*., 2011). Appearance of phosphate group on Serine 47 leads to creation of the binding site for E3 ubiquitin ligase SLIMB and subsequent degradation of PER (Chiu *et al*., 2008 and 2011; Kivimae *et al*., 2008; Garbe *et al*., 2013). This sequence of events closes the loop of the molecular oscillator as in the absence of PER CLK/CYC can accumulate in the nucleus and activate the transcription of circadian genes again.

One of the other genes which transcription is activated by CLK is CLOCKWORK ORANGE (CWO) (Kadener *et al*., 2007), named because of the bHLH motif within its Orange domain (Lim *et al*., 2007a). It serves a role of a modulator of the function of the molecular oscillator rather than being a key element, as in response to reduced CWO levels only some elongation of period length and decrease in amplitude of rhythms were observed (Kadener *et al*., 2002; Lim *et al*., 2007a; Matumoto *et al*., 2007; Richier *et al*., 2008). Its promoter element contains E-box similar to that in *per* or *tim* (Kadener *et al*., 2007). Unlike PER and TIM, however, CWO can bind E-boxes and inhibit transcription of genes from these promoters or outcompete CLK/CYC and prevent it from activating transcription (Kadener *et al*., 2002; Lim *et al*., 2007; Matumoto *et al*., 2007). It also represses its own transcription, however most of the CLK/CYC targets show reduced and blunted peaks for their mRNA profiles for *cwo* loss of function mutants, which suggests that CWO promotes gene activation sometimes and inhibits t at others (Kadener *et al*., 2002; Lim *et al*., 2007a; Matumoto *et al*., 2007; Richier *et al*., 2008).

1.3.4.4 Second feedback loop

The mechanism outlined above does not explain oscillations in *Clk* mRNA (Bae *et al*., 1998; Darlington *et al*., 1998). However, two of the genes also expressed due to transcriptional activation by CLK/CYC appear to be involved in this process. *vrille* (*vri*) and *Par-domain protein 1* (*Pdp1*, member of the Leucine Zipper DNA binding proteins subfamily) mRNAs show daily rhythms in

oscillation and are necessary for behavioural circadian rhythms (Blau and Young, 1999; McDonald and Rosbash, 2001; Cyran *et al*., 2003; Glossop *et al*., 2003).

Both VRI and PDP1 bind to the same region in *Clk* promoter element, called V/P box. Upon binding, VRI causes repression of *Clk* transcription (Cyran *et al*., 2003). PDP1ε, one of the six isoforms of PDP1 and the only one expressed in circadian neurons was shown to be involved in a regulation of circadian rhythms and has presumably an antagonistic effect to VRI, activating *Clk* expression (Reddy *et al*., 2000; Cyran *et al*., 2003; Zheng *et al*., 2009). PDP1 levels peak 3-6 hours after VRI peaks, providing a necessary regulatory mechanism for *Clk* expression in which activation of *Clk* happens when concentration of PDP1ε is high enough to replace VRI at *Clk* promoter (Cyran *et al*., 2003).

In addition to being involved in the main molecular oscillator, it was also suggested that PDP1ε is involved in connecting molecular oscillator to downstream behavioural outputs (Lim *et al*., 2007c). The importance of PDP1ε for the generation of molecular rhythms was later questioned as silencing PDP1ε expression using dsRNA resulted in fairly normal molecular rhythms but arrhythmic behaviour (Benito *et al*., 2007). This was independently confirmed two years later as transgenic expression of CLK in circadian neurons did not rescue the behavioural defects caused by PDP1ε dysfunction (despite restoring PER expression and molecular rhythms), suggesting that PDP1ε links the core molecular oscillator and output pathways as well as being involved in the oscillator itself, though it does not regulate *Clk* expression (Zheng *et al*., 2009). Interestingly, the role of the second loop in the generation of output rhythms might be confirmed by the fact that either over-expressing VRI or mutating PDP1ε resulted in a lower expression of neurotransmitter PDF that has been implied in a generation of output rhythms (Blau and Young, 1999; Zheng *et al*., 2009).

In addition to circadian regulation of *Clk* expression and post-translational regulation of CLK function described above, *Clk* mRNA undergoes post-transcriptional regulation (Lerner *et al*., 2015). The genomic region that is necessary for this is 3' UTR (Lerner *et al*., 2015). *Clk* can also be regulated by

miRNA *bantam,* which causes decrease in CLK levels (Kadener *et al.*, 2009, Lerner *et al.*, 2015).

This second loop of the molecular oscillator appears less important than the primary one. Disruption to rhythmic *Clk* expression resulted in limited impact on circadian rhythms, suggesting that oscillations in *Clk* mRNA are not required for generation of circadian rhythms (Kim *et al.*, 2002).

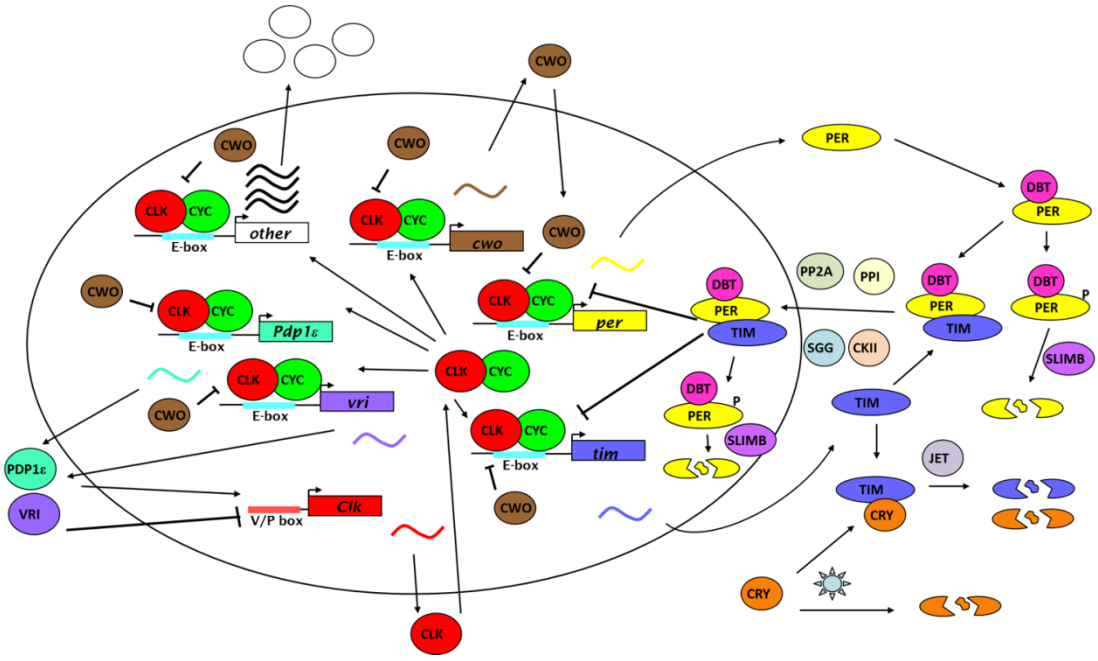


Figure 1.2. Schematic representation of the molecular oscillator in *D. melanogaster.*

Large oval represents nucleus and processes that take place inside: the space around it represents cytoplasm. Small ovals represent proteins, the rectangles represent genes and wavy lines represent mRNA. When an arrow points to a gene, it indicates transcriptional activation; in contrast, a blunt line pointing to a gene represents transcriptional repression. Other arrows are used to represent progression of events. Refer to main text in 1.3.4 for explanation of components and the steps in a pathway.

1.3.4.5 Is transcription necessary for the molecular oscillator?

The molecular oscillator of *D. melanogaster* described here is clearly more complex than the one in cyanobacteria (Figure 1.1). However, one of the most important implications of the generation of rhythms in cyanobacteria is the notion that maybe rhythmic transcription is not required for circadian rhythms (Rosbash, 2009). This is partially supported by the studies in mammalian fibroblasts that have shown that circadian rhythms can be sustained (with a shorter period) even in the absence of fully functional Polymerase II (Dibner *et al*., 2009). Additionally, circadian rhythms in the locomotor rhythmicity of flies could be obtained even in the presence of constant *per* and *tim* levels (Yang and Sehgal, 2001). This is not conclusive, however, as rhythmic expression of other genes (such as *Clk*) might be enough for sustaining molecular oscillations. Moreover, rhythms that were observed with constant *per* and *tim* were not as robust as when they were expressed rhythmically (Hall *et al*., 2007).

1.3.4.6 Molecular oscillator in the periphery

The previously described peripheral oscillators (1.3.3) make use of exactly the same molecular machinery. In fact, many discoveries on organization of the oscillator come from the study of peripheral clocks rather than circadian neurons. There are, however, some differences between the molecular oscillator in a brain and periphery. First, a phase of the various molecular oscillations is different in circadian neurons and various other tissues (Levine *et al*., 2002a). This should not be surprising, given that even different groups of neurons have different phases of oscillation (Kaneko *et al*., 1997) and might reflect an adaptation of different cells and tissues to generating various rhythms.

Another difference between the function of the main molecular oscillator in a brain and periphery is that while the molecular oscillations in a brain persist and sustain rhythmic behaviour for many days in constant darkness, in peripheral tissue rhythms tend to vanish after couple days in constant darkness. It was postulated that a lack of robustness of the peripheral oscillators could be to blame and this could be due to a function of

CRYPTOCHROME (CRY), which might be acting differently in both systems (Krishnan *et al*., 2001; Levine *et al*., 2002a). However, it is more probable that molecular oscillations persist in each cell of a tissue, however they slowly become desynchronised from each other (Nagoshi *et al*., 2004).

1.3.5 Resetting the clock

In most cases, organisms receive environmental time cues from multiple periodicities existing in nature. Two main ones are light/dark cycles and temperature changes (Millar, 2004; Salomé and McClung, 2005). These environmental time cues are called Zeitgebers ("time givers" in German, name coined by Aschoff) and they are responsible for keeping endogenous biological clocks synchronised to the exogenous period of rotation of the Earth around its axis, which is 24 hours (Aschoff, 1960). The entrainment does not happen instantaneously and a time necessary to completely synchronize the circadian rhythm to an environmental cycle depends on a Zeitgeber used, a shift in phase that needs to occur and a type of rhythm that needs entrainment.

There are, however, situations when an external stimulus will not cause a shift in rhythm but only temporary change in the circadian rhythm. This phenomenon is known as masking. Masking can be caused by stimulus acting on certain behaviour rather than on the circadian clock.

1.3.5.1 Light as Zeitgeber

Light is one of the most common and strongest Zeigebers. Even pulses of light as short as 1 minute long can cause a phase shift of up to 4 hours (Egan *et al*., 1999; Busza *et al*., 2004). Even in the input from other contradictory Zeitgebers, organisms preferentially entrain to light (Pittendrigh, 1960). *D. melanogaster* is extremely sensitive to light (Hirsh *et al*., 2010) and even LD cycles with the intensity of 0.03 lux, equivalent to quarter-moon light can entrain flies (Bachleitner *et al*., 2007).

Resetting the clock through light depends on a time of day when a light pulse is administered, which was first demonstrated by Hastings and Sweeney (1958)

in *Gonyaulx polyhedral*. When a light pulse occurs late at subjective night (before the dawn), it advances the clock by mimicking earlier morning. If the light pulse occurs early at night (after the dusk), it delays the phase as if a light portion of day was extended (Hastings and Sweeney, 1958). Light pulse has very limited to no effect during a subjective day. Therefore it appears that sensitivity to a stimulus is regulated by the clock itself. To graphically represent a sensitivity of an organism to stimuli at various times of a day, a phase response curve (PRC) can be plotted. It shows a phase shift in response to a stimulus applied at different times (Pittendrigh, 1967; Levine *et al*., 1994; Suri *et al*., 1998; Dunlap *et al*, 2004).

The light that achieves the most efficient phase shifts in a short duration of time has a wavelength in the blue range (Frank and Zimmerman, 1969; Zimmerman and Goldsmith, 1971; Klemm and Ninnemann, 1976; Suri *et al*., 1998). This is because a blue light causes the most effective TIM degradation in response to light (Suri *et al*., 1998). This, among other evidence, might suggest that the molecular oscillator of flies can be reset by a rapid degradation of TIM as a response to light (Hunter-Ensor *et al*., 1996; Lee *et al*., 1996; Myers *et al*., 1996; Zeng *et al*., 1996; Suri *et al*., 1998; Yang *et al*., 1998; Naidoo *et al*., 1999). Without TIM, PER is vulnerable and becomes phosphorylated and subsequently degraded. If this degradation occurs before midnight, when PER is still accumulated in the cytoplasm, progression of the clock is delayed because PER still needs to enter nucleus. On the other hand, if the degradation of TIM occurs later at night, when PER was already in the nucleus, light advances the clock as CLK/CYC inhibition is removed early (reviewed by Stanewsky, 2003). It is also worth noting that this mechanism is cell autonomous and exists both in the molecular oscillator in the brain and in the peripheral tissue (Plautz *et al*., 1997a and 1997b). However, it is also tissue-specific, meaning that if a photoreception mechanism functions in a particular tissue only, the molecular oscillations and output circadian rhythms only in that tissue are functioning (Emery *et al*., 2000b).

For an entrainment or shift in a locomotor activity or eclosion, information on a light signal or phase has to reach circadian neurons. Light information can be passed to circadian lateral neurons either via a cell-autonomous circadian blue light photoreceptor CRYPTOCHROME (CRY) (Emery *et al*., 1998; Stanewsky *et al*., 1998) or from retinal and extraretinal photoreceptor cells (Stanewsky *et al*.,

1998). In the first case, a light-activated CRY in lateral neurons (LNs) interacts with TIM and initiates a degradation process via a proteasome (Busza *et al.*, 2004; Naidoo *et al.*, 1999). In the second case, TIM degradation also occurs, after light information is passed on to LNs.

In adult *D. melanogaster* visual photoreceptor cells can be found in a retina, ocelli and in an extraretinal structure known as Hofbauer-Buchner eyelet (Helfrich-Förster *et al.*, 2001; Rieger *et al.*, 2003; Veleri *et al.*, 2007). Eyelet photoreceptors send their projections directly to LNs, suggesting that the main function of an eyelet is a clock entrainment (Helfrich-Förster *et al.*, 2001 and 2002). The ocelli were shown to project to Pars Intercerebralis (PI) and compound eyes are signalling to l-LN_vs (Helfrich-Förster *et al.*, 2001; Veleri *et al.*, 2007; Umezaki and Tomioka, 2008).

There is a certain degree of redundancy to both of these pathways, since flies lacking either a functioning visual system or a functional CRY can entrain their locomotor rhythmicity to light but flies lacking both pathways cannot, resulting in flies free-running through the LD cycle (Wheeler *et al.*, 1993; Helfrich-Förster *et al.*, 2001). Interestingly, CRY expression patterns connect both mechanisms, since it is expressed both in photoreceptor cells in a compound eye and in some circadian neurons (Yoshii *et al.*, 2008). This does not mean, however, that there are no alternative light-input pathways, as molecular oscillations in isolated antennae and Malphigian tubules can be synchronised even in an absence of CRY (Krishnan *et al.*, 1999; Ivanchenko *et al.*, 2001; Dolezelowa *et al.*, 2007) and some dorsal neurons retain synchrony to LD cycles even in flies missing photoreceptors and CRY (Veleri *et al.*, 2003).

CRY is a molecule sensitive to blue light and UV belonging to a family including also photolyases - DNA repair enzymes (Kanai *et al.*, 1997; Cashmore, 2003; Ozturk *et al.*, 2007). CRY was first identified as a photoreceptor in plants (Kasemir, 1997) and later homologues were found in insects and mammals (Cashmore, 2003), although mammalian CRYs (type II CRYs) do not work as circadian photoreceptors even though they can probably still sense light (Hattar *et al.*, 2003; Panda *et al.*, 2003; Tu *et al.*, 2004; Hoang *et al.*, 2008), but rather are a component of the main oscillator (Kume *et al.*, 1999; van der Horst *et al.*, 1999).

CRY found in *D. melanogaster* is a type I CRY (Yuan *et al*., 2007). It is expressed in s-LN_vs, l-LN_vs and a subset of LN_ds and DN1s (in both DN1a and six DN1p), but not DN2s and DN3s (Benito *et al*., 2008; Yoshii *et al*., 2009a). CRY's role as a main circadian photoreceptor has been confirmed since its over-expression made the circadian clock more sensitive to light pulses (Emery *et al*., 1998; Emery *et al*., 2000b) and hypomorphic (*crybaby*) or null alleles (cry^{01}) had unusually stable TIM in a presence of light and displayed behavioural rhythmicity in constant light (Stanewsky *et al*., 1998; Emery *et al*., 2000a and 2000b; Dolezelowa *et al*., 2007). In contrast, wild-type flies are displaying behavioural arrhythmicity in constant light due to persistent low levels of TIM. Moreover, tissue-specificity of CRY was demonstrated, meaning that the rescue of CRY in a specific tissue was able to restore molecular rhythms in that tissue only (Emery *et al*., 2000b). For example, restoring CRY in circadian neurons restores a photoperiodic response in behaviour but no oscillations in a peripheral tissue. Finally, a spectral sensitivity of CRY, which is sensitive to blue and violet light (Lin *et al*., 2001), explains a sensitivity of TIM and consequently of an entire clock (Suri *et al*., 1998; Busza *et al*., 2004; Van Vickle-Chavez and Van Gelder, 2007; Hoang *et al*., 2008).

There are two different models proposed for a CRY photosignalling. One model of CRY action suggested that in response to light, there is a redox change of the flavin chromophore which is a cofactor of the CRY. There are 4 tryptophans involved in this: 342, 397 and 420 that form so called Trp triad and 536 (Froy *et al*., 2002; Sancar, 2003; Chaves *et al*., 2011; Zoltowski *et al*., 2011; Levy *et al*., 2013; Czarna *et al*., 2013) that can act as electron donors independently. The charge transfer through a Trp triad or Trp-536 causes the flavin to change from a two-electron oxidized form to anionic semiquinone (Song *et al*., 2007; Ozturk *et al*., 2008; Hoang *et al*., 2008; Vaidya *et al*., 2013). Since this electron transfer resulted also in generation of a separated radical pair, CRY was also proposed to be a magnetoreceptor (Gegear *et al*., 2008; Yoshii *et al*., 2009a). However, recently a second model has been suggested in which the redox state of flavin is not important for a conformational change of CRY as the flavin is in a two-electron reduced state all the time (Emery *et al*., 1998; Song *et al*., 2007; Ozturk *et al*., 2008). It is still not known which of these models is correct.

Regardless of the flavin oxidation/reduction change (Ozturk *et al*., 2014), it is agreed that light causes conformation change of CRY, the main part of which is

a detachment of a C-terminal 20 amino acids from the compact photolyase homology region (PHR) (Dissel *et al*., 2004; Ozturk *et al*., 2011). The PHR is an active domain of CRY while the C-terminal tail is modulating its activity; this is contrary to the process in plants where photolyase domain modulates C-terminal tail that is an active domain (Yang *et al*., 2000; Dissel *et al*., 2004). This conformational change in turn promotes binding of CRY to TIM (a process that does not depend on PER presence in flies, in contrast to yeast and tissue cell culture) (Ceriani *et al*., 1999; Rosato *et al*., 2001; Lin *et al*., 2001; Busza *et al*., 2004; Peschel *et al*., 2009). At the same time, CRY binds two E3 ligases – JETLAG (JET) and RAMSHACKLE (BRWD3)-CRL4 (Koh *et al*., 2006b; Peschel *et al*., 2009; Ozturk *et al*., 2013) that ubiquitinate both TIM and CRY, which makes them more susceptible to proteosomal degradation (Ceriani *et al*., 1999; Naidoo *et al*., 1999; Lin *et al*., 2001; Busza *et al*., 2004). Degradation of TIM is what resets the clock while degradation of CRY is a mechanism by which the clock desensitizes.

Phenotypes of JET and CRY mutants resemble those obtained with a defective CSN (which is responsible for deneddylation of CULLINS in SCF complexes). It was proposed that JET acts downstream of CSN (Knowles *et al*., 2009). However, if CSN is repressing JET, a defective photoresponse observed in CSN mutants is difficult to explain, as more logical output could be hypersensitivity to light. The way to reconcile this was to suggest that JET is also targeted for proteasomal degradation by an extreme CULLIN neddylation (Knowles *et al*., 2009). A defect in a CSN does not affect the molecular oscillator, even though SLIMB (containing a SCN complex) is crucial for PER degradation and setting the pace of the clock, suggesting that levels of SLIMB are more important than those of JET (Knowles *et al*., 2009).

It is important to note that CRY is not necessary for generation of circadian rhythms, making it not part of the core components of the molecular oscillator (Stanewsky *et al*., 1998). However, *cry* mutations can change amplitude of oscillations in the peripheral tissue, suggesting that in some circumstances CRY might be affecting a function of the oscillator (Krisnan *et al*., 1999; Ivanchenko *et al*., 2001). The previously mentioned SGG (1.3.4.3) involved in PER and TIM phosphorylation and degradation making them more sensitive to degradation in response to light is also involved in a degradation of CRY (Martinek *et al*., 2001; Stoleru *et al*., 2007). Most of the time, SGG stabilizes

CRY due to an unknown mechanism. When SGG is over-expressed, a period of circadian rhythms in DD shortens and this phenotype depends on CRY (Stoleru *et al.*, 2007). It has been proposed that CRY can act as PER-dependent repressor of CLK/CYC transcription in peripheral tissue (Collins *et al.*, 2006).

Several genes that might be modulating CRY's photosensitivity have been identified (Dubruille *et al.*, 2009; Benna *et al.*, 2010). A homologue of mammalian TIM called TIM2 (or TIMEOUT) in *Drosophila melanogaster* is one of them (Benna *et al.*, 2010; McFarlane *et al.*, 2010). TIM2 appears to be acting predominantly in optic lobe neurons called T1 basket cells (which might be involved in motion detection (Rister *et al.*, 2007)), however the exact mechanism still remains unknown (Benna *et al.*, 2010). Since T1 cells were shown not to receive input from photoreceptors (Meinertzhagen and O'Neil, 1991), it probably involves the cell-autonomous photoreception mechanism. In addition to its role in photoreception, TIM2 is important for chromosome integrity (Benna *et al.*, 2010).

Another gene involved in the CRY photoreception and affecting TIM degradation is a chromatin remodelling protein KISMET (KIS). KIS interacts with CRY and CRY over-expression can restore the defective light-response phenotype associated with KIS knockdown (Dubruille *et al.*, 2009). One more gene that was shown to impact the photoreception is NOCTURNIN (NOC), which is especially involved in processing light signal early at night (Nagoshi *et al.*, 2010).

1.3.5.2 Temperature as Zeitgeber

Insects, as poikilotherms, are very sensitive to changes in environmental temperature. Therefore it should not come as a surprise that temperature cycles (composed of thermophase and cryophase) can be used as synchronising cues (Pittendrigh, 1954; Wheeler *et al.*, 1993; Glaser and Stanewsky; 2005; Yoshii *et al.*, 2005; Currie *et al.*, 2009). In nature, both temperature and light cycles work together to synchronize circadian rhythms (Boothroyd *et al.*, 2007; Yoshii *et al.*, 2009b). Temperature cycles are a weaker Zeitgeber than light; when flies were moved from LD cycles to temperature cycles with a phase shifted by 6 hours, they required several days to adjust to

the new phase (Currie *et al.*, 2009). Molecular and behavioural rhythms in response to artificially imposed light and temperature cycles have phases shifted by several hours, which is consistent with the fact that in nature there is some delay between a temperature cycle and a light cycle (Boothroyd *et al.*, 2007). Temperature entrainment is cell-autonomous (and probably involves cell-autonomous sensing molecules) as isolated body parts can be entrained to temperature cycles (Glaser and Stanewsky, 2005). However, recently the existence of a non-autonomous mechanism has also been suggested, since isolated brains do not entrain to temperature cycles (Sehadova *et al.*, 2009). This is very similar to mammals, where brains are similarly insensitive to temperature cycles (Buhr *et al.*, 2010).

The mechanism governing temperature synchronization is less explored than that of light entrainment. The first protein that was shown to be involved in temperature entrainment is NO CIRCADIAN TEMPERATURE ENTRAINMENT (NOCTE) (Glaser and Stanewsky, 2005). NOCTE mutants have impaired temperature entrainment of both molecular and behavioural rhythms measured in constant light. Even though an exact role of NOCTE is not known, its broad expression pattern makes it a good candidate for the role of main temperature sensor or at least a member of the temperature entrainment pathway (Sehadova *et al.*, 2009). Since circadian neurons are not sensitive to temperature cycles, a synchronisation of behaviour happens through NOCTE expression in other tissue – chordotonal organs. Interruption of NOCTE expression in this tissue or mutations affecting integrity of chordotonal organs disrupts temperature entrainment (Kwon *et al.*, 2010).

Another potential component of the thermal transduction pathway is NO RECEPTOR POTENTIAL A (NORPA). It is a phospholipase C that is involved in visual phototransduction and its mutations cause disappearance of entrainment to temperature cycles in constant light (LL) and loss of synchrony in the periphery (Glaser and Stanewsky, 2005). The final confirmation of the involvement of NORPA would be confirming its expression in chordotonal organs, which were previously linked to temperature entrainment (Kwon *et al.*, 2010), but it has not been demonstrated yet.

Unexpectedly, photosensor CRY has also been shown to be involved in temperature sensing. In a similar way as the clock can be synchronised to short

pulses of light, it can also be synchronised to a pulses of 37˚C lasting 30 minutes (temperature pulses have to be over 34˚C in order to elicit any response) (Edery *et al.*, 1994a; Kaushik *et al.*, 2007). Even though these pulses cannot advance the phase, delays of up to 2 hours follow temperature pulses at early night. These responses are dependent on CRY which can interact with TIM/PER only after pulses in early night (Kaushik *et al.*, 2007). Interestingly, a mutant was found that has both a lower temperature threshold for phase shifts (30˚C) and can show phase advance – it was one of the original circadian mutants described, per^{L}. It was suggested that this was due to lower threshold for CRY/PER/TIM complex formation in response to temperature pulses. Therefore it is possible that temperature pulses change affinity of CRY for PER/TIM by affecting either conformation of CRY or PER/TIM itself (Kaushik *et al.*, 2007).

The argument against this theory is that CRY does not seem to be necessary for responses to temperature cycles over more natural temperature ranges (17-29˚C) both for the molecular and behavioural rhythms (Stanewsky *et al.*, 1998; Glaser and Stanewsky, 2005; Busza *et al.*, 2007). However, some evidence suggests that it is not that simple, as CRY function and its ability to induce behavioural arrhythmicity in constant light varies with temperature (Kaushik *et al.*, 2007).

1.3.5.3 Different Zeitgebers

Of course light and temperature are not the only factors capable of synchronizing circadian rhythms. Not all Zeitgebers that are effective in vertebrates also work in flies. For example, even though restricted food availability was sufficient to shift a phase of circadian behaviour (despite LD cycles with different phases) in mammals (Damiola *et al.*, 2000; Stephan, 2002), in flies it was without effect on the behavioural activity (Oishi *et al.*, 2004) but can drive rhythmic gene expression in the fat body (Xu *et al.*, 2011). When feeding was restricted to the period when food consumption is normally low, an altered phase of gene expression in the fat body caused desynchronization of internal rhythms and was associated with reduced reproductive fitness, as these flies produced fewer eggs (Xu *et al.*, 2011). Another Zeitgeber that was demonstrated only in a particular group of

organisms was sound, which caused changes to circadian locomotor rhythms in house sparrows (Menaker and Eskin, 1966).

Less well described Zeitgebers are mechanical vibrations. Rhythms of vibration and silence (12hrs:12hrs) could synchronize the locomotor activity of flies. This entrainment requires a functional molecular oscillator in the brain and chordotonal organs and involves also shifting the phase of PER oscillations in circadian neurons (Simoni *et al*., 2014). Chordotonal receptors, are mechanoreceptors that are located in the joints and work as stretch receptors, mediating vibration and proprioreception, so their involvement in this process was not surprising (Kernan, 2007). It remains possible that there is some interaction between the chordotonal organs' role in the synchronization of the circadian clock through temperature and mechanosensory stimuli. It was suggested that a reason for existence of this mechanosensory input pathway might be to allow flies to stay connected to their own activities induced by stimulus. The locomotor activity changes induced by other Zeitgebers might be sensed by the chordotonal organ and used to reset circadian clocks through universal mechanisms (Simoni *et al*., 2014).

Social cues were also shown to function as Zeitgebers (Levine *et al*., 2002b; Lone and Sharma, 2011a and 2011b and 2012). When two populations of flies were mixed together, the smaller one with a defined phase was able to phase shift a larger one. This phase shift was dependent on olfactory cues, therefore meaning that olfactory cues can be also treated as Zeigebers. It has been proposed that the synchronizing molecules are rhythmically produced pheromones (Levine *et al*., 2002b). Pheromones are produced by oenocytes that have functional circadian clocks and at least one enzyme involved in pheromone production – DESATURASE 1 (DESAT1) is weakly rhythmic and regulates the production of at least 4 cuticular hydrocarbons that are involved in courtship (Krupp *et al*., 2008). It is possible that those are the pheromones causing a response to social interactions. Moreover, social interactions were shown to impact the clock in oenocytes, expression of *desat1* and production of those cuticular hydrocarbons (Krupp *et al*., 2008). Recently, an involvement of the ODORANT RECEPTOR 47b (Or47b) receptor neurons in mediating social interactions has been demonstrated, as their ablation or silencing caused disruptions to the synchronizing role of those cues. Out of circadian neurons, the key neurons driving circadian behaviour - the ventrolateral neurons were

shown not to be involved but there is a possibility that dorsal circadian neurons are involved (Lone and Sharma, 2012).

In order to increase the chances for reproduction, animals might need to synchronize their behaviour to that of their potential mate. Therefore it should not be surprising that circadian rhythms in mating also can be altered by interaction of females and males (Sakai and Ishida, 2001; Fujii *et al*., 2007; Hanafusa *et al*., 2013). Moreover, locomotor rhythms of flies also can be modulated depending on other flies they are housed with – flies that were together with flies of the opposite sex, as opposed to flies kept in groups or couples of the same sex, exhibited brief rest around dusk and increased activity during night and early morning, confirming that courtship can act as Zeitgeber (Fujii *et al*., 2007; Hanafusa *et al*., 2013). Even though sexual interactions were unable to shift the phase of locomotor behaviour, they were capable of shifting the molecular rhythms in DN1 neurons, therefore confirming the role of sexual interactions as a weak Zeitgeber (Hanafusa *et al*., 2013). More detailed analysis has revealed that the impact of sexual interactions depends on the clock oscillations in both brain and antennae, especially in males, suggesting that the synchronizing cue is originating in females and received by males through olfaction (and potentially other sensory organs), again suggesting some involvement of pheromones and oenocytes (Fujii *et al*., 2007).

1.3.6 Comparison of the oscillator in flies and mammals

The circadian clock of *D. melanogaster* shows a high degree of similarity to the mammalian clock. The master pacemaker that coordinates rhythmic behaviour and physiology of mammals is the Suprachiasmatic Nuclei (SCN) located in the hypothalamus just above the optic chiasm (Moore and Eichler, 1972; Stephan and Zucker, 1972; Ibuka and Kawamura, 1975). SCN is important for the synchronization of peripheral oscillators that can sustain their oscillations even independently of SCN for days or even weeks (Yoo *et al*., 2004; Hastings *et al*., 2005; Reddy *et al*., 2005). Interestingly, the oscillator in the liver can desynchronise from SCN by as much as 12 hours if an animal has restricted access to food (Damiola *et al*., 2000; Hara *et al*., 2001).

The biggest differences between both systems are in the exact components of the molecular oscillator, however a transcription-translation negative feedback model is common between flies and mammals (Vansteensel *et al.*, 2008; Brown *et al.*, 2012). In the mammalian oscillator, two functional homologues of CLK exist: CLOCK and NPAS2 (NEURONAL PAS DOMAIN-CONTAINING PROTEIN 2) (Antoch *et al.*, 1997; Hida *et al.*, 2000; Takekida *et al.*, 2000; Reick *et al.*, 2001; DeBruyne *et al.*, 2007). The homologue of CYC is called BMAL1 (BRAIN AND MUSCLE ARNT-LIKE PROTEIN) and in contrast to CYC it has a transcriptional activation domain (Gekakis *et al.*, 1998; Bunger *et al.*, 2000). There is not one but three homologues of PER and two of them are crucial for the generation of circadian rhythms (Bae *et al.*, 2001). The degradation of the PERs is mediated through P-TRANSDUCIN REPEAT-CONTAINING PROTEIN (P-TRCP), a homologue of SLIMB (Schirogane *et al.*, 2005; Reischl *et al.*, 2007). In contrast to flies' oscillator, the partner of PERs is CRY1 and CRY2 (Griffin *et al.*, 1999; Kume *et al.*, 1999). Even then, the function of CRYs is different than TIM in flies as CRYs are a main repressor of CLOCK/BMAL1 (Kume *et al.*, 1999).

The main component of the molecular oscillator in mammals, BMAL1/2, forms dimers with CLOCK or NPAS2 (Gekakis, 1998; Reick *et al.*, 2001). This dimer binds to E-boxes and activates the transcription of *Period* (*Per1*-3) and *Cryptochrome* (*Cry1*/2) (Yoo *et al.*, 2005). PER and CRY form complexes in cytoplasm and later translocate to the nucleus where they stops their own transcription by inhibiting CLOCK/BMAl1 (Van Der Horst *et al.*, 1999; Zheng *et al.*, 2001; Yu *et al.*, 2002). PER/CRY is subsequently phosphorylated by CASEIN KINASE Iε (CKIε) and ubiquitinated and degraded by proteasome (Eide *et al.*, 2005; Siepka *et al.*, 2007). This removes an inhibition of CLOCK/BMAL1 allowing the cycle to start again.

In contrast to their role in flies, CRYs are not photoreceptors and photoreception is based on opsins (in retina that are expressed in rods and cones) and melanopsins (Hattar *et al.*, 2003; Panda *et al.*, 2003). There is also mammalian TIM; however it is more similar to TIM2, another TIM protein from the *D. melanogaster*'s molecular oscillator that plays a role in the maintaining chromosomal integrity and light response (Sangoram *et al.*, 1998; Benna *et al.*, 2010). The role of TIM in the circadian oscillator in mammals is still questioned (Gotter *et al.*, 2000; Tischkau and Gillette, 2005) as it might be functioning as a link between the oscillator and cell cycle and checkpoints of DNA damage

through interacting with CRY2 (Unsal-Kacmaz *et al.*, 2005). The mammalian clock also has an equivalent of DBT (CK1ε) and CKII that are involved in the regulation of circadian pace (Lowrey *et al.*, 2000; Xu *et al.*, 2005; Etchegaray *et al.*, 2009; Maier *et al.*, 2009; Lee *et al.*, 2009a).

1.4 Neuroanatomy of the circadian clock

The main molecular oscillator driving locomotor behaviour is located in the brain. It was speculated that it would be possible to find a discrete structure in the brains of flies, similar to what was discovered in mammals in the form of Suprachiasmatic Nuclei (SCN). Studies of an expression of circadian protein, especially PER, have been conducted in order to identify that structure and they have revealed that *per* is expressed in many cells, however it shows oscillations only in about 75-80 cells per brain hemisphere (unless otherwise noted, all numbers of neurons described below are per hemisphere) forming a couple of the cell groups (Ewer *et al.*, 1992; Helfrich-Förster, 1995; Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Schafer *et al.*, 2006). *per* expression in those neurons is necessary and sufficient for generating locomotor activity rhythms in the absence of environmental cues (Ewer *et al.*, 1992; Frisch *et al.*, 1994; Grima *et al.*, 2004). Based on their location, these cells can be divided into seven groups falling into two categories: Lateral Neurons (LN) or Dorsal Neurons (DN).

1.4.1 Different classes of circadian neurons

Lateral Neurons could be divided into four groups. There are 4-6 large ventrolateral neurons (l-LN$_v$s) and five small ventrolateral neurons (s-LN$_v$s) which differ, among other factors, by a size of their soma (Kaneko and Hall, 2000; Helfrich-Förster, 2005). All l-LN$_v$s and 4 out of 5 s-LN$_v$s express PIGMENT DISPERSING FACTOR (PDF) and one s-LN$_v$, called 5th s-LN$_v$ is PDF-negative. These cells were shown to be the most important for generation of circadian rhythms in constant darkness.

l-LN$_v$s send some of their projections ipsilaterally to the optic lobe and arborize ventrally forming the ventral elongation. They also project contralaterally

through a posterior optic tract to an opposite optic lobe (Park and Griffith, 2006; Helfrich-Förster *et al*., 2007b). s-LN_vs have short neurites in accessory medulla (aMe) and are sending their long projections into dorsal protocerebrum, where they form a dorsal horn before they extensively arborize (Kaneko and Hall, 2000; Helfrich-Förster, 2005). Interestingly, this arborisation shows rhythmical changes in fasciculation and defasciculation, which depend on a function of the molecular oscillator (Fernandez *et al*., 2008).

Another group of lateral neurons are the Dorsolateral Neurons (LN_ds), which consist of 4-6 cells (Helfrich-Förster, 2005). They send their projectors into the dorsal protocerebrum. Moreover, ventral ipsilateral projections from LN_ds, together with dorsal ipsilateral projections from s-LN_vs, are meeting fibers from dorsal neurons which complicates distinguishing them (Kaneko and Hall, 2000). However, it appears that some projections target neurons at the opposite side of the brain. These projections loop around anterior optic tract (AOT) and go through the dorsal-fusion-commissure (DFC) and terminate in the contralateral aMe (Helfrich-Förster *et al*., 2007b).

The last group of lateral neurons to be identified were Lateral Posterior Neurons (LPNs) (Shafer *et al*., 2006). They were previously excluded from circadian neurons as they did not show expression of PER but only TIM (Kaneko and Hall, 2000). There are around three or four LPNs per each hemisphere and they are situated close to LN_ds. LPNs make contact with dorsal projections of s-LN_vs (Shafer *et al*., 2006).

Dorsal Neurons are the most abundant group of circadian neurons. They can be divided into three subgroups. There are around 14-17 DN1s which can be further subdivided into 2 anterior (DN1a) and 12-15 posterior (DN1p) (Kaneko and Hall, 2000; Helfrich-Förster, 2005). They are located most medially out of all DNs and most of them send their projections contralaterally through the DFC (Hefrich-Förster *et al*., 2007). DN1as are usually located most dorsally where they do not interact with s-LN_vs projections and send their projections to aMe (Shafer *et al*., 2006).

DN2s and DN3s are less studied. DN2s consist of only 2 cells that are located close to dorsal projections of s-LN_vs and project to DFC (Helfrich-Förster *et al*., 2007b). There are 30-40 DN3s, constituting almost half of circadian neurons. These cells are not homogenous and 3-5 of them are bigger than the rest and

project medially (but do not cross contralaterally) (Shafer *et al.*, 2006; Helfrich-Förster *et al.*, 2007b).

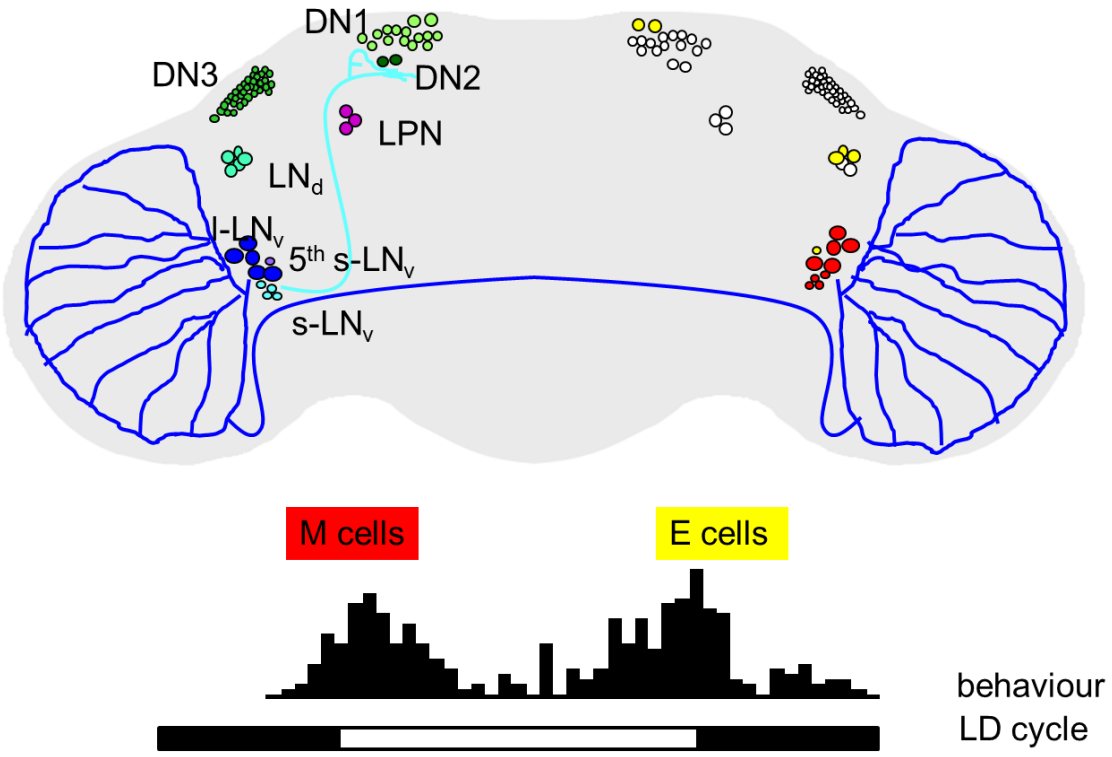


Figure 1.3. Diagram showing location and function of clock neurons in adult *D. melanogaster.*

Left hemisphere shows location of all seven groups of clock neurons: Dorsal Neurons 1-3 (DN1-3), dorsolateral neurons (LN$_d$), Lateral Posterior Neurons (LPN), small ventrolateral neurons (s-LN$_v$) and large ventrolateral neurons (l-LN$_v$). Schematic representation of projections from l-LN$_v$s (dark blue) and dorsal projections from s-LN$_v$s (light blue) are included. Right hemisphere shows the same clock neurons divided according to their function into Morning cells (M) and Evening cells (E), which are responsible for driving morning and evening peak of locomotor behaviour, respectively.

1.4.2 Roles of circadian neurons

The various groups of circadian neurons described above differ not only by their location and number of cells but also by genes that are expressed in them (Nagoshi *et al*., 2010; Kula-Eversole *et al*., 2010). The genes that are expressed in particular groups are indicative of their function. The most obvious example is the fact that PDF is only expressed in the ventrolateral neurons (Renn *et al*., 1999). In addition to PDF, LN_vs also express higher levels of 48-RELATED 2 (FER2), which is a transcription factor essential for a proper development of these neurons (Nagoshi *et al*., 2010). Dorsal neurons are exclusively expressing a specific isoform of NOCTURNIN (NOC), which is important for regulating a response to light since *noc* mutants are displaying behavioural rhythmicity in LL and have different PRC (Nagoshi *et al*., 2010). It is possible that it acts by removing polyA from mRNAs, thus regulating expression of some genes in a mechanism similar to the function of mammalian homologue of NOC (Baggs and Green, 2003). DN1ps are also expressing transcription factor GLASS, crucial for photoreceptor differentiation (Moses *et al*., 1989; Shafer *et al*., 2008).

In addition, not all cells are equally involved in the generation of patterns of locomotor rhythms. Other than testing which cells are responsible for driving the circadian behaviour in constant darkness, researchers have focused on two peaks of activity that occur under standard LD cycles – one of them is around dawn (so called morning peak, M) and the second around dusk (evening peak, E). It is important to note that these peaks are clearly anticipatory and do not represent a simple response to a change in conditions and are governed by a function of the circadian clock (Blanchardon *et al*., 2001; Grima *et al*., 2004; Stoleru *et al*., 2004). The conservation of this pattern from insects to mammals suggests that these peaks might be governed by two different oscillators responsible for each of them (Pittendrigh and Daan, 1976). To determine the contributions of various cells, they were either ablated through an expression of the pro-apoptotic gene *head involution defective (hid)* (Renn *et al*., 1999; Blanchardon *et al*., 2001; Stoleru *et al*., 2004) or rescue of PER expression only in selected cells (Blanchardon *et al*., 2001; Grima *et al*., 2004). This way morning and evening oscillators were created.

When PDF neurons were ablated, a morning peak disappeared and when PER expression was restored only in PDF neurons, M peak was restored (Renn *et al*., 1999; Grima *et al*., 2004; Stoleru *et al*., 2004). By targeting s-LN_vs and l-LN_vs separately, it turned out that rescuing PER expression only in l-LN_vs did not restore M peak, therefore implying that M oscillator consists only of 4 cells per hemisphere – PDF-positive s-LN_vs (Grima *et al*., 2004; Cusumano *et al*., 2009). It appears that this M peak of activity can be caused by a release of PDF that appears mainly during the day when dorsal projections from s-LN_vs are most extensive, potentially contacting most targets (Taghet and Shafer, 2006; Fernandez *et al*., 2008). It was also suggested that light-dependent arousal in morning depends on l-LN_vs (Shang *et al*., 2008; Sheeba *et al*., 2008a). The M cells are also responsible for the generation of circadian rhythms in complete darkness (Park *et al*., 2000).

By ablating all LN_vs, LN_ds and few DN1s (through using the specific genetic driver, *cry-Gal4-13*), both morning and evening peak were removed, however exclusion of LN_vs rescued only M oscillator (Stoleru *et al*., 2004). Selective PER rescue in 5th s-LN_vs and some LN_ds (and possibly also couple DN1s) confirmed that those cells work as E oscillator (Grima *et al*., 2004; Rieger *et al*., 2006).

Even though similar activity pattern exists for flies exposed to temperature cycles, cells responsible for bouts of activity observed in temperature cycles are not identical as a restoration of PER expression in both M and E cells does not fully rescue locomotor activity under temperature cycles. Moreover, a weak evening peak was still observed when flies with ablated M and E cells were analysed in temperature cycles. This implies that some other cells are also involved in regulating locomotor activity in temperature cycles (Busza *et al*., 2007). It was suggested that those cells are DN2s and LPNs as under conflicting LD and temperature cycles those cells, in contrast to others, follow temperature cycles (Miyasako *et al*., 2007). The role of DN2s in temperature synchronization of molecular rhythms in LN_vs has been also suggested in larvae (Picot *et al*., 2009). Interestingly, a pace of entrainment to temperature cycles depends on the presence of and function of the molecular oscillator in PDF-positive neurons, indicating that these neurons prevent instantaneous entrainment to temperature changes (Busza *et al*., 2009).

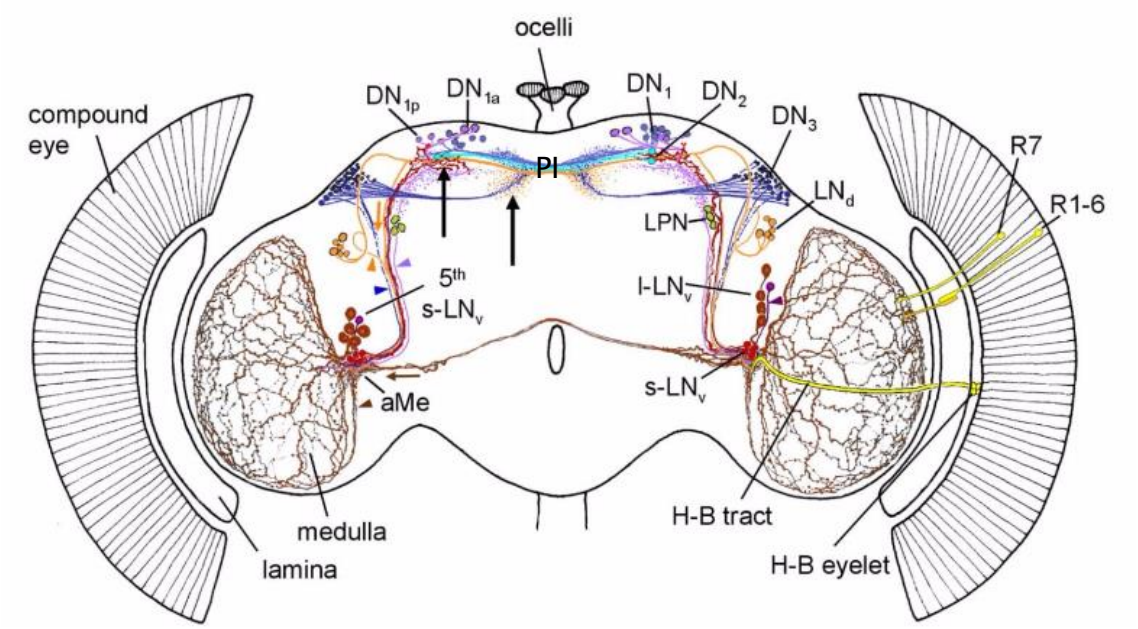


Figure 1.4. Diagram showing neuronal projections from clock neurons (reproduced from Helfrich-Förster *et al.*, 2007a).

In addition to previously mentioned clock neurons, the diagram above shows colour-coded projections from clock neurons. aMe – accessory Medulla, H-B – Hofbauer-Buchner, PI – Pars Intercerebralis. Black arrows point to areas of extensive synaptic contacts.

The function of other circadian neurons is less well understood. As mentioned previously, DN1 neurons are necessary for synchronizing the effect of sexual interactions (Hanufasa *et al.*, 2013). The subset of DN1s, DN1ps, was also shown to be involved in light sensitivity of the circadian clock (Klarsfeld *et al.*, 2004). In addition to their role in temperature entrainment, DN2s are also contributing to light input detection (Klarsefeld *et al.*, 2011). They also contribute to temperature preference (Kaneko *et al.*, 2012). DN3s are contributing to the generation of rhythmic locomotor behaviour under LD conditions (Veleri *et al.*, 2003).

The existence of a distinct morning and evening oscillator was later questioned as it was for example possible to retain M peak in flies that have PER expression everywhere except in PDF neurons. This suggested that M and E oscillators were coupled and that PDF-negative cells could activate PDF-positive

neurons (Stoleru *et al.*, 2004). Moreover, in certain LD protocols, bimodal activity pattern could be retained with functional PER only in 5th s-LN$_v$ and 3 LN$_d$s, indicating that a division of cells into M and E oscillators might be correct only for some conditions and not others (Rieger *et al.*, 2009). It has also been proposed that l-LN$_v$s can take over from s-LN$_v$s, making s-LN$_v$s sufficient but not necessary for generation of M peak (Sheeba *et al.*, 2010).

1.4.3 Glial circadian rhythms

In addition to neurons, glial cells were shown to be involved in the generation of circadian behaviour. Not only is PER expressed in glia (Siwicki *et al.*, 1988) but even when its expression is restricted to glia only, some weak behavioural rhythms could be sustained (Ewer *et al.*, 1992). One of the genes that are rhythmically expressed in glia is *ebony* (*e*), a mutation which is responsible for dark coloration of cuticle (Suh and Jackson, 2007). Mutations in *e* do not affect eclosion rhythms however they do decrease or abolish behavioural rhythms (Newby and Jackson, 1991) through glia, as rescuing *e* expression in glia only restores these defects (Suh and Jackson, 2007). However, the same effect could be also achieved using multiple rounds of back-crossing of the e^1 mutation to wild-type flies, suggesting that maybe the background (other genetic elements) is contributing to a decreased rhythmicity of flies with *e* mutation (Hall *et al.*, 2007).

The exact role of *e* in generation of circadian rhythms is not understood, but it appears to be involved downstream of molecular oscillator as molecular rhythms in *e* mutants persist in circadian neurons and circadian neurons have normal outputs (Suh and Jackson, 2007). It was suggested that since *e* is an enzyme with a capability of conjugating P-Alanine to biogenic amines, it might be involved in metabolic processing of circadian neurotransmitters (Suh and Jackson, 2007).

1.4.4 Neurotransmitters involved in circadian circuit

The function of an entire circadian neural circuit and its ability to drive any circadian output depends on an ability of neurons to communicate with each other and with their targets. It has been proposed that this is due to neurotransmitters released from various groups of circadian neurons.

The first circadian neurotransmitter that was characterized, and which appears to be the most important one, was PIGMENT DISPERSING FACTOR (PDF) (Nässel *et al*., 1991; Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995; Park and Hall, 1998; Renn *et al*., 1999). It shares homology with the PIGMENT DISPERSING HORMONE (PDH) that was discovered in the central nervous systems of arthropods and is involved in a movement of the pigment in the retina of crustaceans to protect them from solar radiation (Nässel *et al*., 1991; Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995). PDF is expressed in a very limited number of cells, namely all l-LN_vs, 4 out of 5 s-LN_vs, 4-6 abdominal ganglionic neurons (that are not circadian) and tree neurons of tritocerebellum (that are disappearing just after eclosion) (Renn *et al*., 1999). Interestingly, levels of PDF that are detected in termini of dorsal projections from s-LN_vs show a daily oscillation pattern which depends on the function of the circadian clock and might represent rhythms in PDF release (Park *et al*., 2000).

PDF is crucial for the generation of circadian rhythms. Even though mRNA of *Pdf* is not rhythmically expressed, it is under circadian regulation, also indirectly from CLK/CYC (Park and Hall, 1998; Blau and Young, 1999; Park *et al*., 2000; Taghert and Shafer, 2006). Majority of null mutants of *Pdf* (*Pdf^{01}*) are arrhythmic in DD (after at most 3 days of some rhythms) with a small percentage of flies displaying weak rhythms with short period. In LD, *Pdf^{01}* flies lack morning peak and have advanced evening peak (Renn *et al*., 2000). This suggests that PDF is necessary to convey timing information from LN_vs to other neurons. It was shown that in addition to causing behavioural arrhythmicity, flies lacking PDF kept in constant darkness lose a synchrony between various circadian neurons, consequently leading to decreased amplitude of molecular rhythms. Therefore PDF is responsible for ensuring synchrony between circadian neurons (Peng *et al*., 2003; Lin *et al*., 2004). The exact role of PDF depends on the target neurons as for some it is necessary for molecular

oscillations and for others it either accelerates or slows down the pace of oscillations (Choi *et al.*, 2009; Yoshii *et al.*, 2009c).

PDF receptor (PDFR) is a class II G-protein coupled receptor (Mertens *et al.*, 2005). Mutations in PDFR phenocopy mutations in PDF (Huyn *et al.*, 2005; Lear *et al.*, 2005; Mertens *et al.*, 2005). PDFR is expressed in many non-circadian and circadian neurons, including s-LN_vs, 3 l-LN_vs and subset of LN_ds, DN1s and DN3s (Huyn *et al.*, 2005; Lear *et al.*, 2005 and 2009; Mertens *et al.*, 2005; Shafer *et al.*, 2008; Im and Taghert, 2010). A subset of DN1s that express PDFR is important for rhythms in DD and morning peak under LD (Zhang *et al.*, 2010). Moreover, since s-LN_vs target a subset of circadian cells, this supports the role that PDF plays in maintaining synchrony between circadian neurons. The other circadian neurons that do not express PDFR might be receiving signals from PDF through communicating with cells that express PDFR. Also, since s-LN_vs express both PDF and PDFR, this suggests an existence of the autocrine feedback (Im and Taghet, 2010). It was also suggested that PDFR needs to be present in both LN_vs and DN1s to maintain synchrony in the LN_vs (Collins *et al.*, 2014).

Levels of PDF in termini of dorsal projections of s-LN_vs oscillate in circadian manner with the highest levels of PER detected about an hour after time of lights-on and a trough after start of subjective night (Park *et al.*, 2000). This suggests that PDF is released rhythmically from s-LN_vs and acts on other neurons to synchronize them. PDF might be modulating the molecular oscillator in various neurons through upregulating PROTEIN KINASE A (PKA) which causes stabilization of PER (Li *et al.*, 2014; Seluzicki *et al.*, 2014). When PDF is received by PDRF, ADENYL CYCLASE increases CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) levels (Shafer *et al.*, 2008; Vecsey *et al.*, 2014), consequently slowing down the pace of the clock. Moreover, PDF also contributes to TIM degradation, especially in cells that lack CRY (Guo *et al.*, 2014; Seluzicki *et al.*, 2014).

In summary, PDF signalling is crucial for generation of M and E bouts of activity and circadian rhythmicity in DD (Renn *et al.*, 1999; Helfrich-Förster *et al*, 2007b; Allada and Chung, 2010; Zhang *et al.*, 2010; Guo *et al.*, 2014). Whether or not this is due to a rhythmic release of PDF is still unknown, however it has been demonstrated that some flies expressing modified PDF lost a rhythmic

imunnoreactivity in dorsal termini and still retained behavioural rhythms (Kula *et al*., 2006). Interestingly, Pdf^{01} flies can display rhythmicity with a short period, if bacterial sodium channel (NaChBac) is expressed in LN_vs (Sheeba *et al*., 2008b), which suggests existence of more neurotransmitters in LN_vs that can take over PDF signalling. Moreover, Pdf^{01} larvae display rhythmic photophobic behaviour, also indicating an existence of other communication mechanisms (Mazzoni *et al*. 2005).

In fact, another neurotransmitter was discovered in the PDF-positive s-LN_vs (and also two LN_ds) which is called SHORT NEUROPEPTIDE F (sNPF) (Johard *et al*., 2009). It was suggested that a role of sNPF might be to repress cAMP synthesis that is promoted by PDF (Vecsey *et al*., 2014). Related to sNPF is a NEUROPEPTIDE F (NPF, a homologue of mammalian NEUROPEPTIDE Y (NPY) involved in, among others, circadian rhythms) that is expressed in three LN_ds only in males in a clock-controlled way (Yannielli and Harrington, 2001, Johard *et al*., 2009). This suggest that NPF might be responsible for mediating the sexual dimorphism of circadian behaviour, which is for example more prominent siesta behaviour found in males (Helfrich-Förster, 2000; Lee *et al*., 2006).

Another neuropeptide is ION TRANSPORT PEPTIDE (ITP) found in one LN_d (that also expresses CRY and NPF) and 5th s-LN_v (Yannielli and Harrington, 2001; Jonard *et al*., 2009; Hermann-Liubl *et al*., 2014). It is secreted cyclically with peaks late in the day and late at night. This can imply that ITP might be involved in the generation of morning and evening peaks of activity (Hermann-Liubl *et al*., 2014). ITP neurons were shown to signal to Pars Intercerebralis (Hermann-Liubl *et al*., 2014).

Circadian neurons are also involved in signalling through some less specific neuropeptides. One of them is IPNamide that is expressed in DN1as (Shafer *et al*., 2006). The peak of expression of IPNamide is late at night and it has been suggested to be involved in the phototransduction (Shafer *et al*., 2006; Verleyen *et al*., 2004). Some DN1ps, both DN1as and some DN3s are also expressing a vesicular glutamate transporter (DvGluT) and metabotropic glutamate receptors (mGluRA) are for example expressed in LN_vs (Hamasaka *et al*., 2007). Flies with mutated glutamate receptors (or expressing RNAi against gene encoding it) had increased morning activity as adults and as larvae had

increased photophobic behaviour, therefore indicating that glutamatergic signal from DNs is inhibitory (Mazzoni *et al*., 2005; Hamasaka *et al*., 2007). Glutamate might be influencing circadian oscillator through cAMP (Collins *et al*., 2014).

LN_vs also express the receptors to γ-AMINOBUTYRIC ACID A ($GABA_A$) and serotonin (5HT) (Hamasaka *et al*., 2005; Hamasaka and Nassel, 2006). Moreover, some of the GABAergic and serotoninergic projections' termini are in close proximity of dendrites of LN_vs (Hamasaka *et al*., 2005; Hamasaka and Nassel, 2006). Serotonin was suggested to be involved in the CRY-dependent circadian photoresponses (Yuan *et al*., 2005). Inhibitory action of GABA on LN_vs induced sleep and decreased response to light during the night (Parisky *et al*., 2008). Finally, given that histaminergic cells from Hofbauer-Buchner eyelet send their projections to the vicinity of LN_vs, it is plausible that histamine contributes to light entrainment (Hamasaka and Nassel, 2006, Veleri *et al*., 2007).

1.4.5 Generation of the output rhythms

The third component of a functional circadian clock is a way to connect molecular oscillator to overt behaviours and physiology. Despite progress that was made in determining components and mechanisms of the molecular oscillator, location of the pacemaker in the brain and input pathways, output pathways remained mysterious for a long time. Gradually, a breakthrough came when some molecules were identified that contribute to the generation of behavioural rhythms, without regulating function of the molecular oscillator.

The first gene that was identified as participating in the generation of output rhythms was *Neurofibromatosis-1* (*Nf1*) (Williams *et al*., 2001). *Nf1* mutants have normal oscillations in *per* and *tim* levels but affected locomotor rhythms. Moreover, they have increased MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) activity, suggesting that NF-1 signals through RAS/MAPK (Williams *et al*., 2001). Moreover, oscillating phospho-MAPK was found in proximity of termini of dorsal s-LN_vs projections, suggesting that PDF might be a signal that is received and causes RAS/MAPK signalling (Williams *et al*., 2001).

Another pathway that was identified as contributing to the generation of circadian rhythms was JAK/STAT pathway. It is regulated by the microRNA miRNA-279 (Luo and Sehgal, 2012). Increased levels of miRNA-279 caused a delay to locomotor rhythms without affecting underlying PER oscillations in circadian neurons. miRNA-279 was shown to target UNPAIRED (UPD), a ligand of JAK/STAT. Neurons expressing UPD can be found close to dorsal projections of s-LN_vs, possibly interacting with each other, again suggesting that s-LN_vs might be generating output rhythms through the JAK/STAT pathway (Luo and Sehgal, 2012).

Pars Intercerebralis (PI), the fly equivalent of hypothalamus, was suggested to be a structure responsible for the generation of output rhythms (de Velesco *et al*., 2007). Not only is it located near projections from several groups of circadian neurons (Kaneko and Hall, 2000; Hall, 2003), it also expresses cyclically and in clock-dependent manner SLOWPOKE BINDING PROTEIN (SLOB), which modulates a calcium-dependent potassium channel SLOWPOKE (Jarmillo *et al*., 2004). However, SLOB appears not to be involved in the generation of locomotor rhythms (Shahidullah *et al*., 2009).

A subset of 16-18 neurons (per brain) in PI has been recently demonstrated to be crucial for the generation of locomotor activity, as their activation resulted in locomotor arrhythmicity in a presence of functional molecular oscillations (Cavanaugh *et al*., 2014). These neurons were complementary to previously shown PI neurons expressing INSULIN-LIKE PEPTIDE (DILP2), which is involved in the regulation of sleep and metabolism in flies, but was excluded from being involved in output rhythms (Rulifson *et al*., 2002; Broughton *et al*., 2005; Crocker *et al*., 2010, Cavanaugh *et al*., 2014). These cells do not interact with dorsal projections from s-LN_vs, however, they form synaptic connections with DN1 neurons that in turn contact s-LN_vs, establishing a pathway through which a signal from s-LN_vs can generate behavioural rhythmicity (Cavanaugh *et al*., 2014). A contact between s-LN_vs and DN1s was suggested before, since DN1s respond to bath application of PDF (Shafer *et al*., 2008) and accelerating clock in s-LN_vs causes similar acceleration in several dorsal neurons, including some of DN1s (Stoleru *et al*., 2005). 6 out of PI neurons specifically express DH44 which is a homologue of the stress hormone CORTICOTROPIN-RELEASING FACTOR (CRF) and 4 additional ones express SIFamide (Cavanaugh *et al*., 2014). All of the above suggest the existence of a simple output pathway, in

which information on time is passed from s-LN_vs probably via PDF through DNs to PI neurons, which then contact other neurons, or release neurotransmitters or hormones.

1.5 Development of rhythms

Despite years of research in the area of chronobiology, one of the aspects of circadian biology remained relatively poorly understood – how circadian clocks develop in an organism. This part of the introduction focuses on organizing existing information on this topic and introducing the research question.

1.5.1 Development of mammalian circadian clock

Mammals do not express behavioural rhythms right after birth. Instead, it takes them from 2-3 weeks (Davis, 1981) to about 2 months in humans (reviewed in Davis and Reppert, 2001). For example, rhythmic melatonin production is detected at the end of the first week postnatal (Tamarkin *et al.*, 1980).

The mammalian pacemaker SCN starts developing very early. For example in rats, it develops gradually from embryonic day (E) 14 to E17 (birth occurs at E21-22), however, there are indications that circadian clock components are present in nuclei of germinal vesicle (GV) oocytes and one-cell- to four-cell-stage embryos (Moore, 1991; Amano *et al.*, 2009). In humans, SCN becomes visible as a structure around the 18^{th}-30^{th} week of pregnancy (Swaab *et al.*, 1990). The first rhythm to appear in SCN is the metabolic activity measured by 2-deoxyglucose uptake which can be observed between E19 and E21 in squirrel monkeys (Reppert and Schwartz, 1983 and 1984). More rhythms follow: around E21 rhythms in vasopressin (AVP) mRNA levels can be detected (Reppert and Uhl, 1987) and at E22 SCN neuronal firing rhythms can be observed (Shibata and Moore, 1987). In sheep, neuronal activity marker *c-fos* starts oscillating in SCN around gestational day 135 (Constandil *et al.*, 1995). These findings suggest that the circadian clock in SCN starts functioning before birth. It is important to note that these rhythms were determined in

animals with rhythmic mothers housed in (or exposed to for a significant period of time) LD cycles, therefore not excluding the possibility that these rhythms are not truly endogenous.

However, other experiments demonstrated that mammals do not require any input from Zeitgebers during development to drive the behavioural rhythms in adulthood. When mice pups heterozygous for circadian mutations were raised at constant darkness by arrhythmic mothers, they were still displaying rhythmic wheel running (Jud and Albrecht, 2006). Moreover, this experiment confirmed that the rhythmicity of mothers raising pups was not important for their behaviour. However, other studies have demonstrated that circadian clock function in mothers is important for the entrainment of the circadian clock in foetuses to LD cycle (Reppert and Schwartz, 1983; Reppert *et al*., 1984) or can provide the entraining cues (Takahasji *et al*., 1982; Ohta *et al*., 2002). For example, rat pups born and raised in DD show oscillations of pineal gene *arylalkylamine-(serotonin) N-acetyltransferase* (*aanat*) (a rate-limiting enzyme in melatonin synthesis controlled by the circadian clock) expression in the pineal gland consistent with oscillations in their mothers (Reppert *et al*., 1984; Klein *et al*., 1997). Similar observations were also described for hamsters (Davis and Gorski, 1985; Davis and Mannion, 1988). Moreover, mothers with ablated SCN have pups that are less synchronous to each other (Reppert and Schwartz, 1986; Davis and Gorski, 1988).

Circadian gene expression can be detected in SCN even before the first metabolic rhythms can be detected (E10-21, but one report indicated as early as E7). Around this time *per1*, *per2*, *cry1*, *clock* and *bmal1* can all be found in SCN at constant levels. Over time, their levels start oscillating, with *per1* showing oscillations in expression levels around E12 and *per2* oscillating from E15-16 (and continues developing until the first day after birth, P1), however the times are highly variable and depend on the organism studied (mice - Shimomura *et al*., 2001; rats -Saxena *et al*., 2007; mice - Wreschnig *et al*., 2014). At E20-P1 strong rhythmic expression of *per1*, *per2* and *bmal1* can be detected and a day later *cry1* joins oscillating genes (Ohta *et al*., 2002 and 2003; Sladek *et al*., 2004; Kovacikova *et al*., 2006; Houdek and Sumova, 2014). All components of circadian machinery are showing high-amplitude expression rhythms only around P10 (Sladek *et al*., 2004; Kovacikova *et al*., 2006). PER1 and PER2 protein levels start oscillating (with low levels and amplitude) in

murine SCN around E18, even if mothers are housed in complete darkness (Ansari *et al*., 2009).

These experiments suggest that oscillations observed in SCN before birth do not require the presence of a fully assembled molecular oscillator. Interestingly, the phase of developing clock is inherited from mothers (Reppert and Schwartz, 1984; Houdek and Sumova, 2014). This can be explained by the fact that the main components of the circadian clock, such as CLOCK or BMAL1, regulating expression of other circadian genes, are present in an embryo very early on (preimplantation) at constant levels and represent the mRNAs inherited from mothers (Johnson *et al*., 2002; Hamatani *et al*., 2004). On the other hand, this could be thanks to melatonin supplied by maternal pineal glands adjusting a phase of the circadian clock of a foetus (Seron-Ferre *et al*., 2012; Houdek *et al*., 2015). Later on, 5 days after birth, LD cycles become a main signal, setting the phase of the circadian clock, however, until this happens, the clock is prone to disturbances by external factors (Reppert *et al*., 1984; Duncan *et al*., 1986). Alternatively, a new study suggested an even earlier presence of circadian oscillations, with rhythms in circadian gene expression starting as soon as neural cells are derived from embryonic cells and neuronal progenitor cells, which might suggest autonomous origin in circadian clocks (Kowalska *et al*., 2010; Yagita *et al*., 2010; Umemura *et al*., 2013). Metabolic rhythms in the form of glucose transporter mRNA expression and uptake of 2-deoxyglucose were present earlier, in undifferentiated cells (Palouse *et al*., 2012). However, these studies were using stable cell lines rather that tissues in live animals, which might yield different results.

Mammalian clocks are capable of receiving signals from the environment through melanopsin-expressing intrinsically photoreceptive retinal ganglion cells (ipRGCs) that project to SCN (Berson *et al*., 2002; Hatter *et al.,* 2002). Melanopsin is expressed from E10.5 in mice and E18 in rats and around the same time, ipRGCs emerge in developing retina (Tarttelin *et al*., 2003; Fahrenkrug *et al*., 2004). ipRGCs become light responsive and connect with SCN around birth, and interestingly a number of ipRGCs decreases gradually afterwards to reach about $1/5^{th}$ of the concentration in adults (Sekaran *et al*., 2005; Tu *et al*., 2005). Since embryonic SCN can entrain to light before birth, it has to occur through some other pathways, with one candidate being maternally inherited melatonin, as injections of melatonin can reset or phase

shift circadian rhythms in hamster pups of mothers with ablated SCN (Davis and Mannion, 1988) or in pregnant rats without pineal gland housed in constant light (Houdek *et al*., 2014).

It remains unknown whether functioning SCN causes any rhythmic outputs before birth. It was suggested that emerging rhythms in SCN are necessary for the maturation of the clock neurons (Vallone *et al*., 2007).

1.5.2 Development of circadian clocks in zebrafish

Surprisingly, the development of the circadian clock was extensively studied not in one of standard model organisms such as flies or rodents, but rather in zebrafish (*Danio rerio*), mainly due to the ease of studying a large number of embryos, transparent bodies and development occurring ex utero in transparent chorion (Mullins *et al*., 1994; Haffter *et al*., 1996; Pando and Sassone-Corsi, 2002; Nüsslein-Volhard and Dahm, 2002; Vallone *et al*., 2005; Vatine *et al*., 2011). Molecular pacemaker function and clock outputs appear early in zebrafish development, which might represent an adaptation to fast development (24 hours of embryo development and larval hatching within 3 days after fertilization) and a need for a functional clock from early on to optimize feeding and avoid predators (Vallone *et al*., 2007).

Behavioural circadian rhythmicity in zebrafish can be detected early during development. Around 4-5 dpf (days postfertilization) circadian rhythms can be detected in diurnal locomotor activity (swimming) (Cahill *et al*., 1998; Hurd and Cahill, 2002; Nüsslein-Volhard and Dahm, 2002; Hirayama *et al*., 2005; Cahill, 2007), sleep-like nocturnal inactivity (Zhdanova *et al*., 200) and arousal threshold (Prober *et al*., 2006). Appearance of these behavioural rhythms is dependent on prior exposure to LD or temperature cycle during the first 4 days of development, with 4 LD cycles producing the best rhythmicity (Cahill *et al*., 1998; Hurd and Cahill, 2002; Lahiri *et al*., 2014). When zebrafish embryos were raised in constant darkness, most larvae were arrhythmic with a minority of larvae displaying weak low-amplitude rhythms with a phase set by the handling of embryos (Hurd and Cahill, 2002). Interestingly, even though zebrafish develop faster in warmer temperatures, the timing of the appearance of these rhythms remained constant, indicating that the developmental stage is

less important than the threshold of a proper number of LD cycles experienced during the first 4 dpf, and reducing this number from 4 resulted in a decrease of amplitude of locomotor rhythms (Hurd and Cahill, 2002; Dekens *et al*., 2003).

The first indication of circadian rhythms at molecular level in zebrafish is in melatonin synthesis and they become apparent around 37-43 hpf (hours postfertilization), however, full diurnal rhythms are observed from 4 dpf. The appearance of this rhythm required transition from light to dark after 20-26 hpf, suggesting that the oscillator begins functioning and is responsive to light around that time (Kazimi and Cahill, 1999). Rhythm in melatonin production is accompanied by the rhythm in *zfaanat2* (*zfaanat2* is expressed mainly in pineal gland and *zfaanat1* mainly in retina (Gothilf *et al*., 1999; Falcón *et al.,* 2003)) expression in pineal gland at 2dpf (*zfaanat2* starts being expressed at 22 hpf and is key enzyme involved in melatonin production) (Gastel *et al*., 1998; Gothilf *et al*., 1999; Falcón *et al*., 2001). The pineal gland is a primary pacemaker in zebrafish (Korf *et al*., 1998; Ziv *et al*., 2005; Ben-Moshe *et al*., 2014), as no impact of anatomical SCN equivalent was found on circadian rhythms (Kaneko *et al*., 2006) and ventral brain containing region giving rise to SCN is not required for generation of circadian rhythms (Cahill, 1996; Noche *et al*., 2011). The rhythm in *zfaanat2* expression persists even after the transfer of zebrafish to constant darkness, confirming that the circadian clock is functional at this stage (Gothilf *et al*., 1999).

The next rhythms are in a frequency of S-phase-positive nuclei (replicating DNA) in tissues (with a peak around the end of the light phase) that appear around 4 dpf and grow in amplitude through larval development (Dekens *et al*., 2003). This suggests that the circadian clock might be contributing to cell proliferation during development. Similarly, rhythmic melatonin production early in development contributes to the stimulation of cell proliferation at night (protecting DNA from UV light) through melatonin receptors (Danilova *et al*., 2004).

The presence of rhythms in *zfaanat2* mRNA expression in the pineal gland on 3 or 4 dpf required at least a single light pulse early in development (0-16 hpf). Moreover, the time of this pulse was able to set a phase of this rhythm (Ziv *et al*., 2005; Ziv and Gothilf, 2006a; Vuilleumier *et a*l., 2006). This means that

information on a phase can be preserved through development and cell divisions. This situation is similar to what is observed in *D. melanogaster*, where light pulses given from early larval stage were sufficient to entrain adult behaviour (Sehgal *et al*., 1992). Subsequent studies have demonstrated that *zfaanat2* mRNA rhythms require expression of *zfper2*, which is upregulated in presence of light (Tamai *et al*., 2004; Ziv *et al*., 2005; Ziv and Gothilf, 2006a and 2006b), suggesting that light-induced *zfper2* expression is crucial for a proper maturation of the clock. The role of *per2* in this light-mediated entrainment of the circadian clock is consistent with its role in other vertebrates, such as mice (Albrecht *et al*., 2001; Zheng *et al*., 2001).

Raising larvae in constant darkness abolished rhythms in melatonin production or S-phase-positive nuclei accumulation, however, an exposure to LD cycle later in development could restore rhythmic melatonin production (Kazimi and Cahill, 1999; Dekens *et al*., 2003). Moreover, circadian genes are expressed at constant levels in zebrafish larvae raised in constant darkness and can start showing oscillations only after exposure to LD or temperature cycles (Lahiri *et al*., 2005). This suggests an alternative explanation - rhythms are present in individual cells but remain undetectable due to a lack of synchrony between these cells. This hypothesis is supported by a discovery that circadian rhythmicity in clock genes expression persisted in individual cells after many days in constant darkness, with some variability in amplitude and period length, however they gradually lose synchrony to other cells and after sufficient time no rhythms at the population levels can be detected (Carr and Whitmore, 2005). Moreover, short light pulses could restore a synchrony between cells (Carr and Whitmore, 2005; Carr *et al*., 2006). Therefore, synchrony rather than initiation of circadian clock function in zebrafish is dependent on a presence of entraining cues during development, suggesting that mammals and flies developed some strategies to ensure synchrony between their cells.

Expression of *zfper1*, another key component of the circadian clock, begins in 1 dpf and adopts a rhythmic pattern on 2 dpf if LD cycle is present in the development (Dekens and Whitmore, 2008). In the case of zebrafish kept in constant darkness during development, circadian rhythms in *zfaanat2* and *zfper1* could be initiated after exposure to temperature cycles from 2-3 dpf (Lahiri *et al*., 2014). Molecular studies revealed that *zfper1* oscillates in

individual cells from 24 hpf with variable phase, resulting in no rhythmicity on an embryo level, providing additional evidence for a theory that either light or temperature Zeitgebers are necessary to synchronize the molecular oscillations in individual cells (Dekens and Whitmore, 2008). This expression requires the presence of CLOCK, however, unlike in the mature zebrafish, *clock1* and *bmal1* are not rhythmically expressed, indicating that the molecular oscillator functions differently during adulthood and development (Dekens and Whitmore, 2008). The rhythms in expression of *clock1* and *bmal1* can be detected from 4-5 dpf, suggesting that other clock outputs that develop around that time rely on a different mechanism involving expression of *clock1* and *bmal1* (Cahill *et al*., 1998; Dekens and Whitmore; 2008).

This sensitivity of the circadian clock in developing larvae requires the presence of functional photoreceptors. Retinal photoreceptors differentiate at 48-50 hpf and visual responses can be detected around the time of hatching (72 hpf) (Branchek, 1984; Branchek and Bremiller, 1984; Larison and Bremiller, 1990; Raymond *et al*., 1995; Easter and Nicola, 1996). Extraretinal photoreception matures even earlier with the expression of photoreceptor markers in photoreceptor pineal cells detected around 24 hpf (Masai *et al*., 1997; Falcon *et al*., 2003). Moreover, a cell autonomous entrainment of clock in peripheral tissue can be detected even earlier, around 10 hpf, as light pulses administered then can initiate circadian gene expression of *zfper2* and *zfcry1* (Ziv and Gothilf, 2006a).

Moreover, even though studies of the *zfper3* expression have suggested that a functional circadian clock and phase of rhythmicity in zebrafish are inherited from mothers through maternal *zfper3* (Delaunay *et al*., 2000), neither of the previous experiments nor attempts to recreate the study have offered any supporting evidence for this hypothesis, as zebrafish raised in constant darkness remained arrhythmic (Kazimi and Cahill, 1999; Hurd and Cahill, 2002; Dekens *et al*., 2003; Kaneko and Cahil, 2005; Lahiri *et al*., 2005). Moreover, *zfper3* oscillations were later shown to appear much later in development than originally suggested (5-6 dpf) and only if larvae were exposed to 5 LD cycles, including one after 4 dpf (Kaneko and Cahil, 2005). *zfper1* oscillations were shown to be present at level of individual cells from 24 hpf irrespectively from a presence of external cues (Dekens and Whitmore,

2008). Even though some circadian transcripts are passed on to offspring, their function remains unknown (Delaunay *et al*., 2003).

1.5.3 Life cycle of *Drosophila melanogaster*

The main difference between *Drosophila melanogaster* as a model organism and mammals is the fact that it is a holometabolous insect and flies undergo complex development with clearly different life stages (in principle resembling development of zebrafish more than the development of mammals). Female flies lay eggs that are fertilised from sperm stored in their spermathecal (Kaufmann and Demerec, 1942). About 12-24 hours (for 25˚C) after oviposition, embryos develop into larvae, which starts feeding immediately. There are three larval instars - L1 to L3 (lasting around 24 hours for L1 and L2 and around 48 hrs for L3), separated by molting – cuticle apolysis and shedding, that differ by size and behaviour - L1 larvae stay on the surface of food, L2 larvae burrow into food and L3 move again to a surface and eventually leave food in preparation for puparitation in response to an increase in the ecdysone hormone (Ashburner and Thompson, 1978; Kaznowski *et al*., 1985; Ashburner *et al*., 2005). It is important to note that the number of cells remains constant from hatching to puparium formation and cells only increase in size (Ashburner and Thompson, 1978; Ashburner *et al*., 2005).

Metamorphosis, a process of transformation from larvae to adult flies begins with puparium formation. After a larvae finds a place, it stops moving, shortens and broadens, everts its anterior spiracles and its larval skin hardens and tans, becoming a pupal case. About 4 hours after the beginning of puparitation, a larval/pupal apolysis begins and is followed about 20-24 hours later by a pupal/adult apolysis (Ashburner, 1976; Chiara *et al*., 1982; Fechtel *et al*., 1989). Subsequently almost all larval cells (with an exception of Malpighian tubules and some nerve cells) undergo autolysis. The adult head emerges after abdominal muscle contractions about 12 hours after puparium formation (Bodenstein, 1965; Fraenkel and Bhaskaran, 1973). Adult cells contained in imaginal discs throughout larval stages either cease to divide on the beginning of metamorphosis or divide during early metamorphosis and then grow and move to form an adult fly (Ashburner, 1976; Fristrom and Fristrom, 1993).

The adult cuticle is formed about 48-50 hours after puparium formation but a process of metamorphosis is not complete until about two days later, when the fully formed adult fly emerges from a pupal case in a process called eclosion (Chiara *et al*., 1982; Thummel, 1995).

There are two neurogenesis periods during development. The first one occurs during embryogenesis and is responsible for a formation of neurons mainly important for larval life (Schmidt *et al*., 1999; Urbach and Technau, 2003; Technau *et al*., 2006). The second one occurs during metamorphosis and involves creation of neurons for an adult life. Moreover, during metamorphosis most of the neurons created during embryogenesis undergo autolysis and new neuronal projections are generated, new synapses created and some of the existing ones are removed by use-dependent axonal pruning (Truman, 1990; Truman *et al*., 1993; Levine *et al*., 1995; Kantor and Kolodkin, 2003; Tessier and Broadie, 2008; Seid and Wehner, 2009).

1.5.4 Circadian rhythms in developing flies

The complex life cycle of *D. melanogaster* described above raises an interesting question on the development of the circadian rhythm and oscillator. The crucial factors are whether the components of the molecular oscillator are present during development and especially if they are rhythmically expressed in neurons that might act as circadian. Another important question to consider is what the role of a functional circadian clock during development might be, and whether it would be connected to a function of the circadian clock in adult flies.

Contrary to rodents, adult flies display circadian behavioural rhythms from birth. In fact, some circadian rhythms can be observed even before hatching, as the first detectable behavioural rhythms in *Drosophila melanogaster* are found in larvae. The photophobic response, which is a light avoidance behaviour demonstrated by larvae, displays circadian variations in sensitivity (Mazzoni *et al*., 2005). In wild-type larvae, a photoperiodic response was the strongest around subjective dawn and the weakest around subjective dusk. Moreover, mutations in clock components disrupted this behaviour (Mazzoni *et*

al., 2005). More detailed explanation of the circadian rhythm in photoperiodic response can be found in 3.1.

These results suggest that the clock has to be functional in larvae. In fact, circadian components can be detected much earlier, from late embryonic stages (Liu *et al*., 1988; Saez and Young, 1988; Zerr *et al*., 1990; Ewer *et al*., 1992; Frisch *et al*., 1994; Kaneko *et al*., 1997; Stanewsky *et al*., 1997a; Kaneko and Hall, 2000). PER is present at late embryonic stages in the ventral nerve chord and brain, reaching about 130 cells at the end of the embryonic stage (Ruiz *et al*., 2010). Several hours after PER is detected, other cells in the brain start expressing CLK (finally reaching around 20 cells) and 6-8 hours after that the same cells start expressing PER, suggesting that CLK expression sets a fate of cells as circadian cells as it starts the process of molecular oscillations (Houl *et al*., 2008). More details on the development of circadian neurons are presented in 5.1.

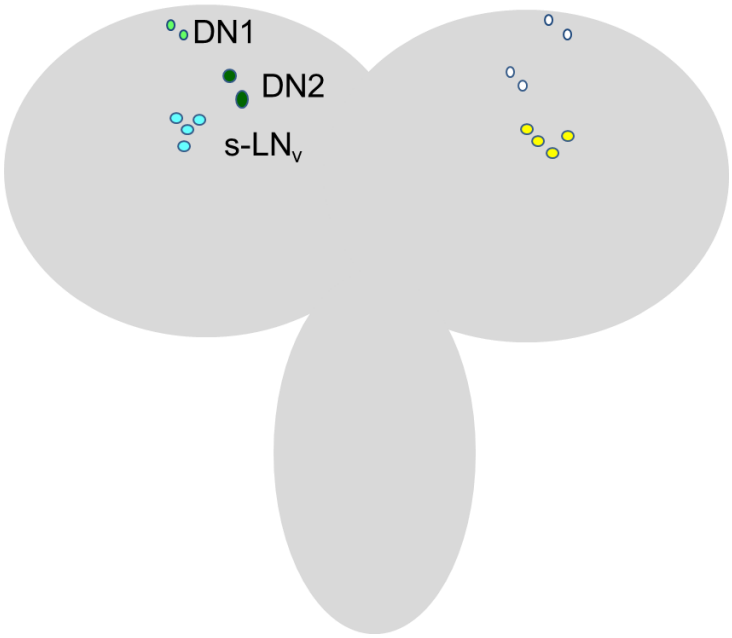


Figure 1.5. Diagram showing location of clock neurons in *D. melanogaster* larvae.

Left hemisphere shows the location of four groups of clock neurons present in larvae: Dorsal Neurons 1-3 (DN1-3) and small ventrolateral neurons (s-LN_v). Yellow cells in the right hemisphere are s-LN_vs that are later (during adulthood) responsible for driving

the Morning peak in LD and are crucial for free-running rhythms in DD.

Neurons that have a functional circadian clock in larvae represent precursors of adult circadian neurons as they are among cells that are not hystolysed during metamorphosis. They form three distinct groups – one group is located laterally and two groups are located dorsally (Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Houl *et al.*, 2008). The dorsal groups consist of 2 dorsal neurons (DN2s precursors) and 2 dorsal neurons (DN1s precursors) (Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Kaneko *et al.*, 2008). The 4 lateral neurons that express PDF represent s-LN_vs. In contrast, l-LN_vs are not present in larvae and differentiate in pupae (Helfrich-Forster, 1997; Kaneko *et al.*, 1997). It is interesting that s-LN_vs precursors are present right from when larvae hatches, as it suggest that these might be the cells that modulate photophobic response in the same way that they regulate locomotor behaviour. Indeed, these cells are potentially able to receive visual input, since the Bowlig's organ, larval photoreceptor, sends its projections to these lateral neurons (Kaneko *et al.*, 1997, 2000b; Malpel *et al.*, 2002; Mazzoni *et al.*, 2005). However, the mechanism of generation of behavioural rhythm appears to be different, as PDF was shown not to be involved in larval rhythms (Mazzoni *et al.*, 2005). More details on the generation of circadian neurons and larval photoreception are available in 5.1 and 3.1 respectively.

The most interesting problem suggested by the findings presented above is the impact of circadian clock during development on circadian function in adulthood. The circadian clock regulates the timing of development as eclosion of adult flies is correlated to the function of the circadian clock (Konopka and Benzer, 1971). Moreover, flies given a brief light pulse at the beginning of their larval instar were able to synchronize their adult behaviour or eclosion to it, not only confirming that a circadian clock works in larvae but also suggesting that somehow the phase information persists through metamorphosis, despite extensive remodelling (Brett, 1952; Sehgal *et al.*, 1992). This suggests the existence of a mechanism preserving circadian clock function through metamorphosis which is supported by protection of the clock even through the cell cycle as daughter cells inherit clock (with some phase shifts) from original

cell after mitosis in cyanobacteria and fibroblasts (Mori *et al*., 1996; Kondo *et al*., 1997; Nagoshi *et al*., 2004).

Contrary to the situation described for zebrafish (1.5.2), experiments in flies have also demonstrated that circadian clock function does not require prior input from Zeigeber, as flies demonstrated rhythmicity even when not exposed to light at all, however they lack synchrony (Sehgal *et al*., 1992). On the other hand, the function of the clock during development appeared to be unnecessary for the adult behavioural rhythms (Ewer *et al*., 1988 and 1990). More information is available in 3.1.

Therefore, it appears that the mechanism of the development of circadian clock function in *Drosophila melanogaster* might be different to the one in mammals or zebrafish.

1.5.5 Impact of circadian clock on development

The fact that circadian clock components and tissues are present during development, and that some rhythms can be detected during development, could suggest that a function of the circadian clock is important for the proper development of organisms. However, the function of a circadian clock is not necessary for normal development since most circadian mutants develop normally (Konopka and Benzer, 1971; Dolatshad *et al*., 2006). This might be somewhat justified by the fact that development in most cases occurs in a reasonably protected environment such as a uterus or chorion. Therefore being able to time physiology according to an external cycle might not be crucial. However, given that experiments conducted in a laboratory use a very simplified environment, in which there are no predators and food is in unlimited quantity, there is a possibility that a selective advantage of a functional clock during development is not uncovered. On the other hand, even in a simplified laboratory environment, mouse circadian clock mutants showed decreased survival of birth and early life (Dolatshad *et al*., 2006).

D. melanogaster larvae do not develop in a protected environment and therefore circadian clock during development could be more important for them. Indeed, circadian clock also regulates the timing of eclosion, which

happens around dawn, even if flies reached maturity earlier. This is the time of day when it is most humid and therefore optimal for eclosion and subsequent expansion of wings (Myers *et al*., 2003). Moreover, the circadian clock was shown to regulate the time of development and also affect an adult lifespan – the faster the clock, the shorter the development and lifespan (Kyriacou *et al*., 1990; Klarsfeld and Rouyer, 1998; more details in the discussion in Chapter 4).

Moreover, in flies, many components of the molecular pacemaker have been shown to be important for some aspects of development, except the main components of the primary transcription-translation loop, including CRY (Hardin, 2005). For example, mutations in kinase DBT (which is also known as DISCS OVERGROWN) result in either a smaller size or the absence of imaginal discs, which leads to lethality to the developing fly (Zilian *et al*., 1999). Another kinase, SGG, is necessary for a proper segment polarity, regulating the β-catenin ARMADILLO distribution (Siegfried *et al*., 1990). Similarly, PP2A regulates β-catenin distribution (Seeling *et al*., 1999). Also SLIMB was shown to be critical for larval survival (Jiang and Struhl, 1998). Finally, some of CLK/CYC targets are important for regulating development. Among circadian clock components, VRI was shown to participate in dorsoventral patterning during embryogenesis (George and Terracol, 1997).

Concluding this section, even from a preliminary analysis of the literature of the development of circadian clock function, it is evident that there is no consistency between various organisms. It appears that development of the neuronal and molecular oscillator and timing of the appearance of circadian outputs depend on the complexity of the life cycle of the organism. Therefore, despite many similarities between a clock function and components, there are still some major organism-dependent differences.

1.6 Project Aims

It is still relatively unknown how the molecular oscillator acts during development to affect adult locomotor behaviour. This thesis describes research on the developmental requirements of the circadian clock function in *Drosophila melanogaster*.

The research can be divided into three main questions:

- **Is functional activity of the circadian clock or its components during development important for circadian locomotor rhythms in adulthood?** To answer this question I performed experiments to test the results from literature suggesting that circadian clock function during development is not necessary for adult behavioural rhythmicity. I tested various methods of reversible disruption to molecular oscillator function to test if levels of various components of molecular oscillator are important for the function of the circadian clock in adulthood. Answer to this question is presented in Chapters 3 and 4.
- **What are the temporal and spatial requirements for the molecular circadian oscillator and/or its components for the generation of adult behavioural rhythmicity?** To answer this question Il provided further characterisation of the developmental requirement for either circadian clock function or the presence of the clock component, as determined in the previous part of research. I tested which stages of development and which tissues/cells during development are connected to locomotor rhythmicity of adults by disrupting the clock at various developmental times and in various locations. This question is answered in Chapter 5.
- **What is the mechanism linking the function of the circadian clock during development and adult locomotor rhythmicity?** The most likely ways in which developmental clock function or clock components can be connected to adult rhythmicity is either through permanent loss or weakening of function of the molecular oscillator or through the change in neuroanatomy. Both of these theories were tested, together with others that became plausible when the answers to the previous questions were uncovered and the results are presented in Chapters 5 and 6.

Results obtained in a course of this research will not only be useful to understand the development of the circadian clock but also to gain deeper understanding of the function of the molecular oscillator *per se*. Depending on the results, it is also possible that they could suggest some mechanisms that are conserved among species, and contribute to better understanding of mammalian clock function or at least suggest complimentary research to be performed in mammals.

Chapter 2: Methods and materials

2.1 Culturing flies

2.1.1 Media

Flies were kept in the glass or plastic vials containing 8-12 ml of solidified fly food media. In some of the experiments the food was the standard cornmeal-agar medium with molasses (3.4.1, 3.4.2, 3.4.3, 4.4.1, 4.3.2, 4.4.3, 4.4.4, 5.4.1, 5.4.2, 5.4.3, 5.4.4, 5.4.5 (Figure 5.9), 5.4.6, 6.4.2, A.1.1 (except Figure A.1.2), A.1.2, A.1.3, A.2.1-A.2.5, A.3.1 – A.3.6 - Experiments A) and in some with sucrose (5.4.5 (Figures 5.10 and 5.11), 6.4.1, 6.4.3, Figure A.1.2, A.2.1, A.4.1 - Experiments B). As an anti-fungal agent, propionic acid and 10% (w/v in 95% ethanol) Tegosept (Genesee Scientific) were used. Food was stored in a fridge prior to use. When it is not indicated differently, flies were raised and kept at room temperature (RT).

Cornmeal-agar medium with molasses:

For 1000 ml of distilled water:

- 10.6 g agar
- 71.4 ml molasses
- 29.4 g yeast
- 71.4 ml cornmeal
- 5.7 ml propionic acid
- 16 ml Tegosept

Cornmeal-agar medium with sucrose:

For 1000 ml of distilled water:

- 6 g agar
- 17.5 g yeast
- 10 g soy flour
- 73.1 g yellow maize meal
- 46.2 g light malt extract
- 80 ml 60% sucrose solution (48 g sucrose + 80 ml water)
- 5 ml propionic acid
- 7 ml Tegosept

2.1.2 Addition of a drug (NaBu) to media

To test how food properties change upon addition of various quantities of liquid, various amounts of water were added to liquid media to obtain 0% (control) 5%, 10%, 15% and 20% diluted food (sucrose media recipe was used). Food was tested for: solidification time (it took slightly longer for the most diluted food), overall consistency, as determined by the touch and desiccation of food (no major differences observed), adhesiveness of food, as determined by a number of adult flies that get stuck in food (no differences observed) and ability to sustain flies' development, as assessed by the number of adult progeny obtained from 20 parents placed in a vial (no differences). Based on these preliminary analyses I have decided that adding a diluted drug to the media would be a viable method of administering a drug.

When a drug (Sodium Butyrate - NaBu) was added to the media, a 1M dilution of drug in water was prepared first. This concentration was chosen so that an addition of liquid to food could be kept to a minimum while staying within solubility limits of NaBu. Final working concentrations of 100 mM and 30mM were prepared when drug was added to food when it cooled down, to avoid thermal degradation of NaBu. Food was stored according to a standard protocol.

Similarly, when cuvettes with drug were needed, I added 1M NaBu in water to a standard agar-sucrose food (recipe in 2.3.1) to obtain a proper working concentration. Just as before, the diluted drug was added to cooled food. Both in the case of vials and cuvettes, the controls had just water added in a same amount.

2.1.3 Stocks

Flies with simple genetic elements used either for experiments or creating more complex lines were either gratuitously received from the fellow *D. melanogaster* researchers or ordered from fly lines repositories. Lines which contained a combination of genetic elements were either created in a laboratory by the author or by other members of the research group.

Tables below do not include lines carrying *dsRNA* elements (*dsRNA* connected to GAL4-responsive *UAS*) that came from either Bloomington Stock Centre at Indiana University (marked as four or five digits referencing the stock number), Vienna *Drosophila* RNAi Centre (marked as five digits starting with "v" referencing the stock number) or Paul Hardin's laboratory (*ds Pdp1ε* line).

