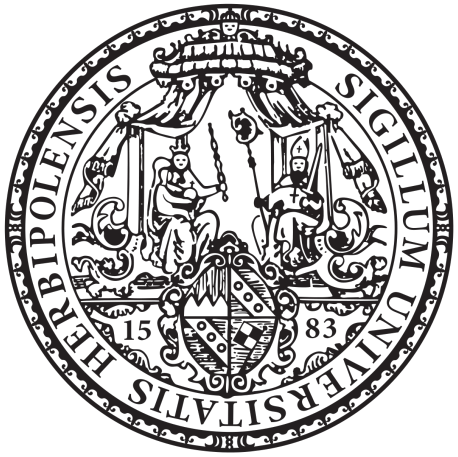

Comparative analysis of insect circadian clocks: a behavioural, anatomical, and molecular study

Vergleichende Analyse der zirkadianen Uhr von Insekten: eine verhaltensbezogene, anatomische und molekulare Studie

Doctoral thesis for a doctoral degree
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Section Integrative Biology,

submitted by

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from

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Summary

Biological clocks are endogenous oscillators that give organisms the sense of time. Insects, as the largest taxonomic group, offer fascinating models to study the evolution of clocks and their adaptation to various environments. Although the laboratory fruit fly, *Drosophila melanogaster*, led the role in the field of circadian biology as it provides a powerful genetic experimental tool, new model insect species need to be established to understand photoperiodic responses and to enable comparative studies. This work reports the behavioural, anatomical, and molecular characterization of the circadian clock of five insect species.

The malt fly *Chymomyza costata* carries a *D. melanogaster*-like clock network, which supports circadian rhythms under rhythmic environment but cannot self-sustain when isolated from external time cues.

The olive fly *Bactrocera oleae* is the major pest of olive plantations and the characterization of its circadian clock will improve future pest management strategies.

The linden bug *Pyrrhocoris apterus*, a well suited model for investigating circadian and photoperiodic timing interactions, shows high degree of homology of the clock network with *D. melanogaster*.

The scuttle flies *Megaselia scalaris* and *Megaselia abdita* represent new fascinating models to study how the clock network controls circadian behaviour.

Overall, this work highlights high degree of homology between different circadian clock systems, but at the same time also dramatic differences in terms of circadian behaviour and neuro-anatomical expression of clock components. These have been mainly discussed in regards to the evolution of clocks in Diptera, and the adaptation of clocks to high latitudes.

Zusammenfassung

Biologische Uhren sind endogene Oszillatoren, mit welchen Organismen die Zeit messen können. Als größte taxonomische Gruppe stellen Insekten eine Vielzahl faszinierender Modelle, um die Evolution und Anpassung von biologischen Uhren an verschiedene Umweltbedingungen zu untersuchen. Obwohl *Drosophila melanogaster* eines der führenden Modelltiere im Feld der Chronobiologie ist, was sich leicht auf die herausragende genetische Manipulierbarkeit der Fliege zurück führen lässt, müssen weitere Insektenarten als Modellorganismen etabliert werden, um anhand vergleichender Studien die Anpassungen an photoperiodische Veränderungen verstehen zu können. Die vorliegende Arbeit beschreibt die Charakterisierung der zirkadianen Uhr von fünf Insektenarten auf molekularer-, anatomischer- und Verhaltens-Ebene.
Die Taufliegenart *Chymomyza costata* besitzt eine *Drosophila*-ähnliche Uhr, die zirkadiane Rhythmen unterstützt solange sich das Tier in einer rhythmischen Umwelt befindet. Allerdings kann die Uhr den Rhythmus nicht selbstständig aufrecht erhalten, wenn die Fliege von externen Zeitgebern isoliert ist.
Die Olivenfruchtfliege *Bactrocera oleae* ist der bedeutendste Schädling auf Olivenplantagen und die Charakterisierung der zirkadianen Uhr dieser Art wird zukünftige Schädlingsbekämpfungsstrategien verbessern.
Die Gemeine Feuerwanze *Pyrrhocoris apterus*, ein gut geeignetes Modell um die Interaktion des zirkadianen und photoperiodischen Timings zu untersuchen, zeigt hohe Homologie zum Uhrennetzwerk von D. melanogaster.
Die Buckelfliegen *Megaselia scalaris* und *Megaselia abdita* repräsentieren neue faszinierende Modelle für die Erforschung wie das Uhrennetzwerk zirkadianes Verhalten steuert.
Zusammengenommen hebt diese Arbeit die hohe Ähnlichkeit zwischen verschiedenen zirkadianen Systemen hervor, zeigt jedoch gleichermaßen gravierende Unterschiede in Bezug auf zirkadianes Verhalten und der neuroanatomischen Expression von Uhrenkomponenten. Die Homologien und Unterschiede werden hauptsächlich in Bezug auf die Evolution biologischer Uhren in Dipteren, sowie der Anpassung der Uhren an höhere geografische Breiten, erörtert.

Contents

1 Introduction

1.1 A rhythmic world

Life on earth has evolved under the selective pressure of rhythmic environmental changes. For example, Earth rotation around its own axis imposes cyclic periods of alternated illumination and darkness, referred as days and nights, which repeat rhythmically day after day with a period of 24 hours (Figure 1). On the other hand, Earth rotation around the Sun on a tilted axis imposes seasonality in non-equatorial regions, which generates changing environmental conditions that repeat year after year with a period of 365.256 days (Figure 1).

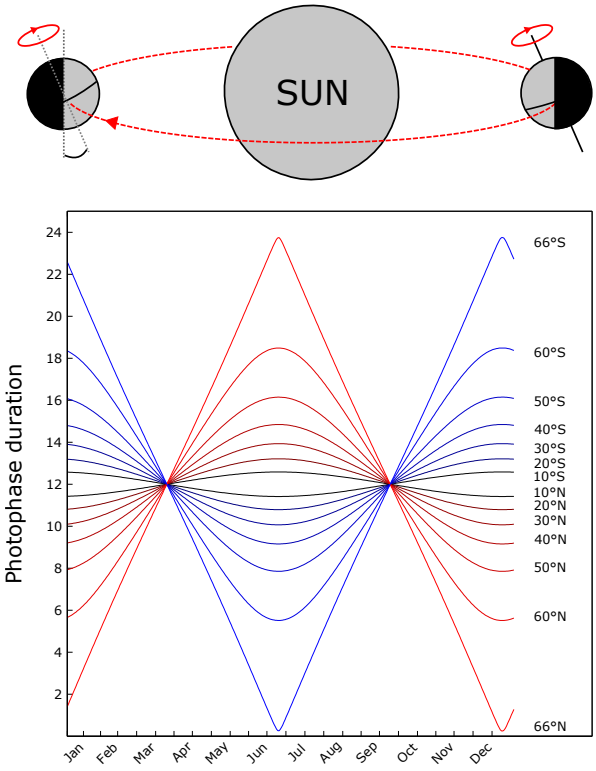

Figure 1: Top: Earth rotation on its own axis generates 24 hours daily rhythms. Earth rotation around the Sun on a 23.4° tilted axis generates annual rhythms. Bottom: Variation of the day/night length ratio at different latitudes across the year (red: Northern hemisphere; blue: Southern hemisphere).