Genetic element:	Description:	Reference and Source:
per^{01}	Null mutant of *per*	Konopka and Benzer, 1971 Donated by M. Young
cyc^{01}	Null mutant of *cyc*	Rutila *et al.*, 1998 Donated by M. Young
tim(UAS)-Gal4	Expresses GAL4 in all clock-bearing cells	Blau and Young, 1999 Donated by M. Young
tim62-Gal4	Expresses GAL4 in all clock-bearing cells	Kaneko *et al.*, 2000b Donated by M. Young
elav^{C155}::Gal4	Expresses GAL4 in all post-mitotic neurons	Lin and Goodman, 1994 Donated by M. Young
Pdf-Gal4	Expresses GAL4 in all PDF-positive neurons	Renn *et al.*, 1999 Bloomington stock
Mai179-Gal4	Expresses GAL4 in all s-LN$_v$s, including the PDF-negative 5th, 3 LN$_d$s and possibly one l-LN$_v$	Grima *et al.*, 2004 Donated by F. Rouyer
cry-Gal4-13	Expresses GAL4 in Morning and Evening cells	Stoleru *et al.*, 2004, Busza *et al.*, 2007 Bloomington stock

Genetic element:	Description:	Reference and Source:
R6-Gal4	Expresses GAL4 in s-LN_vs and much weaker in few (occasionally) of l-LN_vs, as well as couple other non-circadian neurons	Helfrich-Förster *et al.*, 2007a Donated by P. Taghert
c929-Gal4	Expresses GAL4 in a pattern corresponding to DIMMED – in many peptidergic cells and l-LN_vs but no s-LN_vs	Park *et al.*, 2008 Bloomington stock
tub_PGal80^{ts}	Expresses $GAL80^{ts}$ in all somatic cells	McGuire *et al.*, 2003
UAS-per24	Expresses PER in presence of GAL4	Kaneko *et al.*, 2000b, Yang and Sehgal, 2001
UAS-cyc	Expresses CYC in presence of GAL4	Tanoue *et al.*, 2004 Donated by P. Hardin
Pdf-Gal80	Expresses GAL80 in PDF+ neurons	Park *et al.*, 2000 Donated by J. Blau (transgene on third chromosome)
cry-Gal80	Expresses GAL80 in CRY+ neurons	Emery *et al.*, 2000b
tim-luc	Expresses *luciferase* in all clock cells	Stanewsky *et al.*, 1998 Donated by M. Rosbash and J. Hall
UAS-Dcr2	Expresses DICER2 in presence of GAL4	Dietzl *et al.*, 2007 Bloomington stock

Genetic element:	Description:	Reference and Source:
UAS-mCD8::GFP	Expresses GFP in presence of GAL4	Lee and Luo, 1999 Donated by D. Dickinson
UAS - Pdp1ε	Expresses PDP1ε in response to GAL4	Benito *et al.*, 2007 Donated by P.Hardin
UAS – Mef2	Expresses MEF2 in response to GAL4	Blanchard *et al.*, 2010 Bloomington stock

Table 2.1. Main genetic elements used for creation of flies.

Table presenting main genetic elements used to conduct experiments together with a short description of their function. When possible, a source of the particular line used is acknowledged. Table does not include any *dsRNA* lines.

Line:	Description:	Source/Reference:
Canton-S	Wild-type control	Stern and Schaeffer, 1943 Bloomington stock
y w	Wild-type control	Eeken, 1982 Bloomington stock
w^{1118}	Wild-type control	Hazelrigg *et al.*, 1984 Bloomington stock
y w BGluc::60	*luciferase* transgenic transposon in *yw* background	Stanewsky *et al.*, 1997b Donated by J. Hall

Line:	Description:	Source/Reference:
y per^{01} w; tim(UAS)-Gal4/tub$_P$Gal80^{ts}; UAS-per24/+	"*per* rescue"; conditionally rescues PER expression in all circadian cells	Result of a cross of 2 lines below
y per^{01} w; tim(UAS)-Gal4	Expresses GAL4 in circadian cells of *per^{01}* flies	Created by K. Mirowska
y per^{01} w; tub$_P$Gal80^{ts}; UAS-per24	Expresses GAL80^{ts} and has GAL4-inducible *per* transgene in all cells of *per^{01}* flies	Created by J. Currie
y tub$_P$Gal80^{ts} w/FM7c; tim(UAS)-Gal4; UAS-per24	"*per* over-expression"; conditionally over-expresses PER in all circadian cells	Created by H. Wijnen
elav^{C155}::Gal4; UAS-cyc/CyO; cyc^{01} tub$_P$Gal80^{ts}	"*cyc* rescue"; conditionally rescues CYC expression in all post-mitotic neurons	Created by K. Mirowska
tim-luc; tim(UAS)-Gal4; UAS-per24	Expresses *luciferase* and over-expresses PER in all clock-bearing cells	Created by K. Mirowska
y tub$_P$Gal80^{ts}w/tim-luc; tim(UAS)-Gal4; UAS-per	Conditionally over-expresses PER and expresses *luciferase* in all clock-bearing cells	Result of a cross

Line:	Description:	Source/Reference:
y tub$_P$Gal80^{ts}w/FM7c; Pdf-Gal4/CyO; UAS-per24	Conditionally over-expresses PER in PDF+ cells	Created by K. Mirowska
y tub$_P$Gal80^{ts}w/FM7c; Mai179-Gal4/CyO; UAS-per24	Conditionally over-expresses PER in cells targeted by *Mai179-Gal4*	Created by K. Mirowska
y tub$_P$Gal80^{ts}w/FM7c; tim(UAS)-Gal4/Pdf-Gal80; UAS-per24	Conditionally over-expresses PER in all circadian cells except PDF+ neurons	Result of a cross
y tub$_P$Gal80^{ts}w/FM7c; tim(UAS)-Gal4; UAS-per24/cry-Gal80	Conditionally over-expresses PER in all circadian cells except CRY+ neurons	Result of a cross
w; tim(UAS)-Gal4; UAS-per24/Pdf-Gal80	Constitutively over-expresses PER in all circadian cells except PDF+ neurons	Result of a cross
w; tim(UAS)-Gal4; UAS-per24/cry-Gal80	Constitutively over-expresses PER in all circadian cells except CRY+ neurons	Result of a cross
y w/w;Pdf-Gal4/+; UAS-per24/+	Constitutively over-expresses PER in all PDF+ cells	Result of a cross

Line:	Description:	Source/Reference:
y w/w;Mai179-Gal4/+; UAS-per24/+	Constitutively over-expresses PER in all cells targeted by *Mai179-Gal4*	Result of a cross
y w/w;c929-Gal4/+; UAS-per24/+	Constitutively over-expresses PER in all cells targeted by *c929-Gal4*	Result of a cross
y w/w;R6-Gal4/+; UAS-per24/+	Constitutively over-expresses PER in all cells targeted by *R6-Gal4*	Result of a cross
y w/w;+/+; UAS-per24/cry-Gal4-13	Constitutively over-expresses PER in all CRY+ cells	Result of a cross
y w UAS:CD8:GFP/y w $tub_{P}Gal80^{ts}$*; tim(UAS)Gal4; UAS-per24*	Conditionally over-expresses PER and expresses GFP	Result of a cross
w; tim(UAS)Gal4; $tub_{P}Gal80^{ts}$	Line used for conditional expression of genes or *dsRNA* connected to UAS	Created by H. Wijnen

Table 2.2. Main lines used for experiments.

Full genotypes and short description of lines used in experiments presented. For simplicity, only final experimental lines (and not lines used in their creation or lines used as controls) are listed. Origin of a particular line is acknowledged, with "Result of a cross" marking flies that did not come from established fly culture and had to be obtained from a cross before each experiment.

2.1.4 Crosses

At times it was necessary to cross lines to obtain offspring with a novel combination of genetic elements. When it was done to create a new stable line, care was taken to ensure that final flies were either homozygous or had genetic elements on one copy of a chromosome and a balancer chromosome (a chromosome which contains the same set of genes as a chromosome it replaces but in a different order, preventing chromosomes from lining up properly and exchanging genetic material in the process of crossing-over). When an aim was to obtain offspring for use in experiments immediately, they were often heterozygous for certain chromosomes (without the use of balancer chromosomes), but this heterozygosity was always introduced at the last stage of a cross.

To ensure that resulting flies had an anticipated genotype, balancer chromosomes were usually introduced at early stages of a cross, to make sure that it was easy to track genes. This was possible since balancer chromosomes (as well as some other marker chromosomes) contained dominant mutations affecting an appearance of flies and homozygous lethal mutations that prevented flies from having two copies of a balancer chromosome. Therefore it was easy to distinguish flies containing balancer chromosome from flies with two copies of unmarked chromosome.

When flies were crossed, 5-10 (or less when it was difficult to obtain this preferred number) fresh (up to 7 days old if necessary) virgin females of a certain genotype were placed in a new vial and 3-10 young males were added. Flies were left at room temperature for 3-4 days to allow them to mate and females to lay eggs. After that time, adult flies were moved to a fresh vial and the old vial with embryos and young larvae was moved to a temperature at which development was supposed to take place. When there was no need to raise flies at a particular temperature, flies were raised at room temperature. If food in vials was becoming too desiccated, water was added to vials. When flies were ready to eclose, they were monitored for offspring at least twice a day to allow for a collection of virgins or less often if males were to be recovered from the cross. Unused flies were discarded.

2.2 Manipulating gene expression

The nature of experiments envisioned in this project was largely based on being able to switch activity of certain genes on and off at will. This can be achieved by manipulating a level of circadian gene through either restoring arrhythmicity of circadian genes null mutants by expressing transgenic circadian genes or by over-expressing one of these genes. The mechanism of ensuring that correct genes are activated in a certain cells at a proper time that was used in this experiment was the temporal and regional gene expression targeting (TARGET) system (McGuire *et al*., 2003 and 2004).

2.2.1 Spatial control over gene expression

The easiest approach to ensuring that a certain gene is expressed in a given spatial pattern would be to connect a gene of interest to a promoter element restricting expression only to particular cells. This approach is not perfect, as it requires creation of new transgenic elements for every spatial expression patter anew. Moreover, in a case where mis- or over-expression of some genes could result in a phenotype affecting health or fertility of flies, it would be impossible to keep a certain line as a stable stock. The perfect solution is creating flies that have certain genetic element but do not express it until it is needed. This is provided by the TARGET system.

The spatial part of the TARGET system is based on use of transcription activator protein from yeast – GAL4 (Brand and Perrimon, 1993), part of the galactose – mediated gene induction pathway. By placing the *Gal4* gene under action of a specific promoter element (also called driver genes) it is possible to direct GAL4 expression to specific cells or tissues. GAL4 is not expressed in flies, but its expression in those cells or tissues has little or no effect on flies. To activate gene expression, GAL4 needs to bind to and activate enhancer known as UAS (Upstream Activation Sequence). A gene of interest connected to UAS will be present in all cells but will be expressed only when GAL4 is present (Brand and Perrimon, 1993). Therefore these two elements might be carried by two separate flies and combined to produce progeny that contains both of

them, making it possible to have an access to far more genetic combinations and healthy stocks.

It is also possible to exclude some cells or tissues from flies that express a gene of interest due to Gal4/UAS system. This is because a natural repressor of GAL4 exists in yeast, known as GAL80 (Suster *et al*., 2004). GAL80 is a part of the same yeast galactose – mediated gene induction pathway. By introducing it into flies under some specific promoter element, certain groups of cells can be excluded from the ones that express a gene of interest due to the GAL4 presence, providing further refinement of a spatial control of a gene expression. It permitted removal of a group of cells targeted by a promoter element linked to *Gal80* gene from cells expressing GAL4. The elements I used during my research were *Pdf-Gal80* and *cry-Gal80*, which were described in Park *et al*. (2000) and Emery *et al*. (2000b). Also, since the GAL4 is a yeast-derived protein, its activity is correlated to a temperature, with GAL4 more active (and consequently higher expression of the UAS-linked gene) at higher temperatures.

2.2.2 Temporal control over gene expression

Temporal control over when a transgenic gene is expressed can be achieved either by connecting a transgene to a heat-shock promoter (*hs*) and then exposing flies to a pulse of elevated temperature (>30˚C) or by using the TARGET system (McGuire *et al*., 2003 and 2004). An element of the TARGET system that allows temporal control is a temperature sensitive GAL80 ($GAL80^{ts}$) (McGuire *et al*., 2003 and 2004). At elevated temperatures (25-29˚C), $GAL80^{ts}$ is disabled, allowing for GAL4 mediated gene expression. At lower temperatures (17-23˚C), $GAL80^{ts}$ remains active and represses GAL4, so that genes linked to UAS promoter are not expressed. The system is fairly sensitive to changes in temperature and an expression of a gene can be switched on almost immediately and switched off within a day. To make sure this on/off switch can be applied to all cells, I have used tub_PGal80^{ts}, a $Gal80^{ts}$ transgene connected to the systemic *tubulin* (*tub*) promoter.

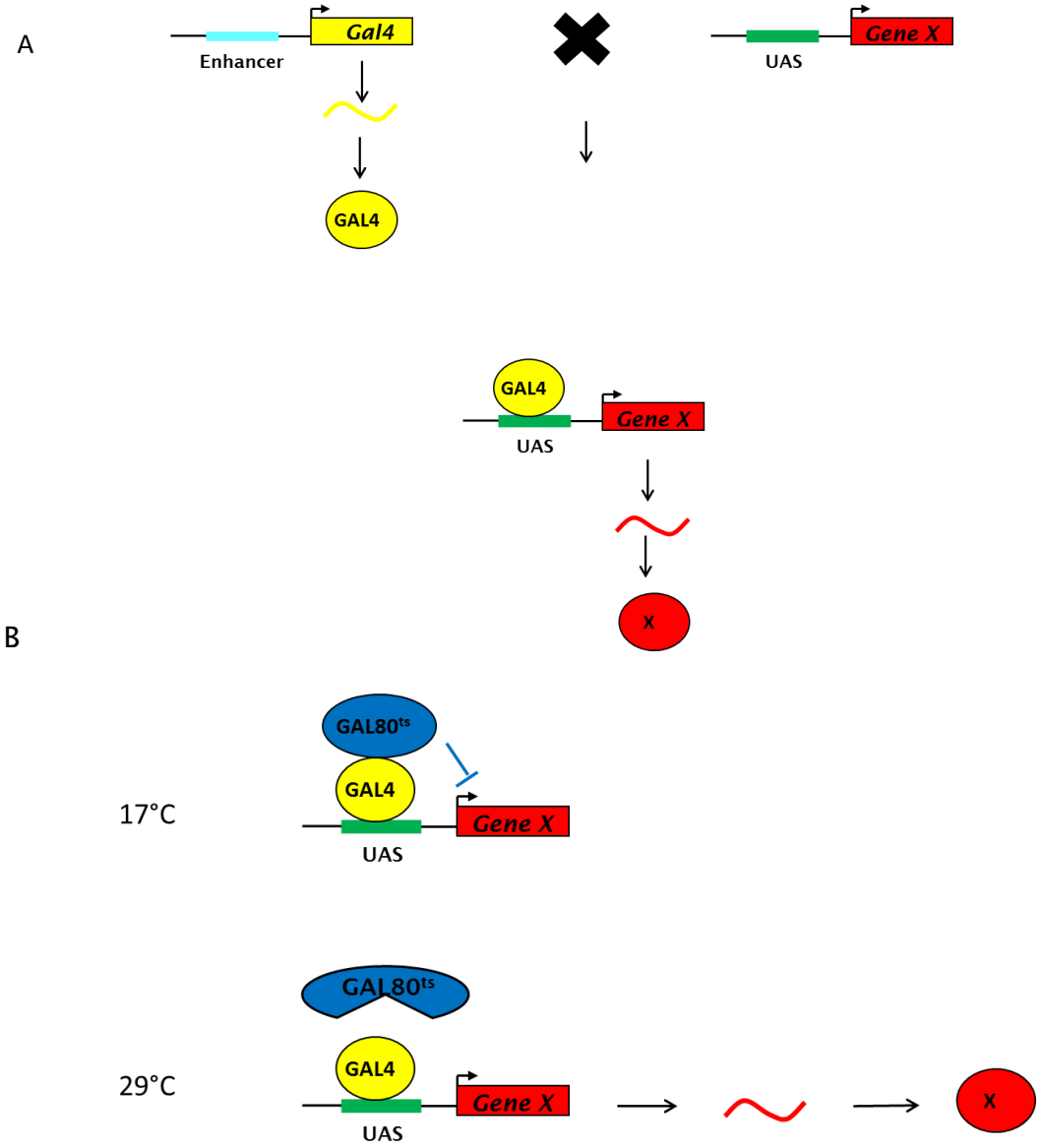


Figure 2.1. Schematic representation of the system used for spatial and temporal control of gene expression.

(A) TARGET system for achieving spatial control, based on combined action of GAL4 and UAS. Expression of a transgene occurs in specific cells or tissues only when GAL4 driver and transgene connected to UAS are combined. (B) Addition of GAL80^{ts} to TARGET system allows temperature-dependent gene expression, as GAL80^{ts} blocks GAL4-mediated expression of transgene only at low temperatures.

2.2.3 Inhibition of gene expression (gene knockdown)

RNA interference (RNAi) decreases levels of a certain protein by inhibiting gene expression. It utilizes *double stranded interfering RNA* (*dsRNA*) which is complimentary to a gene of interest (Fire *et al*., 1998; Van Roessel *et al*., 2002). Additionally, in most cases flies in which RNA interference was supposed to be used were supplemented with *UAS-Dicer-2* (*UAS-Dcr-2*) element. The endoribonuclease DICER-2 initiates the RNAi pathway by cleaving *dsRNA* molecules to shorter, ~20 nucleotide fragments, which later have their strands separated and the guide strand becomes a part of RNA-INDUCED SILENCING COMPLEX (RISC). Adding additional DCR-2 was aimed at increasing activity of RNAi pathway (Dietzl *et al*., 2007).

Flies carrying *Gal4* transgene directed to a specific subset of cells or tissues and *UAS-Dcr-2* were crossed to flies carrying *dsRNA*. Resulting experimental flies were tested and analysed as described in 2.3 separately for females and males, as due to placement of UAS-Dcr-2 on X-chromosome, a gene dosage effect could be observed.

2.3 Behavioural assay

2.3.1 Locomotor analysis

Adult locomotor activity of flies was monitored using *Drosophila* Activity Monitoring System (DAMSystem, TriKinetics, Waltham, MA, USA). Flies (usually 3-4 days post eclosion) anaesthetised with CO_2 were loaded individually into glass cuvettes (5 mm diameter/50-60 mm length) containing standard sugar-agar media (5 % sucrose, 1 % agar) with 0.07% Tegosept (w/v; Genesee Scientific, San Diego, CA, USA prepared in 95% ethanol) added as an antifungal agent. The other end of the cuvettes was closed with a cotton plug, allowing for air exchange.

Flies were analysed in either the Percival I-36VL incubators (Percival Scientific, Perry, IA, USA) maintaining a constant temperature and 70% relative humidity (Experiments A) or in incubators made from lightproof black boxes with water trays, maintaining around 80% relative humidity (Experiments B). During light

phase, flies were exposed to the white fluorescent light of the intensity ~450 $\mu W/cm^2$ (experiments A) or white light from the LED lights (with a sharp spectra peak found at 441 nM and a broader and smaller one at 547 nM) of intensity ~0.97uM/m^2s (experiments B).

When flies were analysed at 12 hours light:12 hours dark cycles, abbreviated as LD, the relative time of a cycle was referred to as ZT time (from Zeitgeber time), with ZT0 being the time of lights switching on, representing dawn, and ZT12 time of lights switching off, representing dusk. When flies were moved to aperiodic environment, where temperature and humidity were kept constant and the lights were permanently switched off, it was known as DD and time was denoted as CT (from Circadian time), with CT0 being consistent with ZT0 of previous light cycle.

Unless otherwise noted, flies were moved from LD to DD just before ZT0 (just before lights should be turned on). The transfer of monitors between incubators or boxes occurred in constant darkness with the help of a lamp emitting purely red light (very sharp and narrow single peak at 631 nM), which could not be detected by flies. When flies were being moved from DD to LD, it was also just before ZT0 as determined by LD cycle.

Data was collected from the *Drosophila* Activity Monitor system with software provided by the DAMSystem manufacturer by pooling activity of flies over 30 minute bins. Subsequently, data was analysed using the ClockLab software (ActiMetrics, Wilmette, IL, USA). First, individual activity records of flies were investigated to exclude flies that didn't survive the entire testing period from further analysis.

2.3.2 Qualitative analysis

To obtain qualitative graphs, all flies of the same genotype, sex and testing conditions were pooled together and a median or average (as noted in analysis) of their activity records was created. This was then used to plot behaviour of flies over time as actograms. In most cases, actograms were double-plotted, meaning that each day was represented twice and two days formed one row on the graphs (top row showing days 1 and 2, second row days 2 and 3 and so

on). Activity of flies within each 30 minute window was represented with black bars and a height of each bar represented the amount of activity.

2.3.3 Quantitative and statistical analysis

To detect a rhythmic component to the behaviour of a fly, a chi-square analysis was performed, which plotted a strength of rhythmic component of a given period for a range selected (in most cases 15-35 hrs) and a threshold line representing the confidence level of $p<0.01$. The period for which the most significant rhythmic component within this range was detected was treated as a period length.

To analyse individual flies, chi-square periodograms were created for individual flies, usually using the first full seven days at constant conditions (unless stated otherwise) and using $p<0.01$ as a confidence level for detecting rhythms. Flies were labelled as rhythmic, weakly rhythmic or arrhythmic based on a height of a peak detected with this analysis. If the peak was not above a threshold, flies were categorized as arrhythmic. Flies for which a rhythmic component higher than a threshold was detected were divided into weakly rhythmic and rhythmic based on their relative rhythmic power, which is a height of the peak divided by a height of threshold for the same period length. When this value was equal to or higher than 1.5, flies were called rhythmic. If this value was less than 1.5, flies were classified as weakly rhythmic.

The distribution of rhythmic, weakly rhythmic and arrhythmic flies was plotted using stacked bar diagrams, with dark blue representing rhythmic, light blue representing weakly rhythmic and red representing arrhythmic flies. A correlation between the distribution of flies and condition being tested (genotype/ developmental conditions/ treatment) was tested with a statistical significance test used in the analysis of contingency tables - Fisher's Exact 2x2, 2x3, 3x2, 3x3 or 4x2 (depending on a number of categories within each group and number of groups compared). When more groups were compared or numbers of flies were sufficiently high, Pearson's chi-square test was performed.

One exception from using only three categories of rhythmicity was when a rhythmicity of flies in LD was tested. To reflect both on a rhythmic strength (calculated as outlined above) and how well flies adhere to 12:12 LD cycle, five categories were created: flies displaying strong behavioural rhythm with a period of 24 hours (±0.5 hour), flies displaying weak rhythms with a period of 24 hours (±0.5 hour), flies displaying strong rhythms of other periodicities, flies displaying weak rhythms of other periodicities and arrhythmic flies. One of these categories was not used as it turned out that no strongly rhythmic flies with periods of rhythmicity other than 24 hours were ever recovered. Further analysis was conducted as described above with an exception that 4 categories had to be compared.

Average period length was calculated for all rhythmic flies (unless noted otherwise) based on results determined during individual analysis. For all values standard deviation and standard error of mean were calculated. Average relative rhythmic power (RRP) was determined for all rhythmic and weakly rhythmic flies (unless arrhythmic flies were also included in analysis with a value of 1 assigned, which would be clearly stated) and plotted as bar diagrams with error bars representing SEM. Scale on the X axis was adjusted to reflect the fact that flies with the relative rhythmic power below 1 were never tested. The association between average period length or relative rhythmic power and property tested was determined using non-parametric Mann-Whitney U-test (when two groups were compared), Kruskal-Wallis test (one-way ANOVA on ranks) when more than two groups were compared with the pairwise comparisons with Wilcoxon Matched-Pairs Signed Rank test or Welch's test (one-way ANOVA, unequal variances t-test) with Games-Howell post-hoc test (as noted next to experiment). The significance level used for all tests was $p<0.05$. When multiple comparisons were performed within the same group, either adjusted significance values were used or Bonferroni correction was used on the significance values.

2.3.4 Analysis of a phase of locomotor rhythmicity

To determine a phase of rhythmicity and its correlation with the time of shift to other conditions, chi-square periodograms of individual flies were plotted as

described above for full 5-7 days of analysis (as detailed) after experimental procedure or interruption. Flies that were classified as arrhythmic were excluded from further analysis as no phase could be assigned to a rhythm that is absent. Using a period length determined for each fly as x –axis, activity profile for each fly was plotted using ClockLab Software.

When activity profile is plotted for LD, it consists of morning and evening peaks separated by a mid-day siesta. In constant conditions, in the majority of cases there is only one broader peak around a time where an evening peak should be, usually with a sharper peak at the end. In cases when more peaks were present, an evening peak was used for phase determination. Two features of activity profile were used to determine a phase: time of an evening peak and offset time, which is a time when a peak has declined to half its original value. The final value of a phase was determined according to the protocol described previously (Currie *et al.*, 2009). First, a value determined from a plot was adjusted to represent phase of fly that would have period length of 24 hrs, using the following formula:

$$\varphi_{ADJ} = {}^{24 \times \varphi_{ORG}}/_{\tau}$$

Where:

ϕ_{ADJ} – adjusted phase value;

ϕ_{ORG} – original phase value;

τ – period length of behavioural rhythm.

Since flies were analysed starting from a first full day of DD, variability in duration of time they spent in DD before analysis has started had to be taken into account to reliably represent a phase of locomotor rhythmicity on a transition day with ZT0 selected as a reference point. If flies were in free-running conditions for n hours before the start of analysis, a correction coefficient was determined from the formula:

$$\varphi_{corr} = {}^{\lfloor n \times (\tau - 24) \rfloor}/_{\tau}$$

Where:

ϕ_{corr} – correction factor;

n – hours in DD before analysis;

τ – period length behavioural rhythm.

$$\varphi = \varphi_{ADJ} + \varphi_{corr}$$

Finally, a final phase value was plotted against the time of shift to constant conditions. To test if there was any correlation between these values, trendlines were fitted to data and two-tailed probability value of Pearson correlation coefficient was determined from R and n. Moreover, where a constant value of phase was suspected, values of phase time were tested with Kruskal-Wallis test to check if they were significantly different from each other. Also, a 2-tailed test was used.

2.4 Longevity experiment

In order to test an average lifespan of flies, male flies were collected within 3-4 days of eclosion. 50 flies of the same condition were analysed, with 10 flies in each vial, 5 vials per condition. Vials were coded so that the only information that was available to the researcher was the type of food on which the flies were housed. Vials were kept at 17˚C with 12:12 LD cycle. Every 2-3 days during light phase vials were inspected and live flies were counted and moved to a vial with fresh food every other time. Any flies that escaped during a change of vial or appeared to die due to getting stuck in food were excluded from the analysis.

Collected data was analysed using the GraphPad Prism 6 (GraphPad Software, Inc.) and when an experiment was finished, data were assigned to proper condition. Survival of flies was plotted using the GraphPad Prism 6 software, which was also used to determine a median survival (day at which half of population had died). Conditions were compared using GraphPad Prism 6 with Log-Rank and Mantel-Cox test.

2.5 Molecular oscillations in peripheral tissue

2.5.1 Preparation of assay plates

To measure gene oscillations in the peripheral tissue, I used transgenic luciferase reporter system. Young flies containing *tim-luciferase*, *luciferase* transgene element linked to *tim* promoter (Stanewsky *et al*., 1998) were anaesthetised with CO_2 and individually loaded into wells in assay plates (Optiplate, Perkin Elmer). Assay plates were 96-well plates with each well filled with 100 µl of solidified standard sugar-agar media (5 % sucrose, 1 % agar) with 0.07% Tegosept (Genesee Scientific, prepared in 95% ethanol) and luciferin (GOLDBIO) added to a final concentration of 11-12 mM (adapted from Brandes *et al*., 1996). To restrict vertical movement of flies, small transparent plastic domes (converted from the PCR tubes caps) were used to cover them. Each dome had two holes punched in it to allow air exchange. Finally, plates were sealed with transparent plastic and holes were punched in plastic to allow air exchange.

To prevent accidental detection of light from flies housed in neighbouring wells, two different techniques were used. In some experiments, black plates were used as I have previously tested that no light is detected in an empty well surrounded by wells with flies emitting light. When white plates were used, flies were loaded into every other well, changing order with each row, so that each well with a fly was surrounded by four empty wells.

2.5.2 Running the experiment

Plates were loaded into the Packard TopCount Multiplate NXT Scintillation Counter which was programmed to measure light produced in each well for 5-17 seconds (as counts per second), depending on experiment and number of plates handled, so that each plate was tested around every hour or two hours. Flies were always exposed first to LD cycle (with ZT0 at 9:00 am) for 3-4 days to entrain their rhythms and subsequently analysed in DD. Every time a plate was taken into the Scintillation Counter for measurements, it was moved to constant darkness for a limited amount of time, even if flies were in light phase

of LD. The measurement room was temperature controlled and TopCount Scintillation Counter was equipped with additional temperature control mechanism to keep the temperature around 17°C. During some experiments temperature in the room rose at times to around 21°C due to a failure in room temperature control system, however this temperature was still recognized by flies analysed as permissive and results obtained did not differ from those from a lower temperature.

2.5.3 Data analysis

Data collected was appended in a table using DigDB (Microsoft) and analysed using BRASS software (created by Dr Paul E. Brown, University of Edinburgh). First, results were plotted as counts per second against CT/ZT time for individual flies. Flies that did not survive until the end of a fifth full day of DD or flies that escaped from under domes (as obvious from visual inspection) were excluded from further analysis. Also, everything before ZT/CT24 and after the end point for analysis was removed. Data for all the remaining flies from the same conditions was averaged and resulted datasets were detrended for amplitude and baseline level and average profile was plotted as a function of counts per second over ZT/CT.

Data for individual flies was analysed using Fast Fourier Transform – Non-Linear Least Squares (FFT-NLLS) over the selected ZT range (that covered 5 full DD days) using 10-35 period range at 95% confidence level ($p<0.05$) with a prior normalisation. FFT-NLLS returned information on period length, phase and amplitude of detected rhythmic component. When no rhythm could be fitted into a given data set, flies were treated as arrhythmic. Flies with a rhythmic component detected were further divided into two categories based on a strength of rhythm as determined by the Relative Amplitude Error (RAE) into rhythmic ($RAE<0.7$) and weakly rhythmic ($RAE>0.7$). For each condition, distribution of rhythmic, weakly rhythmic and arrhythmic gene expression patterns was plotted. Correlation between this distribution and either developmental temperature or genotype was tested with Fisher's Exact 2x3 test. Additionally, an average RAE value for each condition was calculated and

the association of this value with either developmental temperature or genotype was tested with the Mann-Whitney test.

2.6 Immunofluorescence

2.6.1 Flies used

For an experiment analysing gross morphology (Figure A.3.6), flies with conditional *per* over-expression were used. Flies were raised under either permissive (~23˚C) or restrictive (29˚C) conditions. Adult flies were kept at 17˚C LD for over a week and collected either on day 8 at ZT 22 or at day 9 at ZT 4, representing peak and trough, respectively.

For more detailed analysis (Figures 5.12 and A.3.7), conditional *per* over-expression flies were crossed to introduce a *UAS-mCD8::GFP* transgene on X chromosome for fluorescent marking of circadian neurons (Lee and Luo, 1999). To keep the same levels of *per* over-expression as in previous behavioural experiments, flies were homozygous for both *tim(UAS)Gal4* and *UAS-per24* elements and that left me with only X chromosome for introduction of *mCD8::GFP*. Since flies had to retain the $tub_{P}Gal80^{ts}$ element that is also located on X chromosome, this restricted the experiment to using only females.

Final experimental female flies (*yw UAS:CD8:GFP/yw* tub_{P}*Gal80ts; tim(UAS)Gal4; UAS-per24)* were raised under either permissive (room temperature) or restrictive (29˚C) conditions. 3-4 days old flies were collected and placed at 17˚C LD for 6 days for entrainment and subsequently moved to constant darkness at 17˚C. Starting on 2nd day of DD, flies were collected on ice every 6 hours, starting at CT16 (therefore CT4 and CT10 were collected on day 3).

2.6.2 Preparation of brains

Flies were collected into clean vials on ice, which anaesthetised them and prevented degradation of proteins. Vials were kept on ice until flies could be further treated but in no case for longer than an hour. When flies were

collected from DD or dark part of LD cycle, they were then kept on ice in complete darkness.

Working in batches, flies from the same condition were placed on a pre-cooled and cleaned metal block kept on ice. The brains were dissected on the block (as opposed to a drop technique) using some moisture that collects on the block to prevent desiccation and keep tissue in place. The heads of the flies were removed using a sharp micro-scalpel and the bodies were discarded. Using sharp forceps, a proboscis was removed and then using gentle manipulation of two sets of forceps, the brain was removed from the head cuticle. As soon as the brain was extracted and surrounding trachea fragments were removed, the brain was placed in 0.5 ml tube with 100 µl 4% paraformaldehyde kept on ice. The procedure was repeated until around 20-30 intact brains were collected, which lasted for about 20-30 minutes.

The procedure for fixing and staining brains was adapted from Wu and Luo, 2006. When a sufficient number of brains was collected, they were left for further fixing in formaldehyde for 20 minutes at room temperature on a nutator. After 20 minutes, the tube was placed on a rack and the brains were allowed to settle. Paraformaldehyde was aspired and the brains were washed twice in 100 µl of 0.3% (vol/vol) PBT (100 mM Na_2HPO_4/NaH_2PO_4 at pH7.2 with Triton-X), each time gently inverting a vial and allowing the brains to settle before removing the liquid. Three longer, 20 – minute washes with 100 µl of PBT followed where the tubes were placed at nutator. After a final wash, PBT was removed and 100 µl of fresh 5% (vol/vol) normal goat serum (NGS) which acted as a block solution was added and the tubes were placed on nutator for 1-2 hours.

After removal of the block solution, 100 µl of primary antibodies diluted in fresh 5% NGS was added and tubes were placed on nutator at 4˚C for 2-3 days. Following that time, the solution was removed and the same washing procedure as outlined above was used. After a third long wash 100 µl of secondary antibodies solution in fresh 5% NGS was added and the tubes were wrapped in tin foil and placed on nutator at 4˚C for further 2-3 days. The final step of the staining protocol was the removal of secondary antibody solution and washing the brains in PBT using the same procedure as described above. After the last wash, the brains were removed using a cut pipette tip and placed

on a glass slide. Excess liquid was removed and Hard-set mounting medium added. To avoid squishing brains between a slide and a coverslip, edges of a slide were previously painted with a nail polish, creating a slight elevation on which the coverslip could be safely placed. Edges of a cover slip were then sealed with nail polish. Slides were stored in boxes at -18-20˚C until ready for further analysis.

Following concentrations of antibodies were used in the experiments presented in this thesis:

- Rabbit anti-PER 1:187500
- Mouse anti-PDF 1:250
- Rat anti-TIM 1:500
- Chicken anti-GFP 1:1000
- Goat anti-chicken Alexa 488 green 1:250
- Goat anti-mouse Alexa 488 green 1:250
- Goat anti-rat Alexa 594 red 1:250
- Goat anti-rabbit Alexa 568 red 1:250

2.6.3 Immunofluorescence data collection and analysis

Images were collected using spinning disk confocal microscope using x40 oil objective with additional x10 optics. Pictures were taken using green and red channel using stack thickness of 1 µm (analysis of projections) or 0.2 µm (analysis of molecular oscillations). To study gross neuroanatomy of dorsal LN_v projections, images from blinded brains were scored for presence or absence of said projections. LN_vs were identified by staining with PDF.

To analyse molecular oscillations in a group of cells, before taking a picture of each stack these cells were placed in the middle of a field of view. Cells were identified based on their shape, size and location. After pictures of the stacks were taken, images were processed using ImageJ, in a way that prevented a researcher from knowing which stack corresponds to which flies and which timepoint. Each cell was individually selected by drawing around its outline and its size in square pixels and the maximum intensity of average red signal and average green signal for the same picture in a stack were recorded. Using the

same area, a picture was found on which the red and green signal was no longer visible and values recorded were used as a background signal, which was subtracted from the maximum values recorded. Resulting values were used to calculate a median value for all cells of the same type in one hemisphere and 6-10 of these values from different hemispheres were used again to determine a median value for a particular group of cells amongst one experimental set. Additionally, each cell was scored based on a localisation of TIM and assigned one of the following categories: nuclear, cytoplasmic, both or none.

The results of the analysis of TIM cellular localisation were plotted using stacked bar diagrams. A correlation between a distribution of types of TIM cellular localisation for a certain timepoint and developmental temperature was tested using 4x2 Fisher's Exact test. To determine a presence of oscillations in TIM level, value for each timepoint was compared individually to a value for CT10, representing a trough, using a Welch's test with Games-Howell post-hoc analysis. In addition, the same test was used to determine a relationship between the developmental temperature of flies and TIM intensity. Moreover, non-parametric Mann-Whitney or Kruskal-Wallis test was used to determine an association between a number of cells detected and their area for each cell type and experimental condition with individual comparisons performed with Wilcoxon Rank test.

2.7 Gene expression level analysis

To measure levels of transcript, quantitative reverse transcriptase Polymerase Chain Reaction (qRT-PCR) was used. Flies used in the experiment were raised at room temperature and kept as adults at 18˚C for 5 days and subsequently moved to 25˚C. Flies were harvested onto ice just before the shift, 15 and 30 hours after the shiftF. Heads were dissected on pre-chilled clean metal block with a scalpel and transferred to guanidinium thiocyanate buffer. RNAqueous4PCR kit (Ambion) was used to obtain DNAse I-digested total RNA following the protocol recommended by the manufacturer. RNA samples were analyzed using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen) according to the protocol described by manufacturer. Primer pairs

that were used were designed to specifically amplify fragments of the circadian *per*, the transgenic *UAS-per* transcript and the *rp49* control transcript. Expression levels were measured on a SmartCycler system (Cepheid) and ther results were analysed relative to *rp49* using the comparative Cycle threshold (Ct) method (Schmittgen and Livak, 2008).

Primers used

- Gene: *per*

Forward primer: per.5/6s2 5'-CGC CAA CAA CAA GAA ATA CAC GG-3'

Reverse primer: per.6a2 5'-TGA TGA AGG ACG AGT AGA AGG AGG-3'

Concentration: 1 µM

Length of product from cDNA: 89bp

Length of product from DNA: doesn't exist, since forward primer is complementary to exons' fragments flanking the intron

- Gene: transgenic *per*

Forward primer: hsp70TATA 5'-GAG CGC CGG AGT ATA AAT AGA GG-3'

Reverse primer: pUAST-cyc_TCS_R 5'-CTC GTG CCG AAT TCC CAA TTC-3'

Concentration: 1 µM

- Gene: *rp49*

Forward primer: rp49for 5'-AAG ATC GTG AAG AAG CGC ACC AA-3'

Reverse primer: rp49rev 5'-CTG TTG TCG ATA CCC TTG GGC TT-3'

Concentration: 0.4 µM

Length of product from cDNA: 101 bp

Length of product from DNA: 163 bp

Protocol used:

- Stage 1: Hold 60ºC for 180 seconds – to synthesize cDNA
- Stage 2: Hold 95ºC for 300 seconds – to activate the Platinum Taq DNA polymerase
- Stage 3 – Temperature cycle which is repeated 45 times:
    - 95ºC for 15 seconds – to denaturate double stranded fragments
    - 60ºC for 30 seconds – for annealing and elongation
- Stage 4: Hold 40ºC for 60 seconds
- Stage 5: Melting curve analysis – from 60ºC to 95ºCat 0.2ºC/second – to check the specificity of the reaction and the presence of the primers dimers.

Chapter 3: Adult phenotypes resulting from developmental manipulation of PERIOD expression

3.1 Introduction

The earliest manifestation of the circadian clock regulating behaviour of *Drosophila melanogaster* can be observed during the larval stages, as demonstrated by the circadian modulation of the larval photophobic response (Mazzoni *et al.*, 2005). *D. melanogaster* undergo a remarkable change in their response to light - even though adult flies are crepuscular and day-active, feeding larvae prefer darkness to light (Sawin *et al.*, 1994; Sawin-McCormack *et al.*, 1995). The different behaviour of larvae can be explained as a strategy to facilitate finding a suitable environment and increase chances of survival. First to third instar larvae feed voraciously and light avoidance helps them to dig in the food and avoid predators. The switch towards being diurnal occurs prior to puparitation, as a way of repelling flies from forming pupa in the food (Sokolowski, 1980 and 1985; Sokolowski *et al.*, 1984).

How do *D. melanogaster* larvae receive light information in the first place? Contrary to adult flies having two kinds of the photoreceptor cells, in the retina and in an extraretinal structure known as Hofbauer-Buchner eyelet, larvae have visual structure known as Bolwig's Organs (Helfrich-Förster *et al.*, 2002; Sprecher and Desplan, 2008). Two Bolwig's Organs (BO) are located in the larval brain, one in each hemisphere, each one consisting of 12 photoreceptors. BO neurons express *rhodopsin-5* (*rh5*) or *rhodopsin-6* (*rh6*) (Malpel *et al.*, 2002). Axons from BO directly contact the four circadian pacemaker lateral neurons (LN_vs) in each hemisphere (Kaneko *et al.*, 1997, 2000; Malpel *et al.*, 2002; Mazzoni *et al.*, 2005), revealing BO's involvement in the clock entrainment by TIM degradation (Mazzoni *et al.*, 2005; Malpel *et al.*, 2002, 2004). BO is also necessary for the foraging larvae to avoid light, a behaviour which is meant to keep them in the food away from predators (Sawin-McCormack *et al.*, 1995). BO becomes the adult photoreceptive Hofbauer-Buchner eyelet soon after the beginning of pupariation (Tix *et al.*, 1989; Helfrich-Förster *et al.*, 2002).

Mazzoni *et al.* (2005) shows that larvae with either ablated or hyperpolarized circadian neurons displayed a similar impairment in the light avoidance as larvae with no photoreceptor cells. Interestingly, the photophobic behaviour does not require either the presence of CRY (Malpel *et al.*, 2004) or PDF (Mazzoni *et al.*, 2005). Additionally, the degree of the light avoidance was

found to be regulated in a circadian manner. Wild-type larvae were found to be the most sensitive to light towards the subjective dawn and showed minimal sensitivity towards the end of subjective day. In addition to this, mutations in circadian genes were resulting in flies with no detectable rhythms in the photophobic response. Mutations in the positive clock components (such as *Clk* and *cyc*) increased the light sensitivity of larvae and mutations in the negative components (like *per* and *tim*) left larvae insensitive to light, possibly by the hyperpolarization of the neuron's membrane (Mazzoni *et al*., 2005).

Interestingly, larval clock function is connected to clock function in the adulthood. Even though adult flies developing in the constant darkness display locomotor rhythmicity, the phase of the activity of flies is distributed randomly, meaning that flies are active at different times. However, a single 12hr light pulse during the first larval stage (but not during embryonic stage) was sufficient to synchronize the phase of most of flies (Sehgal *et al*., 1992). Light pulses as short as 1 minute delivered at the larval stage were also enough to start rhythmic pattern of eclosion (Brett, 1952). This not only indicates that the clock functions from a very early stage onwards but also signifies that the circadian clock is capable of keeping time throughout development (Sehgal *et al*., 1992; Kaneko *et al*., 2000a).

On the other hand, there are indications that a functional circadian clock during development is not necessary for the adult behavioural rhythmicity. Ewer *et al.* (1988 and 1990) describes the introduction of a *period* transgene, connected to the *heat-shock protein 70* (*hsp70*) promoter in an arrhythmic per^{01} background. Interestingly, flies that received a 37°C heat shock for 30 minutes or flies transferred to an elevated temperature (29°C) as adults (even during only a portion of adulthood) showed rhythmic locomotor behaviour, indicating that even following development in the absence of *period* expression and a functioning clock, adult flies are still capable of showing behavioural rhythms. Careful analysis of the data presented in Ewer *et al*., (1988 and 1990) revealed a more complicated story. Although adult flies exhibited locomotor rhythms, in most cases at least 25% of flies remained arrhythmic. Also, the periods of rhythmicity were unusually long (Ewer *et al*., 1988 and 1990). Thus, the incomplete rescue of rhythmicity might indicate that the circadian clock function during development has some importance. A

sensible hypothesis would be that the circadian clock during the development is necessary for the robustness of the clock and wild-type period lengths.

3.2 Aim

The purpose of the experiments presented in this chapter was to test if the function of the circadian clock during development is required for adult behavioural rhythmicity. To this aim, a different, spatially-targeted, strategy of restoring *per* function in adulthood was used. In addition, I explored possible strategies for improving the rescue of rhythmicity in adult flies, focusing in particular on light conditions during development and the entrainment immediately prior to testing. I tested hypotheses that (1) the light conditions used during development are not important for the rescue of *per* function during adulthood and (2) the low rhythmicity of flies with the rescue of *per* function can be partially explained by the lack of the entraining cues before analysis in the permissive conditions.

3.3 Protocols

3.3.1 Testing the behaviour of *per* rescue flies in various temperatures

Young *per* rescue flies raised at room temperature were analysed in constant darkness at a variety of temperatures ranging from 17˚C to 29˚C as described in 2.3, using the first full five days for the analysis of individual flies. The distribution of the rhythmic, weakly rhythmic and arrhythmic flies in the population was compared between different temperatures using chi-square analysis and compared between experimental and control flies (*Canton-S*) analysed at the same temperature using Fisher's Exact 3x2 test. Relative rhythmic power and period length for *per* rescue flies at different temperatures were compared using the Welch's test with Games-Howell post-hoc tests for pairwise comparisons. Period length of *per* rescue flies was compared to *Canton-S* using Mann-Whitney rank-sum test.

3.3.2 Locomotor activity of flies with conditional *per* rescue

per rescue flies were raised under restrictive conditions (17˚C in constant light). Young flies were analysed first at restrictive conditions (17˚C DD) for 7 days and then moved to permissive conditions (25˚C DD) to check if behavioural rhythmicity could be induced. Flies were analysed individually as described in 2.3. Effects on the distribution of the rhythmic, weakly rhythmic and arrhythmic flies between populations were tested with the Fisher's Exact 3x2 tests. The relationship between the temperature (and PER expression) and relative rhythmic power was tested with the Mann-Whitney test.

3.3.3 Testing the impact of developmental conditions and entrainment on adult locomotor rhythmicity of *per* rescue flies

per rescue flies were raised under either restrictive conditions (17˚C) or permissive conditions (25˚C) in either constant darkness (DD), constant light (LL) or with a light cycle consisting of 12 hours of light and 12 hours of darkness (LD), resulting in total of six developmental conditions. Flies were first in 17˚C DD for either 10 days (for flies raised at 17˚C DD younger flies were collected (1 day old instead of 3-4 days old) so this allowed comparison of flies of the same age) or for 7 days (for flies raised at other conditions) and then they were moved to a permissive temperature (25˚C DD; where transgenic *per* is expressed and should rescue circadian clock function). Some flies had additional entrainment in form of 4 light/dark cycles at either 17˚C or 25˚C between testing at restrictive and permissive condition, resulting in two experimental adult testing protocols ($[17^{\circ}C\ DD]_{7/10}$>$[17/25^{\circ}C\ LD]_{4}$ > [25˚C DD]) and control one with flies moved directly from 17˚C DD to 25˚C DD ($[17^{\circ}C\ DD]_{7/10}$> [25˚C DD]).

Flies were analysed at restrictive (17˚C DD) and permissive (25˚C DD) conditions using 7 full days to determine the presence of rhythms as described in 2.3 with a small modification to determination of relative rhythmic power of flies - I assigned a value of 1 to every arrhythmic fly to better visualize the potential changes at the population level. The values for the relative rhythmic power and period length were compared independently for flies of different

sex and analysed at different temperatures using 2-way ANOVA with developmental protocol and adult entrainment as independent variables and 3-way ANOVA, which separated the developmental protocol into developmental temperature and light conditions. Individual comparisons were also performed. Individual comparisons were performed with the Tukey HSD Post Hoc test. The distribution of the rhythmic, weakly rhythmic and arrhythmic flies between different groups was compared using Fisher's Exact 3x3 test with Bonferroni correction. For the same group period length and relative rhythmic power were compared using the non-parametric Kruskal – Wallis test with Wilcoxon Rank test for individual comparisons.

Wild-type flies used to test the impact of developmental light conditions on adult locomotor activity were raised at 23˚C in different light protocols. Flies raised in constant darkness were processed in constant darkness with only red light used to separate flies. All flies were analysed directly in 23˚C DD conditions to study their free-running rhythms. Flies were analysed in the same way as described above.

3.4 Results

Part of the results presented here was previously published by the Wijnen lab (Goda, Mirowska and Currie *et al.*, 2011) as a result of collaborative research. The behavioural experiments presented in sections 3.3.1 and 3.3.2 were performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen, but since they are crucial to the rest of the story, they are included here.

3.4.1 It is possible to manipulate clock function with the TARGET system

To confirm the observations of Ewer *et al.*, (1988, 1990) we designed a new transgenic fly with conditional rescue of the arrhythmic per^{01} phenotype (Konopka and Benzer, 1971) by an introduction of a single copy *per* cDNA construct (*UAS-per24,* with 24 denoting the particular construct used, Kaneko *et al.*, 2000b; Yang and Sehgal, 2001), expressed in all circadian cells under

control of the previously described *tim(UAS)Gal4* element (Blau and Young, 1999). These flies are homozygous for per^{01} and have one copy of *tim(UAS)Gal4*, *UAS-per24* and the temperature sensitive GAL4 repressor tub_PGal80^{ts} each (McGuire *et al.*, 2003). Therefore, at low temperature, the $GAL80^{ts}$ element remains active, leading to the arrhythmia associated with the per^{01}, whereas at elevated temperatures $GAL80^{ts}$ is inactivated and GAL4 can activate the transcription of the transgenic *per* to restore circadian clock function (Figure 3.1A).

To confirm that transgenic *per* can be expressed at elevated temperature using the approach described above, molecular tests measuring *per* levels were performed as described in 2.7. We were able to observe strong induction of *per* in adult heads following the transition from 18˚C to 25˚C. Contrary to constitutively low *per* transcript levels observed in flies lacking *tim(UAS)Gal4* driver, in experimental flies with conditional rescue of per^{01} phenotype total *per* levels (including per^{01} and labeled as per^{01}/per) and levels of transgenic *per* transcript were increased approximately five-fold and more than forty-fold, respectively, 15 hours after the transfer to permissive conditions (Figure 3.1D).

To confirm the proper functioning of all genetic elements used and to check the possibility of using the system to manipulate circadian behaviour just by changing temperature, we decided to test the adult locomotor behaviour of flies at permissive (high temperature) and restrictive conditions (low temperature). Flies of the genotype described above (Figure 3.1 A; referred to from now on as the "*per* rescue" flies) were raised at room temperature (~23˚C; which allows for some transgenic *per* expression) and analysed in 18˚C (restrictive conditions, where no transgenic *per* is expressed), 25˚C or 29˚C (permissive conditions) and subsequently moved to 25˚C or 29˚C (from 18˚C) or 18˚C (from either 25˚C or 29˚C). Data was analysed as described in 2.3 using full 5 or 6 full days for quantitative analysis.

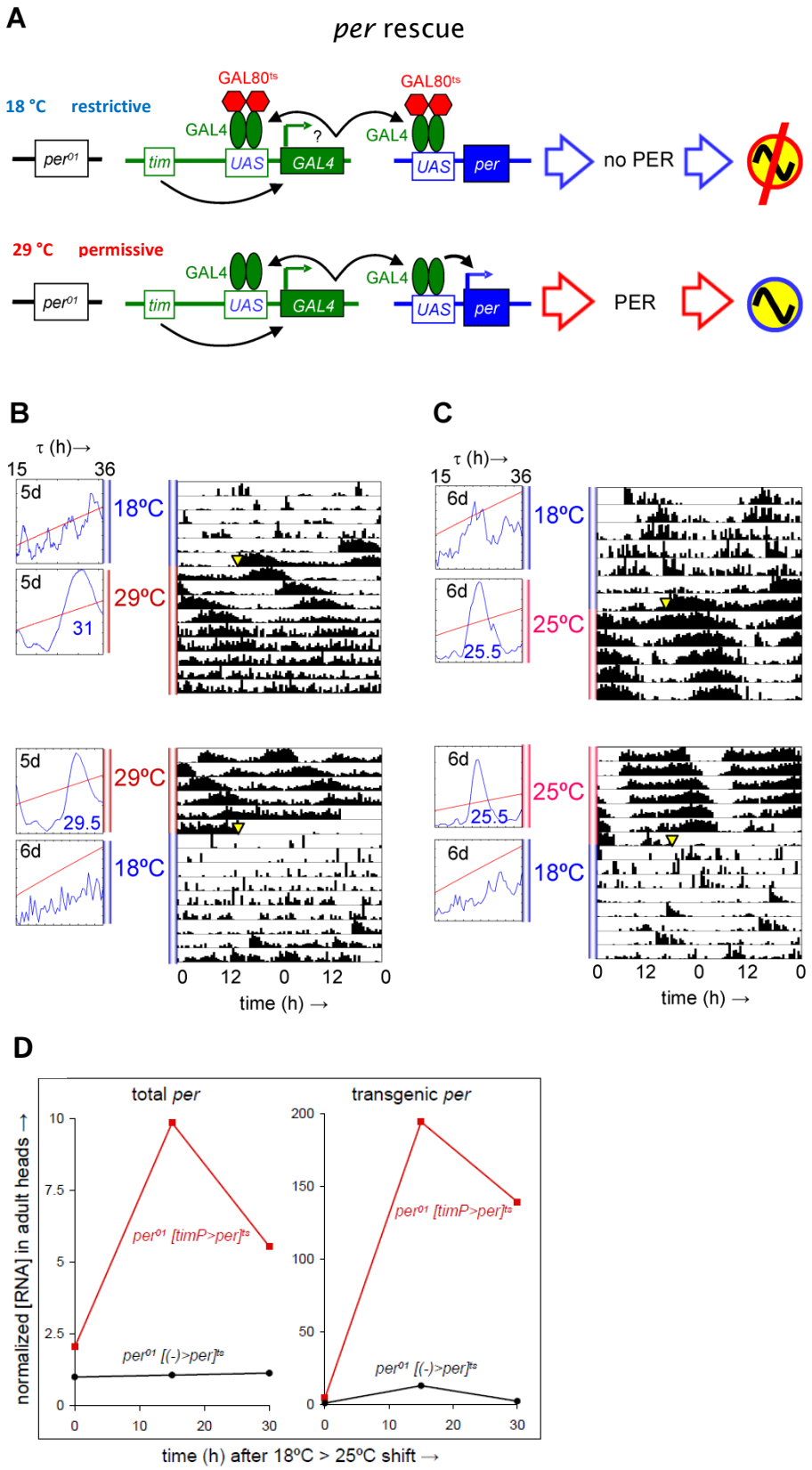


Figure 3.1. Conditional rescue of *per* as a tool for controlling clock function (adapted from Goda, Mirowska, Currie *et al.*, 2011;

experiments performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen).

Using the TARGET system, it is possible to change the state of the clock by changing environmental temperature. (A) Diagram showing the system of conditional expression in the transgenic line tested. Flies with a *y per^{01}w; tim(UAS)-Gal4/tub$_P$Gal80^{ts}; UAS-per24/+* genotype, show arrhythmic behaviour at restrictive conditions as a result of inhibition of GAL4-mediated expression of transgenic *per* by the temperature-sensitive version of the GAL4 repressor GAL80^{ts}. At permissive temperature, GAL80^{ts} is inhibited, allowing GAL4 to drive expression of *UAS-per*, rescuing *per^{01}* arrhythmicity. (B-C) Average (n=8) double-plotted actograms and chi-square periodograms (for 5 or 6 continuous days) for female conditional *per* rescue flies raised at room temperature and moved from restrictive to permissive (top) and from permissive to restrictive (bottom) conditions using 29˚C (B) and 25˚C (C) as permissive conditions. Yellow triangles mark the time of transfer. Red and blue colours are used for the easier visual identification of permissive and restrictive conditions. (D) Total *per^{01}/per* and transgenic *per* expression levels as revealed by qRT-PCR analysis from *per* rescue (*per^{01} [timP>per]^{ts}*) flies and controls without the *tim(UAS)-Gal4* driver (*per^{01} [->per]^{ts}*) immediately before and 15 and 30 h after a shift from 18˚C DD (restrictive) to 25˚C DD (permissive). *per* levels remained low in the flies without *tim(UAS)-Gal4* driver but both the total *per^{01}/per* and transgenic *per* levels were induced at 15 h in *per* rescue flies approximately five-fold and more than forty-fold, respectively.

Visual analysis of actograms showing the mean locomotor activity for females (Figure 3.1B and C) revealed a clear difference between flies analysed at different conditions. At 18˚C flies were virtually arrhythmic and at 25˚C and 29˚C strong circadian locomotor rhythms could be detected. Interestingly, 29˚C caused elongation of the free-running period, however the power of the rhythms remained strong. This mirrored the previously mentioned results described in Ewer *et al.* (1988, 1990). What is important, after the exposure to restrictive conditions (18˚C) flies displayed strong rhythms in permissive conditions within a day after transfer. This data confirms that the TARGET system is a valid approach for manipulating the clock function, as it was possible to readily and reliably change the rhythmicity of flies just by changing the environmental temperature. Therefore *per* rescue flies are a valid tool for studying the relationship between circadian clock function during development and the adult locomotor rhythmicity.

To investigate the function of the *per* rescue line further, we determined the rhythmicity of flies raised at room temperature at various temperatures, ranging from restrictive to permissive as described in 3.3.1 (Figure 3.2). To investigate just the impact of the move of adult flies from room temperature to different temperatures, *Canton-S* (Stern and Schaeffer, 1943) flies were used as controls.

Wild-type *Canton-s* flies were showing anticipated behaviour (Figure 3.2 C), with over 70% of flies displaying locomotor rhythms in all cases. The poorest rhythmicity was observed for them in case of flies analysed at 18˚C, where over 50% of flies were displaying weak locomotor rhythms. There was a highly significant association of the rhythmicity of *per* rescue flies (measured as the percentage of the rhythmic, weakly rhythmic and arrhythmic flies) with temperature (Figure 3.2 A-B, D; Chi-square analysis: females: df=12, $p<10^{-15}$, n=228; males: df=12, $p<10^{-18}$, n=231). Also, the relative rhythmic power showed a significant association with temperature (Welch's test: females: F(6, 42.276)=19.370, $p<10^{-9}$, n=165; males: F(6, 42.705)=20.089, $p<10^{-10}$, n=157). One can also notice that the best rescue of rhythmicity was observed for female *per* rescue flies released to 25-27˚C and male *per* rescue flies showed similar rescue for any temperature between 23˚C and 29˚C (Figure 3.2 D).

When the rhythmicity and the relative rhythmic power of flies with *per* rescue moved to different temperatures were compared to *Canton-S* flies (Figure 3.2 C), no difference between them was observed only for 25˚C. On the other hand, Mann-Whitney analysis of the period length revealed a significant elongation of period in *per* rescue flies at both 25˚C and 29˚C (Mann-Whitney test: females at 25˚C: U=0.000, z=-7.512, p<0.001, n=76; males at 25˚C: U=0.000, z=-7.248, p<0.001, n=73; females at 29˚C: U=0.000, z=-3.587, p<0.001, n=38; males at 29˚C: U=0.000, z=-5.007, p<0.001, n=33). The difference between wild-type flies and experimental flies at 18˚C (Fisher's Exact 3x2 test: females: $p<10^{-5}$, n=117; males: $p<10^{-5}$, n=116) could be easily explained by the fact that at these conditions experimental flies do not have any transgenic *per* expressed or its levels are fairly low. However, the rhythmicity of *per* rescue flies at 29˚C was significantly (Fisher's Exact 3x2 test: females: $p<10^{-5}$, n=83; males: $p<10^{-4}$, n=78) lower than for the control wild-type flies. The remaining rhythmic flies displayed very long periods. This decrease of rhythmicity can be potentially explained by much higher than in wild-type flies levels of PER at elevated temperatures. Previous reports suggested that elevated PER levels due to increased gene dosage are associated with period shortening (Smith and Konopka, 1982; Baylies *et al*., 1987). However, in the case of the *per* rescue line over-expression might be constitutive rather than circadian and this might impair the rhythmicity and cause the period elongation. This hypothesis will be formally addressed later in this chapter.

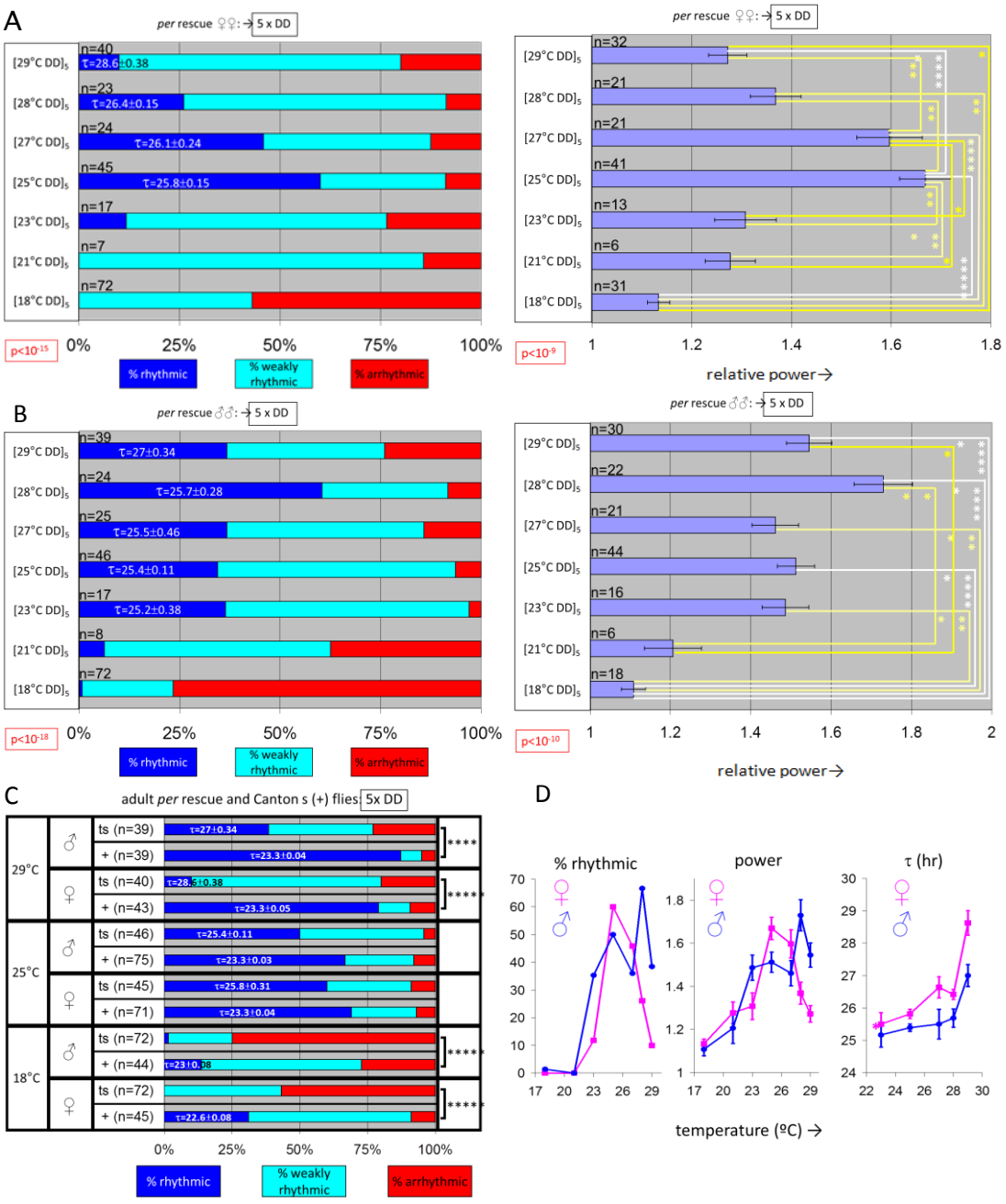


Figure 3.2. Arrhythmicity caused by *per*^{01} can be conditionally rescued by the temperature-dependent transgenic expression of *period* (adapted from Goda, Mirowska, Currie *et al.*, 2011; experiments performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen).

Quantitative analysis of adult locomotor rhythmicity of females (A) and males (B) with conditional *per* rescue raised at room temperature analysed over 5 days and a range of temperatures.

Comparison between the distributions of rhythmicity in the flies' population analysed at different temperatures was performed with chi-square with result written underneath. Welch's test analysis was used for comparison of the relative rhythmic power of flies with the post-hoc Games-Howell test used for pairwise comparisons of developmental treatments, and the results are indicated with * $p<0.05$, ** $p<0.01$, *** $p<10^{-3}$, **** $p<10^{-4}$ and ***** $p<10^{-5}$. (C) Comparison between the distribution of rhythmic, weakly rhythmic and arrhythmic flies between wild-type (*Canton-S*, +) flies and flies with conditional rescue of *per* (ts) at 18˚C, 25˚C and 29˚C With Fisher's Exact 2x3 tests, **** $p<10^{-4}$ and ***** $p<10^{-5}$. (D) Plots showing the changes in the percentage of rhythmic flies, relative rhythmic power (for both rhythmic and weakly rhythmic flies) and period length (tau, for rhythmic flies) determined for full 5 days as a function of environmental temperature for flies with conditional *per* rescue raised at room temperature and analysed in indicated temperatures at constant darkness. Error bars are SEM.

3.4.2 Developmental *per* expression is not necessary for adult locomotor rhythmicity

Since *per* rescue flies offered a reliable way of manipulating circadian clock function, we were ready to check the relationship between developmental *per* expression and adult behavioural rhythms. We hypothesised that it would be possible to rescue the circadian clock function in per^{01} rescue flies by expressing the transgenic *per* during adulthood only in circadian neurons. Flies were raised under restrictive conditions (17˚C in constant light) and analysed both at restrictive (17˚C DD) and permissive (25˚C DD) conditions as described in 3.3.2.

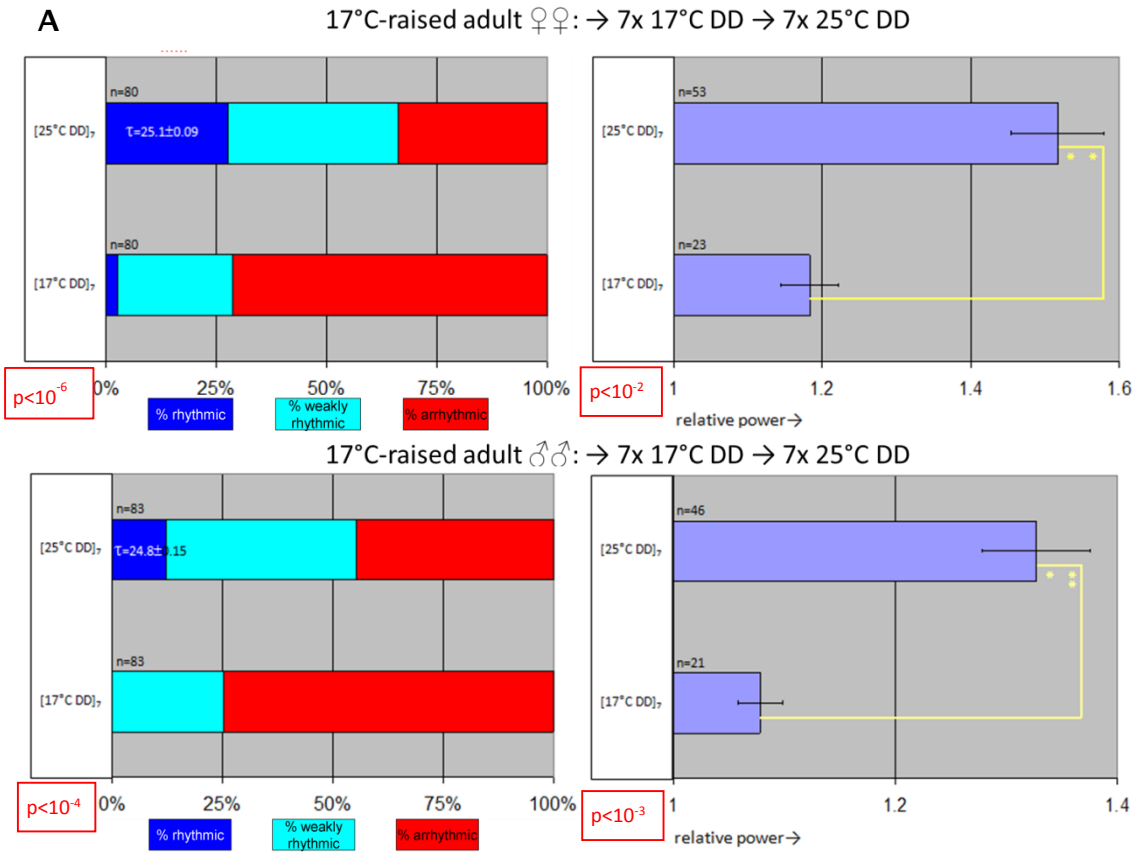


Figure 3.3. PER expression is not required during development for adult locomotor rhythms (adapted from Goda, Mirowska, Currie *et al.*, 2011; experiments performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen).

Behavioural rhythmicity of adult flies was restored after transfer from restrictive to permissive conditions in flies raised with no PER during development. Diagrams represent adult locomotor rhythmicity and relative rhythmic power of circadian locomotor activity of females (top) and males (bottom) with conditional *per* rescue raised under restrictive condition (17˚C LL) and analysed under restrictive conditions (17˚C DD) and subsequent permissive conditions (25˚C DD) for seven consecutive days. Fisher's Exact 2x3 tests were used for comparison of the distribution of rhythmicity in flies' population. For comparisons of the average relative rhythmic power between developmental temperatures Mann-Whitney rank-sum tests were used with ** $p<10^{-2}$, *** $p<10^{-3}$).

The majority of *per* rescue flies analysed at restrictive conditions (17˚C, Figure 3.3) did not show any circadian locomotor rhythms, and remaining flies had low relative rhythmic power. At permissive conditions (25˚C, Figure 3.3), the percentage of arrhythmic flies decreased (however a considerable portion of flies remained arrhythmic at permissive temperature: over 30% for females, over 45% for males) and some flies started to display strong rhythms, as reflected in their relative rhythmic power. Fisher's Exact 3x2 test confirmed a strong association between temperature and the percentage of rhythmic, weakly rhythmic and arrhythmic flies (females: $p<10^{-6}$, n=160; males: $p<10^{-4}$, n=166). An association was also revealed between relative rhythmic power and temperature (Mann-Whitney test: females: U=900.000, z=3.285, $p<10^{-2}$, n=76; males: U=784.000, z=4.068, $p<10^{-3}$, n=67). These results demonstrate that *per* rescue flies were capable of displaying locomotor rhythmicity in permissive conditions (when transgenic *per* is expressed), even when they were raised under restrictive conditions, with no transgenic *per* present. This nicely mirrors the data described by Ewer *et al.* (1988, 1990) and support their conclusion that neither *per* expression nor a functional circadian clock are necessary during the development for adult behavioural rhythmicity.

To exclude the possibility that residual clock function during prior restrictive conditions drove the observed behavioural adult rhythmicity at permissive conditions, we wanted to show that behavioural rhythmicity was initiated upon the expression of PER in adulthood. To do this, we moved adult flies from restrictive to permissive conditions at different times of the day. The first 5 full days were used to determine the rhythmicity, period length and the phase of the offset of rhythmicity according to the method described in the 2.3. The phase of the offset of rhythmicity was then compared to the phase of switch to permissive conditions and trendlines were fitted for all groups of the same gender.

The phase of locomotor rhythms after transfer to permissive conditions was determined by the time of the transfer (Figure 3.4). Quantitative analysis of the phase of the offset of the circadian locomotor activity at 25˚C in relation to the phase of the switch from restrictive to permissive conditions revealed a strong correlation between these two values (two-tailed probability value of Pearson correlation coefficient: $p<0.005$, n=6 for females, $p<0.003$, n=6 for males).

This confirmed that the circadian clock started functioning after flies were moved to permissive conditions.

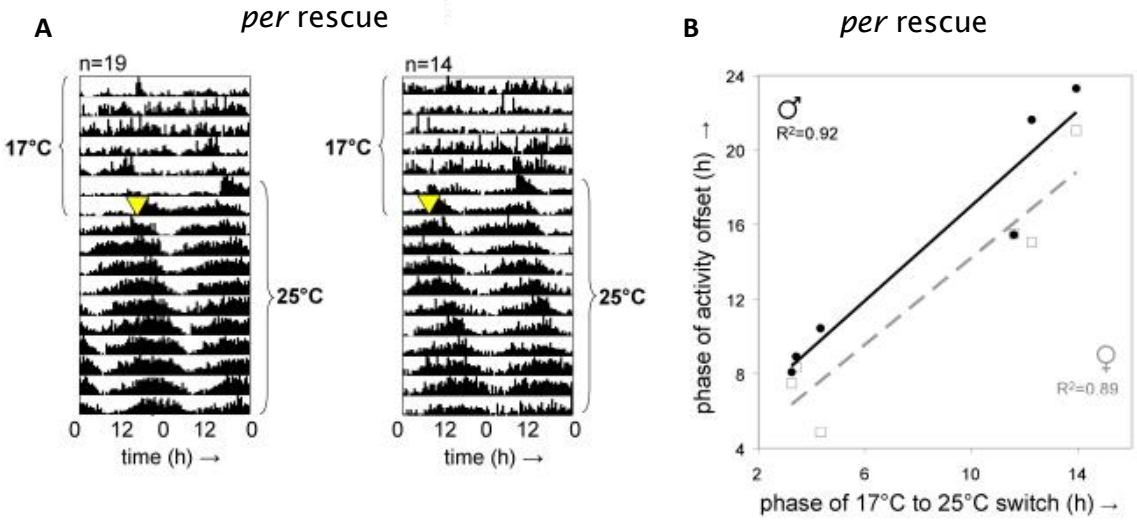


Figure 3.4. The phase of *per* induction in the adulthood sets the phase of adult locomotor rhythmicity (adapted from Goda, Mirowska, Currie *et al.*, 2011; experiments performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen).

The phase of the offset rhythmic locomotor activity of flies with conditional *per* rescue at permissive conditions is connected to the time of the move from restrictive to permissive conditions. (A) The average locomotor activity of female flies raised under restrictive conditions (17˚C LL) analysed at 17˚C DD and then moved to 25˚C DD at the time indicated with the yellow triangle – the left panel shows 19 flies moved at reference phase hour 12.5 and the right one 14 flies moved at reference phase hour 3.25. The relationship between the time of transfer and the phase of activity at 25˚C can be easily observed. (B) Plot showing the correlation between the phase of the offset of circadian locomotor activity at 25˚C and the phase of the switch from restrictive (17˚C) to permissive conditions (25˚C). Black dots represent median data for six groups of males and white squares represent six median groups of females. Trend lines were fitted for each gender and the associated coefficients are shown (from 2-tailed test significance: $p<0.005$ for females, $p<0.003$ for males).

3.4.3 Light treatment during development and entrainment during adulthood, but not the developmental *per* expression, impact adult locomotor rhythmicity of *per* rescue flies

As demonstrated above, it appears that *per* expression during development is not necessary for adult behavioural rhythmicity (Figure 3.3). However the restoration of adult locomotor rhythmicity was never perfect, with a substantial percentage of flies remaining arrhythmic. This can suggest that the clock in adult flies without *per* expression during development, even though functional, is weaker. One possible explanation for this was that the *per* rescue line can restore adult locomotor rhythmicity up to maximum of 55% of rhythmic flies due to the constitutive rather than circadian *per* expression leading to elevated PER levels at points in the circadian cycle where the level is normally lower. Another possibility was that the reduced adult behavioural rhythmicity is associated with the developmental conditions and testing protocol.

As for developmental conditions, *per* rescue flies used in previous experiments were raised in constant light which could have detrimental effect on adult locomotor behaviour. The reason for using this particular light/dark protocol was to further ensure lack of *per* during development. As described in the General Introduction (1.3.5.1), light causes the CRY-mediated TIM degradation, leading to PER degradation and disruption of the molecular oscillator (Zeng *et al*., 1996; Myers *et al*., 1996). This brings the possibility that the light/dark conditions experienced during development impact the behavioural rhythmicity rescue observed upon restoration of clock function.

Secondly, I hypothesised that incomplete restoration of rhythmicity observed previously (Figure 3.3) could be associated with lack of synchrony between various circadian neurons. I was therefore interested in confirming if the addition of entrainment before flies were moved to the permissive condition could improve their rhythmicity. By combining this with the developmental conditions I was hoping to explore the possible causes for the limits to the rescue of per^{01}-caused behavioural arrhythmicity.

To test the impact of both the developmental conditions and adult entrainment I conducted a large scale adult locomotor experiment, raising flies at either restrictive or permissive conditions (25˚C) in various light protocols -either constant darkness (DD), constant light (LL) or with a light cycle consisting of 12

hours of light and 12 hours of darkness (LD). Moreover, adult flies were entrained at either permissive or restrictive conditions. Detailed description of the experimental setup and analysis is included in 3.3.3.

At 17˚C, the majority of flies were arrhythmic and no rhythmic flies were ever recovered (Table 3.1), which nicely mirrored the results described before (Figure 3.3). When flies from all experiments were pooled together (this was possible as there were no differences between various treatments in the adulthood, ANOVA for multiple factors: $F(2, 264)=0.202$, $p=0.818$, $n=282$ for females, $F(2, 258)=0.099$, $p=0.906$ for males), ANOVA analysis has revealed that there was no impact of developmental protocol (combination of development temperature and light protocol) or developmental temperature or light conditions on the relative rhythmic power of flies (ANOVA for multiple factors: females: $F(5, 264)=1.367$, $p=0.237$, $n=282$ for developmental conditions; $F(2, 264)=1.421$, $p=0.243$, $n=282$ for developmental light conditions; $F(1, 264)=1.425$, $p=0.234$, $n=282$ for developmental temperature; males: $F(5, 258)=1.788$, $p=0.131$, $n=276$ for developmental conditions; $F(2, 258)=0.866$, $p=0.422$, $n=276$ for developmental light conditions; $F(1,258)=1.806$, $p=0.180$, $n=276$ for developmental temperature). This was also confirmed by looking at flies from restrictive and permissive conditions separately and comparing the correlation between developmental light protocol and rhythmicity of flies. For flies raised at 17˚C, light/dark condition during development had no effect on both the distribution of the weakly rhythmic and arrhythmic flies (Fisher's Exact 3x2: $p=0.877$, $n=139$ for females and $p=0.058$, $n=141$ for males) and relative rhythmic power which stayed close to the arrhythmic range in all cases (Kruskal- Wallis: $H(2)=0.340$, $p=0.983$, $n=139$ for females; $H(2)=4.086$, $p=0.130$, $n=141$ for males). For flies raised at permissive temperature (25˚C), similarly, developmental light conditions had no effect on the distribution of flies (Fisher's Exact 2x3 test: $p=0.264$, $n=143$ for females; $p=0.324$, $n=135$ for males) and relative rhythmic power (Kruskal-Wallis test: $H(2)=0.299$, $p=0.861$, $n=143$ for females; $H(2)=2.302$, $p=0.316$, $n=135$) (Figure A.1.1).

These results suggested that constant light used during the experiments published in Goda, Mirowska and Currie *et al.*, (2011) and presented in previous sections (Figure 3.3) was not the reason for behavioural arrhythmicity observed when flies were tested at 17˚C but it was rather the lack of *period*.

Moreover, light conditions during development had negligible effect on the arrhythmicity of adults at restrictive temperature.

For *per* rescue flies analysed at permissive conditions without any prior entrainment (Table 3.1) ANOVA revealed no association between relative rhythmic power and developmental conditions (ANOVA for multiple factors: $F(5, 85)=1.969$, $p=0.091$, $n=91$ for females; $F(5, 84)=1.912$, $p=0.101$, $n=90$ for males). This was due to the developmental temperature not having any impact on the rhythmicity of flies (ANOVA for multiple factors: $F(1, 85)=0.607$, $p=0.438$, $n=91$ for females and $F(1, 84)=0.414$, $p=0.522$, $n=90$ for males) but rather due to developmental light conditions (ANOVA for multiple factors: $F(2, 85)=3.622$, $p<0.05$, $n=91$ for females, with LL better than DD: Tukey HSD Post Hoc test: $p=0.024$, $n=61$ and $F(2, 84)=4.139$, $p=0.05$, $n=90$ for males, with DD worse than LL: Tukey HSD Post Hoc test: $p=0.024$, $n=60$). In summary, the lack of the impact of developmental temperature (and *per* expression through this) confirmed my hypothesis that developmental *per* expression is not required for adult locomotor rhythmicity and fit with the previously published data (Ewer *et al.*, 1988 and 1990).

Impact of developmental light conditions was confirmed in a more detailed analysis of flies raised under restrictive or permissive conditions analysed at permissive temperature (Figure A.1.1). In case of flies raised at restrictive temperature, there was a clear difference between the rescue of rhythms of flies raised with different light/dark protocols. Even though the percentage of rhythmic, weakly rhythmic and arrhythmic flies showed significant correlation with the developmental light protocol only for females (Fisher's Exact 3x3 test: $p=0.040$, $n=45$), both sexes showed a trend for the biggest percentage of arrhythmic flies (over 70% in case of females, over 50% in case of males) after development at 17˚C DD and the best rescue of rhythmicity after development at 17˚C LL (less than 20% arrhythmic flies).

Similarly, restoration of rhythmicity in flies with *period* expression both during development and adulthood (raised under permissive conditions and analysed at permissive temperature) was also associated with developmental light conditions (Figure A.1.1). Again, both males and females raised in constant light showed the best rescue of adult behavioural arrhythmicity and the worst rescue was observed when flies were raised at 25˚C DD, even though the

results for the distribution of flies were significant only for females (Fisher's Exact 3x3 test: p=0.027, n=46). Relative rhythmic power was significantly different for both females (Kruskal-Wallis test: H(2)=9.858, p=0.007, n=46 with flies raised in constant darkness significantly worse than flies raised in LD (Wilcoxon Rank test: W=11.467, z=2.501, p=0.037, n=31) and flies raised in LL (Wilcoxon Rank test: W=13.112, z=2.906, p=0.011, n=32) and males (Kruskal-Wallis test: H(2)=6.319, p=0.042, n=42).

These results do not support my hypothesis that light/dark regime used during development of flies with the disrupted circadian clock function does not impact the recovery from adult arrhythmicity associated with *per* rescue. Instead, they suggested that developmental lights conditions were more important than the presence of *per* during development (Table 3.1 and Figure A.1.5) and that flies raised in constant light can restore their rhythms better than those raised in constant darkness. These results were puzzling, because constant darkness or LD conditions do not disrupt the circadian clock whereas constant light does, via CRY-mediated impact on degradation of TIM and PER. I have, however, speculated that this could possibly be associated with the impact of developmental light conditions on synchronous recovery of clock function and might signify that developmental constant light and LD allow a more synchronous recovery upon switch to permissive temperature than constant darkness.

If my hypothesis that the developmental light treatment affects the ability of *per* rescue flies to overcome adult arrhythmicity by promoting synchronous recovery of clock function is true, then developmental light/dark cycle should have no effect on the adult behaviour of wild-type flies that are not experiencing adult arrhythmicity. I hypothesized that in this case, regardless of the developmental history, flies would show clear behavioural rhythmicity.

For this experiment I used two different lines of 'wild-type' flies, *Canton-S* and *ywBGluc::60* (that shares the same *yw* background; Stanewsky *et al.*, 1997b). Both lines were raised at 23˚C in different light protocols and subsequently analysed at 23˚C DD as described in 3.3.3.

As anticipated, the light/dark conditions used during development did not have consistent significant effect on the rhythmicity of either of the wild-type lines used (Figure A.1.2). For *ywBGluc::60* females, there was an association of

the rrp with the developmental light conditions (Kruskal-Wallis test: H(2)=7.400, p=0.025, n=88) with females raised in DD less rhythmic than those raised at LD (there is a significant difference between rrp of females raised in DD and LD: Wilcoxon Rank test: W=18.440, z=2.705, p=0.021, n=57). Additionally, the period of the behavioural rhythms of females was associated with the developmental light conditions (Kruskal-Wallis test: H(2)=19.543, p<0.001, n=54) with females raised in LL with a significantly longer period than the one of flies raised in LD (Wilcoxon Rank test: W=19.002, z=3.479, p=0.002, n=41) and DD (Wilcoxon Rank test: W=17.616, z=3.935, p<0.001, n=30). For males, the period of behavioural rhythms of flies was also associated with developmental light conditions (Kruskal-Wallis test: H(2)=8.236, p=0.016, n=39) with flies raised in LL having longer period than flies from DD (not significant) or LD (Wilcoxon Rank test: W=11.673, z=2.840, p=0.014, n=27).

Moving to investigating the impact of entrainment during adulthood (Table 3.1 and Figures A.1.3 and A.1.4), ANOVA analysis revealed that it impacted the relative rhythmic power of adult flies analysed at 25˚C, regardless of developmental temperature (ANOVA for multiple factors: F(2, 252)=3.466, p=0.033, n=270 for females and F(2, 236)=19.064, p<0.001, n=254 for males). Results varied depending on the developmental light protocol, but overall the best rhythmicity, as manifested either by the rhythmic:weakly rhythmic:arrhythmic ratio or by relative rhythmic power was always observed in case of flies receiving 4 days of entrainment at 25˚C. The smallest improvement was observed for flies raised in LL, however this could be explained by the relatively low (≤35%) percentage of arrhythmic flies in the group without any entrainment. Biggest improvement was observed for flies raised in DD which had the worst rhythmicity without any entrainment. Contrary, in most cases 4 days of entrainment at 17˚C did not improve rhythmicity of flies and in some cases was even decreasing it (Table 3.1 and Figures A.1.3 and A.1.4).

Raised:	Ent:	Analysed:	Sex	n	%R : %WR : %Ar	tau (SEM)	rrp (SEM)
17 LL	[17LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 18.8 : 81.3		1.011 (0.006)
			♂	15	0.0 : 20.0 : 80.0		1.032 (0.022)
		[25 DD]$_7$	♀	16	18.8 : 37.5 : 43.8	25.17 (0.17)	1.260 (0.106)
			♂	15	6.7 : 40.0 : 53.3	25.00	1.160 (0.054)
	[25 LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 12.5 : 87.5		1.021 (0.020)
			♂	16	0.0 : 12.5 : 87.5		1.010 (0.008)
		[25 DD]$_7$	♀	15	40.0 : 40.0 : 20.0	24.67 (0.11)	1.424 (0.094)
			♂	15	46.7 : 26.7 : 26.7	24.79 (0.15)	1.516 (0.142)
	none	[17 DD]$_7$	♀	16	0.0 : 31.3 : 68.8		1.021 (0.010)
			♂	16	0.0 : 6.3 : 93.8		1.001 (0.001)
		[25 DD]$_7$	♀	15	33.3 : 46.7 : 20.0	24.90 (0.10)	1.396 (0.121)
			♂	16	18.8 : 68.8 : 12.5	24.50 (0.00)	1.290 (0.075)
17 LD	[17LD]$_4$	[17 DD]$_7$	♀	15	0.0 : 40.0 : 60.0		1.044 (0.021)
			♂	15	0.0 : 26.7 : 73.3		1.015 (0.010)
		[25 DD]$_7$	♀	15	6.7 : 6.7 : 86.7	25.50	1.076 (0.072)
			♂	14	0.0 : 14.3 : 85.7		1.059 (0.040)
	[25LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 12.5 : 87.5		1.009 (0.006)
			♂	15	0.0 : 60.0 : 40.0		1.033 (0.012)
		[25 DD]$_7$	♀	14	28.6 : 42.9 : 28.6	24.75 (0.25)	1.315 (0.090)
			♂	14	42.9 : 14.3 : 42.9	24.58 (0.27)	1.399 (0.123)
	none	[17 DD]$_7$	♀	15	0.0 : 13.3 : 86.7		1.002 (0.001)
			♂	15	0.0 : 13.3 : 86.7		1.005 (0.004)
		[25 DD]$_7$	♀	15	20.0 : 26.7 : 53.3	25.00 (0.76)	1.224 (0.100)
			♂	15	20.0 : 40.0 : 40.0	24.33 (0.44)	1.292 (0.101)
17 DD	[17LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 25.0 : 75.0		1.008 (0.006)
			♂	16	0.0 : 6.3 : 93.8		1.015 (0.008)
		[25 DD]$_7$	♀	15	6.7 : 26.7 : 66.7	25.00	1.073 (0.041)
			♂	16	0.0 : 6.3 : 93.8		1.072 (0.056)
	[25LD]$_4$	[17 DD]$_7$	♀	15	0.0 : 13.3 : 86.7		1.009 (0.009)
			♂	15	0.0 : 20.0 : 80.0		1.037 (0.028)
		[25 DD]$_7$	♀	13	15.4 : 53.8 : 30.8	25.00 (0.50)	1.226 (0.072)
			♂	12	8.3 : 50.0 : 41.7	24.50	1.168 (0.067)
	none	[17 DD]$_7$	♀	16	0.0 : 37.5 : 62.5		1.039 (0.016)
			♂	16	0.0 : 31.3 : 68.8		1.041 (0.019)
		[25 DD]$_7$	♀	15	20.0 : 6.7 : 73.3	24.17 (0.44)	1.217 (0.109)
			♂	15	6.7 : 40.0 : 53.3	24.50	1.151 (0.053)
25 LL	[17LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 6.3 : 93.8		1.012 (0.012)
			♂	16	0.0 : 6.3 : 93.8		1.004 (0.004)
		[25 DD]$_7$	♀	15	13.3 : 53.3 : 33.3	25.00 (0.00)	1.315 (0.135)
			♂	11	9.1 : 54.5 : 36.4	24.50	1.116 (0.048)
	[25LD]$_4$	[17 DD]$_7$	♀	16	0.0 : 31.3 : 68.8		1.035 (0.016)
			♂	15	0.0 : 13.3 : 86.7		1.019 (0.013)
		[25 DD]$_7$	♀	16	56.3 : 25.0 : 18.8	24.78 (0.12)	1.508 (0.104)

Raised:	Ent:	Analysed:	Sex	n	%R : %WR : %Ar	tau (SEM)	rrp (SEM)
			♂	10	30.0 : 40.0 : 30.0	24.67 (0.17)	1.382 (0.122)
	none	$[17\ DD]_7$	♀	16	0.0 : 25.0 : 75.0		1.026 (0.020)
			♂	16	0.0 : 6.3 : 93.8		1.001 (0.001)
		$[25\ DD]_7$	♀	16	37.5 : 31.3 : 31.3	25.00 (0.13)	1.343 (0.093)
			♂	14	42.9 : 28.6 : 28.6	24.42 (0.15)	1.418 (0.117)
25 LD	$[17LD]_4$	$[17\ DD]_7$	♀	16	0.0 : 18.8 : 81.3		1.007 (0.004)
			♂	16	0.0 : 6.3 : 93.8		1.000 (0.000)
		$[25\ DD]_7$	♀	15	26.7 : 26.7 : 46.7	25.13 (0.24)	1.251 (0.102)
			♂	16	6.3 : 31.3 : 62.5	24.00	1.108 (0.052)
	$[25LD]_4$	$[17\ DD]_7$	♀	16	0.0 : 6.3 : 93.8		1.004 (0.004)
			♂	15	0.0 : 6.7 : 93.3		1.012 (0.012)
		$[25\ DD]_7$	♀	16	25.0 : 68.8 : 6.3	25.13 (0.24)	1.318 (0.057)
			♂	15	46.7 : 33.3 : 20.0	24.21 (0.21)	1.499 (0.121)
	none	$[17\ DD]_7$	♀	15	0.0 : 6.7 : 93.3		1.004 (0.004)
			♂	15	0.0 : 20.0 : 80.0		1.003 (0.002)
		$[25\ DD]_7$	♀	15	33.3 : 26.7 : 40.0	25.20 (0.25)	1.295 (0.087)
			♂	15	40.0 : 40.0 : 20.0	24.42 (0.20)	1.342 (0.094)
25 DD	$[17LD]_4$	$[17\ DD]_7$	♀	16	0.0 : 25.0 : 75.0		1.012 (0.009)
			♂	14	0.0 : 21.4 : 78.6		1.045 (0.024)
		$[25\ DD]_7$	♀	14	7.1 : 50.0 : 42.9	23.50	1.157 (0.052)
			♂	14	0.0 : 28.6 : 71.4		1.059 (0.035)
	$[25LD]_4$	$[17\ DD]_7$	♀	16	0.0 : 0.0 : 100.0		1.000 (0.000)
			♂	12	0.0 : 25.0 : 75.0		1.022 (0.012)
		$[25\ DD]_7$	♀	16	18.8 : 43.8 : 37.5	25.67 (0.17)	1.231 (0.067)
			♂	11	36.4 : 63.6 : 0.0	24.38 (0.24)	1.430 (0.120)
	none	$[17\ DD]_7$	♀	16	0.0 : 6.3 : 93.8		1.005 (0.005)
			♂	16	0.0 : 12.5 : 87.5		1.007 (0.004)
		$[25\ DD]_7$	♀	15	0.0 : 20.0 : 80.0		1.019 (0.011)
			♂	15	6.7 : 40.0 : 53.3	25.00	1.105 (0.049)

Table 3.1. Summary of the results for the analysis of flies with conditional *per* rescue.

Table showing the distribution of rhythmicity in flies' population, the relative rhythmic power, and period length of strongly rhythmic flies, together with the temperature at which flies were raised (Raised:), presence and temperature of entrainment (Ent:) and conditions at which flies were analysed (Analysed:), the number and sex of flies. Flies were kept at 17˚C DD for 10 days (for flies raised at 17˚C DD) or for 7 days (for flies raised at other conditions) and then moved to 25˚C DD with or without entrainment for 4 days at either

restrictive or permissive temperature, as indicated. Average values are displayed together with the Standard Error of Means (SEM).

3.5 Discussion

3.5.1 Limitations of the approach used

A possible critique of the data presented in this (and subsequent) chapter could be the lack of explicit controls for the use of GAL4-UAS system. To ensure that the observed effects come indeed from the transgenic expression of *per*, fly lines carrying either the *Gal4* or the *UAS* constructs separately (*tim(UAS)Gal4* and *UAS-per*), should be tested. This would confirm that there are no adverse effects associated with the insertion site of these transgenes or accumulation of GAL4 in clock neurons.

The GAL4/UAS system is commonly used in *D. melanogaster*'s genetics and is considered a reliable tool for spatial control of transgene expression (Brand and Perrimon, 1993), partially as there are no GAL4 orthologues in *Drosophila* and no UAS elements in genome of *Drosophila* (Liu and Lehmann, 2008), However, there are suggestions that *Gal4* expression has biological effects in *D. melanogaster*. For example, expression of GAL4 in the eye imaginal disk resulted in eye defects and apoptosis (Kramer and Staveley, 2003) and in some instances, raised GAL4 levels resulted in neuron death (Rezaval *et al.*, 2007). These phenotypes might be caused by genomic response to GAL4, as high levels of GAL4 were shown to change the profiles of gene expression, probably based on protein-protein interaction (Liu and Lehmann, 2008). However, this effect is mainly associated with very high expression levels (Rezaval *et al.*, 2007), for example resulting from the use of heat shock (Kramer and Staveley, 2003; Liu and Lehmann, 2008) and it makes sense to always try to perform experiments with drivers giving restricted expression and avoid heat shock. As in my experiments I was using restricted drivers and not using heat shock as a way of inducing expression, I can assume that I am not causing excessive GAL4 levels that could negatively impact behaviour of flies. However, it could be advantageous to check GAL4 levels in the circadian neurons of flies used.

Even though neither the *tim(UAS)Gal4* nor *UAS-per* constructs used have been tested in the experiments described in this chapter for potential behavioural changes caused by the insertion of these transgenes, phenotypic effects associated with the location of these transgenes can be excluded as these are not novel constructs and have been extensively used previously without any reports on nonspecific effects caused by the location of these transgenes. The *tim(UAS)Gal4* construct was shown not to affect behavioural rhythmicity of flies negatively (Blau and Young, 1999; Martinek *et al*., 2001). Similarly, *UAS-per* construct on its own did not impact the rhythmicity of flies (Yang and Sehgal, 2001). Although, additional single transgene tests could not be conducted for the sake of completeness, it would be impossible to test their impact on rhythmicity in per^{01} flies as flies with this mutation are already arrhythmic and no further impairment to rhythmicity could be observed. Therefore, as it was impossible to test that either transgene insertion site or GAL4 expression and accumulation might negatively influence behavioural rhythmicity in this genetic background, these tests were not conducted. Moreover, the most important experiment in this chapter (3.4.3) involved comparing flies of the same genotype that experienced different developmental conditions or treatments as adults, which signifies that any possible impairments associated with the insertion site of the transgenes would affect all conditions.

3.5.2 PER is not required during development for adult behavioural rhythmicity

Since flies raised at 25˚C had a restored circadian clock due to transgenic *per* expression and I demonstrated that adult rescue of rhythmicity was not correlated with developmental temperature, I suggest that the impact of the clock function during development is negligible, if any (Figure A.1.5). Therefore, results described in this chapter provide convincing evidence for the lack of a requirement for developmental *per* expression in order to maintain a functional circadian clock in adult *Drosophila melanogaster* in agreement with Ewer *et al.* (1988 and 1990). Interestingly, even though flies described by Ewer *et al.* (1988 and 1990) displayed behavioural rhythms, these were associated with a very long period. Our research shows that when targeted only to clock-bearing cells, adult restoration of *period* function can result in strong

behavioural rhythmicity with a relatively normal circadian period length (Figure 3.3 and Table 3.1). It was impossible to obtain a perfect rescue of the rhythmicity in case of flies expressing *per* in the adulthood and period lengths recorded were still longer than those of wild-type flies (Figure 3.3 and Table 3.1). Similar observations were also reported before for flies with constitutive *per* rescue with the same *per* transgene as the one we have used – depending on the *tim-Gal4* driver used, only between 34 and 53% of flies showed behavioural rhythmicity and a period length from 24.1 to 24.6 hrs (Kaneko *et al.*, 2000b), consistent with our data.

Three explanations can be suggested for the less incomplete rescue of rhythmicity. One could be that an insufficient level of *per* expression is achieved with the tested construct, which prevents flies from fully regaining their rhythms. The elongation of period length observed would be consistent with this hypothesis, as there is a negative correlation between the level of *per* expression and the period length (Smith and Konopka, 1982; Cote and Brody, 1986; Baylies *et al.*, 1987). However, as is apparent from testing *per* levels (Figure 3.1 E), the incomplete rescue observed does not appear to be simply due to low *per* levels. Moreover, when analysing *per* rescue flies (Figure 3.2 D) it can be noticed that with an increase in temperature above the ambient range, a continuous increase of the period length and a decrease in the percentage of rhythmic flies occurs. As the increased temperature is associated with $GAL80^{ts}$ degradation, and higher levels of GAL4 as well as higher levels of transgene expression (e.g., Morimura *et al.*, 1996), low PER level does not explain the decrease in rhythmicity observed when temperatures are increased above 25˚C as the levels of PER sufficient for the rescue of adult locomotor rhythmicity at 25˚C further increase at higher temperatures. Therefore a possible explanation for the decreased rhythmicity of flies at high temperatures might be excessive PER levels.

Alternatively, some low levels of *per* expression could be required during developmental stages. This is, however, very unlikely, since even flies raised at permissive temperature are not displaying very strong rhythms (Table 3.1). Additionally, Ewer *et al.* (1990) suggests that the expression of *period* during development does not help with the restoration of the rhythmicity.

The more plausible hypothesis is that the intrinsic properties of the line used are preventing flies from displaying stronger rhythms than 55% rhythmic flies. This can be due to suspected negative impact of the constitutively high transgenic *per* level (resulting in elevated *per* levels also during the times where *per* levels should normally drop, i.e. be in a trough phase) on the behavioural rhythmicity in flies studied. I hypothesised that the presumed decreased rhythmicity of PER expression during adulthood decreases behavioural rhythmicity of flies and elongates free-running circadian period length. In wild-type flies *period* mRNA displays oscillations of a 5 to10-fold amplitude, with the peak around the dusk (switch to darkness) and trough 2 hours before lights on (and extending to early morning) (Baylies *et al*., 1987; Hardin *et al*., 1990). These oscillations persist in DD, but with the amplitude decreasing 2 to 3-fold over time. The trough in the *per* expression helps to facilitate the PER downregulation, eliminating the CLK/CYC inhibition and subsequently inducing the expression of the CLK/CYC-controlled genes (Hardin *et al*., 1990). However, *per* mRNA oscillations are not necessary for the PER protein oscillations or behavioural rhythmicity (Frish *et al*., 1994; Vosshall and Young, 1995; Yang and Sehgal, 2001). In the line used for the experiments described in this chapter, a lack of the trough in the oscillation of *per* can result in a delay in PER downregulation leading also to increased period length, just as observed. This can offer an explanation as to why we are not observing period shortening with elevated *per* levels, as suggested by the literature (Smith and Konopka, 1982; Baylies *et al*., 1987).

Another important observation from my experiments is that the rescue of adult arrhythmicity can be improved with exposure to entraining stimuli. The addition of the entrainment in form of four days of light/dark cycles at permissive temperature (where PER was present) usually improved the overall rhythmicity of flies (Table 3.1 and Figures A.1.3 and A.1.4). Entrainment at restrictive conditions, except in case of flies raised in DD (which had a very poor rhythmicity without any entrainment), on the other hand, decreased the rhythmicity of flies (Table 3.1 and Figures A.1.3 and A.1.4). These results suggest that entrainment can improve the rhythmicity of flies with the temperature-mediated rescue of per^{01}-caused behavioural arrythmicity, but only when PER is expressed at the time of the entrainment. When entrainment occurs at restrictive conditions, it even appears to further decrease the

rhythmicity of flies, probably by acting as an extension of the restrictive conditions, making it more difficult for flies to recover. This experiment is also consistent with the hypothesis that the internal desynchrony of the cells can explain the difference between the adult rhythmicity of *per* rescue flies raised in different light/dark cycles, as entrainment could be improving rhythmicity of flies by resetting the phase of all circadian neurons.

Of course, there is a possibility that the entrainment used was too short to fully synchronize the molecular oscillator. To address this issue, an experiment should be performed in which flies receive more than 4 days of entrainment. Also, spending a week in the adulthood at restrictive conditions, where the circadian clock gets arrested might have a detrimental effect on rhythmicity. A way of testing this effect would be to raise flies at 25˚C and study them as adults directly at 25˚C DD.

3.5.3 Light conditions used during development impact the adult behavioural rhythmicity of flies with conditional rescue of *per*^{01}

One of the unexpected results of the experiments presented in this chapter is the difference between the rhythmicity of flies raised in different light conditions (Table 3.1 and Figure A.1.1). For the line with the conditional *per* rescue, light conditions during development showed greater association with the rescue of adult behavioural arrhythmicity that the presence or absence of *per* during development. There was no association between developmental light conditions and the behaviour of flies at restrictive temperature, when *per* was not expressed, since flies could not show any greater arrhythmicity. On the other hand, entrainment during the adulthood improved the rhythmicity of flies, showing that in the presence of entrainment, developmental temperature and light conditions do not contribute much to the rhythmicity of adult flies (Table 3.1 and Figures A.1.3 and A.1.4).

To my knowledge there has been only one study comparing the impact of developmental light conditions on the rhythmicity of flies across all three different light regimens (Power *et al*., 1995a) which concluded that the rhythmicity of *per*$^{+}$ and *per*^{01} flies raised in DD was worse than of those raised in LD or LL, so it is difficult to conclude if my observations suggest a common

property or if they are only specific to the lines tested. Power *et al*. (1995a, 1995b) suggested that rearing *D. melanogaster* for multiple generations in DD disrupted their rhythmicity, a phenotype that could be reversed by treatment with either 1 hr light pulse or 2 LD cycles in adult flies. Rearing flies in LL did not result in any decrease of the circadian rhythmicity when compared to LD-raised flies (Power *et al*., 1995a, 1995b).

Some past studies have supported a notion that raising flies in DD greatly reduces their rhythmicity (Kalmus, 1940, anecdotal evidence from W. Zehring and J. Hall). One formal study described by Dowse and Ringo in 1988, recorded that only 23% of 'Old Town' flies raised in DD displayed wild-type periods and over half of flies did not display any locomotor rhythms, with the rest displaying either very short or long periods. They also noted an emergence of flies with "ultradian" rhythms, which can be a sign of desynchronized oscillators (Dowse and Ringo, 1988). It is important to note, however, that in their experiments they used flies reared in DD for several generations and low-power analysis methods. Malpel *et al.* (2004) also reported that raising wild-type flies in DD decreased the rhythmic power of flies by 30% with no impact on the percentage of the rhythmic flies.

It is, however, unlikely that wild-type flies are sensitive to developmental light protocol to the same extent as per^{01} flies, since in the past we used various flies raised in constant darkness and we observed strong locomotor rhythms for them. Another study also suggested that there were no significant differences in both the percentage of rhythmic flies and the period length between wild-type (*Canton-S*) flies raised in complete darkness (from the early embryo stage) and flies raised in constant darkness interrupted by two full light-dark cycles (Sehgal *et al.*, 1992). In both cases well over 80% of flies displayed rhythmic behaviour with a wild-type period length. In this study, however, flies were kept in DD only for one generation.

Various hypotheses have been formed to explain this lack of consistency between the data. One suggestion was the impact of the length of DD exposure, whether it was just for one generation (meaning parents were raised and kept in LD) or for multiple generations. Another was connected to the differences between the strains of flies used and the tools used for the locomotor rhythmicity analysis. Individual flies raised in DD may display

divergent phases of activity, which can make them appear arrhythmic in an actogram showing average or median activity but they will show rhythmicity when analysed individually. Another possible source of discrepancies was the source of the red light used, with transmittance at either 620 nm (Sehgal *et al.*, 1992) or at 650 nm (Dowse and Ringo, 1989). However in both experiments flies showed no phase coherence, which is a hallmark of a lack of Zeitgebers cues.

It was also shown that keeping flies for several hundred generations in constant light did not disrupt the rhythmicity in the eclosion (Sheeba *et al.*, 1999, 2001, 2002; Paranjpe *et al.*, 2003) and 35 generations in a periodic environment had no effect on the eclosion, oviposition and locomotor activity rhythms (Sheeba *et al.*, 2001).

Preliminary studies of two wild-type lines presented (Figure A.1.2) seem to point to no significant correlation of developmental light conditions and adult locomotor rhythmicity. There is, however, a subtle difference between the rhythmicity and the period length of flies raised at different conditions. Further testing is needed to compare adult locomotor behaviour of wild-type and *per* rescue flies raised in different light protocols together with a detailed analysis of their light-sensitivity, including the status of their *tim[s]/tim[ls]* alleles. *tim[s] and tim[ls]* are two variants of *tim* found in both laboratory stocks and flies caught from the wild (Rosato *et al.*, 1997; Tauber *et al.*, 2007). The *tim[s]* variant produces short *tim* isoform while *tim[ls]* can produce either short or long isoform through alternative start codon. Since CRY binds more efficiently to and preferentially degrades the short TIM, flies carrying *tim[s]* are more light-sensitive (Sandrelli *et al.*, 2007; Peschel *et al.*, 2009). If *per* rescue flies are less sensitive to light, they might remain rhythmic in constant light at permissive conditions. As a precaution, it might be recommended to conduct the experiments using the same developmental light conditions for all groups tested.

To find the possible explanation for the decreased rhythmicity of flies raised in DD in comparison to flies raised in LL, detailed analysis of the impact of light on the molecular oscillator is also required. If the light is present after the degradation of the TIM, it leads to the JETLAG-dependent degradation of CRY (Lin *et al.*, 2001; Peschel *et al.*, 2007; Peschel *et al.*, 2009). CRY is not

degraded before TIM because JET has a higher affinity to TIM than CRY (Peschel *et al.*, 2009). Without CRY, TIM and PER can function again. This means that in the presence of constant light degradation of TIM occurs first, subsequently CRY is degraded which leads to presence of some PER and TIM, but not their oscillations. This suggests a possibility that the molecular clock of the larvae raised in DD, LL and LD can be at different states, with either some residual oscillator function or stopped at different phases. A detailed molecular analysis of larvae clock is required to address this possibility.

The data presented in this chapter is consistent with my assumption that the difference in restored adult rhythmicity between *per* rescue flies raised in different light/dark protocols (Table 3.1 and Figure A.1.1) is associated with the initial internal synchrony between timekeeping neurons. In this case, addition of adult entrainment prior to release into permissive conditions should help restore rhythmicity. If my hypothesis that this impact of light conditions during development on the rhythmicity of flies exists only for the line with the conditional *per* rescue is true, the possible explanation can be that in DD the cells in the pacemaker of flies are in the arrhythmic or asynchronous state and short light pulse associated with loading flies into *Drosophila* Activity Monitors is not strong enough to synchronize them, which explains why the added entrainment days helped to improve the rhythmicity. Data also suggests that prolonged adult exposure to aperiodic environment might be detrimental to the locomotor rhythmicity of flies (Table 3.1), which again can be improved by the addition of entrainment. In case of LD-raised flies, cells are synchronous, so when flies are moved to constant darkness they all resume activity with the same phase. In LL all cells have the same state associated with the molecular degradation of *per*, so when flies are moved to DD, all cells start from the same point. This hypothesis can be tested by immunofluorescence analysis of the levels of various circadian proteins in different groups of circadian neurons of flies raised under different light conditions.

Chapter 4: Analysis of developmental CLK/CYC activity required for adult behavioural rhythmicity

4.1 Introduction

Previously published results (Goda, Mirowska and Currie *et al.*, 2011) together with the data presented in Chapter 3 (Figure 3.3, Table 3.1) support the idea that a functional clock during development is irrelevant for adult behavioural rhythmicity. However, it would be incorrect to treat the oscillator during development and the oscillator in adult fly as separate entities, since *D. melanogaster* retains the circadian phase information through development - the phase of light pulses given at early larval stages determine the phase of adult locomotor activity (Sehgal *et al.*, 1992). This points to the importance of the developmental oscillator for adult locomotor rhythmicity.

My conclusions in Chapter 3 are based on the phenotype of circadian arrest through depletion of PER. Therefore, I was curious if similar results would be obtained using different ways of disrupting the clock. Since the oscillator relies on the balance between the levels of various transcripts and proteins, stopping the clock at different phases might lead to completely different physiological states.

As described in General Introduction (1.3.4), main components of the molecular oscillator are PER, TIM, CLK and CYC. With no functional PER present, CLK/CYC is not phosphorylated and remains bound to E-box for longer. Therefore per^{01} mutant flies exhibit moderate to high levels of *per*, *tim*, *vri* and other CLK/CYC targets (Sehgal *et al.*, 1994; Blau and Young, 1999; Hao

et al., 1999; Claridge-Chang *et al*., 2001). Another way of stopping the clock would be to cause a decrease in CLK/CYC activity. A developmental requirement for CLK/CYC activity could be demonstrated by testing the adult locomotor rhythmicity of flies raised with decreased CLK/CYC levels. Given that CLK/CYC is a transcriptional activator of multiple genes containing E-boxes (Darlington *et al*., 1998; Abruzzi *et al*., 2011), this way of stopping the clock is also associated with a change in the level of other genes normally activated by CLK/CYC. If the circadian clock during development is not required for adult locomotor rhythmicity, there should be no difference in the adult rhythmicity of flies raised with a clock arrested in different states.

4.2 Aim

The goal of the experiments presented in this chapter was to determine if a clock arrest caused by a lack of CLK/CYC activity during development impacted adult locomotor rhythmicity. As I already established that a ticking clock *per se* was not developmentally needed for adult circadian locomotor behaviour (since lack of *per* expression during development did not cause irreversible adult arrhythmia), these experiments on clock arrest with manipulation of CLK/CYC activity specifically addressed the possibility of a developmental requirement for CLK/CYC in this context. Data presented in this chapter aims to contribute to our understanding of the formation of the circadian oscillator and its function during development.

4.3 Protocols

4.3.1 Analysis of the behaviour of flies with conditional *per* over-expression

Flies with conditional *per* over-expression were raised under permissive conditions of 17˚C DD, room temperature (RT, ~23˚C) and restrictive conditions of 29˚C. 3-4 days old flies were tested as described in 2.3 in permissive conditions of 17˚C LD for seven days followed by 17˚C DD to check for the

impact of PER over-expression during development on adult locomotor rhythms. In one condition flies raised at 17˚C were exposed to 29˚C as adults for the same duration of time as flies raised at 29˚C (7 days); afterwards they were treated and analysed as described above. The presence of rhythms during first full 7 days in DD was determined as described in 2.3.

4.3.2 Analysis of the behaviour of *cyc* rescue flies

cyc rescue flies were raised under permissive conditions (29˚C) and as adults were analysed at permissive (29˚C DD) or restrictive (17-18˚C DD) conditions according to procedure described in 2.3 Flies received no entrainment prior to testing and the first full seven days at each condition were used for the analysis. To compare the distribution of flies analysed at different temperatures, Fisher's Exact 3x3 test was performed. An association between the temperature and the relative rhythmic power was analysed using Welch's tests with Games-Howell post-hoc analyses.

4.3.3 Comparison of the behaviour of flies with or without developmental *cyc* rescue

cyc rescue flies were raised either under permissive (29˚C) or restrictive (~23˚C) conditions and were analysed first at permissive conditions (29˚C DD), followed by restrictive conditions (17˚C DD), to check if the conditional *cyc* rescue resulted in the changes in the locomotor behaviour of flies. Median actograms were created as described in 2.3.2 and the full six days at both conditions were used to analyse the quality of behavioural rhythms as described in 2.3.3.

4.3.4 Analysis of the behaviour of flies with developmental *cyc* rescue exposed to restrictive conditions as adults

cyc rescue flies raised at 29˚C were analysed first at 17˚C DD (for Figure 4.2 C) or LD (for Figure 4.2 F-G) for 3-7 days and then were released to either 29˚C or

17˚C DD. Behaviour of flies was tested using the first full seven days at each DD conditions as described in 2.3. Association between the rhythmicity of the population and the experimental conditions was tested by Fisher's Exact 3x2 test. Average relative rhythmic power was analysed with the Mann-Whitney test.

4.3.5 Analysis of the relationship between the phase of rhythmicity of *cyc* rescue flies and the time of their switch to permissive conditions

cyc rescue flies were raised at permissive temperature (29˚C) until they were 3-4 days old. The controls were w^{1118} flies raised at room temperature. Flies were first placed at 17˚C LD (restrictive conditions for *cyc* rescue flies; LD ensured that flies were synchronous) for 7 days and subsequently moved to 29˚C DD (permissive conditions for *cyc* rescue). Flies were moved to permissive conditions 4 or 6 hours apart, starting at ZT0 (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 or ZT0, ZT6, ZT12 and ZT18). The first full seven days of 29˚C DD (except for flies moved at ZT0, for which the days used were 2-8 to use flies of the same age as in other cases) were used to determine the phase of the rhythmicity as described in 2.3.4. The phase time (the phase of the peak or the offset in the DD) was plotted against the time of the move from restrictive to permissive conditions and the correlations were tested by the determination of the trendline and two-tailed probability value of Pearson correlation coefficient. Moreover, to confirm whether phase is associated with the time of shift, I compared values with a non-parametric Kruskal-Wallis test.

4.3.6 Analysis of the behaviour of *cyc* rescue flies in LD at permissive temperature

cyc rescue flies were raised under permissive (29˚C LD), restrictive conditions (17˚C LL or 17˚C DD) and intermediate conditions (RT and 25˚C LD). Young flies were analysed either at 25˚C LD or 29˚C LD as described in 2.3. Rhythmicity of flies in the LD cycles was determined as described in 2.3.2 and 2.3.3, using the first six full days at each conditions Since data obtained for flies raised at 17˚C

LL and 17˚C DD were virtually indistinguishable, these groups were combined into one. Additionally, data for flies raised at RT (~23˚C) were combined with data for flies raised at 25˚C LD and will be referred to as RT.

The distribution of flies across different categories was compared using Fisher's Exact 3x3 test, grouping together flies displaying rhythms with a period close to the environmental τ cycles (24hr rhythmic) to form one category; flies displaying weak rhythms with other period lengths (other weakly rhythmic) as a second category; and flies with no rhythms at all (arrhythmic) as the third one. Pairwise comparisons of distribution of flies across all four categories were performed with Fisher's Exact 4x2 tests. Average period length and relative rhythmic power were calculated for all rhythmic and weakly rhythmic flies and tested for effects of developmental conditions with non-parametric Kruskal-Wallis or Mann-Whitney tests.

cyc rescue flies raised at either restrictive (17˚C) or permissive temperature (29˚C) analysed at 25˚C LD were also compared to *yw;;cyc*^{01} and constitutive *cyc* rescue (*elav*^{C155}*::Gal4; UAS-cyc/CyO; cyc*^{01}) flies raised at room temperature (data collected by Min-Ho Kim) and analysed at 25˚C LD as described in 2.3.2 and 2.3.3. The association between the distribution of flies and the genotype was tested using Fisher's Exact 4x2 test, grouping together flies displaying strong rhythms with period close to the environmental τ cycles (24hr rhythmic), weak rhythms with the period close to the environmental τ cycle (24hr weakly rhythmic), weak rhythms with other period lengths (other weakly rhythmic) as one category and flies with no rhythms at all (arrhythmic) as the second one. Pairwise comparisons of distribution of flies across all four categories were performed with Fisher's Exact 4x2 tests. Period lengths and relative rhythmic powers of all rhythmic and weakly rhythmic flies were compared with non-parametric Kruskal-Wallis test.

4.3.7 Analysis of the behaviour of *cyc* rescue flies in LD at restrictive temperature

cyc rescue flies raised under permissive conditions (29˚C) and control *Canton-S* flies raised at room temperature were analysed at 17˚C LD (which is restrictive

temperature for *cyc* rescue flies) as described in 2.3. The first six full days at LD were used to determine the rhythmicity of individual flies as described in 2.3.2 and 2.3.3. The distribution of flies across different categories of rhythmicity was compared between *cyc* rescue and *Canton-S* flies with 4x2 Fisher's Exact Test. Average period length and relative rhythmic power calculated for all rhythmic and weakly rhythmic flies were tested for association with the genotype with non-parametric Kruskal-Wallis test.

4.4 Results

Some of the results presented here were previously published by the Wijnen lab (Goda, Mirowska and Currie *et al.*, 2011). They were obtained during collaborative research and since they are crucial for the story, are included as results. The behavioural experiments presented in sections 4.3.1 and 4.3.2 were partially performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen, but I contributed data to them.

4.4.1 Over-expressing PER during development causes irreversible adult arrhythmia

In order to uncover the function of various clock components during development, I decided to arrest the clock in an opposite state to the per^{01} arrest described in Chapter 3. The easiest way to achieve this with the genetic elements already described in Chapter 3 (Figure 3.1) was to use flies with conditional PER over-expression. Instead of using arrhythmic per^{01} flies, this time my experimental flies had a per^{+} background. As previously, a *per* cDNA construct (*UAS-per24*) was introduced and expressed in all circadian cells by way of previously described *tim(UAS)Gal4*. Temporal control of transgenic *per* expression was achieved via a previously described $tub_{P}Gal80^{ts}$ element (Figure 4.1 A). Both females and males used in the experiments had one copy of an X chromosome containing $tub_{P}Gal80^{ts}$ and were homozygous for both *tim(UAS)Gal4* and *UAS-per24*. The resulting flies had wild-type PER levels when kept at low temperature (17-23˚C, permissive) and elevated PER levels resulting

from the transgenic PER expression at higher temperatures (25-29˚C, restrictive).

Flies with conditional *per* over-expression were raised under either restrictive (29˚C) or permissive (17-23˚C) conditions and analysed in permissive conditions (17˚C) as described in 43.1 Flies raised at 17˚C or room temperature (Figure 4.1 B) displayed robust rhythms with strong relative rhythmic powers and only slightly longer period lengths when analysed at permissive temperature (17˚C). However, when I analysed flies raised at restrictive temperature of 29˚C at permissive conditions, I noticed adult behavioural arrhythmia (Figure 4.1 B). There was a striking difference compared with the results obtained previously with conditional rescue of *per* function (Figure 3.3).

To test if this observation was just the result of exposing flies to elevated levels of PER, or if it was a truly developmental phenotype, I exposed adult *per* over-expressing flies raised at permissive temperature to restrictive conditions as described in 4.3.1. At 29˚C, when *per* was actively over-expressed, female flies were mainly arrhythmic. However, upon return to permissive conditions behavioural rhythms were rescued. The periods of these flies (which are longer than for wild-type flies) mirror the periods already described in Chapter 3 (Figure 3.3, Table 3.1) and can be explained by the slightly elevated PER levels. At first glance, results of this experiment appear to disprove our previous theory that adult flies do not require a functional circadian clock during development to display locomotor rhythmicity.

It is important, however, to recognize the difference between the arrests of the circadian clock caused by depleting PER and by its over-expression. As mentioned in the introduction, PER functions in the molecular oscillator as the negative regulator of the master CLK/CYC heterodimer. Therefore, it is reasonable to assume that the phenotype observed was not a direct result of the circadian clock disruption during development, but rather a consequence of the CLK/CYC inhibition during development. This in turn suggests that CLK/CYC is necessary during development for adult locomotor rhythmicity.

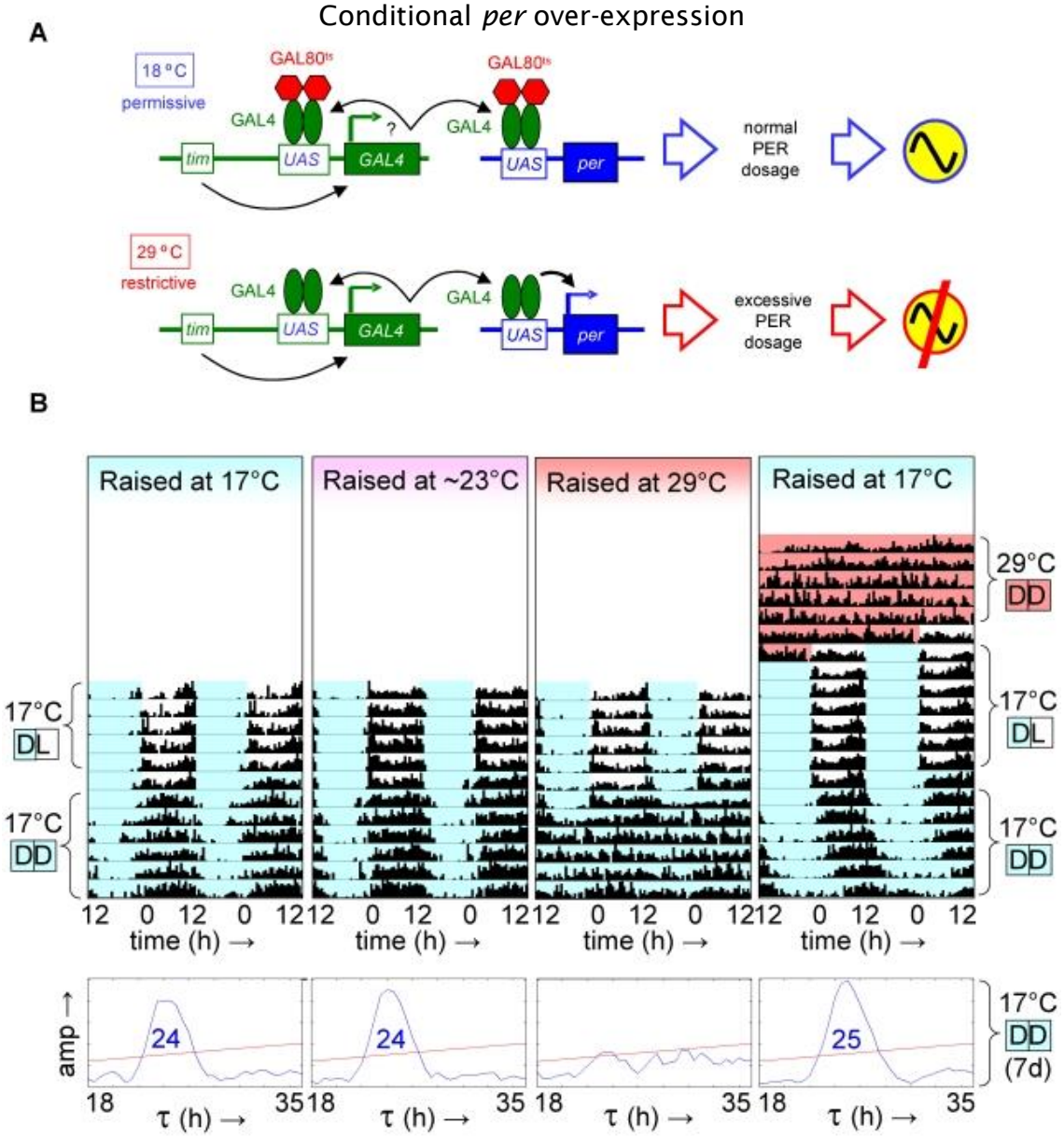


Figure 4.1. Developmental over-expression of *per* disrupts adult circadian behaviour, while the phenotype of adult *per* over-expression is reversible (adapted from Goda, Mirowska, Currie *et al.*, 2011; experiments performed and analysed by Min-Ho Kim, Jake Currie and Dr Herman Wijnen, with author of this thesis contributing data).

Transgenic flies with conditional over-expression of *per* display a behavioural arrhythmicity when analysed under restrictive conditions (29˚C), which can be reverted under permissive conditions (17˚C). However, developmental exposure to restrictive conditions results in irreversible behavioural arrhythmia. (A) Diagram showing genetic

basis of the conditional *per* over-expression. Flies with a *y tub$_P$Gal80^{ts} w/(FM7c or Y); tim(UAS)-Gal4; UAS-per* genotype (referred to as conditional *per* over-expression), display rhythmic locomotor behaviour at permissive temperature (17-23˚C), because they experience regular *per* levels as transgenic *per* expression is prevented by GAL80^{ts}. At restrictive temperature GAL80^{ts} is ineffective and GAL4 can induce expression of transgenic *per,* leading to PER over-expression and interfering with normal circadian clock function. (B) Comparison of the locomotor activity of flies raised at different temperatures. The top four panels are double-plotted actograms representing median locomotor activity of female flies during LD and subsequent DD conditions at permissive temperature (17˚C). The white, light blue, and red background colours represent 17˚C light, 17˚C dark, and 29˚C dark conditions, respectively. The lower four panels show chi-square periodograms for the median data reflecting circadian rhythmicity in 17˚C DD with the p=0.01 threshold line in red. Numbers in blue correspond to the single significant period length found in the circadian range (18–35 h). First two panels from the left show flies raised under permissive conditions of 17˚C or ~23˚C for which there was no obvious effect on adult circadian behaviour. The third panel shows the data for flies with developmental PER over-expression (raised at 29˚C) which results in adult behavioural arrhythmia. The last actogram illustrates behaviour of flies raised at permissive temperature and exposed to restrictive conditions (29˚C DD portion of the actogram) as adults. The behavioural arrhythmicity seen under restrictive conditions is reversed by the shift to permissive conditions.

However, raising flies at two different temperatures introduced another variable that had to be considered, namely the developmental impact of temperature on flies with conditional PER over-expression. This suggests an alternative hypothesis concerning the impact of the combined developmental temperature and genetic background on aging. Some connections have been found pointing to the relationship between the developmental conditions and the lifespan of flies (de Moed *et al.*, 1998, 1999; Ashburner *et al.*, 2005). It is

also well established that as flies age, their locomotor activity deteriorate (Driver, 2000; Koh *et al*., 2006a; Krishnan *et al.* 2009, Rakshit *et al*., 2012; Luo *et al*., 2012). Therefore, developmental temperature might potentially reduce the lifespan of the fly. It could be argued that the phenotype we observe while analysing flies with conditional *per* over-expression raised at 29˚C corresponds to a natural age-related decline in the locomotor rhythmicity. It was also possible that the developmental PER over-expression could impact the lifespan of flies, leading to the same age-related decline in locomotor rhythmicity. To address these issues, I designed a simple longevity experiment to check the implications of raising flies in the elevated temperature and raising flies in the absence of a functional clock (Appendix A.2.1).

Development at 29˚C shortened the lifespan of flies compared with flies raised at 17˚C (Figure A.2.1). This result confirmed previously published observations on the impact the temperature experienced during pre-adult stages has on the survival of flies (Alpatov and Pearl, 1929; Lamb, 1968; Burcombe and Hollingsworth, 1970; Lints and Lints, 1969, 1971b; Chippindale *et al*., 1994; Zwaan *et al*., 1995, Yadav and Sharma, 2014). Moreover, developmental temperature affected both the lifespan of the conditional *per* over-expression flies and wild-type flies and there was no significant difference between the median lifespan of those groups of flies.

Results for experimental flies raised at 29˚C revealed that over 80% of flies survived for more than 20 days, which was the age at which we usually studied them when maintained under constant darkness. Flies which did not survive until the last day of the analysis (usually day 18-19) were disregarded. Therefore flies that were close to the end of their life (dying within the next 10 days, which might have resulted in them displaying deteriorated locomotor rhythms due to their age) were included in the analysis, constituting around 20% of the total sample. When we excluded the flies previously discarded from analysis, they accounted for no more than 25% of the results. Therefore my data suggest that the phenotype associated with developmental *per* over-expression cannot be explained by age-related changes, as flies survived far longer than the time period when they were analysed.

Taken together, these results suggest that developmental *per* over-expression causes the irreversible adult arrhythmia that cannot be explained by the age-

related deterioration of locomotor activity. I have also shown that elevated developmental temperature largely affects the lifespan of flies, however a disrupted locomotor clock during development does not have a significant effect on the lifespan.

4.4.2 Conditional suppression of *cyc* expression during development leads to irreversible adult locomotor arrhythmia

To test the hypothesis that active CLK/CYC dimer is necessary during development for the generation of adult behavioural rhythms, we decided to test if rescue of the function of *cyc* only during adulthood restores adult locomotor rhythms. We created a line in the cyc^{01} (Rutila *et al.*, 1998) background in which we achieved a temperature – controlled (via tub_PGal80^{ts}) expression of transgenic *cyc* (from *UAS-cyc* element; Tanoue *et al.*, 2004) in all postmitotic neurons, using the *elavC155::Gal4* driver (Lin and Goodman, 1994). Resulting flies were homozygous for the cyc^{01} and tub_PGal80^{ts}, containing one copy of the *UAS-cyc* element and were hemizygous for the *Gal4* driver. These flies are referred to as *cyc* rescue flies. At restrictive low temperature (17-23˚C), the transgenic *cyc* is not expressed leading to molecular oscillator arrest; whereas at the permissive temperature (25-29˚C), the transgenic *cyc* should restore the function of the molecular oscillator (Figure 4.2 A).

To confirm if the changes in the molecular state of the clock correspond to changes in the behaviour of flies, we checked whether the changes in temperature correspond to the changes in the locomotor activity of flies. Flies raised with a functioning molecular oscillator (at permissive temperature) were analysed at three different conditions: 17˚C, 18˚C and 29˚C as described in 4.3.2.

While flies analysed at 29˚C displayed strong rhythms, a majority of flies analysed at either 17˚C or 18˚C were arrhythmic and remaining flies showed very weak rhythms. For female flies (Figure 4.2 D-E), temperature was clearly associated with the rhythmicity of the individual flies (Fisher's Exact 3x3 test: $p<10^{-3}$, n= 111). Welch's tests revealed that the relative rhythmic power showed

a strong association with the developmental temperature (Welch's test: $F(2, 12.214)=7.034$, $p<10^{-2}$, $n=48$). These results confirmed that it is possible to manipulate the function of the molecular oscillator, and hence the locomotor behaviour of flies, by conditional restoration of *cyc* expression by changing testing temperature

Next experiments checked if there were differences in the locomotor behaviour of flies raised with or without transgenic *cyc* expression (therefore with or without a functioning molecular oscillator). *cyc* rescue flies were raised under either permissive conditions of 29˚C DD or restrictive conditions of room temperature (~23˚C) and analysed as described in 4.3.3.

Unfortunately, the survival of flies was poor, but even with the low numbers a striking difference between both groups of flies was observed. Flies raised at permissive temperature displayed a strong rhythmicity when analysed at 29˚C and behavioural arrhythmia at 17˚C (Figure 4.2 B). However, flies raised under restrictive conditions did not display any significant locomotor rhythms when analysed at permissive conditions. This experiment was later performed multiple times on both genders of flies, resulting in the same results (some of them are presented in 5.4.1).

To confirm that the phenotype observed in Figure 4.2 B was developmental, we exposed flies raised under permissive conditions to restrictive conditions when adults as described in 4.3.4. Even after prolonged adult exposure to restrictive conditions (during which flies did not show any locomotor rhythmicity), *cyc* rescue flies raised under permissive conditions displayed strong behavioural rhythms upon return to permissive conditions (Figure 4.2 C, F-G). Flies expressing transgenic *cyc* as adults (analysed at permissive conditions - 29˚C) were significantly more rhythmic than flies without the transgenic *cyc* expression (analysed at restrictive conditions – 17-18˚C) (Fisher's Exact 3x2 test: $p<10^{-21}$, $n=191$ – Figure 4.2 F). A similar significant decrease was found for the relative rhythmic power (Welch's test: $F(1, 103.930)=143.443$, $p<10^{-3}$, $n=109$). This suggests that lack of CLK/CYC activity prior to testing is important only when it occurs during development. Taken together, results presented in this chapter confirmed our hypothesis that it is the activity of CLK/CYC and not a functional circadian clock *per se* that is required during the development for adult behavioural rhythmicity.

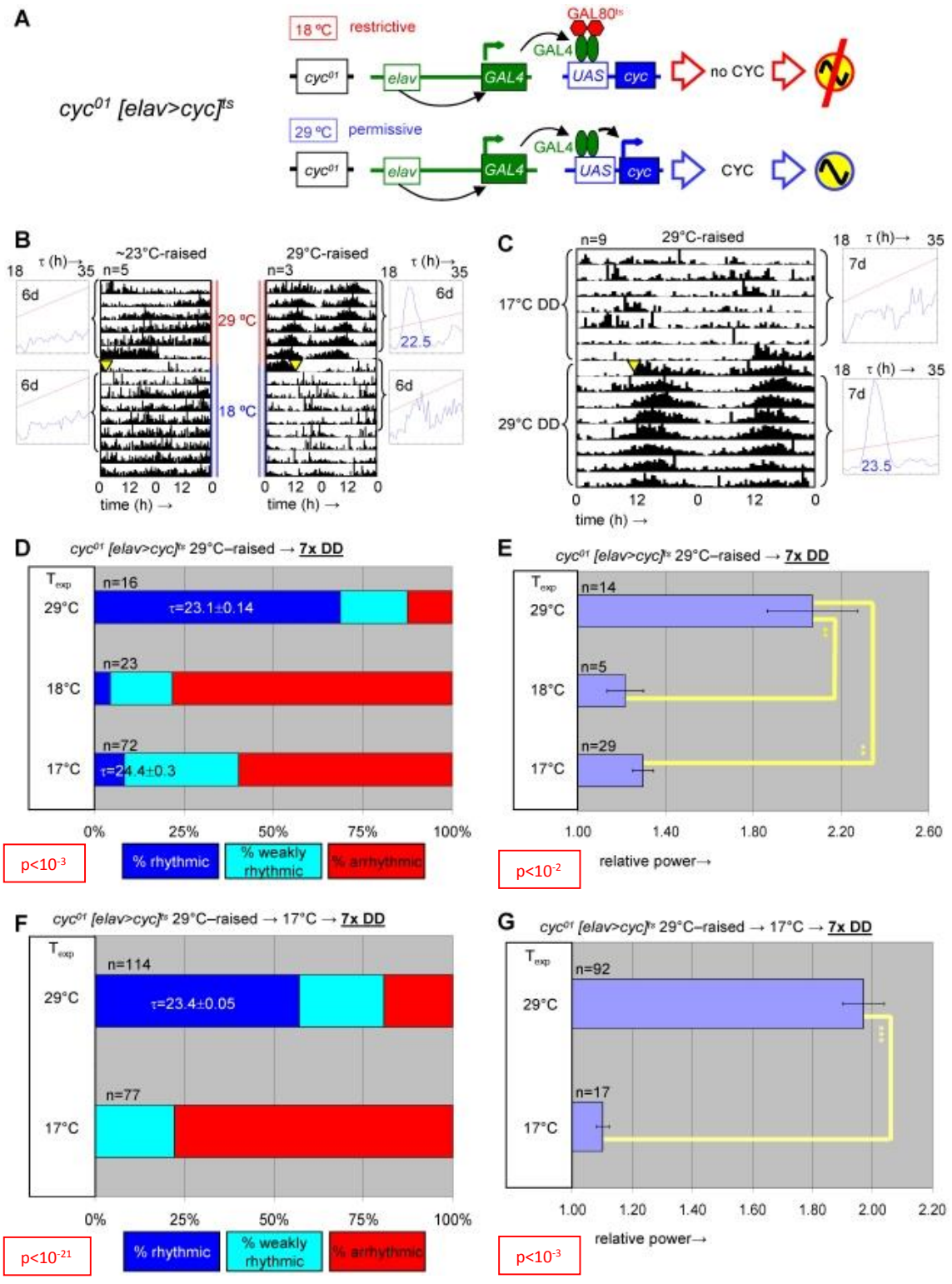


Figure 4.2. Developmental depletion of *cyc* disrupts adult circadian behaviour, while the phenotype of adult depletion of *cyc* is reversible (adapted from Goda, Mirowska, Currie *et al.*, 2011; experiments performed and analysed by Min-Ho Kim, Jake Currie

and Dr Herman Wijnen, with author of this thesis contributing data).

Transgenic flies with conditional *cyc* rescue display a behavioural arrhythmicity when analysed under restrictive conditions (17˚C), which can be reverted at permissive conditions (29˚C). However, developmental exposure to restrictive conditions results in irreversible behavioural arrhythmia. (A) Diagram showing genetic basis of the line tested. Flies with the genotype *elav^{C155}::Gal4; UAS-cyc/CyO; cyc^{01} tub$_{P}$Gal80^{ts}* flies (referred to as flies with conditional *cyc* rescue) allow for a conditional rescue *cyc^{01}* in all postmitotic neurons when at permissive temperature (29˚C), but maintain a disrupted clock at lower temperatures (17–25˚C). (B) Example double-plotted actograms and chi-square periodograms (with the p=0.01 threshold line in red and single significant period length found in the circadian range (18–35 h) in dark blue) illustrating average locomotor activity at 29˚C DD and subsequent 18˚C DD conditions for females raised at either restrictive (~23˚C) or permissive temperature (29˚C), respectively. (C) The double-plotted actogram showing the average behaviour of female flies raised under permissive (29˚C) conditions analysed at restrictive (17˚C DD) and subsequent permissive (29˚C DD) conditions. (D–G) Diagrams showing distribution of rhythmic, weakly rhythmic and arrhythmic flies (D, F) and average relative rhythmic power (E,G; error bars are SEM) for the analysis of adult locomotor behaviour of flies with conditional *cyc* rescue raised under permissive conditions (29˚C). Flies were analysed at either permissive (29˚C) or one of two restrictive conditions (17˚C, 18˚C) (D,E) or permissive (29˚C) or restrictive (17˚C) conditions after adult exposure to restrictive conditions (≥3 days 17˚C) (F,G). For comparison between distribution of flies and temperature of testing Fisher's Exact test was used. The Welch's test (E) or Mann-Whitney test (G) were used for comparison of the average relative rhythmic power with temperature with post-hoc Games-Howell tests for pairwise comparisons of developmental treatments, ** $p<0.01$, *** $p<10^{-3}$.

4.4.3 Adult *cyc* rescue flies raised under permissive conditions respond to photocycles presented at restrictive conditions, but this does not improve their rhythmicity in restrictive conditions

Further analysis of the *cyc* rescue line was performed to characterize the temperature-dependence of adult behavioural rhythms in *cyc* rescue flies raised under permissive conditions. To obtain some insight into the molecular state of the clock, I decided to focus on three questions of particular interest. The first was whether there is some residual circadian locomotor rhythmicity after switch to restrictive conditions. This could inform me about the molecular state and dynamics of the circadian oscillator. I speculated that there is some residual clock function due to accumulation of *cyc* after short exposures to restrictive conditions in adulthood, but that longer adult exposure to restrictive conditions would render flies completely behaviourally arrhythmic in the restrictive conditions. A related question was whether adult behavioural rhythms can still be rescued at permissive conditions after prolonged exposure to restrictive conditions when adult. The final question is about the possibility of some rescue of circadian locomotor rhythmicity apparent after LD exposure and general sensitivity to LD cycles at restrictive conditions. I suspected that exposure to LD conditions would not impact the rhythmicity of conditional *cyc* rescue flies in restrictive conditions.

A series of experiments described in Appendix A.2.2- A.2.4 confirmed, that the circadian locomotor rhythmicity of flies disappears within several days after moving to restrictive conditions (Figures A.2.2 – A.2.4). This suggests that weak residual circadian locomotor rhythmicity can persist after switching to restrictive conditions. The precise amount of time depended on whether LD was present prior to the switch – LD seemed to slightly extend the period during which flies displayed some rhythmicity. On the other hand, prolonged adult exposure to restrictive conditions did not disrupt adult circadian locomotor behaviour (Figures A.2.2 – A.2.3). Surprisingly, even though exposure to LD cycles did not significantly improve the rhythmicity of flies at restrictive conditions, it was still sufficient to entrain behaviour of flies switched into permissive conditions (Figures A.2.2 – A.2.3).

In Chapter 3 I demonstrated that the phase of adult behavioural rhythmicity depended on the time of the transfer of *per* rescue flies from restrictive to

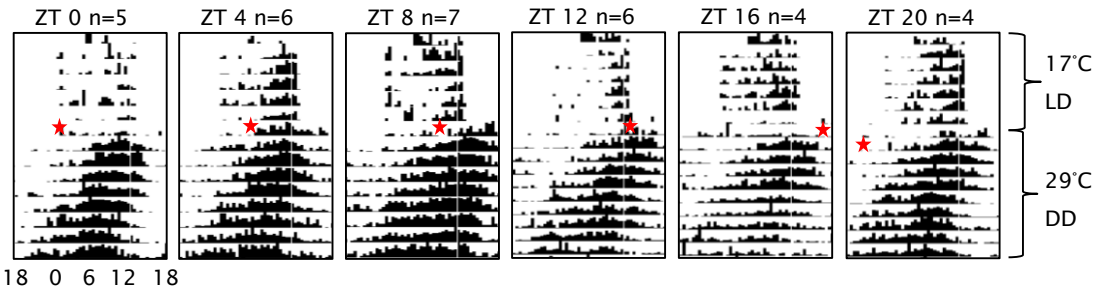
permissive conditions (Figure 3.4). It was interesting to check if the same was true for flies with conditional *cyc* rescue raised under permissive conditions. I speculated that the time of the switch of flies from restrictive to permissive conditions would determine the phase of locomotor rhythms.

It is worth describing the possible outcomes before I discuss the actual results. Three forces impact the phase of rhythmicity in this experiment. First, there is the start of the *cyc* expression – if this was the only factor influencing the phase of the locomotor activity, then we would have a perfect positive correlation between the phase and the time of the move, as flies would be starting their rhythmicity in response to the cyc expression, resulting in a fixed duration of time between the move to permissive conditions and the offset of behavioural activity. Second is the influence of the LD cycle – if this was the only factor then we would have a stable phase regardless of the time of the shift, with the possible exception of the phase of rhythmicity of flies moved during the light phase, which would be earlier than for other points, indicating that the locomotor activity of flies is determined by the LD experienced. Third is the temperature shift, which cannot be uncoupled from either, and might result in a lack of perfect correlation. It is also possible that we have the combination of different effects, in which case the phase will not display any of the patterns described above.

Phase of the rhythmicity of *cyc* rescue flies raised at permissive temperature (29˚C) and control w^{1118} flies raised at room temperature was determined as described in 4.3.5. Data for the time of the peak of activity were excluded from the analysis due to large variation, partially resulting from the difficulty in assigning the peak to some traces. In addition, the survival of w^{1118} flies was very poor and the only case for which phase could be determined across all timepoints was for females moved every 6 hours. Therefore I strongly recommend that in the future other control flies are used and the data are validated.

A

cyc rescue ♀♀



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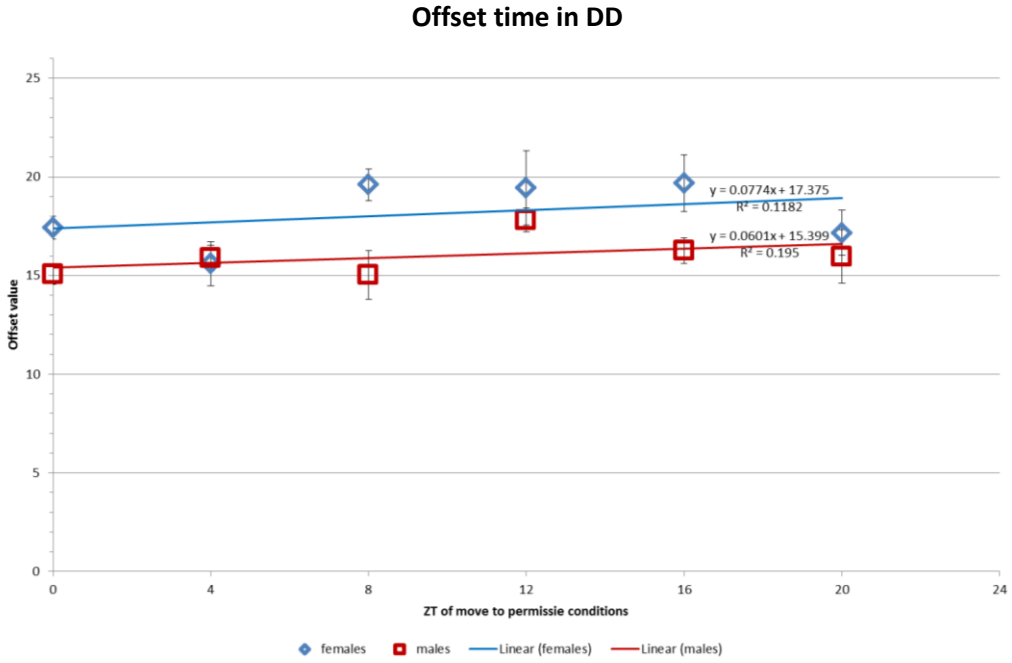

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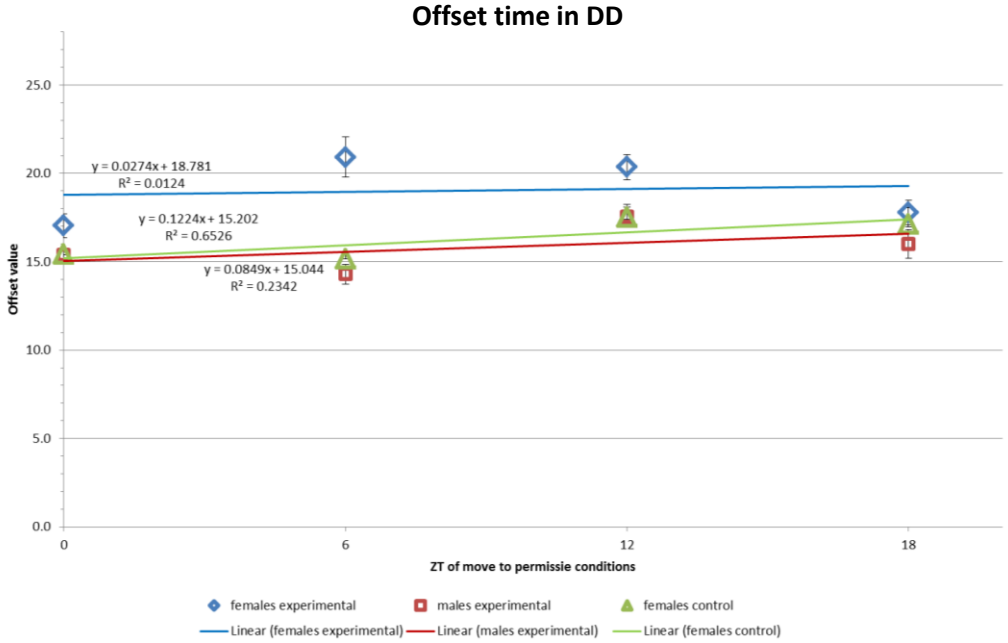


Figure 4.3. *cyc* rescue flies raised under permissive conditions behave rhythmically in permissive conditions with the phase connected to prior LD cycle.

The phase of the offset of the locomotor activity at 29˚C shows a clear dependence on the phase of the LD cycle and not on the time of the move to permissive conditions. (A) Sample actograms showing the mean activity of female *cyc* rescue flies following a shift from 17˚C LD to 29˚C 4 hours apart. Time of shift (marked by the red star) and the number of flies (n) are indicated above. Actograms are plotted over a 24h period, starting at ZT18. (B and C) Scatter plots showing the relationship between the average offset phase in DD and the time of the shift for two independent repeats of the experiment. Red points are used for *cyc* rescue males, blue for *cyc* rescue females and green are for *w1118* control females. Error bars represent the SEM. There is a stable relationship between the phase of prior LD cycle and the offset phase of rhythmicity in DD.

From the data collected for the wild-type flies (Figure 4.3 C), I could see no correlation (n=4, R^2=0.6526 value indicate that the fit is not perfect; two-tailed probability value of Pearson correlation coefficient: p=0.192) with a slope close to 0 (0.122), indicating that the offset of rhythmicity happens around 14-15 hours after ZT0, regardless of the time of the shift. However, Kruskal-Wallis analysis of individual (and not average) values confirmed that the offset phase was not perfectly linear (Kruskal-Wallis test: H(4)=11.057, p=0.026, n=24), which was most likely caused by earlier phase of offset for flies moved at ZT6. This is consistent with the phase advance observed with earlier transfer from the light to dark portion of the cycle. As expected, the offset of behavioural rhythmicity of control flies was determined almost exclusively by the prior LD cycle, with a small variation probably caused by both the temperature change and the duration of the last light or dark phase. This is consistent with the clock functioning in the control flies both at 17˚C and at 29˚C.

For the experimental *cyc* rescue flies (Figure4.3) there was a remarkable similarity between the independent repeats (Figure 4.3 B and C). In both cases I observed a close correlation with the linear trend of the slope close to 0 (for

females, slope=0.027, R^2=0.012, n=4 and slope=0.077, R^2=0.118, n=6; for males slope=0.085, R^2=0.234, n=4 and slope=0.060. R^2=0.195, n=6) and no correlation of the offset time with the time of shift (two-tailed probability value of Pearson correlation coefficient: p=0.505, n=6 and p=0.889, n-4 for females; p=0.381, n=6 and p=0.516, n=4 for males). This was confirmed by a Kruskal-Wallis analysis of individual values that revealed no association between offset time and time of shift in females (Kruskal-Wallis test: H(6)=8.819, p=0.184, n=36 and H(4)=9.313, p=0.054, n=33) and some association for males, where in one experiment shift at ZT12 delayed phase (Kruskal-Wallis test: H(6)=13.490, p=0.036, n=57; possible outlier) and in another shift at ZT6 advanced phase (Kruskal-Wallis test: H(4)=10.215, p=0.037, n=42). Female flies were also displaying the offset of rhythmicity a couple of hours later than males (CT17-18 instead of ~15). These results were unexpected as they suggest that regardless of the time of the move, flies displayed similar activity phases. Therefore I concluded that the phase of rhythmicity was not determined by the time of the shift from restrictive to permissive conditions, but rather by the prior LD cycles.

4.4.4 Developmental depletion of *cyc* disrupts adult locomotor behaviour in the light/dark cycle

Previous experiments describe in detail the phenotype of *cyc* rescue flies raised under permissive conditions (Figures 4.2, 4.3, A.2.2-A.2.4). As described below, flies raised under restrictive conditions were not only arrhythmic as adults in constant darkness at permissive conditions, but also displayed disrupted behaviour in LD.

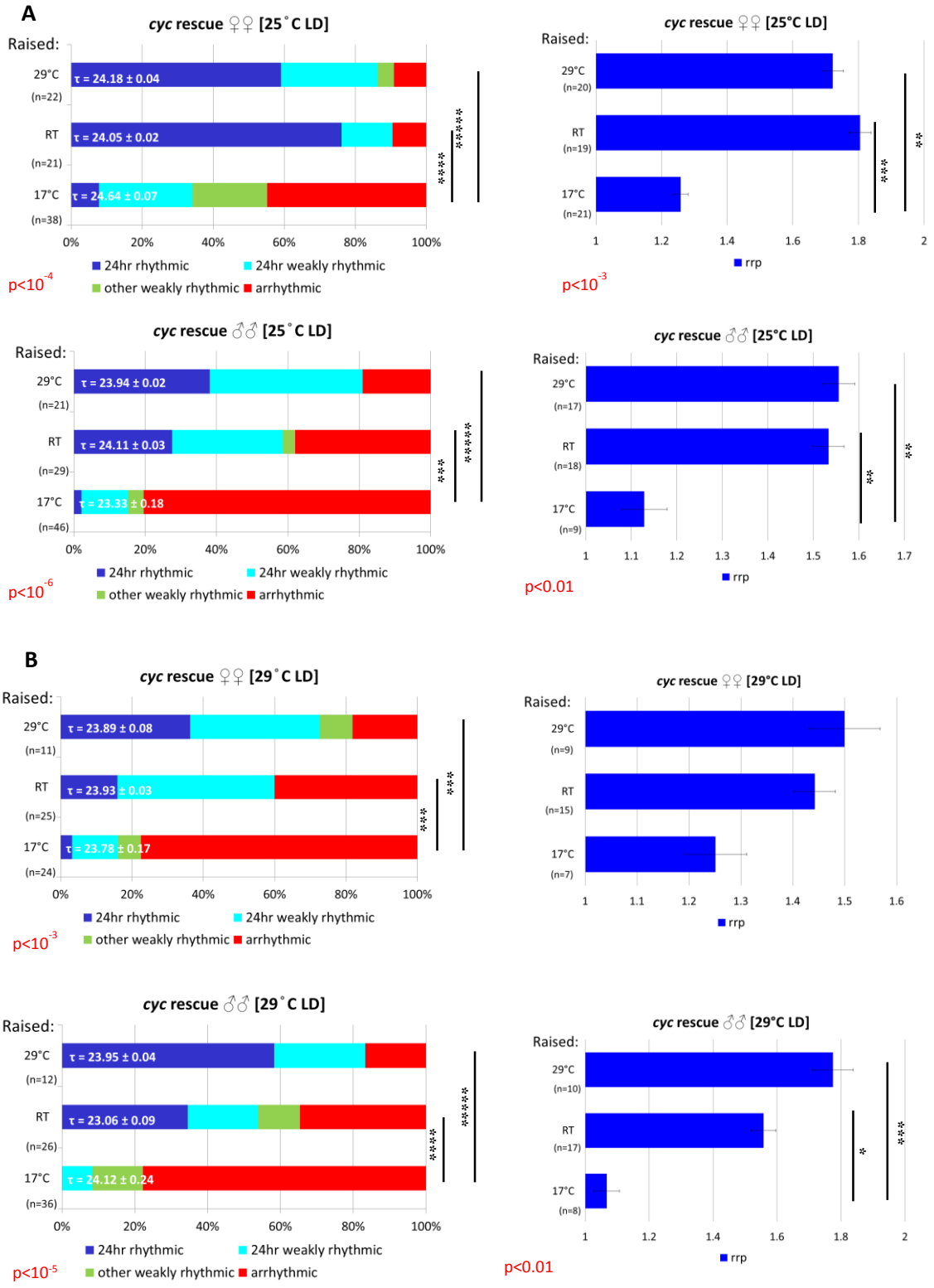


Figure 4.4. Impact of developmental *cyc* depletion on the locomotor behaviour in LD cycles at permissive temperature.

Flies raised with developmental *cyc* depletion show aberrant locomotor behaviour at permissive conditions in the presence of

light/dark cycles. Quantitative analysis of rhythmicity of individual flies raised under different conditions and analysed at 25˚C LD (A) and 29˚C LD (B). Stacked bar diagrams (left) represent the percentage of flies displaying different rhythmicity. The numbers of flies and the period of activity of all flies displaying rhythms (with Standard Error of Mean values) are included on the diagrams. The correlation between distribution of flies and their developmental temperature was tested using Fisher's Exact 3x3 test. Pairwise comparisons of distribution of flies across all four categories were performed with Fisher's Exact 4x2 tests. Bar diagrams on the right show the average of the relative rhythmic power (rrp) for all rhythmic and weakly rhythmic flies with the error bars showing SEM. The correlations between the period length and rrp and the developmental temperature were tested with the non-parametric Kruskal-Wallis test with Wilcoxon Rank test used for individual comparisons. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<10^{-4}$; ***** $p<10^{-5}$.

To analyse this phenomenon, *cyc* rescue flies were raised under permissive (29˚C LD), restrictive (17˚C LL, 17˚C DD) or intermediate conditions (RT and 25˚C LD) and their behaviour at 25˚C or 29˚C LD was tested as described in 4.3.6. For both conditions analysed the difference between flies raised at 17˚C and 29˚C was striking (Figures 4.4 and A.2.5). RT-raised flies showed an intermediate phenotype, which was not significantly different from that for 29˚C. At 25˚C LD (intermediate temperature), adult flies raised at 29˚C were exclusively diurnal with the vast majority of flies displaying a period of around 24 hours. Females tended to stay active during mid-day, while males exhibited a siesta. Anticipation of lights-off was present; however in females the lights-on anticipation appeared absent. Flies raised at room temperature were also active mainly during the light portion, with low proportions of arrhythmic flies. On the other hand, the majority of flies raised at 17˚C were arrhythmic (Figures 4.4 and A.2.5). There was a significant difference between flies raised at 29˚C and 17˚C (Fisher's Exact 4x2 test: $p<10^{-4}$, n=60 for females and 10^{-6}, n=67 for males) and between flies raised at 25˚C and 17˚C (Fisher's Exact 4x2 test:

$p<10^{-6}$, n=59 for females and 10^{-3}, n=75 for males), but not between flies raised at RT and at 29˚C. Also the relative rhythmic power for flies was significantly different(Kruskal-Wallis test: females: H(2)=20.733, p<0.001, n=60 for overall comparison - p<0.01, n=41 for flies raised at 29˚C and 17˚C and p<0.001, n=40 for flies raised at RT and 17˚C; males: H(2)=11.726, p<0.01, n=44 (n=26) for overall comparison - p<0.01, n=26 for flies raised at 29˚C and 17˚C and p<0.01, n=27 for flies raised at RT and 17˚C).

At 29˚C LD, flies raised at 29˚C displayed strong rhythmicity of around 24 hours (Figures 4.4 and A.2.5). Both males and females showed anticipation of lights-on and lights-off and displayed a siesta mid-day (Figure A.2.5). Similarly to what happened at 25˚C LD, flies raised at 17˚C were largely arrhythmic (and females even had significantly worse rhythmicity at 29˚C LD than at 25˚C LD; Fisher's Exact 4x2 test: p<0.05, n=62). Interestingly, few flies that displayed rhythms of around 24 hours were predominantly active during the dark portion of the cycle. Also, among females raised at room temperature and analysed at 29˚C LD, compared to those analysed at 25˚C LD, a much larger proportion of flies did not show any rhythms (Fisher's Exact 4x2 test: p<0.001, n=46). The lights-off anticipation was present, however flies were either as active during the dark portion as during the light one (females) or even more active during the dark portion (males). The statistical analysis of the rhythmicity of individual flies has revealed a difference between the rhythmicity of flies raised at 17˚C and at 29˚C (Fisher's Exact 4x2 test: $p<10^{-3}$, n=35 for females and $p<10^{-5}$, n=48 for males) and flies raised at 17˚C and at 25˚C (Fisher's Exact 4x2 test: $p<10^{-2}$, n-49 for females and $p<10^{-4}$, n=62 for males) but not between flies raised at RT and 29˚C. Despite a clear trend in the data for relative rhythmic power, due to relatively low numbers of flies, no association between developmental temperature and rrp was found for female flies. On the other hand, rrp of males raised at 17˚C was different than rrp of both flies raised at RT and 29˚C (Kruskal-Wallis test: H(2)=12.881, p<0.01, n=35 for overall comparison - p<0.05, n=25 for flies raised at RT and 17˚C and p<0.001, n=18 for flies raised at 29˚C and 17˚C). The difference between flies raised at 17˚C and RT/25˚C raised flies might be explained by some expression of the transgenic *cyc* at intermediate temperature. Taken together, the data presented here suggest that the developmental disruption of CLK/CYC function leads to adult

behavioural arrhythmia not only in free-running conditions but also under LD cycle.

To demonstrate that these disruptions due to the behaviour of the *cyc* rescue flies correspond to the developmental CLK/CYC state, rather than CLK/CYC function during the analysis, I decided to test *cyc* rescue flies raised under permissive conditions (29˚C) and analysed at restrictive conditions (17˚C) as described in 4.3.7. Both *cyc* rescue and *Canton-S* flies aligned their rhythmicity with the light portion of the day, generating periods of around 24 hours (Figure 4.5). Despite somewhat different activity profiles for both groups (*cyc* rescue flies have extended lights-off anticipation), adult disruption of circadian clock through interruption of CLK/CYC function did not appear to affect the light masking behaviour and diurnal (or rather crepuscular) behaviour of flies.

Together, data presented in Figures 4.2 and 4.4 provide some evidence that developmental CLK/CYC function might be necessary for both establishing rhythmic adult behaviour in constant darkness and in regular light/dark cycles.

If my hypothesis that the changes to the behaviour in LD were caused during development was true, then partial rescue of *cyc* function during adulthood should not improve the rhythmicity of flies, leaving *cyc* rescue flies similar to flies without any rescue of *cyc* - *yw;;cyc^{01}*. On the other hand, the fact that at 25˚C the rescue of CLK/CYC was incomplete should leave *cyc* rescue flies raised with a functional clock (at 29˚C) no more arrhythmic than flies with constitutive *cyc* rescue - *elav^{C155}::Gal4; UAS-cyc/CyO; cyc^{01}*.

This hypothesis could be confirmed from the results (Figure 4.6), since conditional *cyc* rescue flies raised at 17˚C were indistinguishable from *yw;;cyc^{01}* flies, but significantly different from flies with constitutive *cyc* rescue (Fisher's Exact 4x2 test: $p<0.01$, n=53 for females and $p<10^{-10}$, n=62 for males for distribution of flies) or conditional *cyc* rescue flies raised at 29˚C (Fisher's Exact 4x2 test: $p<0.001$, n=60 for females and $p<10^{-6}$, n=67 for males for the distribution of flies). In addition, despite the fact that at 25˚C the levels of transgenic *cyc* decreased in the conditional *cyc* rescue flies raised at 29˚C, the rhythmicity of individual flies and their rrp was very similar to those for flies with constitutive *cyc* recue and significantly different from *yw;;cyc^{01}* flies (Fisher's Exact 4x2 test: $p<0.001$, n=35 for females and $p<0.001$, n=37 for

males for distribution of flies). To confirm the results, however, the levels of *cyc* expression during both development and adulthood should be tested for all genotypes used.

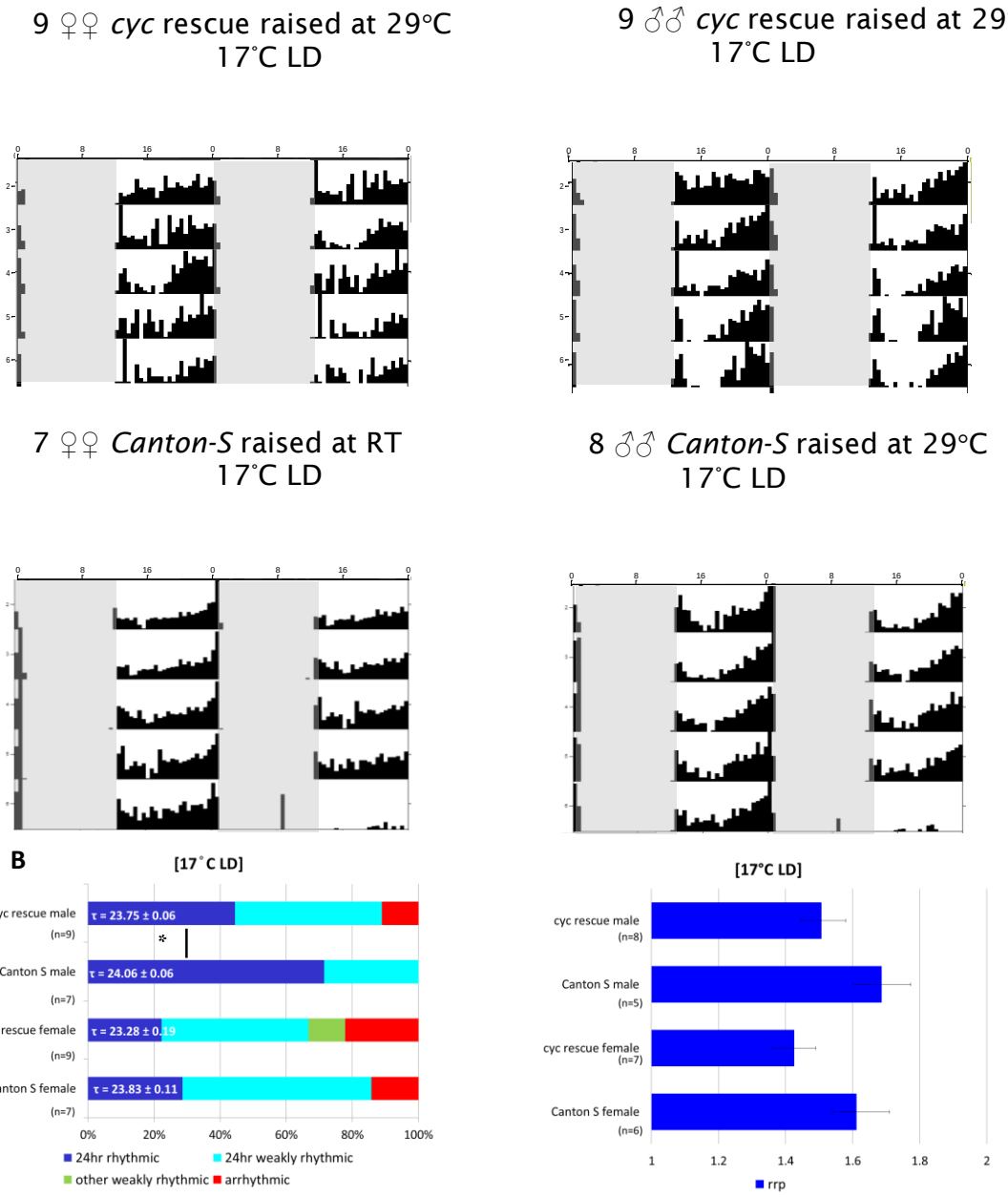


Figure 4.5. Adult *cyc* depletion does not disrupt locomotor behaviour in LD cycles.

cyc rescue flies raised at permissive temperature display rhythmic behaviour with a period close to the environmental τ cycle at restrictive conditions. (A) Double-plotted actograms showing the median locomotor behaviour of the *cyc* rescue flies raised at 29˚C (top) and *Canton-S* control flies (bottom) analysed at 17˚C LD. Numbers are indicated above actograms. Shading on actograms represents the periods of darkness. There is no statistical difference between *cyc* rescue flies and *Canton-S* flies. (B) Distribution of the rhythmicity of fies and average relative rhythmic power for the analysis of the rhythmicity of individual flies raised under different conditions. The numbers of flies and the period of activity of all flies displaying rhythms (with Standard Error of Mean values) are included on the diagrams. *cyc* rescue females and males were compared individually to controls with 2x4 Fisher's Exact tests for the distribution of flies. The correlation between the rrp or period length and the developmental temperature was tested with the non-parametric Kruskal-Wallis test with Wilcoxon Rank test used for individual comparisons. * $p<0.05$.

To summarise, flies raised under restrictive conditions (which includes RT or 25˚C) were not only unable to display rhythmicity in constant darkness (Figure 4.2), they also displayed aberrant behaviour in LD cycle (Figure 4.4). It was interesting that the behaviour seemed even worse at 29˚C than at 25˚C, which can potentially be explained by the fact that keeping flies at 29˚C shortens their lifespan, negatively impacting their rhythmicity. Also, the increased nocturnality observed at 25˚C (Figure A.2.5) can create impression that the rhythmicity of flies is better than it actually is. The disruption of LD behaviour of flies also seemed to be linked with the developmental, rather than adult, function of CLK/CYC (Figures 4.4 – 4.6).

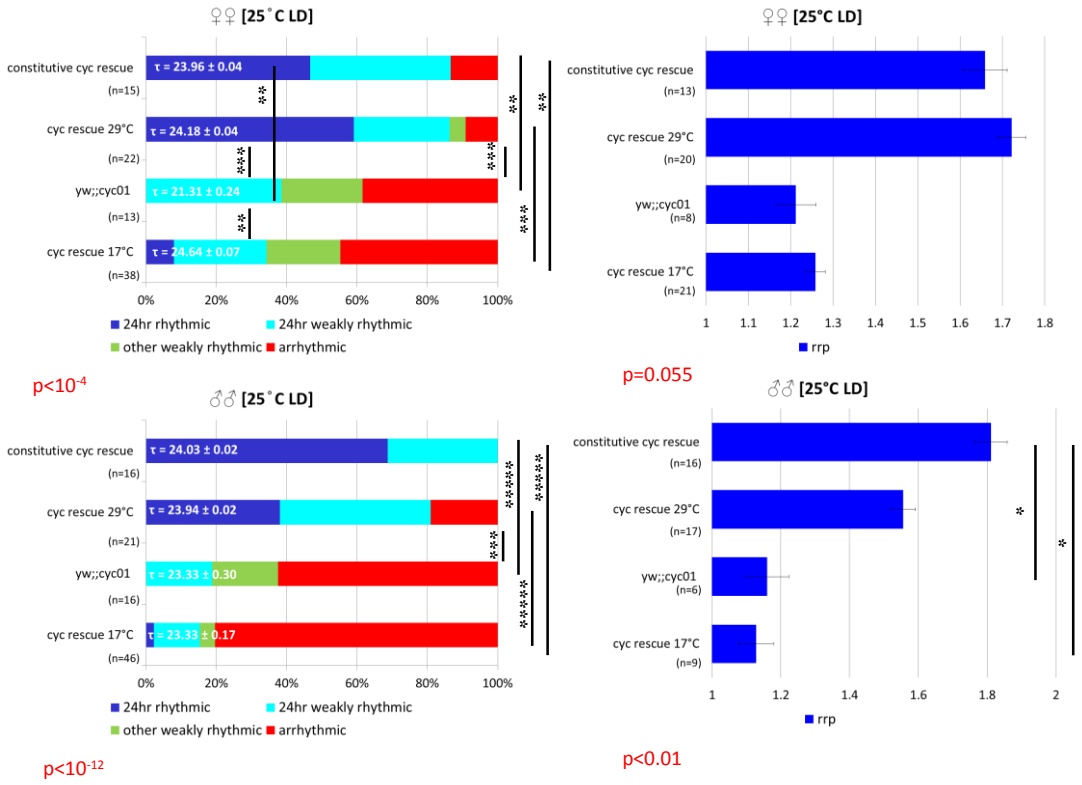


Figure 4.6. Impact of developmental and adult *cyc* depletion on locomotor behaviour in LD cycles.

Quantitative analysis of the rhythmicity of individual flies of different genotypes raised at RT compared to *cyc* rescue flies raised at 29˚C and 17˚C. Stacked bar diagrams (left) show the distribution of flies with different strength of rhythmicity in the population. The period of locomotor activity at LD of all rhythmic and weakly rhythmic flies (with Standard Error of Mean values) together with the number of flies used for the analysis are included on the diagrams. 2x4 Fisher's Exact tests were used to compare the distribution of flies. Pairwise comparisons of distribution of flies were performed with Fisher's Exact 4x2 tests. On the right side, bar graphs represent the average relative rhythmic power (rrp) of all rhythmic and weakly rhythmic flies with the error bars showing SEM. Period lengths and relative rhythmic powers of all rhythmic and weakly rhythmic flies were compared with non-parametric Kruskal-Wallis test with Wilcoxon Rank test used for individual comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ***** $p<10^{-5}$.

4.5 Discussion

4.5.1 CLK/CYC function is required during development for adult behavioural rhythmicity

As discussed in the previous chapter (3.5.1), no controls were run to check the levels of GAL4 expression achieved or impact of the insertion position of the transgene on the rhythmicity of the flies. Flies that were compared to each other had the same transgenes present, which meant that the same insertion site bias affected all relevant conditions. Moreover, in spite of extensive previous use of these *Gal4* and *UAS* constructs (Tanoue *et al*., 2004, Lin and Goodman, 1994) in analyses of circadian behaviour, no impact of insertion site on behavioural rhythmicity has been reported.

The connection between the circadian clock in developing *D. melanogaster* and the behavioural rhythmicity of adult flies has not been widely tested previously. Data presented in Chapter 3 suggested that developmental *period* expression was not necessary for the adult locomotor rhythmicity (Figure 3.3 and Table 3.1). As PER is the key component of the molecular oscillator, I have also concluded in Chapter 3 that a functioning molecular oscillator during development is not required for behavioural rhythmicity in adulthood. However, I demonstrated in this chapter that the function of one of the oscillator components, CLK/CYC, is required during development for adult locomotor rhythmicity (Figures 4.1 B and 4.2 F and G). This finding was confirmed using two independent genetic manipulations, namely conditional temperature-dependent over-expression of the CLK/CYC inhibitor PERIOD and conditional temperature-dependent depletion of CYC, leading to similar changes at the molecular level. Using these two independent strategies helped to eliminate the alternative explanation of developmental temperature irreversibly affecting adult behaviour, since the elevated temperature of 29˚C was acting once as a restrictive and once as a permissive conditions, and adult rhythmicity was not associated with development at 29˚C, but rather the state and function of molecular oscillator during development.

Despite the obvious adult arrhythmia resulting from the absence of CLK/CYC function during development, the molecular state of the oscillator is more difficult to infer. I conclusively showed that the clock can regain its robustness

after adult, rather than developmental, CLK/CYC depletion (Figure 4.2). However, it is unknown if the molecular oscillator completely stops functioning under adult versus developmental restrictive conditions, and how long it takes.

Interestingly, even with clear prior behavioural arrhythmia, adult *cyc* rescue flies restarted their activity in permissive conditions with a phase tightly correlated to the LD cycles experienced under restrictive conditions (Figure 4.3). The experiment studying the relationship between the phase of rhythmicity with the time of transfer from restrictive to permissive conditions presented in Figure 4.3 had an obviously different design to the previously mentioned *per* rescue experiment (Figure 3.4), in which flies were moved directly from DD at restrictive conditions to DD in permissive conditions. Therefore I cannot conclude that the underlying reason for the discrepancies observed is caused by the altered molecular state of the clock in these two cases. A follow up experiment would be to run each of the experiments again with the design of the other experiment.

A possible explanation as to why the phase of the offset of locomotor rhythmicity in adult *cyc* rescue flies is closely correlated to prior environmental conditions is that despite their behavioural arrhythmia in restrictive conditions, the molecular clock of *cyc* rescue flies retains function that is uncoupled from behavioural rhythms under restrictive conditions. A reason for this could be low levels of *cyc* expression even at restrictive conditions, which may not be enough to sustain behavioural rhythmicity, but can cause low amplitude rhythmic oscillations in the levels of circadian genes. In that case, a clock-dependent light-mediated phase resetting mechanism involving CRY or TIM may persist.

Confirming this hypothesis involves performing experiments to test the molecular state of the oscillator. One of them would be testing if oscillations persist in *cyc* rescue adult heads under restrictive conditions for transcript levels of clock-controlled genes, such as *period*, *timeless*, *vrille* (*vri*) and *PAR domain protein1* (*Pdp1*) through a qPCR. At the same time the levels of CRY could be tested, but I suspect that regardless of the oscillations in clock-controlled genes they will be elevated, based on the elevated CRY levels during the dark phase reported previously in the Clk^{Jrk} mutant flies (Kumar *et al.*, 2012), which can be considered molecularly similar to cyc^{01} flies. The

persistence of oscillations in CRY mutants suggests that CRY can still be degraded by light (Lin *et al.*, 2001) and in the presence of even low amplitude molecular oscillations, it could reliably set the phase of the locomotor activity in darkness at permissive temperature. Confirming this could involve entraining flies to red light (instead of white which has a significant blue component). Red light would not activate the CRY-mediated pathway (Hanai and Ishida, 2009; Cusumano *et al.*, 2009) at restrictive temperature and therefore the results of the phase analysis after switching flies to permissive conditions at various ZT would show if CRY is mediating the phase – setting observed in the experiment described in 4.3.3. An even more elaborate approach would involve genetically manipulating the *cyc* rescue flies so that they carry an additional *cry* mutation and testing them using the same protocol, to see if the correlation between the phase of behaviour and the lights-on still exists.

An alternative hypothesis is that light can moderate the arrhythmic state in some way that sets the phase of the clock once the clock starts working. To distinguish between these possibilities a simple experiment can be designed. *cyc* rescue flies could be raised at permissive temperature and then monitored in the 17˚C LD for 3 days followed by release to 17˚C DD prior to a subsequent move at different ZTs to 29˚C. Data obtained from this experiment should be compared to the data presented in section 4.3.3. If there is a similarity, we can speculate that *cyc* rescue flies indeed retain some residual rhythmicity. If the relationship between the offset phase and the time of the shift to permissive temperature becomes more stable, then we can presume it is a clock-independent light effect.

Lastly, my results suggest that developmental CLK/CYC inhibition in addition to affecting free-run of adult flies, can also increase nocturnality of flies or render flies insensitive to the presence of light/dark cycles, but only in cases when the depletion during development is more complete (Figures 4.4 and 4.6). Similar observations have been made before, Kumar *et al.* (2012) reported increased levels of nocturnality in Clk^{Jrk} mutant flies. Since Clk^{Jrk} can be considered similar to cyc^{01} flies in terms of CLK/CYC activity, I speculate that similar mechanism might be involved in both cases. Increased dopamine signalling in the Clk^{Jrk} flies, resulting from loss of CLK-mediated regulation of

Tyrosine Hydroxylase, is acting through CRY to increase the locomotor activity at night. My results, however, seem to point to the combination of the developmental and adult clock function impacting the behaviour in LD, confirming the role of the CLK/CYC in regulating both the dopamine and CRY pathway and hence modulating the wakefulness and activity of flies.

The reason for the different results obtained with the *per* rescue and *cyc* rescue flies is the different molecular state of flies. Flies with per^{01} mutation lack the CLK/CYC inhibition and hence experience intermediate to high levels of other clock-regulated transcripts (Sehgal *et al.*, 1994; Blau and Young, 1999; Hao *et al.*, 1999; Claridge-Chang *et al.*, 2001). Contrary to this, flies with elevated PER levels, or lacking functional CLK/CYC, have down-regulated levels of clock-controlled genes (Allada *et al.*, 1998; Rutila *et al.*, 1998; Blau and Young, 1999; Hao *et al.*, 1999; Claridge-Chang *et al.*, 2001).

So what makes CLK/CYC so special during development? First of all, the difference between PER and CLK/CYC is their apparent importance for setting up the function of the molecular oscillator. During development, the first circadian protein present in the brain is PER, however it is CLK that can be detected first in the neurons that become circadian, followed by PER three to six hours later (Houl *et al.*, 2008). Moreover, CLK was demonstrated to induce ectopic clocks in flies (Kilman and Allada, 2009). This might signify that the developmental CLK/CYC expression is necessary to start the function of the molecular oscillator.

The main difference between the function of PER and CLK/CYC is that, contrary to PER (Huang *et al.*, 1993), CLOCK and CYC are transcription factors that form a heterodimer and bind to E-box (CACGTG) elements in the promoter region of target genes (Hao *et al.*, 1997; Darlington *et al.*, 1998; Allada *et al.*, 1998; Rutila *et al.*, 1998). They activate the transcription of multiple genes, including circadian genes like *period*, *timeless*, *vrille* (*vri*) and *PAR domain protein1* (*Pdp1*) (Blau *et al.*, 1999; Glossop *et al.*, 2003). This is confirmed by the phenotype observed in cyc^{01} mutants – in addition to behavioural arrhythmia they display a lack of molecular oscillations and generally express low levels of these genes (Rutilla *el al.*, 1998). However, since adult arrhythmia caused by either PER over-expression or CYC depletion in flies raised under permissive conditions was reversible, it seems to point to the developmental function of

this complex outside maintaining the function of the molecular oscillator. This is confirmed by the fact that the oscillator function *per se* was not necessary during development for the adult behavioural rhythms, as evident from the *per* rescue data (Figure 3.3).

A large number of genes activated by CLK/CYC are not directly implied in the functioning of the molecular oscillator (Abruzzi *et al*., 2011) and since some of these genes are important for other physiological processes (and some have as yet unknown function), I can speculate that the developmental deficiency of one or more of these genes could be what is causing the adult behavioural phenotype resulting from developmental CLK/CYC inhibition or absence. CLK/CYC targets would be expected to be expressed at constitutive elevated levels during development at restrictive conditions in *per* rescue flies (as in PER absence elevated levels of CLK/CYC would result in longer expression of its targets), but not *cyc* rescue flies (where CLK/CYC is absent at restrictive conditions).

CLK/CYC- regulated candidate genes could either permanently modify the properties of the oscillator, or change the organization of the neuronal circadian circuit. Park *et al*., (2000) shows that cyc^{01} and Clk^{Jrk} flies have both neuro-anatomical defects in the LN_vs and lower PDF expression, which might be pointing to the developmental role of CLK/CYC in ensuring proper circuit organization or neurotransmitter release.

One hypothesis is that disruption of CLK/CYC function in development interferes with PDF expression. Since PDF is involved in generation of behavioural rhythms in both LD and DD (Renn *et al*., 1999; Helfrich-Förster *et al*., 2000), changes in its level might lead to behavioural arrhythmia. This is also further supported by the finding that PDF levels in s-LN_vs in cyc^{01} flies are low (Goda, Mirowska, Currie *et al*., 2011). In fact, the abnormal behavioural patterns observed in LD cycles in the case of *cyc* rescue flies raised under restrictive conditions (Figure 4.4) seem to point to this possibility. There is also an apparent titration effect, in which flies raised at lower restrictive temperatures display a phenotype more severe than flies raised at higher restrictive temperatures, which could suggest that the level of PDF expression is connected to the extent of interruption of CLK/CYC function during development. However, the phenotype observed with Pdf^{01} flies, or flies with

ablated PDF neurons (Renn *et al*., 1999) is less severe than the one reported in this chapter for flies with developmental CLK/CYC disruption (Figures 4.2-4.4), suggesting that other neurons or neurotransmitters are also involved.

Improper neuronal connectivity could potentially lead to the asynchrony between the clock neurons or influence the output pathway without disrupting the cell-autonomous function of molecular oscillator. In fact, flies with constitutive CLK/CYC manipulations (Clk^{ar} flies and cyc^{01} flies with *cyc* function rescue only in PDF-positive neurons) have been shown previously to display separation of the behavioural and molecular (Allada *et al*., 2003; Peng *et al*., 2003) phenotypes. This underlying mechanism might be suggested by the fact that upon transfer to permissive conditions flies were showing the rhythmicity connected to the phase of prior LD cycle. This would suggest that CLK/CYC manipulation in adulthood only disrupts some aspect of the output pathway temporarily while developmental disturbance of CLK/CYC function causes irreversible changes.

The developmental changes to the neuronal circuit involved in receiving signals through PDF expression could also result in uncoupling behavioural rhythms from molecular oscillations. PDF release from the s-LN_vs is implied to be part of the output pathway (Yoshii *et al*., 2009c; Cavannaugh *et al*., 2014). Stereotypical projections from s-LN_vs extend towards the dorsal part of the brain (Helfrich-Förster, 1995) and contact DN1 neurons, which in turn contact some of pars intercerebralis (PI) neurons (Cavannaugh *et al*., 2014). PI neurons then regulate the locomotor activity rhythms by releasing DH44, a corticotropin-release factor (CRF) homologue (Cavannaugh *et al*., 2014). Additionally, the same projections undergo circadian changes in the remodelling of the axonal termini (Fernández *et al*., 2008, Sivachenko *et al*., 2013) and in their synaptic contacts. Changes to these processes would cause the disruption of the output rhythms, without changing the function of the molecular oscillator within the neurons. It is possible that CLK/CYC affects either of these processes, leading to the uncoupling of the molecular oscillations in the ventrolateral neurons and behavioural rhythms. Interestingly, one of the CLK/CYC-regulated genes, *Myocyte Enhancer Factor 2* (*Mef2*) is not only enriched in the ventral lateral neurons (Nagoshi *et al*., 2010) but was also shown to be required for the daily fasciculation-defasciculation (contractions

and expansions of projections) cycle through regulating transcription of *Fasciclin-2* (*Fas2)*, providing a way in which CLK/CYC can be involved in neuronal remodelling (Sivachenko *et al*., 2013).

4.5.2 Impaired clock function does not affect the survival of flies

Data presented in A.2.1 relate to the impact of the developmental temperature and function of the clock during development on adult longevity. Developmental conditions can affect lifespan through their impact on the duration of development (de Moed *et al*., 1998, 1999; Ashburner *et al*., 2005). The optimum developmental temperature giving rise to the shortest development but also the shortest lifespan is 29˚C (Loeb and Northrop, 1917), which was restrictive temperature for flies with conditional *per* over-expression. Despite some evidence for the lack of the impact of developmental time or temperature on adult lifespan (Economos and Lints, 1986, Zwaan *et al*., 1991, 1995; Nunney, 1996), most of the literature agrees that flies raised at higher temperatures develop faster (Zwaan *et al*., 1992) and live for a shorter time (Alpatov and Pearl, 1929; Lamb, 1968; Burcombe and Hollingsworth, 1970; Lints and Lints, 1969, 1971a; Chippindale *et al*., 1994; Zwaan *et al*., 1995, Yadav and Sharma, 2014).

The effect of elevated temperature on the lifespan of *per* over-expression flies could be ignored if it was not for the fact that the circadian clock function depends on the health and age of the fly. Since the duration of fly development is connected with ageing, one could conclude that the developmental rate can therefore influence the function of the circadian clock, making 29˚C a particularly bad condition for studying adult behavioural rhythms. Therefore, there was a possibility that the impaired locomotor rhythmicity associated with *per* over-expression flies raised at 29˚C might represent this age-dependent disruption of rhythms.

Faster development of flies is connected not only to a decreased lifespan, but also a shortening of the period length of behaviour. Slower development of flies is associated with both increased longevity and longer period length, as per^{+} flies develop faster than per^{L}, but slower than per^{s} (Kyriacou *et al*., 1990)

and flies selected for faster development have shorter periods (Yadav and Sharma, 2013a, 2013b, 2014). Moreover, in a screen of 32 genomic deficiencies, 18 genomic regions were found that correlated both to developmental time and period length (Takahashi *et al.*, 2013). Koh *et al.* (2006a) report that with age the strength of the sleep/wake cycle and length of activity bouts decrease and fragmentation of sleep increases. This can also be modulated by changing the environmental temperature. Driver (2000), Krishnan *et al.* (2009), Rakshit *et al.* (2012) and Luo *et al.* (2012) provide a detailed description of what happens to circadian clocks as flies age – behaviour becomes disrupted and period lengthens. At the molecular level, *period*, *timeless*, *vrille* (*vri*) and *PAR domain protein1* (*Pdp1*) were expressed at lower level. This decrease in the levels of circadian genes was found not to be associated with reduced CLK/CYC function (Rakshit *et al.*, 2012). Period lengthening was also reported in ageing mice (Valentinuzzi *et al.*, 1997), however, most research on mammals and humans reveals an association between period shortening and ageing (Pittendrigh and Daan, 1974; Witting *et al.*, 1994; Monk *et al.,* 2005; Aujard *et al.*, 2006). Molecular changes do not appear to be conserved between organisms, since studies on ageing mammals and zebrafish revealed either reduction (Weinert *et al.*, 2001; Kolker *et al.*, 2003; Zhdanova *et al.*, 2008); Nakamura *et al.*, 2011) or normal profiles of gene expression (Asai *et al.*, 2001; Yamazaki *et al.*, 2002), depending on the organism and organ tested.

On the other hand, functional circadian clocks have been shown to be necessary for ensuring a normal lifespan for a wide variety of organisms. Some scientists have speculated that the match between the period of locomotor behaviour and the environmental conditions might extend the lifespan (Pittendrigh and Minnis, 1972). Some studies found no connection between the function of the circadian clock and longevity (Konopka *et al.*, 1987), but the consensus is that there is a connection between clock function and lifespan. For example, neurodegeneration-prone flies kept in constant light (which causes disruption in the circadian rhythms in wild-type flies, but not in the hypomorphic or null CRY mutants (cry^{b}, cry^{01}) (Konopka *et al.*, 1989; Stanewsky *et al.*, 1998; Emery *et al.*, 2000a and 2000b; Dolezelowa *et al.*, 2007)) died much sooner than control flies (Krishnan *et al.*, 2012). These experiments did not rule out the possibility that other factors affect the lifespan of flies. One

could be that most flies used to demonstrate the relationship between clock function and lifespan are also neuroanatomical mutants, and the results of just disrupting the function of the clock are difficult to uncouple from the neurodegeneration effects on ageing. Moreover, some pleiotropic effects of *per* are not related to the circadian clock function and elevated *per* levels could potentially influence aging. Finally, it is also possible that constant light has other effects on the longevity of flies, not mediated through the circadian clock function. Therefore, to uncouple the impact of constant light on the circadian clock function, it might be interesting to check the impact of constant light on the lifespan of hypomorphic or null CRY mutants.

Disruption of circadian clock function is said to phenocopy ageing and might lead to a reduced lifespan in *D. melanogaster* (Klarsferd and Rouyer, 1998; Hendricks *et al*., 2003; Kumar *et al*., 2005). Knockout of circadian genes or chronic jet-lag leads to premature ageing and increased mortality in mammals (Antoch *et al*., 2008; Hurd and Ralph, 1998; Bunger *et al*., 2005; Kondratov *et al*., 2006; Kondratov *et al*., 2007; Lee *et al*., 2006; Davidson *et al*., 2006; Dubrovsky *et al*., 2010; Yu and Weaver, 2011). Particularly, null mutations in *per* gene (Krishnan *et al*., 2009), *cyc* or *Clk* (Hendricks *et al*., 2003) accelerate ageing, however, in the case of *per* it was only when flies were challenged with oxidative stress. However, in all of these cases the disruption of the circadian clock was both developmental and during adulthood. Moreover, since *cyc* and *Clk* mutants also affect neuroanatomy (Park *et al*., 2000) and per^{0} required additional oxidative stress, it might signify that this may not be through the circadian oscillator impairment only. My data suggest a lack of the connection between the function of the circadian clock during development and longevity of flies. This supports the theory that the reduction in the lifespan of flies with an impaired circadian clock is due to the lack of rest or sleep rather than function of the clock *per se* (Hendricks *et al*., 2003).

No definite conclusions could be made based on the analysis of one clock mutant with one control line. Moreover, in *per* over-expressing flies PER was supplemented both at peak and trough, and therefore it might also have counteracted any possible age-dependent dampening in PER oscillations, leading to a more stable oscillator. In addition to this, unfortunately, *yw* control flies used did not share the exact background as the experimental flies.