In order to cope with such environmental rhythms, all taxonomic groups have evolved a series of timekeeping mechanisms which are grouped under the name of biological clocks, or endogenous clocks. The advantages of having internal clocks is clear, as they give fitness advantage helping organisms to cope with such dynamic aspect of ecology, and allowing them to predict and therefore anticipate cyclic changes

(Enright, 1970). It is now well known that both circadian and seasonal rhythms are under the control of molecular mechanisms.
The field of biology that study such rhythmic aspect of lives is named *chronobiology*.

1.2 Chronobiology: the biology of time

Biological clocks are endogenous timers that allows organisms to synchronize to local environment. They generate endogenous rhythms that are generally classified by the length of their period (τ) in:

- ultradian rhythms: rhythms with τ significantly shorter than 24 hours;
- circadian rhythms: rhythms with τ of approximately 24 hours;
- infradian rhythms: rhythms with τ significantly longer than 24 hours;
- circannual rhythms: rhythms with τ of approximately 1 year.

Among these, circadian clocks are the most studied and best characterized, from bacteria to humans (Dunlap, 1999). Circadian clocks control several daily vital biological processes such as sleep, cell cycles, immunity, reproduction, etc. Photoperiodic clocks (or circannual clocks) control instead seasonal aspects of lives such as migration, reproduction, hibernation, etc. The interaction between these two distinct timekeeping mechanisms and how they cooperate in measuring time on two different scales is largely unknown in insects. Nevertheless, insects are great models to study the evolution of biological clocks because of their relatively easy husbandry (low-cost maintenance, short generation time, etc.) and, at the same time, their high degree of similarity with higher systems like humans.

This thesis deals predominately with circadian clocks, but several parts are discussed in regard to the interactions between circadian and photoperiodic timers. Hereafter, an introduction of both timing systems is given.

1.3 Circadian clocks

Circadian clocks are ubiquitous time-keeping systems which are present in virtually all life forms, from bacteria to humans (Dunlap, 1999). They are generally described by three main properties:

1. they are generated by endogenous oscillators, which self-sustain under certain constant conditions;

2. they synchronize to environmental changes, by perceiving external time cues such as light and temperature;

3. they are temperature compensated, which allows them to maintain a stable intrinsic rhythm.

In the most simplified model, circadian clocks can be represented by three distinct components: 1. input, 2. clockwork, and 3. output (Figure 2). The clockwork is composed of a master oscillator plus several peripheral oscillators which generate circadian rhythms. The master oscillator receives temporal information from the environment, such as light, temperature, social cues, etc. and synchronizes with them. The rhythm generated at the level of the clockworks is transmitted to downstream outputs pathways that control behaviour, physiology, metabolism, etc.

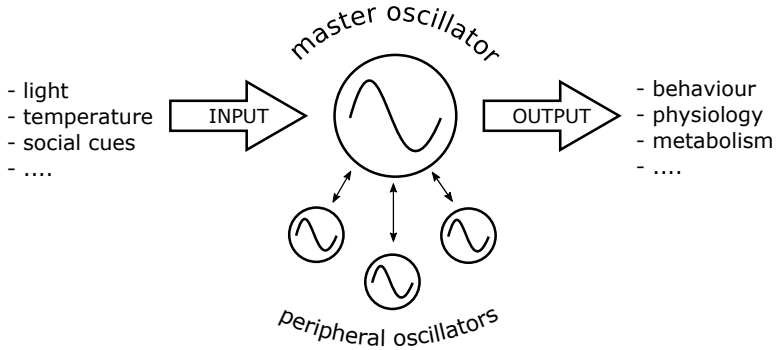

Figure 2: Schematic organization of circadian clocks. The clockwork, composed by a master oscillator and several peripheral oscillators, receives environmental information as input signals, such as light, temperature, social cues etc. The rhythm generated at the level of the clockwork is transmitted to downstream pathways that control behaviour, physiology, metabolism, etc.

In particular, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus* are the two systems where circadian clocks are best described for invertebrate and vertebrate models. Hereafter, a specific introduction of the fly and the mouse circadian clocks is given.

1.4 The circadian clock of *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae) is the species that has most contributed to the current understanding of how a circadian clock ticks in an insect model (Weiner, 1999). Pioneering experiments by Ron Konopka and

Seymour Benzer using chemical mutagenesis screenings for abnormal circadian behaviour isolated the first clock mutants (Konopka and Benzer, 1971). These mutations, which all mapped at the same locus, led to the subsequent identification and characterization of the first clock gene, *period* (*per*). After *per* discovery, other clock genes, that take part in the molecular clock mechanism in both invertebrate and vertebrate systems, have been described (Reppert, 1998; Hardin, 2005; Helfrich-Förster, 2017). This subsequent characterization of other molecular players revealed also the dynamics of the circadian clock, which relies upon interlocked autoregulatory transcriptional/translational feedback loops (TTFL), that are the basis of the cell-autonomous oscillation (Hardin et al., 1990; Glossop et al., 1999). To be mentioned, the 2017 Nobel Prize in Physiology or Medicine was awarded to Jeffrey Hall, Michael Rosbash, and Michael Young "for their discoveries of molecular mechanisms controlling the circadian rhythm" (Ledford and Callaway, 2017).

1.4.1 The fly TTFL

In the most simplified model, the *Drosophila* circadian molecular oscillator is described by two main feedback loops that are interlocked (Figure 3).

- A first feedback loop involves the clock genes *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*), *Clock* (*Clk*), and their respective products.

  CLK and CYC form heterodimers in the cytoplasm, subsequently enter the nucleus and bind to the E-box regulatory elements in the promoters of the clock genes *per* and *tim*, activating their transcription. Consequently, *per* and *tim* mRNA levels rise and are translated in the cytoplasm, where they are subjected to post-translational modification, dimerize, and after a while enter the nucleus as a complex. In the nucleus, PER/TIM complexes interfere with the transcriptional activity of CLK/CYC complexes. Doing so, they negatively regulate their own expression. Subsequent PER and TIM destabilization and degradation stop the repression on CLK/CYC activity, and a new transcriptional-translational cycle restarts.

- A second feedback loop involves the clock genes *cycle* (*cyc*), *Clock* (*Clk*), *Vrille* (*Vri*), *PAR Domain Protein 1*ε (*Pdp1*ε), and their respective products.

  Vri and *Pdp1*ε carry E-box regulatory elements in their promoters, therefore their expression is also activated by the active CLK/CYC complex. VRI accumulates earlier than PDP1ε and it represses the expression of *Clk*, acting at the

level of VP-boxes (VRI/PDP regulatory elements, Emery and Reppert, 2004) present in *Clk* promoter region. PDP1ε instead accumulates later than VRI and promotes *Clk* expression. Their synergistic activity generates circadian transcription of *Clk*, which as soon as it accumulates, it dimerizes with CYC, enters the nucleus, and a new transcriptional-translational cycle restarts.