Therefore I cannot exclude the possibility that one of the sites of transgene insertion or another difference in genetic background affected longevity. It might be worth repeating the experiment with multiple control lines, with a combination of genetic elements used in the conditional *per* over-expression line. For better confidence in the data, ideally, I would like to test also the behaviour of the control flies to demonstrate that despite comparable lifespan, wild-type flies do not exhibit irreversible adult arrhythmia following development at 29°C.

Chapter 5: Spatial and temporal characterization of the developmental CLK/CYC requirement

5.1 Introduction

In *D. melanogaster*, the vast majority of studies on the circadian clock function involve analysis of the behavioural rhythms of adult flies. However, it is important to recognize that the state of the clock in adulthood can be influenced by the state of the clock during development. Previous chapters focused on the connection between these two states. In Chapter 3, I demonstrated that *per* expression in the clock cells is not required during development for rhythmic locomotor behaviour in adult flies (Figure 3.3). Chapter 4 showed that, on the other hand, the presence of functional CLK/CYC in the clock cells during development is necessary for adult circadian locomotor rhythms (Figures 4.1 and 4.2).

What is lacking from the results presented in Chapter 4 is a more detailed analysis of the developmental CLK/CYC requirement. I was particularly interested in when and where CLK/CYC was necessary during development. Narrowing down the temporal and spatial requirements for the CLK/CYC expression during development would help me to understand how the molecular oscillator is assembled. Additionally, it could also help to identify the exact CLK/CYC function during development.

D. melanogaster passes through three successive life forms – embryo, larvae and adult. Since *D. melanogaster* is a holometabolous insect, larvae do not

resemble the adults. *D. melanogaster* grows fast from the embryo stage (going through three larval instars, L1-L3) to the size of an adult. Upon reaching the required size, larvae transform into an immobile pupal stage, associated with metamorphosis. Metamorphosis in all holometabolous insects is a dramatic process, during which the larval tissue organisation is replaced with the adult one. This means that almost all muscles are histolysed and most tissues replaced (Tissot and Stocker, 2000).

Two distinct times of neurogenesis can be distinguished in the life of *D. melanogaster* – the first occurs during embryonic development (at stage 9 of 17 embryonic stages; Doe, 1992), giving rise to neurons necessary for larval functions, which, to a large extent, are destroyed during metamorphosis; the second starts during larval stages and progresses to early metamorphosis and involves forming neurons necessary for adult life. A large number of adult neurons are derived from larval neurons after extensive modifications, which adds further complexity to the scope of changes in the nervous system during metamorphosis (reviewed in Truman, 1990; Truman *et al.*, 1993; Levene *et al.*, 1995). Therefore, understanding if CLK/CYC is important for the generation of adult behavioural rhythms during one or both periods of neurogenesis might help delineate its function.

As described in detail in the General Introduction (1.4.1), the molecular oscillator of adult flies consists of around 150 neurons (Figures 1.3 and 1.4). As briefly mentioned previously (1.5.4), circadian proteins have been detected in flies from the late embryonic stages (Liu *et al.*, 1988; Saez and Young, 1988; Zerr *et al.*, 1990; Ewer *et al.*, 1992; Frisch *et al.*, 1994; Kaneko *et al.*, 1997; Stanewsky *et al.*, 1997a; Kaneko and Hall, 2000). The first cells expressing PER appear in central ventral chord and in the brain as early as embryonic stage 12. At embryonic stage 16, CLK can be detected in some cells in the brain (Houl *et al.*, 2008). Around 6-8 hours later, the same cells start expressing PER (Houl *et al.*, 2008). This delay is consistent with the delay between *per* mRNA and protein accumulation in adults (Hardin *et al.*, 1990; Zerr *et al.*, 1990), suggesting that the appearance of CLK in these cells starts the assembly process for the molecular oscillator in these cells (Houl *et al.*, 2008). It is important to notice, that whereas *per* is reportedly expressed in a variety of different neurons, including ventral nerve chord (James *et al.*, 1986; Liu *et al.*,

1988), CLK expression is limited only to oscillator neurons (Houl *et al.*, 2008). Other neurons expressing *per* do so even in Clk^{Jrk} mutant flies, confirming that early PER expression does not depend on CLK (Houl *et al.*, 2008). Around the same time (embryonic stage 16-17) the synaptic activity in the central neurons develops (Baines and Bate, 1998), suggesting that the neuronal networks become functional at or shortly after that time (Houl *et al.*, 2008; Sehgal *et al.*, 1992).

Overall, in the brain of stage 16 embryos around 130 cells express *per*, around 160 express *tim* and 20 cells express both (Ruiz *et al.*, 2010). Location of these 20 cells is similar to the cells co-expressing *per* and *Clk* described by Houl *et al.* (2008) and it seems that these cells are the same circadian cells that can be detected during the larval stage (Kaneko *et al.*, 1997; Kaneko and Hall, 2000). It has been suggested that expression of *per* and *tim* in non-circadian neurons in embryos might indicate that they have other functions, possibly neuronal development (Ruiz *et al.*, 2010).

At the early larval stages three distinct groups of circadian neurons can be seen in each hemisphere of the brain – 4 small ventral lateral neurons (s-LN_vs), 2 dorsal neurons 2s (DN2s) and 2 dorsal neurons 1s (DN1s) (Kaneko *et al.*, 1997; Kaneko and Hall, 2000). CLK stays expressed in these cells from larval stage 1 and PER oscillates with a peak around ZT21, except in 2 DN2s, where it appears later than in other cells and remains in antiphase to the signal in s-LN_vs and DN1s, with the peak around ZT9 (Houl *et al.*, 2008). The LN_vs receive the information about the external time from the CRY protein and also from the input from the Bolwig's Organ and mediate the circadian sensitivity of the larval photophobic response (Kaneko *et al.*, 1997, 2000; Malpel *et al.*, 2002; Mazzoni *et al.*, 2005).

During later development additional circadian neurons can be observed (Helfrich-Förster, 2003). While some reports point to the decrease in the level of circadian proteins in the pupal stage (Kaneko *et al.*, 1997; Kaneko and Hall, 2000), RNA-Seq data from Flybase suggests that during metamorphosis the levels of *Clk* in the whole organism go slowly up until they reach stable levels in adults, while levels of *cyc* increase until third day post white pupae stage when they decrease and stabilize in or and later increase again in females (modENCODE Temporal Expression Data available on Flybase). It was also

recently showed that, in addition to oscillating in 4 s-LN_vs, 2 DN1s and 2 DN2s, both CLK and CYC are expressed in ~60 (per hemisphere) remaining circadian neurons (except l-LN_vs) at a constant level from mid- to late L3 larvae (Liu *et al.*, 2015). l-LN_vs start expressing CLK and CYC around the middle of metamorphosis (Liu *et al.*, 2015).

Four s-LN_vs neurons express PDF from the early larval stage (~6 hrs after hatching, 1st larval stage) all the way through adulthood and send their projections to the dorsal regions of the brain, terminating in the vicinity of DN1s and DN2s (Helfrich-Förster, 1995 and 1997, Figure 1.4). During metamorphosis (at 50% of completion), four to six larger cells (future l-LN_vs) start expressing PDF, arborize to the surface of the second optic neuropil, medulla and accessory medulla and send their projections contralaterally through the posterior optic tract (Helfrich-Förster, 1997, Figure 1.4).

5.2 Aim

The goal of this chapter was to uncover temporal and spatial requirements of the developmental CLK/CYC function. Understanding which circadian neurons require CLK/CYC presence during development and at which time is interesting on its own but it is also a good starting point for uncovering the role that CLK/CYC plays during development. My hypothesis was that CLK/CYC was required in PDF neurons from larval stages through metamorphosis, since these cells are responsible for circadian modulation of behaviour (Mazzoni *et al.*, 2005). Moreover, this is when flies can first be entrained by light pulses (Sehgal *et al.*, 1992) and when neurogenesis occurs (Doe, 1992), suggesting that this time could be connected to the formation of circadian oscillator. The idea that PDF neurons will be the most sensitive to CLK/CYC during development was supported by the fact that s-LN_vs are responsible for driving the locomotor behaviour of adult flies in DD (Renn *et al.*, 1999; Park *et al.*, 2000).

5.3 Protocols

5.3.1 Temporal analysis of the CLK/CYC requirement

Flies with conditional *per* over-expression or *cyc* rescue flies were allowed to lay eggs in vials at room temperature. After 3 days, some vials were left at room temperature while others were moved to 29˚C. Each of these two groups was further divided into 3 groups. For the first group, larvae at wandering larvae stage were selected and moved to opposite conditions. For the second group, early white pupae were selected and moved to opposite conditions. The third group was left in the original conditions for entire development (Figures 5.1E and 5.3 E). After flies eclosed, they were analysed either at 17˚C in LD cycles for 7 days and subsequently moved to constant darkness at 17˚C (for *per* over-expression) or entrained to LD cycles at 25˚C for 7 days and subsequently moved to 29˚C DD (for *cyc* rescue flies) as described in 2.3.1.

Flies were analysed as described in 2.3.2 and 2.3.3, using the first full seven days in DD for individual analyses of flies. As there were no obvious difference between flies moved at the wandering larvae and white pupae stages I decided to pool both groups together, creating only 4 distinct categories. The distribution of rhythmic, weakly rhythmic and arrhythmic flies between different categories was compared using the chi-square analysis for *per* over-expression flies (numbers of flies validated use of this method over Fisher's Exact test) or with pairwise Fisher's Exact 3x2 tests for *cyc* rescue flies (as numbers were too low for reliable chi-square analysis). The impact on the relative rhythmic power or period length by the developmental treatment of fly was determined using Welch's test with Games-Howell post-hoc analysis.

5.3.2 Monitoring the function of the circadian oscillators in the peripheral tissue

Parental flies were crossed at room temperature and removed after 3-4 days and vials with embryos and early larvae were divided into two subgroups: one was left at room temperature and the second was moved to restrictive conditions (29˚C). 3-4 days after eclosion flies were collected and tested in the

luciferase reporter assay as described in 2.5. Flies were first given 3-4 days of LD entrainment at permissive conditions (~17˚C) and subsequently analysed in 17˚C DD. Experimental flies with conditional *per* over-expression were compared to positive control flies over-expressing *per* constitutively (no tub_PGal80^{ts} element) and negative controls lacking the *tim-luc* construct.

5.3.3 Conditional *per* over-expression in restricted groups of cells

Developing flies were seeded at room temperature and then either kept at room temperature (permissive conditions) or moved to 29˚C (restrictive conditions). Young flies was entrained in 17˚C LD for eight days and subsequently released to and analysed in 17˚C DD as described in 2.3, using first full 7 days in the DD were used to determine the presence of rhythms using the chi-square periodograms.

Flies of the same gender and genotype but raised at different temperatures were compared to each other; to test for the correlation of the distribution of rhythmic, weakly rhythmic and arrhythmic flies with the developmental temperature Fisher's Exact 3x2 tests were used and the average period length and relative rhythmic power were compared using non-parametric Mann-Whitney tests.

5.3.4 *per* over-expression in all PDF-negative and CRY-negative neurons

Flies were raised either at 23˚C or 29˚C and as adults first entrained to LD cycles for seven days and then released into constant darkness where their behaviour was analysed as described in 2.3, Association between the presence of the *Gal80* element and the rhythmicity of flies was tested with the Fisher's Exact 2x3 test, comparing experimental flies with the *Gal80* element to negative controls not carrying one or to positive controls without the *tim(UAS)Gal4* driver (that should have normal levels of PER and intact behavioural rhythmicity; *w;If/CyO;UASper24/X-Gal80*, where *X* is either *Pdf or cry*). Period length and relative rhythmic power were compared using non-parametric Kruskal-Wallis tests.

5.3.5 Constitutive *per* over-expression in restricted groups of cells

Flies were raised at either 23˚C or 29˚C and their locomotor behaviour was tested couple days after their eclosion as described in 2.3. Flies were first entrained to LD cycles at either 23˚C, 25˚C or 29˚C for 5-11 days and then released into either 23˚C or 29˚C DD. The presence of locomotor rhythms was analysed as described in 2.3 using first full seven days in DD. Association between the presence of the rhythmicity of flies and the pattern of *per* over-expression was tested with the Fisher's Exact 3x2 test, comparing flies to the controls used (line that showed the weakest phenotype for the preliminary experiment (Figure 5.9) or true control (Figures 5.10 and 5.11) and also comparing flies with the *per* over-expression in the s-LN_vs (*R6-Gal4>per*) and l-LN_vs (*c929-Gal4>per*). Period length and relative rhythmic power were compared using non-parametric Kruskal-Wallis test, running pairwise Wilcoxon Rank tests between all lines and then selecting comparisons of control flies to other lines and comparison of *R6-Gal4>per* to *c929-Gal4>per*.

5.4 Results

I received some help with collecting the data for this chapter from a group of talented undergraduate students who I had a pleasure to supervise. Virginia Baker helped generate flies and perform the experiments presented in 5.3.3, Gloribell Bonilla contributed greatly to the experiments presented in 5.3.1 and Albert Nieh helped gather data for preliminary experiment in 5.3.5. In addition, data presented in A.3.5 was collected and analysed by Jake Currie.

5.4.1 CLK/CYC function is required during metamorphosis for establishing adult behavioural rhythmicity

Previous experiments described in the Chapter 4 were based on flies with CLK/CYC function impairment either by inhibition of CLK/CYC via PER over-expression or knockdown of *cyc* during development from the first/early second larval stage (flies were always seeded at room temperature and moved

to permissive or restrictive conditions within a week, most often after 3 or 4 days, after the parents were transferred to a fresh vial). It was interesting therefore to check if within the whole development certain periods were more sensitive to CLK/CYC presence than others. To test this, I performed a simple experiment as described in 5.3.1, raising flies in various developmental protocols combining permissive and restrictive conditions and testing flies in permissive conditions (Figure 5.1 E).

The rhythmicity of flies spending their entire development in either permissive (23˚C) or restrictive (29˚C) conditions supported the results presented in Chapter 4 (Figure 4.1), with no rhythmic flies among both female and male flies raised under restrictive conditions and strong rhythmicity of flies raised under permissive conditions (Figure 5.1 A - D). Statistical analysis revealed a strong association between the developmental conditions experienced by fly and both the distribution of the rhythmicity (Chi-square test: df=6, $p=10^{-23}$, n=166 for females; df=6, $p=10^{-13}$, n=130 for males) and the relative rhythmic power (Welch's test: F(3, 43.098)=50.116, $p=10^{-13}$, n=97 for females; F(3, 30.638)=31.769, $p=10^{-8}$, n=81 for males). Interestingly, flies that were moved to permissive conditions after reaching the wandering larvae/white pupae stage were almost indistinguishable from flies that were in permissive conditions for the entire development (no statistical difference between both groups for either relative rhythmic power or the rhythmicity of the population), suggesting that PER expression levels during metamorphosis determine the behavioural phenotype of flies. Similarly, flies moved to restrictive conditions around the beginning of the metamorphosis were not significantly different form flies that were in restrictive conditions for the entire time.

In a complementary experiment (Figure 5.2), flies raised under the same conditions as described above were analysed first at restrictive conditions of 29˚C and subsequently moved to 17˚C LD for 7 days for entrainment and finally released at 17˚C DD. Data was analysed in the same way as described in 5.3.1.

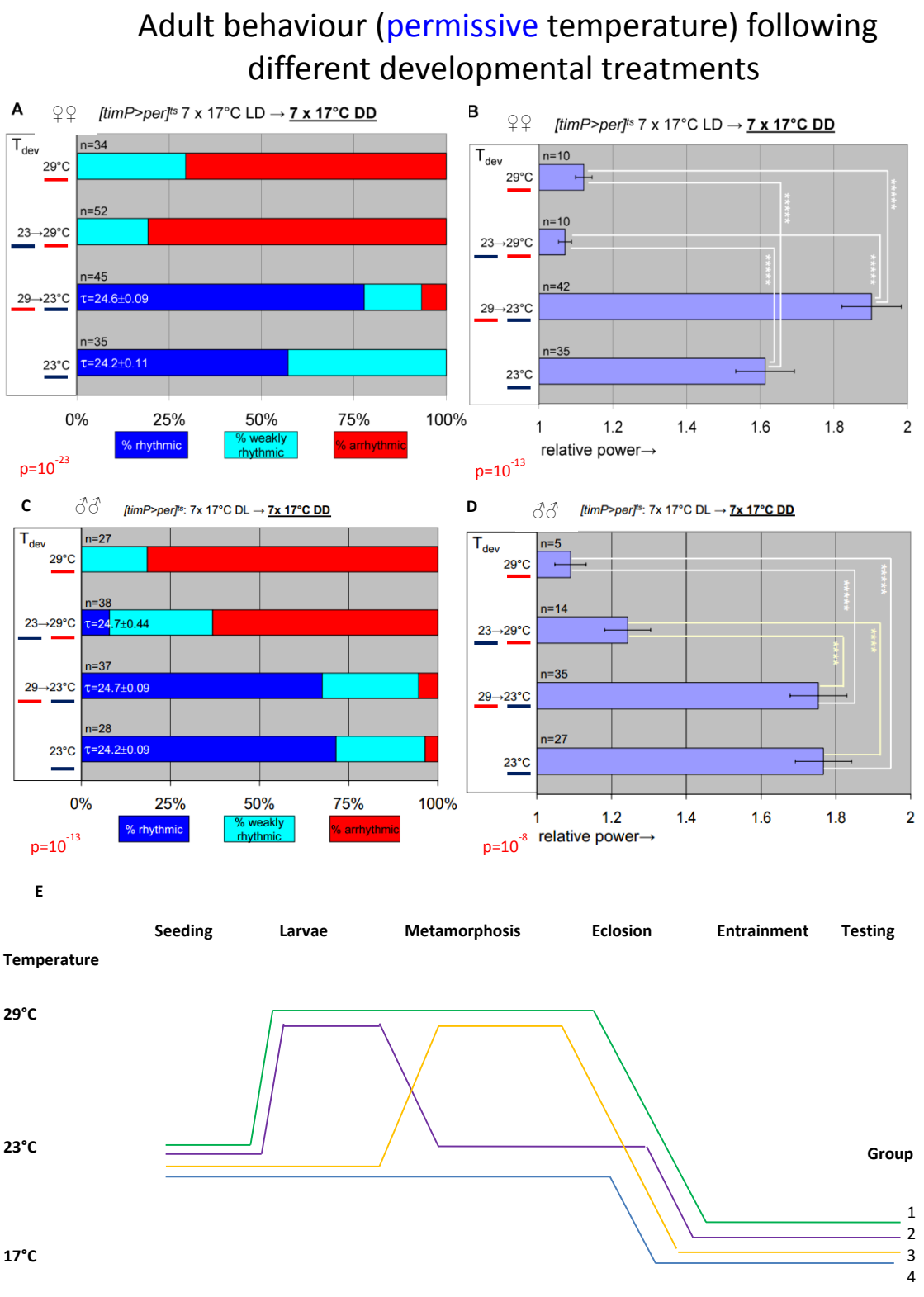


Figure 5.1. PER over-expression during metamorphosis in all clock-bearing cells caused adult behavioural arrhythmia (A-D adapted from

Goda, Mirowska, Currie *et al.*, 2011; data partially collected by Gloribell Bonilla).

Analysis of the behavioural rhythms of adult flies with conditional *per* over-expression (denoted here as *[timP>per]*^{ts}) tested at permissive conditions (17˚C DD after a week of entrainment to 17˚C LD) revealed that CLK/CYC inhibition prior to metamorphosis (29 > 23˚C) did not impact the rhythmicity of flies but inhibition during metamorphosis (23 > 29˚C) did. Experimental protocols are schematically presented in (E) and in (A-D) red or blue lines were used to mark restrictive and permissive conditions respectively. On the left, stacked bar diagrams show the distribution of rhythmic, weakly rhythmic and arrhythmic females (A) and males (C) in the population in permissive conditions. Numbers of flies analysed and the period length (±SEM) of rhythmic flies are included on the graph. Groups were compared with chi-square analysis. On the right, bar charts show the relative rhythmic power of all females (B) and males (D) displaying behavioural rhythms. Numbers of flies are included on the graphs and error bars represent SEM. Flies were compared with the Welch's test with post-hoc Games-Howell analysis. **** $p<10^{-4}$, ***** $p<10^{-5}$.

At restrictive conditions of 29˚C DD (Figure 5.2 A and B) there was no difference between the distributions of female flies based on their rhythmicity following different raising protocols, with the large majority of females not displaying any significant rhythms. Possibly due to higher levels of *Gal80*^{ts} expression in males (on account of the fact that the transgene is inserted into X chromosome) there was a highly significant association between the developmental conditions and the rhythmicity of flies (Chi-square test: df=6, $p<10^{-5}$, n=106), with flies raised at permissive temperatures exhibiting long-period rhythms. These longer than wild-type rhythms suggested that the function of circadian oscillator was affected by adult over-expression of *per* but the lower levels of over-expression in males due to higher levels of GAL80^{ts} persisting from the development at room temperature were not sufficient to cause arrhythmicity. This elongation of rhythms observed in adult-only

exposure to restrictive conditions also nicely mirrored the results presented in Chapter 4 (Figure 4.1).

Upon transfer to free-running permissive conditions (Figure 5.2 C-F), exactly the same pattern was found as observed in flies with no prior adult exposure to restrictive conditions (Figure 5.1). A highly significant association between developmental treatment and rhythmicity was discovered for both females (Chi-square test: df=6, $p<10^{-9}$, n= 131) and males (Chi-square test: df=6, $p<10^{-5}$, n=93). Rhythmic flies exposed to permissive conditions during metamorphosis displayed period lengths close to 24 hrs. Additionally, the distribution of relative rhythmic power of rhythmic and weakly rhythmic flies confirmed the association between the developmental conditions and the robustness of rhythmicity (Welch's test: F(3, 21.328)=13.748, $p<10^{-4}$, n=62 for females and F(3, 18.795)=13.731, $p<10^{-4}$, n=46). Based on all of these results I concluded that the CLK/CYC function is required during metamorphosis for establishing adult locomotor rhythmicity and its function prior to the metamorphosis may not be necessary for establishing rhythms in the behaviour.

To confirm that CLK/CYC activity is required during metamorphosis for adult circadian behaviour, I also tested flies with conditional *cyc* rescue as described in 5.3.1. Flies were again raised using combination of restrictive and permissive conditions and tested in permissive conditions (Figure 5.3 E), but this time 29°C was permissive temperature and room temperature was restrictive condition.

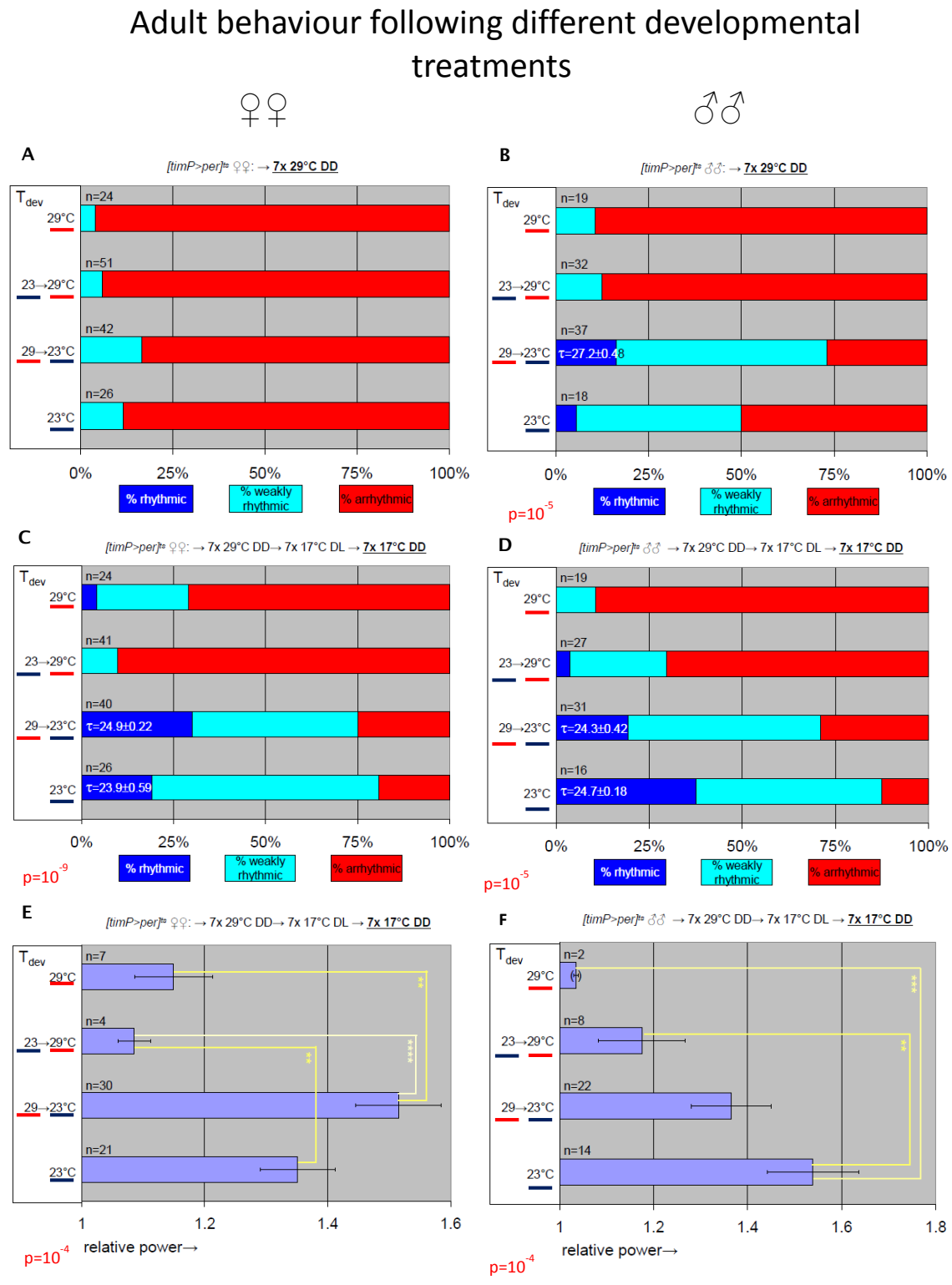


Figure 5.2. Analysis of the adult behaviour of flies with PER over-expression during metamorphosis in all clock-bearing cells (figure adapted from Goda, Mirowska, Currie *et al.*, 2011; data partially collected by Gloribell Bonilla).

Analysis of behavioural rhythms of adult flies with conditional *per* over-expression (denoted here as *[timP>per]*^{ts}) tested at restrictive conditions (29˚C DD, A-B) and subsequent permissive conditions (17˚C DD after an additional week of entrainment to 17˚C LD, C-F) confirmed that CLK/CYC inhibition during metamorphosis (23 > 29˚C) caused a decrease in adult rhythmicity while CLK/CYC inhibition prior to metamorphosis (29 > 23˚C) did not produce the same phenotype. (A-B) Stacked bar diagrams showing the distribution of rhythmic, weakly rhythmic and arrhythmic females (A) and males (B) in the population in restrictive conditions. Groups were compared with chi-square analysis. (C-F) Diagrams showing the distribution of rhythmic, weakly rhythmic and arrhythmic flies in the population (C and D) and bar charts showing the relative rhythmic power of all females (E) and males (F) displaying behavioural rhythms. Numbers of flies are included on the graphs and error bars represent SEM. Distribution of flies in the population was compared with the chi-square analysis. Average relative rhythmic power and period length were compared with the Welch's test with post-hoc Games-Howell analysis. **** $p<10^{-4}$, ***** $p<10^{-5}$.Addition of adult *per* over-expression did not change the overall conclusion that developmental *per* over-expression during metamorphosis corresponds with adult behavioural arrhythmicity. ** $p<10^{-2}$, *** $p<10^{-3}$, **** $p<10^{-4}$, ***** $p<10^{-5}$.

Adult behaviour (permissive temperature) following different developmental treatments

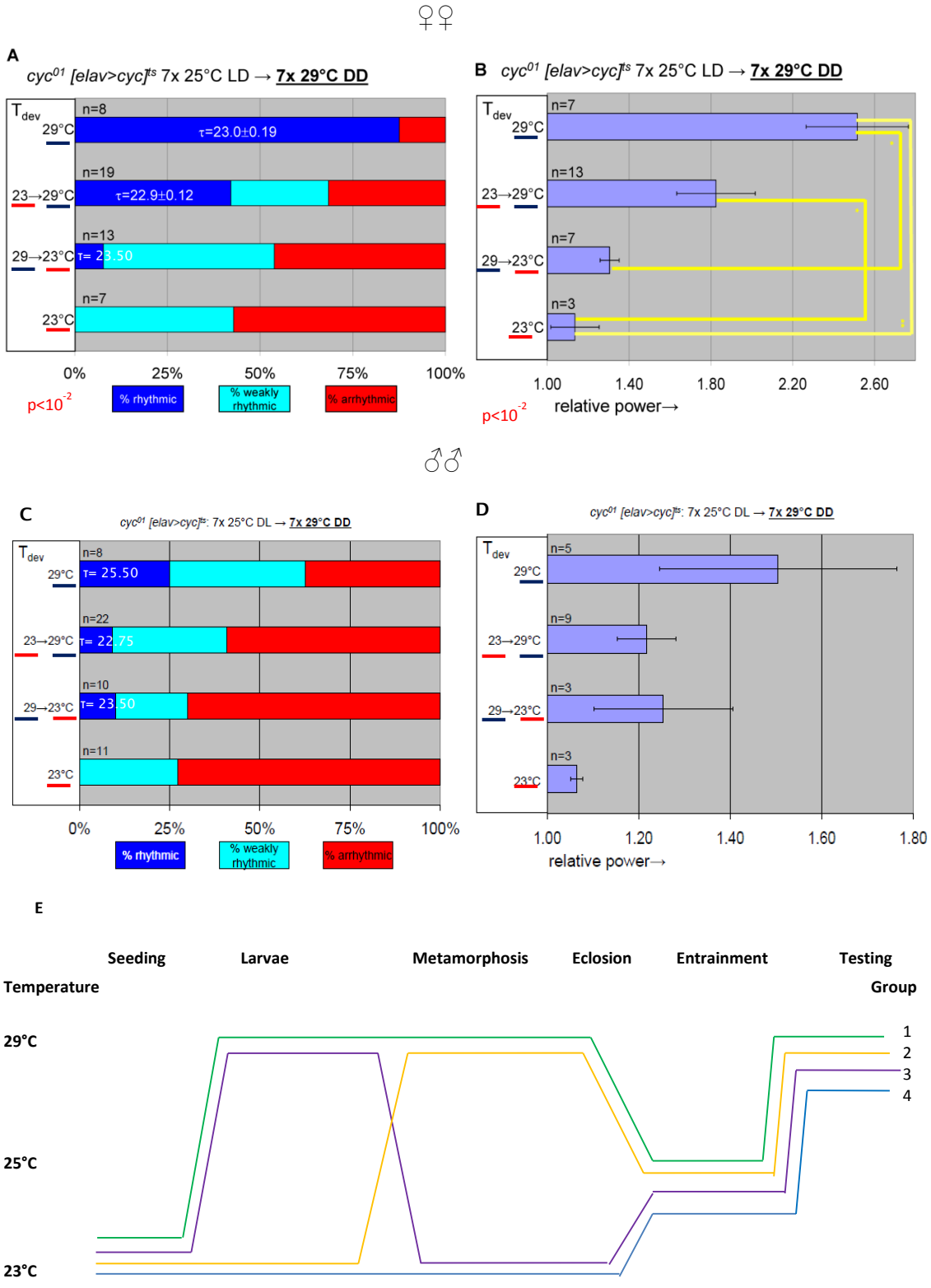


Figure 5.3. CLK/CYC depletion during metamorphosis in all clock-bearing cells caused adult behavioural arrhythmia (A-D adapted from

Goda, Mirowska, Currie *et al.*, 2011; data partially collected by Gloribell Bonilla).

Analysis of behavioural rhythms of adult flies with conditional *cyc* rescue (denoted here as $cyc^{01}[elav>cyc]^{ts}$) tested at permissive conditions (29˚C DD after a week of entrainment to 25˚C LD) confirmed that CLK/CYC activity is required during metamorphosis (23˚C and 29 > 23˚C) but not during the prior development (29˚C and 23 > 29˚C). Experimental protocols are schematically presented in (E) and in (A-D) red or blue lines were used to mark restrictive and permissive conditions respectively. On the left, stacked bar diagrams show the distribution of rhythmic, weakly rhythmic and arrhythmic females (A) and males (C) in the population in permissive conditions. Numbers of flies analysed and the period length (±SEM) of rhythmic flies are included on the graph. Groups were compared with pairwise Fisher's Exact 2x3. On the right, bar charts show the relative rhythmic power of all females (B) and males (D) displaying behavioural rhythms. Numbers of flies are included on the graphs and error bars represent SEM. Flies were compared with the Welch's test with post-hoc Games-Howell analysis. * $p<0.05$, ** $p<0.01$.

The changes in the distribution of the rhythmicity of the population of *cyc* rescue flies (Figure 5.3) were more gradual, but there was still an association between the developmental treatment and rhythmicity that was statistically significant in females (lowest value from pairwise Fisher's Exact 3x2 tests: $p<10^{-2,}$ n=47) but not in males (lowest value from pairwise Fisher's Exact 3x2 tests $p>0.158$, n=51), possibly due to low numbers. Analysis of flies kept in permissive and restrictive conditions for the entire development mirrored the data presented in the Chapter 4 (Figure 4.2), with the majority of flies raised under permissive condition displaying very strong rhythms and only weakly rhythmic and arrhythmic flies among flies raised under restrictive conditions. Flies that were moved from permissive to restrictive conditions at the beginning of the metamorphosis had only marginally better rhythmicity than flies kept in restrictive conditions throughout development. Flies that spent only their pre-metamorphosis development in restrictive conditions had

slightly impaired rhythmicity when compared to flies raised under permissive conditions, however the difference between the two was not statistically significant. Relative rhythmic power of rhythmic and weakly rhythmic flies nicely mirrored the trends observed in the distribution of flies of different rhythmicity, with a significant association between the developmental treatment and the power of rhythm for females (Welch's test: $F(3, 8.017)=9.145$, $p<10^{-2}$, $n=30$) but again same trend but no significant association for males (Welch's test: $F(3, 5.822)=2.610$, $p=0.15$, $n=20$), again, possibly due to low n. The apparent difference in the significance between males and females could be possibly explained by the small differences in the levels of *cyc* expression resulting from the insertion of the *Gal4* transgene on the X chromosome. Overall, data obtained supported my conclusions that CLK/CYC function during metamorphosis is necessary for establishing adult behavioural rhythmicity.

5.4.2 Peripheral clocks are not affected by developmental *per* over-expression with *tim(UAS)Gal4* but become disrupted with stronger developmental over-expression

As mentioned in the General Introduction (1.3.3), multiple tissues in *D. melanogaster* express oscillating clock proteins and regulate some physiological functions in a circadian manner (Plautz *et al.*, 1997a). It was therefore interesting to test whether inhibition of CLK/CYC function during development irreversibly affected the function of these peripheral oscillators to the same extent as it affected the molecular oscillator in the brain (which gives rise to the behavioural rhythmicity).

To monitor gene expression oscillations in vivo I used a luciferase reporter system. I introduced a transgenic *luciferase* reporter gene under control of a *tim* promoter element (Stanewsky *et al.*, 1998) into two lines of flies with conditional *per* over-expression: one with the *tim(UAS)-Gal4* driver (previously described *per* over-expressing line, Figure 4.1) and a second with *tim62-Gal4* driver (Kaneko *et al.*, 2000b) (experimental flies were heterogenous for *tim62-Gal4* driver as when they were crossed to flies that did not have the *tim62-Gal4* transgene on the second chromosome, but rather *tim(UAS)Gal4/CyO*, so flies

with *CyO* had be used for experiment), both directing transgenic *per* expression into clock cells. The resulting flies (*y w tim-luc/y w* tub_PGal80^{ts}*;tim(UAS)-Gal4; UASper* or *y w tim-luc/y w* tub_PGal80^{ts}*;tim62-Gal4/CyO; UASper)* expressed luciferase in the spatiotemporal pattern dictated by *tim* gene expression. This method allowed me to monitor expressions in peripheral tissues rather than the brain because when the luciferase signal is collected from the whole fly, most of it originates in the 'periphery' (compound eyes, proboscis, antennae, thorax, abdomen, legs and even wings) (Plautz *et al.*, 1997a and 1997b).

Since both the *tim-luc* element and tub_PGal80^{ts} transgene were located on the X chromosome, only female progeny could be used for the experiment. However, since these flies were a result of a cross, it was possible to recover also positive control flies expressing *tim-luc* and over-expressing *per* in a conditional way (*y w tim-luc/FM7c;tim(UAS)-Gal4; UASper* or *y w tim-luc/FM7c;tim62-Gal4; UASper*). Behavioural analysis of experimental flies with conditional *per* over-expression revealed that presence of *tim-luc* did not impact the adult arrhythmicity of flies raised under restrictive conditions and analysed at permissive (Figure A.3.1). Although this was tested only for flies with *tim(UAS)Gal4* (as not enough flies with *tim62-Gal4* were recovered), it is unlikely that it would be different for flies with *tim62-Gal4*. Moreover, analysis of behaviour of flies with *tim62-Gal4* – driven *per* over-expression revealed that there was no difference between flies with different *Gal4* drivers (Figure A.3.1).

Adult peripheral molecular oscillations following developmental *per* over-expression (permissive temperature) *tim-luc in vivo* luciferase assay

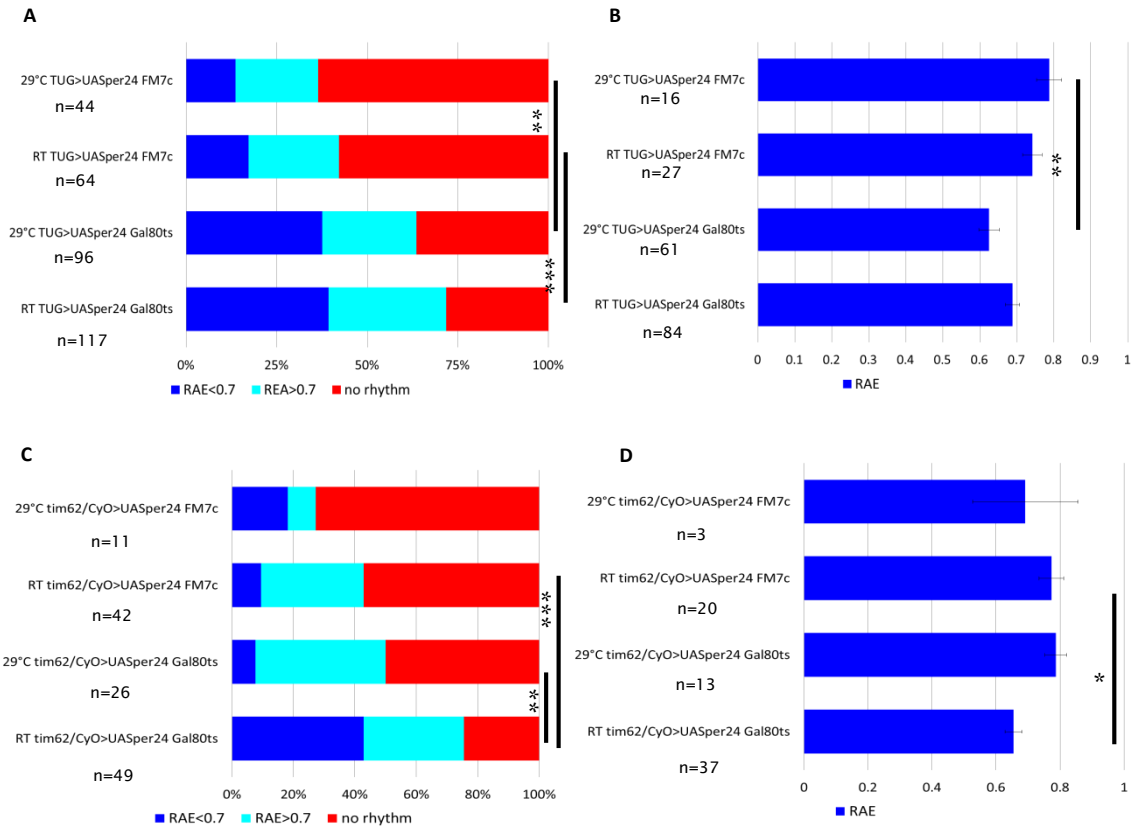


Figure 5.4. Developmental *per* over-expression can impact molecular oscillations across whole adult flies – results from *tim-luc in vivo* luciferase assay.

(A and C) Stacked bar diagrams representing the prevalence of flies with rhythmic (RAE<0.7), weakly rhythmic (RAE>0.7) and arrhythmic *tim* oscillations with the numbers of flies tested. Associations between either developmental conditions or genotype and distribution of flies were tested with pairwise Fisher's Exact 2x3 tests. (B and D) Average RAE of flies displaying any rhythms in the *tim* oscillations with the numbers of flies and error bars representing SEM included. Flies were compared with non-parametric Man-Whitney test. * p<0.05, ** p<0.01, *** p<0.001.

Experimental (conditional *per* over-expression) and control flies (constitutive *per* over-expression) were raised either under permissive or restrictive conditions and analysed at permissive conditions as described in 5.3.2. Flies with constitutive *per* over-expression driven by either *Gal4* transgene (two top rows in Figure 5.4 A and C) exhibited disruption of circadian gene expression, as indicated by the large percentage of flies with the arrhythmic luciferase activity. Moreover, there was no significant difference between the relative numbers of flies with rhythmic, weakly rhythmic and arrhythmic luminescence raised at different temperatures. Thus, I concluded that temperature experienced by flies during development (and hence *per* levels during development) apparently did not impact the adult circadian expression profiles of flies.

There was also no statistical difference between flies with or without *tim(UAS)Gal4* - driven PER over-expression during development (two bottom rows in Figure 5.4 A and B; Fisher's Exact 323 test: $p=0.414$, $n=213$ for the distribution of flies). Moreover, for both temperatures flies with conditional *per* over-expression were clearly different than flies with constitutive *per* over-expression (Figure 5.4 A; Fisher's Exact 3x2 test: $p<0.01$, $n=140$ for 29˚C raised flies; $p<0.001$, $n=181$ for RT raised flies; Mann – Whitney test on RAE: $U=703.500$, $z=2.706$, $p<0.01$, $n=77$ for flies raised at 29˚C).). Based on these experiments I hypothesised that the developmental PER over-expression did not disrupt the circadian gene expression pattern in the periphery in adult flies.

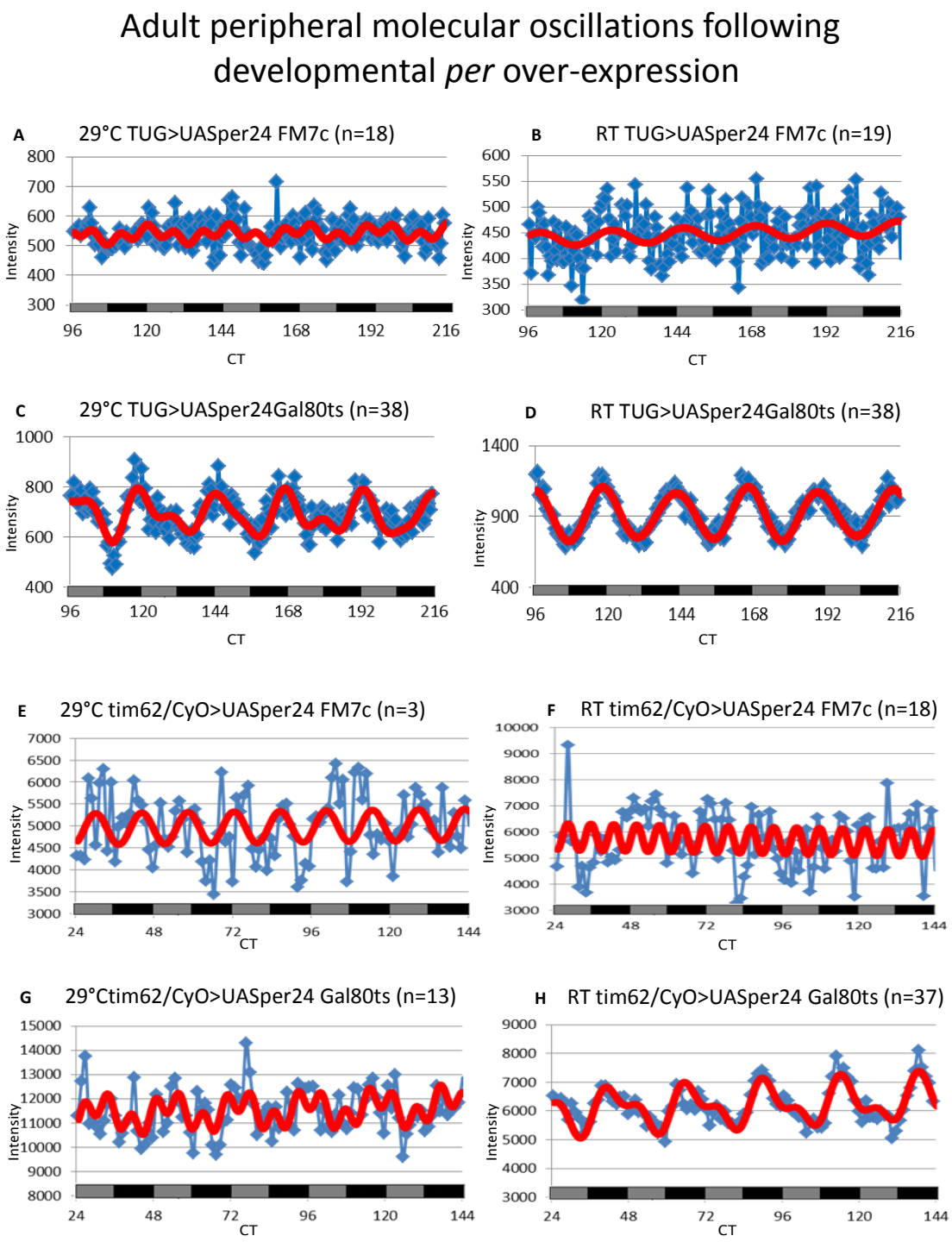


Figure 5.5. Sample traces of *tim* oscillations over 120 hours in DD.

Detrended and normalized median *tim* oscillation profiles for flies with conditional (Gal80ts) (C-D and G-H) and constitutive (FM7c) (A-B and E-F) *per* over-expression in all clock neurons, driven by *tim(UAS)-Gal4* (TUG) (A-D) and *tim62-Gal4* (E-H). Red lines represent

lines fitted with the Fourier Transform analysis. X axis: CT time over 5 consecutive days of testing with the grey and black bars marking the relative day and night portions. Y axis: intensity of luciferase signal.

Results for *tim62-Gal4*-driven *per* over-expression (Figure 5.4 C and D) were not following the same pattern (as in Figure 5.4 A and B). In case of flies raised at room temperature, there was however a highly significant association between rhythmicity of *tim* expression and genotype (Figure 5.4 C, constitutive or conditional *per* over-expression; Fisher's Exact 3x2 test: $p<10^{-3}$, n=91) accompanied by an association of RAE with the genotype (Figure 5.4 D, Mann-Whitney test: U=469.000, z= 2.440, $p<0.05$, n=57). However, for flies raised at 29°C there was no association between either rhythmicity of *tim* expression or RAE and the genotype. Additionally, flies with conditional *per* over-expression showed an association of developmental temperature and rhythmicity of *tim* expression (Fisher's Exact 3x2 test: $p<0.01$, n=75), but not for RAE (Figure 5.4 C and D).

The difference between the data obtained in this experiment with different *Gal4* drivers might suggest that the *Gal4* transgenes cause various levels of GAL4 expression (and consequently PER over-expression). Despite many reports of using *tim(UAS)Gal4* to target gene expression to peripheral tissues (Rush *et al.*, 2006), one report has suggested that *tim(UAS)Gal4* drives GAL4 expression rather weakly in photoreceptors (Sathyanarayanan *et al.*, 2004), which might suggest that the strength of GAL4 expression (and consequently PER over-expression) in the peripheral tissues may affect the function of molecular oscillator in peripheral clocks.

Taken together, these results (Figure 5.4) suggested that a stronger over-expression of *per* in the peripheral tissue during development could cause irreversible impairment to the function of the molecular oscillators in these tissues in adulthood. Therefore peripheral tissues also appeared sensitive to developmental CLK/CYC function, however to a lesser extent than the main molecular oscillator in the brain.

5.4.3 CLK/CYC is required in PDF-positive neurons during development.

In the previous experiments I demonstrated that CLK/CYC was required during metamorphosis for establishing adult behavioural rhythmicity (Figures 5.1 - 5.3). What remained to be uncovered was in which clock neurons this function of CLK/CYC was necessary. Since all circadian neurons are either present or being formed (l-LN$_v$s) during metamorphosis (Liu *et al.*, 2015, no group could be excluded from the study. The spatial characterization of the developmental CLK/CYC requirement is the aim of the next sections presented in this chapter.

Since the previously described line with conditional *per* over-expression caused CLK/CYC inhibition in all clock-bearing cells (Figure 4.1), the approach chosen was to generate two new transgenic lines with spatially restricted patterns of the *per* over-expression:

- *Pdf-Gal4>per* over-expression, where CLK/CYC inhibition occurs only in 18 ventrolateral neurons expressing PDF (but not in the PDF-negative 5th s-LN$_v$) (Renn *et al.*, 1999)
- *Mai179-Gal4>per* over-expression targets all s-LN$_v$s, including the PDF-negative 5th, 3 LN$_d$s and possibly one l-LN$_v$ (Grima *et al.*, 2004)

To check if developmental *per* over-expression in the more restricted spatial pattern could also affect adult locomotor rhythmicity, flies from these lines were prepared, raised under permissive or restrictive conditions and analysed in permissive conditions as described in 5.3.3. Qualitative analysis of the actograms of flies with developmental *per* over-expression in PDF neurons (Figure 5.6 A) revealed a difference between flies raised at different temperatures. Female flies raised at RT displayed strong behavioural rhythms, however its period length drastically changed after about a week at DD (Figures 5.6 A and A.3.2 A). While periodograms of average male showed an overall decrease of rhythmicity, average female flies raised at 29˚C displayed complex rhythmic pattern (Figures 5.6 A and A.3.2 A). Surprisingly, it was not a result of different sub-populations of flies each displaying different period length since many individual flies were showing more than one peak. To analyse the complexity of rhythmicity additional analysis was carried (Figure A.3.2B).

As can be seen on Figure 5.6 B, for both female and male *pdf-Gal4>per* over-expression flies a there was an association between the developmental temperature and the adult behavioural rhythmicity of flies (Fisher's Exact 3x2 test: $p=0.001$, $n=35$ for females, $p<0.001$, $n=29$ for males). Similar to the results obtained previously with the *tim(UAS)Gal4* (Figure 4.1), flies raised with normal *per* levels in PDF neurons during development displayed strong behavioural rhythms as adults in permissive conditions while flies with developmental over-expression of *per* in the PDF neurons were mainly arhhythmic or weakly rhythmic (Figure 5.6 A and B). More of female than male flies displayed weak behavioural rhythms, however, as visible on the actogram depicting the activity of the average female (Figure 5.6 A), these flies were actually displaying split rhythms with two different period lengths (since for the most of flies the period length from the strongest rhythmic component was closer to the wild-type, there was no significant difference between the period lengths value of flies raised at different temperatures). The mean relative rhythmic power of female flies raised at 29˚C was also significantly lower than the relative rhythmic power of females raised at room temperature (Mann-Whitney test: $U=44.000$, $z=-2.472$, $p<0.05$, $n=29$), suggesting that the robustness of the rhythms was decreased for all flies (Figure 5.6 B). The same trend existed for males, however, due to a much smaller number of rhythmic and weakly rhythmic flies the difference was not statistically significant. There was also an association between the period length and the developmental temperature for male flies (Mann-Whitney test: $U=24.000$, $z=2.438$, $p<0.05$, $n=14$).

More detailed analysis (Appendix, A.3.2) revealed that the change in the period length observed for flies raised at RT might be a result of decrease in the number of rhythmic flies displaying circadian period length and instead more visible component from flies with weak rhythms displaying shorter period length (Figure A.3.2 B). Moreover, I was able to confirm that for both females (more prominent) and males (less prominent) raised at 29˚C there were three components to the rhythm: one with a period in a circadian range, one around 20-22 hrs and one over 26 hours. This complexity of rhythm was less visible during the second week in DD.

Another experiment was designed to test the effect of either just adult or both adult and developmental l *per* over-expression with the constructs used and the influence of this additional adult *per* over-expression on the behavioural rhythmicity in permissive conditions (Figure 5.6 C and D). To do so, flies were first loaded into 29˚C DD (since this was conducted in the light, all flies should be synchronised to the same phase) for a full seven (for *Mai179-Gal4> per* over-expression) or eight (*Pdf-Gal4> per* over-expression) days, and were subsequently moved to 17˚C LD for 7 days for entrainment and then to 17˚C DD. Flies were analysed in the same way as described in 5.3.3, with separate analyses conducted for 29˚C DD and 17˚C DD.

When analysed at 29˚C, it could be clearly noticed that regardless of the developmental temperature, the rhythmicity of *Pdf-Gal4>per* over-expression flies was impaired to some extent (Figure 5.6 C). It was surprising though that in the case of flies raised with normal *per* levels, a large proportion of flies remained rhythmic and only the addition of developmental *per* over-expression in PDF neurons was sufficient to reduce the rhythmicity of flies (there was an association between the developmental temperature and the rhythmicity for females with Fisher's Exact 3x2 test: $p<0.05$, $n=37$ but despite the same trend, no correlation was observed for males, with Fisher's Exact 2x3 test: $p=0.144$, $n=27$). Analysis of the mean relative rhythmic power further confirmed the impact of developmental *per* over-expression in the PDF neurons, with both females and males displaying an association between the developmental temperature and relative rhythmic power (Mann-Whitney test: $U=14.000$, $z=-2.340$, $p<0.05$, $n=20$ for females; $U=10.000$, $z=-2.312$, $p<0.05$, $n=15$ for males).

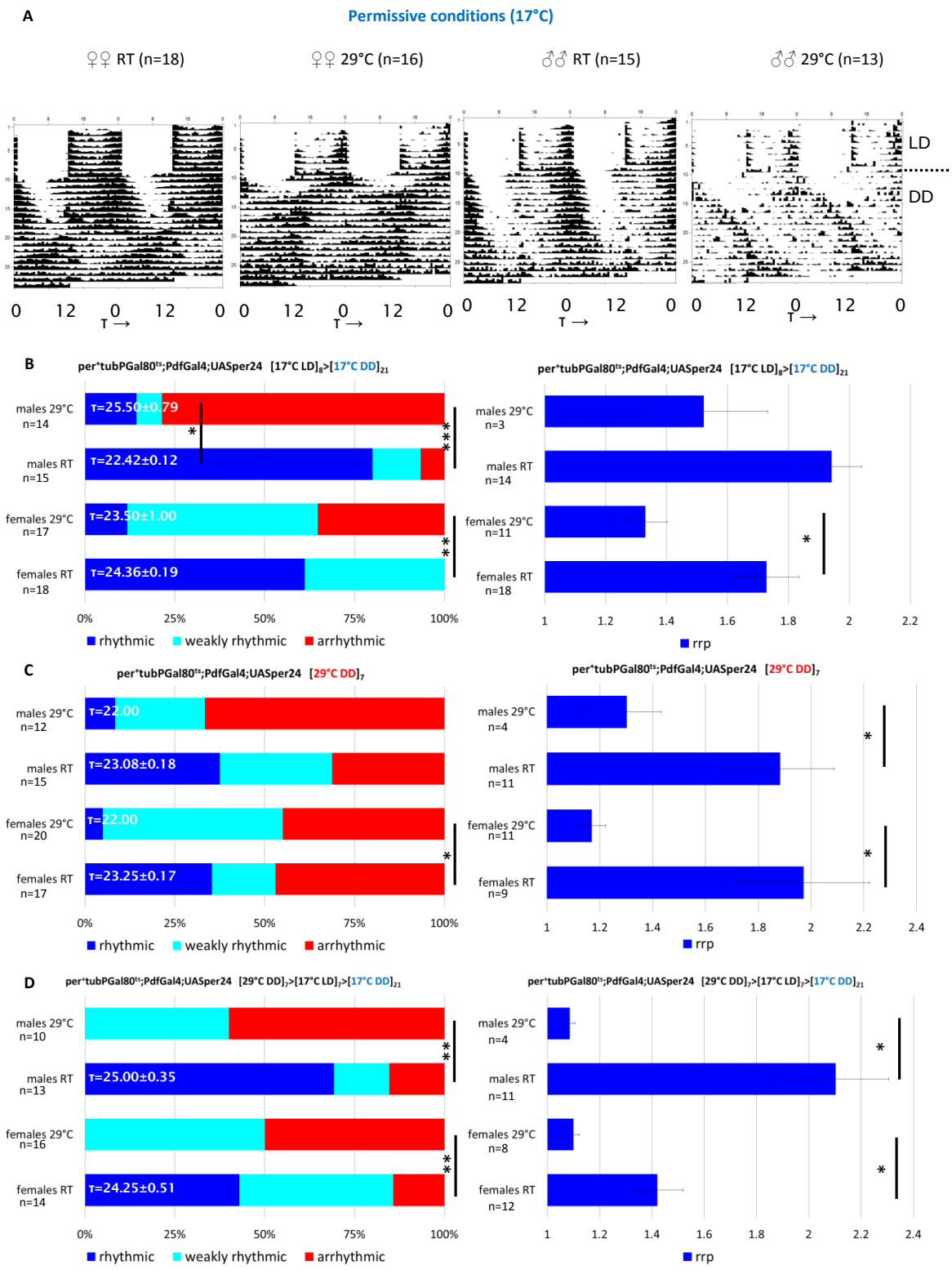


Figure 5.6. Developmental *per* over-expression in PDF neurons caused an irreversible decrease in adult behavioural rhythmicity (data partially collected by Virginia Baker).

Flies with conditional expression of transgenic *per* driven by *Pdf-Gal4* (raised at 29˚C) were significantly less rhythmic than flies with the same genotype raised at room temperature, with reduced transgenic *per* expression. (A) Average double-plotted actograms (top) and periodograms (bottom). Raising temperature and number of flies are indicated. Period length estimated from the average actograms is included on periodograms. (B-D) Quantification of rhythmicity of individual flies in multiple conditions. On the left, stacked bar diagrams represent the percentage of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies analysed and the period length (±SEM) of rhythmic flies are included on the graph. Flies raised at different temperatures were compared with Fisher's Exact 2x3 tests (males and females separately) and the results are indicated next to graphs. Period lengths of rhythmic flies were compared with non-parametric Mann-Whitney tests and are indicated next to values. On the right, bar diagrams show the average relative rhythmic power of flies displaying behavioural rhythms. Numbers of flies are included on the graphs and error bars represent SEM. Flies raised at different temperatures were compared with the non-parametric Mann-Whitney test. When analysed at permissive temperature (B and D), a clear difference between flies raised at RT and 29˚C could be observed, with the latter far less rhythmic. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Upon transfer to permissive conditions, robust behavioural rhythmicity was restored for *Pdf-Gal4>per* over-expression flies raised at room temperature, confirming that impairment of rhythmicity associated with adult *per* over-expression in PDF neurons was reversible (Figure 5.6 D). On the other hand, flies that were raised at 29˚C and additionally exposed to 29˚C as adults displayed a further reduction of rhythmicity, with no rhythmic flies recovered (Figure 5.6 D). There was a significant association between the developmental temperature and both the distribution of rhythmic, weakly rhythmic and arrhythmic flies (Fisher's Exact 3x2 test: $p<0.01$, $n=30$ for females; $p<0.01$, $n=23$ for males) and the relative rhythmic power (Mann-Whitney test:

U=21.000, z=-2.083, p<0.05, n=20 for females; U=0.000, z=-2.872, p<0.01, n=15 for males).

Flies with developmental *per* over-expression driven by *Mai179-Gal4* did not show irreversible adult behavioural rhythmicity comparable to the one observed with *tim(UAS)-Gal4* or *Pdf-Gal4* (Figures A.3.3 and A.3.4). Moreover, even when analysed in restrictive temperature, majority of flies still displayed locomotor rhythms (Figures A.3.3 and A.3.4). It is, however, unknown whether this represents lack of developmental sensitivity of the neurons targeted by *Mai179-Gal4* to CLK/CYC inhibition or is caused by low levels of *per* expression achieved with the use of that *Gal4* driver.

5.4.4 CLK/CYC function in PDF negative neurons is not required for adult behavioural rhythmicity

Having shown that *per* over-expression in PDF neurons during metamorphosis leads to irreversible adult arrhythmicity (Figure 5.6), I wanted to investigate if the developmental PER over-expression in all PDF-negative neurons affected adult behavioural rhythmicity. Additionally, I also tested the effect of the developmental PER over-expression in all CRY-negative neurons. In order to do this, I generated conditional *per* over-expression flies which, in addition to all genetic elements in a conditional *per* over-expression line, carried *Pdf-Gal80* (on the second chromosome, so flies were heterozygous for *tim(UAS)Gal4*) or *cry-Gal80* (on third chromosome, so flies were heterozygous for *UAS-per24*) constructs described previously (Park *et al.*, 2000; Emery *et al.*, 2000b).

My results (Figure A.3.5) showed that the addition of either *Pdf-Gal80* or *cry-Gal80* to flies with conditional *per* over-expression did not rescue adult locomotor rhythmicity. The easiest explanation could be that the developmental *per* over-expression in PDF-negative or CRY-negative neurons was detrimental to adult behavioural rhythmicity. However, since my results indicated that GAL80 expression in addition to expression of $GAL80^{ts}$ did not further modify behaviour, alternative explanation can be suggested. In PDF or CRY neurons of the lines tested two different forms of GAL80 protein were present – normal GAL80 and the temperature-sensitive version $GAL80^{ts}$. No biochemical studies were ever carried on what happens in the cell when both

forms are present, however anecdotal evidence seemed to be pointing to the temperature sensitive version of GAL80^{ts} outcompeting the GAL80. Therefore I assumed that *per* over-expression was never really specifically blocked in these neurons.

To test the effect of *per* over-expression outside PDF or CRY neurons I created flies that lacked *tub$_P$Gal80^{ts}* (and therefore lacked temperature control of transgenic *per* expression) and instead had either *Pdf-Gal80* or *cry-Gal80* (introduced into third chromosome for consistency). Resulting flies were constitutively over-expression *per* in all circadian neurons except those expressing GAL80 and were homozygous for *tim(UAS)Gal4* and heterozygous for both *UAS-per24* and *Gal80* element. Control flies had either TM3-Ser^1 or TM6B-Tb^1 balancer chromosome instead of the *Gal80* element.

In the first experiment (Figures 5.7 A and 5.8 A), flies were raised at standard room temperature and analysed at 23˚C DD. To increase PER over-expression during development, in two remaining experiments flies were raised at 29˚C and analysed either at 23˚C DD (Figures 5.7 B and 5.8 B) or at 17˚C DD (Figures 5.7 C and 5.8 C) to improve the survival of flies. Behavioural rhythms of flies were tested as described in 5.3.4.

Constitutive *per* over-expression in all circadian cells (Figures 5.7 and 5.8) caused in most cases locomotor arrhythmicity of flies, with only small number of flies displaying weak rhythms (with only one case of male flies with considerable rhythmicity). Positive control flies did not display strong behavioural rhythms reliably in all cases, which might be due to the *Irregular facet* (*If)* mutation that they carry which might affect the eyes of flies or affected health of flies. Because of that, in some cases the comparisons to these flies were not really informative.

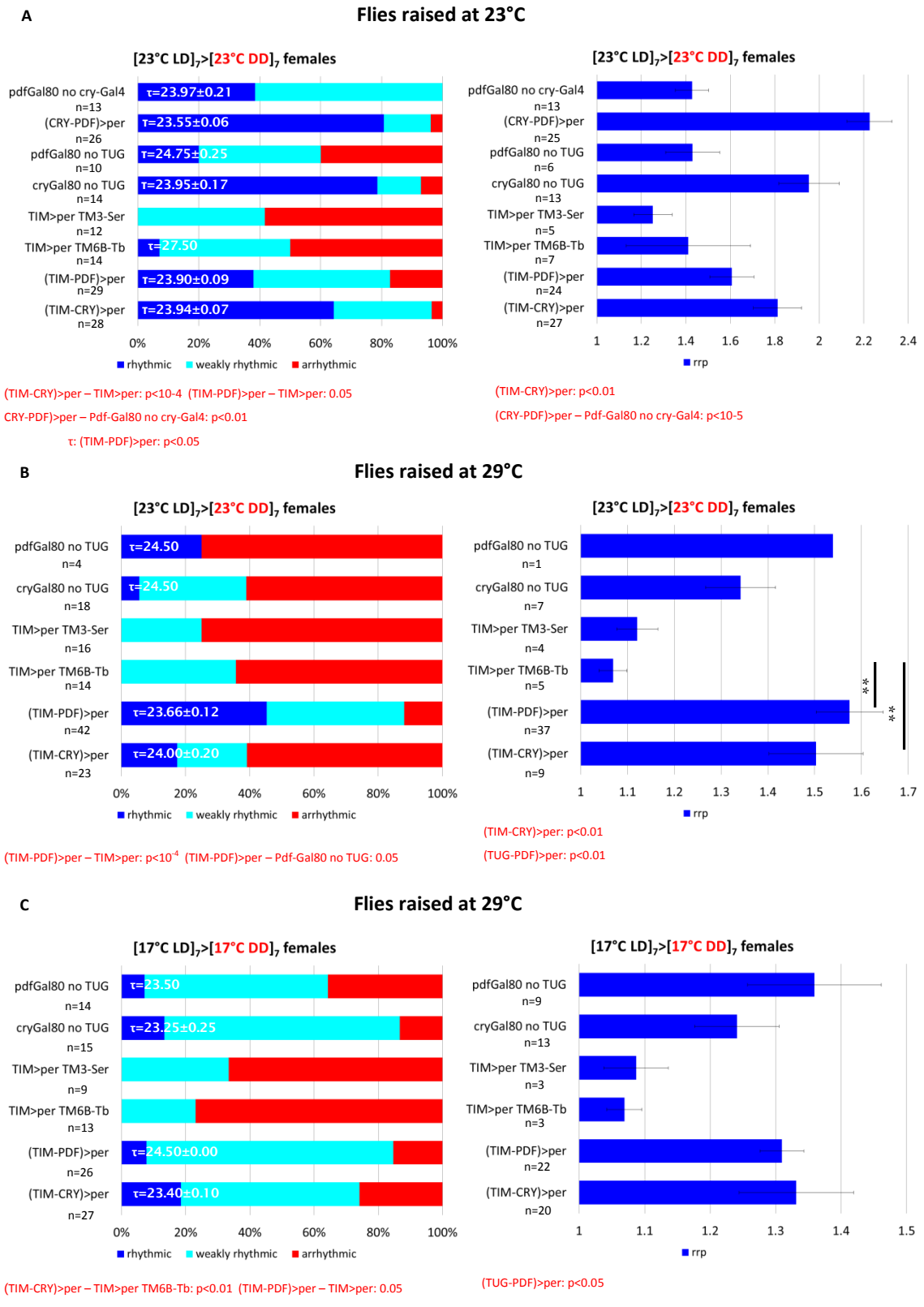


Figure 5.7. Normal levels of *per* expression in PDF-positive or CRY-positive cells rescue adult locomotor behaviour of flies – results for females.

Constitutive *per* over-expression in all PDF-negative ((TIM-PDF)>per) or CRY-negative ((TIM-CRY)>per) circadian cells impacted adult locomotor behaviour to a lesser degree than constitutive *per* over-expression in all circadian cells. Quantitative analysis of individual flies raised in either 23˚C (A) or 29˚C (B and C) and analysed at either 23˚C (A and B) or 17˚C (C), to test a variety of potential expression combinations. Stacked bar diagrams on the left show the distribution of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies and the average period length (±SEM) of the rhythmic flies are included on the graph. Flies with the *Gal80* element were compared to flies with no *Gal80* element (TIM>per), which served as negative controls and positive control flies with no Gal4 driver causing *per* over expression (Pdf-Gal80 no TUG or cry-Gal80 no TUG). The first test contains additional analysis of *per* over-expression in all CRY-positive (driven by *cry-Gal4-13* driver) PDF-negative neurons ((CRY-PDF)>per) and their controls lacking *cry-Gal4-13* driver (Pdf-Gal80 no cry-Gal4). Distributions of flies of different genotypes were compared individually with pairwise Fisher's Exact 3x2 tests and the results are listed below each graph (for the comparison to negative controls only the higher of two p values is listed). To test the associations between the period lengths and the genotype, the non-parametric Kruskal-Wallis tests were used with Wilcoxon Rank test used for individual comparisons, comparing experimental flies to appropriate control without the driver (cry-Gal80 no TUG in case of (TUG-CRY)>per flies, Pdf-Gal80 no TUG in case of (TUG-PDF)>per flies) and both negative controls (comparing 3 or 4 categories together) and the overall significances are included below graphs. Bar diagrams on the right show the average relative rhythmic power, with the numbers of flies tested included in the graph and error bars representing SEM. The same groups were compared using non-parametric Kruskal-Wallis tests with Wilcoxon Rank test used for individual comparisons, and the overall significance is included at the bottom while individual significant differences are marked with a black bar, * $p<0.05$, ** $p<0.01$.

In the case of flies raised at room temperature (Figure 5.7 A), there was a significant association between the distribution of rhythmic, weakly rhythmic and arrhythmic flies and the genotype of female flies, with both flies with the *Pdf-Gal80* transgene (two independent Fisher's Exact 3x2 tests: $p<0.05$, n=41 and n=43) and *cry-Gal80* transgene (two independent Fisher's Exact 3x2 tests: $p<0.05$, n=40 and n=42) more rhythmic than the negative controls, but not different from positive control. Also, for *cry-Gal80* females the relative rhythmic power was associated with the genotype of flies (Kruskal-Wallis test: $H(3)=7.887$, $p<0.05$, n=52) but there were no variations in the period length (Figure 5.7 A). For the *Pdf-Gal80* though there was no association between the genotype of flies and the relative rhythmic power but there was an association of the period length with the genotype (Kuskal-Wallis test: $H(2)=7.302$, $p<0.05$, n=14).

The situation was slightly different for males raised at room temperature (Figure 5.8 A), where only the distribution of rhythmic, weakly rhythmic and arrhythmic flies was associated with the genotype when *cry-Gal80* flies were analysed (two independent Fisher's Exact 3x2 tests: $p<0.05$, n=41) but not when *Pdf-Gal80* flies were tested (two independent Fisher's Exact 3x2 tests: $p>0.131$, n=42). It was supported by the analysis of the relative rhythmic power and period length, which showed an association with the genotype of flies for *cry-Gal80* (Kruskal-Wallis test: $H(2)=12.264$, $p<0.01$, n=51 for relative rhythmic power; $H(2)=16.375$, $p<0.001$, n=36 for the period length) but for flies with *Pdf-Gal80* only an association between the period length and the genotype (Kruskal-Wallis test: $H(2)=10.638$, $p<0.01$, n=15).

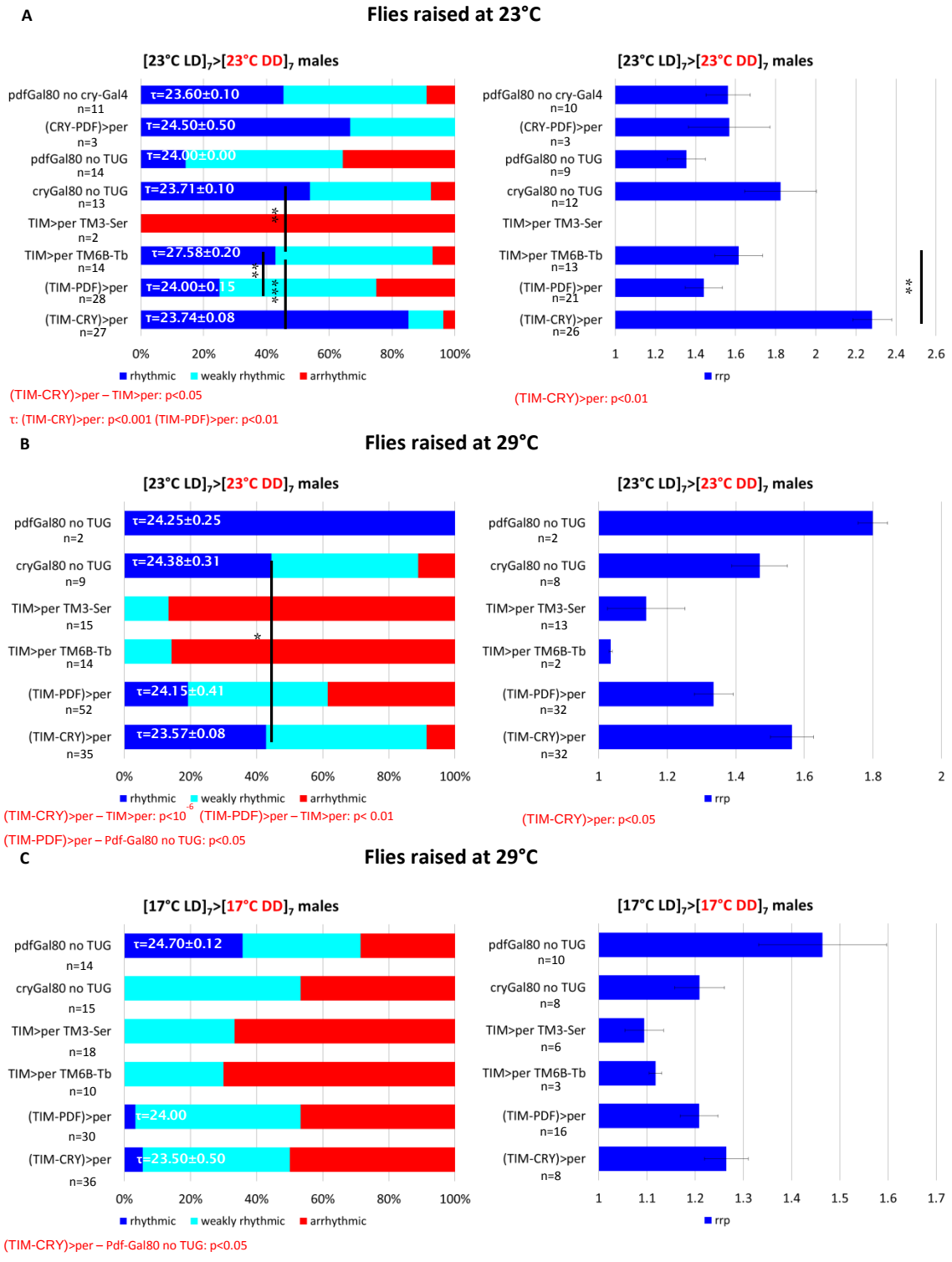


Figure 5.8. Normal levels of *per* expression in PDF-positive or CRY-positive cells rescue adult locomotor behaviour of flies – results for males.

Adult locomotor arrhythmicity caused by constitutive *per* over-expression in all circadian cells could be rescued by blocking PER over-expression in all PDF-positive ((TIM-PDF)>per) or all CRY-positive ((TIM-CRY)>per) circadian cells. Quantitative analysis of individual flies raised at either 23˚C (A) or 29˚C (B and C) and analysed in either 23˚C (A and B) or 17˚C (C). On the left hand side, stacked bar diagrams show the distribution of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies and the average period length (±SEM) of the rhythmic flies are included on the graph. Flies with the *Gal80* element were compared to flies with no *Gal80* element (TIM>per), which served as negative controls and positive control flies with no Gal4 driver causing *per* over expression (Pdf-Gal80 no TUG or cry-Gal80 no TUG). The first test contains additional analysis of *per* over-expression in all CRY-positive (driven by *cry-Gal4-13* driver) PDF-negative neurons ((CRY-PDF)>per) and their controls lacking *cry-Gal4-13* driver (Pdf-Gal80 no cry-Gal4). Distributions of flies of different genotypes were compared individually with pairwise Fisher's Exact 3x2 tests and the results are listed below each graph (for the comparison to negative controls only the higher of two p values is listed). To test the associations between the period lengths and the genotype, the non-parametric Kruskal-Wallis tests were used with Wilcoxon Rank test used for individual comparisons, comparing experimental flies to appropriate control without the driver (cry-Gal80 no TUG in case of (TUG-CRY)>per flies, Pdf-Gal80 no TUG in case of (TUG-PDF)>per flies) and both negative controls (comparing 3 or 4 categories together) and the overall significances are included below graphs. On the right, bar diagrams represent the average relative rhythmic power, with the numbers of flies tested included in the graph and error bars representing SEM. The same groups were compared using non-parametric Kruskal-Wallis tests with Wilcoxon Rank test used for individual comparisons, and the overall significance is included at the bottom while individual significant differences are marked with a black bar, * $p<0.05$, ** $p<0.01$.

Unexpected impaired rhythmicity observed for female positive controls (with no *tim(UAS)-Gal4* element) raised at 29˚C and analysed at 23˚C (Figure 5.7 B) could be explained by development at 29˚C affecting the health of flies or more advanced age of flies used. Additionally, female flies with the *cry-Gal80* element raised at 29˚C and analysed at 23˚C (Figure 5.7 B) did not show a significant increase in the number of rhythmic females when compared to controls (two independent Fisher's Exact 3x2 tests: $p>0.283$, n=37 and n=39). However, addition of this transgene improved the robustness of the rhythms, as indicated by the association of the relative rhythmic power and the genotype (Kruskal-Wallis test: $H(3)=12.004$, $p<0.01$, n=25). In fact, the relative rhythmic power was almost identical to the relative rhythmic power of female flies with the *Pdf-Gal80*, which was also showing an association with the genotype of flies (Figure 5.7 B; Kruskal-Wallis test: $H(3)=13.965$, $p<0.01$, n=47). In addition to this, female flies with *Pdf-Gal80* raised at 29˚C and analysed at 23˚C (Figure 5.7 B) were displaying a highly significant association between the genotype of flies and the percentage of rhythmic, weakly rhythmic and arrhythmic flies (two independent Fisher's Exact 3x2 tests: $p<10^{-4}$, n=56 and n=58).

The situation was much simpler for males raised at 29˚C and analysed at 23˚C (Figure 5.8 B), where there was an association between the genotype and the adult locomotor rhythmicity both for flies with the *cry-Gal80* (two independent Fisher's Exact 3x2 tests: $p<10^{-6}$, n=49 and n=50) and *Pdf-Gal80* (two independent Fisher's Exact 3x2 tests: $p<0.01$, n=66 and n=67). Additionally, flies with the *cry-Gal80* had also a higher relative rhythmic power, compared to controls (Kruskal-Wallis test: $H(3)=9.261$, $p<0.05$, n=44). There was also a significant difference between the period length and the genotype uncovered (Mann-Whitney test: $U=51.000$, $z=2.552$, $p<0.05$, n=19) but this is due to the unusually long period length of positive control flies (Figure 5.7 B).

Results obtained with flies raised at 29˚C and analysed at 17˚C DD (Figures 5.7 C and 5.8 C) showed the same trend as the ones for flies analysed at 23˚C, however the overall rhythmicity of flies decreased, possibly due to a more dramatic temperature difference between the developmental and testing conditions. Even though there was an association between the genotype and the distribution of flies for both females (Figure 5.7 C) with *Pdf-Gal80* (two independent Fisher's Exact 3x2 tests: $p<0.05$, n=35 and n=39) and *cry-Gal80*

(Fisher's Exact 3x2 test: $p<0.01$, $n=40$ when compared to the controls with TM6B-Tb[1], but not with TM3-Ser[1], possibly due to their low number), no such correlation was found for male flies (Figure 5.8 C). Also, the only significant difference of relative rhythmic power was found for female flies with *Pdf-Gal80* (Figure 5.7 C; Kruskal-Wallis test: $H(3)=10.609$, $p<0.05$, $n=37$).

Based on all the results described I concluded that constitutive *per* over-expression outside either PDF neurons or CRY-positive neurons did not decrease the rhythmicity of flies to the same extent as *per* over-expression only in PDF neurons. In light of these results it was highly unlikely that *per* over-expression in the same subset of cells only during metamorphosis could irreversibly affect adult behavioural rhythmicity. Therefore this experiment provided additional evidence to the previously described experiment (Figure 5.6) showing that developmental *per* over-expression in just PDF-positive cells is sufficient for causing irreversible adult arrhythmicity.

5.4.5 CLK/CYC is required in s-LN$_v$s neurons for establishing adult behavioural rhythmicity

PDF-positive neurons are composed of two distinct populations of neurons – small ventrolateral (s-LN$_v$s) and large ventrolateral (l-LN$_v$s) neurons (General Introduction, 1.4.1). They do not differ only by their cell body size and pattern of projections but also play different roles in the circadian clock. Therefore I was interested to check if there was also a difference between these clusters of neurons in terms of CLK/CYC developmental function requirement. I hypothesised that restricting the GAL4 expression just to one subset of the cells during development might not be sufficient to reveal any differences, so I used flies with the constitutive *per* over-expression. I hypothesised that if constitutive *per* over-expression limited to a certain subgroup of circadian neurons was not sufficient to cause adult behavioural arrhythmia, it would be impossible for the developmental *per* over-expression in the same neurons to cause adult behavioural arrhythmia in permissive conditions. Even though this experiment would not result in a positive confirmation as to which cells require CLK/CYC function during development, since with the constitutive *per* over-expression the developmental effect cannot be separated from the adult one, it

could provide me with the information on the cells that are not sensitive to *per* over-expression. Based on these results it might be possible to eliminate cell cluster from the group of cells requiring CLK/CYC function.

I tested flies with constitutive *per* over-expression in various subsets of neurons:

- *Pdf-Gal4>per* over-expression, where transgenic *per* is expressed only in 18 ventrolateral neurons expressing Pigment Dispersing Factor (but not in the PDF-negative 5th s-LN$_v$) (Renn *et al.*, 1999),
- *Mai179-Gal4>per* over-expression, that direct the transgenic *per* expression to all s-LN$_v$s, including the PDF-negative 5th, 3 LN$_d$s and possibly one l-LN$_v$ (Grima *et al.*, 2004),
- *cry-Gal4-13>per* over-expression, with which I could direct *per* over-expression to both Morning and Evening cells - l-LN$_v$s, s-LN$_v$s, 5th s-LN$_v$ (PDF-negative), some or all of the LN$_d$s (conflicting reports on the actual number of cells), 2 IPNamide-positive DN1s (Stoleru *et al.*, 2004; Busza *et al.*, 2007) and perhaps 2 additional DN1s and two DN3s (Shafer *et al.*, 2006).
- *R6-Gal4>per* over-expression, with the strongest *per* expression in s-LN$_v$s and much weaker in few (occasionally) of l-LN$_v$s, as well as couple other non-circadian neurons (Helfrich-Förster *et al.*, 2007a)
- *c929-Gal4> per* over-expression, which reflects the expression pattern of the DIMMED, targeting many peptidergic cells and l-LN$_v$s but no s-LN$_v$s, both during development and adulthood (Park *et al.*, 2008).

(As an important side note, both *c929-Gal4* and *R6-Gal4* are inserted at exactly the same location (Hewes *et al.*, 1999; Hewes *et al.*, 2000).)

In the first preliminary experiment, flies heterozygous for the *Gal4* driver and *UAS-per24* transgene were raised at 23˚C, entrained at 25˚C LD for 11 days and analysed in 29˚C DD.

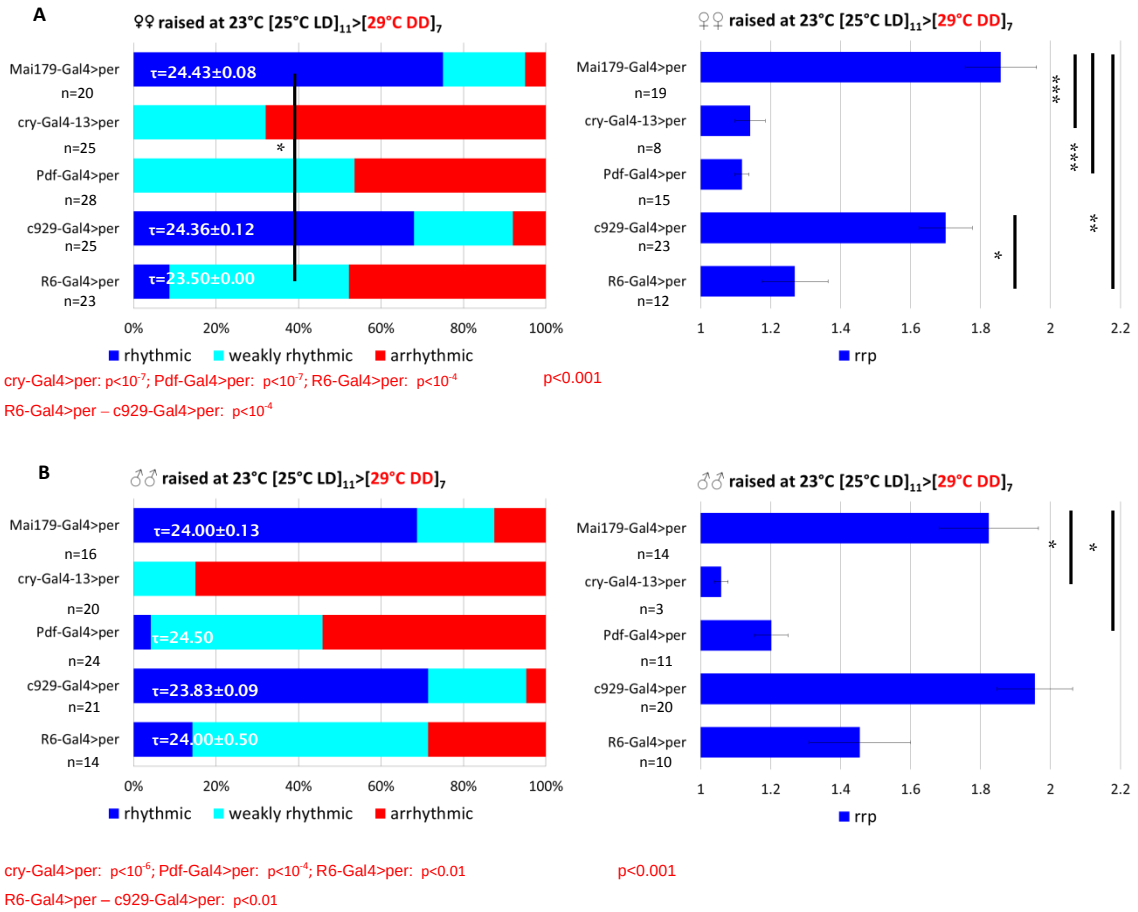


Figure 5.9. Constitutive *per* over-expression in l-LN$_v$s does not impact adult behavioural rhythmicity (data collected by Albert Nieh).

Constitutive *per* over-expression in all morning both morning and evening cells resulted in decreased rhythmicity of flies. Moreover, there was a difference between flies with *per* over-expression directed to s-LN$_v$s (*R6-Gal4>per*) and l-LN$_v$s (*c929-Gal4>per*). Quantitative results from the analysis of the adult behavioural rhythmicity of individual adult females (A) and males (B) raised at room temperature and tested at 29˚C following 11 days of entrainment at 23˚C. On the left hand side, stacked bar diagrams represent the percentage of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies analysed and the period length (±SEM) of the rhythmic flies are included on the graphs. The distributions of flies were compared individually to the one of *Mai179-Gal4>per* flies with the Fisher's Exact 3x2 tests. Same test

was performed to compare *R6-Gal4>per* to *c929-Gal4>per* flies. Results of all tests are summarised below the graphs. Bar graphs on the right represent the average relative rhythmic powers calculated for rhythmic and weakly rhythmic flies with the error bars representing SEM. Period length and relative rhythmic power were compared using non-parametric Kruskal-Wallis tests and the results of the overall comparison are summarised below the graphs with the individual significant results of comparison of *Mai179-Gal4>per* to other lines, as well the comparison of *R6-Gal4>per* to *c929-Gal4>per* labelled on the graphs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Over-expressing *per* using the *Mai179-Gal4* (*Mai179-Gal4>per*) driver did not impair adult locomotor rhythmicity and therefore was treated as the negative control to which all other flies were compared (Figure 5.9). True negative control flies were not recovered at that particular time. However, the period length of the rhythmic flies appeared elongated for females, preventing reliable comparison of period length values. Not surprisingly, analysis of the results (Figure 5.9) confirmed that constitutive *per* over-expression in all Morning (*Pdf-Gal4>per*) or both Morning and Evening cells (*cry-Gal4>per*) reduced the strength of the rhythmicity of adult flies (when compared to *Mai179-Gal4>per* with Fisher's Exact 3x2 test: *Pdf-Gal4>per*: $p<10^{-7}$, n=48 for females and $p<10^{-4}$, n=40 for males; *cry-Gal4>per*: $p<10^{-7}$, n=45 for females and 10^{-6}, n=36 for males). Also, the rhythmicity of remaining weakly rhythmic flies was much lower (Wilcoxon Rank test: W=-37.712, z=-4.800, *Pdf-Gal4>per*: $p<0.001$, n=34 for females and W=-19.929, z=-2.929, $p<0.05$, n=25 for males; *cry-Gal4>per*: W=-36.579, z=3.879, $p<0.001$, n=27 for females and W=-28.929, z=-2.693, $p<0.05$, n=17 for males).

Interestingly, data suggested that constitutive *per* over-expression in l-LN_vs (*c929-Gal4>per*) did not result in a decrease in adult rhythmicity (when compared to *Mai179-Gal4>per* with Fisher's Exact 3x2 test: p=1, n=45 for females and p=0.748, n=37 for males) or lower relative rhythmic power (Figure 5.9). On the other hand, over-expressing *per* in just the s-LN_vs (*R6-Gal4>per*) caused large impairment in the adult behavioural rhythmicity as manifested by both lower proportion of rhythmic flies (Fisher's Exact 3x2 test: $p<10^{-4}$, n=43

for females, $p<0.01$, n=30 for males) and also some decrease of the relative rhythmic power (Wilcoxon Rank test: W=-30.829, z=-3.737, $p<0.01$, n=31 for females). There was a clear difference between flies with the constitutive *per* over-expression in l-LN_vs and in s-LN_vs (Figure 5.9; for the distribution: Fisher's Exact 3x2 test: $p<10^{-4}$, n=48 for females and $p<0.01$, n=35 for males; for the relative rhythmic power: Wilcoxon Rank test: W=-25.380, z=-3.186, $p<0.05$, n=35 for females). Based on these results I concluded that the CLK/CYC function during development was not required in l-LN_vs and the cells sensitive to CLK/CYC presence during metamorphosis were s-LN_vs.

To confirm results from Figure 5.9, I repeated the same experiment, this time using proper negative controls, which lacked any *Gal4* driver (*->per*). I tested flies raised at both RT and 29˚C and analysed at both RT and 29˚C to modulate the levels of *per* over-expression during development and during testing. Flies were analysed as described previously.

Flies with constitutive *per* over-expression in all circadian neurons, obtained with *tim(UAS)Gal4* driver (*TUG>per*), which was tested only for one condition (Figures 5.10 C and 5.11 C), were clearly different than control flies (two independent Fisher's Exact 3x2 tests: $p<10^{-5}$, n=60 and $p<10^{-6}$, n=61 for females and $p<0.05$, n=52 and $p<10^{-4}$, n=71) - almost predominantly arrhythmic, with small proportion of weakly rhythmic flies with low relative rhythmic powers (only males were significantly different than the control – Wilcoxon Rank test: W=55.000, z=1.993, $p<0.05$, n=37 for flies with TM6 and W=43.167, z=2.574, $p<0.01$, n=41 for flies with Sb^1) and only one rhythmic female fly with a very short period length.

As expected from the results with conditional *per* over-expression driven by *Mai179-Gal4* (Figures A.3.3 and A.3.4), in most cases *Mai179-Gal4>per* flies were showing comparable rhythmicity to control flies (Figures 5.10 and 5.11). In two cases they were even more rhythmic than controls - female flies raised at 23˚C and analysed at 29˚C (Figure 5.10 B) were more rhythmic than control flies (Fisher's Exact 3x2 test: $p<0.05$, n=27, since control flies demonstrated unusually poor rhythmicity, possibly due to impaired health) and were treated as control flies for the comparisons of distribution of female flies for that conditions and males raised at 29˚C and analysed at 23˚C had both more rhythmic flies (Fisher's Exact 3x2 test: $p<0.01$, n=72) and higher relative

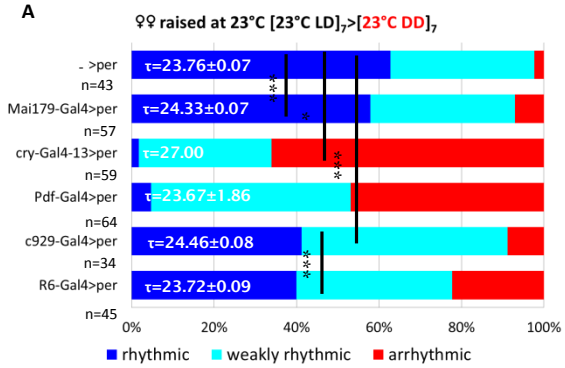
rhythmic power (Wilcoxon Rank test: W=-32.500, z=-3,147, p<0.01, n=57). In about half of the cases analysed, the period length of the rhythmic flies was significantly shorter (Wilcoxon Rank test: W=-30.300, z=-4.388, p<0.001, n=60 for females raised and analysed at RT; W=-17.650, z=-2,259, p<0.05, n=23 for females raised at 29˚C and analysed at RT; W=-36.341, z=-3.932, p<0.05, n=33 for males raised and analysed at RT; W=-9.562, z=-2.043, p<0.05, n=22 for males raised at RT and analysed at 29˚C). Also, in case of females raised and analysed at 29˚C (Figure 5.10 D) flies displayed significantly lower rhythmicity than the controls (Fisher's Exact 3x2 test: $p<10^{-7}$, n=64), which was also accompanied by the reduced relative rhythmic power (Wilcoxon Rank test: W=41.712, z=4,454, p<0.001, n=41). As in case of the *Pdf-Gal4>per*, these differences reflected the association between the rhythmicity of flies with the combination of the developmental and testing conditions (females showed difference for relative rhythmic power –Kruskal-Wallis test: H(3)=14.932, p<0.01, n=107 and males displayed association for both period length – Kruskal-Wallis test: H(3)=43.076, p<0.001, n=68 and relative rhythmic power - Kruskal-Wallis test: H(3)=15.810, p<0.001, n=85).

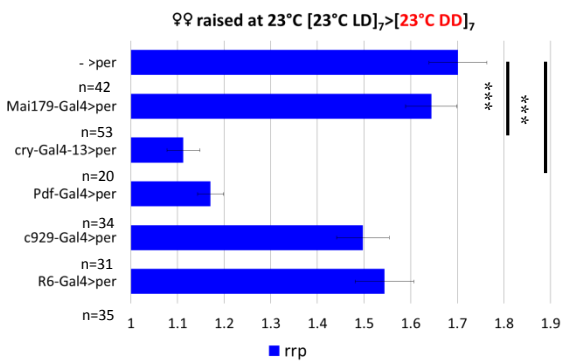
The line that showed consistently the poorest rhythmicity, which was indistinguishable from the results obtained for flies with *per* over-expression in all circadian cells (*TUG>per*), was again the *cry-Gal4>per* line (Figures 5.10 and 5.11). There were almost no rhythmic flies in all conditions (significant in comparison of the distribution of flies to control for all conditions with four independent Fisher's Exact 3x2 tests: $p<10^{-4}$, n=102, n=36, n=69, n=60 for females and $p<10^{-5}$, n=80, n=34, n=75, n=56 for males) and very low relative rhythmic power of remaining rhythmic and weakly rhythmic flies with significant difference for three conditions for females (Wilcoxon Rank test: W=104.746, z=6.198, p<0.001, n=62 for flies raised and analysed at RT; W=61.763, z=2.820, p<0.01, n=43 for flies raised at 29˚C and analysed at RT; W=39.759, z=4.030, p<0.001, n=38 for flies raised and analysed at 29˚C) and for all conditions for males (Wilcoxon Rank test: W=88.623, z=4.457, p<0.001, n=31 for flies raised and analysed at RT; W=42.250, z=3,571, p<0.001, n=10 for flies raised at RT and analysed at 29˚C; W=41.667, z=2.484, p<0.05, n=41 for flies raised at 29˚C and analysed at RT; W=44.152, z=3.837, p<0.01, n=28 for flies raised and analysed at 29˚C). The rhythmicity of flies was fairly consistent across all tested conditions, with no significant association between

the relative rhythmic power of flies and the developmental and testing protocol (Kruskal-Wallis test: $H(3)=5.965$, $p=0.133$, $n=42$ for females; $H(3)=0.729$, $p=0.866$, $n=30$ for males).

Pdf-Gal4>per flies also displayed impaired behavioural rhythmicity (Figures 5.10 and 5.11), which was in most cases still significantly lower than of the control flies (for the comparison of distribution of flies to controls, females were showing significant difference for all conditions analysed with independent Fisher's Exact 3x2 tests: $p<0.01$, $n=107$, $n=36$, $n=73$, $n=56$ and males were significantly different only when analysed at 29˚C with two independent Fisher's Exact 3x2 tests: $p<0.05$, $n=41$ and $n=57$). Except one condition (Figures 5.10 C and 5.11 C), for which the period length of flies was significantly lower (for flies raised at 29˚C and analysed at 23˚C; Wilcoxon Rank test: $W=32.250$, $z=4.128$, $p<0.001$, $n=25$ for females; $W=18.375$, $z=2.502$, $p<0.05$, $n=10$ for males), the relative rhythmic power of rhythmic and weakly rhythmic flies was lower than for controls (for females it was significant for two conditions Wilcoxon Rank Rest: $W=88.478$, $z=6.165$, $p<0.001$, $n=76$ for flies raised and analysed at RT; $W=39.300$, $z=3.434$, $p<0.01$, $n=34$ for flies raised and analysed at 29˚C; for males it was significant for three conditions: Wilcoxon Rank test: $W=30.979$, $z=2.086$, $p<0.05$, $n=55$ for flies raised and analysed at RT; $W=28.132$, $z=3.277$, $p<0.01$, $n=25$ for flies raised at RT and analysed at 29˚C; $W=23.501$, $z=2.610$, $p<0.01$, $n=40$ for flies raised and analysed at 29˚C). As obvious from Figures 5.10 and 5.11, the rhythmicity of flies with the constitutive *per* over-expression in PDF neurons depended on the developmental and testing conditions (for females relative rhythmic power was associated with the conditions: Kruskal-Wallis test: $H(3)=18.064$, $p<0.001$, $n=66$ and for males the period length changed with the conditions: Kruskal-Wallis test: $H(3)=18.992$, $p<0.001$, $n=27$). The difference between the results obtained for the *per* over-expression in the PDF-positive and CRY-positive neurons might be suggesting that some other CRY-positive and PDF-negative neurons require developmental CLK/CYC function during development for establishing adult locomotor rhythmicity.

A



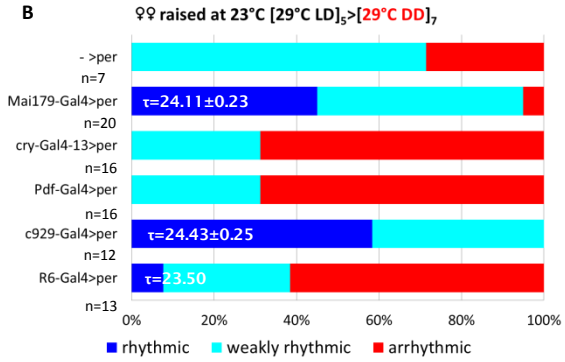


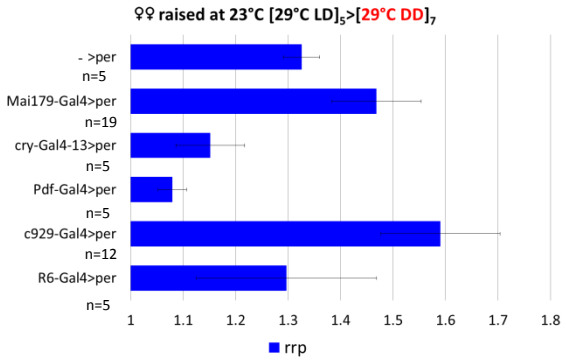
compared to ->per:

cry-Gal4>per: $p<10^{-15}$; Pdf-Gal4>per: $p<10^{-12}$; R6-Gal4>per: $p<0.01$

$p<0.001$

B





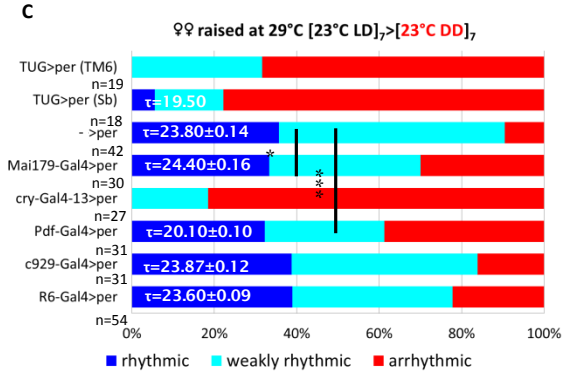
compared to Mai179-Gal4>per:

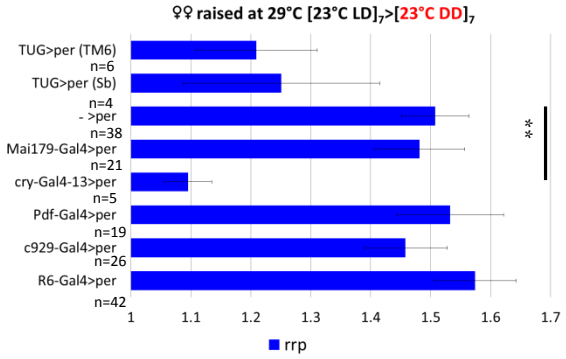
->per: $p<0.05$; cry-Gal4>per: $p<10^{-4}$; Pdf-Gal4>per: $p<10^{-4}$; R6-Gal4>per:

$p<0.01$ R6-Gal4>per – c929-Gal4>per: $p<0.01$

$p<0.01$

C





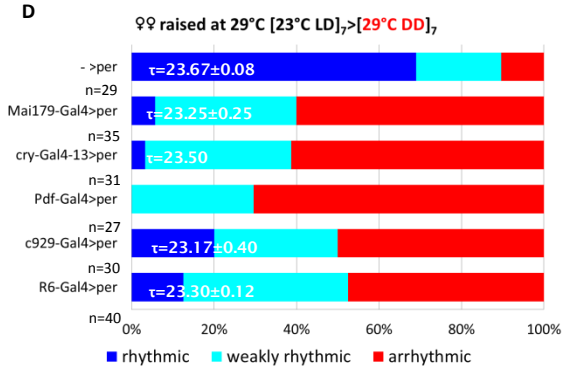
compared to ->per:

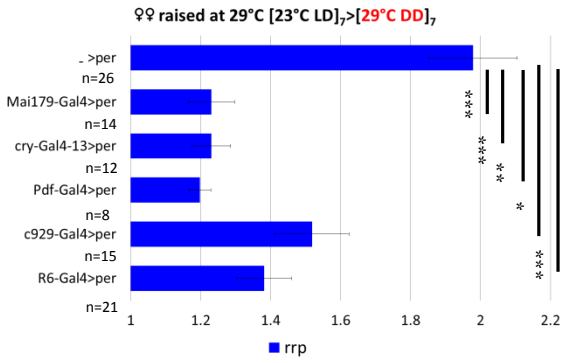
TUG>per (TM6B-Tb): $p<10^{-5}$ TUG>per(Sb): $p<10^{-6}$; cry-Gal4>per: $p<10^{-9}$;

Pdf-Gal4>per: $p<0.01$

$p<0.05$ - cry-Gal4>per: $p<0.01$;

D





compared to ->per:

Mai179-Gal4>per: $p<10^{-7}$; cry-Gal4>per: $p<10^{-7}$; Pdf-Gal4>per: $p<10^{-8}$;

c929-Gal4>per: $p<0.001$; R6-Gal4>per: $p<10^{-5}$

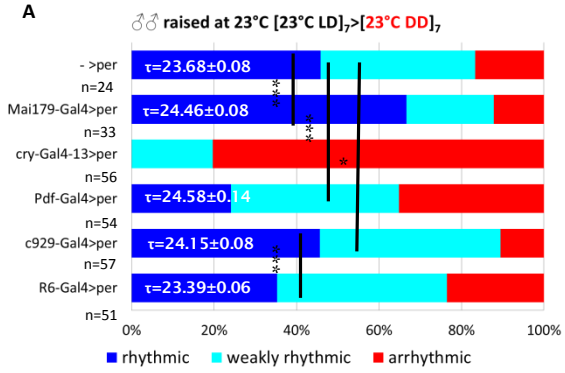
$p<0.001$ – Mai179-Gal4>per: $p<0.001$; cry-Gal4>per: $p<0.001$;

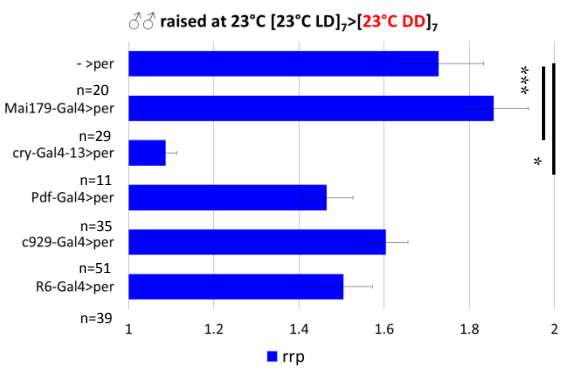
Pdf-Gal4>per: $p<0.01$; R6-Gal4>per: $p<0.001$; c929-Gal4>per: $p<0.05$

Figure 5.10. Difference between adult locomotor rhythmicity of flies with constitutive *per* over-expression in l-LN$_v$s and s-LN$_v$s depends on the developmental conditions and testing protocol – results for females.

Female flies with constitutive *per* over-expression directed to a particular subset of cells were raised at either RT (A and B) or 29˚C (C and D) and tested at either 23˚C (A and C) or 29˚C (B and D), following entrainment to LD cycles. Stacked bar diagrams on the left represent the results from the individual analysis of adult behavioural rhythmicity and depict the percentage of rhythmic, weakly rhythmic and arrhythmic flies. The distributions of flies were compared individually to control flies or *Mai179-Gal4>per* flies (B, where the numbers and rhythmicity of the control flies were compromised probably due to impaired health at the time of testing) with the Fisher's Exact 2x3 test. *R6-Gal4>per* were compared to *c929-Gal4>per* flies. On the right hand side, bar graphs show the average relative rhythmic powers of both rhythmic and weakly rhythmic flies with the error bars representing SEM. Values for period length and relative rhythmic power were compared to each other using non-parametric Kruskal-Wallis test and the results of the overall comparison and the significant results from the comparison of all flies to *->per* and also of the *R6-Gal4>per* to *c929-Gal4>per* are summarised below the graphs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

A



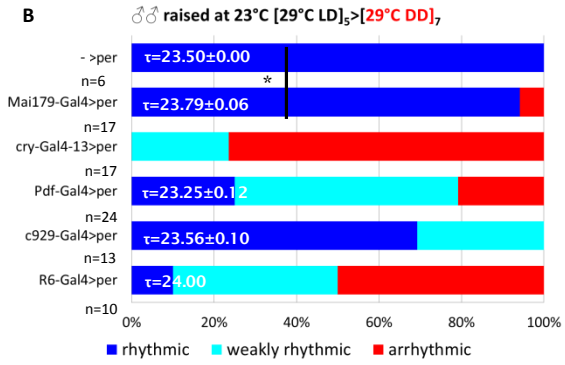


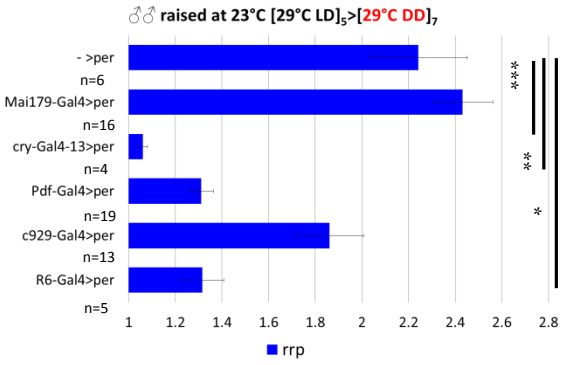
compared to ->per:

cry-Gal4>per: $p<10^{-9}$;

p<0.001

B





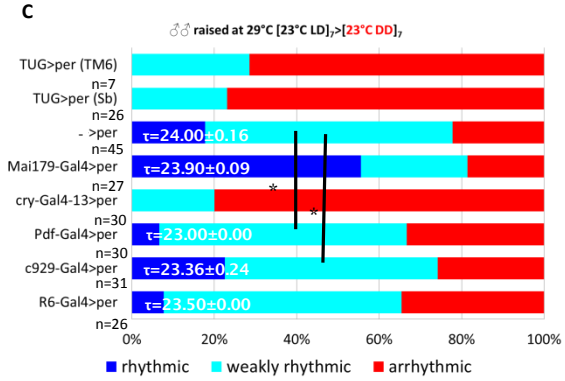
compared to Mai179-Gal4>per:

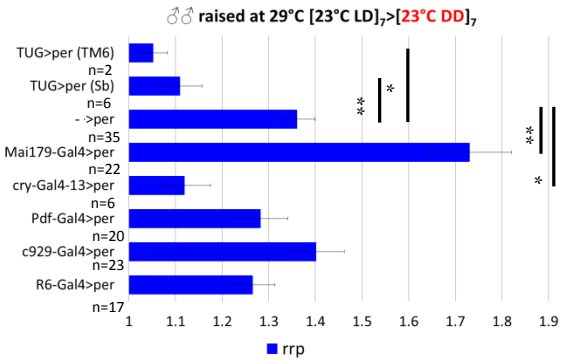
cry-Gal4>per: $p<10^{-5}$; Pdf-Gal4>per: p<0.01; R6-Gal4>per: p<0.01

R6-Gal4>per – c929-Gal4>per: p<0.01

p<0.001

C



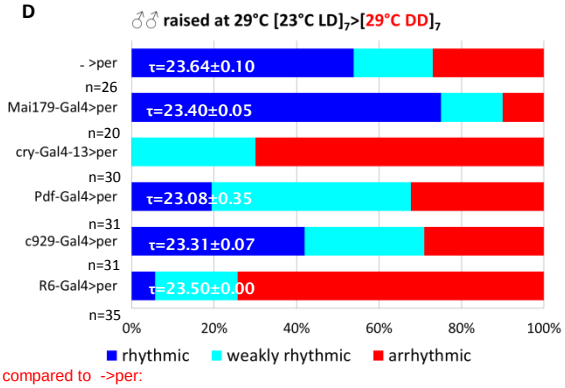


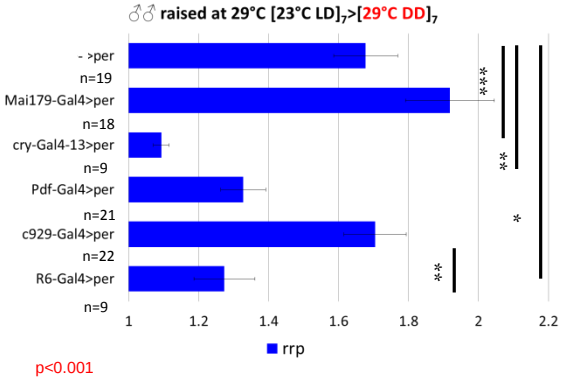
compared to ->per:

TUG>per (TM6B-Tb): p<0.05; TUG>per (Sb): $p<10^{-4}$; Mai179-Gal4>per: p<0.01; cry-Gal4>per: $p<10^{-5}$

p<0.001

D





compared to ->per:

cry-Gal4>per: $p<10^{-5}$; Pdf-Gal4>per: p<0.05; R6-Gal4>per: $p<10^{-4}$ R6-Gal4>per – c929-Gal4>per: p<0.001

p<0.001

Figure 5.11. Developmental conditions and testing protocol influence the adult locomotor rhythmicity of flies with constitutive *per* over-expression in l-LN$_v$s and s-LN$_v$s – results for males.

Male flies with constitutive *per* over-expression directed to a particular subset of cells were raised at either RT (A and B) or 29˚C (C and D) and tested at either 23˚C (A and C) or 29˚C (B and D), following entrainment to LD cycles. Stacked bar diagrams on the left represent the results from the individual analysis of adult behavioural rhythmicity and depict the percentage of rhythmic, weakly rhythmic and arrhythmic flies. The distributions of flies were compared individually to control flies with the Fisher's Exact 2x3 test. *R6-Gal4>per* were compared to *c929-Gal4>per* flies. On the right hand side, bar graphs show the average relative rhythmic powers of both rhythmic and weakly rhythmic flies with the error bars representing SEM. Values for period length and relative rhythmic power were compared to each other using non-parametric Kruskal-Wallis test and the results of the overall comparison and the significant results from the comparison of all flies to *->per* and also of the *R6-Gal4>per* to *c929-Gal4>per* are summarised below the graphs. * p<0.05, ** p<0.01, *** p<0.001.

Data suggested that developmental over-expression of *per* in s-LN$_v$s affected rhythmicity of flies but *per* over-expression in l-LN$_v$s is not sufficient to cause adult behavioural arrhythmicity (Figures 5.10 and 5.11). *c929-Gal4>per* flies displayed only difference between the period length for flies raised and analysed at 23˚C (Wilcoxon Rank test: W=-37.648, z=-4.296, p<0.001, 41 for females (Figure 5.10 A) and p>0.05, n=37 for males (Figure 5.11 A)) and for the females raised and analysed at 29˚C (Figure 5.10 D) where both the distribution of flies (Fisher's Exact 3x2 test: p<0.001, n=59) and the relative rhythmic power (Wilcoxon Rank test: W=19.126, z=2.089, p<0.05, n=27) showed significant difference in comparison to controls. There were also some differences between the period length of *c929-Gal4>per* males and controls raised at 29˚C and analysed at 23˚C (Figure 5.11 C; Wilcoxon Rank test: W=10.089, z=2.089-p<0.05, n=15). The rhythmicity of these flies was again

dependent on the developmental and testing conditions, with period length of females (Kruskal-Wallis test: H(3)=14.637, p<0.01, n=39) and both period length (Kruskal-Wallis test: H(3)=29.191, p<0.001, n=55) and relative rhythmic power (Kruskal-Wallis test: H(3)=10.255, p<0.05, n=107) showing a correlation with the conditions (Figures 5.10 and 5.11). This could have indicated that the levels of *per* over-expression might influence the results, however the higher PER levels during adulthood associated with the testing at 29˚C corresponded to stronger rhythmicity.

In contrast, the rhythmicity of flies with the constitutive *per* over-expression in l-LN_vs (with *R6-Gal4*) was not associated with the developmental and testing conditions (Figures 5.10 and 5.11; relative rhythmic power: Kruskal-Wallis test: H(3)=6.082, p=0.108, n=104 for females and H(3)=3.329, p=0.344, n=70 for males; period length: Kruskal-Wallis test: H(3)=5.333, p=0.149, n=45 for females and H(3)=4.469, p=0.215, n=23 for males) and in most of the conditions analysed was worse than of control flies. For females, the distribution of flies was different for all conditions except flies raised at 29˚C and analysed at 23˚C (Figure 5.10 A, B and D; three independent Fisher's Exact 3x2 test: p<0.01, n=88, n=33, n=69) and in one of these cases (flies raised and analysed at 29˚C) also the relative rhythmic power was different (Wilcoxon Rank test: W=28.790, z=3.525, p<0.001, n=47). For males, the distribution of flies was different when flies were analysed at 29˚C (Figure 5.11 B and D; two independent Fisher's Exact 3x2 tests: p<0.01 n=27 and n=61) which was also accompanied by the significantly lower relative rhythmic power (Wilcoxon Rank test: W=26.700, z=2.406, p<0.05, n=11 for flies raised at RT and analysed at 29˚C; W=28.264, z=2.456, p<0.05, n=28 for flies raised and analysed at 29˚C).

Coming to the two most important comparison, in all cases but two (females raised at 29˚C or 23˚C and tested at 23˚C (Figure 5.10 A and C)) flies with *per* over-expression in s-LN_vs were less rhythmic than flies with the *per* over-expression in l-LN_vs, as demonstrated by both percentage of rhythmic, weakly rhythmic and arrhythmic flies and relative rhythmic power (however in most cases the differences were not significant). For males analysed at 29˚C, the distribution of the rhythmic, weakly rhythmic and arrhythmic flies was significantly different when compared to *c929-Gal4>per* (Figure 5.11 B and D; two independent Fisher's Exact 3x2 tests: p<0.01, n=23 and n=66). Differences

between the average relative rhythmic power for flies with *per* over-expression in these neurons were significant only for males both raised and analysed at 29˚C (Figure 5.11 D; Wilcoxon Rank test: W=-29.364, z=-2.610, p<0.01, n=31). For females however, the only significant difference between the *c929-Gal4>per* and *R6-Gal4>per* was when flies were raised at 23˚C and analysed at 29˚C (Figure 5.10 B, Fisher's Exact 3x2 test: p<0.01, n=25), which happens to be same as in case of flies analysed in the preliminary experiment. Taking all of the results into account, it is plausible that the s-LN_vs are more sensitive to the developmental CLK/CYC levels than l-LN_vs.

5.4.6 CLK/CYC inhibition during development disrupts the molecular oscillations in s-LN_vs

I suspected that developmental CLK/CYC inhibition might lead to adult behavioural arrhythmicity through impairment of neuroanatomy, which could mean either changes to the cell number, morphology or connectivity. Preliminary analysis of neuroanatomy performed by Jake Currie (Figure A.3.6) revealed no changes in the gross morphology of circadian neurons in flies with developmental *per* over-expression. Moreover, dorsal projections from s-LN_vs appeared normal and reached dorsal protocerebrum. That experiment, however, did not provide a quantitative analysis of the morphology of the cells or test the function of the molecular oscillator in these cells. To test these, I conducted an immunofluorescence analysis of adult brains from flies with or without developmental PER over-expression (*ywUAS:CD8:GFP/yw tub$_P$Gal80^{ts}; tim(UAS)Gal4; UAS-per24* females were raised under either permissive (room temperature) or restrictive (29˚C) conditions) at various times of the day as described in 2.6.

Analysis of LN_ds (Figure 5.12) revealed no significant differences in the oscillation patterns for flies with and without developmental *per* over-expression. In both cases rhythms in TIM intensity were present with large amplitude (Welch's test: F(3, 18.505)=18.460, p<0.001, n=41 for flies raised at RT; F(3, 15.501)=21.989, p<0.001, n=40 for flies raised at 29˚C, also indicated by the significant differences between values for CT 16, CT 22 and CT 4 versus CT 10). The peak in TIM intensity appeared a bit earlier for flies with

developmental *per* over-expression, however due to the sampling size, the exact phase advance could not be determined. There was a significant difference in TIM expression between flies raised at different temperatures at CT 4 (Mann-Whitney: U=9.000, z=-3.099, p<0.01, n=20) and CT 10 (Mann-Whitney: U=21.000, z=-2,887, p<0.01, n=21). A highly significant association between TIM localization and developmental temperature was found for all timepoints except CT 16 (Fisher's Exact 4x2 test: $p<10^{-5}$, n=94 for CT 4; $p<10^{-5}$, n=106; for CT 10 $p<10^{-3}$, n=106 for CT 22). These significant differences in the cellular localisation of TIM seemed, however, more connected to a possible phase shift than to a disruption of rhythms. On the basis of these results I concluded that the molecular rhythms in LN_ds persisted in spite of developmental *per* over-expression.

As for PDF-positive neurons, oscillations in l-LN_vs persisted with high amplitude and (Welch's test: F(3, 10.387)=9.707, p<0.01, n=24 for flies raised at RT; F(3, 9.045)=16.240, p<0.01, n=24 for flies raised at 29˚C) and were not significantly impacted by developmental *per* over-expression (Figure 5.12). There was a trend for the amplitude of the oscillations in the brains of flies with developmental *per* over-expression to be even higher (as for flies raised at 29˚C all timepoints were significantly different from the trough CT 10 value and for flies raised at RT only CT 4 was different than CT 10). Also, the cellular localisation, despite some differences (Fisher's Exact 4x2 test: $p<10^{-5}$, n=58 for CT 10; $p<10^{-5}$; n=57 for CT 22), was largely similar. These results could be interpreted as evidence that the molecular rhythms in l-LN_vs also persisted despite developmental *per* over-expression.

s-LN_vs displayed circadian rhythms in TIM intensity only for flies raised at room temperature (Figure 5.12), without developmental *per* over-expression (Welch's test: F(3, 12.081)=24.225, p<0.001, n=32 with CT 22 and CT 4 significantly different than CT 10). No significant difference between any of the values and CT 10 was, however, found for flies raised with developmental *per* over-expression (raised at 29˚C). TIM levels for these flies remained constant around the trough values of flies raised at RT. Also, when TIM cellular localization was compared, for every timepoint there was a significant difference between the distributions of flies raising at different temperatures (Fisher's Exact 4x2 test: $p<10^{-3}$, n=8 for CT 4; p<0.05, n=45 for CT 10; $p<10^{-3}$, n=54 for CT 16; $p<10^{-5}$,

n=81 for CT 22). This indicated that, in contrast to LN_ds and l-LN_vs, s-LN_vs were affected by the developmental *per* over-expression.

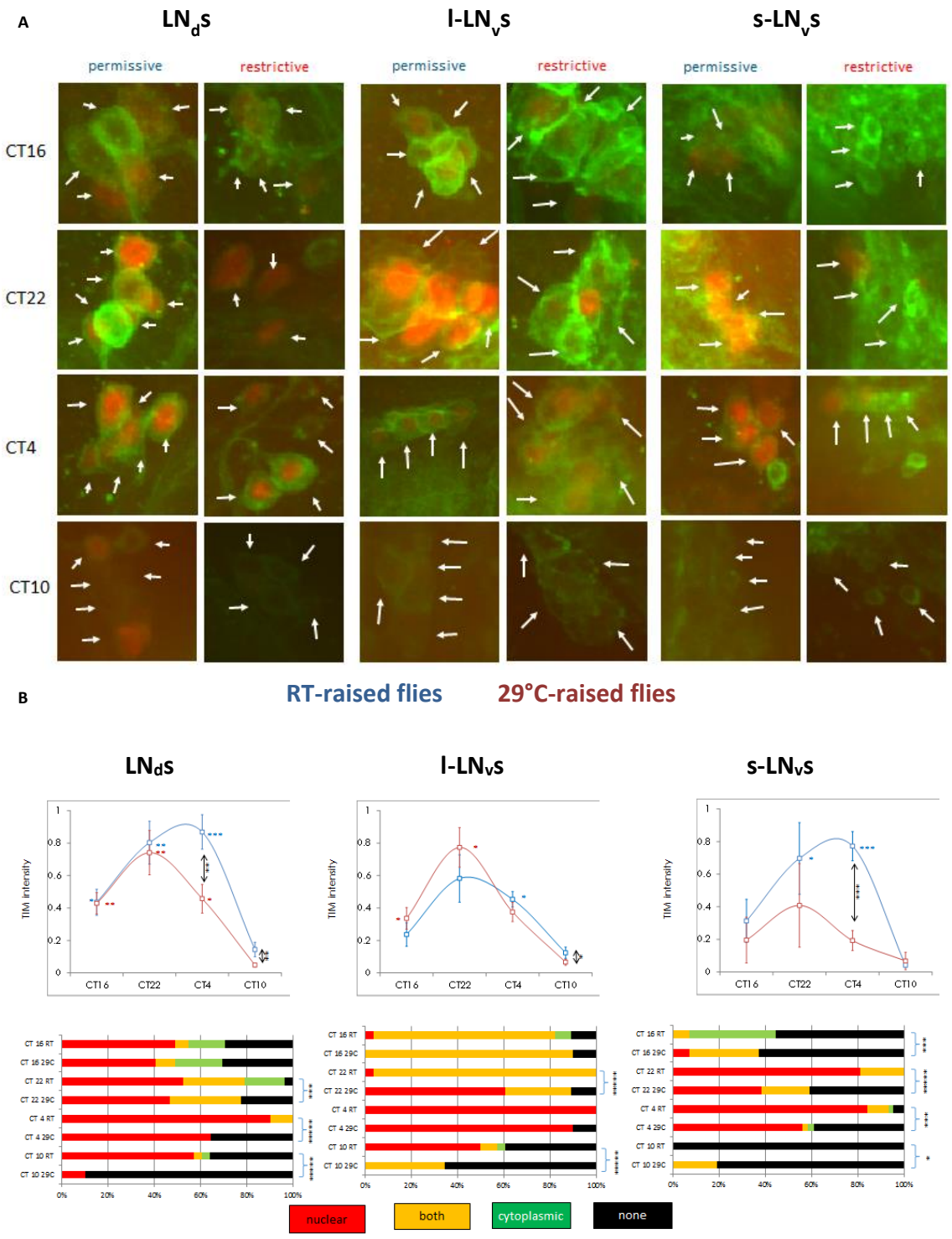


Figure 5.12. Developmental *per* over-expression affects molecular oscillations in s-LN_vs but not in LN_ds or l-LN_vs.

(A) Representative pictures showing TIM protein rhythms (at permissive conditions) in LN_ds, l-LN_vs and s-LN_vs neurons of flies with developmental PER over-expression. GFP (green) was used to mark the circadian cells, with arrows pointing to the cell bodies. TIM signal is in red. Restrictive and permissive denotes flies raised at 29˚C and RT, respectively. (B) Quantification of TIM staining intensity (top) and its cellular localisation (bottom) for all groups of cells for flies raised at RT (blue line) and 29˚C (red line). Error bars represent SEM. Coloured asterisks denote significant differences between the trough (CT10) and other timepoints detected with the Welch's test with Games-Howell post-hoc analysis and are an indication of oscillations. Black asterisks denote the difference between flies raised under permissive and restrictive conditions (calculated with Welch's test with Games-Howell post-hoc analysis for the TIM intensity and with Fisher's Exact 4x2 test for the distribution). Data comes from cells from 6-10 hemispheres, cell n numbers: LN_ds= 45-57, s-LN_vs = 21-44, l-LN_vs = 28-30. * $p<0.05$, ** $p<0.01$, *** $p<10^{-3}$, **** $p<10^{-4}$ and ***** $p<10^{-5}$.

Contrary to the results described in A.3.6, I discovered some subtle differences in the cell morphology between flies raised at room temperature and 29˚C (Figure A.3.7). Even though the number of cells detected for each group of neurons was consistent for RT-raised and 29˚C-raised flies for all timepoints, there was a highly significant correlation between the area of cells and the circadian time for all groups of cells for both flies raised at RT and 29˚C. These results might suggest that there were in fact some subtle differences between the morphology of different circadian neurons caused by the developmental *per* over-expression. However, these results are difficult to explain and need to be repeated using the conditional *cyc* rescue flies before they can be fully trusted.

5.5 Discussion

5.5.1 CLK/CYC activity during metamorphosis ensures adult locomotor rhythmicity

I demonstrated in the previous chapters that developmental CLK/CYC activity is important for adult locomotor activity of *D. melanogaster* (Figures 4.1 and 4.2). In this chapter I further described this developmental requirement by testing which cells required developmental CLK/CYC activity for their adult timekeeping function. The first part of this chapter demonstrated that inhibition of CLK/CYC activity by means of conditional *per* over-expression during (but not prior to) metamorphosis resulted in irreversible adult arrhythmia (Figures 5.1 and 5.2). This was also confirmed with flies with conditional *cyc* rescue (Figure 5.3), whose CLK/CYC function was restored prior to (but not during) metamorphosis, as these flies had disrupted adult locomotor rhythmicity in permissive conditions. Whether this can be extended to imply that CLK/CYC heterodimer presence is not important remains unknown as molecular studies should be conducted to test levels of CLK/CYC in developing conditional *cyc* rescue flies.

During adult *per* over-expression, female flies showed adult locomotor arrhythmia independent of previous developmental treatment (Figure 5.2 A and B). Males, however, revealed a difference between flies with prior *per* over-expression during metamorphosis, which displayed behavioural arrhythmia, and flies with either no prior *per* over-expression or over-expression that ceased before metamorphosis, which showed the persistence of weakened and lengthened behavioural rhythms (Figure 5.2 A and B). This discrepancy between genders could be linked to the genotype of flies, since due to the location of *tub$_P$Gal80^{ts}* element on the X chromosome, males might have had higher levels of GAL80^{ts}.

These results implied that CLK/CYC activity prior to metamorphosis was not required for adult locomotor behaviour. This might suggest that CLK/CYC is required developmentally either in the neurons formed during metamorphosis (l-LN$_v$s, Liu *et al.*, 2015) or for some aspects of the reorganization of the circadian neurons that survive from larval stage. l-LN$_v$s precursors appear during late larval stage L3 (Kaneko *et al.*, 1997; Helfrich-Förster *et al.*, 2007a

and 2007b), at the beginning of metamorphosis the number of circadian cells increases and cells develop projections until at 30-40% pupal development l-LN_vs and LN_ds can be distinguished. These cells continue to migrate towards their respective adult positions until ~50-60% pupal development. Around the same time, l-LN_vs start expressing circadian proteins and PDF, followed by the circadian protein expression in LN_ds at 70% pupal development (Kaneko and Hall, 2000; Helfrich-Förster *et al.*, 2007a).

However, in the light of the results presented in this chapter, it is very unlikely that the first hypothesis is true, since the developmental requirement for CLK/CYC activity was mapped to s-LN_v neurons (Figures 5.9 – 5.11). This would support the notion that during metamorphosis changes occur in s-LN_vs to adapt their function to the adult behavioural rhythmicity and the lack of CLK/CYC function affects this process.

Processes that could be potentially affected by the CLK/CYC inhibition during metamorphosis are the activity-dependent synaptic and axonal pruning. Larval s-LN_vs have dendritic arbors in the central part of the optic neuropil that later becomes accessory medulla (Helfrich-Förster, 1997) and presynaptic sites in the dorsal protocerebrum (Helfrich-Förster *et al.*, 2007b). Synaptogenesis and synaptic pruning are important processes shaping the final morphology of adult s-LN_vs synaptic arbors (Gatto and Broadie, 2009b). These processes happen during metamorphosis (reviewed in Truman, 1990; Truman *et al.*, 1993; Levene *et al.*, 1995) and depend on the presence of FMR1 (Fragile X Mental Retardation) protein during pupal days 3-4 (Gatto and Broadie, 2009b). Absence of FMR1 led to overextension, overgrowth, mistargeting past the normal area in the protocerebrum and additional expansive synaptic arborisation at the termini (Dockendorff *et al.*, 2002; Morales *et al.*, 2002; Reeve *et al.*, 2005 and 2008; Sekine *et al.*, 2008; Gatto and Broadie, 2009a and 2009b) and over-expression of FMR1 resulted in a collapse of the dorsal neuronal arborisation (Reeve *et al.*, 2005).

The fact that CLK/CYC activity during metamorphosis was shown to be necessary for adult behavioural rhythms did not exclude the possibility that CLK/CYC activity prior to metamorphosis is important for larval timekeeping or even rhythmic eclosion. For example, low CLK/CYC activity alters excitability of LN_vs and DN1s, but it was not shown if these changes require persistent

CLK/CYC inhibition (Collins *et al.*, 2012). One interesting experiment would be to look at the impact of CLK/CYC inhibition during embryonic and larval stages at the circadian modulation of the larval photophobic response. Even though it was demonstrated that larvae with mutations in either CLK or CYC have increased light sensitivity leading to aberrant photophobic response (Mazzoni *et al.*, 2005), it would be interesting to know whether if a correct function of these proteins could be restored for testing it would still be true. It is possible that CLK/CYC inhibition during prior development affects the function of the circadian oscillator in larvae in constant darkness by either disrupting the molecular oscillations in the circadian neurons directly or altering electrical excitability of the membranes of the circadian neurons, which was shown to be required for those oscillations (Nitabach *et al.*, 2004). Conducting the suggested experiment could help us to distinguish permanent changes from reversible ones.

5.5.2 CLK/CYC inhibition during development affects function of peripheral oscillators based on the strength of inhibition

The analysis of the molecular oscillation in the circadian gene expression in the periphery resulted in some conflicting findings (Figure 5.4). Data obtained with constitutive *per* over-expression was consistent with what was already reported (Kaneko *et al.*, 2000b). First, developmental *per* over-expression driven by *tim(UAS)Gal4* did not seem to affect the function of the peripheral oscillators. However when the driver was switched to *tim62-Gal4*, rhythms in the periphery were affected by developmental *per* over-expression. This might suggest that the latter driver triggers stronger peripheral expression.

Molecular oscillations in the 'periphery' were believed to diminish quickly in DD (Plautz *et al.*, 1997a and 1997b; Stanewsky *et al.*, 1997b; Giebultowicz *et al.*, 2000). Data presented in this chapter (Figures 5.4 and 5.5) challenged this as it extended previous reports that TIM oscillations persist in the peripheral tissues for 3 days in constant darkness (Grima *et al.*, 2012) by showing their persistence for at least 5 days. Additionally, the fact that flies with *tim(UAS)Gal4* – driven *per* over-expression raised at restrictive temperatures displayed behavioural arrhythmicity and lack of molecular oscillations in the

s-LN$_v$s and yet showed rhythmic TIM expression in the peripheral tissues for 5 consecutive days in the constant darkness suggested that these rhythms are independent of the function of the molecular oscillations in these neurons.

The caveat of the results presented and conclusions was that expression patterns have not been verified. Therefore the explanation of the differences observed by different levels of GAL4 expression is purely speculative. It is unknown to what extent PER is over-expressed and how it accumulates in peripheral tissues and how long it persists there. For a full understanding of the differences observed, some experiments comparing GAL4 and PER levels in the periphery should be therefore conducted. This could be achieved by performing qRT-PCR on *per* over-expression flies with either *Gal4* driver using primers to detect *Gal4* and *per* levels.

5.5.3 CLK/CYC activity is required in PDF neurons for adult locomotor rhythmicity

As briefly mentioned in the previous sections of this discussion, developmental CLK/CYC activity requirement was mapped to PDF-positive neurons. First of all, I demonstrated that both constitutive and developmental *per* over-expression driven by *Pdf-Gal4* led to a large decrease in adult locomotor rhythmicity (Figures 5.6, 5.9-5.11). Complex rhythms observed in flies over-expressing *per* in PDF neurons (Figures 5.6 and A.3.2) resembled the behaviour of flies with hyperpolarisation of PDF neurons (Sheeba *et al.*, 2007). On the other hand, constitutive *per* over-expression in all circadian neurons except PDF neurons was shown not to affect circadian rhythmicity to the same extent (Figures 5.7 and 5.8). Similarly, flies constitutively over-expressing *per* in circadian neurons other than CRY-positive neurons (both Morning and Evening cells) also displayed the persistence of adult behavioural rhythms (Figures 5.7 and 5.8). Thus, it can be concluded that CLK/CYC function during development is necessary in the morning oscillator cells for establishing adult behavioural rhythmicity.

However, it was not possible to demonstrate that developmental *per* over-expression outside PDF or CRY positive neurons leads to normal rhythmicity of

adult flies (Figure A.3.5) as I suspect that combining GAL80 and GAL80^{ts} is not a reliable way of providing both spatial and temporal control over gene expression. The experiment that could address the original question would be a creation of the *per* over-expression flies lacking *tub$_P$Gal80^{ts}* and containing instead *Pdf-Gal80^{ts}* or *cry-Gal80^{ts}*. Using them would have allowed me to study the true effect of conditional blocking of *per* over-expression in these particular groups of neurons. Unfortunately, such genetic elements have not been created yet and the time constraints of this work prevented me from making them myself. Therefore it is my strong recommendation that whoever continues working on this project creates these lines and performs experiments on them.

PDF neurons were previously shown to be the key circadian neurons in the brain, as demonstrated by the fact that experiments involving their genetic ablation or electrical silencing resulted in severe disruption of adult behavioural rhythms (Renn *et al.*, 1999; Nitabach *et al.*, 2002 and 2005; Lin *et al.*, 2004). They were suggested to be responsible for maintaining synchrony across circadian neurons (Lin et al., 2004; Nitabach *et al.*, 2005) by providing a circadian signal to PDF-sensitive neurons (Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2000; Park *et al.*, 2000; Peng *et al.*, 2003; Lin *et al.*, 2004). For example, release of PDF caused TIM degradation in a CRY-independent pattern in five specific cells from the evening oscillator (Guo *et al.*, 2014). Yoshii *et al.* (2009c) suggested that depending on the target cell, PDF is necessary for either maintaining molecular oscillations, keeping cells synchronous or regulating speed of oscillations. Therefore I speculated that possibly the results describing behaviour of flies with constitutive *per* over-expression reported in the literature might reflect more on the developmental effects, as adult *per* over-expression in PDF neurons led to reversible adult arrhythmia.

In addition to this, flies with developmental *per* over-expression in all circadian cells (Figure 4.1) were less rhythmic as adults in permissive conditions that flies with *per* over-expression only in the PDF neurons (Figure 5.6). Therefore these results might suggest that other cells might be also sensitive to CLK/CYC inhibition to a lesser degree.

PDF neurons consist of s-LN$_v$s and l-LN$_v$s which have very distinctive properties and functions. Analysis of the constitutive *per* over-expression in various

subsets of circadian neurons (Figures 5.9 – 5.11) has offered insight in the spatial requirements for CLK/CYC activity during metamorphosis, as if some cells could be shown not to be important for adult behavioural rhythmicity with constitutive *per* over-expression, it would be impossible for the same cells to cause any adult impairment of locomotor rhythmicity if *per* over-expression in them was only developmental. This way it was possible to exclude a large number of clock cells. However, there existed a possibility that the phenotype observed was adult only.

per over-expression in l-LN_vs affected the adult behavioural rhythmicity of flies to a far lesser extent than *per* over-expression in the s-LN_vs. The relatively strong adult behavioural rhythms observed despite constitutive over-expression of *per* in l-LN_vs makes it unlikely that developmental *per* over-expression in them might be responsible for the phenotype observed when *per* is over-expressed in all PDF neurons. Flies with *per* over-expression driven by *R6-Gal4* were consistently displaying stronger phenotypes than those with *per* over-expression driven by *c929-Gal4* (Figures 5.9 – 5.11), even though *R6-Gal4* is thought to be a weaker driver (for example, *R6-Gal4* was described as weaker than *Pdf-Gal4* in Shafer and Taghert, 2009), which might explain the lack of more significant difference between the results for s-LN_vs and l-LN_vs.. Therefore this suggested that s-LN_vs are more sensitive to CLK/CYC activity during metamorphosis for generation of adult circadian rhythms.

Although *Mai179-Gal4* also targets s-LN_vs, among other neurons (Grima *et al.*, 2004), *Mai179-Gal4* – driven constitutive *per* over-expression did not offer the confirmation of this conclusion (Figures A.3.3 and A.3.4). This might, however, be due to a lower level of GAL4 expression in s-LN_vs with this driver, not sufficient to cause enough CLK/CYC inhibition or to the fact that when *Mai179-Gal4* was used to restore per^{01} by driving *UAS-per*, it did not cause stable PER oscillations in the s-LN_vs (Grima *et al.*, 2004). Moreover, it is possible that this driver was not functional, which can be tested by using it to express GFP and checking expression pattern. This control experiment should be performed to check whether this is the case. Additionally, data obtained has confirmed that *per* over-expression in the CRY-positive neurons (l-LN_vs, s-LN_vs including the 5^{th} one, some LN_ds and DN1s) led to behavioural arrhythmicity (Figures 5.9 – 5.11).

5.5.4 Absence of CLK/CYC manifests itself in s-LN$_v$s

Focusing on the role of CLK/CYC in the circadian neurons in the brain, especially in PDF neurons, I first demonstrated the lack of effect of developmental *per* over-expression on the gross morphology of circadian neurons (Figure A.3.6). Most importantly, dorsal projections from s-LN$_v$s appeared intact. A caveat of this conclusion was that this analysis was only qualitative. Another experiment using flies with *cyc* function blocked in PDF neurons showed however that CLK/CYC absence in PDF neurons was connected to the morphology of dorsal projections, since they were absent in 25% and stunted in the 37% of analysed brains (Goda, Mirowska and Currie *et al.*, 2011). Neuro-anatomical defects of LN$_v$s were previously described for the *Clk^{Jrk}* and *cyc^{01}* flies, and they were also accompanied by a reduction in PDF expression (Park *et al.*, 2000) This might suggest that in case of flies with developmental *per* over-expression there was some residual CLK/CYC activity which was enough to sustain the projections. The theory of the non-complete CLK/CYC inhibition could also explain why the phenotypes observed with conditional *cyc* rescue flies were generally more severe than those obtained with *per* over-expression. This warrants repeating experiments performed only with the *per* over-expression line using the conditional *cyc* rescue flies. These experiments would test the impact of developmental *cyc* depletion on molecular oscillations in the 'periphery', spatially map the requirement for developmental *cyc* expression and investigate neuroanatomy and molecular oscillations in circadian neurons of adult flies with developmental *cyc* deletion.

The presence of the dorsal projections (Figure A.3.6) does not necessarily mean that they function properly. Termini of the dorsal projections show circadian rhythms in their complexity and the fasciculation/defasciculation (Fernández *et al.*, 2008; Sivachenko *et al.*, 2013) and also in the levels of anti-PDF immunoreactivity (Helfrich-Förster *et al.*, 2000; Park *et al.*, 2000, Nitabach *et al.*, 2005). Since these properties were not tested, I cannot fully confirm that the PDF projections are not affected by developmental CLK/CYC inhibition.

Developmental *per* over-expression impaired TIM oscillations in s-LN$_v$s but not l-LN$_v$s or LN$_d$s of adult flies placed at permissive temperature, leaving them at constantly low levels (Figure 5.12). This could offer an explanation for the higher sensitivity of s-LN$_v$s to CLK/CYC activity impairment during

development. Lack of *cyc* expression observed in cyc^{01} mutants also resulted in molecular arrhythmia and constantly low expression levels for CLK/CYC targets, such as TIM (Rutila *et al.*, 1998).

Additionally, these results highlighted another difference between s-LN_vs and l-LN_vs. s-LN_vs are responsible for driving the morning peak in LD and also the rhythmicity in constant darkness (Renn *et al.*, 1999; Blanchardon *et al.*, 2001; Stoleru *et al.*, 2004; Sheeba *et al.*, 2009) and are considered the main pacemaker neurons. They are involved in the regulation of the phase of other clock neurons (Yoshii *et al.*, 2009c). PDF-negative 5^{th} s-LN_v and 3 LN_ds are responsible for driving the evening peak (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Rieger *et al.*, 2006) and rhythmic behaviour for some genotypes in constant light (Picot *et al.*, 2007). Cells forming the evening oscillator receive resetting signals from the morning oscillator (Stoleru *et al.*, 2005).

l-LN_vs were shown to be involved in the control of sleep and arousal in flies (Agosto *et al.*, 2008; Parisky *et al.*, 2008; Kula-Eversole *et al.*, 2010, Shand *et al.*, 2011; Gmeiner *et al.*, 2013) and also in promoting the activity of flies in response to light, especially in the morning (Shang *et al.*, 2008; Sheeba *et al.*, 2008a and 2008b; Fogle *et al.*, 2011). They have also been implicated in the adaptation to the seasonal changes by regulating the phase of the evening peak across various photoperiods (Potdar and Sheeba, 2012).

It was also suggested that CLK/CYC acts differently in s-LN_vs and l-LN_vs, for example it regulates the expression of *pdf* only in s-LN_vs (Park *et al.*, 2000; Helfrich-Förster *et al.*, 2000). Since there is only 50% identity between the genes expressed in s-LN_vs and l-LN_vs (Kula-Eversole *et al.*, 2010), this leaves a rather significant potential for other genes regulated differently by CLK/CYC. The unique sensitivity of PDF expression in s-LN_vs to CLK/CYC function suggests a possible mechanism by which developmental CLK/CYC inhibition could cause adult arrhythmicity – by permanent dysregulation of PDF production. However, qualitative analysis of the dorsal projections of s-LN_vs has detected PDF in them.

The existence of TIM molecular oscillations in l-LN_vs after 2 days in DD at permissive conditions (Figure 5.12) not only supports the hypothesis that l-LN_vs do not require CLK/CYC during the development, but it also contradicts

the previous observation that PER and TIM oscillations in l-LN$_v$s degrade rapidly in the constant darkness (Herzog *et al.*, 1998; Helfrich-Förster *et al.*, 2001; Blanchardon *et al.*, 2001; Yang and Sehgal, 2001; Shafer *et al.*, 2002; Peng *et al.*, 2003; Veleri *et al.*, 2003; Lin *et al.*, 2004; Grima *et al.*, 2004; Helfrich-Förster *et al.*, 2007a), however, *tim* mRNA rhythms persist (Peng *et al.*, 2003; Veleri *et al.*, 2003; Stoleru *et al.*, 2004 and 2005 and Sellix *et al.*, 2010). Despite lack of circadian protein oscillations, l-LN$_v$s display also stable robust circadian rhythms in electrical activity (Sheeba *et al.*, 2008b; Cao and Nitabach, 2008) and Klarsfeld et al. (2004) also reported the persistence of robust PER oscillations in l-LN$_v$s lasting 2.5 day after the transfer to DD, so within the time experiment described here was conducted.

In summary, data presented in this chapter indicates that CLK/CYC is necessary in the s-LN$_v$s during metamorphosis for the generation of the adult locomotor rhythms. Not only constitutive CLK/CYC inhibition in these neurons affected rhythmicity of flies more than CLK/CYC inhibition in l-LNvs, also s -LN$_v$s were the only among circadian neurons tested that did not display strong molecular oscillations following developmental CLK/CYC inhibition. This suggests a possible underlying mechanism for the behavioural arrhythmia, however, it is still unknown how developmental CLK/CYC inhibition can cause disappearance of molecular oscillations. Moreover, it appears that developmental CLK/CYC inhibition might lead to smaller circadian neurons (Figure A.3.7). To gain full confidence in molecular and neuroanatomical phenotypes reported in this chapter, these experiments should be repeated using conditional *cyc* rescue flies. This would serve as independent confirmation and offer definite confirmation that the phenotype observed is caused by the lack of CLK/CYC activity and not accumulation of PER.

Chapter 6: Determination of mechanisms underpinning CLK/CYC function during development – preliminary data

6.1 Introduction

Molecular oscillations that are driving circadian behaviour are also impacted by multiple post-translational modifications, such as protein acetylation, phosphorylation and methylation. Depending on the target, these modifications can either change gene expression via changing the activity of transcription factors or impact chromatin structure by modification to histone proteins (Struhl, 1998; Hall, 2003).

A chromatin is composed of DNA tightly wrapped around two pairs of each of highly conserved core histones: H2A, H2B, H3 and H4. Histone acetylation is regulated by enzymes called histone acetyltransferases (HATs), which add acetyl groups onto specific lysine residues in amino termini of histones. On the

other hand, histone deacetylases (HDACs) recruit various co-factors and remove acetyl groups (Ahringer, 2000; Ng and Bird, 2000). The DNA backbone has an overall negative charge and histones are positively charged, which ensures electrostatic attraction between them. Adding acetyl groups to the lysine residues neutralizes the positive attraction and loosens the chromatin structure (Hong *et al.*, 1993; Luger *et al.*, 1997). When a substantial amount of acetyl groups is added, the chromatin becomes much more accessible to enzymes, a state that is associated with active transcription. Histone deacetylation, on the other hand, blocks the access of the transcriptional machinery to the DNA and is also recognized by chromatin-interacting proteins that favour the formation of heterochromatin (Hansen and Wolffe, 1992; Wolffe, 1997; Struhl, 1998; Goodman and Mandel, 1998; Luo and Dean, 1999; Strahl and Allis, 2000; Gregory *et al.*, 2001; Orphanides and Reinberg, 2002; Lande-Diner and Cedar, 2005; Eskland *et al.*, 2010; Milon *et al.*, 2012).

HDACs are highly conserved in organisms and play an important role in the regulation of gene expression (Frye, 2000; Gregoretti *et al.*, 2004; Yang and Seto; 2008). So far, four different classes of HDACs have been identified based on the homology of the catalytic domains to HDACs in yeast (Gray and Ekström, 2001; Khochbin *et al.*, 2001). Classes I, II and IV represent the zinc-dependent or classic HDACs, while class III are NAD^+-dependent proteins (deRuijter, 2003; Verdin *et al.*, 2003, Blander and Guarante, 2004). Class I HDACs includes RPD3 in yeast, *D. melanogaster* and human, dHDAC3 in *D. melanogaster*, HDAC1, HDAC2, HDAC3 and HDAC8 in human (reviewed in Marks and Dokmanovic, 2005; Carey and La Thangue, 2006) and they form complexes with SIN3 (Kasten *et al.*, 1997). Class II HDACs are large (~130kDa) proteins and include HDA1, HOS1-3 in yeast, HDAC4 and HDAC6 in *D. melanogaster* and HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 in human (reviewed in Marks and Dokmanovic, 2005; Carey and La Thangue, 2006) and they can interact with MEF2 transcription factors to inhibit transcription driven by MEF2 (Wang *et al.*, 1999; Miska *et al.*, 2001). Members of class III are SIR2 from yeast, *C. elegans* and *D. melanogaster*, dSIRT2, dSIRT4, dSIRT6 and dSIRT7 in *D. melanogaster* and SIRT1-7 (reviewed in Marks and Dokmanovic, 2005; Carey and La Thangue, 2006) and are involved in the regulation of longevity, apoptosis and skeletal myogenesis (Guarente, 2001;

Luo *et al.*, 2001; Fulco *et al.*, 2003). Class IV consists of HDA11 in *C.elegans* and HDAC11 in D. *melanogaster* and humans (Gao *et al.*, 2002).

Changes in the chromatin structure have been previously shown to be critical for the regulation of transcription in other systems (Strahl and Allis, 2000; Fry and Peterson, 2001; Jenuwein and Allis, 2001; Orphanides and Reinberg, 2002; Jason *et al.*, 2002; Lachner and Jenuwein, 2002; Nathan *et al.*, 2003; Nowak and Corces, 2004). Circadian rhythms in histone acetylation have been first studied in mammals. They were shown to be present at the promoters of the core clock genes in mouse liver, where rhythmic acetylation was shown at H3, but not H4 (Etchegaray *et al.*, 2003) and later on also in mouse heart (Curtis *et al.*, 2004) and fibroblasts, also at H4 (Naruse *et al.*, 2004). This was also associated with RNA polymerase II binding, suggesting that *Per1* and *Per2* transcription is regulated by the chromatin state (Etchegaray *et al.*, 2003; Naruse *et al.*, 2004; Curtis *et al.*, 2004). Similar rhythms in acetylation have also been show for D element-binding protein (DBP) (Ripperger and Schibler, 2006) and clock-controlled output genes (Feng *et al.*, 2011).

In vivo, CLK/BMAL1 was shown to precipitate with the HAT p300 (Etchegaray *et al.*, 2003; Curtis *et al.*, 2004; Ripperger and Schibler, 2006), which is a part of the coactivator transcriptional complex participating in the acetylation of H3 (Fry and Peterson, 2001; Jenuwein and Allis, 2001; Orphanides and Reinberg, 2002), and CREB-binding protein (CBP), a transcriptional coactivator (Takahata *et al.*, 2000). CRY, which is a mammalian equivalent of PER/TIM, was also shown to decrease HAT activity by disrupting the CLK/BMAL1-p300 interaction (Etchegaray *et al.*, 2003; Naruse *et al.*, 2004).

In *D. melanogaster*, the rhythms in CLK-CYC binding to E-boxes in *per* intron 1 were found to be accompanied by the rhythms in acetylation of histone H3-K9 (with a peak at ZT10) and trimethylation of histone H3-K4 (peaking at ZT16) (Taylor and Hardin, 2008). These rhythms were correlated with the transcriptional activation by CLK/CYC, since they were absent in cyc^{01} flies with constantly low levels of acetylation. Since the same modifications had been previously discovered for mammals, this suggested that circadian histone modifications are conserved among species (Taylor and Hardin, 2008). These rhythms in H3-K9 acetylation and H3-K4 methylation were also associated with

RNA polymerase II binding to *tim* promoter and transcriptional elongation at *per* promoter, where Pol II was constitutively bound (Taylor and Hardin, 2008).

Interestingly, mammalian CLOCK was shown to have an intrinsic HAT activity (Doi *et al*., 2006). It was speculated that this could also be the case for the fly CLK, since its carboxy-terminal Q-rich domain shares a high degree of sequence similarity with the carboxy-terminal domain of ACTR, a member of SRC HAT family, which was implied to have HAT activity (Chen *et al*., 1997). Moreover, there exists a homology between CLK homologues, including CLK in *D. melanogaster* and BMAL1, and acetyl-coenzyme A binding motifs within the MYST HAT family (Doi *et al*., 2006).

In addition to this, CLK in *D. melanogaster* has been shown to interact with the CREB-binding protein (CBP) orthologue NEJIRE, which was shown in one study to act as a transcriptional coactivator (Hung *et al*., 2007) but its over-expression was shown to have inhibitory effect on CLK/CYC mediated transcription in another report (Lim *et al*., 2007b). It was suggested by the authors of that study (Lim *et al*., 2007b) that this was an example of the differences between the transcriptional regulation in flies and mammals. However, Taylor and Hardin (2008) suggested that it could be explained as the circadian feedback compensation and support NEJIRE function as coactivator.

CLOCK/BMAL1 was also shown to form complexes with JumonjiC (JmjC) and ARID (AT-rich interaction domain) domain-containing histone lysine demethylase 1a (JARID1a) (DiTacchio *et al*., 2011). JARID1a increases histone acetylation not by having HAT activity, but by inhibiting the function of histone deacetylase 1 and also causing the increase in the gene transcription driven by CLOCK/BMAL1 in a demethylase-independent way (DiTacchio *et al*., 2011). Similarly to the decrease of *Per2* expression observed when JARID1a is inhibited in mammals, inhibition of *little imaginal disks* (*lid*), JARID homolog in *D. melanogaster,* leads to lower *per* expression (DiTacchio *et al*., 2011), probably due to RPD3 HDAC inhibition (Lee *et al*., 2009b).

6.2 Aim

In the previous chapters, I have demonstrated that CLK/CYC function during metamorphosis (Figures 5.1 – 5.3) is required in the PDF-positive neurons (Figures 5.6, 5.9-5.11) for the generation of adult behavioural rhythmicity. I have also shown that developmental inhibition of CLK/CYC leads to abrogation of adult molecular oscillations in the s-LN_vs (Figure 5.12). The precise mechanism linking the function of CLK/CYC during development to the generation of aberrant adult rhythmicity, however, remained unknown. The overall goal of the research described in this chapter is to investigate mechanisms underlying CLK/CYC function during the development by testing the following three hypotheses: 1). A developmental lack of CLK/CYC activity causes epigenetic changes to the fly genome, preventing future circadian gene activation. 2) CLK/CYC activity during development is necessary for the activation of specific target genes that connect to the behavioural rhythmicity in the adulthood. 3) Phenotypes associated with the developmental *per* over-expression can be reversed by constitutive light-mediated induction of CRYPTOCHROME. The last part of the chapter aims at finding the potential gene effectors through which CLK/CYC might affect adult circadian rhythmicity during development.

6.3 Protocols

6.3.1 Inhibition of histone deacetylases in flies with developmental *per* over-expression

Conditional *per* over-expression flies were divided into two groups, one of which was allowed to mate in vials containing NaBu (100 mM or 30 mM) and another in vials with control food (standard medium described in Methods and Materials diluted with water instead of NaBu to the same concentration). After embryos were found in vials, parents were removed and flies were allowed to develop at restrictive temperature (29˚C) or, in case of flies tested with 40 mM, either permissive (23˚C) or restrictive temperature. Within two days of hatching, flies were loaded into cuvettes for analysis of the locomotor rhythms.

Half of flies from each group were tested in the cuvettes containing 100mM NaBu in the sucrose-agar medium and another half was added to the control cuvettes with water added in place of NaBu, bringing the number of experimental groups up to four. Flies were first entrained to LD cycles at 17˚C for seven days and subsequently moved to constant darkness at 17˚C for the analysis of their free-running rhythms. Activity for individual flies was analysed as described in 2.3. The distribution of rhythmic, weakly rhythmic and arrhythmic flies between populations was tested with the 2x2 Fisher's Exact tests (for 100 mM NaBu), 3x2 Fisher's Exact test (for 30 mM NaBu to compare distribution of flies raised at different temperature with the same developmental and adult treatment) or 4x2 Fisher's Exact test or 4x3 Chi-square tests (for 30 mM NaBu to compare flies raised at the same temperature with different NaBu treatments). The association of the average relative rhythmic power with the conditions was tested with the non-parametric Mann-Whitney or Kruskal-Wallis test.

6.3.2 Impact of 30 mM NaBu on the longevity of flies

Flies with conditional *per* over expression and control wild-type flies (*y w*) were seeded at room temperature in vials with 30 mM NaBu and on control food diluted with water to the same concentration. After three days, flies were moved to other vials with the same food and the first set of vials was left at room temperature. 3 days later, flies were discarded from the second set of vials and it was moved to 29˚C.

Flies from both conditions eclosed at the same time and young old male flies from each genotype, type of food and developmental temperature were collected and divided into two further groups – half were placed in fresh vials (10 males/vial) with 30 mM NaBu and the other half was added to the water-diluted food. Vials were kept at 17˚C LD. The survival of flies was analysed as described in 2.4 using all log rank for trend, Mantel-Cox and Gehan-Breslow-Wilcoxon comparisons. To compare impact of individual treatments, all conditions were compared to each other using Gehan-Breslow-Wilcoxon and Mantel-Cox test.

6.3.3 Locomotor behaviour of *per* over-expression flies raised at constant light

Flies with conditional *per* over-expression were raised at either 29˚C LL or 29˚C DD and tested in two different protocols, both involving 7 days of entrainment at 17˚C LD and subsequent testing at permissive conditions (17˚C DD). In one of the protocols flies were additionally kept as adults at restrictive conditions (29˚C DD) for 7 days before entrainment at 17˚C LD. Locomotor rhythms were tested using first full seven (or six in case of the analysis at 29˚C DD) days of DD as described in 2.3. The distribution of flies raised in LL and DD was compared using Fisher's Exact 2x2 test. The average relative rhythmic power values were compared with the non-parametric Mann-Whitney test.

6.3.4 Locomotor behaviour of flies with developmental PER over-expression in PDF-negative circadian neurons raised at constant light

Flies with conditional PER over-expression in PDF-negative neurons (described previously in 5.4.4) were raised at RT (permissive conditions), 29˚C DD (restrictive conditions) or 29˚C LL (restrictive conditions). Flies were analysed at permissive conditions of 17˚C and at restrictive conditions of 29˚C, in both cases both in both LL and DD, as described in 2.3, using first full seven days at each condition. Flies were moved directly to testing conditions, without prior light/dark entrainment due to anticipated poor survival of flies raised at 29˚C.

Association between genotype and the distribution of rhythmic, weakly rhythmic and arrhythmic flies was tested for flies raised under the same conditions with Fisher's Exact 3x2 tests and the association between the raising conditions and the distribution for flies of the same genotype was tested with Fisher's Exact 3x3 tests. Where average period lengths or relative rhythmic power could be determined, their association with the genotype was tested with the non-parametric Mann-Whitney test and the association between the period length or rrp and the developmental conditions was tested with either non-parametric Mann-Whitney or Kruskal-Wallis test.

6.3.5 Knockdown of the candidate genes (first part of the screen)

Male flies from selected *dsRNA* lines (*dsRNA* connected to GAL4-responsive *UAS*) coming from either Bloomington Stock Centre at Indiana University (5-digit stock number included), Vienna *Drosophila* RNAi Centre (5-digit stock number starting with "v" included) or Paul Hardin's laboratory (*ds Pdp1εB* line, with B denoting specific *dsRNA* used) were crossed to virgins flies which expressed *Gal4* driver in all clock-containing cells (*tim(UAS)-Gal4,* annotated as *TUG*) in a *Dicer-2* (*Dcr-2*) background to ensure the best knockdown. Flies were raised at room temperature and as adults entrained for 7 days in a 12:12 LD cycles at either 23˚C (*Dcr-2;TUG>ds Pdp1εB, Dcr-2;CyO>ds Pdp1εB* and some *Dcr-2;tim(UAS)Gal4/CyO* control flies) or 25˚C (rest of *Dcr-2;tim(UAS)Gal4/CyO* control flies and all other lines) and then moved into constant darkness at the same temperature.

Flies were analysed using first full seven days at constant conditions as described in 2.3. Distribution of rhythmic, weakly rhythmic and arrhythmic flies among experimental flies from each genotype (annotated as *Dcr-2;TUG>ds RNA*) was compared both to control flies expressing *dsRNA* element but without *Gal4* driver (*Dcr-2;CyO>ds RNA*) and to *Dcr-2;tim(UAS)-Gal4/CyO* flies using 3x2 Fisher's Exact tests. The average period length and relative rhythmic power were compared between flies expressing *dsRNA* with or without GAL4 using Mann-Whitney test and between flies with the expressed *dsRNA* element and *Gal4* driver line without *dsRNA* with Wilcoxon Rank test with Bonferroni correction.

6.3.6 Conditional knockdown of the candidate genes (second part of the screen)

Flies carrying *dsRNA* element (as described in 6.3.5) were crossed with flies carrying *tim(UAS)-Gal4* and $tub_{P}Gal80^{ts}$ in a *Dcr-2* background. Offspring were raised under permissive conditions (17˚C or RT) or restrictive conditions (29˚C). Adult flies were entrained for 7 days to the 12:12 LD cycles at permissive temperature (17˚C) and subsequently released into constant darkness at permissive temperature. One group of flies raised at 17˚C was additionally

exposed to 29˚C DD for 7 days before entrainment and subsequent testing at permissive conditions. Locomotor behaviour of flies was analysed using first full seven days at 17˚C as described in 2.3. Distribution of rhythmic, weakly rhythmic and arrhythmic flies was compared using Fisher's Exact 3x2 tests and average relative rhythmic power and period length were compared between groups using either Mann-Whitney test or Kruskal-Wallis test with pairwise Wilcoxon Rank test. Following comparisons were performed (where RT, 17˚C and 29˚C denote raising conditions and 17˚C>29˚C>17˚C denotes flies raised at 17˚C and exposed to 29˚C as adults): RT-29˚C, RT-17˚C, 17˚C-29˚C, 17˚C-17˚C>29˚C>17˚C, 29˚C-17˚C>29˚C>17˚C.

6.3.7 Locomotor behaviour of flies with conditional over-expression of *per* and the candidate gene

Flies with developmental *per* over-expression were crossed to either flies with the gene of interest linked to *UAS* or to flies with the *UAS-transgene* combined with either *TUG* or *UAS-per*. Control flies were either flies coming from crossing *per* over-expressing flies to w^{1118} flies or from crossing *per* over-expressing flies to lines with UAS-transgenes and selecting flies with a balancer chromosome in place of the gene of interest. Flies were raised either at permissive (RT) or restrictive temperature (29˚C). Adult flies were first entrained to LD cycle at permissive conditions (17˚C) for 7 days and subsequently moved to constant darkness at 17˚C to study the function of their molecular oscillator. The first full seven days of constant conditions were used to analyse flies individually as described in 2.3. Distribution of flies was compared between flies of the same genotype raised at different temperatures with Fisher's Exact 3x3 tests or between experimental flies and control flies raised at the same temperature either with 3x2 Fisher's Exact tests (for flies heterozygous for *TUG* and *UAS-per*) or with chi-square 4x3 tests for females and Fisher's Exact 3x2 tests for males (for flies homozygous either for *TUG* or *UAS-per*). Association of the average period length of all rhythmic flies and average relative rhythmic power with the genotype or developmental temperature of flies was compared with either non-parametric Mann-Whitney tests (when 2 groups were compared) or Kruskal-Wallis tests.

6.4 Results

6.4.1 Blocking histone deacetylases does not restore the behavioural rhythmicity of flies with developmental PER over-expression

In order to address the possibility that developmental CLK/CYC inhibition affected adult behavioural rhythmicity through chromatin modifications, I tested whether chemical inhibition of HDAC during development improved adult rhythmicity of flies with conditional *per* over-expression. I used sodium butyrate (NaBu), which is a simple fatty-acid that inhibits a wide range of HDACs in D. *melanogaster*, including most of the zinc binding enzymes from class I, II and IV, but not HDAC6 (Hubbert *et al.*, 2002; Cho *et al.*, 2005; Witt *et al.*, 2009; Chuang *et al.*, 2009). It does not target *Sir2* (Cho *et al.*, 2005). Another advantage of NaBu was that it could be easily administered to both larvae and adults as an aqueous solution added to the food.

Since some reports (Dey-Guha and Kar, 2001) suggested that a large concentration of NaBu was required to elicit strong responses I performed the initial experiment using 100 mM NaBu concentration in the food. Conditional *per* over-expression flies were raised at restrictive temperature of 29˚C and tested at permissive conditions (17˚C) as described in 6.3.1.

During the development of flies, I did not observed a significant difference between numbers of larvae in vials with different foods. However, there were fewer pupae in the vials containing NaBu. Two days after flies eclosed, I found a lot of adult flies in the vials with water, but only a few flies in the vials with NaBu. There were some empty pupal cases and dead adults at the bottom of the vials (which were not stuck in food). Additionally, the majority of the pupae were darker, which was an indication that the imago has died. Therefore, I concluded that the high concentration of NaBu might affect the development of flies at both larval and pupal stages.

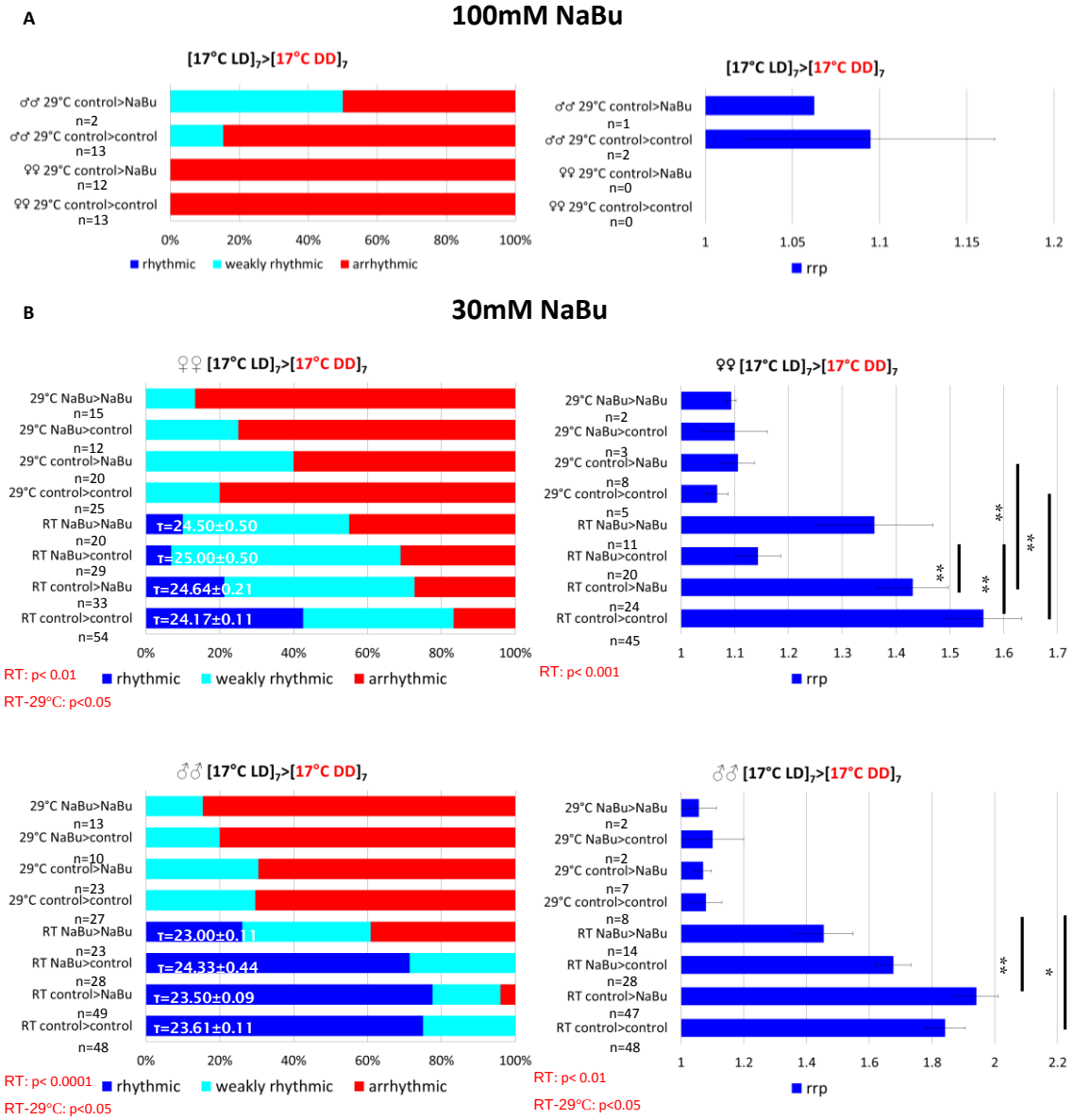


Figure 6.1. Inhibition of HDACs did not improve the rhythmicity of flies with conditional *per* over-expression.

Individual analysis of the conditional *per* over-expression flies raised at either RT (permissive conditions) or at 29˚C (restrictive). Flies were either raised and tested on the standard food (control/control), were treated with NaBu only during the development (NaBu/control), treated with NaBu only as adults (control/NaBu) or received NaBu both during development and adulthood (NaBu/NaBu) either at 100 mM concentration (A) or at 30 mM (B). Stacked bar diagrams on the

left show the distribution of the rhythmic, weakly rhythmic and arrhythmic flies for each of the conditions tested, with the number of flies and the average period length (±SEM) of the rhythmic flies included on the graphs. Experimental knockdown flies were compared to controls of the same sex (A) or flies raised at 29˚C were compared of flies raised at RT (B) of the same gender and NaBu treatment using Fisher's Exact 2x2 or 3x2 test. On the right side, bar diagrams represent the average relative rhythmic power of rhythmic and weakly rhythmic flies with the error bars representing SEM. Period lengths and rrp were compared between the same groups using non-parametric Mann-Whitney (for comparison of two group only) or Kruskal-Wallace test (for comparison of more than two groups). Results of all the comparisons are below the graphs with the condition or group showing the significant result named or, if all conditions showed statistical difference, the largest p value for all four conditions. Individual significant results for the rrp values are marked with the black line * $p<0.05$, ** $p<0.01$.

As no *per* over-expression flies exposed to NaBu during development were recovered, I decided to use only the data on behaviour of flies exposed to 100mM NaBu during adulthood to check if this could impact their adult rhythmicity. To obtain data on the impact of HDACs inhibition during development, I repeated the experiment as described in 6.3.1 using lower concentration of NaBu – 30mM.

As mentioned previously, the results obtained with 100 mM NaBu (Figure 6.1 A) were incomplete, especially for males, for which only low numbers were recovered after the adult exposure to NaBu. Results obtained for females suggested that the adult HDAC inhibition was not sufficient to restore the adult behavioural arrhythmia observed in flies with developmental *per* over-expression.

Neither developmental nor adult treatments with 30 mM NaBu improved the adult behavioural rhythmicity of flies with developmental *per* over-expression (Figure 6.1 B). In all cases no rhythmic flies were recovered and weakly

rhythmic flies had very low relative rhythmic power. When compared to flies raised at room temperature undergoing the same NaBu treatment, in all cases there was a significant association between the distribution of the rhythmic, weakly rhythmic and arrhythmic flies and the developmental temperature (4 independent Fisher's Exact 3x2 tests: females: from $p < 0.05$ to $p < 10^{-7}$, n=36, 38, 72 and 75; males: $p < 0.05$ to $p < 10^{-15}$, n=35, 41, 53 and 79).

In all cases, the biggest differences due to developmental temperature were observed for flies raised with no NaBu treatment and the smallest were found for flies exposed to NaBu during both development and adulthood (Figure 6.1 B). NaBu did not reverse adult locomotor arrhythmia of flies with developmental *per* over-expression and the most apparent impact of NaBu was a negative effect on adult locomotor rhythmicity. Especially when flies were exposed to NaBu during development (females) or both during development and adulthood (males), the rhythmicity of flies decreased when compared to flies with no NaBu exposure (Figure 6.1 B). For both females and males raised at RT, the distribution of rhythmic, weakly rhythmic and arrhythmic flies showed significant association with NaBu treatment (Chi-square test, df=6: $p < 0.01$, n=136 for females and $p < 0.0001$, n=148 for males). Also, the relative rhythmic power showed similar correlation (Independent samples Kruskal – Wallis: $H(3)=19.923$, $p < 0.001$, n=100 for females, $H(3)=15.507$, $p < 0.01$, n=137 for males). Interestingly, the period length of the rhythmic flies did not vary with the NaBu treatment.

As the treatment with NaBu decreased the strength of adult behavioural rhythmicity (but not the period length) and results showing potential toxicity of 100 mM NaBu (Figure 6.1 A), I wondered if the behavioural phenotype after NaBu exposure was associated with a shortened life span (for information on the correlation between the health of flies and locomotor rhythms see discussion in 4.5.1). Therefore, I decided to test the impact of 30 mM of NaBu on the longevity of *per* over-expressing flies and control wild-type flies (*y w*) as described in 6.3.2.

Data revealed a clear association between the NaBu treatment and the survival of flies with conditional *per* over-expression (Figure 6.2). For *per* over-expression flies raised at RT (Figue 6.2 A), all three measures returned $p < 0.0001$ (Mantel-Cox, df=3, $p < 0.0001$, n=200; log rank for trend, df=1,

p<0.0001, n=200; Gehan-Breslow-Wilcoxon, df=3, p<0.0001, n=200). It seems that adult NaBu treatment was detrimental to the health of flies, since flies with adult NaBu treatment died first (median survival 55.5 day; when compared to flies with no NaBu exposure, Mantel-Cox, df=1, p<0.0001, n=100 and Gehan-Breslow-Wilcoxon, df=1, p<0.0001, n=100). This seemed improved by the addition of developmental NaBu treatment, as it increased median survival to 101 days (when compared to flies with adult NaBu exposure, Mantel-Cox, df=1, p<0.0001, n=100 and Gehan-Breslow-Wilcoxon, df=1, p<0.0001, n=100). Interestingly, developmental NaBu treatment did not increase lifespan of flies on its own as a median survival of 114 days was observed for both flies with or without developmental NaBu exposure when flies were on control food as adults (Mantel-Cox, df=1, p=0.362, n=100 and Gehan-Breslow-Wilcoxon, df=1, p=0.347, n=100). This can suggest the existence of a 'pre-selection' effect, meaning that developmental NaBu exposure selectively kills weaker flies, and only stronger flies eclose and therefore they might live longer.

A similar pattern was observed for flies with conditional *per* over-expression raised at 29°C (Figure 6.2 B). Again, all trends were clearly different from each other (Mantel-Cox, df=3, p<0.0001, n=200; log rank for trend, df=1, p<0.0001, n=200; Gehan-Breslow-Wilcoxon, df=3, p<0.0001, n=200). Flies with only adult exposure to NaBu died first (median survival of 16 days). Again, median survival of flies treated with NaBu during development prior to adult exposure was increased to 34.5 days (when compared to flies with NaBu exposure only during adulthood, Mantel-Cox, df=1, p<0.0001, n=100 and Gehan-Breslow-Wilcoxon, df=1, p<0.0001, n=100). Moreover, flies exposed to NaBu during both development and adulthood were once more almost indistinguishable from flies not exposed to NaBu at all (median survival 34.5 and 46 days, respectively; Mantel-Cox, df=1, p=0.152, n=100 and Gehan-Breslow-Wilcoxon, df=1, p=0.213, n=100). Contrary to the results for RT, however, developmental - only NaBu exposure clearly extended survival (when compared to flies with no NaBu exposure, median survival 51 and 46 days, respectively; Mantel-Cox, df=1, p<0.01, n=100 and Gehan-Breslow-Wilcoxon, df=1, p<0.01, n=100).

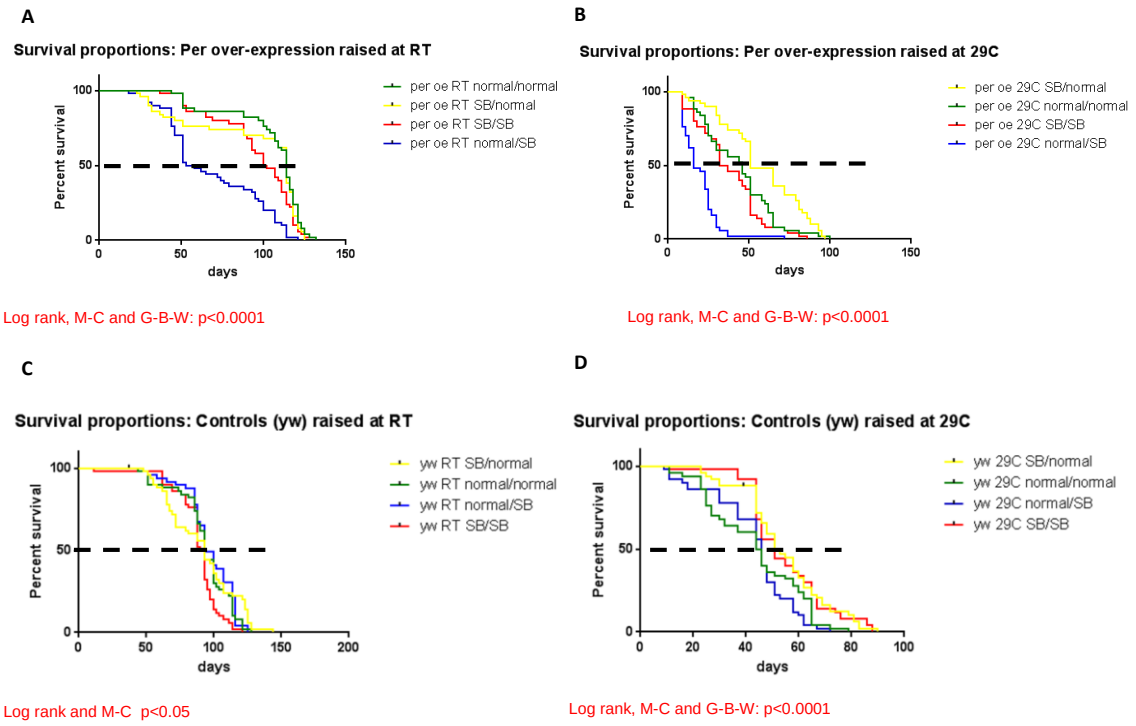


Figure 6.2. Impact of NaBu treatment on survival of flies with or without developmental *per* over-expression.

When analysed at 17˚C, survival of male flies with conditional *per* over-expression (A and B, per oe) was associated with both developmental temperature and NaBu treatment. Survival of flies with *per* over-expression (A and B) and control flies (*y w*) (C and D) raised at either RT (A and C) or 29˚C (B and D). Mantel-Cox and Gehan-Breslow-Wilcoxon were used to check for the correlation between treatment and survival. The dashed horizontal line marks median survival (the time point coinciding with 50% survival). Developmental and treatment conditions on the legend as "developmental/treatment" are indicated - with "SB" representing NaBu and "normal" denoting control food. For all groups tested, n=49 or 50. P values below the graphs correspond to comparison of all 4 conditions; "log rank" means long rank for trend, "M-C" means Mantel-Cox and "G-B-W" means Gehan-Breslow-Wilcoxon.

Except the anticipated differences in the survival of flies raised at different temperatures, there seemed to be less impact of the different NaBu treatments on survival of *y w* control flies (Figure 6.2 C and D). In case of flies raised at RT (Figure 6.2 C), even though both Mantel-Cox (df=3, p=0.0163, n=199) and log rank for trend (df=1, p=0.0189, n=199) reveal significant difference between trends, the traces are grouped close together (median survival of flies raised on NaBu and kept on normal food as adults was 91.5 days, in all other treatment groups it was 93 days). For the *y w* control flies raised at 29˚C (Figure 6.2 D), the differences are more prominent and all measures of significant difference return p<0.0001 (Mantel-Cox, df=3, p<0.0001, n=199; log rank for trend, df=1, p<0.0001, n=199; Gehan-Breslow-Wilcoxon, df=3, p<0.0001, n=199). The median survival for flies never exposed to NaBu was 45 days, for flies exposed to NaBu only during adulthood 46 days and for flies exposed to NaBu during development (with or without adult exposure) 51 days. From the trace analysis, it seems that the developmental NaBu treatment extends the lifespan (when compared to flies not exposed to NaBu; Mantel-Cox, df=1, p<0.01, n=99; Gehan-Breslow-Wilcoxon, df=1, p<0.01, n=99) but adult treatment is neutral.

To summarise, data presented in this section does not support the hypothesis that the adult behavioural arrhythmia in flies with developmental *per* over-expression is not caused by the developmental histone deacetylation as inhibition of HDAC during development did not improve adult behavioural rhythmicity (Figure 6.1). Interestingly, NaBu treatment was shown to affect lifespan of flies (Figure 6.2).

6.4.2 Constant light during development does not reverse adult behavioural arrhythmicity caused by developmental PER over-expression

As described in detail earlier (1.3.5.1), light causes changes to the molecular oscillator, in part by the action of the circadian photoreceptor CRY (Emery *et al*., 1998; Emery *et al*., 2000b). After CRY undergoes a conformational change (Lin *et al*., 2001; Ozturk *et al*., 2011), it binds to TIM and activates its proteosomal degradation (Myers *et al*., 1996; Ceriani *et al*., 1999; Naidoo *et*

al., 1999; Busza *et al*., 2004). In the absence of TIM, PER can be also proteosomally degraded following DBT-mediated phosphorylation (Lee *et al*., 1996; Zeng *et al*., 1996; Grima *et al*., 2002; Ko *et al*., 2002). As flies with no PER during development were able to display adult behavioural rhythmicity (Figure 3.3), I hypothesised that the use of the constant light during development might enhance PER degradation and therefore rescue adult behavioural rhythms of *per* over-expressing flies.

Flies with conditional *per* over-expression were raised at either 29˚C LL or 29˚C DD and analysed at either permissive (17˚C) or restrictive (29˚C) temperature as described in 6.3.3. Unfortunately poor health of flies raised at 29˚C led to lack of data for females for some condition and poor statistical power for other. Interestingly, contrary to my hypothesis, the analysis revealed that adult rhythms are disrupted by developmental *per* over-expression regardless of environmental light treatment (Figure 6.3). There were no differences between the rhythmicity of either females or males raised in different light conditions, both in terms of the distribution of flies over rhythmic categories (Fisher's Exact 2x2 test: p=0.455, n=37 for females analysed at 17˚C (A); p=0.170, n=24 for males analysed at 17˚C (A), p=0.456, n=35 for males analysed at 29˚C (B) and p=0.633, n=29 for males analysed at 17˚C after adult exposure to 29˚C (C)) and in terms of average relative rhythmic power (Mann-Whitney test: U=3.000, z=0.707, p=1.000, n=5 for females analysed at 17˚C (A); U=4.000, z=0.000, p=1.000, n=6 for males analysed at 17˚C (A), U=99.000, z=0.000, p=1.000, n=29 for males analysed at 29˚C (B)). Moreover, it turned out that regardless of developmental light conditions, flies have shown a significant association between testing condition and rhythmicity (for the distribution of flies, Fisher's Exact 2x3 test: females raised in LL - $p<10^{-10}$, n=112; males raised in DD – $p<0.001$, n=40; males raised in LL – $p<10^{-5}$, n=48). The unexpected result was that flies analysed at restrictive conditions displayed stronger rhythms than those analysed at permissive conditions.

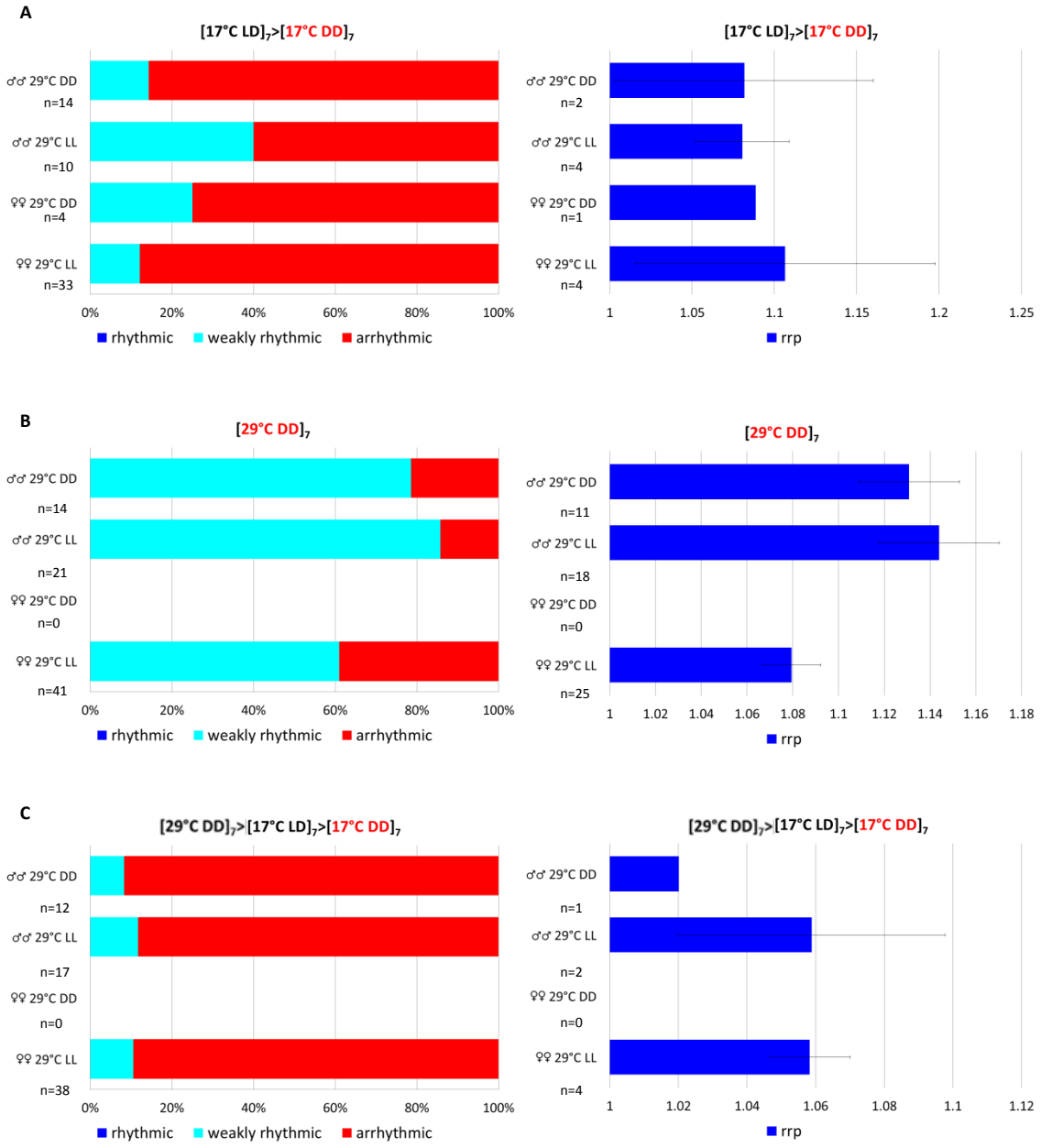


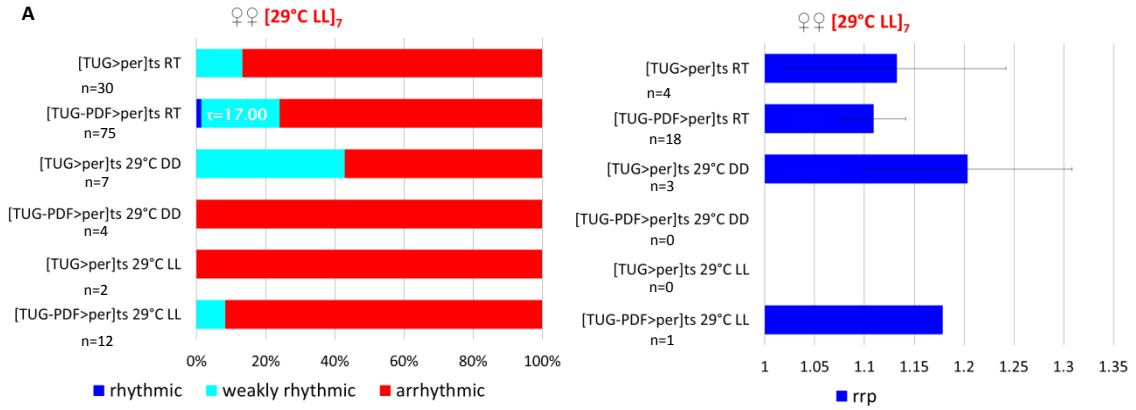
Figure 6.3. Constant light during development does not restore the adult behavioural arrhythmicity of flies with conditional *per* over-expressions.

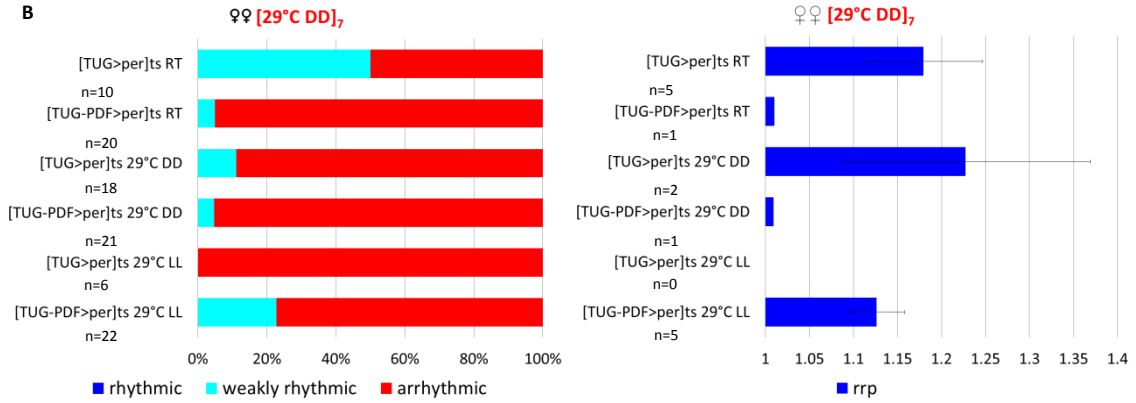
Flies with conditional *per* over-expression raised under restrictive conditions of 29˚C were mainly arrhythmic or very weakly rhythmic as adults, regardless of the light conditions during development. Quantitative results of the comparison between *per* over-expressing flies analysed at permissive conditions of 17˚C DD (A), restrictive conditions of 29˚C DD (B) and 17˚C DD following adult exposure to

restrictive conditions (C). Results on the left hand side are the stacked bar diagrams representing the percentage of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies tested for each condition are included on the graphs. Correlation between the distribution of flies and the developmental conditions were analysed by the Fisher's Exact 2x2 test. On the right hand side, bar diagrams show the average relative rhythmic power determined for all weakly rhythmic flies, with the numbers of flies used included on the graph and error bars representing SEM. An association between the average relative rhythmic power and the developmental conditions was tested with the non-parametric Mann-Whitney test.