Importantly, there are many other genes that carry E-box or VP-box regulatory sequences in their promoters and therefore these genes are subjected to rhythmic expression. These genes are called *clock controlled genes* (*ccgs*) and they are the molecular bases to transmit the self-generated rhythm to downstream output pathways.
The current model of the *D. melanogaster* TTFL is actually more complex and it involves several other clock genes which generate additional feedback loops and complexity. In addition, the equilibrium between gene expression and protein activity is finely tuned by several processes acting at both post-transcriptional and post-translational levels. Nevertheless, this additional complexity is not necessary for understanding this thesis, therefore it is here omitted. The reader is invited to refer at the following review articles for a deeper view on the *Drosophila* TTFL (Glossop et al., 1999; Hardin, 2011, Özkaya and Rosato, 2012, Hardin and Panda, 2013, Helfrich-Förster, 2017).

1.4.2 The *Drosophila* clock neural network

The molecular mechanism of the TTFL provides cell-autonomous oscillations (Figure 3), but it requires to be part of a neural network to function properly. The master clock of *D. melanogaster* is located in specialized groups of neurons in the brain. It consists of approximately 150 clock neurons, symmetrically distributed across the two hemispheres in the lateral and dorsal brain (Siwicki et al., 1988; Ewer et al., 1992; Zerr et al., 1990; Helfrich-Förster, 1995; Hermann-Luibl and Helfrich-Förster, 2015). Based on their position, their morphology, and the expression of specific components, clock neurons are generally clustered into several subgroups (Figure 4). The projections of these neurons have been described by extensive neuroanatomical studies (Helfrich-Förster, 1997; Helfrich-Förster et al., 2007b; Schubert et al., 2018), and the so-called clock network is represented in Figure 4A.
There are seven clock neuronal clusters:

1. The small-lateral-ventral clock neurons (s-LN_v) are a group of 4 cells with the somata housed in the accessory medulla (AME), that send projections to

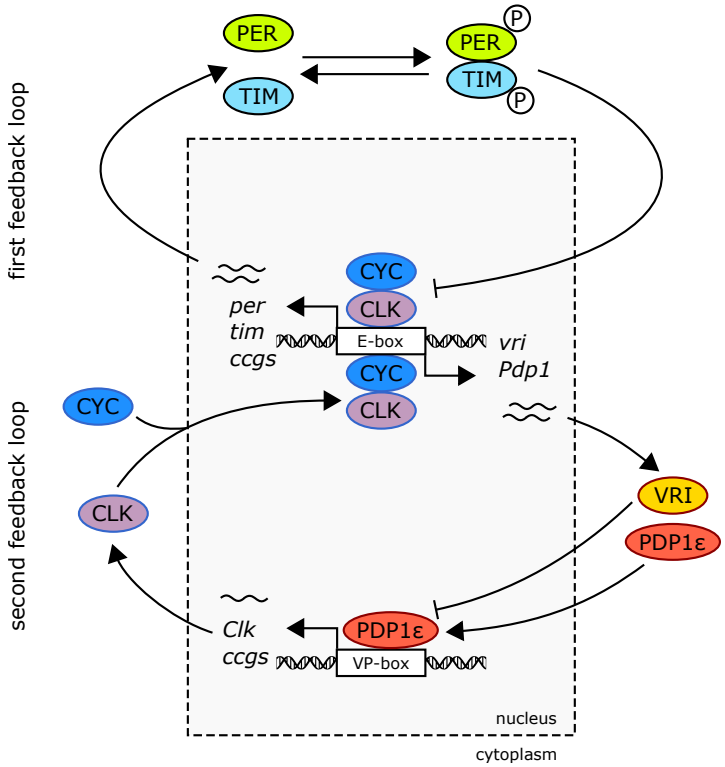

Figure 3: Schematic model of the *D. melanogaster* TTFL. The CLK/CYC complex binds to the E-box regulatory elements in the promoters of *per*, *tim*, *Vri*, and *Pdp1* initiating their transcription. PER and TIM accumulate in the cytoplasm and dimerize. As they enter the nucleus as a complex, they repress the transcriptional activity of CLK/CYC. VRI and PDP1ε act at the level of the VP-box regulatory elements in the promoter of *Clk*. VRI accumulates earlier that PDP1 and represses *Clk* expression; subsequently PDP1 accumulates and instead activates *Clk* expression. In the cytoplasm, CLK dimerizes with CYC, then enters the nucleus and binds to the E-box regulatory elements, and a new cycle restarts. These two feedback loops are interlocked and generate rhythmic expression of the so called clock controlled genes (*ccgs*). *Adapted from Emery and Reppert, 2004.*

the dorsal protocerebrum (Figure 4A). The s-LN$_v$ express CRY, PDF, PDF receptor, and sNPF (Figure 4B).

The 5th of the small-lateral-ventral clock neurons (5th s-LN$_v$) is a single cell that differentiate from the other 4 s-LN$_v$ because it does not express PDF (Figure 4B). It expresses instead CRY, PDF receptor, ITP, and NPF (Figure 4B). Its projections in *Drosophila* adults are similar to the other 4 s-LN$_v$ (Kaneko and Hall, 2000; Helfrich-Förster et al., 2007b; Schubert et al., 2018).

2. The large-lateral-ventral clock neurons (l-LN$_v$) are a group of 4 cells, which in-

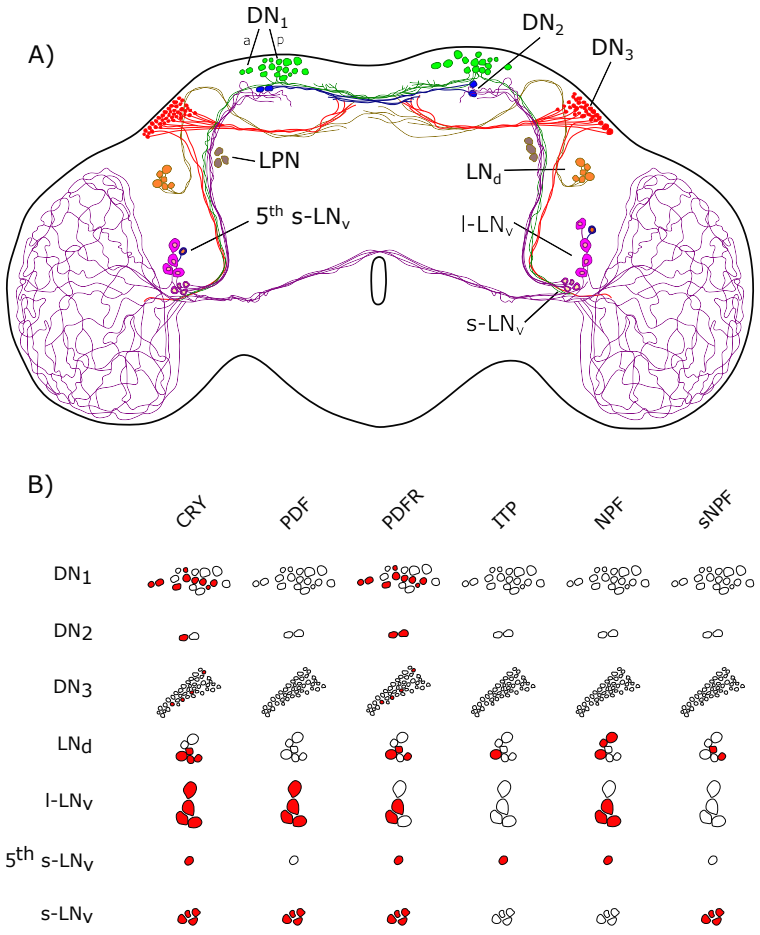

Figure 4: A) Schematic representation of the clock neuronal network in the brain of *D. melanogaster*. The clock expressing neurons are clustered depending on their morphology and their position across the protocerebrum. These are the following: DN_1 (DN_{1a} (2) and DN_{1p} (∼15), DN_2 (2), DN_3 (∼50), LPN (3), LN_d (6), l-LN_v (4), 5^{th} s-LN_v (1), and s-LN_v (4). B) Neurochemistry of the *D. melanogaster* clock neurons. CRY, PDF, PDF receptor (PDFR), ITP, NPF, and sNPF expression in single clock neurons is highlighted in red. The clusters represent the left hemisphere in Figure 4A. *Adapted from Helfrich-Förster et al., 2007a.*

nervate the surface of the distal medulla and the ipsilateral accessory medulla via the posterior optic commissure (POC)(Helfrich-Förster et al., 2007b). They differentiate from the s-LN_v because of the size of their somata and their more dorsal localization. All the l-LN_v express CRY and PDF. PDF receptor is expressed in half of them (Im and Taghert, 2010) only in very young flies (Paul Taghert, *personal communication*). NPF is expressed in 3 out of 4 of the l-LN_v (Figure 4B).

3. The lateral-dorsal clock neurons (LN_d) are an heterogeneous group of 6 cells

which innervate the dorsal protocerebrum. The expression of CRY, PDF receptor, ITP, NPF, and sNPF in the LN_d is represented in Figure 4B.

4. The lateral-posterior clock neurons (LPN) are a group of 3 cells which are involved in temperature entrainment (Kaneko and Hall, 2000; Helfrich-Förster, 2005a; Yoshii et al., 2005; Miyasako et al., 2007). Their projections are unknown so far.

5. The dorsal clock neurons 1 (DN_1) are a group of approximately 17 cells, which are further subdivided into a more anterior cluster (DN_{1a}, 2 cells) and a posterior cluster (DN_{1p}, ∼15 cells). They project to the dorsal protocerebrum and ventrally to the AME. PDF receptor and CRY are expressed in all the DN_{1a} and in half of the DN_{1p}.

6. The dorsal clock neurons 2 (DN_2) are a group of 2 cells with the somata in close proximity to the dorsal PDF^+ projections from the s-LN_v. They express the PDF receptor and one of the two cells expresses also CRY (Im and Taghert, 2010; Yoshii et al., 2008).

7. The dorsal clock neurons 3 (DN_3) are a group of approximately 50 cells which project to the dorsal protocerebrum and ventrally to the AME. CRY and PDF receptor are co-expressed in few of the DN_3 (Figure 4B). (Helfrich-Förster, 2003; Helfrich-Förster et al., 2007b).

This neural network generates rhythmic signals, that time essential brain functions. In particular, the clock network highly innervates the dorsal brain, which includes regions like the pars intercerebralis (PI), the pars lateralis (PL), the mushroom bodies (MB), and the central complex (CC) which are known to be centres of locomotion, sleep, metabolism, and other physiological processes that are under circadian control.

Importantly, clock neuronal groups are heterogeneous (Figure 4B), indicating specific functions. For example, despite being the main clock resetting factor (see page 10), cryptochrome (CRY) is not expressed in all clock neurons. CRY expression has been reported in all LN_v (s-LN_v, 5^{th} s-LN_v, and l-LN_v), in 4 LN_d, in few DN_3, in 1 DN_2, and in half of the DN_1 (Yoshii et al., 2008; Benito et al., 2008). Clock neurons that express or do not express CRY entrain differently, and it is the cooperation between the different neural clusters that allows to reset the whole circadian network (Benito et al., 2008; Yoshii et al., 2008; Yoshii et al., 2010; Lamba et al.,

2014). Engineered flies that lack CRY (cry^{01}) exhibit circadian rhythmicity under LL (Emery et al., 2000; Doleželova et al., 2007). This can be explained by the continuous light activation of CRY which leads to the constant degradation of TIM within the clock cells (Ceriani et al., 1999) In addition, it has been shown that these engineered flies exhibit low morning activity, and can delay their evening peak under long photoperiods more than wild-type flies (Rieger et al., 2003; Kistenpfennig et al., 2018).

The clock neuropeptide *pigment dispersing factor* (PDF) is expressed in all LN_v (s-LN_v and l-LN_v), except the 5^{th} s-LN_v (Helfrich-Förster, 1995; Kaneko and Hall, 2000; Figure 4B). It is also expressed in four large neurons (l-Ab) and in four small neurons (s-Ab) in the abdominal ganglion, although these cells do not express the clock proteins (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1997; Shafer and Taghert, 2009). PDF is required for the control of the output of the circadian network and for the interactions and the coordination of the different clock neural clusters in the brain (Lin et al., 2004). PDF appears to be rhythmically secreted at the dorsal projections of the s-LN_v (Park et al., 2000; Gorostiza et al., 2014). Flies lacking PDF (pdf^{01}) are unable to anticipate the lights-on transition, they exhibit advanced evening activity, and show high percentage of arrhythmicity when released in DD (Renn et al., 1999).

The receptor of PDF (PDFR, encoded by the gene *han*) is a G-protein-coupled receptor (GPCR) that is expressed in about half of the clock neurons, as well as in few neurons which are not part of the clock network (Figure 4B). It is expressed in the 4 s-LN_v, in the 5^{th} s-LN_v, in half of the l-LN_v and the LN_d, in the DN_2, in the DN_{1a}, and in half of the DN_{1p} (Figure 4B). Interestingly, all the clock cells that express PDFR also express CRY (Im and Taghert, 2010). A mutation at the *han* locus (han^{5304}) partially phenocopy the behaviour of pdf^{01} flies (Mertens et al., 2005; Lear et al., 2005; Hyun et al., 2005; Im and Taghert, 2010).

The *ion transport peptide* (ITP) is expressed in the 5^{th} s-LN_v and in 1 LN_d (Figure 4B). It is additionally expressed in some other neurosecretory cells in the *Drosophila* protocerebrum, called *ipc* (ITP-immunoreactive protocerebrum neurons), which are not clock neurons. Also ITP appears to be released rhythmically in the dorsal protocerebrum (Dircksen et al., 2008; Johard et al., 2009; Hermann-Luibl et al., 2014).

The *neuropeptide F* (NPF) and the *short-neuropeptide F* (sNPF) are two neuropeptides expressed in the clock network. Specifically, NPF is expressed in the 5^{th} s-LN_v, in some l-LN_v, and in half of the LN_d (Figure 4B). sNPF is expressed in the s-LN_v

and in 2 LN_d (Figure 4B). The function of these two neuropeptides in the clock network is not completely clear yet, but they are thought to take part in the output repertoire of the clock and in the communication between neural subgroups (Lee et al., 2006; Johard et al., 2009; Hermann et al., 2012).