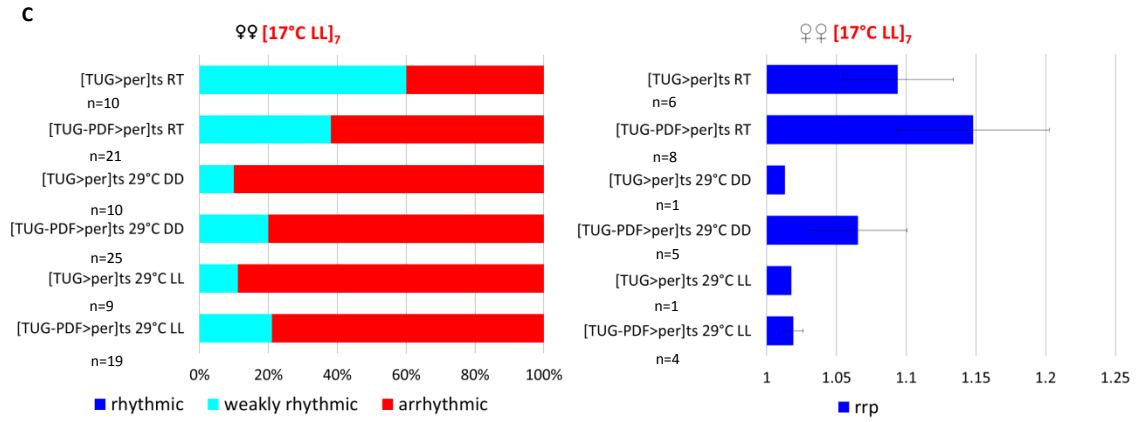
While looking for the possible explanation for the lack of effect of constant light during development on the adult rhythmicity of *per* over-expression flies, I encountered a paper that suggested that clock function, which is normally disrupted in constant light, can be restored in the presence of selective PER over-expression in PDF-negative clock neurons (Stoleru *et al*., 2007). Therefore I hypothesized that combining constant light exposure with targeted *per* over-expression in non-PDF clock neurons during development would result in adult behavioural rhythmicity. Thus, I decided to test the adult behavioural phenotype arising after combining developmental exposure to constant light with developmental over-expression of *per* in non-PDF clock cells.

Flies with conditional PER over-expression in PDF-negative neurons (as described in 5.4.4) were raised at permissive or restrictive conditions (with either constant light or constant darkness) and analysed at either permissive or restrictive conditions, either at LL or DD, as described in 6.3.4. When analysed at constant light (Figures 6.4 A and C and 6.5 A and C), presence of *Pdf-Gal80* appeared to make no difference, as the majority of the adult flies analysed were arrhythmic, both at permissive and restrictive temperatures. At 29˚C LL (Figures 6.4 A and 6.5 A), the rare rhythmic flies recovered had period lengths outside of the normal circadian range (17.00 hrs for females and 31.50 hrs for males). No difference between either the distribution of flies or relative rhythmic power and genotype or developmental conditions was found. At 17˚C LL (Figures 6.4 C and 6.5 C), again, the majority of flies were arrhythmic.

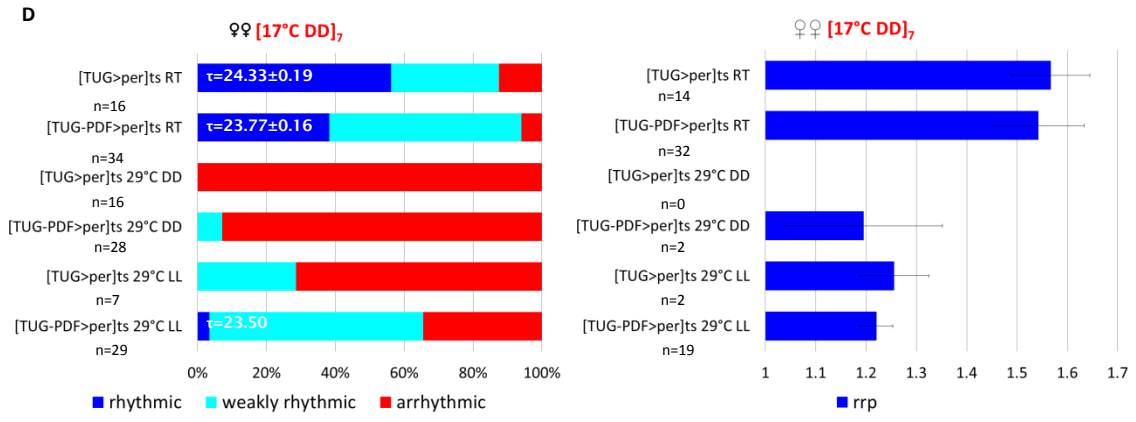




RT: p<0.01 [TUG>per]ts: p<0.05



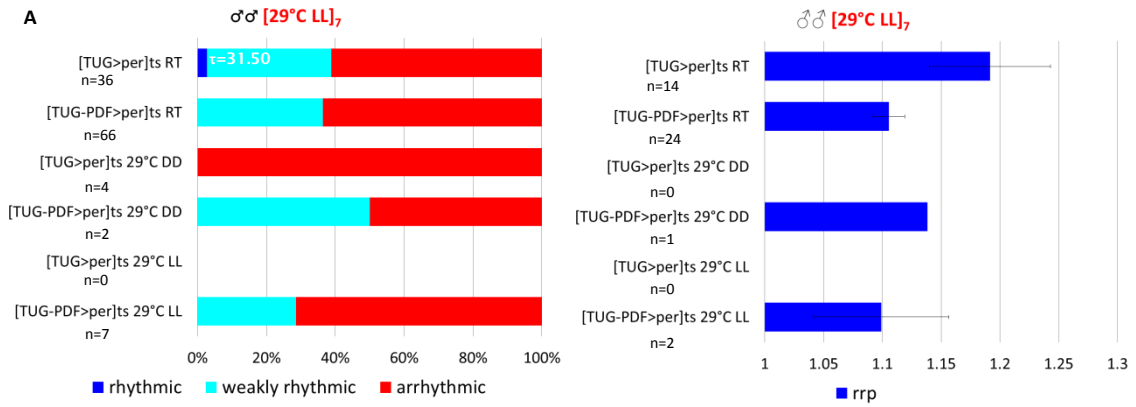
[TUG>per]ts: p<0.05

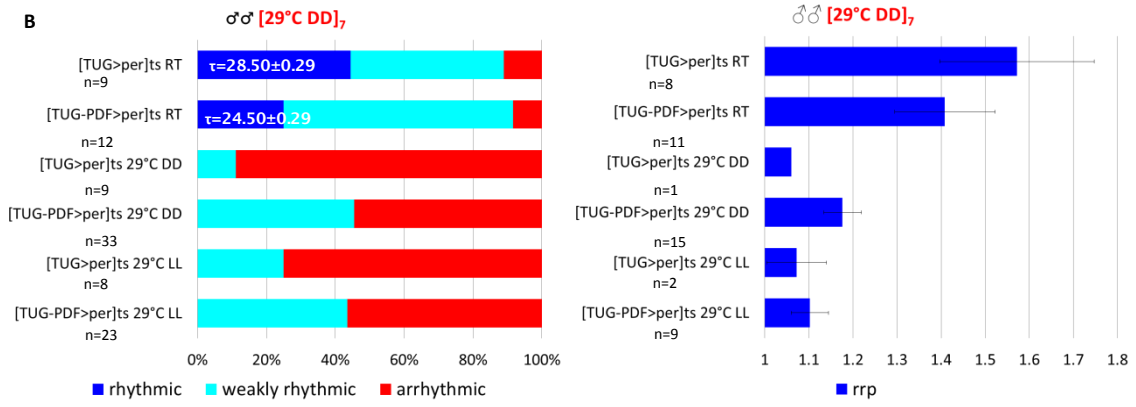


[TUG>per]ts: $p<10^{-6}$, [TUG-PDF>per]ts: $p<10^{-12}$

Figure 6.4. Constant light during development does not rescue adult rhythmicity of flies with conditional *per* over-expression outside PDF+ neurons – results for females.

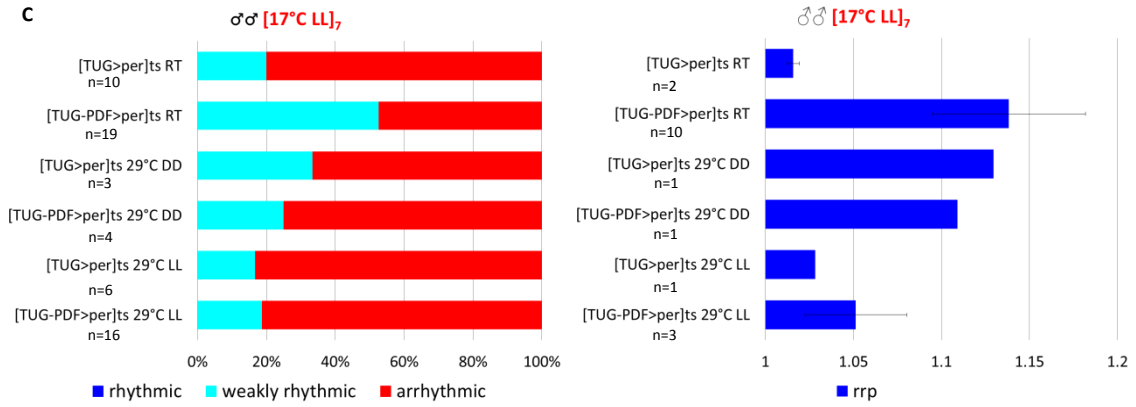
Quantitative adult locomotor rhythmicity analysis of flies with *per* over-expression in all circadian neurons ([TUG>*per*]^{ts}) and in the PDF-negative circadian neurons ([TUG-PDF>*per*]^{ts}) raised under permissive conditions (RT) and restrictive conditions in constant darkness (29˚C DD) and constant light (29˚C LL). Flies were analysed at restrictive temperature with the addition of constant light (29˚C LL, A), restrictive temperature in constant darkness (29˚C DD, B), permissive temperature with constant light (17˚C LL, C) and permissive temperature in constant darkness (17˚C DD, D). On the left, stacked bar diagrams show the percentage of rhythmic, weakly rhythmic and arrhythmic flies, with the numbers of flies tested for each condition included on the graphs. Also included are the average period length values for the rhythmic flies (±SEM). Flies were tested for the association between the distribution of flies raised under the same conditions and the genotype with the Fisher's Exact 2x3 test and for the association between the distribution and developmental conditions for flies with the same genotype with the Fisher's Exact 3x3 test and the results of the significant correlations are listed below graphs. To compare the period length or the average period length association with the genotype of flies, non-parametric Mann-Whitney tests were employed and the association between the average period length and the developmental conditions was tested with the non-parametric Kruskal-Wallis test with Wilcoxon Rank test for individual comparisons. Bar diagrams on the right show the values of average relative rhythmic power of rhythmic and weakly rhythmic flies with the error bars representing SEM and the number of flies tested written on the graphs.

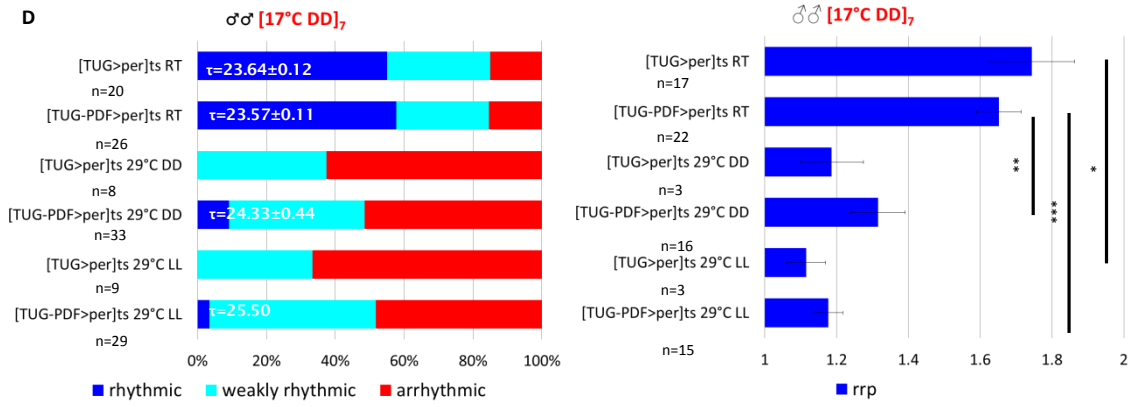




[TUG>per]ts: p<0.01, [TUG-PDF>per]ts: p<0.01

[TUG-PDF>per]ts: p<0.05





[TUG>per]ts: p<0.05, [TUG-PDF>per]ts: $p<10^{-4}$

[TUG>per]ts: p<0.05, [TUG-PDF>per]ts: p<0.001

Figure 6.5. *per* over-expression in all circadian cells except PDF-positive neurons does not rescue adult behavioural arrhythmicity associated with constant light – results for males.

Quantitative adult locomotor rhythmicity analysis of flies with *per* over-expression in all circadian neurons ([TUG>*per*]^{ts}) and in the PDF-negative circadian neurons ([TUG-PDF>*per*]^{ts}) raised under permissive conditions (RT) and restrictive conditions in constant darkness (29˚C DD) and constant light (29˚C LL). Flies were analysed at restrictive temperature with the addition of constant light (29˚C LL, A), restrictive temperature in constant darkness (29˚C DD, B), permissive temperature with constant light (17˚C LL, C) and permissive temperature in constant darkness (17˚C DD, D). On the left, stacked bar diagrams show the percentage of rhythmic, weakly rhythmic and arrhythmic flies, with the numbers of flies tested for each condition included on the graphs. Also included are the average period length values for the rhythmic flies (±SEM). Flies were tested for the association between the distribution of flies raised under the same conditions and the genotype with the Fisher's Exact 2x3 test and for the association between the distribution and developmental conditions for flies with the same genotype with the Fisher's Exact 3x3 test and the results of the significant correlations are listed below graphs. To compare the period length or the average period length association with the genotype of flies, non-parametric Mann-Whitney tests were employed and the association between the average period length and the developmental conditions was tested with the non-parametric Kruskal-Wallis test with Wilcoxon Rank test used for individual comparisons. Bar diagrams on the right show the values of average relative rhythmic power of rhythmic and weakly rhythmic flies with the error bars representing SEM and the number of flies tested written on the graphs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

At restrictive conditions (29˚C DD) results varied from males (Figure 6.5 B) to females (Figure 6.4 B). For females, the majority of flies were arrhythmic, with no rhythmic flies recovered (Figure 6.4 B). Females raised at RT displayed an association between the genotype and the distribution of flies, with more weakly rhythmic flies without *Pdf-Gal80* element (Fisher's Exact 2x2 test: $p<0.01$, n=30). Due to the improved rhythmicity of females without *Pdf-Gal80* raised at RT, there was also an association between developmental conditions and the distribution of flies observed for them (Fisher's Exact 2x3 test: $p<0.05$, n=34). On the other hand, males raised at RT did not show an association between the genotype and the rhythmicity of flies but had a much smaller percentage of arrhythmic flies with a good number of rhythmic flies recovered (Figure 6.5 B). Due to this improved rhythmicity of males raised at RT, both flies with *Pdf-Gal80* and without *Pdf-Gal80* showed an association between the developmental conditions and the rhythmicity of flies (Fisher's Exact 3x3 test: $p<0.01$, n=68 for flies with *Pdf-Gal80;* $p<0.01$, n=26 for flies without *Pdf-Gal80*). Due to a higher number of flies, a significant association between relative rhythmic power and developmental conditions was only found for flies with *Pdf-Gal80* (Kruskal-Wallis test: $H(2)=6.561$, $p<0.05$, n=36) (Figure 6.5 B).

When analysed at permissive conditions (17˚C DD), flies raised at RT had better rhythmicity than flies from other conditions, with a good proportion of rhythmic flies displaying circadian period lengths and low percentage of arrhythmic flies (Figures 6.4 D and 6.5 D). Flies without *Pdf-Gal80* raised under restrictive conditions (29˚C) displayed similar rhythmicity, regardless of light conditions during development. It appeared, however, that in the presence of *Gal80* element, females raised in LL had better rhythmicity than females raised in DD, as demonstrated by a reduction in the percentage of arrhythmic flies (Fisher's Exact 3x2 test: $p<10^{-5}$, n=57) but not by an increase in the relative rhythmic power (Figure 6.4 D). This was, however, not observed for males (Figure 6.5 D). When analysed for the association of the rhythmicity with the developmental conditions, it was present for both flies with and without *Pdf-Gal80* (Fisher's Exact 3x3 test: flies with *Pdf-Gal80*: $p<10^{-12}$, n=91 for females and $p<10^{-4,}$ n=88 for males; flies without *Pdf-Gal80*: $p<10^{-6}$, n=39 for females and $p<0.05$, n=37 for males when analysed for the distribution of flies. Moreover, males showed an association between the relative rhythmic power and the developmental conditions for both flies with *Pdf-Gal80* (Kruskal- Wallis

test: H(2)=22.607, p<0.001, n=53) and without *Pdf-Gal80* (Kruskal-Wallis test: H(2)=9.154, p<0.05, n=23).

Overall, these results suggest that *Pdf-Gal80* in this genotype with developmental *per* over-expression does not have the same effect as in the experiments described by Stoleru *et al*. (2007). This might be due to a presence of *tubP-Gal80^{ts}* and its hypothetical dominant impact on GAL4 activity at both restrictive and permissive temperatures (as discussed at 5.4.3).

6.4.3 Screen for genes mediating developmental requirement for CLK/CYC activity

6.4.3.1 Design of the screen

The General Introduction described the function of CLK and CLK/CYC heterodimer as a transcriptional activator, acting through the bHLH domain and targeting E-box sequences (CACGTG) in promoter elements (1.3.4.2 and 1.3.4.3). Therefore, it was reasonable to assume that the developmental requirement for CLK/CYC function might mean that the function of one or more of the downstream targets (direct or indirect) of CLK/CYC is required during development for the adult behavioural rhythmicity. A manipulation of this effector gene in clock neurons during metamorphosis should lead to adult locomotor rhythms defects. One of the aims of this chapter is to identify such elements.

A CLK/CYC effector gene that is crucial in metamorphosis for establishing adult circadian clock function should have three key properties. Firstly, it has to be expressed in clock neurons in the fly's brain, especially in PDF+ neurons, which were shown to require CLK/CYC during development to establish adult behavioural rhythmicity (Figure 5.6). Secondly, this effector gene should be present in said cells during metamorphosis, as I shown that CLK/CYC is required during metamorphosis (Figures 5.1 and 5.3). And finally, if I focus on direct CLK/CYC targets, it has to be activated by CLK/CYC.

Lists of both genes that are expressed in PDF+ neurons (Nagoshi *et al*., 2010; Kula-Eversole *et al*., 2010) and that are CLK/CYC targets (Abruzzi *et al*., 2011)

are currently available. Of course multiple core clock genes described previously in the General Introduction are also CLK/CYC targets, including *per, tim, cwo, vri* and *Pdp1ε*. From previous experiments I knew that *per* can be excluded from the target list, since it was not required during development to establish adult locomotor rhythmicity (Figure 3.3).

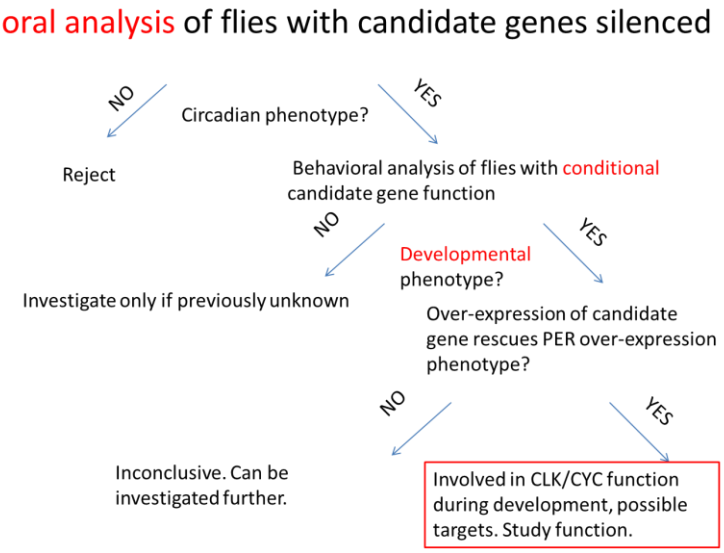
Given the role of CLK/CYC as a transcriptional activator of many genes and the fact that developmental CLK/CYC role appeared independent of its function in the molecular oscillator, I speculated that one role of CLK/CYC is to activate a specific gene(s) during metamorphosis. To find these CLK/CYC effectors I designed a simple three- step screen. In the first part of this screen, I identified potential CLK/CYC effectors by comparing the list of genes that are expressed in the PDF+ neurons (Nagoshi *et al.*, 2010; Kula-Eversole *et al.*, 2010) with a lists of CLK/CYC targets (Abruzzi *et al.*, 2011). I did not test any genes that were shown previously to be not involved in the generation of the circadian rhythms and genes for which *dsRNA* element was not present in the stock centres. For genes selected, *dsRNA* constructs were ordered from stock centres, either Bloomington Stock Centre or Vienna *Drosophila* Resource Centre.

The first step of the screen was just to find out if a downregulation of the candidate gene in all circadian cells (driven by *tim(UAS)Gal4*) resulted in an adult locomotor behaviour phenotype. *UAS-Dcr2* element was added to increase gene downregulation. For genes that showed phenotype different than control lines, more *dsRNA* lines were ordered and they were analysed in the second stage of the screen.

The second part of the screen was concerned with separating genes with an adult function from genes with a developmental one. To facilitate this, *dsRNA* lines for genes of interest were crossed to *UAS-Dcr2;tim(UAS)Gal4; tub$_P$Gal80^{ts}* line and flies were raised at different temperatures. When flies were raised under permissive conditions (room temperature or 17˚C), they served as controls, since no *dsRNA* should be expressed and the gene of interest will not be downregulated. Flies raised under restrictive conditions (29˚C) had a downregulation of the gene of interest during development. The most interesting group were flies raised under permissive conditions and then exposed to 29˚C for a week as adults (17˚C >29˚C), since they represented flies

with prior adult (but not behavioural) gene silencing. This experiment was performed only on flies that showed statistical differences (or a trend for differences) between flies raised under permissive or restrictive conditions.

A



B

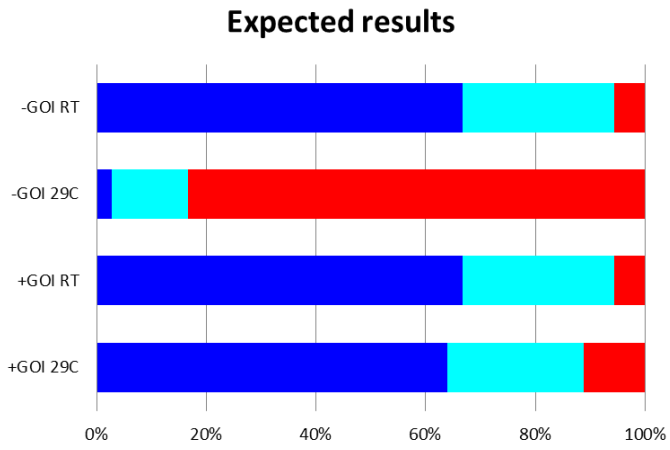


Figure 6.6. Design of the screen to identify CLK/CYC targets.

(A) Three-stage screen to identify CLK/CYC effectors that are required during development to establish adult circadian rhythms. (B) Anticipated results for a successful rescue of developmental *per* over-expression locomotor rhythmicity defect (last part of the screen). Distribution of rhythmic (dark blue), weakly rhythmic (light blue) and arrhythmic (red) flies in a population of flies analysed at permissive temperature. Without the over-expression of the gene of

interest (GOI), flies with developmental *per* over-expression should be predominantly rhythmic when raised at permissive temperature (room temperature, RT) and mainly arrhythmic when raised at restrictive temperature (29˚C). Addition of the UAS-Gene_of_interest (GOI) should not affect the rhythmicity of flies raised under permissive conditions and restore the rhythmicity of flies raised under restrictive conditions.

The final part of the screen was designed to confirm if the gene of interest was indeed a CLK/CYC effector required during development to establish functional circadian rhythms in adult *Drosophila melanogaster*. I hypothesised that if adult locomotor behaviour phenotype observed with a developmental CLK/CYC inhibition was a reflection of a developmental requirement for a particular gene of interest, then when I over-express this gene during development in flies with conditional PER over-expression (leading to CLK/CYC inhibition), I might be able to reverse the phenotype and see adult locomotor rhythmicity in these flies. However, there is a possibility that an over-expression of a gene previously implied to play a role in a generation of circadian rhythms in locomotor behaviour (as indicated in the part 1 and 2 of the screen) might result in a phenotype on its own.

6.4.3.2 Screen for the circadian phenotype of the gene candidates

The aim of the first part of the screen was to identify novel genes involved in the generation of the circadian rhythms. 21 different *dsRNA* elements were tested; some of them were against the same genes. Adult locomotor behaviour of flies with the knockdown of the gene of interest in all circadian cells was tested as described in 6.3.5.

Four out of 21 tested lines (*Pdp1ε, Mef2, Fer2, trx)* showed the strongest disruption to the circadian rhythmicity, which was characterized by a significant difference between experimental flies and both kinds of controls used for either the distribution of flies in the population or rrp (Table 6.1). Out of these, three were previously linked to circadian clocks (*Pdp1ε, Mef2, Fer2)*

and to my knowledge involvement of the fourth one (*trx)* in the generation of the circadian rhythms was not previously studied.

PAR domain protein 1 was previously discovered to be a part of a molecular oscillator. Female flies with the *PAR domain protein 1 ε* (*Pdp1ε)* knockdown in all clock cells were excluded from the analysis since a parental line containing the *dsPdp1ε* element was found to have a circadian phenotype severely extending period length. I was able to determine this was due to a mutation on the X chromosome, which meant that female flies were unreliable. This was not a problem for males, however. Male flies with a knockdown of *Pdp1ε* gene displayed statistically significant increase in a number of arrhythmic flies to almost 90 % (Fisher's Exact 3x2 test: $p=1.565x10^{-10}$, n=68 compared to control without a *Gal4* driver (called undriven), $2.989x10^{-21}$, n=62 compared to the driver). No strongly rhythmic flies were observed and the remaining flies categorized as weakly rhythmic had a relative rhythmic power close to arrhythmic flies (1.099 with the SEM of 0.034, Mann-Whitney test: U=52.000, z=2.944, p<0.001, n=27 for comparison with the undriven control; Wilcoxon Rank test: W=-109,679, z=-3.149, p<0.001, n=40 for comparison to the driver). These results confirmed involvement of *Pdp1ε* in the generation of circadian rhythms, which was also described previously in literature (Cyran *et al*., 2003).

Interestingly, in addition to abolishing rhythmicity in constant conditions, visual inspection revealed that *Pdp1ε* knockdown resulted in the unexpected phenotype in LD conditions (Figure 6.7). As could be seen on an actogram, flies had lost their bimodal activity pattern and instead, they were rhythmic predominantly during dark portion of LD. To my knowledge, this is the first time such phenotype was observed for flies with changed *Pdp1ε* expression.

Severe circadian defect was also uncovered for flies with *48-related 2* (*Fer2*) knockdown in all clock cells (Figure 6.7, Table 6.1). *Fer2* has been identified as enriched in ventral lateral neurons and involved in generation of circadian rhythms (Nagoshi *et al*., 2010). Among both females and males no rhythmic flies were recovered and increased number of arrhythmic flies was observed – over 70% of females were arrhythmic (Fisher's Exact 3x2 test: $p<10^{-4}$, n=19 compared to undriven control; $p<10^{-4}$, n=51 compared to the driver) and all males were arrhythmic (Fisher's Exact 3x2 test: p<0.01, n=11 compared to undriven control; $p<10^{-5}$, n=39 compared to the driver). Relative rhythmic

power of remaining females was significantly lower than of controls (1.104 with the SEM of 0.054, Mann-Whitney test: U=24.000, z=2.449, p<0.02, n=11 for comparison with the undriven control; Wilcoxon Rank test: W=-105.750, z=-3.140, p<0.01, n=27 for comparison to the driver). Unfortunately, a lot of male flies displayed early mortality, lowering the n number. Data obtained confirmed previously reported involvement of *Fer2* in a generation of circadian rhythms.

Less severe phenotype than both previously described (for *Pdp1ε* or *Fer2*) was observed for flies with *Myocyte enhancer factor 2* (*Mef2*) *dsRNA* expressed in all clock cells (Figure 6.7, Table 6.1). *Mef2* was included in this study since it was both identified as CLK/CYC target (Abruzzi *et al.*, 2011) and as enriched in the ventral lateral neurons (Nagoshi *et al.*, 2010). I observed a decrease in the number of flies displaying rhythmic behaviour under constant conditions for both females to just below 15% (Fisher's Exact 3x2 test: $p<10^{-4}$, n=30 compared to undriven control; p<0.01, n=54 compared to the driver) and males to 40% (Fisher's Exact 3x2 test: p<0.01, n=27 compared to undriven control; $p<10^{-3}$, n=48 for the driver). This was accompanied by a decrease in relative rhythmic power for both females (Mann-Whitney test: U=158.000, z=3.918, $p<10^{-4}$, n=26 for comparison with the undriven control; Wilcoxon Rank test: W=-102,205, z=-4.686, p<0.001, n=35 for comparison to the driver) and males (Mann-Whitney test: U=139.000, z=2.932, p<0.01, n=26 for comparison with the undriven control; Wilcoxon Rank test: W=-81.224, z=-2.962, p<0.01, n=22 for comparison to the driver). As can be seen from the number of flies classified as rhythmic, there was no dramatic increase in a number of arrhythmic flies, with only slightly over 20% arrhythmic females and no arrhythmic males. Visual inspection of actograms (Figure 6.7) also suggested unusual for this screen shortening of a period length, however it turned out not to be statistically significant when compared to controls with undriven version of *dsMef2*, possibly due to low number of flies.

The last gene for which I saw a strong phenotype (Figure 6.7, Table 6.1) was previously not linked to circadian clocks. *Trithorax* (*trx*) was selected to be a part of this screen because it was identified as one of CLK/CYC targets (Abruzzi *et al.*, 2011). Both females and males with a downregulation of *trx* in all clock cells are completely arrhythmic (Fisher's Exact 3x2 test: females: $p<10^{-3}$, n=20 compared to undriven control; $p<10^{-6}$, n=49 compared to the

driver; males $p < 10^{-5}$, n=21 compared to undriven control; $p < 10^{-8}$, n=43 compared to the driver). At least 60 % of *trx* undriven control flies were strongly rhythmic, indicating that results observed were not due to the genetic background. Due to a complete arrhythmicity of flies, period length and relative rhythmic power could not be compared to controls.

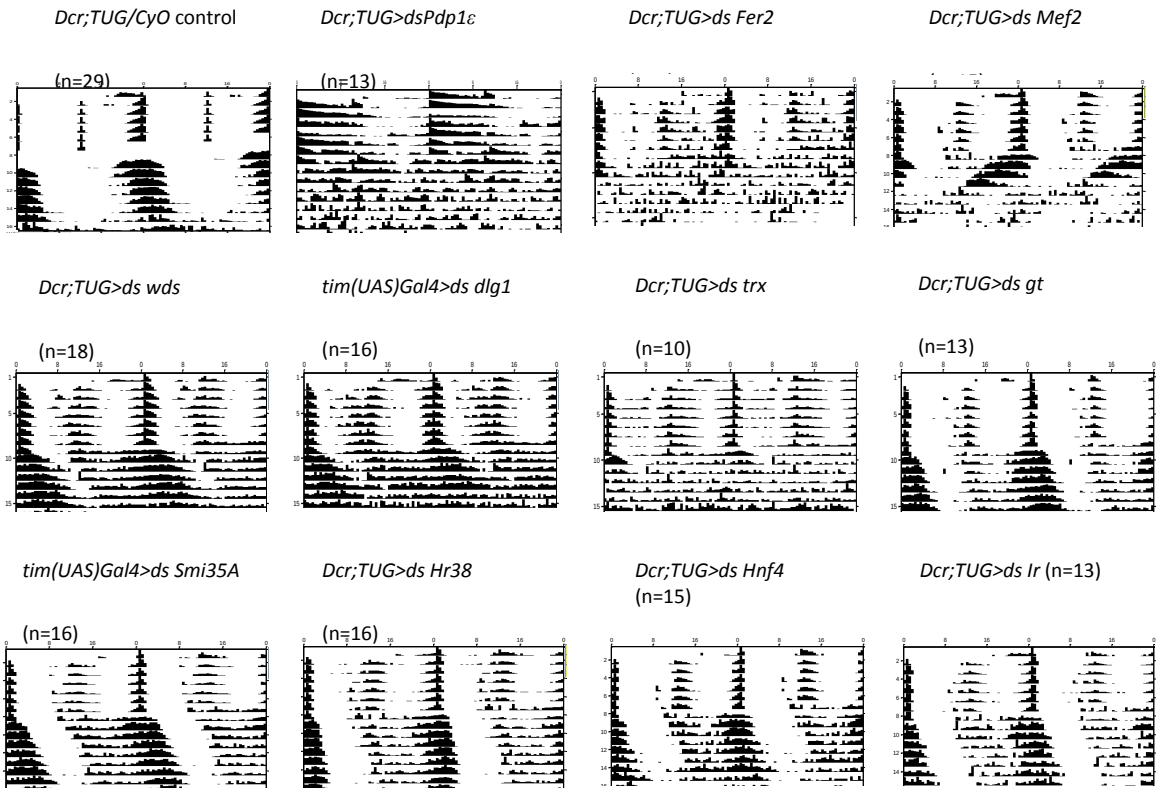


Figure 6.7. Sample results of the knockdown of genes from the screen.

Double-plotted actograms depict the median rhythmicity of male flies with a knockdown of the gene of interest in all circadian cells raised at room temperature. Flies were entrained to 12:12 light/dark cycle for 7 days at 23˚C or 25˚C and then released into constant darkness.

Six genes tested (*smi35A, wds, dlg1, Hnf4, Hr38, Ir*) produced an intermediate phenotype which was sufficient to include most of them in the next stage of the screen (Figure 6.7, Table 6.1). Knockdown of *Smell impaired 35A* (*smi35A*, CLK/CYC target) (Abruzzi *et al.*, 2011) in the clock cells resulted in a decrease in the number of strongly rhythmic females to 33.3% but with no arrhythmic flies (Fisher's Exact 3x2 test: $p<0.01$, n=30 compared to undriven controls; $p<0.05$, n=55 compared to the driver), which was also accompanied by a decrease of relative rhythmic power of rhythmic flies (Mann-Whitney test: U=176.000, z=2.634, $p<0.01$, n=30 for comparison with the undriven control; Wilcoxon Rank test: W=-69,550, z=-3.528, $p<0.001$, n=39 for comparison to the driver). Also, females had a longer period than flies without *smi35A* knockdown (Mann-Whitney test: U=10.500, z=-2.224, $p<0.05$, n=18 for comparison with the undriven control). Males, however, did not display reduced rhythmicity or relative rhythmic power but instead displayed elongation of period in comparison to undriven control (Mann-Whitney test: U=0.000, z=-4.197, $p<10^{-5}$, n=25 for comparison with the undriven control; Wilcoxon Rank test: W=48.214, z=2.215, $p<0.05$, n=19 for comparison to the driver). Similar pattern of sexual dimorphism was quite common in this screen.

Will die slowly (*wds*) knockdown in the clock cells resulted in a sexual dimorphism as well (Figure 6.7, Table 6.1), with no strongly rhythmic and almost 60% of arrhythmic females (Fisher's Exact 3x2 test: $p<0.01$, n=23 compared to undriven controls; $p<10^{-4}$, n=52 compared to the driver) with a relative rhythmic power of remaining weakly rhythmic flies also significantly lower at 1.14 (Mann-Whitney test: U=42.000, z=2.600, $p<0.01$, n=14 for comparison with the undriven control; Wilcoxon Rank test: W=-102.417, z=-3.789, $p<0.001$, n=32 for comparison to the driver). Males displayed a quite dramatic period lengthening (Mann-Whitney test: U=0.000, z=-3.325, $p<0.001$, n=15 for comparison with the undriven control; Wilcoxon Rank test: W=-48.929, z=-2.266, $p<0.05$, n=19 for comparison to the driver) with a reduction in the relative rhythmic power significant only for comparison to the driver line (Wilcoxon Rank test: W=-55.395, z=-2.178, $p<0.05$, n=22) or population rhythmicity distribution different than only the driver line (Fisher's Exact test: $p<10^{-3}$, n=27). It is worth noticing, however, that parental line had relatively poor rhythmicity, with only around 65% of strongly rhythmic flies (Table 6.1).

Discs, large homologue 1 (*dlg1*) was included in the screen since not only it is a CLK/CYC target but also its mRNA shows circadian oscillations (Abruzzi *et al.*, 2011). I have analysed two different lines (33629 and 33620) for this gene and both gave a very similar phenotype (Figure 6.7, Table 6.1). Females for both constructs tested displayed complete loss of strongly rhythmic flies from the population (from ~70% of strongly rhythmic flies in control undriven lines (Fisher's Exact 3x2 test: for 33629: $p<10^{-4}$, n=24 compared to undriven control; $p<10^{-5}$, n=50 compared to the driver; for 33620: p<0.01, n=22 compared to undriven control; $p<10^{-3}$, n=51 compared to the driver) and a decline in the relative rhythmic power (for 33629: Mann-Whitney test: U=28.000, z=2.223, p<0.05, n=16 for comparison with the undriven control; for 33620: Mann-Whitney test: U=83.000, z=3.220, p<0.001, n=13 for comparison with the undriven control; Wilcoxon Rank test: W=-96.542, z=-4.300, p<0.001, n=32 for comparison to the driver). In case of males, for both lines tested I saw a decrease in the percentage of rhythmic flies, significant only for comparison with a driver line (Fisher's Exact 3x2 test: for 33629 $p<10^{-4}$, n=24 and for 33620 $p<10^{-3}$, n=24). I could also see a significant period lengthening, in case of 33629 significant when compared to both controls (Mann-Whitney test: U=0.000, z=-2.785, p<0.01, n=10 for comparison with the undriven control; Wilcoxon Rank test: W=67.929, z=2.707, p<0.01, n=12 for comparison to the driver) and in case of 33620 the lengthening was only significant when compared to undriven control (Mann-Whitney test: U=2.000, z=-2.594, p<0.01, n=12), which has an unusual short period, indicating possible background mutations affecting period length, rather than an effect of gene knockdown (Table 6.1). Relative rhythmic power values for males were also decreased for 33629 (Mann-Whitney test: U=75.000, z=2.083, p<0.05, n=20 for comparison with the undriven control; Wilcoxon Rank test: W=-88.679, z=-3.355, p<0.001, n=19 for comparison to the driver; 33620). Because of these differences between both lines described above, I decided to include *dlg1* in the next stage of the screen but exclude the 33620 line, which might be suffering from some genetic background issues.

Hepatocyte nuclear factor 4 (*Hnf4*) was shown by Abruzzi *et al.* (2011) to be a CLK/CYC target. *Hnf4* knockdown in clock cells resulted in a large decline in the rhythmicity of female population (Figure 6.7, Table 6.1), as demonstrated by a decrease in the percentage of strongly rhythmic flies and larger

percentage of arrhythmic flies (Fisher's Exact 3x2 test: $p<0.01$, n=32 for undriven line but not significant compared to driver). It was also accompanied by a decrease in the relative rhythmic power (Mann-Whitney test: U=166.000, z=2.719, $p<0.01$, n=29 for comparison with the undriven control; Wilcoxon Rank test: W=-77.365, z=-3.751, $p<0.001$, n=37 for comparison to the driver). Male flies, on the other hand, showed rhythmicity comparable to undriven controls (Table 6.1), but significantly less rhythmic than a driver control (Fisher's Exact 3x2 test: $p<0.05$, n=48 for distribution of flies; Wilcoxon Rank test: W=-65.824, z=-2.401, $p<0.05$, n=22 for rrp and comparison to the driver). In addition, strongly rhythmic flies had a significantly longer period than undriven controls (Mann-Whitney test: U=10.000, z=-2.960, $p<0.01$, n=21 for females; Mann-Whitney test: U=2.000, z=-4.140, $p<10^{-4}$, n=22).

Among the two lines studied for the knockdown of *Hormone receptor-like in 38* (*Hr38*) in the clock cells (29378 and 29377), both caused overall changes in the distribution of flies in the population only for females (which was significant only for the 29377 line –Fisher's Exact 3x2 test: $p<0.05$, n=26 compared to undriven controls; $p<0.05$, n=53 compared to the driver) (Figure 6.7, Table 6.1). Knockdown of *Hr38* with the 29377 line also demonstrated an overall decrease in the relative rhythmic power for females (Mann-Whitney test: U=116.000, z=3.077, $p<0.001$, n=23 for comparison with the undriven control; Wilcoxon Rank test: W=-55.508, z=-2.772, $p<0.01$, n=35 for comparison to the driver) and an elongation of period for males as compared to undriven controls (Mann-Whitney test: U=10.500, z=-3.801, $p<10^{-4}$, n=25). Knockdown of *Hr38* with the 29376 resulted in a period elongation for all flies as compared to undriven controls (Mann-Whitney test: U=0.000, z=-3.974, $p<10^{-4}$, n=20 for females and Mann-Whitney test: U=2.000, z=-4.596, p<10-6, n=28 for males). Female flies also had a lower relative rhythmic power than undriven controls but not driver line (Mann-Whitney test: U=165.000, z=2.203, $p<0.05$, n=30).

Inward rectifier (*Ir*) was identified as highly enriched in the PDF+ clock neurons (Nagoshi *et al.*, 2010). In my screen both females and males showed a decline in the overall rhythmicity of the populations (Figure 6.7, Table 6.1), however they were statistically significant only for males (Fisher's Exact 3x2 test: $p<0.05$, n=28 compared to undriven controls and $p<10^{-4}$, n=55 compared to the driver). There was also a decrease in relative rhythmic power (females:

Mann-Whitney test: U=132.000, z=2.436, p<0.05, n=26 for comparison with the undriven control; Wilcoxon Rank test: W=-77.365, z=-3.751, p<0.001, n=37 for comparison to the driver and males; Wilcoxon Rank test: W=-96.896, z=-3.451, p<0.001, n=20 for comparison to the driver). Both females and males showed a severe period elongation to 25.0 hrs (Mann-Whitney test: U=0.000, z=-3.097, p<0.001, n=14 for comparison with the undriven control; Wilcoxon Rank test: W=41.286, z=2.310, p<0.05, n=27 for comparison to the driver) and 24.6 (Mann-Whitney test: U=0.000, z=-2.768, p<0.01, n=10), respectively. This period lengthening was interesting enough to take flies to a next stage of the screen.

Nine genes tested were excluded from a further analysis due to a lack of or weakness of the circadian phenotype after their knockdown in all clock cells. For *Ampiphysin* (*Amph*, CLK/CYC target (Abruzzi *et al*., 2011)) and *hairy* (*h*) genes, I was able to show that their downregulation does not lead to any major circadian locomotor rhythms defects (other than lower rrp for females with *Amph* knockdown (Wilcoxon Rank test: W=-56.517, z=-2.731, p<0.01, n=34 for comparison to the driver) and period shortening for females with h knockdown (Wilcoxon Rank test: W=-32.484, z=-2.563, p<0.01, n=34 for comparison to the driver), indicating a genetic background phenotype) (Figure 6.7, Table 6.1). Despite some statistically significant differences (mainly for males), *giant* (*gt*) and *puckered* (*puc*, which both a CLK/CYC target according to Abruzzi *et al.* (2011) and enriched in clock cells according to Nagoshi *et al*. (2010)) were also treated as not connected to the circadian rhythms since this significance was caused by a low rhythmicity of control lines, indicating a genetic background issues (Table 6.1). Similar was also true for *short neuropeptide F precursor* (*sNPF*), for which males with *dsRNA* element driven where more rhythmic than undriven controls (Fisher's Exact 3x2 test: $p<10^{-2}$, n=22), and had period length longer than undriven *dsRNA* (Mann-Whitney test: U=6.000, z=-2.483, p<0.05, n=18). Females, meanwhile, had lower relative rhythmic power (Mann-Whitney test: U=127.000, z=2.179, p<0.05, n=26 for comparison with the undriven control; Wilcoxon Rank test: W=-45.186, z=-2.386, p<0.05, n=37 for comparison to the driver). Also, for *Insulin-like peptide 3* (*Ilp3*), a period elongation observed was only significant for males when compared to undriven *dsRNA* element (Mann-Whitney test: U=13.000, z=-3.577, p<0.001, n=24) (Table 6.1).

I have also excluded *Chronologically inappropriate morphogenesis* (*chinmo*, CLK/CYC target (Abruzzi *et al.*, 2011)) from the further analysis, despite statistically significant decrease in a rhythmicity in females (Fisher's Exact 3x2 test: $p<0.05$, n=54 compared to undriven controls; $p<0.05$, n=29 compared to the driver) and decrease in rrp for females (Mann-Whitney test: U=9.000, z=-2.320, $p<0.05$ n=16 for comparison with the undriven control; Wilcoxon Rank test: W=-58.250, z=-2.382, $p<0.05$, n=32 for comparison to the driver) since this phenotype was relatively weak and there was no significant change in rhythmicity of females (Table 6.1). I excluded also CG7945, showing a very similar decrease in the rhythmicity in females (Fisher's Exact 3x2 test: $p<0.01$, n=30 compared to undriven controls) but no decrease in a relative rhythmic power. (Table 6.1). Another excluded line, *Sirtuin2* (*Sir2*), showed only a statistically significant decrease in a rhythmicity for females (Fisher's Exact 3x2 rest: $p<0.05$, n=32 compared to undriven controls for distribution of flies and a Mann-Whitney test: U=176.000, z=2.214, $p<0.05$, n=31 for comparison with the undriven control for relative rhythmic power) and the elongation of period length in males, but only when compared to undriven control flies (Mann-Whitney test: U=15.500, z=-3.371, $p<0.001$, n=24l) (Table 6.1).

Genotype	gender	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG/CyO controls	F	40	62.5 : 27.5: 10.0	24.39 (0.167)	2.294 (0.135)
Dcr2;TUG/CyO controls	M	33	90.9 : 6.8 : 2.3	24.18 (0.100)	2.149 (0.150)
Dcr2;TUG > ds Pdp1ε;ds Pdp1ε	F	64	0.0 : 18.8 : 81.2[a,b]	N/A	1,101[b] (0,025)
Dcr2;CyO > ds Pdp1ε;ds Pdp1ε	F	24	12.5 : 12.5 : 75.0	25,67 (0,167)	1,352 (0,059)
Dcr2;TUG > ds Pdp1ε;ds Pdp1ε	M	39	0.0 : 10.3 : 89.7[a,b]	N/A	1,099[a,b] (0,034)
Dcr2;CyO > ds Pdp1ε;ds Pdp1ε	M	29	58.6 : 20.7 : 20.7	23,44 (0,059)	2,025 (0,152)
Dcr2;TUG > ds Fer2 (28697)	F	11	0.0 : 27.3 : 72.7[a,b]	N/A	1,104[a,b] (0,054)
Dcr2;CyO > ds Fer2 (28697)	F	8	87.5 : 12.5 : 0.0	23,43 (0,071)	2,066 (0,150)
Dcr2;TUG > ds Fer2 (28697)	M	6	0.0 : 0.0 : 100.0[a,b]	N/A	N/A
Dcr2;CyO > ds Fer2 (28697)	M	5	60.0 : 40.0 : 0.0	23,33 (0,167)	1,757 (0,227)
Dcr2;TUG > ds Mef2 (28699)	F	14	14.3 : 64.3 : 21.4[a,b]	22,75 (1,750)	1,248[a,b] (0,086)
Dcr2;CyO > ds Mef2 (28699)	F	16	87.5 : 6.2 : 6.3	23,54 (0,082)	2,040 (0,106)
Dcr2;TUG > ds Mef2 (28699)	M	15	40.0 : 60.0 : 0.0[a,b]	23,08[b] (0,569)	1,530[a,b] (0,091)
Dcr2;CyO > ds Mef2 (28699)	M	12	91.7 : 0.0 : 8.3	23,54 (0,106)	2,102 (0,132)
Dcr2;TUG > ds trx (33703)	F	9	0.0 : 0.0 : 100.0[a,b]	N/A	N/A
Dcr2;CyO > ds trx (33703)	F	11	63.6 : 27.3 : 9.1	23,71 (0,101)	2,021 (0,204)
Dcr2;TUG > ds trx (33703)	M	10	0.0 : 0.0 : 100.0[a,b]	N/A	N/A
Dcr2;CyO > ds trx (33703)	M	11	63.6 : 36.4 : 0.0	23,50 (0,000)	1,834 (0,192)
Dcr2;TUG > ds dlg1 (33629)	F	10	0.0 : 20.0 : 80.0[a,b]	N/A	1,208[a] (0,054)
Dcr2;CyO > ds dlg1 (33629)	F	14	71.4 : 28.6 : 0.0	24,00 (0,197)	1,768 (0,090)
Dcr2;TUG > ds dlg1 (33629)	M	16	31.2 : 43.8 : 25.0[b]	25,20[a,b] (0,200)	1,348[a,b] (0,095)
Dcr2;CyO > ds dlg1 (33629)	M	8	62.5 : 37.5 : 0.0	23,60 (0,100)	1,792 (0,180)
Dcr2;TUG > ds dlg1 (33620)	F	11	0.0 : 72.7 : 27.3[a,b]	N/A	1,147[a,b] (0,050)
Dcr2;CyO > ds dlg1 (33620)	F	11	72.7 : 27.3 : 0.0	23,75 (0,094)	2,055 (0,183)
Dcr2;TUG > ds dlg1 (33620)	M	14	42.8 : 28.6 : 28.6[b]	24,33[a] (0,211)	1,718 (0,178)
Dcr2;CyO > ds dlg1 (33620)	M	10	60.0 : 30.0 : 10.0	22,92 (0,300)	1,689 (0,212)
Dcr2;TUG > ds wds (32952)	F	12	0.0 : 41.7 : 58.3[a,b]	N/A	1,144[a,b] (0,078)
Dcr2;CyO > ds wds (32952)	F	11	63.6 : 18.2 : 18.2	23,71 (0,149)	1,810 (0,150)
Dcr2;TUG > ds wds (32952)	M	18	50.0 : 44.4 : 5.6[b]	24,89[a,b] (0,162)	1,611[b] (0,106)
Dcr2;CyO > ds wds (32952)	M	9	66.7 : 11.1 : 22.2	23,50 (0,000)	2,068 (0,263)
Dcr2;TUG > ds smi35A (35393)	F	15	33.3 : 66.7 : 0.0[a,b]	24,90[a] (0,292)	1,464[a,b] (0,065)
Dcr2;CyO > ds smi35A (35393)	F	15	86.7 : 13.3 : 0.0	24,04 (0,165)	1,788 (0,098)
Dcr2;TUG > ds smi35A (35393)	M	16	75.0 : 25.0 : 0.0	24,75[a,b] (0,075)	1,870 (0,108)
Dcr2;CyO > ds smi35A (35393)	M	12	91.7 : 8.3 : 0.0	23,50 (0,095)	1,969 (0,118)
Dcr2;TUG > ds Hr38 (29377)	F	13	23.1 : 61.5 : 15.4[a,b]	23,50 (0,000)	1,435[a,b] (0,086)
Dcr2;CyO > ds Hr38 (29377)	F	13	76.9 : 15.4 : 7.7	23,30 (0,082)	2,086 (0,172)
Dcr2;TUG > ds Hr38 (29377)	M	15	80.0 : 13.3 : 6.7	24,12[a] (0,109)	2,018 (0,102)
Dcr2;CyO > ds Hr38 (29377)	M	15	86.7 : 13.3 : 0.0	23,31 (0,090)	1,945 (0,092)

Genotype	gender	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG > ds Hr38 (29376)	F	16	43.8 : 43.8 : 12.5	24,71[a] (0101)	1,724[a] (0,127)
Dcr2;CyO > ds Hr38 (29376)	F	16	81.3 : 18.8 : 0.0	23,42 (0,052)	2,176 (0,144)
Dcr2;TUG > ds Hr38 (29376)	M	16	87.5 : 12.5 : 0.0	24,36[a] (0,063)	2,137 (0,125)
Dcr2;CyO > ds Hr38 (29376)	M	16	87.5 : 12.5 : 0.0	23,36 (0,082)	2,062 (0,114)
Dcr2;TUG > ds Ir (25823)	F	15	33.3 : 53.3 : 13.4	25,00[a,b] (0,000)	1,427[a,b] (0,083)
Dcr2;CyO > ds Ir (25823)	F	13	69.2 : 30.8 : 0.0	23,61 (0,200)	1,949 (0,159)
Dcr2;TUG > ds Ir (25823)	M	13	30.8 : 69.2 : 0.0[a,b]	24,62[a] (0,125)	1,390[b] (0,085)
Dcr2;CyO > ds Ir (25823)	M	14	42.8 : 28.6 : 28.6	23,42 (0,083)	1,624 (0,131)
Dcr2;CyO > ds Hnf4 (29375)	F	16	37.5 : 43.7 : 18.8[a]	24,33[a] (0,211)	1,471[a,b] (0,088)
Dcr2;CyO > ds Hnf4 (29375)	F	16	93.8 : 6.2 : 0.0	23,50 (0,098)	2,051 (0,128)
Dcr2;CyO > ds Hnf4 (29375)	M	15	66.7 : 33.3 : 0.0[b]	24,35[a] (0,107)	1,675[b] (0,098)
Dcr2;CyO > ds Hnf4 (29375)	M	16	75.0 : 25.0 : 0.0	23,54 (0,042)	1,979 (0,128)
Dcr2;TUG > ds Sir2 (32481)	F	16	68.8 : 31.2 : 0.0[a]	23,82 (0,122)	1,749[a] (0,106)
Dcr2;CyO > ds Sir2 (32481)	F	16	93.8 : 0.0 : 6.2	23,50 (0,069)	2,197 (0,117)
Dcr2;TUG > ds Sir2 (32481)	M	16	87.5 : 12.5 : 0.0	24,14[a] (0,082)	2,106 (0,135)
Dcr2;CyO > ds Sir2 (32481)	M	16	62.5 : 31.3 : 6.2	23,55 (0,117)	1,763 (0,139)
Dcr2;TUG > ds chinmo (33638)	F	14	35.7 : 21.4 : 42.9[a,b]	24,30 (0,300)	1,575 (0,135)
Dcr2;CyO > ds chinmo (33638)	F	15	73.3 : 26.7 : 0.0	23,54 (0,142)	2,120 (0,182)
Dcr2;TUG > ds chinmo (33638)	M	15	86.6 : 6.7 : 6.7	23,73[b] (0,122)	2,427 (0,211)
Dcr2;CyO > ds chinmo (33638)	M	14	92.9 : 0.0 : 7.1	23,50 (0,098)	2,486 (0,146)
Dcr2;TUG > ds CG7945 (28779)	F	14	35.7 : 35.7 : 28.6[a]	24,00 (0,387)	1,606[b] (0,164)
Dcr2;CyO > ds CG7945 (28779)	F	16	93.8 : 6.3 : 0.0	23,87 (0,077)	2,039 (0,150)
Dcr2;TUG > ds CG7945 (28779)	M	16	68.8 : 25.0 : 6.3	24,09 (0,148)	1,816[b] (0,155)
Dcr2;CyO > ds CG7945 (28779)	M	16	68.8 : 31.3 : 0.0	23,68 (0,102)	1,760 (0,112)
Dcr2;TUG > ds sNPF (25867)	F	15	40.0 : 46.7 : 13.3	24,25 (0,250)	1,502[a,b] (0,065)
Dcr2;CyO > ds sNPF (25867)	F	13	76.9 : 23.1 : 0.0	23,75 (0,112)	1,874 (0,140)
Dcr2;TUG > ds sNPF (25867)	M	14	100.0 : 0.0 : 0.0[a]	24,07[a,b] (0,103)	2,207 (0,095)
Dcr2;CyO > ds sNPF (25867)	M	8	50.0 : 50.0 : 0.0	23,67 (0,204)	1,848 (0,174)
Dcr2;TUG > ds puc (34392)	F	20	35.0 : 55.0 : 10.0	24,07 (0,230)	1,634 (0,139)
Dcr2;CyO > ds puc (34392)	F	10	30.0 : 60.0 : 10.0	24,00 (0,289)	1,486 (0,167)
Dcr2;TUG > ds puc (34392)	M	15	80.0 : 20.0 : 0.0[a]	24,42[a] (0,083)	2,104[a] (0,153)
Dcr2;CyO > ds puc (34392)	M	14	35.8 : 57.1 : 7.1	23,40 (0,100)	1,561 (0,136)
Dcr2;TUG > ds gt (34631)	F	13	23.1 : 53.8 : 23.1	24,17 (0,167)	1,376[b] (0,150)
Dcr2;CyO > ds gt (34631)	F	11	54.5 : 45.5 : 0.0	23,83 (0,167)	1,626 (0,124)
Dcr2;TUG > ds gt (34631)	M	14	85.8 : 7.1 : 7.1	24,38[a] (0,065)	2,436[a] (0,153)
Dcr2;CyO > ds gt (34631)	M	10	50.0 : 20.0 : 30.0	23,50 (0,000)	1,561 (0,186)
Dcr2;TUG > ds Ilp3 (33681)	F	16	56.2 : 31.3 : 12.5	23,89 (0,162)	1,751 (0,138)
Dcr2;CyO > ds Ilp3 (33681)	F	15	53.3 : 46.7 : 0.0	23,69 (0,210)	1,717 (0,134)
Dcr2;TUG > ds Ilp3 (33681)	M	16	81.2 : 18.8 : 0.0	24,12[a] (0,083)	2,055 (0,151)

Genotype	gender	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;CyO > ds Ilp3 (33681)	M	15	73.3 : 26.7 : 0.0	23,45 (0,106)	1,971 (0,173)
Dcr2;CyO > ds h (34326)	F	16	81.2 : 12.5 : 6.3	23,69[b] (0,090)	1,920 (0,109)
Dcr2;CyO > ds h (34326)	F	16	75.0 : 12.5 : 12.5	23,54 (0,096)	2,179 (0,146)
Dcr2;CyO > ds h (34326)	M	16	81.2 : 18.8 : 0.0	23,96 (0,105)	1,960 (0,110)
Dcr2;CyO > ds h (34326)	M	16	75.0 : 25.0 : 0.0	23,67 (0,094)	1,894 (0,126)
Dcr2;TUG > ds Amph (28048)	F	12	33.3 : 50.0 : 16.7	24,25 (0,144)	1,423 (0,087)
Dcr2;CyO > ds Amph (28048)	F	10	70.0 : 10.0 : 20.0	23,64 (0,180)	1,951 (0,170)
Dcr2;TUG > ds Amph (28048)	M	10	70.0 : 30.0 : 0.0	23,93 (0,170)	1,799 (0,170)
Dcr2;CyO > ds Amph (28048)	M	14	57.1 : 28.6 : 14.3	23,44 (0,148)	1,670 (0,099)

Table 6.1. Results of the screen for a circadian phenotype resulting from a downregulation of potential target genes in all clock-containing cells.

Distribution of rhythmic, weakly rhythmic and arrhythmic flies, period length of rhythmic flies (Tau) and relative rhythmic power of rhythmic and weakly rhythmic flies (rrp) for females (F) and males (M) with a downregulation of a particular gene (*Dcr;TUG>dsRNA*, marked by a gene name and stock number) were compared to control flies without *Gal4* driver (*Dcr;CyO>dsRNA*, significant difference highlighted and marked as a) and driver line (*Dcr;TUG/CyO*, significant difference highlighted and marked by b). Numbers of flies tested and SEM for values calculated are included in the table. Association between the genotype of flies and the distribution of rhythmic, weakly rhythmic and arrhythmic flies were tested with 3x2 Fisher's Exact tests. Association between a period length or relative rhythmic power and the genotype of flies was determined with Mann-Whitney tests.

6.4.3.3 Conditional knockdown of potential target genes

In this part of the screen, I included genes that were identified in the previous step of the screen as resulting in either a strong or intermediate circadian phenotype when knocked down in all clock cells (Figure 6.7, Table 6.1). For some of them, more lines carrying *dsRNA* elements were ordered from the stock centres mentioned previously. These lines were crossed to flies carrying

tim(UAS)-Gal4 and *tub$_P$Gal80^{ts}* in a *Dcr-2* background. Resulting offspring should experience a knockdown of the gene of interest when placed in restrictive conditions (29˚C) and regular levels of gene expression at permissive temperature (17˚C or RT).

The first part of this phase of experiment was comparing experimental flies raised under permissive conditions (RT) with those from restrictive (29˚C) conditions as described in 6.3.6. If no difference was observed between flies raised under permissive and restrictive conditions, I assumed that this specific gene was not required developmentally.

To confirm the suspected developmental phenotype, it had to be demonstrated that flies with no developmental knockdown of the particular gene remain unaffected if this gene is knocked down during adulthood for the same amount of time. To demonstrate this, two additional conditions were tested. The most important was raising flies in permissive conditions (17˚C) and moving them to constant conditions at restrictive temperature (29˚C) for a week, as described in 6.3.6. The control group was raised at 17˚C and was not exposed to 29˚C. This comparison allowed to check if adult knockdown of the gene produces a reversible adult behavioural arrhythmia only at restrictive conditions.

There were 7 lines that were not tested further than the initial comparison between RT and 29˚C (*ds Ir* "v28430", *ds Ir* "25823", *ds Ir* "v107389", *ds wds*, *ds smi35A* "v101376", *ds dlg1* "35286", *ds dlg1* "33629") as their phenotype obtained either suggested that the genetic construct used was not strong enough to cause impairment of circadian function when combined with *Gal80^{ts}* or that this particular gene is acting primarily in adulthood (Table 6.2). One of *Hnf4* lines tested (v12692) was also excluded from further analysis since the only statistically significant difference was an elongation of a period length that was occurring in males (Mann-Whitney test: $U=175.000$, $z=3.178$, $p<0.01$, $n=29$). This could suggest that v12692 was weaker than 29375, with a lower level of *ds Hnf4* expression (Table 6.2).

Out of the two lines tested for *dlg1* knockdown, 35286 did not show any differences between flies raised at RT and 29˚C (Table 6.2). The other one, 33629, showed some decrease in rhythmicity for both males and females; however the results were only statistically significant for females (Fisher's

Exact 3x2 test: $p<0.001$, n-38 for a distribution of flies and Mann-Whitney test for the relative rhythmic power: $U=58.000$, $z=-2.810$, $p<0.01$, $n=33$) (Table 6.2). Because of these discrepancies between genders and lines tested, I decided to exclude *dlg1* from the further screen.

Three different lines were tested for the knockdown of *Ir*, with one (25823) showing no difference at all between flies raised at 29˚C and controls from RT (Table 6.2). For the second one (v28430), the only significant difference was for a distribution of female flies (Fisher's Exact 3x2 test: $p<0.05$, $n=29$), with more rhythmic flies being raised at 29˚C and slight decrease in period length for males (Mann-Whitney test: $U=32.000$, $z=-2.478$, $p<0.05$, $n=23$) (Table 6.2). The third line analysed (v107389) was again showing significant differences only for females, but this time flies from RT had both more rhythmic flies (Fisher's Exact 3x2 test: $p<10^{-4}$, $n=32$) and higher relative rhythmic power (Mann-Whitney test: $U=6.000$, $z=-3.795$, $p<10^{-4}$, $n=24$) (Table 6.2). For the same reasons as described above for *dlg1*, I decided not to test *Ir* further.

wds was demonstrated to have a developmental function (Hollman *et al.*, 2002) and my results suggested it might also have a developmental circadian function, since there was a significant decrease in a number of rhythmic and increase in the percentage of arrhythmic flies among flies raised at 29˚C for both females (Fisher's Exact 3x2 test: $p<0.05$, $n=32$) and males (Fisher's Exact 3x2 test: $p<0.05$, $n=31$) (Table 6.2). It was also associated with a decreased relative rhythmic power of the males raised at 29˚C (Mann-Whitney test: $U=17.000$, $z=-3.722$, $p<0.001$, $n=28$). While these results could potentially suggest that *wds* is important during development for a generation of circadian rhythms, since females raised at RT were quite poorly rhythmic, it suggested possible genetic background issues (Table 6.2).

Smi35A gave promising results, suggesting that it might be indeed necessary during development for a generation of adult circadian behaviour as both females and males raised at 29˚C demonstrated weaker rhythmicity (Table 6.2)with a significantly less rhythmic females (Fisher's Exact 3x2 test: $p<0.01$, $n=32$) and males (Fisher's Exact 3x2 test: $p<0.05$, $n=31$). Moreover, a relative rhythmic power was decreased for both females (Mann-Whitney test: $U=30.000$, $z=-2.725$, $p<0.01$, $n=26$) and males (Mann-Whitney test: $U=49.000$, $z=-2.619$, $p<0.01$, $n=30$). Unfortunately during further testing of this line no useful data

was recovered due to a technical fault and lack of a possibility of repeating this experiment in the course of this dissertation. It should, however, be noted that this might be an interesting experiment to perform.

Knockdown of *Hnf4* with the second tested line, 29375, showed a trend for a decrease in rhythmicity for females (which was not significant for distribution of flies but significant for relative rhythmic power: Wilcoxon Rank test: $W=11.571$, $z=2.515$, $p<0.05$, $n=24$) (Table 6.2). This impairment of rhythmicity was more prominent for males (Table 6.2), where the difference between the distribution of flies was statistically significant (Fisher's Exact 3x2 test: $p<0.001$, $n=30$) and relative rhythmic power was significantly decreased (Wilcoxon Rank test: $W=14.357$, $z=2.466$, $p<0.05$, $n=23$). I decided to further study this line in hopes of determining whether *Hnf4* is required during adulthood or development. Unfortunately, both females and males raised under permissive conditions of 17˚C that were exposed to restrictive conditions of 29˚C during adulthood and controls always kept at permissive conditions showed very poor rhythmicity, which was comparable to the results seen previously for flies raised at 29˚C (Table 6.2). In fact, males raised at RT were significantly different than those raised at 17˚C both in terms of distribution of flies (Fisher's Exact 3x2 test: $p<10^{-4}$, $n=34$) and relative rhythmic power (Wilcoxon Rank test: $W=18.682$, $z=3.713$, $p<0.001$, $n=27$) and females had significantly different relative rhythmic power (Wilcoxon Rank test: $W=14.971$, $z=2.586$, $p<0.01$, $n=15$). This could suggest a problem with either the line itself or its response to exposure to lower temperature during development rather than provide useful information on function of this gene.

A very similar problem was encountered with a *Hr38* knockdown (Table 6.2). Again, both males and females raised at 29˚C had weaker rhythmicity than flies raised at RT, but the results were significant only for males (Fisher's Exact 3x2 test: $p<0.01$, $n=31$ for a distribution of flies and Wilcoxon Rank test: $W=26.376$, $z=3.360$, $p<0.001$, $n=26$ for relative rhythmic power). As with *Hnf4*, flies raised at 17˚C had also very poor rhythmicity, which was far worse than for flies raised at RT (distribution of flies - Fisher's Exact 3x2 test: $p<0.001$, $n=47$ for females; $p<10^{-4}$, $n=48$ for males; relative rhythmic power – Wilcoxon Rank test: $W=18.611$, $z=3.624$, $p<0.001$, $n=35$ for females and $W=31.675$,

z=4.867, p<0.001, n=39 for males). This suggested either issues with the line its response to lower temperature.

Both females and males with the downregulation of *trx* had significantly decreased percentage of rhythmic flies when compared to flies raised at RT(with over 90% of arrhythmic flies raised at 29˚C; Fisher's Exact 3x2 test: p<0.001, n=32 for females and $p<10^{-7}$, n=31 for males) and relative rhythmic power (Wilcoxon Rank test: W=20.000, z=2.257, p<0.05, n=13 for females and W=24.375, z=2.185, p<0.05, n=17 for males) (Table 6.2). Unfortunately, raising flies at 17˚C resulted in a large decrease of rhythmicity when compared to flies from RT for both females (Fisher's Exact 3x2 test: p<0.05, n=37 for a distribution of flies and Wilcoxon Rank test: W=11.333, z=3.018, p<0.01, n=21 for relative rhythmic power) and males (Fisher's Exact 3x2 test: p<0.05, n=33 for distribution of flies, Mann-Whitney test: U-175.000, z=3.178, p>0.01, n=15 for the period length). This time, however, males raised and kept at 17˚C were still more rhythmic than those exposed to 29˚C as adults (Fisher's Exact 3x2 test: $p<10^{-4}$, n=43 for a distribution of flies and Wilcoxon Rank test: W=20.975, z=3.782, p<0.05, n=20 for relative rhythmic power). These results are inconclusive since they suggest issues with the line for females and possibly adult function of *trx* for males.

As described in the previous part of the screen (6.4.3.2), results obtained with female flies with *Pdp1ε* knockdown had to be disregarded due to overall poor rhythmicity and unusually long period even in control flies without GAL4 expression. In males, there was a clear significant difference between rhythmicity of flies raised at RT and 29˚C (Fisher's Exact 3x2 test: p<0.01, n=22 for a distribution of flies) (Table 6.2). Flies raised at 17˚C were not significantly different than those from RT (Fisher's Exact 3x2 test: p=0.330, n=36 for the distribution) and therefore they were as well different than those from 29˚C (Fisher's Exact 3x2 test: p<0.001, n=28 for the distribution). There was a clear statistical difference between flies that were raised at 17˚C and exposed to 29˚C as adults and flies that were at 17˚C the entire time (Fisher's Exact 3x2 test: $p<10^{-4}$, n=46 for a distribution and W=15.645, z=3.175, p<0.01, n=27 for relative rhythmic power).

Three different *Fer2* lines were tested (v17851, v103217 and 28697). For all of them, raising flies in 29˚C resulted in a large decrease in a rhythmicity of flies

(distribution of flies: Fisher's Exact 3x2 test: females: $p<0.01$, n=31 for v103217; no significance for v17851 and $p<10^{-4}$, n=32 for 28697; males: $p<10^{-6}$, n=29 for v103217; $p<0.001$, n=31 and $p=10^{-6}$, n=31 for 28697; relative rhythmic power: Wilcoxon Rank test: W=16.838, z=3.112, $p<0.01$, n=26 for v17851 males and W=15.214, z=2.079, $p<0.05$, n=15 for 28697 males) (Table 6.2). The lack of similarity of these results warranted further tests on all three lines. Unfortunately, for females from both v103217 and v17851 raising flies at 17˚C resulted in a decreased rhythmicity and no differences between addition of adult exposure to 29˚C or lack of it. This was not, however, the case for 28697, where females raised and kept at 17˚C remained more rhythmic than both flies raised at 29˚C (Fisher's Exact 3x2 test: $p<0.01$, n=27 for distribution of flies) and flies raised at 17˚C and exposed to 29˚C as adults (Fisher's Exact 3x2 test: $p<0.01$, n=27 for distribution of flies) (Table 6.2). Similarly, raising males at 17˚C resulted in a decrease in rhythmicity of flies, with distribution of flies raised at RT different than of those raised at 17˚C for both v103217 (Fisher's Exact 3x2 test: $p<0.05$, n=47) and v17851 (Fisher's Exact 3x2 test: $p<0.01$, n=37). In addition to this, flies raised at 17˚C were significantly different than raised at 29˚C for both v103217 (Fisher's Exact 3x2 test: $p<10^{-8}$, n=440 and 28697 (Fisher's Exact 3x2 test: $p<0.01$, n=23), but not for v17851, where the distribution of flies was comparable to that of flies raised at 29˚C. In two out of three cases, addition of adult exposure to 29˚C resulted in a further decrease of rhythmicity of flies (Fisher's Exact 3x2 test: $p<10^{-7}$, n=55 for v103217; $p<0.01$, n=28 for 28697; Wilcoxon Rank test: W=19.000, z=2.746, $p<0.05$, n= 16 for relative rhythmic power of v103217) (Table 6.2).

Two different lines were analysed for *Mef2* (v15550 and 28699) and for all of them there was a significant decrease in a proportion of rhythmic flies and increase in the amount of arrhythmic ones (Fisher's Exact 3x2 test: $p<10^{-7}$, n=28 for v15550 females, $p<10^{-4}$, n=22 for v15550 males, $p<0.05$, n=32 for 28699 females and $p<10^{-5}$, n=31 for 28699 males) when flies were raised at 29˚C (Table 6.2). In addition, females from 28699 had significantly lower relative rhythmic power (Wilcoxon Rank test: W=14.267, z=2.635, $p<0.05$, n=13). When flies from the line v15550 were raised at 17˚C, rhythmicity of flies was largely reduced as compared to RT-raised flies (Fisher's Exact 3x2 test: $p<0.01$, n=29 for females and $p<10^{-3}$, n=41 for males for a distribution; Mann-

Whitney test: U=62.000, z=-3.370, p<0.001, n=38 for relative rhythmic power of males), but they were still different than flies raised at 29˚C (Fisher's Exact 3x2 test: p<0.05, n=25 for females and $p<10^{-3}$, n=31 for males for a distribution). Addition of adult exposure to 29˚C did not impact overall rhythmicity of flies since controls were already quite arrhythmic. For the second line (28699), flies raised at 17˚C were comparable to flies raised at RT and significantly different than flies raised at 29˚C for the distribution of flies (Fisher's Exact 3x2 test: p<0.05, n=35 for females; $p<10^{-7}$, n=32 for males). Surprisingly, addition of adult exposure to 29˚C did not impact the phenotype further, except for a shortening of period length observed for males from 28699 line (Wilcoxon Rank test: W=11.125, z=3.670, p<0.01, n=8), which might suggest that *Mef2* is necessary during development for generation of adult behavioural rhythms.

genotype	gender	Treatment	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG;tub$_P$Gal80^{ts} > ds Fer2 (v103217)	F	RT	16	12.5 : 37.5 : 50.0	24,00 (0,500)	1,273 (0,091)
Dcr2;TUG;tub$_P$Gal80^{ts} > ds Fer2 (v103217)	F	29˚C	15	0.0 : 0.0 : 100.0[a,c]	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	F	17˚C	31	9.7 : 38.7 : 51.6	24,50 (0,000)	1,335 (0,081)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	F	17˚C >29˚C	20	0.0 : 20.0 : 80.0	N/A	1,123 (0,051)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	M	RT	16	62.5 : 31.2 : 6.3[a]	24,15 (0,150)	1,754 (0,125)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	M	29˚C	13	0.0 : 0.0 : 100.0[a,c]	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	M	17˚C	31	25.8 : 67.7 : 6.5	24,31 (0,210)	1,377 (0,050)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v103217)	M	17˚C >29˚C	24	0.0 : 20.8 : 79.2[a]	N/A	1,028[a] (0,006)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	F	RT	15	20.0 : 53.3 : 26.7 [a]	23,67 (0,167)	1,255 (0,082)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	F	29˚C	15	0.0 : 53.3 : 46.7	N/A	1,192 (0,035)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	F	17˚C	22	4.5 : 27.3 : 68.2	25.50	1,219 (0,083)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	F	17˚C >29˚C	6	0.0 : 16.7 : 83.3	N/A	1.081
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	M	RT	16	100.0 : 0.0 : 0.0[a]	24,53 (0,125)	1,868 (0,053)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	M	29˚C	15	40.0 : 26.7 : 33.3[c]	24,50 (0,250)	1,506[c] (0,080)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (v17851)	M	17˚C	21	52.4 : 23.8 : 23.8	24,64 (0,118)	1,609 (0,064)
Dcr2;TUG;tub$_P$Gal80^{ts} > ds Fer2 (v17851)	M	17˚C >29˚C	5	40.0 : 40.0 : 20.0	24,25 (0,250)	1,597 (0,209)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	F	RT	16	43.7 : 37.5 : 18.8	23,50 (0,000)	1,601 (0,115)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	F	29˚C	16	0.0 : 6.3 : 93.7[a,c]	N/A	1.093
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	F	17˚C	11	27.3 : 36.4 : 36.4	24,00 (0,289)	1,340 (0,076)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	F	17˚C >29˚C	16	0.0 : 6.3 : 93.7[a]	N/A	1.014
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	M	RT	15	53.3 : 40.0 : 6.7	23,62 (0,157)	1,652 (0,100)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	M	29˚C	16	0.0 : 6.3 : 93.7[a,c]	N/A	1.010[c]
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	M	17˚C	7	28.6 : 42.8 : 28.6	24,25 (0,250)	1,384 (0,076)

genotype	gender	Treatment	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Fer2 (28697)	M	17°C >29°C	21	0.0 : 19.0 : 81.0[a]	N/A	1,072 (0,040)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	F	RT	16	12.5 : 87.5 : 0.0[a]	24,00 (0,500)	1,310 (0,052)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	F	29°C	12	0.0 : 0.0 : 100.0[a,c]	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	F	17°C	13	0.0 : 46.2 : 53.8	N/A	1,187 (0,060)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	F	17°C >29°C	27	0.0 : 18.5 : 81.5	N/A	1,116 (0,048)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	M	RT	16	68.8 : 31.2 : 0.0[a]	24,41 (0,113)	1,699[a] (0,103)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	M	29°C	6	0.0 : 0.0 : 100.0[a,c]	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	M	17°C	25	8.0 : 80.0 : 12.0	24,75 (0,250)	1,270 (0,036)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (v15550)	M	17°C >29°C	1	0.0 : 0.0 : 100.0	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	F	RT	16	12.5 : 50.0 : 37.5	23,50 (0,000)	1,370 (0,050)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	F	29°C	16	0.0 : 18.8 : 81.2[a,c]	N/A	1,049[c] (0,019)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	F	17°C	19	10.5 : 47.4 : 42.1	24,50 (0,500)	1,324 (0,069)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	F	17°C >29°C	12	0.0 : 33.3 : 66.7	N/A	1,120 (0,086)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	M	RT	16	31.2 : 50.0 : 18.8	23,60 (0,245)	1,455 (0,077)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	M	29°C	15	0.0 : 0.0 : 100.0[a,c]	N/A	N/A
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	M	17°C	16	50.0 : 43.7 : 6.3	24,12 (0,157)	1,625 (0,110)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Mef2 (28699)	M	17°C >29°C	23	34.8 : 47.8 : 17.4[b]	22,75[a] (0,164)	1,402 (0,061)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	F	RT	16	37.5 : 37.5 : 25[a].0	23,67 (0,211)	1,572[a] (0,089)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	F	29°C	16	0.0 : 6.3 : 93.7[a,c]	N/A	1.017[c]
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	F	17°C	21	4.8 : 38.1 : 57.1	24.00	1,195 (0,069)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	F	17°C >29°C	29	0.0 : 24.1 : 75.9	N/A	1,146 (0,046)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	M	RT	16	68.8 : 31.2 : 0.0[a]	23,73[a] (0,079)	1,591 (0,059)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	M	29°C	15	0.0 : 6.7 : 93.3[a,c]	N/A	1.002[c]

genotype	gender	Treatment	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	M	17˚C	17	23.5 : 64.7 : 11.8	24,38 (0,125)	1,382 (0,081)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds trx (33703)	M	17˚C >29˚C	26	0.0 : 19.2 : 80.8[a]		1,034[a] (0,018)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	F	RT	16	18.8 : 43.7 : 37.5	25,00 (0,764)	1,518 (0,184)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	F	29˚C	14	0.0 : 7.1 : 92.9[c]	N/A	1.013
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	F	17˚C	25	4.0 : 36.0 : 60.0	25.50	1,188 (0,067)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	F	17˚C >29˚C	22	0.0 : 18.2 : 81.8	N/A	1,045 (0,022)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	M	RT	15	53.3 : 26.7 : 20.0	24,06 (0,305)	1,871 (0,204)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	M	29˚C	7	0.0 : 14.3 : 85.7[a,c]	N/A	1.146
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	M	17˚C	21	38.1 : 52.4 : 9.5	23,62 (0,157)	1,519 (0,096)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Pdp1ε	M	17˚C >29˚C	25	0.0 : 32.0 : 68.0[a]	N/A	1,055[a] (0,017)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	F	RT	16	62.5 : 31.2 : 6.3[a]	23,70 (0,082)	1,582[a] (0,077)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	F	29˚C	13	23.1 : 46.2 : 30.7	23,50 (0,000)	1,506[a] (0,132)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	F	17˚C	31	6.5 : 54.8 : 38 .7	23,50 (0,000)	1,167 (0,048)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	F	17˚C >29˚C	26	0.0 : 30.8 : 69.2[a,b]	N/A	1,049[b] (0,011)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	M	RT	16	81.2 : 12.5 : 6.3[a]	24,00 (0,127)	2,089[a] (0,119)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	M	29˚C	15	20.0 : 53.3 : 26.7[c]	24,83 (0,167)	1,367[c] (0,091)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	M	17˚C	32	15.6 : 59.4 : 25.0	24,20 (0,200)	1,282 (0,051)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hr38 (29376)	M	17˚C >29˚C	26	26.9 : 42.3 : 30.8	23,93 (0,170)	1,392 (0,076)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	F	RT	16	50.0 : 37.5 : 12.5	23,25 (0,134)	1,669[a] (0,098)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	F	29˚C	15	13.3 : 53.4 : 33.3	24,25 (0,750)	1,280[c] (0,076)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	F	17˚C	9	11.2 : 44.4 : 44.4	24.00	1,202 (0,106)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	F	17˚C >29˚C	23	4.3 : 34.8 : 60.9	23.00	1,220 (0,053)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	M	RT	16	68.8 : 31.2 : 0.0[a]	23,73 (0,170)	1,888[a] (0,138)

genotype	gender	Treatment	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	M	29˚C	14	7.1 : 42.9 : 50.0^{c}	23.50	1,271 ^{c} (0,088)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	M	17˚C	18	5.6 : 55.5 : 38.9	23.50	1,200 (0,065)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (29375)	M	17˚C >29˚C	18	0.0 : 55.6 : 44.4	N/A	1,181 (0,046)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v28430)	F	RT	16	12.5 : 81.2 : 6.3	24,25 (0,750)	1,323 (0,052)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v28430)	F	29˚C	13	46.2 : 38.4 : 15.4^{c}	23,92 (0,239)	1,672 (0,149)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v28430)	M	RT	16	75.0 : 25.0 : 0.0	24,50 (0,062)	1,680 (0,054)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v28430)	M	29˚C	16	68.7 : 31.3 : 0.0	24,14^{c} (0,118)	1,867 (0,139)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (25823)	F	RT	16	18.7 : 56.3 : 25.0	23,83 (0,333)	1,402 (0,108)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (25823)	F	29˚C	16	6.3 : 37.5 : 56.2	23.00	1,448 (0,251)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (25823)	M	RT	16	25.0 : 56.3 : 18.7	24,00 (0,289)	1,462 (0,052)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (25823)	M	29˚C	16	12.5 : 68.7 : 18.8	24,50 (0,500)	1,349 (0,100)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v107389)	F	RT	16	81.2 : 0.0 : 18.8	24,04 (0,144)	1,935 (0,092)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v107389)	F	29˚C	16	6.3 : 62.5 : 31.2^{c}	24.50	1,176^{c} (0,063)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v107389)	M	RT	16	68.8 : 31.2 : 0.0	24,45 (0,157)	1,774 (0,122)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Ir (v107389)	M	29˚C	16	50.0 : 50.0 : 0.0	24,00 (0,211)	1,536 (0,098)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds wds (32952)	F	RT	16	12.5 : 75.0 : 12.5	23,25 (0,250)	1,340 (0,080)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds wds (32952)	F	29˚C	16	6.3 : 37.5 : 56.2^{c}	23.50	1,358 (0,170)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds wds (32952)	M	RT	15	86.6 : 6.7 : 6.7	24,35 (0,104)	2,230 (0,117)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds wds (32952)	M	29˚C	16	37.5 : 50.0 : 12.5^{c}	24,75 (0,281)	1,425^{c} (0,100)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (v12692)	F	RT	16	68.7 : 18.8 : 12.5	23,59 (0,176)	1,826 (0,127)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (v12692)	F	29˚C	15	53.3 : 26.7 : 20.0	24,06 (0,199)	1,940 (0,174)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (v12692)	M	RT	16	93.7 : 6.3 : 0.0	23,87 (0,103)	2,180 (0,099)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds Hnf4 (v12692)	M	29˚C	16	87.5 : 12.5 : 0.0	24,54^{c} (0,143)	1,946 (0,113)

genotype	gender	Treatment	n	%r : %wr : %ar	Tau (SEM)	rrp (SEM)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds smi35A (v101376)	F	RT	16	68.7 : 25.0 : 6.3	23,59 (0,061)	1,786 (0,108)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds smi35A (v101376)	F	29˚C	16	12.5 : 56.2 : 31.3^{c}	23,50 (0,000)	1,391^{c} (0,150)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds smi35A (v101376)	M	RT	16	62.5 : 37.5 : 0.0	24,35 (0,198)	1,618 (0,068)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds smi35A (v101376)	M	29˚C	15	20.0 : 73.3 : 6.7^{c}	24,17 (0,441)	1,360^{c} (0,079)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (35286)	F	RT	16	56.2 : 37.5 : 6.3	23,33 (0,083)	1,583 (0,116)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (35286)	F	29˚C	14	35.7 : 35.7 : 28.6	23,60 (0,245)	1,651 (0,157)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (35286)	M	RT	16	62.5 : 37.5 : 0.0	23,65 (0,130)	1,830 (0,161)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (35286)	M	29˚C	16	50.0 : 25.0 : 25.0	23,88 (0,157)	1,810 (0,141)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (33629)	F	RT	16	87.5 : 12.5 : 0.0	23,75 (0,101)	2,106 (0,123)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (33629)	F	29˚C	22	22.7 : 54.6 : 22.7^{c}	23,60 (0,100)	1,584^{c} (0,113)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (33629)	M	RT	15	73.4 : 13.3 : 13.3	24,14 (0,118)	1,889 (0,133)
Dcr2;TUG; tub$_P$Gal80^{ts} > ds dlg1 (33629)	M	29˚C	26	46.2 : 34.6 : 19.2	23,92 (0,083)	1,677 (0,125)

Table 6.2. Results of the screen with conditional gene knockdown in all clock-containing cells.

Distribution of rhythmic, weakly rhythmic and arrhythmic flies, period length of rhythmic flies (Tau) and relative rhythmic power of rhythmic and weakly rhythmic flies (rrp) for females (F) and males (M) with a downregulation of a particular gene (*Dcr;TUG;tub$_P$Gal80ts>dsRNA*, marked by gene name and stock number) analysed at permissive conditions of 17˚C. Flies were raised at restrictive temperature (29˚C) and two different permissive ones (RT and 17˚C). In one condition, flies raised at permissive temperature were exposed to restrictive temperature as adults (17˚C>29˚C). Numbers of flies tested and SEM for values calculated are included in the table. Any association between a genotype of flies and a distribution of rhythmic, weakly rhythmic and arrhythmic flies was tested with 2x3 Fisher's Exact tests. Associations between period length or relative rhythmic power and genotype of flies were

determined either with Mann-Whitney tests or Kruskal-Wallis tests with Wilcoxon Rank tests for individual comparisons. All conditions were compared to flies raised at 17˚C (significant differences highlighted and marked as a), flies raised at 29˚C were compared to those from RT (significant differences highlighted and marked as c) and flies with adult gene knockdown (17˚C >29˚C) were compared to flies with developmental gene knockdown (raised at 17˚C) with significant differences highlighted and marked as b.

6.4.3.4 Attempts to rescue adult arrhythmia associated with developmental *per* over expression by supplementing a gene of interest

For this final part of the screen, I decided to focus my attention on two strongest candidates for mediating CLK/CYC function during development: *Pdp1ε* and *Mef2,* knockdown of which showed consistent disruption to adult locomotor rhythmicity (Figure 6.7, Tables 6.1 and 6.2). Due to the lack of reliable *UAS-transgene* constructs that could be used to cause over-expression of *trx* and F*er2,* I could not test these genes in this part of the screen. Moreover, *Fer2* was suggested previously to be epistatic to CLK/CYC (Nagoshi *et al.*, 2010) and its knockdown resulted in a stronger phenotype (Figure 6.7, Table 6.1) than the one associated with developmental *per* over-expression (Figure 4.1).

In the initial attempt at this experiment, flies with developmental *per* over-expression were directly crossed to flies with the gene of interest linked to *UAS* or w^{1118} controls. All resulting flies were heterozygous for both *TUG* driver, *per* transgene and the *Mef2* or *Pdp1ε*. One line with *Mef2* transgene and two independent *Pdp1ε* transgenes, one located on the second and other on the third chromosome were analysed. Flies were raised either under permissive (RT) or restrictive (29˚C) conditions and analysed at 17˚C as described in 6.3.7.

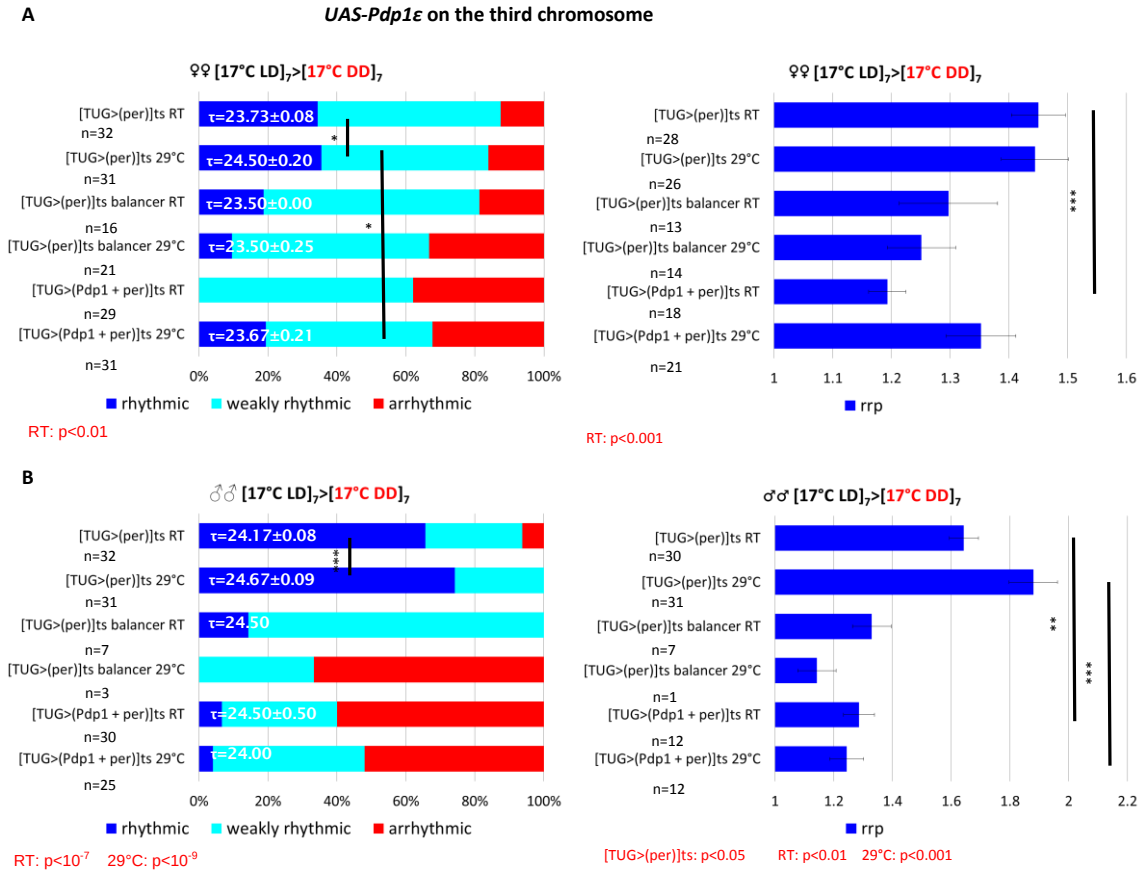

A
UAS-Pdp1ε on the third chromosome
♀♀ [17°C LD]7>[17°C DD]7
[TUG>(per)]ts RT
n=32
[TUG>(per)]ts 29°C
n=31
[TUG>(per)]ts balancer RT
n=16
[TUG>(per)]ts balancer 29°C
n=21
[TUG>(Pdp1 + per)]ts RT
n=29
[TUG>(Pdp1 + per)]ts 29°C
n=31
τ=23.73±0.08
τ=24.50±0.20
τ=23.50±0.00
τ=23.50±0.25
τ=23.67±0.21
0%
20%
40%
60%
80%
100%
rhythmic
weakly rhythmic
arrhythmic
RT: p<0.01
♀♀ [17°C LD]7>[17°C DD]7
n=28
n=26
n=13
n=14
n=18
n=21
1
1.1
1.2
1.3
1.4
1.5
1.6
rrp
RT: p<0.001
B
♂♂ [17°C LD]7>[17°C DD]7
τ=24.17±0.08
τ=24.67±0.09
τ=24.50
τ=24.50±0.50
τ=24.00
n=32
n=31
n=7
n=3
n=30
n=25
rhythmic
weakly rhythmic
arrhythmic
RT: p<10-7 29°C: p<10-9
♂♂ [17°C LD]7>[17°C DD]7
n=30
n=31
n=7
n=1
n=12
n=12
1
1.2
1.4
1.6
1.8
2
2.2
rrp
[TUG>(per)]ts: p<0.05 RT: p<0.01 29°C: p<0.001
C
UAS-Pdp1ε on the second chromosome
♀♀ [17°C LD]7>[17°C DD]7
τ=23.73±0.08
τ=24.50±0.20
τ=24.17±0.44
τ=24.04±0.12
τ=23.83±0.10
n=32
n=31
n=15
n=16
n=32
n=24
rhythmic
weakly rhythmic
arrhythmic
RT: p<10-6 29°C: p<0.05 [TUG>(per)]ts balancer: p<0.001
♀♀ [17°C LD]7>[17°C DD]7
n=28
n=26
n=14
n=12
n=10
n=17
1
1.2
1.4
1.6
1.8
2
2.2
rrp
[TUG>(Pdp1 + per)]ts: p<0.05 [TUG>(per)]ts balancer: p<0.01 RT: p<0.001
29°C: p<0.01
D
♂♂ [17°C LD]7>[17°C DD]7
τ=24.17±0.08
τ=24.67±0.09
τ=23.78±0.12
τ=23.81±0.21
τ=24.12±0.24
n=32
n=31
n=16
n=16
n=12
n=32
rhythmic
weakly rhythmic
arrhythmic
RT: p<0.001 29°C: p<10-6
♂♂ [17°C LD]7>[17°C DD]7
n=30
n=31
n=15
n=16
n=6
n=22
1
1.2
1.4
1.6
1.8
2
2.2
rrp
[TUG>(per)]ts: p<0.05 RT: p<0.01 29°C: p<0.001

Figure 6.8. Adult locomotor rhythmicity of flies with developmental *per* and *Pdp1ε* over-expression.

Quantitative analysis of rhythmicity of individual females (A and C) and males (B and D) from the experiments involving two different *Pdp1ε* transgenes (insertion on the third chromosome (A and B), and insertion on the second chromosome (C and D)) raised at either restrictive (29˚C) or permissive (RT) temperature. Stacked bar diagrams on the left show the distribution of flies with the n numbers included. Flies with the same genotype were compared with Fisher's Exact 3x2 tests. Experimental flies ([TUG>(Pdp1 + per)]ts) were compared to control flies ([TUG>(per)]ts balancer and [TUG>(per)]ts) with Fisher's Exact 3x3 tests and results of these tests are summarised below the graphs. Average period lengths were calculated for all rhythmic flies and are included on graphs with SEM. On the right, bar diagrams represent average relative rhythmic power calculated for rhythmic and weakly rhythmic flies with error bars representing SEM and numbers of flies included on the graphs. Association of period length or relative rhythmic power with developmental temperature was tested with non-parametric Mann-Whitney tests and with a genotype using non-parametric Kruskal-Wallis tests with Wilcoxon Rank test for individual comparisons. Overall significance for the relative rhythmic power is included below the graph. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The anticipated results (Figure 6.6) were clear differences between control flies, with flies raised at 29˚C displaying worse rhythmicity than flies from RT, as indicated either by the changes in a percentage of rhythmic, weakly rhythmic and arrhythmic flies or a relative rhythmic power. I hypothesised that if either *Mef2* or *Pdp1ε* is the CLK/CYC target that is required during development to establish adult locomotor rhythmicity, flies with an expression of the respective transgene raised at 29˚C will display rhythmicity comparable to flies from RT. I was also expecting that in all cases flies raised at RT should display similar rhythmicity while the differences in rhythmicity of flies raised at

29˚C would depend on whether the addition of the transgene rescues adult arrhythmicity of flies (Figure 6.6).