1.4.3 Entrainment of the *Drosophila* clock

The circadian clock synchronizes to daily cycles by perceiving external temporal cues, such as light, temperature, social interactions, etc. (Figure 2). These inputs are called *Zeitgebers* (from the German, *timegivers*). In chronobiology, the synchronization of the clock to Zeitgebers is termed *entrainment*.

Light entrainment

D. melanogaster expresses the blue light sensitive photoreceptor cryptochrome (CRY) in about half of the clock neurons (Figure 4B). CRY transmits light information to the clock in a cell-autonomous way. Upon exposure to light, CRY goes through conformational changes and becomes active. The active form of CRY binds to TIM, and competes for the binding of TIM by PER. Consequently, TIM is ubiquitinated by the protein Jetlag (JET) and directed to proteosomal degradation. At the level of the TTFL, PER without TIM cannot enter the nucleus until the onset of darkness, therefore its repression on CLK/CYC transcriptional activity is delayed. In addition, the *cry* gene carries E-box regulatory elements in its promoter, consequently it is rhythmically expressed (Stanewsky et al., 1998; Emery et al., 1998; Emery et al., 2000; Ozturk et al., 2011).

Although CRY expression within the clock neurons is the main responsible factor for the clock resetting, *Drosophila* circadian clock receives light information from multiple pathways. The fly photoreceptor organs, such as the compound eye, the ocelli, and the H-B eyelets, synergistically contribute to the light input (Helfrich-Förster et al., 2001; Rieger et al., 2003). Such organs are known to form connections, direct or indirect, to the clock PDF^+ neurons (Helfrich-Förster et al., 2002; Helfrich-Förster, 2002; Schlichting et al., 2016). Only flies that lack all photoreceptors are not entrainable by light (Hofbauer and Buchner, 1989; Helfrich-Förster et al., 2001).

Temperature entrainment

As well as to light-dark cycles, the fly circadian clock is able to synchronize to temperature cycles (thermophase-cryophase, TC cycles). Nevertheless, the molecular mechanism upon which temperature information is translated into the clock

network is not completely understood. It was shown that temperature cycles can entrain and shift the *Drosophila* clock also at very low amplitudes (Wheeler et al., 1993; Glaser and Stanewsky, 2005). Interestingly, temperature cycles can entrain the *Drosophila* clock also under constant illumination (Wheeler et al., 1993; Yoshii et al., 2005). Temperature information is perceived by pheripheral thermosensors, which are present for example in the antennae and in the chordotonal organs of the fly (Sayeed and Benzer, 1996; Sehadová et al., 2009). As for LD cycles, it was shown that clock neurons respond differently also to TC cycles. In general, it seems that mainly the CRY^- clock neurons (in particular LPN and DN_2) are responsible for temperature entrainment.

1.4.4 *Drosophila* behavioural rhythm

The rest-activity rhythm of *D. melanogaster* is generally assessed by monitoring fly locomotor activity (Rosato and Kyriacou, 2006; Pfeiffenberger et al., 2010).
Under artificial cycles of 12 hours of light followed by 12 hours of dark (LD 12:12) at constant temperature (usually 20°C or 25°C), *Drosophila* exhibits strong and reliable locomotor daily rhythm. This daily activity is characterized by two main components:

1. morning activity (M-peak), which anticipates the lights-on transition and it is associated to dawn;

2. evening activity (E-peak), which anticipates the lights-off transition and it is associated to dusk.

These two activity components are separated by a period of inactivity, in the middle of the light phase, named "*siesta*".
Thanks to the powerful genetic tools available in *D. melanogaster*, it is now well know that these two peaks of activity are under the control of different subsets of clock neurons (Figure 5). Specifically, the M-peak is modulated by the PDF^+ s-LN_v, and the CRY^+ DN_1p (Grima et al., 2004; Stoleru et al., 2004; Zhang et al., 2010). The E-peak is modulated by the 5^{th} s-LN_v and 3-4 LN_d (Picot et al., 2007; Stoleru et al., 2007; Zhang et al., 2010). These subsets of cells are distinct oscillators, which in some cases do not run in parallel. The proposed dual oscillator model is further explained in a next section (see page 19).
It was shown that M- and E-oscillators respectively track dawn and dusk when photoperiod is increased or decreased simulating seasonal changes (Majercak et al., 1999; Yoshii et al., 2004; Rieger et al., 2012; Stoleru et al., 2007). In other words,

seasonal changes in photoperiod modulates the phase angle ($\Psi_{M,E}$) of the two oscillators. This has been shown to happen because of the acceleration of the M-cells and the deceleration of the E-cells. Importantly, there is a limit above which the two oscillators of *Drosophila* cannot move further apart, which is reached under extremely long photoperiods (i.e LD 20:4) (Rieger et al., 2012). This highlights that the two oscillators are coupled together and that they are not only two independent entities (Helfrich-Förster, 2017).

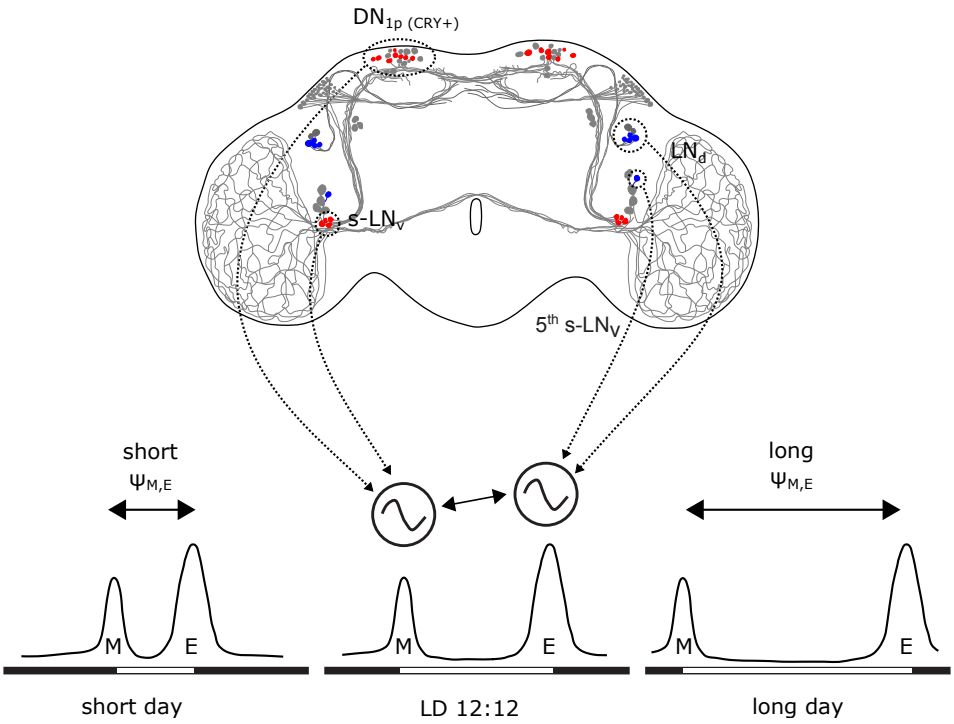

Figure 5: Simplified representation of the morning (M) and evening (E) components in *D. melanogaster* bimodal activity. The two activity bouts are controlled by separate neuronal clusters: M-cells (PDF^+ s-LN_v and the CRY^+ DN_1p) control the M-peak; E-cells (5^{th} s-LN_v and 3-4 LN_d) control the E-peak. Under short or long days, the two activity components track dawn and dusk, decreasing or increasing the phase angle ($\Psi_{M,E}$) of the two oscillators.