Unfortunately, what became obvious from the analysis of the results (Figures 6.8 and 6.9) was that the experiment could not result in any meaningful answer. When looking at percentage of the rhythmic, weakly rhythmic and arrhythmic flies, in majority of cases analysed, control flies did not appear to show correlation of their rhythmicity with a developmental temperature, with a notable exception of female flies with a balancer on the second chromosome (used as a control for *Mef2* and one of *Pdp1ε*), for which, surprisingly, there were more rhythmic flies raised at 29˚C, which also resulted in their higher relative rhythmic power than flies raised at RT. The control flies that resulted from crossing *per* over-expression flies with w^{1118} were consistently displaying period lengthening associated with development at 29˚C, even though in males it was associated with moderately significant higher relative rhythmic power. Taken together, these results might imply that *per* over-expression achieved in flies heterozygous for both *TUG* and *UAS-per* was not sufficiently high to result in an adult locomotor impairment associated with the developmental CLK/CYC inhibition. This was supported by an elongation of the period observed in one line of control flies. Unfortunately, it turned out that in the case of such low levels of *per* over-expression, the rhythmicity of flies is fairly dependent on the genetic background.

With the above in mind, the results obtained when the *Mef2* or *Pdp1ε* was added (Figures 6.8 and 6.9) were not fully informative. It appeared that in most cases their addition did not result in any significant change to the rhythmicity of flies. The two cases where moderate significance was found were the females with the addition of *Mef2* transgene and females with the addition of the *Pdp1ε* transgene located on second chromosome. In both of these cases, however, again flies raised at 29˚C were having higher relative rhythmic power than flies from RT (Mann-Whitney test: U=98.000, z=-2.565, $p<0.01$, n=43 for *Mef2*; U=36.000, z=-2.460, $p<0.05$, n=27 for *Pdp1ε*), which might again be a result of the genetic interactions of the background.

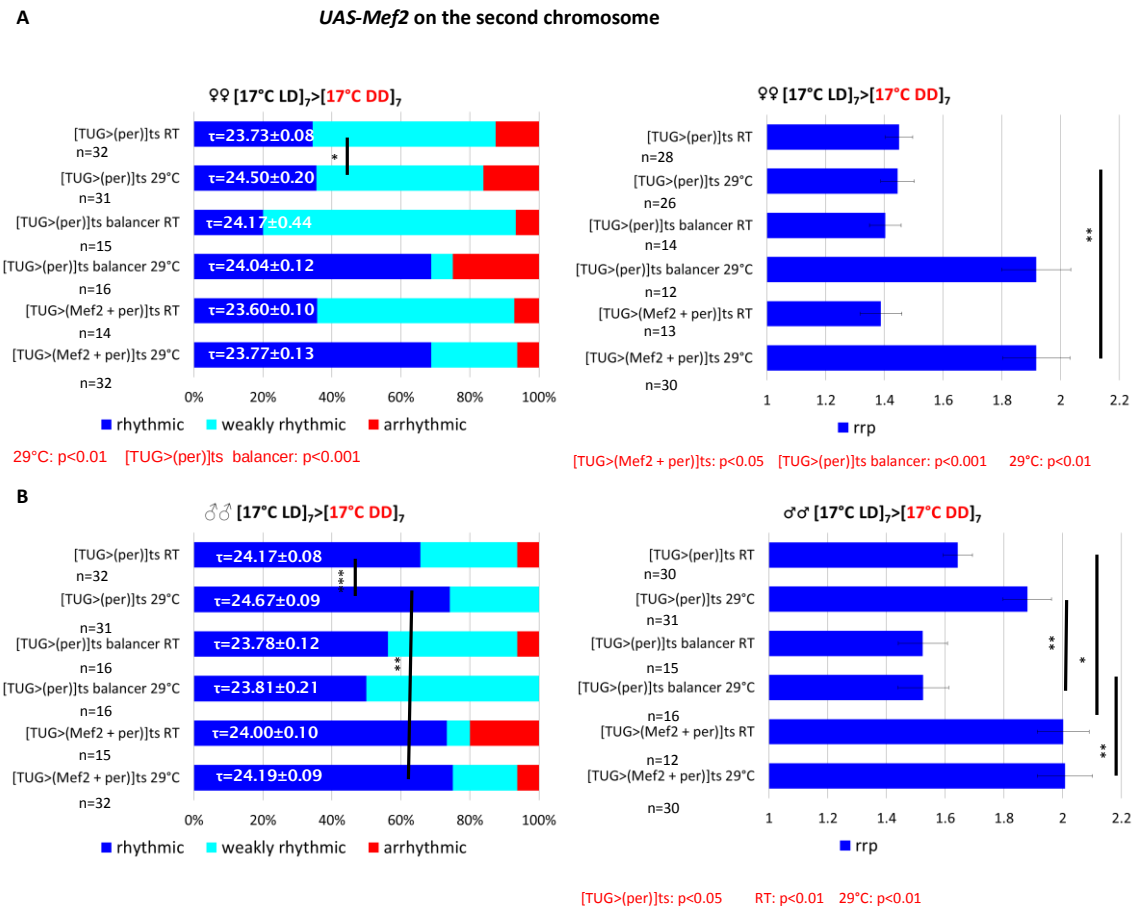


Figure 6.9. Adult locomotor rhythmicity of flies with developmental *per* and *Mef2* over-expression.

Quantitative analysis of the experimental female (A) and male (B) flies combining both *per* and *Mef2* over-expression ([TUG>(Mef2 + per)]ts) and control flies resulting both from the cross to the same line ([TUG>(per)]ts balancer) and cross to w^{1118} ([TUG>(per)]ts) raised at either restrictive (29˚C) or permissive (RT) temperature. Stacked bar diagrams on the left show the distribution of flies with the n numbers included. Flies with the same genotype were compared with Fisher's Exact 3x2 tests. Experimental flies ([TUG>(Mef2 + per)]ts) were compared to control flies ([TUG>(per)]ts balancer and [TUG>(per)]ts) with Fisher's Exact 3x3 tests and results of these tests are summarised below the graphs. Average period lengths were calculated for all rhythmic flies and are included on graphs with SEM. On the right, bar diagrams represent average relative rhythmic power calculated for rhythmic and weakly rhythmic flies

with error bars representing SEM and numbers of flies included on the graphs. Association of period length or relative rhythmic power with developmental temperature was tested with non-parametric Mann-Whitney tests and with a genotype using non-parametric Kruskal-Wallis tests with Wilcoxon Rank test for individual comparisons. Overall significance for the relative rhythmic power is included below the graph. *. * $p<0.05$, ** $p<0.01$.

One of the reasons why the results described above could be not very informative was a low level of *per* over-expression achieved when flies were heterozygous for both *TUG* and *UAS-per*. I therefore decided to increase levels of *per* over-expression by testing flies heterozygous for only one of these elements and homozygous for another. This involved generating flies that combined the *UAS*-linked transgene with either *TUG* or *UAS-per*. This time I had to focus on testing only one line with *UAS-Pdp1ε* (since the other one was unhealthy) and introduced one more line with *UAS-Mef2*. To obtain experimental and control flies, these newly created lines were crossed to *per* over-expressing flies. The experimental flies were flies containing tub_P-$Gal80^{ts}$, either two copies of *TUG* or *UAS-per* (depending on the location of the transgene tested) and a single copy of *UAS-transgene*. Control flies had a balancer chromosome instead of *UAS-transgene*. Additionally, female controls were created lacking tub_P-$Gal80^{ts}$ element and either constitutively over-expressing *per* alone or over-expressing *per* together with the gene of interest. Data was collected and analysed as described in 6.3.7.

This time the expected results were that females with the constitutive *per* over-expression shall display poor rhythmicity, which should serve as a control that *per* over-expression levels are sufficient to affect adult locomotor rhythmicity. Therefore, the control flies should display an association between developmental temperature and rhythmicity of flies. As outlined in Figure 6.6, my hypothesis was that an over-expression of one of the transgenes would restore the rhythmicity of flies raised at 29˚C.

Fortunately, this time flies with the constitutive *per* over-expression were showing very poor rhythmicity, which indicated that the inhibition of CLK/CYC

obtained was sufficient to impact locomotor rhythmicity (Figures 6.10 and 6.11). Levels of *per* over-expression obtained in the new lines contributed to control flies with the conditional *per* over-expression displaying an association between the rhythmicity of adult locomotor behaviour and developmental. The difference was statistically significant for both controls tested with *UAS-Mef2* (for distribution of flies Fisher's Exact 3x2 test: $p<10^{-5}$, n=35 for females and $p<10^{-4}$, n=54 for males; for relative rhythmic power Mann-Whitney test: U=82.000, z=2.758, p<0.04, n=23 for females and U=176.000, z=2.227, p<0.05, n=35) (Figure 6.11) and for males tested with *UAS-Pdp1ε* (Fisher's Exact 3x2 test: p<0.05, n=16) but not for females (Fisher's Exact 3x2 test: p=0.077, n=15) (Figure 6.10). Lack of statistical significance of the results obtained for control female flies for *UAS-Pdp1ε* was a consequence of low numbers of flies recovered, since a trend in both distribution of flies and relative rhythmic power persisted (Figure 6.10).

Addition of *UAS-Pdp1ε* to *per* over-expressing flies during development did not cause any rescue of adult locomotor rhythmicity (Figure 6.10). The distribution of rhythmic, weakly rhythmic and arrhythmic flies was significantly different for both females (Fisher's Exact 3x2 test: p<0.01, n=38) and males (Fisher's Exact 3x2 test: $p<10^{-7}$, n=50), and the relative rhythmic power was also showing same differences (Mann-Whitney test: U=157.000, z=2.845, p<0.01, n=29 for females, U=237.000, z=3.440, p<0.001, n=35 for males). Unfortunately, the same was also true for flies with the addition of the *UAS-Mef2* transgene (Figure 6.11). For the first line tested (17230), females raised at 29˚C displayed significantly lower relative rhythmic power (Mann-Whitney test: U=191.000, z=1.981, p<0.05, n=33), associated also with a shorter period length (Mann-Whitney test: U=18.000, z=2.416, p<0.05, n=9). There were significantly more arrhythmic males raised at 29˚C than at RT (Fisher's Exact 3x2 test: p<0.05, n=30) and remaining rhythmic and weakly rhythmic flies had lower rhythmic power (Mann-Whitney test: U=42.000, z=2.082, p<0.05, n=15). For the second line analysed (43412), females raised at 29˚C had more arrhythmic flies (Fisher's Exact 3x2 test: p<0.05, n=56) and males from the same temperature had both more arrhythmic flies (Fisher's Exact 3x2 test: $p<10^{-4}$, n=58) and lower relative rhythmic power (Mann-Whitney test: U=252.000, z=3.932, $p<10^{-4}$, n=36) (Figure 6.11).

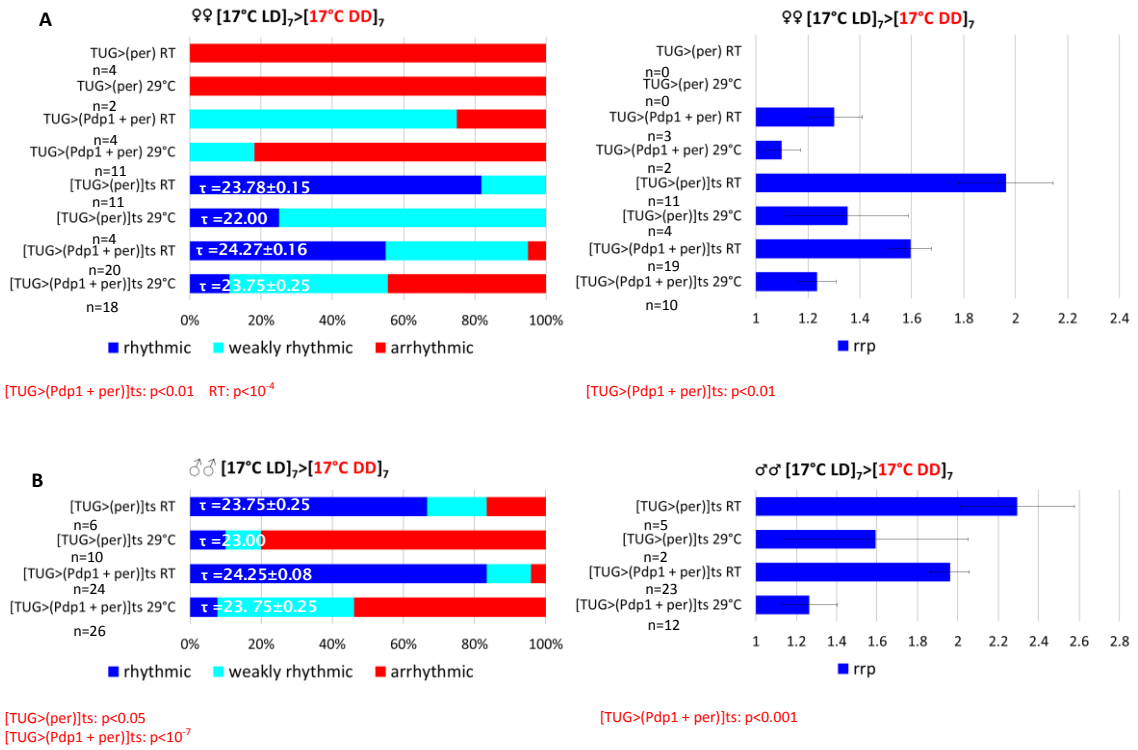


Figure 6.10. *Pdp1ε* over-expression does not rescue adult behavioural arrhythmia caused by developmental CLK/CYC inhibition.

Quantitative results of individual analysis of females (A) and males (B) raised at either restrictive (29˚C) or permissive (RT) temperature Stacked bar diagrams on the left show the distribution of flies with the n numbers included. Average period lengths were calculated for all rhythmic flies and are included on graphs with SEM. On the right, bar diagrams represent average relative rhythmic power calculated for rhythmic and weakly rhythmic flies with error bars representing SEM and numbers of flies included on the graphs. Associations between the distribution and the developmental temperature of flies with the same genotype were analysed with the Fisher's Exact 2x3 tests. Flies raised at the same temperature were compared with either Chi-square 4x3 tests (females, A) or Fisher's Exact 2x3 tests (males, B) Results of both tests are summarised below the graphs. Association of the period length or relative rhythmic power with the developmental temperature or with the genotype were tested with non-parametric Mann-Whitney or Kruskal- Wallis test.

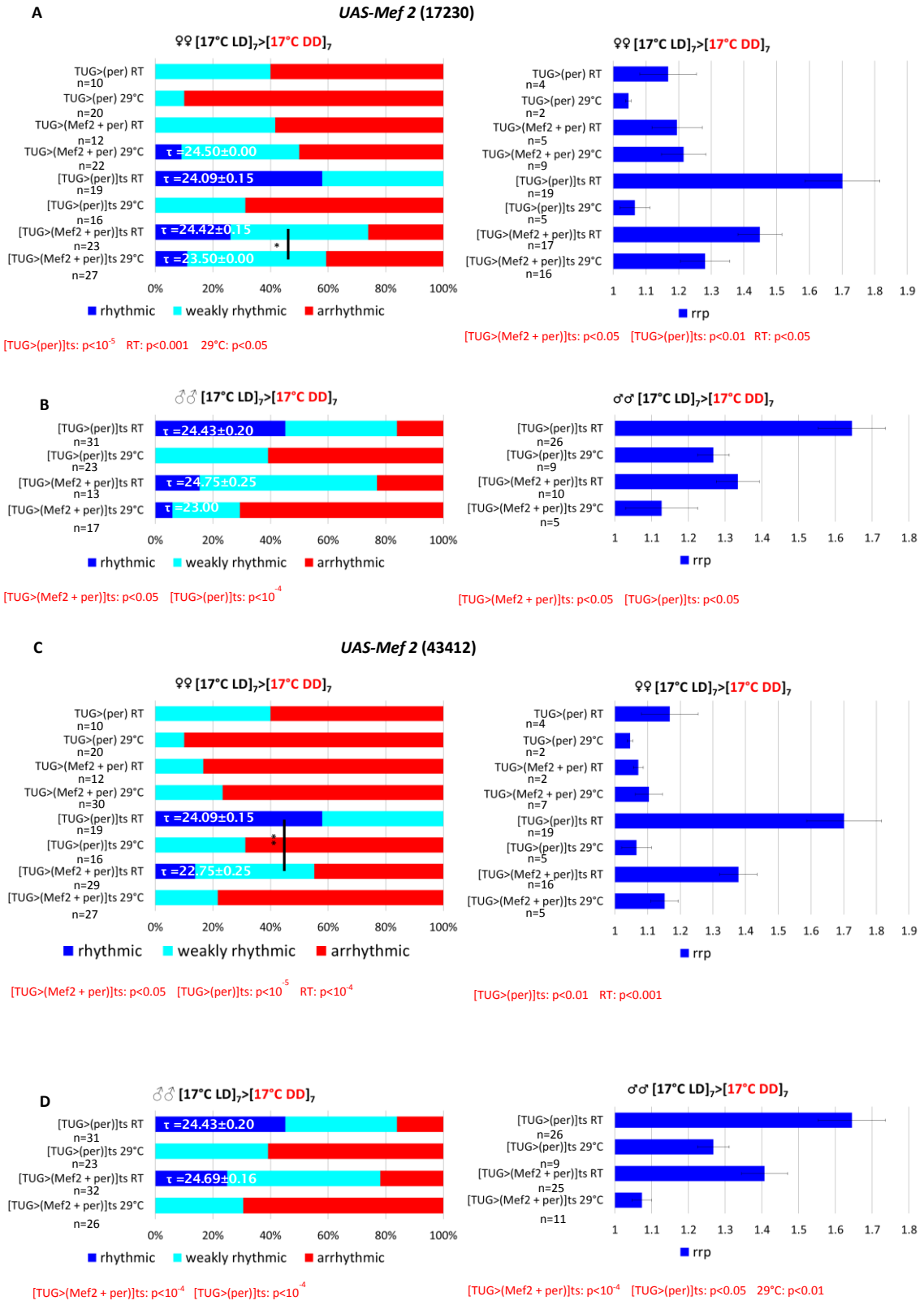


Figure 6.11. Developmental over-expression of *Mef2* does not rescue adult locomotor arrhythmicity caused by developmental *per* over-expression.

Two different *UAS-Mef2* lines were tested – 17230 (A and B) and 43412 (C and D) and graphs present quantitative results of analysis of adult locomotor rhythmicity of individual female (A and C) and male (B and D) flies raised at either restrictive (29°C) or permissive (RT) temperature. Stacked bar diagrams on the left show the distribution of flies with the n numbers included. To test for the associations between the distribution and the developmental temperature of flies with the same genotype the Fisher's Exact 3x2 tests were used and to see it the rhythmicity of flies raised at the same temperature depends on their genotype chi-square 4x3 test was used for females and Fisher's Exact 3x2 test was used for males. Results of both tests are summarised below the graphs. Average period lengths of all rhythmic flies were calculated (included on graphs with SEM). On the right, bar diagrams represent average relative rhythmic power calculated for rhythmic and weakly rhythmic flies with error bars representing SEM and numbers of flies included on the graphs. Associations of the period length or relative rhythmic power with genotype or developmental temperature were tested with non-parametric Mann-Whitney or Kruskal-Wallis tests (with individual comparisons with Wilcoxon Rank tests) and the significant results are marked on the graphs with overall significance for rrp below graphs. * $p<0.05$, ** $p<0.01$.

6.5 Discussion

6.5.1 Developmental PDF knockdown could be causing irreversible adult locomotor arrhythmia

In Chapter 5 I demonstrated that developmental CLK/CYC inhibition restricted to PDF-positive neurons caused irreversible adult arrhythmia (Figure 5.4). Moreover, this adult arrhythmia in response to *per* over-expression in circadian neurons was accompanied by disappearance of molecular oscillations in s-LN_vs (Figure 5.12). Since a rhythmic secretion of PDF from dorsal projections of s-LN_vs is required to synchronize circadian neurons across the brain in the free-

run conditions and generate rhythmic locomotor behaviour (Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2000; Park *et al.*, 2000; Peng *et al.*, 2003; Lin *et al.*, 2004; Stoleru *et al.*, 2005; Guo *et al.*, 2014) and PDF cycling in termini appears clock-dependent (Helfrich-Förster *et al.*, 2000), I speculated that adult behavioural arrhythmia observed in flies with developmental *per* over-expression might be a result of the impaired PDF release during development.

The knockdown of PDF resulted in decreased rhythmicity in females and a shorter period length in males (Figure A.4.1 A). As the results were not identical to the results reported for pdf^{01} flies, where the absence of PDF, similarly to the absence of LN_vs, caused behavioural arrhythmicity in constant darkness within 2-3 days, decrease in the amplitude of *tim* mRNA oscillations and a loss of synchrony in PER cycling in some circadian neurons (Renn *et al.*, 1999; Peng *et al.*, 2003; Lin *et al.*, 2004), it suggested that using *dsPdf* did not fully block the PDF signalling. However, the results obtained (Figure A.4.1 A) suggested that some degree of PDF impairment was achieved. As a complete knockdown of PDF generated a phenotype similar to ablation of PDF-positive neurons (Renn *et al.*, 1999), I did not expect developmental CLK/CYC inhibition causing complete disruption to PDF pathway, but rather only a partial inhibition of PDF signalling. Therefore, I tested if similar behavioural impairment could be achieved if *Pdf* is silenced only during development.

Surprisingly, at restrictive temperature, when *Pdf* was still actively silenced, flies were much more arrhythmic than previously described flies without tub_PGal80^{ts}, with percentages of weakly rhythmic flies close to those reported by Renn *et al.* (1999) for pdf^{01} flies (Figure A.4.1 B. Moreover, the addition of *Pdf* knockdown during development seemed to further reduce rhythmicity of flies (Figure A.4.1 B). When the knockdown of PDF was restricted only to development (Figure A.4.1 C), female flies were indistinguishable from those with the PDF knockdown during both development and adulthood (Figure A.4.1 B). On the other hand, males that experienced PDF knockdown during development had a largely reduced rhythmicity (Figure A.4.1 C).

The observed difference between females and males tested at permissive temperature might be a result of differences in the DICER expression levels. Since *UAS-Dcr2* is located on the X chromosome, males are expressing higher quantities of it than females. An alternative explanation for the lack of

consistency between results might be that 23˚C was not as permissive as I assumed and allowed some degradation of $GAL80^{ts}$, leading to *Pdf* knockdown. This might explain a lack of difference between the 23˚C-raised flies analysed at 23˚C (Figure A.4.1 C) and 29˚C (Figure A.4.1 B).

It is difficult to make the final conclusions on the basis of these results (Figure A.4.1). It is possible that a more informative comparison would have been between flies raised at 17˚C and 29˚C. However, preliminary testing might suggest that the developmental silencing of *Pdf* expression could be one of the factors contributing to the phenotype observed in flies with developmental CLK/CYC inhibition (Figure 4.1). The impairment of PDF signalling could not only account for behavioural arrhythmicity of adult flies (Figure 4.1), but also offers a possible explanation for the phase advance of TIM oscillations in LN_ds (Figure 5.12), as it was demonstrated previously that PDF maintains synchrony across circadian neurons and the lack of PDF signalling can cause phase advance in LN_ds (Lin *et al.*, 2004). Of course, there is a possibility that some other neurotransmitters that might be involved in relaying a signal from LN_vs to other neurons and impacting behaviour (Kaneko *et al.*, 2000b; Blanchandron *et al.*, 2001, Umezaki *et al.*, 2011) are also affected by CLK/CYC inhibition. Therefore, the phenotype observed with only PDF knockdown (Figure A.4.1) should be less severe than the one observed with developmental *per* over-expression.

With regards to the methods used in A.4.1, it is unknown if the process of gene silencing, once started, could be terminated. If it could not, it would be impossible to distinguish developmental effects from adult ones by assigning developmental impact on adult behaviour to the cases at which silencing still takes place.

6.5.2 Action of HDACs during development is not responsible for adult arrhythmicity of flies with developmental *per* over-expression

It was suggested that the inhibition of HDACs during development might change adult gene expression patterns (McDonald *et al.*, 2013). Therefore I hypothesised that histone acetylation status during development might be

responsible for adult behavioural arrhythmia observed in flies with CLK/CYC inhibition. Unfortunately, neither developmental nor adult administration of NaBu rescued adult arrhythmicity of flies (Figure 6.1). This could suggest that chromatin modifications are not involved in mediating adult arrhythmicity after developmental CLK/CYC inhibition. This might imply that the changes to the histone state are linked to the transcriptional activity of CLK/CYC and in its absence are not taking place. Alternatively, no permanent changes on promoters of the circadian genes are taking place during development even when CLK/CYC does not bind these promoters.

This first hypothesis is plausible, given that Doi *et al.* (2006) has shown that HATs (including p300/CBP) were unable to either acetylate histones or activate transcription without CLK HAT activity present. When CLK/CYC forms complex with PER and TIM and is released from E-boxes, HAT activity of CLK is stopped and acetylation of histones decreases (Doi *et al.,* 2006). Therefore, blocking HDACs in flies with developmental *per* over-expression might not lead to an increase of histone acetylation, since CLK/CYC is not present at E-boxes.

Of course, other histone modifications could be also involved in regulating the circadian clock. For example, histone methylation associated with a function of PROTEIN ARGININE METHYL TRANSFERASE 5 (PRMT5), which adds methyl groups to arginine residues (Bedford and Richard, 2005), was shown in *D. melanogaster* to affect circadian rhythms in locomotor activity of flies as well as splicing of *per* and several other clock-associated genes (but not their molecular oscillations) (Sanchez *et al.*, 2010). Mutations in PRMT5 were also affecting gene expression levels, with over 300 genes upregulated and 85 genes downregulated, many of which were CLOCK- regulated (McDonald and Rosbash, 2001; Sanchez *et al.*, 2010).

There is also a possibility that HDACs involved in changing the chromatin state at circadian genes in the absence of CLK/CYC during development belong to a group of deacetylases not targeted by NaBu. However, until now, the only histone deacetylases that were shown to be involved in generation of circadian rhythms in *D. melanogaster* were RPD3, belonging to class I (DiTacchio *et al.*, 2011), over-expression of which can upregulate *Pdf* and *tim* (Cho *et al.*, 2005), and HDAC4, member of class IIa, as well as potentially some other members of class IIa (Fogg *et al.*, 2014). For example, HDAC3 over-expression was also

causing upregulation of *tim*, suggesting that it might be also involved in regulation of circadian rhythms (Cho *et al.*, 2005). Most importantly, all of these HDACs are targeted by NaBu (Hubbert *et al.*, 2002; Cho *et al.*, 2005; Witt *et al.*, 2009; Chuang *et al.*, 2009). Interestingly, in a cell culture, class IIa HDACs can act also as E3 SUMO (Small Ubiquitin-Related Modifier) ligases, promoting SUMOylation of some proteins, including MEF2 (Zhao *et al.*, 2005a).

There exists a possibility that NaBu concentration used was insufficient or a mode of its delivery was incorrect. However, NaBu was shown to elicit a response even at 10 mM (Pallos *et al.*, 2008), which is three times lower than the concentration used in my experiments (Figure 6.1 B). In studies describing feeding NaBu to adult flies (at 10 mM), that concentration was shown to affect the phenotype of flies (Chen *et al.*, 2002; Zhao *et al.*, 2005a and b; Pallos *et al.*, 2008; St. Laurent *et al.*, 2013; McDonald *et al.*, 2013). Despite the fact that most of the experiments involving treating larvae with NaBu involved washing larvae in aqueous NaBu solution for a limited time (Chen *et al.*, 2002; Zhao *et al.*, 2005a and b), it was safe to assume that a considerable amount of NaBu is both going to penetrate larval cuticle and be ingested by them, suggesting that this method of adding NaBu to food is a valid way to administer NaBu.

On the other hand, it seems that NaBu treatment was toxic to flies not only at higher, but also at lower concentration (Figure 6.2). Surprisingly, this was not observed for the wild-type flies (Figure 6.2), suggesting that the impact of NaBu on the health of flies was linked to a genotype of flies. In case of flies with conditional *per* over-expression, data suggested that developmental NaBu treatment was either neutral or even beneficial to flies while adult exposure to NaBu was negatively impacting health of flies (Figure 6.2).

Moreover, at lower concentration (30 mM) NaBu affected locomotor rhythmicity of flies raised at permissive temperature (Figure 6.1 B). Even though it was possible that this counteracted any positive effect that blocking HDACs either during development or adulthood might have on rescuing developmental *per* over-expression of flies raised at 29˚C, it was very unlikely that histone deacetylation plays a major part in generating the phenotype observed in flies with developmental *per* over-expression.

100 mM NaBu, which in my experiments largely impacted health of flies (Figure 6.1 A) has been used by other research group without similar issues (Dey-Guha and Kar, 2001), however in my case flies were also exposed to elevated temperature. It is possible that the combination of these two factors might be especially detrimental to health of flies.

The impact of NaBu on longevity has been studied previously. Feeding NaBu to larvae was shown to extend lifespan of adult flies, but only when the strain of flies was short lived (Zhao *et al.*, 2005b). In case of strains of flies with normal lifespan, it was demonstrated that NaBu increases longevity when fed to flies in transition or senescent spans of life (towards the end of their life), but decreases the lifespan when administered during entire larval and/or adult life (McDonald *et al.*, 2012). Similarly, NaBu extended lifespan of *C. elegans*, which might indicate a conserved mechanism underlying this phenomenon (Zhang *et al.*, 2009). These changes to the lifespan might be the consequence of the fact that HDACs might be causing both a downregulation as well as an upregulation of multiple genes, including those involved in physiological processes (such as metabolism), cellular processes (such as cell differentiation), developmental processes, behaviour and even ageing itself (Cho *et al.*, 2005). However, to date, only *Sir2* was linked to any of them (ageing) in both *C. elegans* and *D. melanogaster* (Guarente, 2000; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). Therefore it is completely reasonable to assume that HDACs inhibition by NaBu might be interfering with some of these processes, leading to decreased healthspan or lifespan of flies.

6.5.3 Constant light during development does not restore the adult rhythmicity of flies with developmental *per* over-expression

Since developmental *per* over-expression leads to adult irreversible behavioural arrhythmia (Figure 4.1), I hypothesized that the adult locomotor rhythmicity could be restored if PER was sufficiently degraded during development. One way to degrade PER is to introduce constant light during development. Light is causing a conformational change to CRY (Lin *et al.*, 2001; Ozturk *et al.*, 2011), causing CRY to bind to TIM and triggering proteosomal degradation (Myers *et al.*, 1996; Ceriani *et al.*, 1999; Naidoo *et al.*, 1999; Busza *et al.*, 2004). After

TIM degradation, PER proteosomal degradation (following a DBT-mediated phosphorylation occurs) (Lee *et al*., 1996; Zeng *et al*., 1996; Grima *et al*., 2002; Ko *et al*., 2002). Therefore, in LL *per* mRNA cycling is eliminated within 3 to 4 days from the beginning of the exposure and resulting *per* level is a median of values observed at LD (Qiu and Hardin, 1996). Consequently, PER protein levels are also constitutively low (Zerr *et al*., 1990; Price *et al*., 1995).

Exposing wild-type flies to LL leads to behavioural arrhythmicity within a day or two (Konopka *et al*., 1989; Zerr *et al*., 1990; Power *et al*., 1995a; Qiu and Hardin *et al*., 1996; Stoleru *et al*., 2004). In flies deficient in CRY or with impaired CRY-dependent TIM degradation, no disruption to behavioural rhythmicity is observed (Emery *et al*., 2000b; Peschel *et al*., 2006; Koh *et al*., 2006b; Picot *et al*., 2007) and PER molecular oscillations persist in s-LN_vs and some *Pdf*-negative LNs up to five days (Rieger *et al*., 2006; Picot *et al*., 2007). Interestingly, even when cry^b flies lacked PER in PDF neurons, they were still displaying behavioural rhythms in LL (Picot *et al*., 2007). Detailed analysis has revealed that in the case of cry^b flies, two rhythmic components to their behaviour are present after several days at LL – a major one with a period of around 25 h and a smaller one, with a short period of around 22.5 h (Yoshii *et al*., 2004; Rieger *et al*., 2006). Also, over-expression of SGG in clock neurons, including evening cells, but not PDF-positive neurons, resulted in over a half of flies displaying behavioural rhythms in LL, which was a consequence of an increased CRY stability (Stoleru *et al*., 2007).

Murad *et al.* (2007) described that flies with constitutive *per* over-expression were behaviourally rhythmic in LL, with longer than circadian, but strong nonetheless, rhythms with a period of around 26.8 hours (longer than in case of rhythms of the same flies in DD, when τ =25.7 hrs). This elongation of a period length might suggest that, even though the behavioural rhythms were present, flies that over-express *per* were partially sensitive to light. Obviously, that study (Murad *et al.,* 2007) cannot reflect what happens when flies with developmental *per* over-expression are raised in LL. However, it suggests that LL causes changes to flies with *per* over-expression that result in presence of molecular rhythms in some circadian neurons and emergence of behavioural rhythms.

I hypothesized that in case of flies with *per* over-expression, constant light during development would decrease *per* levels, reverting flies to a state closely resembling per^{01}. As I have demonstrated in Chapter 3, flies experiencing decreased *per* levels during development could still exhibit adult behavioural rhythmicity (Figure 3.3). Therefore my assumption was that flies with developmental *per* over-expression raised in LL should display adult behavioural rhythmicity.

Surprisingly, my data has revealed that light conditions used during development had no impact on adult rhythmicity of flies with developmental *per* over-expression (Figure 6.3). Regardless of conditions analysed, no rhythmic flies were recovered. Unexpectedly, more flies were displaying weak rhythms under restrictive than permissive conditions, even though their rrp was similar (Figure 6.3). It is difficult to know the precise reason behind these results. It can be assumed that the poor rhythmicity of flies was not a result of developmental LL exposure but rather a consequence of *per* over-expression. As demonstrated previously in Chapter 3, raising flies in LL did not result in a decrease of behavioural rhythmicity of flies (Table 3.1), which was also confirmed by the literature (Power *et al*., 1995b).

There are two possible explanations for the poor rhythmicity of flies with developmental *per* over-expression raised in LL. The first one is that CLK/CYC inhibition persists in these flies even in a presence of constant light. This might suggest that in the case of elevated PER levels, light-dependent PER degradation is not sufficient (or not fast enough) to bring PER levels below the threshold level necessary to cease the CLK/CYC inhibition. Therefore, a molecular analysis of circadian transcripts and proteins should be performed in *per* over-expression larvae raised in LL to test the hypothesis that the developmental LL exposure does not cause sufficient degradation of PER.

The second explanation for the arrhythmicity of flies with developmental *per* over-expression raised in LL might be that *per* over-expression in LN_vs is processed differently and still affects behavioural rhythmicity. Therefore, behavioural rhythms could be restored in LL only by excluding *per* over-expression in these cells. The state of the circadian clock in LN_vs has been especially important for the behavioural rhythmicity of flies in LL as on one hand, restoring CRY expression only in these cells (so restoring a proper state

of the clock) decreased behavioural rhythmicity observed in flies lacking CRY (Emery *et al*., 2000b; Murad *et al*., 2007). On the other hand, it was suggested that SGG or *per* over-expression (and therefore perturbations to molecular clock) in the PDF-positive neurons led to either decreased adult rhythmicity or adult arrhythmicity in LL while SGG or *per* over-expression in PDF-negative circadian neurons left flies behaviourally rhythmic in LL (it is worth noting that these results are opposite to what Murad *et al*., (2007) presented), further suggesting that PDF neurons have unique properties in LL (Stoleru *et al*., 2007). It was speculated that this was due to PDF-positive cells impacting internal synchronization between neurons even at LL (Stoleru *et al*., 2007).

In addition to this, it was suggested that behavioural rhythms found in *per* over-expressing flies in LL were not driven by LN_vs (as the molecular rhythms in LN_vs were abolished in LL), but rather by DN1s, a subset of which showed molecular oscillations of circadian proteins with periods longer than circadian by several hours (Murad *et al*., 2007). This suggests that LN_vs are not important for generation of rhythms in LL. This was supported by Picot *et al.* (2007), who proposed that when CRY signalling is absent or reduced, three LN_ds and PDF-negative 5^{th} s-LN_v are driving the rhythmicity in LL. Picot *et al.* (2007) concluded that these results are not excluding each other, but rather suggest that different subsets of PDF-negative cells drive rhythmic behaviour in LL in response to different clock manipulations.

I hypothesized that protecting PDF neurons from *per* over-expression during development in LL might restore adult rhythmicity in DD at permissive temperature, similarly as it restores rhythmicity in adult flies analysed at LL. However, my results (Figures 6.4 and 6.5) demonstrated that exclusion of developmental *per* over-expression from PDF-positive neurons did not impact rhythmicity of adult flies analysed in permissive DD. Moreover, flies with both developmental and adult *per* over-expression in PDF-negative circadian neurons were mainly arrhythmic in LL (Figures 6.4 and 6.5), clearly in disagreement with Stoleru *et al.* (2007). One of the reasons for this discrepancy between my data and the results described by Stoleru *et al.* (2007) could be a higher light intensity used in my experiments than in that study (1600 ± 200 lux).

The more plausible explanation for this discrepancy is the different genotype of flies used in both studies. To introduce temporal expression control mechanism into flies with *per* over-expression, I used a transgene for *Gal80ts*. Therefore I encountered a previously mentioned (5.4.4) issue with using GAL80ts and GAL80 simultaneously in the same cells. According to the anecdotal evidence, temperature sensitive version of $GAL80^{ts}$ can outcompete GAL80. Therefore I could assume that *per* over-expression was never really blocked in PDF neurons. In this case, LL arrhythmicity of flies with either adult, developmental or adult and developmental *per* over-expression are consistent with results presented by Stoleru *et al.* (2007). However, since behavioural results presented by Murad *et al.* (2007) and Stoleru *et al.* (2007) for flies with *per* over-expression are opposite, it is impossible to make definite conclusions about my results.

6.5.4 *Pdp1ε*, *Fer2* and *Mef2* are the most plausible CLK/CYC targets necessary during development for establishing adult behavioural rhythmicity

I hypothesised that CLK/CYC, a key transcriptional regulator in the molecular oscillator (Allada *et al*., 1998; Rutila *et al*., 1998; Zhao *et al*., 2003) is involved in regulating expression of gene(s) of interest that could ultimately be responsible for establishing adult locomotor rhythmicity. In fact, the first studies using *Drosophila S2* cells and fly heads, identified 28 direct CLK target genes, including many transcription factors (Kadener *et al.,* 2007). With an advancement of molecular techniques, further ~1500 CLK targets were discovered, majority of which are regulated with a very similar mechanism as core clock genes (Menet *et al*., 2010; Abruzzi *et al*., 2011). About 60% of these targets show circadian oscillations (but this is suspected to be an underestimate). Moreover, 575 genes were shown to be targets of CYC, majority of them were also a target of CLK and PER, which acts as a repressor (Abruzzi *et al*., 2011). This uncovers a possibility for the existence of many unknown genes involved in the circadian clock function.

By selecting several genes from the list of CLK/CYC targets that were published (Menet *et al*., 2010; Abruzzi *et al*., 2011) and cross-referencing them to genes

that might be enriched (or even showing rhythmic expression) in PDF-positive neurons (Nagoshi *et al.*, 2010; Kula-Eversole *et al.*, 2010), I selected multiple genes for the screen for CLK/CYC effector gene(s). Obviously, genes selected by me represent only a minute fraction of all genes that deserve testing. My recommendation would be that, resources and time permitting, more of these genes are tested and positive results should be investigated further, since even if they turn out not to be involved in CLK/CYC – mediated generation of adult behavioural rhythmicity, there is a possibility to uncover novel components of circadian machinery. However, it should be kept in mind that due to gene redundancy or essential developmental role of some of these genes, it is possible that some of these genes will never be identified (Nagoshi *et al.*, 2010)

Even in a limited sample of genes tested, knockdown of most of them resulted in some impairment of adult circadian rhythms (Figure 6.7 and Table 6.1). Taking into account an important and unique function of CLK/CYC in the molecular oscillator (Allada *et al.*, 1998; Rutila *et al.*, 1998; Zhao *et al.*, 2003), it should not come as a surprise that multiple CLK/CYC targets are involved in a generation of circadian rhythms. While most of the genes with the strongest phenotypes were previously shown to be involved in circadian rhythms, there is still a potential to discover more targets of CLK/CYC that have circadian function, as evident by the identification of *trx*.

trx was included in the screen since it was shown to be CLK/CYC target (Abruzzi *et al.*, 2011). It was not linked previously to either circadian rhythms or locomotor activity. Surprisingly, knockdown of *trx* gene resulted in a complete arrhythmicity of flies (Figure 6.7 and Table 6.1). *Trx* was shown to be involved in axon guidance and is also a component of TAC1 trithorax group (trxG) (Berger *et al.*, 2008). Genes from the trxG act as gene activators, binding to specific regions of DNA and causing histone modifications that promote an open chromatin configuration (Petruk *et al.*, 2006; Czermin *et al.*, 2002; reviewed in Schuettengruber *et al.*, 2007 and Steffen and Ringrose, 2014). trxG genes also mediate epigenetic inheritance of chromatin states during development (reviewed in Muller and Kassis, 2006 and Schwartz and Pirrotta, 2007). Therefore, one could speculate that the phenotype associated with knockdown of *trx* is caused by the removal of an activation signal for a certain

gene and consequently a lack of expression of that gene. Unfortunately, it was impossible to make any conclusions whether *trx* was more important during development or adulthood (Table 6.2).

The three genes that came as the strongest candidates for being effector genes responsible for determining adult behavioural rhythmicity were *Pdp1ε*, *Fer2* and *Mef2*. Simple knockdown of all of them resulted in a substantial decrease of rhythmicity (Figure 6.7 and Table 6.1). It also appeared that *Pdp1ε* and *Fer2* were acting predominantly during adulthood and *Mef2* was acting mainly during development (Table 6.2).

Pdp1ε is a basic leucine zipper (bZip) transcription factor expressed rhythmically during development and in adult heads that is necessary for a proper growth (but not embryonic development) by being involved in an endoreplication and mitosis (Lin *et al.*, 1997; Reddy *et al.*, 2000; McDonald and Rosbash, 2001; Ueda *et al.*, 2002; Reddy *et al.*, 2006). It is one of the eight tissue-specific isoforms obtained through alternative splicing of *Pdp1* gene (Reddy *et al.*, 2000; Grumbling and Strelets, 2006). *Pdp1ε* has been known to be involved in generation of circadian rhythms for over 10 years – flies heterozygous for $Pdp1^{P205}$ had elongated period, however flies homozygous for the same mutation were severely developmentally delayed and rarely lived to adulthood (Cyran *et al.*, 2003). Reddy *et al.* (2006) confirmed that *Pdp1* mutants show developmental lethality, delayed development and affected growth. Direct target of CLK/CYC (McDonald and Rosbash, 2001), *Pdp1ε* binds to V/P boxes and has an opposite effect to *vri* and activates transcription of *Clk* (Cyran *et al.*, 2003). Subsequent studies disputed the role of *Pdp1ε* in the main molecular oscillator, suggesting that it is rather a component of the output pathway or it affects lateral neuron morphology (Benito *et al.*, 2007 and Lim *et al.*, 2007c showed that higher or lower *Pdp1ε* levels lead to disrupted behaviour but not changes in *Clk* or other circadian genes expression). *Pdp1ε* is currently considered to play a minor part in *Clk* regulation but is confirmed to be a part of the output pathways, regulating, among others, expression of *takeout* (Zheng *et al.*, 2009; Benito *et al.*, 2010). It has been suggested that *Pdp1* links the circadian rhythms with nutritional conditions of animal and is regulated by the nutritional state (Reddy *et al.*, 2006), feeding and metabolism (Benito *et al.*, 2010), male courtship (Benito *et al.*, 2010) and also that it links

clock to xenobiotic metabolism through detoxification effector genes (Beaver *et al.*, 2010). *Pdp1ε* was a suitable candidate for the screen described in 6.4.3 as it has been implied to be involved in a formation of neural connections between s-LN_vs and their targets (Lim *et al.*, 2007c).

My data suggested that both developmental and adult knockdown of *Pdp1ε* were causing irreversible behavioural arrhythmicity (Table 6.2). Two possibilities might explain this – knockdown of *Pdp1ε* at any stage (only development, only adulthood or both) might be detrimental for the behavioural rhythmicity of flies, or only adult knockdown of *Pdp1ε* is causing a behavioural arrhythmicity or the results observed for flies with developmental knockdown were associated with persisting expression of *dsPdp1ε* that was downregulating the gene also during adulthood.

Knockdown of *Fer2* produced the most severe phenotype, with no rhythmic flies remaining (Figure 6.7 and Table 6.1). It was even more severe that the phenotype observed with developmental *per* over-expression (Figure 4.1) which suggested that downregulation of *Fer2* is probably not responsible for that phenotype. This is consistent with previous reports finding that *Fer2* mutants show phenotype stronger than any of *Clk* mutants and as such *Fer2* probably upstream of *Clk* (Nagoshi *et al.*, 2010) and therefore it is unlikely to be a CLK/CYC target. *Fer2* encodes basic helix-loop-helix protein and has been identified as enriched in LN_vs and involved in the generation of circadian rhythms, as flies with mutations in *Fer2* were arrhythmic both at DD and LD (Nagoshi *et al.*, 2010). In addition, *Fer2* is involved in neurogenesis - flies with mutated *Fer2* had fewer LN_d and LN_v neurons (Nagoshi *et al.*, 2010) and dopaminergic neurons in the protocerebral anterior medial (PAM) and protocerebral anterior lateral (PAL) clusters (Bou Dib *et al.*, 2014). Interestingly, the numbers of neurons in these clusters increase until metamorphosis, but they never reach wild-type values in adult flies and further neuronal loss continued for at least 28 days (Bou Dib *et al.*, 2014).

Mef2 is an essential transcription factor that has been first identified as a regulator of expression of muscle genes (Gossett *et al.*, 1989). It is also involved in the myogenic differentiation (Black and Olson, 1998; Sandmann *et al.*, 2006; Naya and Olsen, 2007; Elgar *et al.*, 2008; Soler *et al.*, 2012) and in the process of myoblast identification (Bour *et al.*, 1995; Lilly *et al.*, 1995;

Ranganayakulu *et al.*, 1995; Molkentin *et al.*, 1995). It has been also shown to: be a regulator of many developmental programs (Potthoff and Olson, 2007); have anabolic function in fat body and immune response (Clark *et al.*, 2013) and be an oncogene involved in T cell acute lymphoblastic leukaemia (Homminga *et al.*, 2011) and in the proliferation and metastatic behaviour associated with cancer through Notch signalling (Pallavi *et al.*, 2012). In mammals, *Mef2* is also expressed in neurons, where it targets, among others, genes involved in the synaptic development (Flavell *et al.*, 2008). Some *D. melanogaster* embryonic and adult brain neurons also express *Mef2* (Taylor *et al.*, 1995; Schultz *et al.*, 1996). Although *Mef2* role in most of the neurons is unknown, over-expression of *Mef2* in clock neurons leads to a slower molecular oscillations and progressive desynchronization of s-LN_vs while knocking down *Mef2* dampens molecular oscillations, suggesting that it regulates core clock gene expression and/or influences clock neurons' communication (Blanchard *et al.*, 2010). Results obtained in my genetic screen (Figure 6.7, Tables 6.1 – 6.2) further confirm *Mef2* role in sustaining circadian rhythms Moreover, my data suggests that it might be necessary predominantly during development to establish adult behavioural rhythmicity, as adult exposure to 29˚C did not reduce rhythmicity of flies (Table 6.2).

The genotype of flies used at all stages of the screen could cause some differences between female and male flies. At the first two stages of the screen, location of *UAS-Dcr2* on the X chromosome was causing higher DICER expression levels in males than in females, which could potentially result in a stronger knockdown, and consequently stronger phenotype, in males. In addition to this, as mentioned previously in 6.5.1, there is no certainty that a process of gene silencing can be happening only during development (in other words, that *dsRNA* expression can be stopped). Therefore, the condition thought to be developmental gene knockdown could represent realistically knockdown of a gene during both the development and adulthood. Thus, flies showing impaired rhythmicity at 17˚C when they were raised at restrictive temperature of 29˚C (Table 6.2) might be indeed still experiencing genetic knockdown. However, looking at results obtained in males with the conditional knockdown of *Mef2* (line 28699), it appears that adult exposure to 29˚C does not cause infinite gene downregulation, as flies had rhythmicity comparable to control flies (Table 6.2). Therefore, if adult gene silencing can be stopped,

there is a high chance that the developmental silencing can be also terminated when flies are moved from a restrictive temperature of 29˚C to 17˚C for testing, especially since they are also given 7 days of entrainment at permissive temperature (that could potentially help to remove *dsRNA*).

The first attempt to rescue adult behavioural arrhythmia associated with developmental CLK/CYC inhibition or impairment with over-expression of gene candidates was unsuccessful (Figures 6.8 and 6.9). Unfortunately, levels of *per* over-expression achieved in flies heterozygous for both driver and *per* transgene were insufficient to cause adult behavioural arrhythmicity. Instead, only some period elongation was observed, which is consistent with some mild impairment to circadian clock function (Figures 6.8 and 6.9). With higher levels of *per* over-expression, simultaneous over-expression of either *Mef2* or *Pdp1ε* did not reverse adult behavioural arrhythmicity (Figures 6.10 and 6.11). This might suggest that neither of these genes is a CLK/CYC effector responsible for establishing adult locomotor rhythmicity. However, it has to be acknowledged that the situation in these experimental flies is far more complex than just using two genes to cancel each other out. No precise control over levels of over-expression exists and it is possible that the arrhythmicity is caused by over-expression of the candidate gene rather than by the lingering effect of developmental *per* over-expression.

Chapter 7: General discussion

7.1 Principal Findings

This research set out to uncover the relationship between the function of the circadian clock and its elements during development and adult circadian locomotor behaviour. The question whether circadian clock function during development is necessary for adult behavioural rhythms has been asked before and the answer was that developmental *per* was not essential for adult rhythmicity (Ewer *et al.,* 1988 and 1990). Research presented in this thesis used novel combinations of genetic elements that could regulate the function of a circadian clock in a temperature-sensitive manner to confirm and extend this finding.

In the first series of experiments, I was able to confirm that adult flies do not require the presence of *per* during development for generation of normal adult locomotor rhythms (Chapter 3). Adult entrainment with LD cycles at a

permissive temperature helped restore rhythmic locomotor behaviour in DD after development in the absence of *per*.

Different results were obtained for flies deprived of functional CLK/CYC heterodimer during development (Chapter 4). Both in the case of flies with developmental inhibition of CLK/CYC through *per* over-expression and flies lacking CYC during development, adult flies were unable to exhibit adult locomotor rhythmicity under permissive conditions. Moreover, flies with developmental depletion of CYC showed disrupted adult behaviour in LD conditions.

The developmental requirement for CLK/CYC function during development was temporally mapped to metamorphosis and to a group of 18 (per brain) PDF-positive neurons (Chapter 5). It was also suggested that among those, 8 s-LN_vs were particularly sensitive to developmental CLK/CYC presence. Moreover, developmental depletion of CLK/CYC in all clock neurons caused permanent reduction of molecular oscillations in these neurons.

Finally, possible mechanisms by which CLK/CYC during development might influence adult locomotor rhythms were tested (Chapter 6). Preliminary work indicated that developmental histone deacetylation does not appear to be involved, however it is possible that developmental interruption of PDF signalling might be involved. Potential CLK/CYC targets necessary during expression for ensuring adult behavioural rhythmicity were tested, however over-expression of either of the two strongest candidates did not restore adult circadian rhythmicity of flies with developmental CLK/CYC inhibition.

7.2 General discussion

The general discussion is structured by areas of circadian biology or development of *D. melanogaster* to which my research contributes. Individual results are discussed in more detail in respective chapters. Where more detailed discussion of the implications of my research was included previously, respective sections are clearly mentioned.

7.2.1 Role of development in generation of adult behavioural rhythmicity

Results presented in Chapter 3 provide convincing evidence that developmental *per* expression is not necessary during development for adult rescue of rhythmicity. Even though the preliminary experiments demonstrated that the rescue of rhythmicity observed in flies with conditional *per* rescue was less than perfect (Figure 3.3), with a large percentage of flies remaining arrhythmic, it was later demonstrated that this could be largely improved by the addition of four days of entrainment at permissive temperature before testing at permissive conditions (Table 3.1 and Figure A.1.3). It would be interesting to test whether longer entrainment prior to testing can lead to even better rescue of behavioural rhythmicity. Since PER is necessary for the circadian clock function (Konopka and Benzer, 1971), results described in Chapter 3 demonstrate that circadian clock function during development is not essential for a functional circadian clock in adult *Drosophila melanogaster*.

Similar findings were described previously in literature using per^{01} flies with transgenic *period* expressed from the *heat-shock protein 70* (*hsp70*) promoter (Ewer *et al.,* 1988 and 1990) as in that study also a partial rescue of adult rhythmicity was observed. However, my research narrowed down *per* expression to circadian, rather than all, cells, resembling better a situation which occurs in wild-type flies. This could potentially account for the difference between these studies, as Ewer *et al.* (1988 and 1990) reported flies with an abnormally long period (even if transgenic *per* expression was restricted to adulthood) while in my research period length was closer to wild-type value (about 2 hours longer). Therefore, restoration of *per* function only in clock-bearing cells resulted in more complete rescue of adult behavioural rhythmicity that was not dependent on developmental *per* expression.

However, both in my research and in the research presented by Ewer *et al.* (1988 and 1990), *per* was not expressed in a circadian manner (associated with 2-3 fold changes in *per* mRNA level in DD (Baylies *et al.*, 1987; Hardin *et al.*, 1990)) but rather constitutively. This constitutive pattern of *per* expression at a higher level (and, consequently, elevated *per* levels during times where *per* levels should normally drop, i.e. be in a trough phase) might be responsible for the reduced adult behavioural rhythmicity and period elongation, as similar

phenotypes were also observed for flies constitutively expressing *per* (Kaneko *et al*., 2000b). A trough in *per* expression is necessary in early morning for a downregulation of PER and subsequent induction of CLK/CYC target genes (Hall *et al*., 2000). Lack of that trough in *per* expression may lead to a delay in PER downregulation and CLK/CYC activating expression of its downstream genes, which would account for weaker molecular oscillations and longer period. Therefore the situation in the line used is different than in the case of flies with increased *per* levels due to insertion of more wild-type *per* transgenes, which explains discrepancies with previous reports that demonstrated that elevated *per* levels were associated with period shortening (Smith and Konopka, 1982) while decreased *per* levels or expression resulted in period elongation (Konopka and Benzer, 1971; Baylies *et al*., 1987; Martinek and Young, 2000). In the case of a strong circadian regulation of *per* expression, PER nuclear entry and repression of CLK/CYC function is advanced with elevated *per* levels and delayed when *per* levels are lower.

Interestingly, my results suggested the impact of developmental light conditions on the behavioural rhythmicity of adult flies (Figures A.1.1 and A.1.2). It is currently unknown if this would be identical for all flies or if this is a property of flies with impaired circadian clock or even only flies lacking *per*, as it was demonstrated primarily for the line with conditional *per* rescue. My results demonstrated that DD during development largely reduced adult rhythmicity, a finding consistent with Kalmus (1940), Dowse and Ringo (1988) and Power *et al*. (1995a and 1995b). The first two studies used various wild-type flies while the last one used both per^{+} and per^{01} (for detailed description of their results see 3.4.2). However, both anecdotal and published evidence suggests that raising flies in DD does not affect their rhythmicity (for example: Sehgal *et al*., 1992). It is possible that the differences between lines and various results described in the literature might be explained by light-sensitivity (governed by the status of their *tim[s]/tim[ls]* alleles) (Rosato *et al*., 1997; Tauber *et al*., 2007; Sandrelli *et al*., 2007; Peschel *et al*., 2009) contributing to how strongly light can impact molecular oscillator (detailed explanation in 3.4.2).

Despite the fact that the functional circadian clock itself during development is not important for adult behavioural rhythms, I was able to demonstrate that

function of a key component of the molecular oscillator, CLK/CYC, is essential during development for future adult behavioural rhythms (Figures 4.1 and 4.2). This was demonstrated using two separate methods, one in which CLK/CYC could be inhibited in a temperature-dependant manner through over-expression of PER (Figure 4.1) and another in which formation of the heterodimer is prevented due to conditional *cyc* depletion (Figure 4.2). It has been demonstrated before that both increasing PER levels or decreasing CYC levels negatively impact CLK/CYC function (Rutila *et al*., 1998; Kaneko *et al*., 2000b; Yang and Sehgal, 2001), therefore I could assume that both lines used cause reduction in CLK/CYC activity. These two lines had opposite restrictive and permissive, eliminating the possible impact of developmental temperature on adult behavioural rhythmicity. Contrary to locomotor arrhythmia resulting from adult CLK/CYC inhibition or CYC depletion, which was reversible, the same molecular changes during development resulted in irreversible adult arrhythmicity that persisted even after prolonged entrainment at permissive temperatures (Figures A.2.2-A.2.3). Therefore I could conclude that the adult locomotor arrhythmicity observed in flies with developmental *per* over-expression or *cyc* depletion represented a requirement for CLK/CYC function during development.

One possible limitation of my results is that despite making assumptions about the state of a molecular clock during development in these different fly lines, I never tested it directly. There are some indications that the circadian clock is fully stopped in flies with the conditional *per* rescue, as upon transfer to permissive temperature, flies assumed phase associated with the time of the shift (Figure 3.4). However, flies with conditional *cyc* rescue resumed rhythmicity in permissive conditions with the phase associated with the environmental time experienced prior to testing rather than the time of shift to permissive conditions (Figure 4.3), indicating that they might retain some molecular oscillations, for example due to low levels of *cyc* expression in restrictive conditions, which is not enough to sustain free-running rhythms. Alternatively, this can suggest an existence of a residual oscillator that is LD-entrainable at adult restrictive conditions in spite of behavioural arrhythmia in DD at this temperature. No such studies whatsoever were conducted on flies with developmental *per* over-expression and therefore they should be carried out in the future.

Therefore the most obvious follow-up experiment would be to test the molecular state of the oscillator in all three lines. This could be done by checking the levels of core circadian transcripts and proteins present in brains (or heads), such as *period*, *timeless*, *vrille* (*vri*) and *Par domain protein1* (*Pdp1*) and CRY with a qPCR and Western blot. For the best comparison, this should be performed on flies raised under both permissive and restrictive conditions and analysed at either permissive or restrictive temperatures.

In light of my results pointing to a developmental requirement for CLK/CYC but not PER activity, it is interesting to revisit results showing that even though PER is present in s-LN_vs earlier than CLK, molecular oscillations are absent from them until CLK is expressed (Houl *et al.*, 2008). This can be interpreted as the requirement for developmental CLK/CYC function for establishing the function of the molecular oscillator. Together with my results showing that even when CLK/CYC functions during adulthood, molecular oscillations are absent in s-LN_vs (Figure 5.12), it suggests that if these molecular oscillations are not started prior to metamorphosis, they cannot be reliably started at all.

Finally, as my results show that CLK/CYC function but not functional circadian clock is necessary during adulthood for the adult locomotor rhythmicity, it suggests that there is an important role for CLK/CYC during development that is not connected to its main function as a clock component. This might be because CLK/CYC, as a transcriptional activator, binds to E-box elements (Hao *et al.*, 1997; Darlington *et al.*, 1998; Allada *et al.*, 1998; Rutila *et al.*, 1998) and activates transcription of circadian and also many non-clock genes (Blau *et al.*, 1999; Glossop *et al.*, 2003; Abruzzi *et al.*, 2011). Alternatively, this might be due to other levels of circadian components, as per^{01} flies have intermediate to high levels of clock-regulated transcripts (Sehgal *et al.*, 1994; Blau and Young, 1999; Hao *et al.*, 1999; Claridge-Chang *et al.*, 2001) while cyc^{01} or *per* over-expressing flies have down-regulated levels of clock-controlled genes (Allada *et al.*, 1998; Rutila *et al.*, 1998; Blau and Young, 1999; Hao *et al.*, 1999; Claridge-Chang *et al.*, 2001).

The two most plausible genes that could cause behavioural and molecular arrhythmicity in response to CLK/CYC inhibition or CLK depletion during development are *Pdp1ε* and *Mef2*. Behavioural arrhythmicity in their absence, over-expression or associated with their mutation was previously demonstrated

in literature (Cyran *et al*., 2003, Blanchard *et al*., 2010); and I was able to show that while *Pdp1ε* acted possibly during adulthood, *Mef2* seemed to be important during development (Table 6.2). In a yet unknown mechanism, lower levels of *Mef2* caused dampening of molecular oscillations (Blanchard *et al*., 2010) which is consistent with what I observed in s-LN$_v$s, which might imply that it is somehow involved in the regulation of core clock genes expression. Mechanism of *Pdp1ε* action is better understood as it binds to V/P boxes and activates transcription of *Clk* (Cyran *et al*., 2003). However, multiple reports have demonstrated that decreased *Pdp1ε* levels did not cause changes in the circadian gene expression (Benito *et al*., 2007 and Lim *et al*., 2007c), which might serve as a confirmation that it is necessary for the generation of behavioural rhythms in adulthood as it is a component of the output pathway.

7.2.2 Adult wakefulness and activity of flies might depend on CLK/CYC function during development

Interestingly, my results suggest that CLK/CYC function is necessary during development not only for adult free-running rhythms but also for conventional behaviour of flies in light/dark cycles (Figures 4.4 and 4.6). This supports previous findings that flies with inactive CLK (*Clk^{Jrk}*) have increased levels of nocturnality (Kumar *et al*., 2012). This could be linked to increased dopamine levels in these flies (which is a consequence of loss of CLK-mediated regulation of gene encoding TYROSINE HYDROXYLASE (TH), a rate limiting enzyme necessary for dopamine production) which acts through CRY to increase nocturnal arousal (Kumar *et al*., 2012). However, that study did not focus on distinguishing developmental versus adult implications of disrupting CLK/CYC function whereas my experiment suggests that it is the combination of developmental and adult CLK/CYC function that impacts the adult behaviour in LD. Together, this suggests a role of CLK/CYC in modulating the wakefulness and activity of flies.

To confirm to what extent and how developmental CLK/CYC activity contributes to wakefulness and arousal of adult flies, further studies are recommended. First, LD behaviour of flies with developmental *per* over-expression needs to be tested in various combinations of different

developmental and testing conditions. Presumably, if CLK/CYC function is important for LD behaviour, it should be possible to confirm this in a third independent manner. Moreover, dopamine and TH levels should be tested in flies with developmental CYC depletion and CLK/CYC inhibition.

Even though my results indicated that s-LN_vs might be more sensitive to developmental CLK/CYC function than l-LN_vs (Figures 5.9 – 5.11), this was because the tested property in these experiments was a free-running rhythm. Given that previous research linked l-LN_vs with control of sleep and arousal in flies (Agosto *et al.*, 2008; Parisky *et al.*, 2008; Kula-Eversole *et al.*, 2010, Shand *et al.*, 2011; Gmeiner *et al.*, 2013), it offers the possibility that the impairment of behavioural rhythms in LD observed in flies with developmental *cyc* depletion are a result of impaired CLK/CYC function in l-LN_vs.

7.2.3 Importance of metamorphosis for adult locomotor rhythmicity

I was able to demonstrate that the inhibition of CLK/CYC activity through *per* over-expression or depletion of *cyc* during (but not prior to) metamorphosis caused irreversible adult arrhythmia (Figures 5.1 and 5.2). In separate experiments, I demonstrated that out of all 150 circadian neurons in the brain, a lack of CLK/CYC function during development in just 18 PDF-positive neurons irreversibly impacted adult behavioural rhythmicity (Figures 5.6 – 5.8). This suggested that in spite of molecular oscillations existing in s-LN_vs, DN2s and two DN1s (Kaneko *et al.*, 1997; Kaneko and Hall, 2000) prior to metamorphosis, these molecular rhythms, crucial for larval rhythms in photophobic response (Mazzoni *et al.*, 2005) are not important for adult behavioural rhythms.

7.2.4 Differences between s-LN_vs and l-LN_vs

Interestingly, my results pointed to s-LN_vs rather than l-LN_vs (which start developing during late larval stage L3 and continue through early metamorphosis (Kaneko *et al.*, 1997; Helfrich-Förster *et al.*, 2007a, Liu *et al.*, 2015)), as the clock cells that require function of CLK/CYC for the generation

of adult circadian rhythms (Figures 5.9-5.11) and also demonstrate abnormal molecular oscillations in adulthood following developmental *per* over-expressions, with constantly low levels of TIM (Figure 5.12). This suggests a possible mechanism of generation of adult behavioural arrhythmicity – absence of molecular oscillations in key clock neurons responsible for driving free-running rhythms.

Mapping the development CLK/CYC requirement for establishing adult rhythmicity to s-LN$_v$s is consistent with their main role in driving adult locomotor rhythms in constant darkness (Helfrich-Förster, 1997; Renn *et al.,* 1999; Emery *et al*., 2000b). Sensitivity to CLK/CYC function during development is therefore another property contributing to differences between s-LN$_v$s and l-LN$_v$s. Only about half of the genes expressed in each neuron group are the same between them, with the other half determining their unique properties and functions (Kula-Eversole *et al*., 2010), such as their regulation of different aspects of rhythmic locomotor behaviour, with s-LN$_v$s driving the morning peak and free-running rhythms (Renn *et al*., 1999; Blanchardon *et al*., 2001; Stoleru *et al*., 2004; Sheeba *et al*., 2009) and l-LN$_v$s contributing to the evening peak (Grima *et al*., 2004; Stoleru *et al*., 2004; Rieger *et al*., 2006).

My results suggest that during metamorphosis, some of the processes crucial for generation of adult rhythms occur either before l-LN$_v$s are fully formed and start expressing circadian proteins (~50-60% pupal development, (Kaneko and Hall, 2000; Helfrich-Förster *et al*., 2007a, Liu *et al*., 2015)) or they occur specifically in s-LN$_v$s in the latter portion of metamorphosis, adapting their function to the adult behavioural rhythmicity. Distinguishing between these possibilities is not an easy task but some preliminary insights could be potentially achieved by repeating experiments similar to the one described in 5.3.1, however shifting flies around the midpoint of metamorphosis. However, the limitation of this approach is that at the moment we cannot be sure of how much time is required to translate the change in temperature into true molecular change. This would require detailed molecular studies of the level of *per*, *tim*, *vri* and *Pdp1*ε as proxy for measuring CLK/CYC function.

7.2.5 Neurogenesis and circadian projections

As during metamorphosis existing projections are pruned or rerouted and adult neurons and neuronal connections are formed I hypothesised that CLK/CYC function during development is necessary for establishing proper dorsal projections from s-LN_vs and in its absence intercellular communication is affected. In wild-type flies, long projections from s-LN_vs extend to the dorsal protocerebrum, where they form a dorsal horn and extensively arborize (Kaneko and Hall, 2000; Helfrich-Förster, 2005). They contact DN1 neurons which in turn establish connections with neurons in PI, forming a pathway to convey information from the molecular oscillator to influence behaviour (Shafer *et al.*, 2008; Cavanaugh *et al.*, 2014).

I speculated that in the absence of proper neuronal connections phase information might not be passed on from s-LN_vs to downstream neurons, even if the cell-autonomous oscillations persist in neurons. This kind of neuroanatomical defect was suggested previously in the literature, with Clk^{ar} flies and cyc^{01} flies with *cyc* function rescue only in PDF-positive neurons showing separation between molecular and behavioural rhythms (Allada *et al.*, 2003; Peng *et al.*, 2003) and Clk^{Jrk} and cyc^{01} flies displaying neuroanatomical defects in LN_vs (Park *et al.*, 2000).

In light of all the results obtained, this model can be rejected as the main explanation for the adult arrhythmia observed as preliminary studies showed that the neuroanatomy of flies with developmental *per* over-expression appears normal and dorsal projections from s-LN_vs appeared intact (Figure A.3.6). This was different from our previous observation that blocking *cyc* function in PDF neurons resulted in affected morphology of dorsal projections – dorsal projections were absent in 25% and stunted in the 37% of analysed brains (Goda, Mirowska and Currie *et al.*, 2011). This suggests that developmental CYC depletion or CLK/CYC inhibition produces less severe phenotypes than constitutive *cyc* depletion, possibly due to some low levels of CLK/CYC activity in flies with only developmental manipulations to CLK/CYC.

However, a presence of a connection is not equivalent to its proper function as two phenomena at the termini that were not tested are associated with proper function. A rhythmic fasciculation/defasciculation of termini is important for

the behavioural rhythmicity (Fernández *et al.*, 2008; Sivachenko *et al.*, 2013). These rhythms can be affected either by abolishing molecular oscillations in s-LN_vs or independently through depletion of genes that are crucial for this process. Therefore not surprisingly my results indicate that affecting these rhythms by knocking down *Fer2* or *Mef2* abolished adult locomotor rhythms (Table 6.1). The fact that the phenotype for *Fer2* was stronger than the one for flies with developmental *per* over-expression, and previous reports showing that *Fer2* mutants have more severe phenotype than any *Clk* mutants, suggests that *Fer2* is located upstream, rather than downstream of *Clk* (Nagoshi *et al.*, 2010). On the other hand, *Mef2* appears downstream of CLK/CYC and is also involved in the fasciculation/defasciculation rhythms of s-LN_vs. The third strongest CLK/CYC target gene candidate, *Pdp1ε,* could also influence proper function of the dorsal projections from s-LN_vs as it has been shown to be involved in the formation of neural connections between s-LN_vs and their targets (Lim *et al.*, 2007c).

Moreover, my data demonstrated that in the case of flies with developmental CLK/CYC inhibition there was a consistent decrease in the number of clock neurons discovered and their size (Figure A.3.7). This is probably a side effect of a lack of functional CLK/CYC that could be mediated by *Fer2* that was shown to be involved in neurogenesis (Nagoshi *et al.*, 2010; Bou Dib *et al.*, 2014). Alternatively, it has recently been proposed that two CLK/CYC targets (Abruzzi *et al.*, 2011), LONGITUDINALS LACKING (LOLA) and PHOSPHOINOSITIDE-DEPENDENT KINASE 1 (PDK1) are involved in the development of s-LN_vs and in the absence of either one of them less cells with smaller cell bodies were found. In addition, flies displayed behavioural arrhythmicity (Uryu and Niwa, Short Communication during European Biological Rhythms Society/World Congress of Chronobiology meeting, Manchester, UK, 2-6 August 2015). These results might lead towards an understanding of how CLK/CYC can regulate the organization of the neuronal circuit and how neuroanatomical defects in LN_vs and lower PDF expression in cyc^{01} and Clk^{JRK} flies are developed.

7.2.6 Developmental role of PDF and PDF neurons

My results indicated that restricting developmental CLK/CYC inhibition to PDF neurons is sufficient to cause irreversible adult behavioural arrhythmicity (Figure 5.6). This suggests that even in the presence of functional molecular oscillator in all other clock neurons, PDF neurons can influence the overall behavioural rhythms. This is consistent with literature where PDF neurons were suggested to be responsible for maintaining synchrony across circadian neurons (Lin *et al.*, 2004; Nitabach *et al.*, 2005) by relaying phase information to PDF-sensitive neurons (Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2000; Park *et al.*, 2000; Peng *et al.*, 2003; Lin *et al.*, 2004).

One rhythmic property at neuronal termini that could be responsible for adult behavioural arrhythmicity is PDF levels change and potentially PDF release (Helfrich-Förster *et al.*, 2000; Park *et al.*, 2000, Nitabach *et al.*, 2005). As rhythmic PDF secretion is necessary for the generation of rhythms in both LD and DD (Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2000), I speculated that disrupted PDF signalling, which might either be a consequence of disrupted molecular oscillations in s-LN_vs or developmental PDF signalling inhibition, would result in behavioural arrhythmia.

Our research pointed to a decreased level of PDF in s-LN_vs in cyc^{01} flies (Goda, Mirowska, Currie *et al.*, 2011), therefore I speculated that developmental disruption to PDF signalling might affect the process of PDF signalling in adulthood. Preliminary testing did not exclude this possibility of at least one of the factors possibly being responsible or the phenotype of flies with developmental CLK/CYC inhibition (Figure A.4.1). However, PDF signalling cannot be the only thing responsible for the phenotype as the phenotype of flies with developmental CLK/CYC inhibition or *cyc* depletion was more severe than for the flies deprived of PDF (Pdf^{01}) or PDF neurons (Renn *et al.*, 1999).

7.2.7 Novel genes involved in generation of adult locomotor rhythmicity

Even though the core molecular mechanism governing the generation of behavioural rhythms is known, there are still discoveries of novel genes

involved in locomotor rhythmicity to be made. It was shown previously that about 60% of ~1500 CLK targets are expressed rhythmically (Menet *et al.*, 2010; Abruzzi *et al.*, 2011). When I was looking for CLK/CYC targets that were downregulated during development and could possibly cause irreversible adult arrhythmicity, I included in the screen some CLK/CYC targets of unknown function. Even in that very limited sample, I was able to find a novel gene that when downregulated, caused adult behavioural arrhythmicity – *trithorax* (Table 6.1). In light of my findings, it might be interesting to test even more of CLK/CYC targets.

trx was previously shown to be involved in axon guidance (Berger *et al.*, 2008) and histone modifications to cause open chromatin configuration (Petruk *et al.*, 2006; Czermin *et al.*, 2002; reviewed in Schuettengruber *et al.*, 2007 and Steffen and Ringrose, 2014) and also epigenetic inheritance of chromatic states (reviewed in Muller and Kassis, 2006 and Schwartz and Pirrotta, 2007). This could suggest that *trx* is therefore involved in the generation of adult circadian rhythms through ensuring proper expression of downstream gene(s).

However, I was able to exclude the possibility that developmental CLK/CYC inhibition prevents it from interacting with DNA and permanently affects histone acetylation status, therefore affecting adult gene expression patterns (Figure 6.2). This might suggest that either *trx* function is not required developmentally for adult behavioural rhythmicity or that a different mechanism is employed. Alternatively, my results on developmental histone acetylation/deacetylation might be incorrect. To exclude this possibility, a histone acetylation/deacetylation status should be tested as some research suggests that CLK/CYC activity might need to be present for HATs acetylating histones (Doi *et al.*, 2006).

In summary, my results confirm that CLK/CYC plays a crucial role not only in the molecular mechanism driving circadian oscillations, but also is indispensable during development for ensuring behavioural rhythmicity in adulthood. Moreover, I confirmed that among genes activated by CLK/CYC transcriptional activity are still some uncharacterized genes that might be involved in the generation of circadian behavioural rhythmicity.

Appendices

Appendix A

List of figures in Appendix A

A.1 Supplementary data to Chapter 3

A.1.1 Developmental light treatment impacts adult locomotor rhythmicity of *per* rescue flies

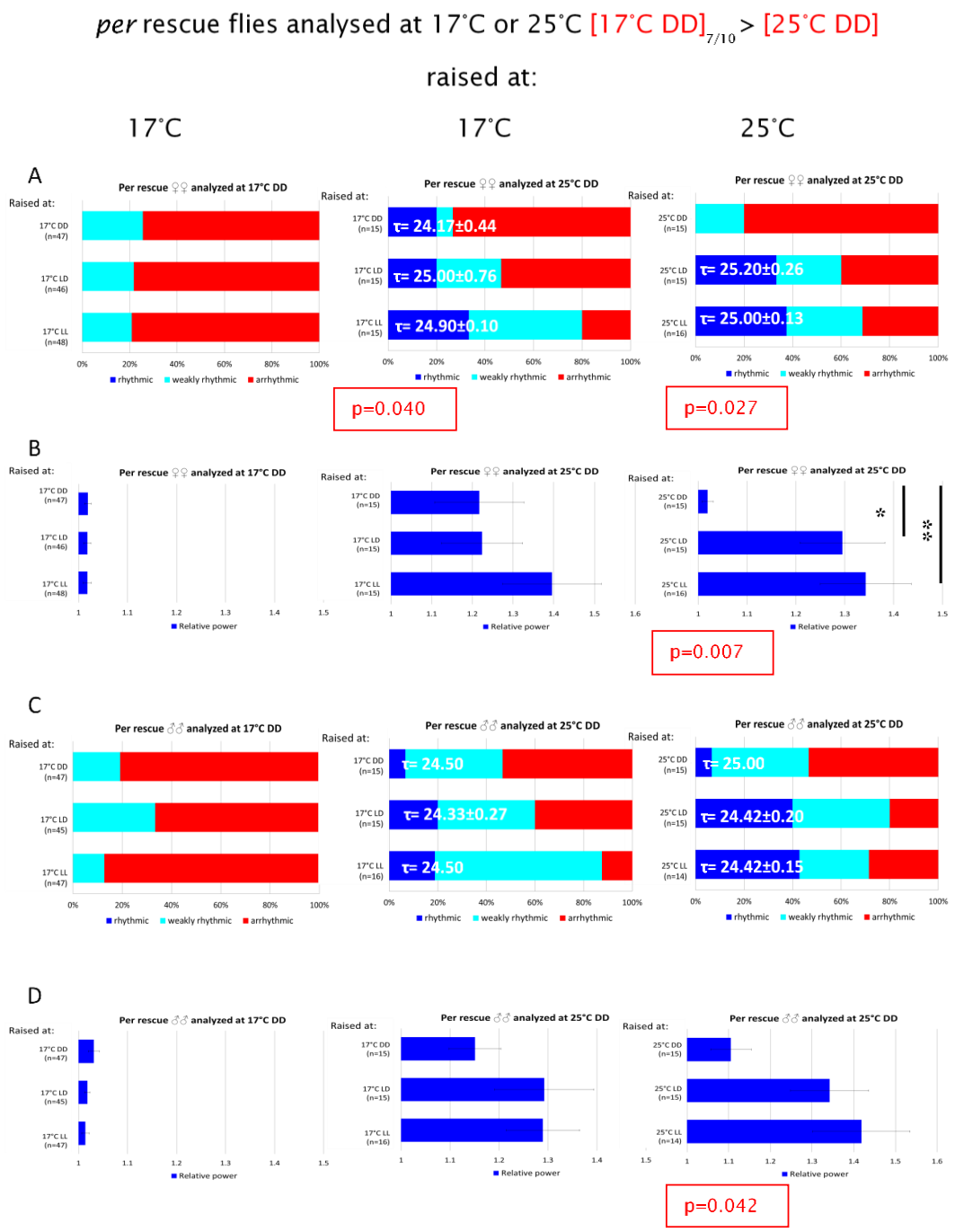


Figure A.1.1. Light conditions during development affect the behaviour of flies with *per* knockdown at permissive conditions.

per rescue flies (*per*^{01} flies which can express transgenic *period* in all clock bearing cells only at elevated temperature) were raised either under restrictive conditions (17˚C) or at permissive temperature (25˚C) and analysed as adults at both restrictive and permissive conditions (7 or 10 days 17˚C DD and then moved to permissive conditions). Stacked bar diagrams (A, C) show the distribution of rhythmic, weakly rhythmic and arrhythmic females (A) and males (C), with n numbers indicated and period lengths and SEM of rhythmic flies indicated. Fisher's Exact 3x3 tests were used for comparisons. Bar graphs (B, D) show the average relative rhythmic power for all females (B) and males (D), with error bars indicating SEM. To compare the values, non-parametric Kruskal – Wallis test was performed. * $p<0.05$; ** $p<0.01$.

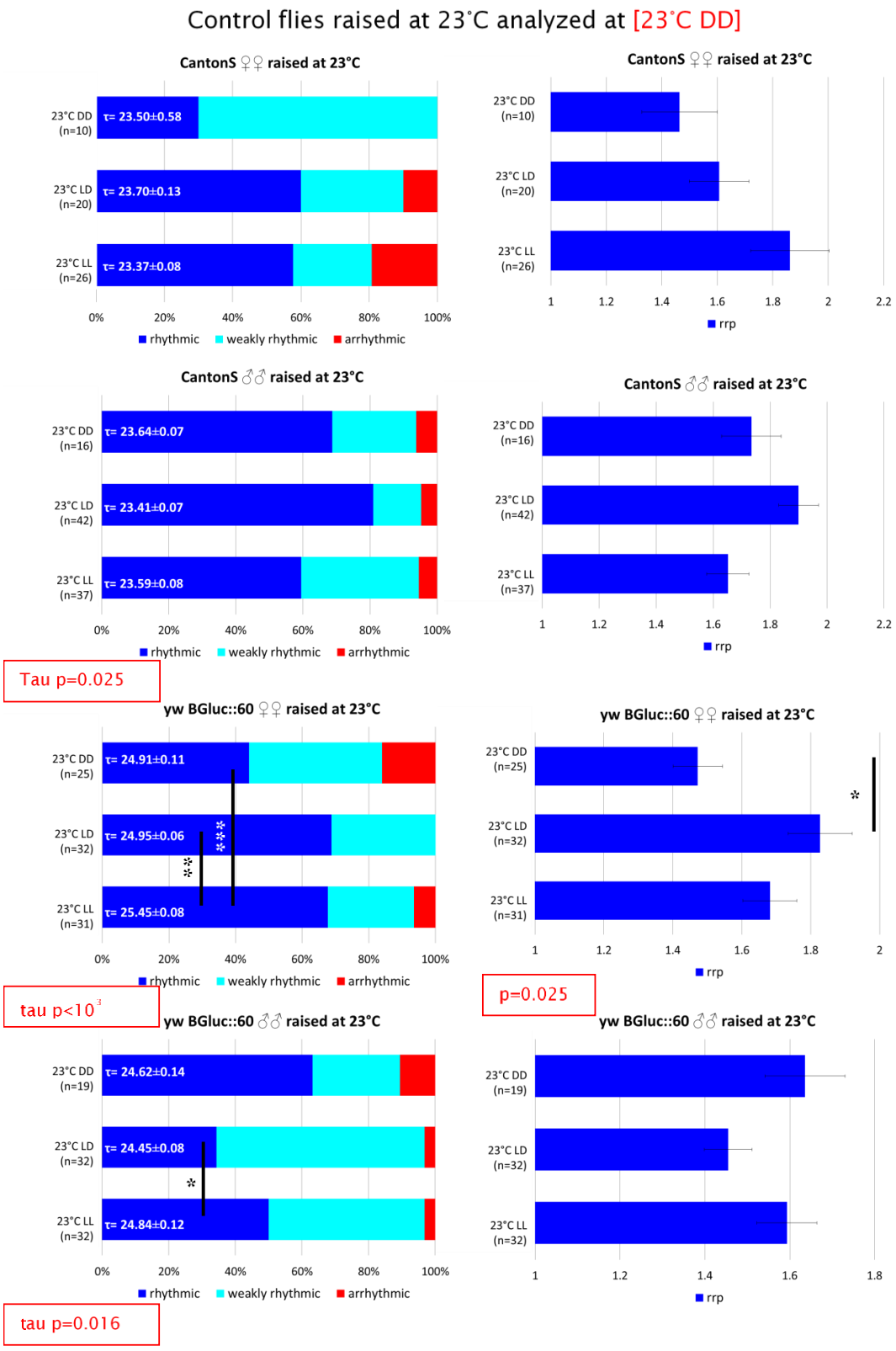


Figure A.1.2. Light conditions during development do not significantly affect the adult behaviour of control flies.

Canton-S and *ywBGluc::60* flies were raised at the room temperature and as adults were analysed in constant darkness at the same temperature. Stacked bar diagrams on the left show the distribution of the rhythmic, weakly rhythmic and arrhythmic flies, with n numbers and period lengths and SEM of the rhythmic flies indicated. Fisher's Exact 3x3 test was performed to compare flies raised at different developmental light conditions. Bar charts on the right show the relative rhythmic power of all flies, with error bars indicating SEM. To compare the values, the non-parametric Kruskal – Wallis tests were performed. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

A.1.2 Four days of entrainment at permissive temperature improve the adult behavioural rhythmicity of *per* rescue flies

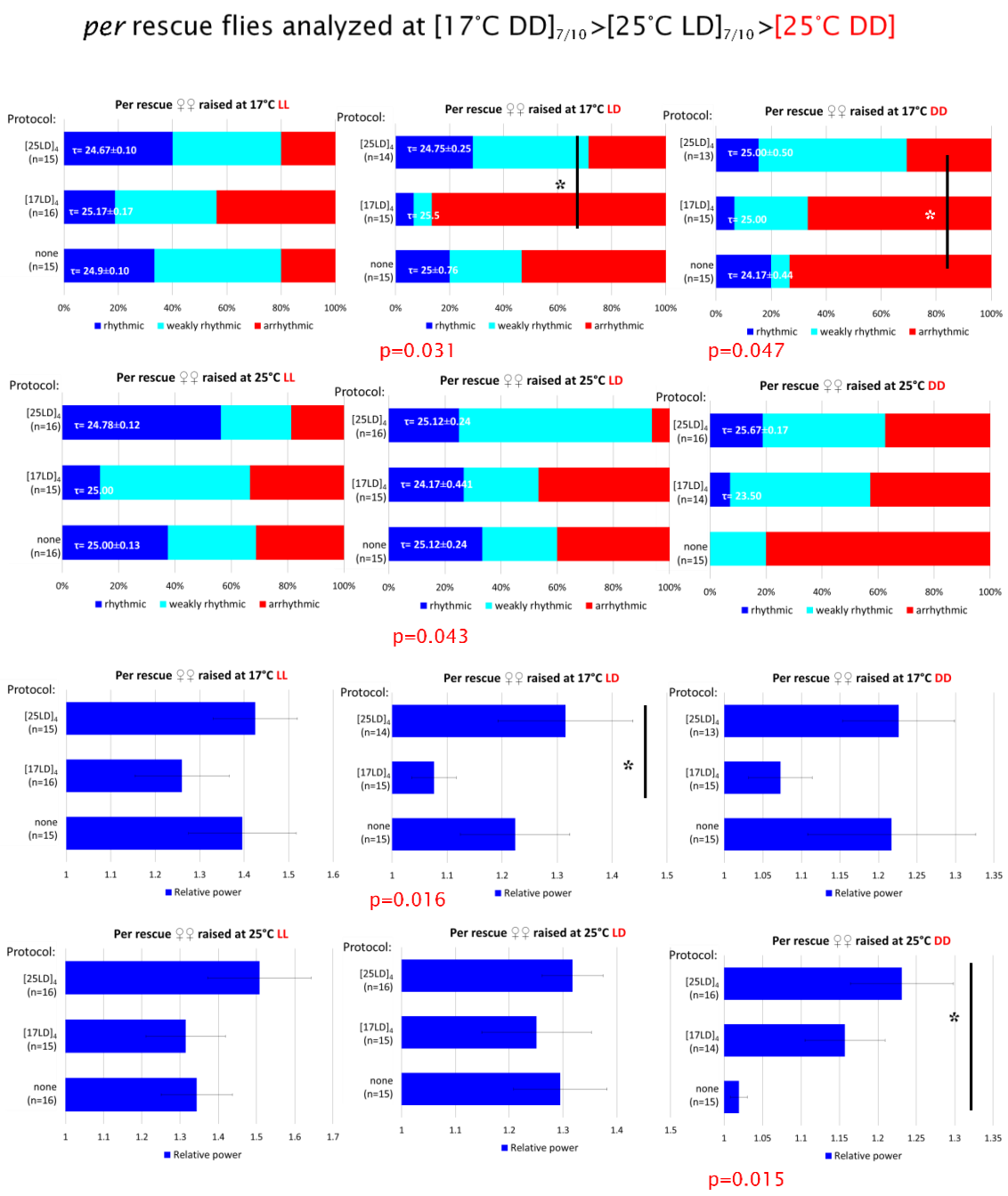


Figure A.1.3. Entrainment at permissive temperature improves the rescue of adult behavioural arrhythmicity of females with conditional *per* rescue.

per rescue females (*per*^{01} flies which can express transgenic *period* in all clock bearing cells only in elevated temperature) were raised under either restrictive conditions (17˚C; where no transgenic *per* is expressed; top and third row) or under permissive conditions (25˚C; where transgenic *per* is expressed; second and bottom row) at either constant light (LL), light/dark cycle (LD) or constant darkness (DD) (marked in red) and as adults were kept for 7 or 10 days at17˚C DD and then either moved directly to 25˚C DD or with 4 days of entrainment at either 25˚C LD or 17˚C LD. Flies were analysed at permissive conditions of 25˚C DD. The top two rows are stacked bar diagrams showing the distribution of rhythmic, weakly rhythmic and arrhythmic flies, with n numbers and period lengths (±SEM) of the rhythmic flies indicated. Fisher's Exact 3x3 test was used to compare different treatments. The bottom two rows of bar charts show the relative rhythmic power of all flies, with error bars indicating SEM. To compare the values, the non-parametric Kruskal – Wallis test was performed. * $p<0.05$; ** $p<0.01$.

per rescue flies analyzed at [17˚C DD]$_{7/10}$>[25˚C LD]$_{7/10}$>[25˚C DD]

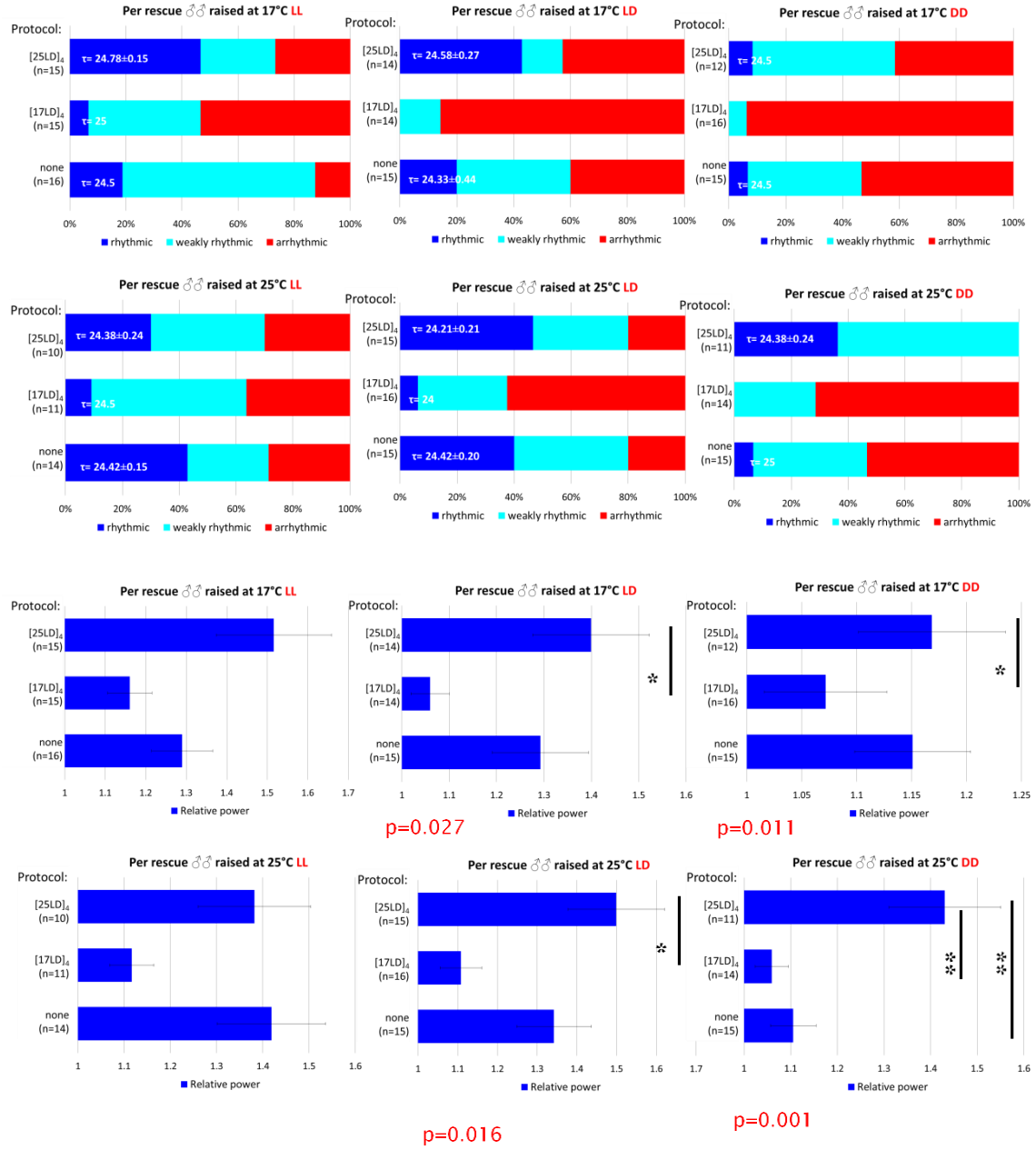


Figure A.1.4. Entrainment at permissive temperature improves the rescue of adult behavioural arrhythmicity of males with conditional *per* rescue.

per rescue males (*per*^{01} flies which can express transgenic *period* in all clock bearing cells only in elevated temperature) were raised under either restrictive conditions (17˚C; where no transgenic *per* is

expressed; top and third row) or at permissive conditions (25˚C; where transgenic *per* is expressed; second and bottom row) in either constant light (LL), light/dark cycle (LD) or constant darkness (DD) (marked in red) and as adults were kept for 7 or 10 days at 17˚C DD and then either moved directly to 25˚C DD or with 4 days of entrainment at either 25˚C LD or 17˚C LD. Flies were analysed at permissive conditions of 25˚C DD. The top two rows are stacked bar diagrams showing the distribution of the rhythmic, weakly rhythmic and arrhythmic flies, with n numbers and period lengths (±SEM) of the rhythmic flies indicated. Fisher's Exact 3x3 test was used to compare different treatments. The bottom two rows of bar charts show the relative rhythmic power of the all flies, with error bars indicating SEM. To compare the values, the non-parametric Kruskal – Wallis test was performed. * $p<0.05$; ** $p<0.01$.

A.1.3 PER is not required during development for adult behavioural rhythmicity.