When *D. melanogaster* is exposed to constant darkness (DD), the circadian rhythm in locomotor activity is self-sustained and "free-runs" with a defined period (τ) that is a readout of the pace of the TTFL. The free-running rhythm of *D. melanogaster* under DD can be recorded up to many weeks. The PDF^+ s-LN_v are required for the function of the circadian clock under constant darkness (Shafer and Taghert, 2009; Menegazzi et al., 2017), as they were shown to display autonomous and sustained molecular oscillation in DD (Veleri et al., 2003; Roberts et al., 2015).

When *D. melanogaster* is instead exposed to constant light (LL), the rhythm in

locomotor activity is lost. From a pure molecular point of view, this can be explained by the continuous light activation of CRY that leads to the constant degradation of TIM within the clock cells; consequently the fly TTFL without TIM cannot self-sustain (Ceriani et al., 1999; Peschel et al., 2009). To confirm this, engineered flies that lack CRY (cry^{01}) exhibit circadian rhythmicity under LL (Doleželova et al., 2007). Interestingly, flies show circadian rhythms under LL also when the circadian components *per*, *tim*, or *Shaggy* (*Sgg*) are overexpressed within the clock neurons (Stoleru et al., 2007; Murad et al., 2007).

These studies also indicate that the M-cells are responsible for driving circadian rhythm under DD (Stoleru et al., 2007; Murad et al., 2007; Picot et al., 2007), whereas the E-cells drive circadian rhythm under LL in cry^{0} background (Stoleru et al., 2007; Murad et al., 2007; Picot et al., 2007).

1.5 The circadian clock of *Mus musculus*

After the discovery of the *Drosophila* clock gene *period*, researchers have looked for its counterpart in higher systems, for example in mice (*M. musculus*) and humans. It was found that there is a high degree of conservation between the fly and the mouse clock, regarding the mechanism upon which the clock runs as well as for the clock genes involved (Yu and Hardin, 2006). Nevertheless, phylogenetics suggest that circadian clocks probably evolved multiple times, because clocks of different organisms are composed of different genes setups, and because different clocks seem to exist within the same organism (Brown et al., 2012). Compared to the *Drosophila* clock, the mammalian circadian system went through several events of gene loss and duplication.

The mouse TTFL

As for *Drosophila*, the mouse circadian molecular oscillator can be simplified in two main feedback loops that are interlocked.

- A first feedback loop involves the clock genes *Period* (*Per1, Per2*), *Cryptochrome* (*Cry1, Cry2*), *Brain and Muscle ARNT-like 1* (*Bmal1*) and *Clock* (*Clk*), and their respective products.

  CLK and BMAL1 form heterodimers in the cytoplasm, subsequently enter the nucleus, and bind to the E-box regulatory elements in the promoters of the clock genes *Per* and *Cry*, activating their transcription. Consequently, *Per* and *Cry* mRNA levels rise and are translated in the cytoplasm, where they

dimerize and enter the nucleus as a complex. In the nucleus, PERs/CRYs complexes interfere with the transcriptional activity of CLK/BMAL1 complexes. Doing so, they negatively regulate their own expression. Subsequent PERs and CRYs degradation stops the repression on CLK/BMAL1 activity and a new transcriptional-translational cycle restarts.

- A second feedback loop involves the clock genes *Bmal1*, *Clk*, *retinoid-related orphan receptor* (Rora) and *Rev-erb*α, and their respective products.

  Rora and *Rev-erb*α carry E-box regulatory elements in their promoters, therefore their expression is also activated by the active CLK/BMAL1 complex. REV-ERBα and RORa control the circadian transcription of *Bmal1*, which as soon as it accumulates it dimerizes with CLK, enters the nucleus, and a new transcriptional-translational cycle restarts.

As for *D. melanogaster*, the rhythm generated by the TTFL is transmitted to downstream pathways thanks to transcriptional control over the so-called clock controlled genes (*ccgs*). The reader is invited to refer to the following publications for a deeper understanding of the mouse TTFL system (Dunlap, 1999; Emery and Reppert, 2004; Sancar, 2004; Partch et al., 2014).

In contrast to the fly model, vertebrate CRYs are transcriptional repressors and they lack photoreceptive properties (Kume et al., 1999; Sancar, 2004). These CRYs belongs to the type 2 family (both mammalian *Cry1* and *Cry2* are type-2 CRY, or CRY2), on the contrary *Drosophila* CRY belongs to the type 1 family (CRY1) (Cashmore, 2003; Yuan et al., 2007). In this case, nomenclature of CRYs could be confusing; in this work I refer to CRY1 for the *Drosophila* type and to CRY2 for the vertebrate type. BMAL1 is the mammalian ortholog of the gene *cyc* in *Drosophila* (Rutila et al., 1998; Hogenesch et al., 1998). The second feedback loop of *M. musculus* involves the clock proteins *Rora* and *Rev-erb*α , which are not derived from PDP1ε and VRI, but are functional homologous (Preitner et al., 2002; Sato et al., 2004).

In mice and humans, the master circadian clock is located in the *Suprachiasmatic Nucleus* (SCN) of the hypothalamus. The SCN is composed by approximately 10000 to 50000 neurons (depending on the species) that receive light information through the retinohypothalamic tract and through the photopigment melanopsin (Lowrey and Takahashi, 2011).