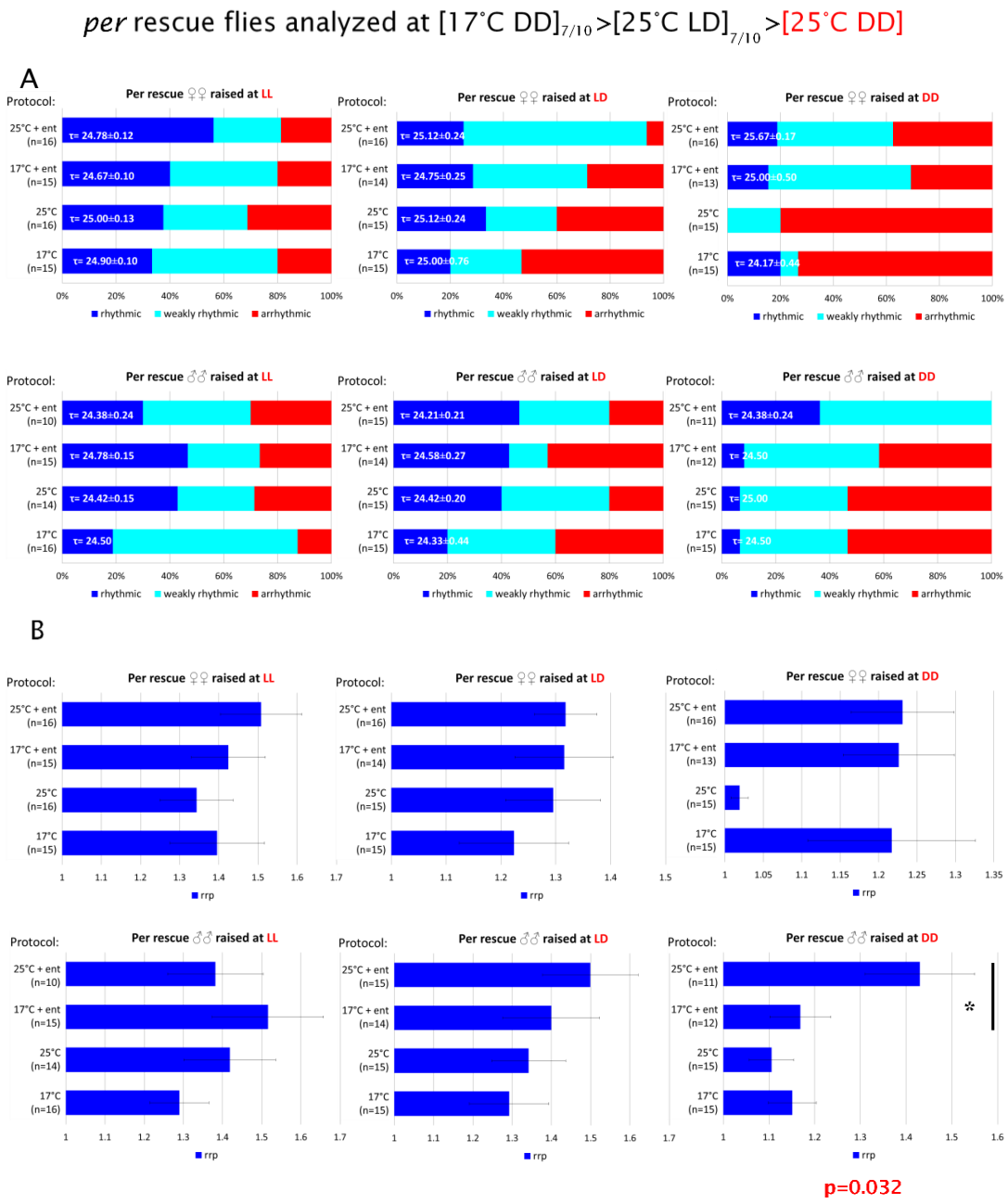


Figure A.1.5. PER expression during development does not affect the adult behavioural rhythmicity of flies with conditional *per* rescue.

per rescue flies (*per*^{01} flies which express transgenic *period* in all clock bearing cells only at elevated temperature) were raised under either restrictive conditions (17˚C; where transgenic *period* is not

expressed) or permissive conditions (25˚C; where transgenic *per* is expressed and rescues per^{01}-caused arrhythmicity) in various developmental light conditions (marked in red) and as adults were kept for 7 or 10 days at 17˚C DD and then either moved directly to 25˚C DD (bottom two rows on each graph, labelled with the developmental temperature) or received 4 days of entrainment at 25˚C LD and then moved to 25˚C DD (top two rows on each graph, labelled as the developmental temperature + ent). Flies were analysed at permissive conditions (25˚C DD). (A) Stacked bar diagrams showing the distribution of the rhythmic, weakly rhythmic and arrhythmic flies for females (top row) and males (bottom row), with n numbers and period lengths and SEM of the rhythmic flies indicated. Fisher's Exact 2x3 test was used to compare flies raised at different temperatures (but with the same treatment as adults). (B) The relative rhythmic power of all flies, with error bars indicating SEM. To compare the values, nonparametric Mann-Whitney test was performed. * $p<0.05$

A.2 Supplementary data to Chapter 4

A.2.1 Flies with conditional *per* over-expression do not exhibit reduced lifespan

A.2.1.1 Protocol

Flies with the conditional *per* over expression and control wild-type flies (*yw,* Eeken, 1982) were seeded at room temperature (~23˚C). After three days, flies were moved to another vials and the first set of vials was left at room temperature. After a further 3 days adult flies were removed from the second set of vials, which were then moved to 29˚C. Both groups were raised in identical 12 hr light: 12 hr dark (LD) conditions. Flies from both conditions hatched 6-7 days after the second set was moved to 29˚C and 3-4 day old male flies were collected and placed into fresh vials (10 males/vial), which were then kept at 17˚C temperature under 12 hr light: 12 hr dark, which is the condition in which the locomotor data presented in 4.4.1 were collected. The survival of flies was analysed as described in 2.4.

A.2.1.2 Results

Raising flies at 29˚C instead of standard room temperature indeed affected their longevity at 17˚C (and presumably at other conditions as well) (Figure A.2.1). For experimental flies with developmental *per* over-expression the difference in the median survival was 69 days between the RT-raised and 29˚C-raised ones (both Mantel-Cox, df=1 and Gehan-Breslow-Wilcoxon, df=1 $p<0.0001$, n=100). However, very similar results were observed with the control wild-type flies, where the difference was 47 days (both Mantel-Cox, df=1 and Gehan-Breslow-Wilcoxon, df=1 $p<0.0001$, n=100).

Interestingly, there was no significant difference between median survival of control flies and flies with developmental *per* over-expression raised at 29˚C (Mantel-Cox, df=1 $p=0.799$, n=100; Gehan-Breslow-Wilcoxon, df=1 $p=0.756$, n=100), suggesting that the observed decline in the rhythmicity of flies with developmental *per* over-expression was not simply due to a genotype-specific effect on health or ageing (Figure A.2.1). Surprisingly, for flies raised at room temperature, flies carrying but not expressing the transgenes survived longer

than control flies, with median survival increasing from 93 days observed for controls to an impressive 114 days for transgenic flies (Mantel-Cox, df=1 p=0.0001, n=100; Gehan-Breslow-Wilcoxon, df=1, p>0.0001, n=100).

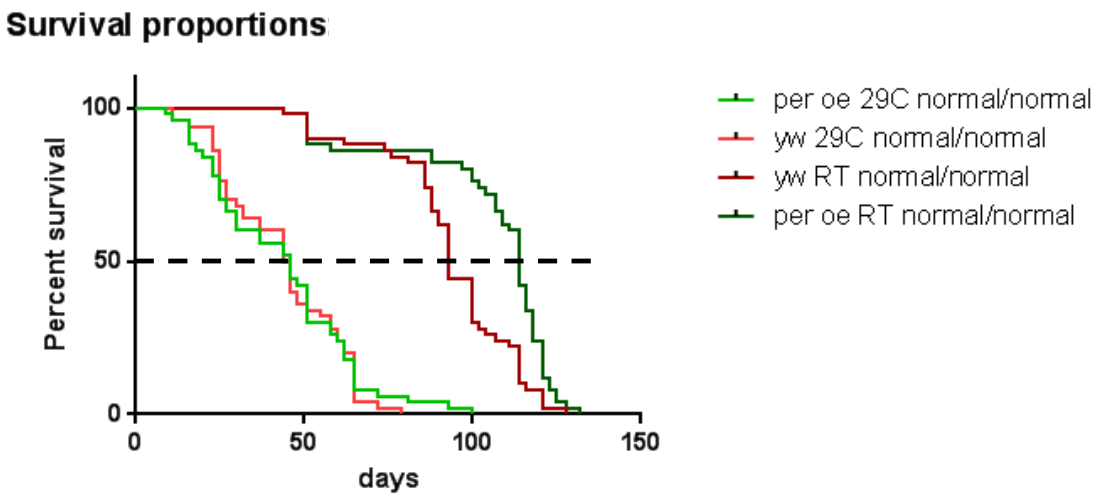


Figure A.2.1. Effects of temperature and elevated *per* levels during development on the lifespan of flies at 17˚C.

Wild-type (*yw*) and *per* over-expression (per oe) male flies were raised at either 29˚C (29˚C), where flies experience elevated *per* levels, or room temperature (RT) and were kept at 17˚C LD as adults. The dashed line denotes the median survival, which is a time at which 50% of the population remain alive. There is no difference between experimental and control flies raised at 29˚C but for flies raised at room temperature experimental flies survive significantly longer. Raising flies at 29˚C clearly reduces the lifespan for both experimental and control flies.

A.2.2 Temperature sensitivity of adult behavioural rhythms after development at permissive conditions.

To test if there is some residual circadian locomotor rhythmicity after switch to restrictive conditions and if adult entrainment can lead to rescue of circadian locomotor rhythmicity, I decided to test the behaviour of flies raised under permissive conditions moved to either permissive or restrictive conditions following variable exposure to LD at restrictive conditions in the adulthood.

A.2.2.1 Protocol

3-4 day old *cyc* rescue flies raised under permissive conditions (29˚C DD) were loaded into *Drosophila* Activity Monitors and were placed in 17˚C LD for 3, 6 or 9 days and subsequently moved into either 17˚C DD or 29˚C DD. Due to a poor health of flies raised at 29˚C DD and then analysed at 29˚C DD, only the first full five days were used for the chi-square period analysis as described in 2.3. Flies were tested for an association between the distribution of rhythmic:weakly rhythmic:arrhythmic flies and testing temperature by Fisher's Exact 3x2 test and for an association between distribution of flies and the length of entrainment with Fisher's Exact 3x3 test. Period length and relative rhythmic power were compared using non-parametric Kruskal – Wallis test for comparisons between the length of entrainment and the Mann-Whitney test for comparisons between testing temperature.

A.2.2.2 Results

Data obtained for males and females was very similar therefore I combined it. Visual inspection of actograms depicting the median activity of all flies revealed a clear difference between flies moved to 17˚C DD and 29˚C DD (Figure A.2.2.A). This was confirmed by the analysis of individual flies (Figure A.2.2.B), showing that flies moved to 29˚C DD were significantly more rhythmic than flies moved to 17˚C DD, both in terms of the distribution of flies and the relative rhythmic power (where test could be reliably performed).

For flies exposed to only 3 days of 17˚C LD, some weak rhythmicity was visible at the beginning of 17˚C DD (this was significantly stronger than after 6 or 9

days of entrainment, which was associated with longer time in restrictive conditions, Fisher's Exact 3x2 test: $p=0.016$, $n=16$ for distribution of flies) and it appeared that flies only became arrhythmic after couple days. This was confirmed by the individual analysis of flies – about 44% of flies remained weakly rhythmic and displayed low relative rhythmic power. This was still significantly lower than in case of flies moved to 29°C DD (Fisher's Exact 3x2 test: $p<0.05$, $n=31$ for the distribution of flies and Mann-Whitney test: $U=53.000$, $z=2.276$, $p<0.05$, $n=16$ for rrp). Therefore I concluded that 3 days of the absence of *cyc* expression in adult flies is enough to dampen but not completely remove the behavioural rhythmicity.

This residual rhythmicity of flies moved to 17°C was absent in flies exposed to 6 LD cycles. Flies analysed at 17°C DD were almost completely arrhythmic and on the other hand, even after 6 days at restrictive conditions, flies were showing strong rhythms at permissive conditions (Fisher's Exact 3x2 test: $p<10^{-7}$, $n=40$). The comparison of the relative rhythmic power (of both rhythmic and weakly rhythmic flies) was not reliable because among both genders, only one fly was displaying weak rhythm. Therefore I could hypothesise that 6 days of adult exposure to restrictive conditions was enough to deplete the residual *cyc* and render flies arrhythmic at restrictive conditions but was not sufficient to prevent circadian clock from starting their function once flies were placed at permissive temperature.

The longest adult LD exposure was used to test the possibility that the prolonged LD exposure, even at restrictive conditions, might help to even partially restore the rhythmicity of flies with conditional *cyc* rescue raised under permissive conditions displayed at restrictive conditions. Both visual analysis of the median activity actograms and the analysis of individual flies revealed no improvement in the rhythmicity of flies analysed at restrictive conditions after 9 days of LD cycles at restrictive conditions as they were still significantly worse than those moved to permissive conditions (Fisher's Exact 3x2 test: $p<0.05$, $n=39$ for the distribution of flies; Mann-Whitney test: $U=32.000$, $z=2.717$, $p<0.01$, $n=12$ for rrp). Even though it might appear as if the prolonged exposure to restrictive conditions decreased the rhythmicity of flies analysed at permissive conditions (as compared to flies with only 3 or 6 days of adult exposure to restrictive conditions), the distribution of flies, rrp and period length was not significantly worse (Fisher's Exact 3x3 test:

p=0.060, n=52 for the distribution of flies; Kruskal-Wallis test: H(2)=0.012, p=0.994, n=32 for rrp; Kruskal-Wallis test: H(2)=3.443, p=0.179, n=21).

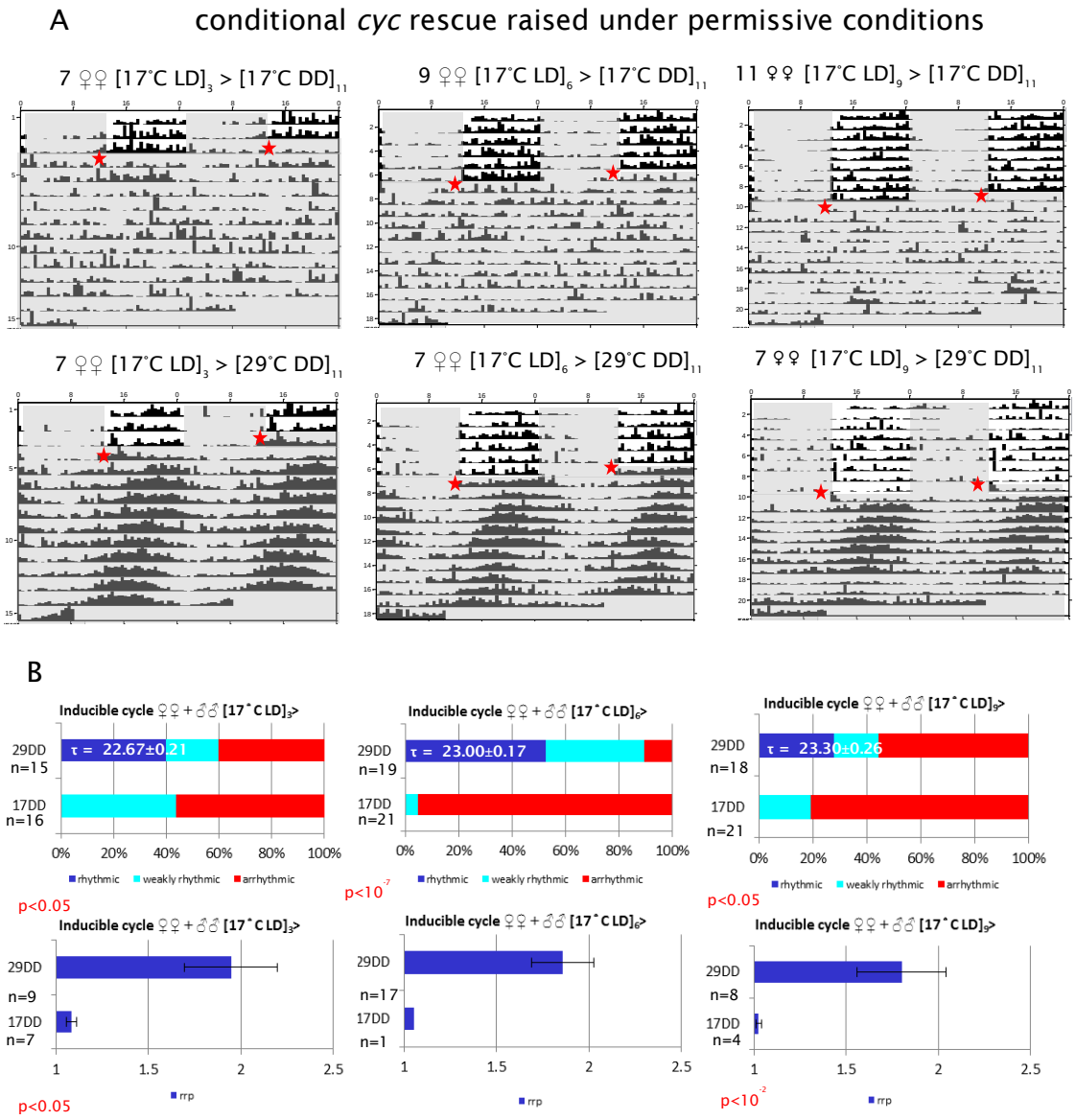


Figure A.2.2. Impact of the exposure to 17°C LD on the locomotor behaviour of cyc rescue flies.

cyc rescue flies raised under permissive conditions become arrhythmic short time after adult exposure to arrhythmic conditions, indicating that weak residual circadian locomotor rhythmicity persists after switch to restrictive conditions. Moreover, even prolonged LD exposure did not induce behavioural rhythmicity in

flies analysed at restrictive conditions. (A) Double-plotted actograms showing the median locomotor behaviour of *cyc* rescue female flies exposed to 17˚C LD for three, six or nine days and subsequently released to either 17˚C DD – restrictive conditions (top) or 29˚C DD – permissive conditions (bottom). Shading on actograms represents the periods of darkness and stars mark the time of the switch between the conditions. (B) Quantitative analysis of the rhythmicity of individual flies analysed at either restrictive (17˚C DD) or permissive (29˚C DD) conditions. Stacked bar diagrams (top) represent the percentage of the rhythmic, weakly rhythmic and arrhythmic flies in the analysed population. The n numbers of flies and the period rhythms of rhythmic flies are included on the diagrams. The association of the distribution of flies with the testing temperature was tested with Fisher's Exact 3x2 test and the association of the distribution of flies with the length of entrainment with Fisher's Exact 3x3 test. Bar diagrams on the bottom show the average relative rhythmic power (rrp) of rhythmic and weakly rhythmic flies with the error bars showing SEM. Differences between the rrp for the length of the entrainment was tested with the nonparametric Kruskal-Wallis test and differences between rrp for flies analysed at different temperatures was tested with Mann-Whitney test.

A.2.3 Inhibition of CLK/CYC function in adults has a reversible effect on circadian behaviour.

Since the results for flies with conditional *cyc* rescue raised under permissive conditions after 9 days of LD at restrictive conditions suggest that long exposure to restrictive conditions might cause some impairment to adult behavioural rhythmicity at permissive conditions, I decided to test this further. I was mainly interested in whether after prolonged exposure to adult restrictive conditions behavioural rhythms can still be rescued at permissive conditions. Moreover, I was interested in whether flies can still remain sensitive to LD at restrictive conditions.

A.2.3.1 Protocol

Flies with conditional *cyc* rescue were raised under permissive conditions of 29˚C DD. 3-4 days old flies were loaded into the *Drosophila* Activity Monitors into restrictive conditions of 17˚C DD to check if these flies are capable of displaying some residual locomotor activity. After 7 full days flies were moved into 17˚C LD for 5 days for entrainment and then released into either 29˚C DD or 17˚C DD (transition points are denoted as stars on the actograms). The first full 7 days in the last condition were used to analyse the rhythmicity of flies as described in 2.3. Association between the distribution of rhythmic:weakly rhythmic:arrhythmic flies and temperature were compared using Fisher's Exact 2x3 test. Period length and relative rhythmic power were compared using non-parametric Kruskal – Wallis test.

A.2.3.2 Results

Visual analysis revealed that flies raised under permissive conditions were able to display some residual rhythmicity in restrictive conditions at the very beginning of the experiment (stronger in males than in females) even when no transgenic *cycle* was expressed (Figure A.2.3.A). This confirmed the previously reported data (Figure A.2.2) and could signify that the *cyc* is stable enough to sustain circadian rhythms for some time even when no new *cyc* is being expressed. However, this was not the case for flies exposed to restrictive

conditions for 12 days, after which time any residual rhythmicity was gone. This could suggest that in the absence of new *cyc* being produced, locomotor rhythms can be sustained for no more than around 7 days.

Even after 12 days of exposure to restrictive conditions flies raised at permissive temperature displayed relatively strong circadian rhythms in permissive conditions (Figure A.2.3). In contrast, flies analysed at restrictive temperature were almost exclusively arrhythmic. There was a correlation between the temperature at which flies were analysed and their rhythmicity (Fisher's Exact 3x2 test: $p<10^{-3}$, n=33 for males and $p<10^{-3}$, n=29 for females). These results seemed to suggest that adult *cycle* expression prior to the testing condition is not required. This data confirms and extends data presented in A.2.2, indicating that rhythms at permissive temperature can be resumed even after long adult exposure to restrictive conditions. Moreover, 17°C LD appeared to be still able to entrain flies. This suggests that either some residual TIM is present even at restrictive conditions or that light-dependent CRY degradation is sufficient to entrain flies.

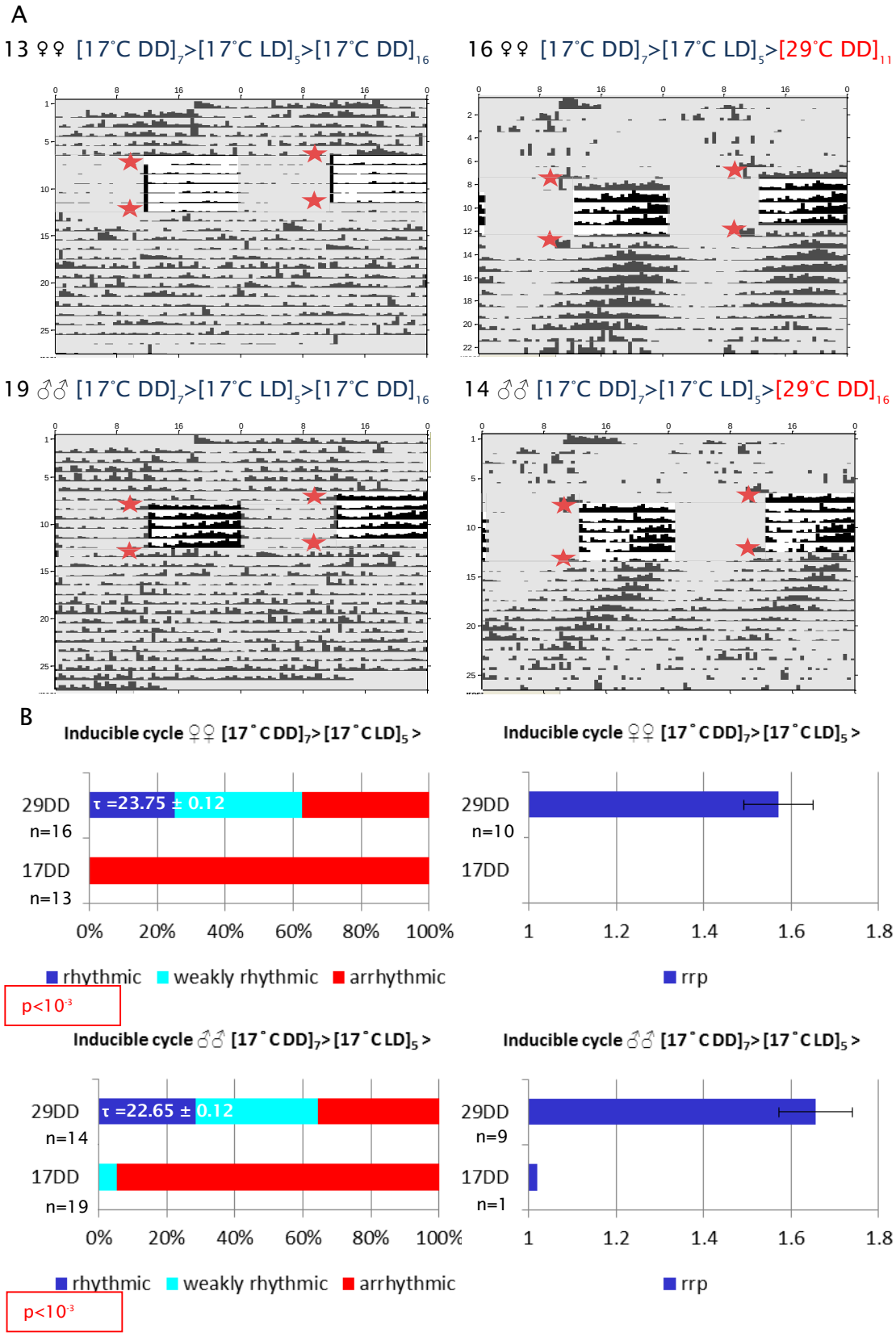


Figure A.2.3. Flies with conditional rescue of *cyc* expression show strong rhythms at permissive conditions even after long adult exposure to restrictive conditions.

Flies with the conditional rescue of the *cyc* function raised under permissive conditions show no adult locomotor rhythmicity in restrictive conditions but are still rhythmic at permissive conditions even after prior 12 days of the exposure to restrictive conditions. (A) Median actograms for females (top) and males (bottom) showing the locomotor behaviour of flies analysed at 17˚C DD for seven days, then at 17˚C LD for five days and subsequently moved into either 17˚C DD – restrictive conditions (left) or 29˚C DD – permissive conditions (right). The shaded areas on actograms represent the dark portion and stars show the time when flies were moved from one condition to another. For simplicity, restrictive conditions are written in blue, and permissive in red, in the legends. (B) Quantitative analysis of the rhythmicity of individual flies analysed at either restrictive (17˚C DD) or permissive (29˚C DD) conditions. Stacked bar diagrams (on the left) represent the percentage of rhythmic, weakly rhythmic and arrhythmic flies in the analysed population. The n numbers of flies and the period rhythms of rhythmic flies are included on the diagrams. The association of the distribution with the temperature was tested with 2x3 Fisher's Exact test. Bar diagrams on the right show the average relative rhythmic power (rrp) of rhythmic and weakly rhythmic flies with the error bars showing SEM.

A.2.4 Without prior LD entrainment, *cyc* rescue flies become arrhythmic within three days of constant darkness at the restrictive temperature.

The data presented in A.2.2 showed that *cyc* rescue flies raised under permissive conditions were displaying some residual locomotor rhythmicity when placed at restrictive conditions following 3 days of adult exposure to LD cycles at restrictive conditions. This raised a question about the duration of time the circadian rhythmicity can persist in *cyc* rescue flies after a shift from permissive (29˚C) to restrictive (17˚C) conditions. To address this, I analysed old flies raised at a permissive temperature and released them directly to constant darkness at restrictive conditions. Since flies were raised in a constant environment (29˚C DD), they were loaded into monitors in the presence of lights to allow them to synchronize.

Visual inspection of the median actograms (Figure A.2.4.A with ZT 0 corresponding to the time of the shift) suggested that, in the case of female flies it took around 5-6 days for the rhythms to go away in restrictive conditions. A similar or slightly longer time was necessary for male flies placed at restrictive conditions to become arrhythmic. For the quantitative analysis I tested flies for the presence of rhythms during overlapping intervals of 3, 4 and 5 days, to determine the smallest window over which rhythms might be reliably detected.

Since I noticed that the data obtained with the analysis of 3, 4 and 5 days was very similar, I decided to use a 3 day interval as it potentially allowed for the best resolution. For females, even during the first 3 days, there was no significant rhythmicity detected. For any three days analysed, there were only 10-30% weakly rhythmic flies and these were not the same flies, i.e. flies that were rhythmic during days 2-4 were not displaying rhythmicity during days 1-3. Moreover, residual rhythmicity of flies in all cases was very low (Figure A.2.4.B). On the basis of this data I can conclude that females become arrhythmic in aperiodic restrictive conditions within 2-3 days. On the other hand, a good percentage (only slightly smaller than what was observed in A.2.2) of males displayed weak rhythmicity during days 1-3. Even more males were rhythmic during days 2-4, with 3 out of 5 males rhythmic during days 1-3 staying rhythmic. This increase was also associated with the trend towards

higher relative rhythmic power for days 2-4, compared to 1-3. For the days 3-5, the number of rhythmic flies decreases, just as relative rhythmic power. This might suggest that males stayed rhythmic in the restrictive conditions for longer than females, around 3-4 days.

The difference between the results of the quantitative analysis of flies (especially females) after three days of exposure to a periodic environment (Figure A.2.2) and flies analysed in constant darkness right away (Figure A.2.4) was that in the case of the latter they were moved to the aperiodic environment following only a short light exposure.

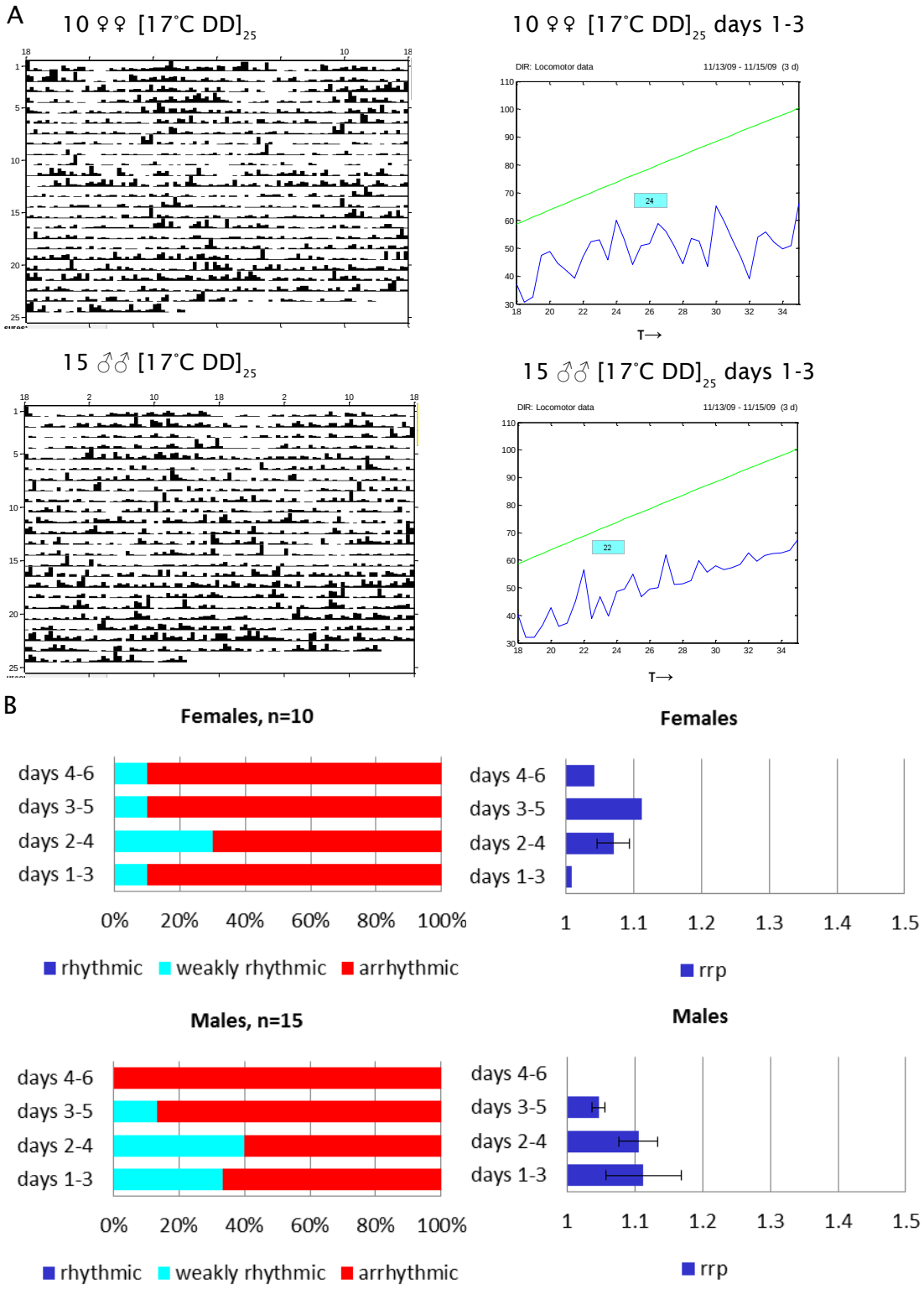


Figure A.2.4. Flies with conditional *cyc* rescue raised under permissive conditions display very weak residual rhythms for no more than 4 days in restrictive conditions.

Even during the first couple of days under restrictive conditions the rhythmicity of adult locomotor behaviour is clearly reduced, with females remaining almost constantly arrhythmic and males displaying some very weak rhythms during the first four days. (A) Median actograms (left) and chi-square periodograms (right) for females (top) and males (bottom) showing the locomotor behaviour of the cyc rescue flies raised at 29˚C and analysed at restrictive conditions of 17˚C DD. (B) Quantitative analysis of the rhythmicity of individual flies analysed at restrictive conditions over different time ranges. Stacked bar diagrams (on the left) represent the percentage of rhythmic, weakly rhythmic and arrhythmic flies in the analysed time window. Numbers of flies are marked over the diagrams. Bar diagrams on the right show the average relative rhythmic power (rrp) of rhythmic and weakly rhythmic flies with the error bars showing SEM. Numbers are: 1, 1, 3, 1 (from the top for females) and 0, 2, 6, 5 (from the top for males).

A.2.5 Developmental *cyc* depletion impairs behaviour of flies in LD conditions

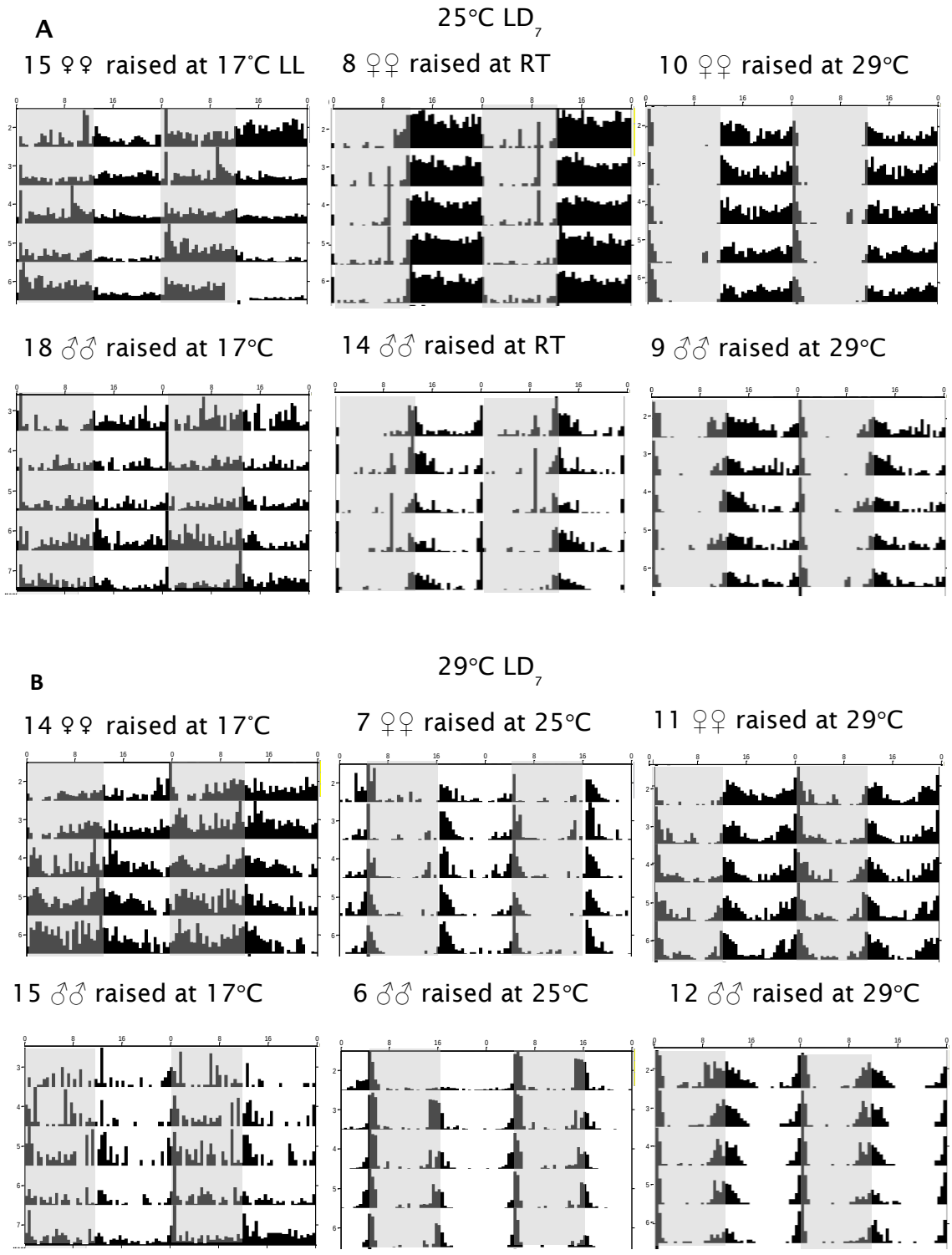


Figure A.2.5. Impact of developmental *cyc* depletion on the locomotor behaviour in LD cycles at permissive temperatures - actograms.

Flies raised with developmental *cyc* depletion show severe impairment to their locomotor behaviour at the permissive conditions in the presence of light/dark cycles. Double-plotted actograms showing the median locomotor behaviour of the *cyc* rescue flies raised at different temperatures (as indicated in above actograms, together with number of flies) analysed at 25˚C LD (A) and 29˚C LD (B). Shading on actograms represents the periods of darkness. At 25˚C LD, flies raised at 17˚C are mainly arrhythmic or nocturnal. Flies raised at 17˚C and analysed at 29˚C LD are almost completely arrhythmic and the few remaining rhythmic flies do not show any preference for activity during the light portion of the day.

A.3 Supplementary data to Chapter 5

A.3.1 Flies with developmental *per* over-expression and introduced *tim-luc* exhibit adult locomotor arrhythmicity in permissive conditions.

To confirm that flies used for the determination of molecular oscillations in peripheral tissues displayed the same adult phenotype following developmental *per* over-expression, young adult flies with *tim(UAS)Gal4*- driven *per* over-expression and *tim-luc* transgene (*y w tim-luc/y w tubPGal80^{ts};tim(UAS)-Gal4; UASper*) were tested in the same or similar conditions as used during the experiments presented in 5.4.2. Unfortunately it was impossible to collect enough flies with the *tim62-Gal4* – driven *per* over-expression and *tim-luc*, so instead I tested flies of the same genotype but without *tim-luc*, which were a stable stock (*y w tubPGal80^{ts};tim62-Gal4/CyO; UASper*).

A.3.1.1 Protocol

Flies were tested either in the incubator kept at 17˚C or at the same room as experiments presented in 5.4.2 were conducted. Flies were first entrained to LD cycle for 7 days and subsequently released to constant darkness. The first full seven days were used to determine the presence of rhythms as described in 2.3. Distribution of flies was compared using Fisher's Exact 2x3 tests and relative rhythmic power and period lengths were compared using Mann-Whitney tests.

A.3.1.2 Results

Behavioural analysis of flies with *tim(UAS)Gal4* – driven *per* over-expression in an incubator (Figure A.3.1 A and B) confirmed that the addition of the *tim-luc* transgene did not affect the phenotype caused by developmental *per* over-expression. While flies with constitutive *per* over-expression were mainly arrhythmic regardless of the developmental temperature (Fisher's Exact 3x2 test: p=0.99, n=37), developmental *per* over-expression largely affected adult

rhythmicity of flies (Fisher's Exact 3x2 test: $p<10^{-6}$, n=45). Therefore flies without developmental *per* over-expression (raised at RT) were different to flies with constitutive *per* over-expression raised at RT (Fisher's Exact 3x2 test: $p<10^{-8}$, n=43), while flies with developmental *per* over-expression (raised at 29˚C) were similar to flies with constitutive *per* over-expression raised at the same temperature (Fisher's Exact 3x2 test: p=0.21, n=39). A further conclusion was that the addition of *per* over-expression in adulthood during testing further impaired the rhythmicity of flies (as seen in comparison of flies with constitutive and conditional *per* over-expression), suggesting a possibility that CLK/CYC inhibition during development only leaves some residual clock function.

Analysis of flies from the testing room revealed that the conditions in the testing room (Figure A.3.1 C and D) did not differ from the conditions used in incubator and flies experiencing either constitutive or developmental *per* over-expression were predominantly behaviourally arrhythmic, while flies raised without *per* over-expression were displaying strong behavioural rhythms when analysed in the testing room. Statistical analysis indicated an association between the developmental temperature and the rhythmicity of flies with conditional *per* over-expression (Mann-Whitney test: U=75.000, z=2.083, p<0.05, n=20), but there was no statistical significance due to low numbers of flies. There was, however, an indication that conditional *per* over-expressing flies are different than flies with constitutive expression of *per* raised at the same temperature, with flies raised at room temperature displaying different distribution of categories of rhythmicity (Fisher's Exact 3x2 test: p<0.05, n=23) and different relative rhythmic power (Mann-Whitney test: U=2.000, z=-2.041, p<0.05, n=11). The low numbers of flies were probably the result of a lower humidity in the testing room in comparison to the incubator.

Similar results were obtained for flies with *tim62-Gal2* – driven *per* over-expression (Figure A.3.1 E and F). As for this genotype both males and females were tested, but there were no controls with constitutive *per* over-expression, flies of the same sex raised at different temperatures were compared to each other. Also, there were not enough flies analysed in the testing room that survived long enough to allow reliable analysis, so only flies analysed in an incubator were used. Distribution of flies was significantly different for females (Fisher's Exact 3x2 test: $p<10^{-5}$, n=51) but not for males (Fisher's Exact 3x2

test: p=0.20, n=41). Similarly, rrp showed association with developmental temperature for females (Mann-Whitney test: U=175.000, z=2.997, p<0.01, n=32) but not males (Mann-Whitney test: U=140.000, z=1.760, p=0.082, n=30).

Adult locomotor behaviour following developmental *per* over-expression (permissive temperature)

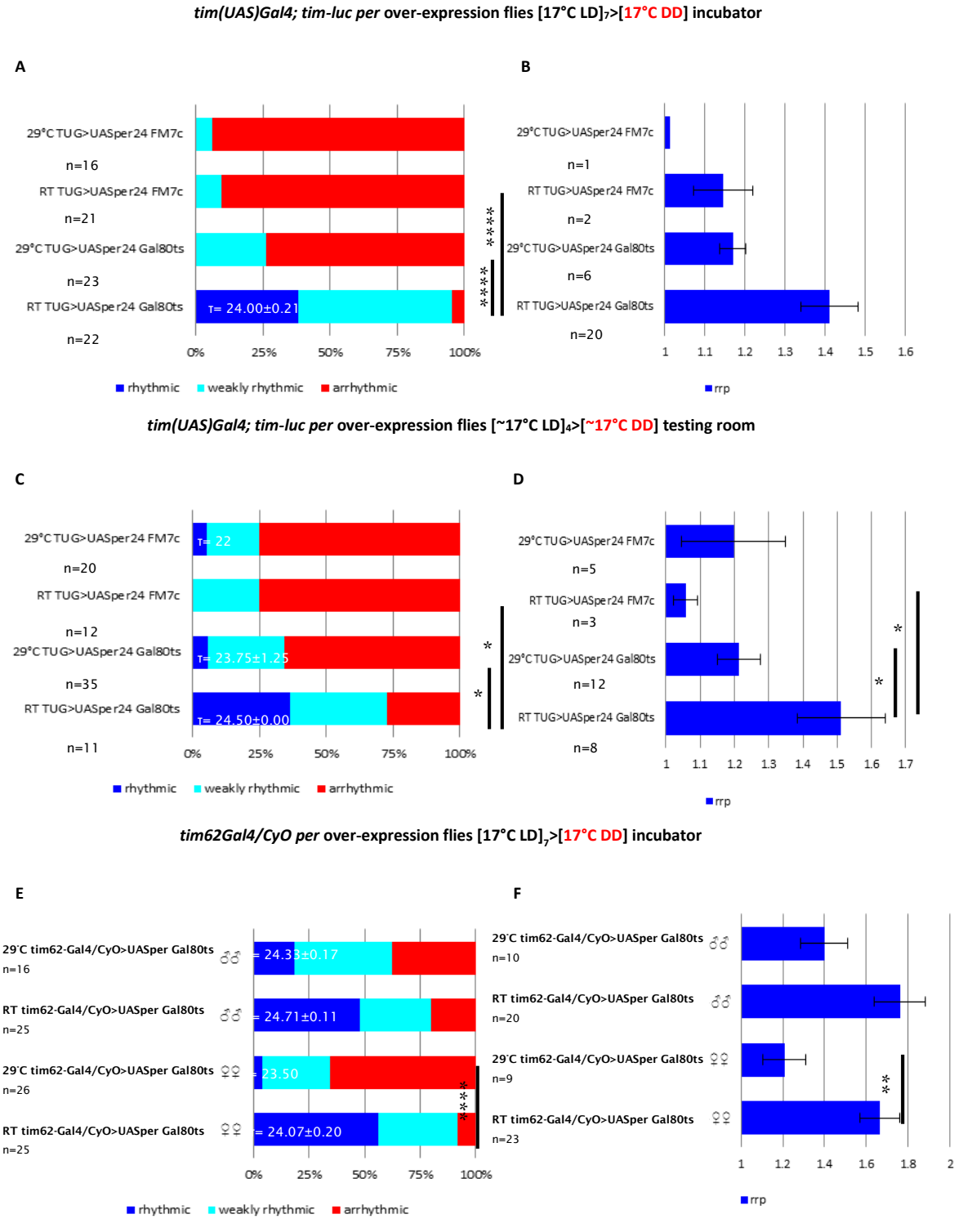


Figure A.3.1. Adult locomotor rhythmicity of *per* over-expression flies used for determination of oscillations in the periphery.

For all lines analysed, in all conditions, developmental *per* over-expression was negatively impacting adult behavioural rhythmicity. Quantification of rhythmicity of *tim(UAS)Gal4* – driven (A-D) and *tim62-Gal4* – driven (E-F) *per* over-expression flies in multiple conditions. Stacked bar diagrams represent the percentage of rhythmic, weakly rhythmic and arrhythmic flies (A, C, E). Numbers of flies analysed and the period length (±SEM) of rhythmic flies are included on the graph. Fisher's Exact 2x3 tests were used to compare either flies of the same genotype raised at a different temperature, or flies with constitutive and temperature-dependent *per* over-expression raised at the same temperature, and the results are indicated next to graphs. Period lengths of rhythmic flies were compared with non-parametric Mann-Whitney tests. Bar diagrams (B, D, F) show the average relative rhythmic power of flies displaying behavioural rhythms. Numbers of flies and error bars representing SEM are included on graphs. Mann-Whitney test was used to compare the same groups of flies and results are included on graphs. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

A.3.2 Analysis of the complexity of adult behavioural rhythms of flies with conditional *per* over-expression in PDF neurons.

Additional analysis was conducted to study two phenomena observed on actograms of flies with developmental *per* over-expression in PDF neurons. To study how the behaviour of flies changes depending on the length of time spent at DD, analysis was conducted separately for the first and second week of DD. Also, to test complexity of rhythms, modified data analysis protocol was used.

A.3.2.1 Protocol

Average chi-square periodograms were created separately for individual flies for first and second week of DD as described in 2.3, using 15-34 hrs period range with the threshold line set for p=0.01. Then all individual flies were tested for the presence of two peaks on individual chi-square periodograms (with the same period length range and confidence level) and values for their period length and relative power were collected. Each fly was classified as either rhythmic (single significant peak on their chi-square periodogram with rrp>1.5), weakly rhythmic (single significant peak with rrp<1.5), arrhythmic (no significant peak) and complex (more than one significant peak). I have noticed that in some cases a second peak was very close to a threshold line but not crossing it so I analysed flies again using p=0.05 and if this analysis resulted in two significant peaks, I was classifying flies as displaying complex rhythms. Distribution of flies was compared using Fisher's Exact 4x2 tests.

To see how each type of rhythmicity contributed to various peaks visible on the periodogram, I analysed average period length of flies displaying one significant peak (both rhythmic and weakly rhythmic flies) and flies displaying complex rhythms, where I divided all period lengths discovered into three categories: τ<22.5 hrs, 23 hrs>τ>26 hrs and τ>26 hrs and analysed the average period length for each of these categories separately.

A.3.2.2 Results

First of all, analysis of the average chi-square periodograms (Figure A.3.2 A) confirmed a shift in period length happening in females raised at RT after about a week of DD. This was not associated with largely increased complexity of flies but rather it seems that it might be a consequence of relatively stronger rhythmicity of flies displaying shorter period. Also, it could be observed on chi-square actograms that both females (more prominent) and males (less prominent) raised at 29°C display three components to the rhythm: one with a period in a circadian range, one around 20-22 hrs and one over 26 hours. This complexity of rhythm was confirmed by the analysis of individual flies, where in the case of females, complex rhythms were present in almost half of all flies. Complex rhythms were less prevalent during the second week in DD, when they disappeared from males, but were still present in females.

Appendix A

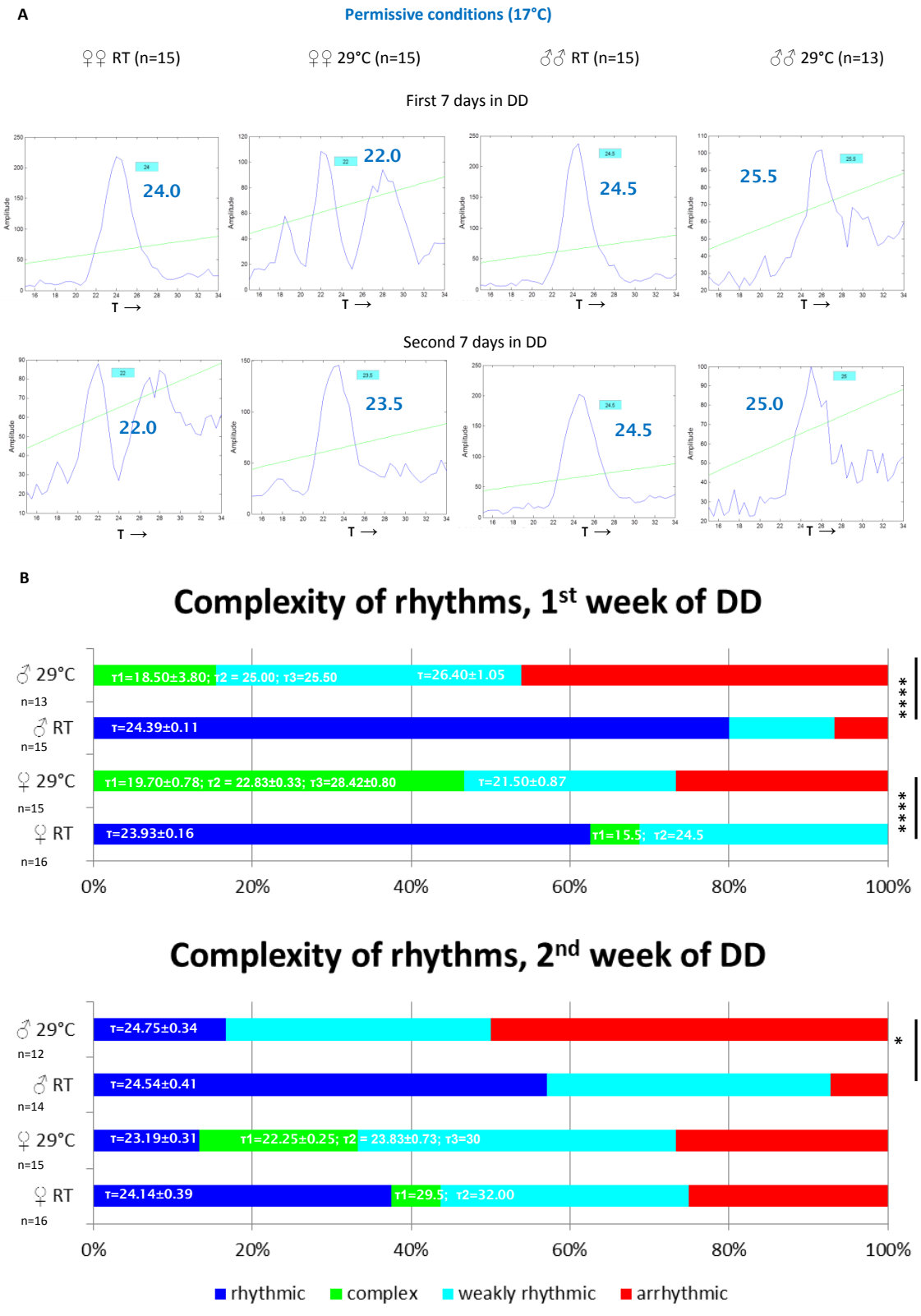


Figure A.3.2. Analysis of complexity of rhythms in flies with developmental *per* over-expression in PDF neurons.

(A). Average chi-square periodograms of flies with (raised at 29˚C) and without (raised at RT) developmental *per* over-expression in PDF neurons. Number of flies tested and period length of detected peak are marked. (B). Stacked bar diagrams representing distribution or rhythmic, weakly rhythmic, arrhythmic flies and flies with complex rhythms. Numbers of flies and period length of rhythmic and weakly rhythmic flies (marked as τ) and period lengths of various components of the complex rhythms (marked as τ1, τ2, τ3) together with SEM included. Flies raised at different temperatures were compared using Fisher's Exact 2x3 test. * $p<0.05$, **** $p<0.0001$.

A.3.3 Flies with developmental *per* over-expression driven by *Mai179-Gal4* exhibit reversible adult locomotor arrhythmicity in permissive conditions.

Creation and analysis of flies is described in 5.4.3. It was possible to obtain both flies heterozygous (balanced over *CyO*) and homozygous for *Mai179-Gal4*. Unfortunately, it was really difficult to analyse data for flies homozygous for *Mai179-Gal4* (Figure A.3.3), since the vast majority of them died early. Heterozygous flies with only one copy or *Mai179-Gal4* (balanced over *CyO*) survived better and allowed for a better comparison of flies raised at different temperatures (Figure A.3.4). Data obtained for either of these conditional *per* over-expression lines was different than for *per* over-expression driven either by *tim(UAS)Gal4* (Figure 4.1) or *Pdf-Gal4* (Figure 5.6). For female homozygous and heterozygous flies, there was no association between developmental temperature and either distribution of flies with different rhythmicity, relative rhythmic power or period length. Heterozygous males, however, displayed an association between raising temperature and the distribution of rhythmic, weakly rhythmic and arrhythmic flies (Fisher's Exact 3x2 test: $p<0.05$, $n=25$), but flies that remained weakly rhythmic and rhythmic had a comparable relative rhythmic power and period length.

Interestingly, even when actively over-expressing *per* with *Mai179-Gal4* driver at the 29˚C, the behavioural rhythmicity of both homozygous and heterozygous flies was not significantly different (Figures A.3.3 B and A.3.4 B). The only case in which there was a trend towards arrhythmicity was for homozygous females. The difference between homozygous and heterozygous females raised and analysed at 29˚C (Fisher's Exact 3x2 test: $p<0.05$, $n=27$) might be indicative of the difference in the expression of *per* resulting from either one or two copies of *Mai179-Gal4*. Unfortunately, the numbers of males alive for the whole experiment were very low. Moreover, even heterozygous *Mai179-Gal4* > *per* over-expression flies did not survive well through the 17˚C DD portion of the experiment (Figures A.3.3 C and A.3.4 C). However, it seemed that the addition of developmental *per* over-expression driven by *Mai179-Gal4* did not reduce the rhythmicity of flies at permissive temperature, as there was no correlation between the developmental temperature and the distribution of rhythmic, weakly rhythmic and arrhythmic flies, except for heterozygous males (Fisher's Exact 3x2 test: $p<0.05$, $n=16$, however for one

condition compared only two flies were recovered). Taking these results together with the previously described results for flies without adult exposure to 29˚C (Figures A.3.3 A and A.3.4 A) and relative robustness of the rhythmicity of flies while analysed at 29˚C DD (Figures A.3.3 B and A.3.4 B), it was very unlikely that a larger number of flies would lead to detection of additional significant differences.

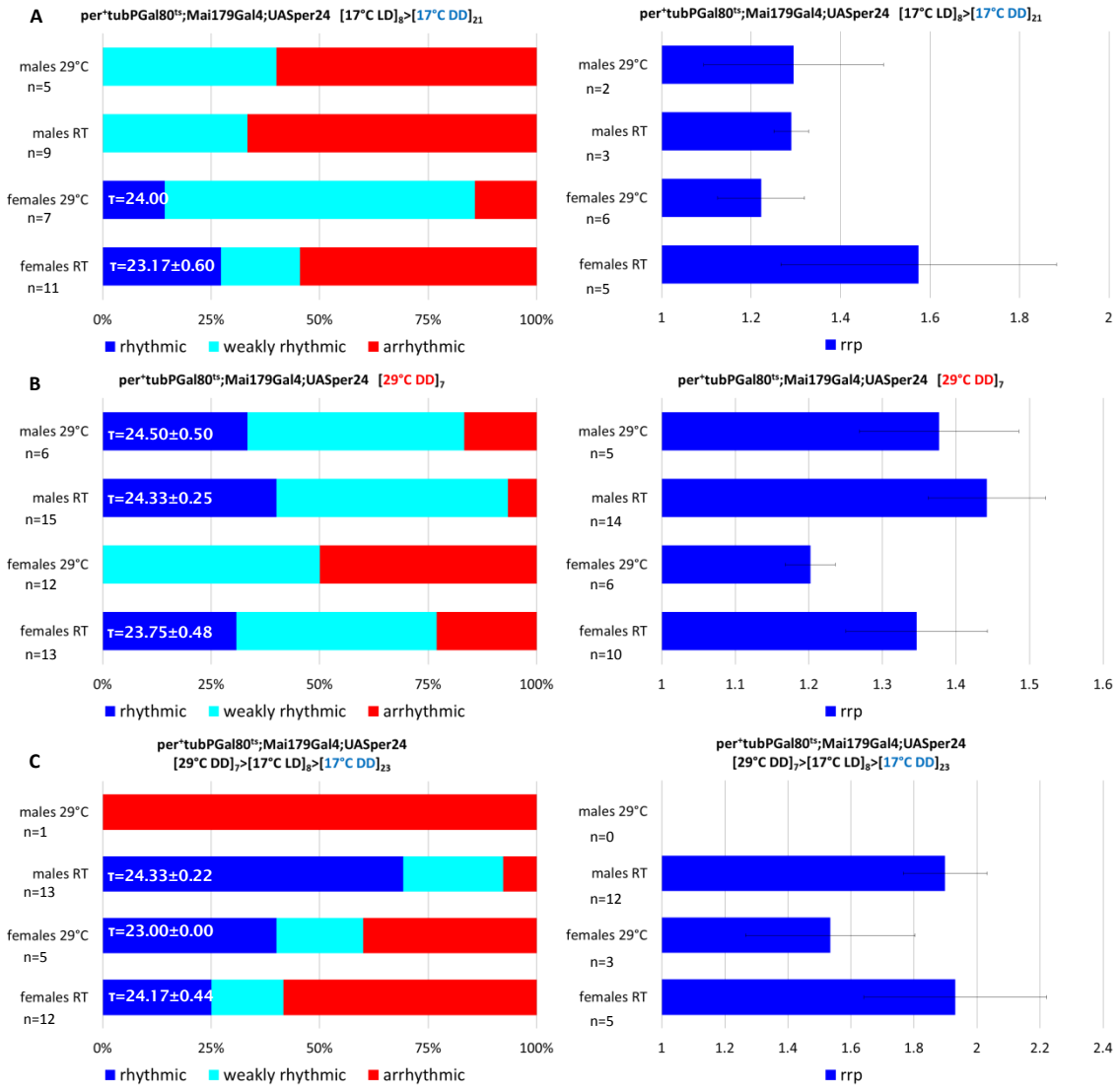


Figure A.3.3. Developmental *per* over-expression driven by *Mai179-Gal4* causes reversible adult arrhythmia – results for homozygous flies.

For all conditions analysed, flies with conditional expression of the transgenic *per* driven by *Mai179-Gal4* driver (raised at 29˚C) showed comparable rhythmicity to flies with the same genotype raised at room temperature, with no transgenic *per* expression. Quantification of the rhythmicity of individual flies analysed at permissive conditions (A), restrictive conditions (B) or permissive conditions following adult exposure to restrictive conditions for at least 7 days (C). Stacked bar diagrams on the left show the percentage of rhythmic, weakly rhythmic and arrhythmic flies, with numbers of flies and the period length (±SEM) of rhythmic flies included on the graph. On the right, bar diagrams depict the average relative rhythmic power of rhythmic and weakly rhythmic flies with numbers of flies included on the graphs and error bars representing SEM.

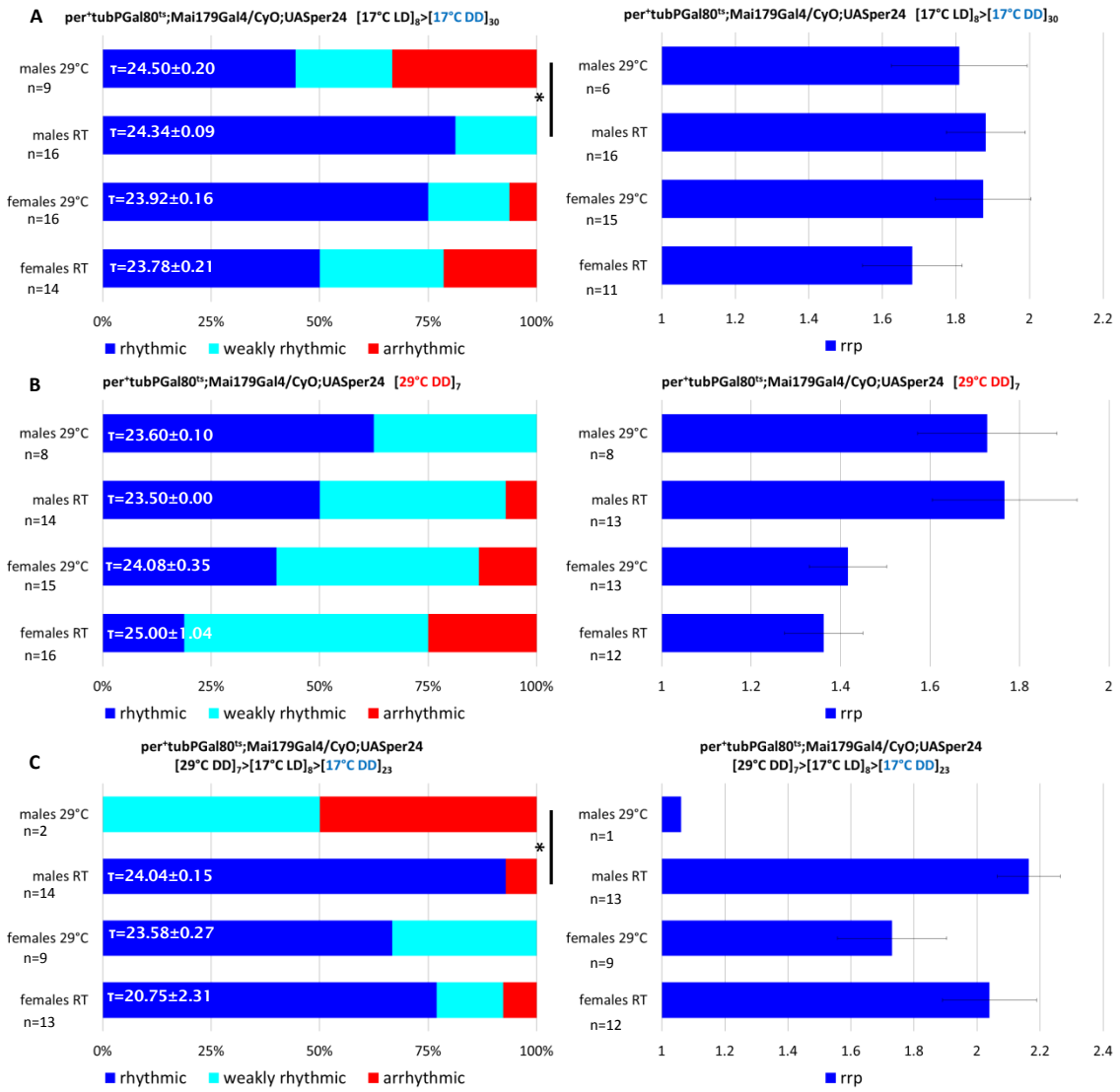


Figure A.3.4. Developmental *per* over-expression driven by *Mai179-Gal4* was unlikely to result in irreversible adult arrhythmia – results for heterozygous flies.

Over-expression of *per* driven by one copy of *Mai179-Gal4* during adulthood and development, did not significantly affect adult behavioural rhythmicity. Quantitative analysis of the rhythmicity of individual flies analysed at permissive conditions (A), restrictive conditions (B) or permissive conditions following adult exposure to restrictive conditions for at least 7 days (C). Stacked bar diagrams on the left demonstrate the percentage of rhythmic, weakly rhythmic and arrhythmic flies. Flies raised at different temperatures were compared with Fisher's Exact 3x2 test. On the right, bar diagrams

show the average relative rhythmic power of rhythmic and weakly rhythmic flies with error bars representing SEM and numbers of flies included on the graphs. Flies raised at different temperatures were compared with Mann-Whitney test. * $p<0.05$.

A.3.4 Attempted conditional *per* over-expression in all circadian neurons except morning or morning and evening cells

A.3.4.1 Protocol

Conditional *per* over-expression flies carrying *Pdf-Gal80* (on the second chromosome, so flies were heterozygous for *tim(UAS)Gal4*) or *cry-Gal80* (on third chromosome, so flies were heterozygous for *UAS-per24*) constructs described previously (Park *et al*., 2000; Emery *et al*., 2000b) were raised at either permissive temperature (RT) or at restrictive conditions (29˚C). Control flies had either CyO instead of *Pdf-Gal80* or TM6B-Tb' instead of *cry-Gal80*. 3-4 day old flies were loaded into *Drosophila* Activity Monitors and flies were either directly moved to 17˚C DD (*Pdf-Gal80*) or entrained first to 17˚C LD for seven days and subsequently moved to 17˚C DD. The first full seven days in DD were used to check flies for the presence of rhythms as described in 2.3. To determine the association between either the developmental temperature or the presence of the *Gal80* element and the rhythmicity of flies, Fisher's Exact 2x3 test was used, comparing experimental flies to controls raised under the same conditions and also flies with the same genotype raised at different temperatures to each other. Period length and relative rhythmic power for the same pairs were compared using the non-parametric Mann-Whitney test.

A.3.4.2 Results

Unfortunately, the analysis has revealed that the presence of the *Gal80* expression in a *tubP-Gal80^{ts}*-containing *per* over-expressing flies did not seem to have any impact on the adult behaviour of flies (Figure A.3.5). In all cases analysed, there was no association between the presence of the *Pdf-Gal80* element and the rhythmicity of flies (Figure A.3.5 A). The only difference observed was the elongation of the period length observed in females without *Pdf-Gal80* raised at RT (Mann-Whitney test: U=88.500, z=1.326, p=0.043, n=22). As expected, there was an association between the developmental temperature and the rhythmicity of control flies without the *Pdf-Gal80* (Fisher's Exact 3x2 test: $p<10^{-6}$, n=32 for females and p<0.001, n=28 for males for the distribution of flies with different rhythmic power and Mann-Whitney test: U=46.000, z=2.170, p<0.05, n=20 for the relative rhythmic power for males

and no test performed for females). The same association was found for flies with *Pdf-Gal80* (Fisher's Exact 3x2 test: $p<10^{-5}$, n=62 for females and $p<10^{-3}$, n =59 for the distribution of rhythmic, weakly rhythmic and arrhythmic flies and Mann-Whitney test: U=284.000, z=3.193, p=0.001, n=38 for the relative rhythmic power in males).

Unfortunately, due to technical difficulties, no data was recovered for males with the *cry-Gal80* insertion (Figure A.3.5 B), however, based on the *Pdf-Gal80* results I have assumed that there should not be any significant difference between both genders. Similar to the results described above with the *Pdf-Gal80*, flies raised at 29˚C showed no association between the presence of *cry-Gal80* and the distribution of the rhythmic, weakly rhythmic and arrhythmic flies (Fisher's Exact 3x2 test: p=0.690, n=23) or the relative rhythmic power (Mann-Whitney test: U=32.000, z=-0.293, p=0.813, n=17). Surprisingly, for flies raised at room temperature such association existed both for the distribution of flies (Fisher's Exact 3x2 test: p<0.001, n=30) and relative rhythmic power (Mann-Whitney test: U=125.000, z=2.944, p=0.002, n=26). However, these associations point to the control flies as more rhythmic than flies with *cry-Gal80*, which is the opposite of what could be expected. Due to this unusual rhythmicity of control line, while no association between the developmental temperature and the distribution of flies with different rhythmicity or relative rhythmic power was found for flies carrying *cry-Gal80*, it existed for control flies (Fisher's Exact 3x2 test: p<0.01, n=16 for the distribution of flies and Mann-Whitney test: U=56.000, z=3.240, $p<10^{-3}$, n=15 for relative rhythmic power).

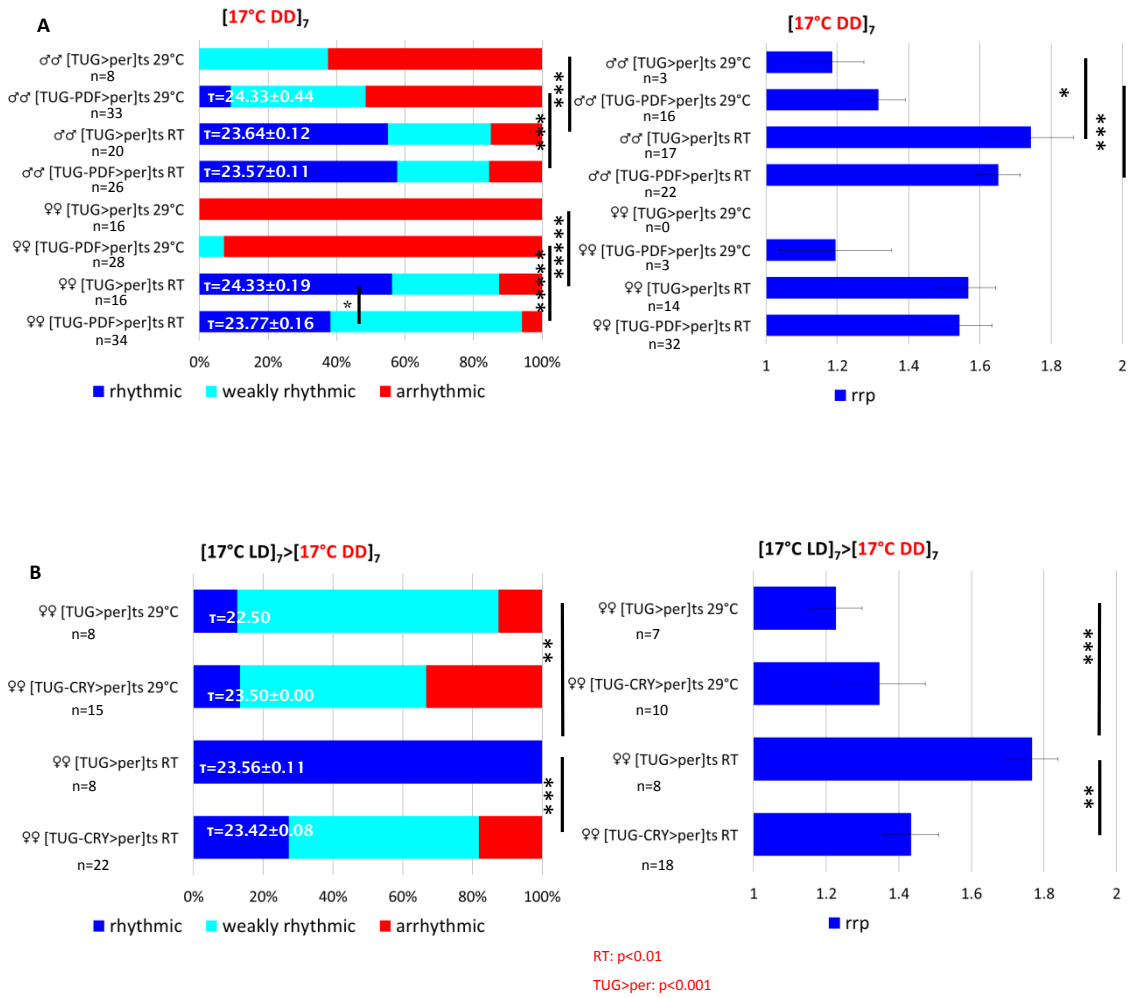


Figure A.3.5. Addition of *Pdf-Gal80* or *cry-Gal80* element did not improve the rhythmicity of flies with developmental *per* over-expression.

Flies over-expressing *per* in all PDF-negative circadian cells ([TUG-PDF>per]ts; (A) and all CRY-negative circadian cells ([TUG-CRY]ts; (B) in a conditional manner were no different than flies with developmental *per* over-expression in all circadian cells ([TUG>per]ts). On the left, stacked bar diagrams depict the distribution of rhythmic, weakly rhythmic and arrhythmic flies. Numbers of flies and the average period length (±SEM) of rhythmic flies are included on the graph. Distribution of flies of different genotype but raised at the same temperature, or of flies with the same genotype but raised at different temperatures, was done with Fisher's Exact 2x3 tests and the significant results are marked next to each graph. Period lengths were compared with the non-

parametric Mann-Whitney tests for the same groups. On the right hand side, bar diagrams represent the average relative rhythmic power, with the numbers of flies tested included in the graph and error bars representing SEM. Values for the same groups as described previously were tested with the non-parametric Mann-Whitney. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ***** $p<10^{-5}$.

A.3.5 Dorsal projections are intact in flies with developmental CLK/CYC inhibition

Since adult behavioural rhythmicity is driven by the molecular oscillator in the brain and previous experiments pointed to ventrolateral neurons as the primary cells requiring CLK/CYC function during metamorphosis (Figures 5.1, 5.3 and 5.6), an obvious question was whether there were any changes in the morphology of these cells or their projections that could potentially suggest the mechanism of CLK/CYC action during development. Brains of flies with conditional *per* over-expression raised at either restrictive or permissive temperature were prepared and stained for PER and PDF according to the protocols described in 2.6. Brains were harvested from flies kept at permissive temperature (17˚C) under standard 12:12 LD conditions on day 8 (ZT 22) and day 9 (ZT 4). Images analysed qualitatively, to check if the gross neuroanatomy of the circadian circuit was similar and if the dorsal projections from LN_vs were intact.

Ln_v cells were identified by the co-staining with the PDF and other groups of cells were identified based on their location. As visible from the representative pictures (Figure A.3.6), developmental *per* over-expression seemed not to cause any gross morphological changes in the clock neurons. The position, number or size of the LN_v, LN_d, and DN cells were comparable and all the changes could be explained by the normal small variations between individual flies. Flies raised at 23˚C displayed lower levels of PER staining due to the fact that these flies were collected at ZT 4, during the trough in the PER expression, while flies raised at 29˚C were collected at ZT 22, when PER levels were still elevated. In addition to this, the dorsal projections from the s-LN_vs appeared normal, reaching the dorsal protocerebrum. Due to the nature of the staining I was unable to conclude if the shapes of the termini of the projections were comparable. Based on this qualitative analysis I concluded that the developmental *per* over-expression did not affect the morphology of the circadian circuit in the brain.

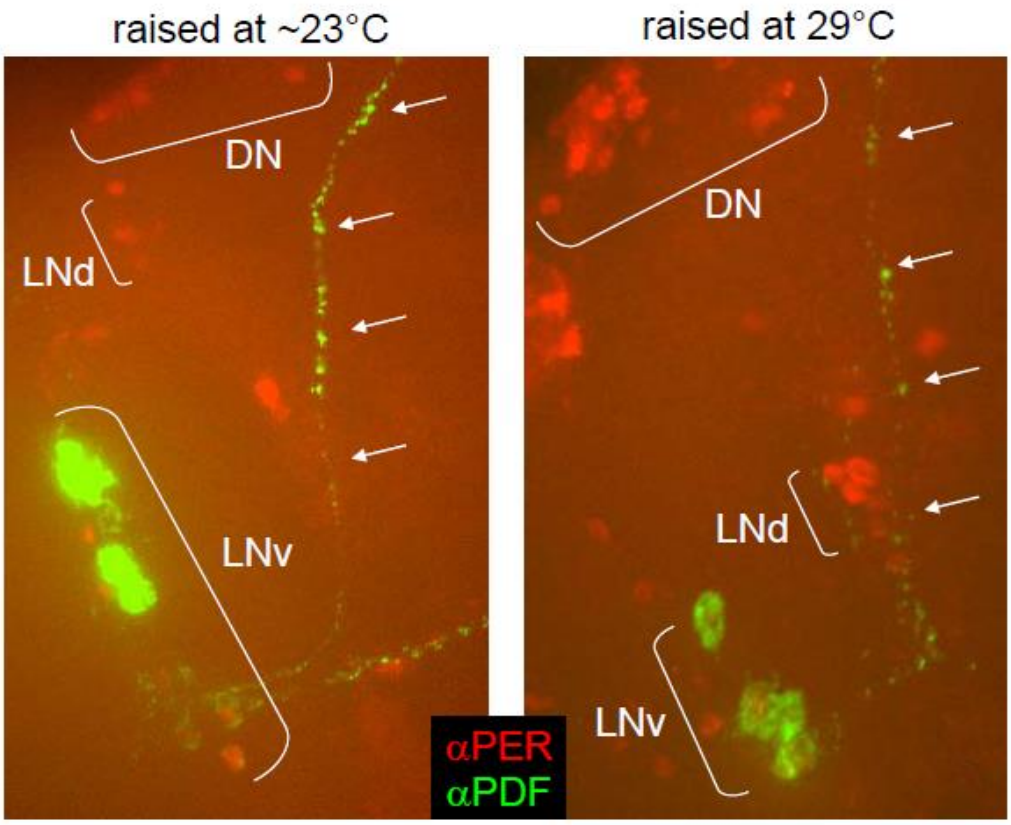


Figure A.3.6. Developmental *per* over-expression does not affect the gross morphology of the circadian neurons, including the dorsal projections of s-LN$_v$s (adapted from Goda, Mirowska and Currie *et al.*, 2011; data collected and analysed by Jake Currie).

LN$_v$, LN$_d$ and DN neurons and dorsal projections from the s-LN$_v$s did not show any differences between flies raised with developmental *per* over-expression and without it. Representative images for the brains of flies raised at room temperature and at 29°C. Circadian neurons were detected with anti-PER antibodies (in red) and anti-PDF antibody (green) was used specifically to mark LN$_v$s and their projections. White arrows point to the dorsal projections from s-LN$_v$s which appear similar for both conditions.

A.3.6 Developmental *per* over-expression might be affecting the size of circadian neurons.

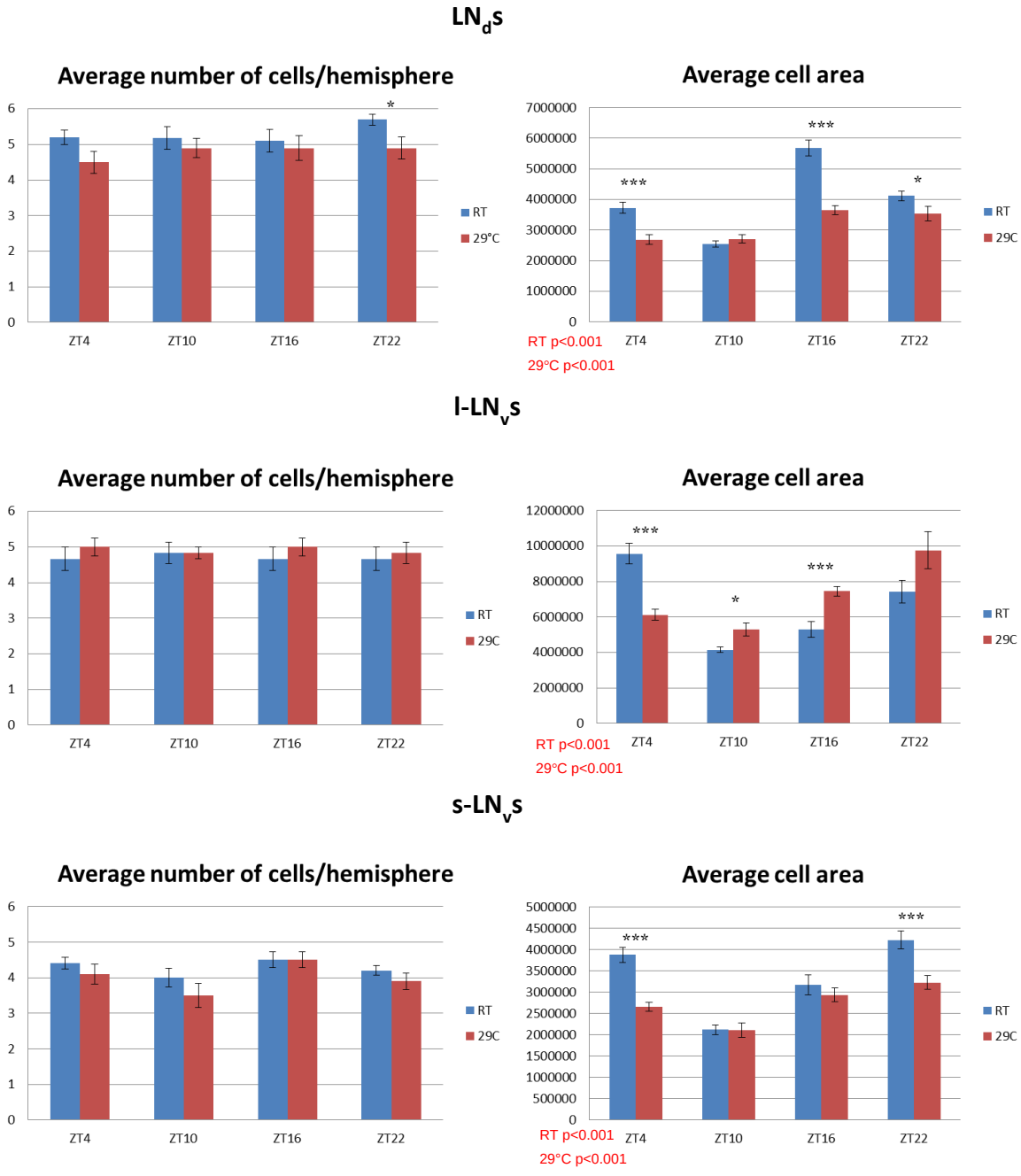


Figure A.3.7. Developmental *per* over-expression affects size of circadian neurons.

On the left, bar charts demonstrate the average number of cells detected per hemisphere. On the right hand side, bar charts show the average area of a particular circadian neuron in square pixels.

Flies with conditional PER over-expression were raised under permissive (RT, ~23˚C; marked in blue) and restrictive (29˚C; marked in red) conditions. Error bars represent SEM. Comparisons of the number or area of cells between timepoints for flies raised at each temperature were performed with non-parametric Kruskal-Wallis tests (results below graphs, marked by developmental temperature) while significant differences between flies raised at different temperatures were tested with non-parametric Mann-Whitney tests and are marked on graphs. Data comes from cells from 6-10 hemispheres, cell n numbers: LN_ds= 45-57, s-LN_vs = 21-44, l-LN_vs = 28-30. * $p<0.05$, ** $p<0.01$, *** $p<10^{-3}$.

The number of cells detected for each group of neurons was fairly consistent for RT-raised and 29˚C-raised flies for all timepoints (Figure A.3.7), with only significantly less cells found for LN_ds of flies raised at 29˚C at CT 22 (Mann-Whitney test: U=20.500, z=-2.425, p=0.023, n=20). When it came to the area of cells, there was a highly significant correlation between the area of cells and the circadian time for all groups of cells for both flies raised at RT and 29˚C (Kruskal-Wallis test: H(3)=96.570, p<0.001, n=217 for LN_ds from RT; H(3)=24.364, p<0.001, n=192 for LN_ds from 29˚C; H(3)=53.866, p<0.001, n=138 for s-LN_vs from RT; H(3)=21.123, p<0.001, n=128 for s-LN_vs from 29˚C; H(3)=44.552, p<0.001, n=113 for l-LN_vs from RT; H(3)=26.595, p<0.001, n=118 for l-LN_vs from 29˚C). This could be explained by the changes in the signal level affecting the selection of the outline of the cells. However, for more than half of the cases the area of cells detected for flies raised at 29˚C was significantly lower than those for RT-raised (Mann-Whitney test: U=592.000, z=-4182, p<0.001, n=97 for LN_ds at CT 4; U=389.500, z=-5.930, p<0.001, n=100 for LN_ds at CT 16; U=1005.000, z=-2.481, p<0.05, n=106 for LN_ds at 22; U=136.000, z=-4.419, p<0.001, n=58 for l-LN_vs at CT 4; U=314.000, z=-5.263, p<0.001, n=86 for s-LN_vs at CT 4; U=442.000, z=-3.564, p<0.001, n=81 for s-LN_vs at CT 22) and significantly higher than for RT-raised only for two cases (Mann-Whitney test: U=578.000, z=2.450, p<0.05, n=58 for l-LN_vs at CT 10 and U=679.000, z=4.038, p<0.001, n=58 for l-LN_vs at CT 16).

A.4 Supplementary data to Chapter 6

A.4.1 Developmental PDF knockdown might impair adult locomotor rhythmicity

Previously, I demonstrated that the developmental CLK/CYC inhibition in PDF neurons resulted in irreversible adult arrhythmia, pointing to the PDF neurons as an important site for developmental CLK/CYC function (Figure 5.6). Additionally, developmental *per* over-expression was shown to affect the molecular oscillations in the s-LN_vs (Figure 5.12). Given that PDF neurons are crucial for the generation of behavioural rhythms in the constant darkness (Renn *et al.*, 1999; Nitabach *et al.*, 2002; Lin *et al.*, 2004, Nitabach *et al.*, 2005) and that they are doing so by rhythmic release of the PDF neuropeptide from the dorsal s-LN_v projections, I wondered if developmental *per* over-expression might cause permanent impairment in PDF release. Analysis of cyc^{01} flies conducted previously revealed a loss of PDF staining in a large number of the dorsal s-LN_v projections (Goda, Mirowska, Currie *et al.*, 2011). Even though the previous experiment looking at the same projections in *per* over-expression flies confirmed presence of PDF in the termini (Figure A.3.6), the lack of quantification did not exclude a possibility that developmental *per* over-expression affects PDF secretion. Therefore, I wanted to test if the silencing of the *Pdf* gene only during development might be resulting in an irreversible adult arrhythmia.

To find the answer, I combined a GAL4-inducible double-stranded *Pdf* RNA transgene (*UAS-dsPdf*) with a transgene targeting *Gal4* expression to the PDF neurons (*Pdf-Gal4*). To increase the level of *Pdf* knockdown, I also added an *UAS-Dcr-2* element, which produces DICER-2, an element in the RISC pathway (Dietzl *et al.*, 2007). The resulting flies had a decreased production of PDF protein. To check if the achieved PDF level decrease was sufficient for causing behavioural changes, I compared the behaviour of the experimental flies with that of controls lacking the *Pdf-Gal4* element (with CyO balancer chromosome instead).

A.4.1.1 Protocol

UAS-Dcr-2/w or Y;(Pdf-Gal4 or CyO)/+;UAS-dsPdf/+ flies were raised at the room temperature on a standard food. Flies were first entrained to a standard 12:12 LD cycle at 23˚C and subsequently moved to constant darkness at the same temperature. The first full seven days at the constant conditions were used to analyse the locomotor behaviour of the individual flies as described in 2.3. The distributions of the rhythmic, weakly rhythmic and arrhythmic flies were compared between the experimental flies and the controls of the same sex with the Fisher's Exact 3x2 tests. Average relative rhythmic power and period length were compared with the non-parametric Mann-Whitney test.

A.4.1.2 Results

Comparing to controls, knocking down *Pdf* expression indeed affected the behaviour of flies (Figure A.4.1 A). Experimental females were significantly more arrhythmic than the control population (Fisher's Exact 3x2 test: $p<0.05$, $n=32$) and the remaining rhythmic and weakly rhythmic flies had largely decreased rhythmic power (Mann-Whitney test: $U=62.000$, $z=2.635$, $p<0.01$, $n=17$). On the other hand, males showed only slight increase in the number or arrhythmic and weakly rhythmic flies and no change in the relative rhythmic power. The remaining rhythmic males did exhibit significantly shorter period lengths than the control flies (Mann-Whitney test: $U=96.500$, $z=2.940$, $p<0.01$, $n=22$). These results served as a confirmation that knocking down *Pdf* affects the behavioural rhythmicity of flies and therefore can be used to test the effects of the developmental effect of PDF signalling disruption on the adult behavioural rhythms.

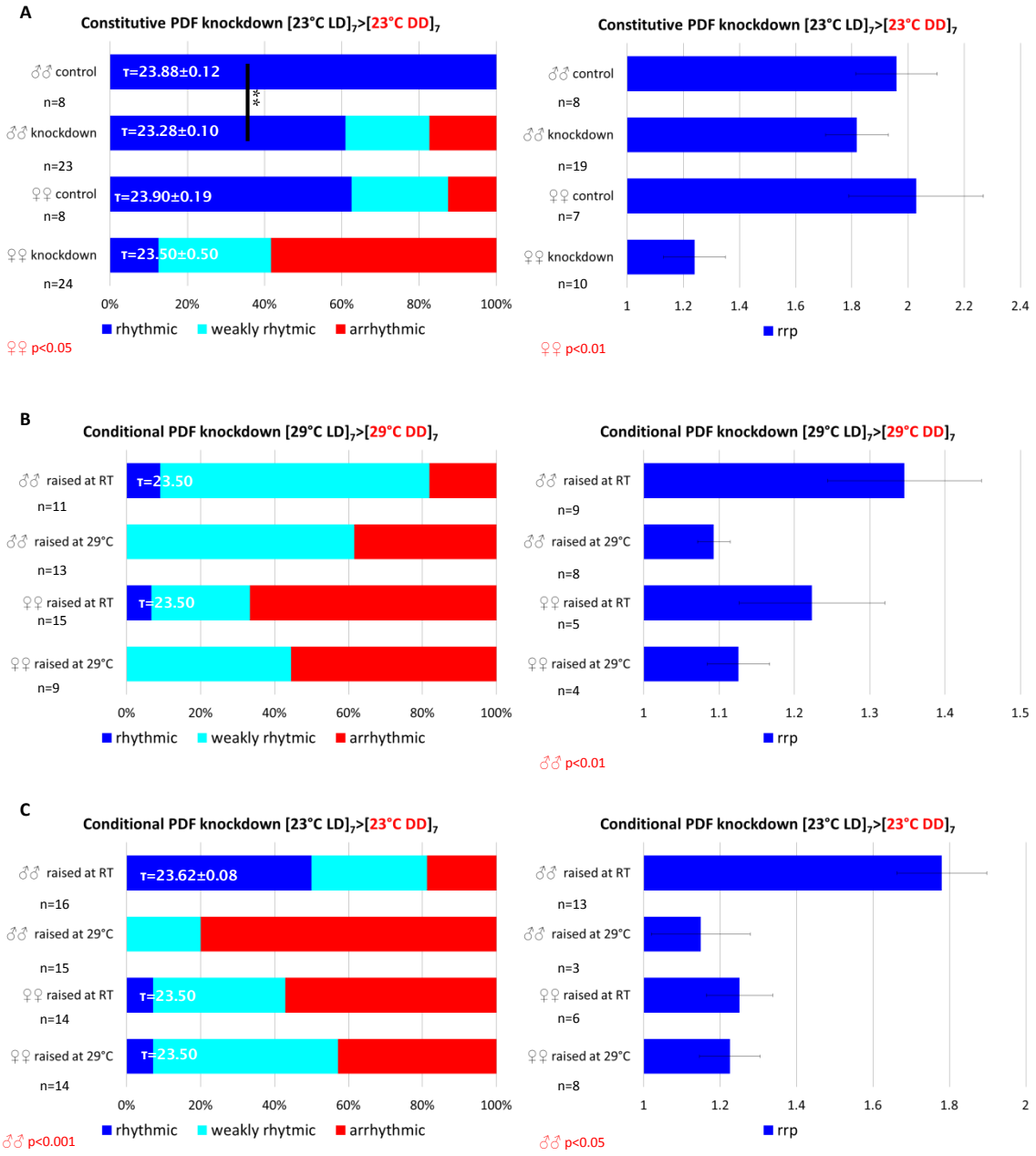


Figure A.4.1. Both developmental and adult *Pdf* knockdown disrupt adult locomotor rhythmicity.

(A) Constitutive *Pdf* knockdown revealed that the *dsPdf* element used was capable of affecting the adult locomotor activity, albeit to a smaller extent than anticipated. "Knockdown" denotes flies expressing *dsPdf* in the PDF-positive neurons in constitutive manner and "control" are flies with just the *dsPdf* transgene. (B) and (C) Males with developmental knockdown of *Pdf* were less rhythmic than control flies raised at RT. The same trend was less consistent for females, which had poor rhythmicity regardless of the raising

temperature and resembled the females tested at restrictive conditions, suggesting that 23˚C represents a (semi-)restrictive condition for females. On the left, stacked bar diagrams show the percentage of rhythmic, weakly rhythmic and arrhythmic flies, with the numbers of flies analysed and the period length (±SEM) of the rhythmic flies indicated on the graph. To test for the difference in the distribution of flies resulting from the *Pdf* knockdown, experimental flies were compared to control ones using Fisher's Exact 3x2 test and the significant results are summarised below the graph. Association between circadian period length and the *Pdf* knockdown was tested using non-parametric Mann-Whitney test with the significant differences indicated with a black line, ** $p<0.01$. On the right, bar diagrams show the average relative rhythmic power of the rhythmic and weakly rhythmic flies with the error bars representing SEM and the numbers of flies used for the analysis indicated. Experimental flies were compared to controls with the non-parametric Mann-Whitney test and significant results are indicated below the graphs.

To be able to test the developmental impact of knocking down *Pdf* I introduced *tub_P-Gal80^{ts}* to the line described above, creating a *UAS-Dcr-2/w or Y;(Pdf-Gal4 or CyO)/+;UAS-dsPdf/tub_P-Gal80^{ts}* line. Resulting flies experience knockdown of *Pdf* only at elevated temperature. Therefore, to study the impact of developmental knockdown of *Pdf*, I compared flies raised under either restrictive conditions of 29˚C or 23˚C, which was assumed to be permissive temperature.

A.4.1.3 Protocol

Parent flies were allowed to mate and lay eggs at room temperature and eggs were subsequently moved to permissive and restrictive temperature in such way to allow me to collect offspring from both conditions at the same time. Adult flies were entrained for seven days to the LD cycle and subsequently moved to DD at either permissive temperature of 23˚C or restrictive

temperature of 29˚C. Free-running rhythms of flies were analysed as described in 2.3. Distribution of rhythmic, weakly rhythmic and arrhythmic was compared between flies raised at different temperatures using Fisher's Exact 2x3 test. Average period length and relative rhythmic power of flies raised at different temperatures were compared using non-parametric Mann-Whitney test.

A.4.1.4 Results

As predicted in the previous experiment, when analysed at restrictive temperature flies demonstrated reduced rhythmicity (Figure A.4.1 B). There was no significant correlation between the distribution of rhythmic, weakly rhythmic and arrhythmic flies and the temperature at which flies were raised (Fisher's Exact 3x2 test: $p=0.785$, $n=24$ for females; $p=0.386$, $n=24$ for males). This was also visible when looking at the relative rhythmic power, where the values for the 29˚C-raised flies were lower than for the 23˚C-raised flies (the difference was statistically significant for males with Mann-Whitney test: $U=6.000$, $z=-2.887$, $p<0.01$, $n=17$ but due to a low number of flies there was no significance detected for females).

When analysed at permissive temperature (Figure A.4.1 C), there was an apparent difference between females and males. Unexpectedly, there were no differences between females raised at 23˚C and 29˚C when I compared the distribution of rhythmic, weakly rhythmic and arrhythmic flies, the relative rhythmic power and period length. Males, on the other hand, showed a clear correlation between the developmental temperature and the distribution of rhythmic, weakly rhythmic and arrhythmic flies (Fisher's Exact 3x2 test: $p<0.001$, $n=31$). Also, relative rhythmic power was also showing correlation with the developmental temperature (Mann-Whitney test: $U=3.000$, $z=-2.220$, $p<0.05$, $n=16$).

Unfortunately, these results revealed a problem with the experiment itself, as it is unclear whether PDF knockdown was reversible. Therefore, it is impossible to ascertain that flies had only developmental PDF inhibition. An experiment to address this would be to look at levels of PDF in circadian neurons following development and adult exposure to various temperatures. To test whether knockdown was reversible one would have to check if PDF levels increase after

transfer from restrictive to permissive temperature. Additionally, it is uncertain whether 23˚C can be indeed treated as permissive conditions, as for example the lack of significant difference between females raised at different conditions might suggest that these temperatures are not as different as one would like them to be.

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