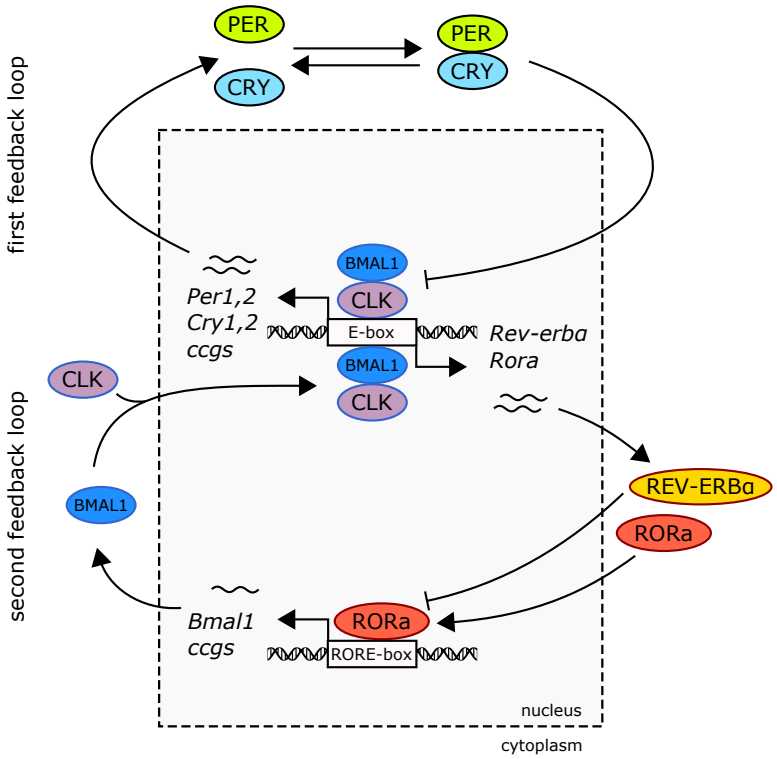

Figure 6: Schematic model of the *M. musculus* TTFL. The CLK/BMAL1 complex binds to the E-box regulatory elements in the promoters of *Per*, *Cry*, *Rev-erbα*, and *Rora* initiating their transcription. PER and CRY accumulate in the cytoplasm and dimerize. As they enter the nucleus as a complex, they repress the transcriptional activity of CLK/BMAL1. *REV-ERBα* and RORa act at the level of the RORE-box regulatory elements in the promoter of *Bmal1* regulating its expression. In the cytoplasm, BMAL1 dimerizes with CLK, then enter the nucleus and bind to the E-box regulatory elements, and a new cycle restarts. These two feedback loops are interlocked, and generate rhythmic expression of the so called clock controlled genes (*ccgs*). *Adapted from Emery and Reppert, 2004.*

1.6 The circadian clock in "non-model" insect species

The fruit fly *Drosophila melanogaster* is a great model organism because of the availability of a vaste number of genetic tools and overexpression/knockout systems (Brand and Perrimon, 1993). Nonetheless, insects are the most diverse group of organisms on earth (Mayhew, 2007) and the choice of *D. melanogaster* as "the insect model" for chronobiology primarily relies on its leading role in 20th century genetics. Several studies have investigated circadian clocks in other insect species and revealed that the general organization of the clockwork in other orders might diverge drastically. These new findings are challenging the *Drosophila*-centric view of how circadian clocks work for an insect model (Tomioka and Matsumoto, 2015;

Lam and Chiu, 2017).

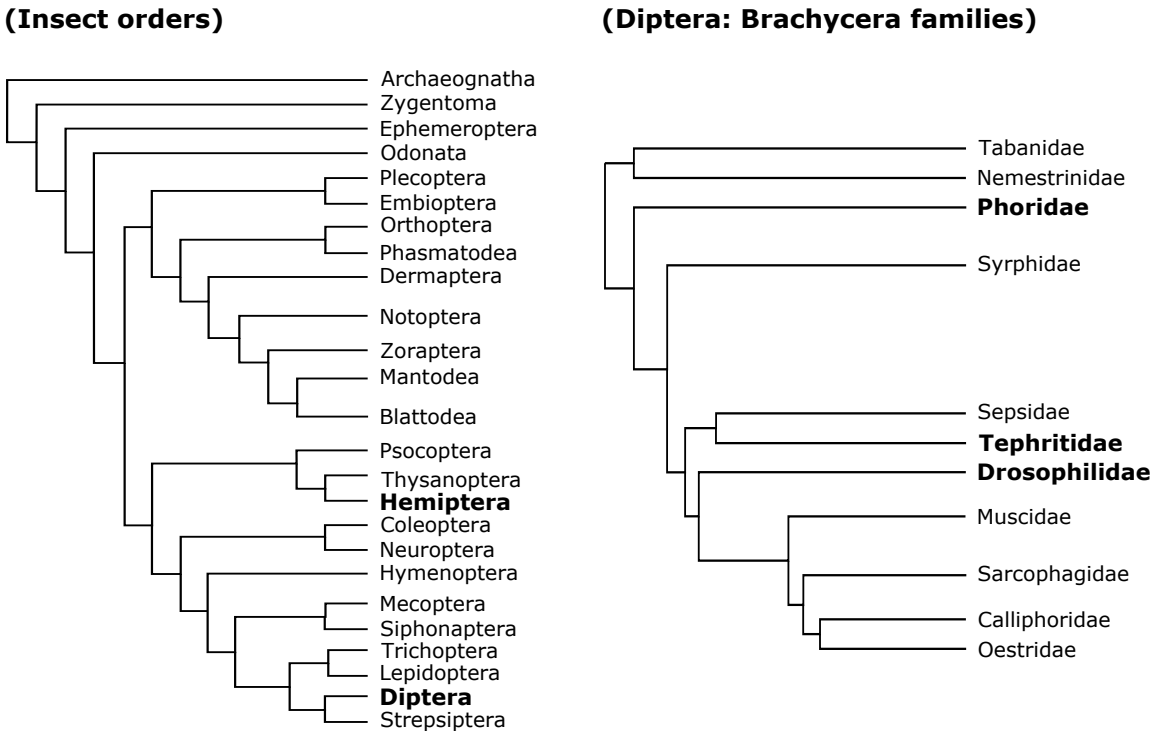

Figure 7: Insect orders and families of the Diptera Brachycera suborder. Highlighted are taxa considered in this work. *Adapted from Mayhew, 2007 and Pu et al., 2017.*

The diverse composition of the TTFL of insects

Currently, the molecular clock of non-drosophilids species is thought to be composed of a mixture between a fly (*D. melanogaster*) and a mammal (*M. musculus*) TTFL (Rubin et al., 2006; Chang et al., 2003; Tomioka and Matsumoto, 2015; Lam and Chiu, 2017). Based on partial genomic annotations and functional studies, three main differences in the organization of the clock system among insects can be summarized.

- The first main difference found between *D. melanogaster* (or drosophilids as a family) and other non-Drosophilidae species is the presence and the role of cryptochromes. Two *Cry* families have been described in insects: 1. CRY1 (*Drosophila*-like); 2. CRY2 (vertebrate-like). CRY1 is photosensitive and functions primarily in the photic entrainment of the *Drosophila* master clock (Stanewsky et al., 1998, Emery et al., 1998). CRY2 seems to lack photoreceptive properties and works instead as a potent transcriptional repressor (Zhu

et al., 2005). CRY1 and CRY2 are both ancient proteins and it was proposed that the ancestral model of the clock carried both cryptochrome orthologues. Subsequent radiations in insects have lost either one of the two forms (Yuan et al., 2007).

- A second main difference is the presence of the *timeless* gene. Two *tim* families have been described in insects: 1. *tim1* (involved in the clock); 2. *tim2* (also called *timeout*, not involved in the clock). TIM2 seems to be the ancestral form, as it is highly conserved among all deutorestomes (Benna et al., 2000). *Tim1* probably emerged from *tim2* duplication. It acts as partner of PER in the *Drosophila* TTFL, but it has been lost in some evolutionary lineages (i.e. Hymenoptera, Rubin et al., 2006).

- A third main difference is the role and the expression of *Clk* and *cyc* (*Bmal1* in mammals). In particular, this involves the presence of the transactivation domain (BCTR domain, termed after Chang et al., 2003), either on CLK or on CYC (BMAL1). *D. melanogaster* carries the transactivation domain on CLK (multiple poly-Q sites at the C-terminus), and the level of CLK protein is known to oscillate in antiphase with PER and TIM (Lee et al., 1998), whereas its partner CYC is constitutively expressed (Bae et al., 2000). On the opposite, insects that carry the transactivation domain on CYC (BMAL1-type) show oscillation of CYC as the main positive regulator in the TTFL and constitutive *Clk* expression (Meireles-Filho et al., 2006; Chahad-Ehlers et al., 2017).

Based on these points, Yuan et al., 2007 proposed that the clock must have evolved at least four types of mechanisms in insects (Figure 8).

1. Diptera (if only Drosophilidae are considered) carry a *Drosophila*-like clockwork (Figure 3). CLK and CYC are the positive regulators, whereas PER acts as the main negative regulator in the loop together with TIM. The main light input to the clock goes through CRY1-TIM pathway (Peschel et al., 2009; see section 1.4).

2. Lepidoptera carry a clock with all the *Drosophila* component (PER, TIM, CLK, CYC, CRY1) plus the vertebrate CRY2. CLK and CYC (BMAL1-type) are the positive regulators, whereas CRY2 seems to have overtaken the role as main transcriptional repressor from PER (Chang et al., 2003; Yuan et al., 2007; Zhu et al., 2008). The light input to the clock might work through CRY1-TIM pathway or opsins (Reppert, 2006).

3. Hymenoptera have lost the *Drosophila* TIM and CRY1, but they carry the vertebrate CRY2 (Rubin et al., 2006; Ingram et al., 2012). CLK and CYC (BMAL1-type) are the positive regulators whereas CRY2 seems to be the main transcriptional repressor (not PER). The light input to the clock might work through vertebrate-like opsins.

4. Coleoptera might not carry the *Drosophila* CRY1 but they carry the vertebrate CRY2. CLK and CYC (BMAL1-type) are the positive regulators whereas CRY2 seems to be the main transcriptional repressor. The light input to the clock might work on CRY independent TIM degradation (Yuan et al., 2007).

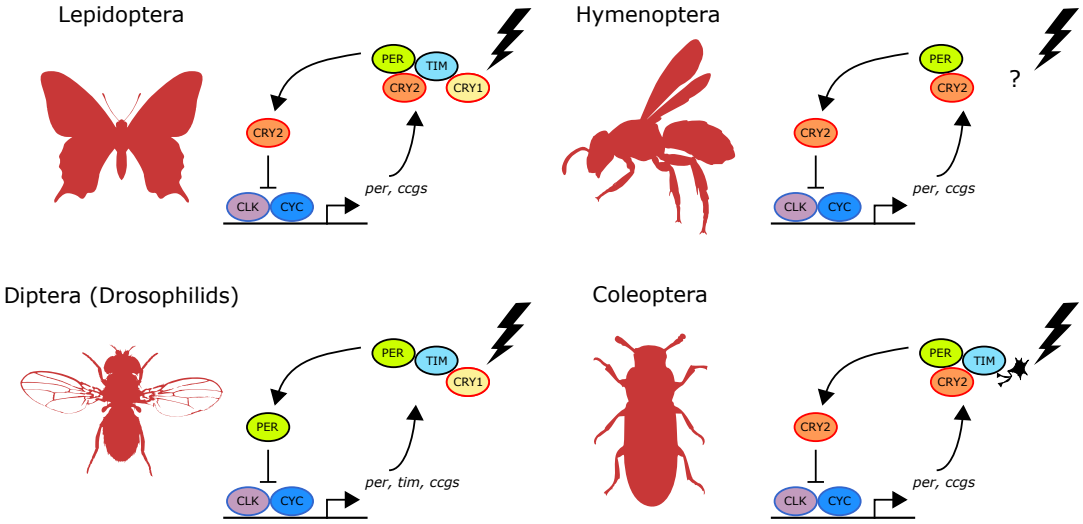

Figure 8: Schematic representation of the TTFL models in different insect orders proposed by Yuan et al., 2007. *Adapted from Yuan et al., 2007.*

1.7 The clock network of drosophilids from high latitudes

Although the TTFL of drosophilids (Diptera: Brachycera) rely most likely on the same TTFL mechanism (Figure 3 and Figure 8), recent works have highlighted striking differences in the organization of the clock at the neuronal level.

For example, the fruit fly *Drosophila virilis* expresses PDF only in the l-LN_v and not in the s-LN_v (Bahn et al., 2009), differently than *D. melanogaster* which expresses PDF in both clusters (Figure 4). *D. virilis* shows reduced morning anticipatory behaviour and weak rhythms under DD, phenocopying the behaviour of *D. melanogaster* where the expression of PDF is manipulated in the s-LN_v (Renn et al., 1999; Grima et al., 2004; Stoleru et al., 2004). A following study expanded this view on several other drosophilids (*D. simulans*, *D. yakuba*, *D. ananassae*, *D. triauraria*,

D. pseudoobscura, *D. willistoni*, *D. virilis*, *D. ezoana*, *D. littoralis*) and showed that this difference in PDF expression in the LN_v is pervasive (Hermann et al., 2013). In addition, a variation in the expression of the circadian photoreceptor CRY within the LN_v was also reported. In contrast to *D. melanogaster* (Figure 4), *D. ezoana*, *D. littoralis*, *D. virilis*, and *D. pseudoobscura* completely lack CRY expression within the l-LN_v (Hermann et al., 2013). Most interestingly, species like *D. ezoana* and *D. littoralis* were collected at high latitude (Northern Finland) and therefore these species are naturally exposed to more extreme photoperiodic conditions than the equatorial *D. melanogaster*. Such difference in the expression of PDF and CRY in the LN_v was correleted with the ability of flies to adapt to long photoperiods (Menegazzi et al., 2017). Under long photoperiods, flies from high latitudes (*D. ezoana* and *D. littoralis*) differ from flies from low latitudes (*D. melanogaster*) in their activity pattern. Equatorial species show a sharp morning activity peak and an evident "siesta" in the middle of the day followed by the evening peak. It was shown that these flies are limited in extending their evening activity under very long photoperiods (Rieger et al., 2012; Prabhakaran and Sheeba, 2012; Menegazzi et al., 2017). High latitude species like *D. ezoana* and *D. littoralis* show instead low morning activity and a broad evening activity. Contrary to *D. melanogaster*, these flies are able to delay their evening peak to track dusk also under very long days (Menegazzi et al., 2017). When exposed to constant darkness (DD), *D. melanogaster* exhibit a self-sustained circadian rhythm, whereas it becomes arrythmic under constant light (LL). *D. ezoana* and *D. littoralis* show instead arrhythmicity under DD, and slightly increased rhythmicity under LL (Menegazzi et al., 2017). The same behaviour was also described for another Holarctic species like *D. montana* (Kauranen et al., 2012; Kauranen et al., 2016).

1.8 The dual oscillator model

Several organisms exhibit two components in their daily activity rhythms, one associated with dawn and the other with dusk (Aschoff and Wever, 1962). In 1976, Colin Pittendrigh and Serge Daan proposed the *dual oscillator model* to explain the "splitting" phenomenon observed in the circadian rhythms of rodents and other animals (Pittendrigh and Daan, 1976). This model proposed that two oscillators, characterized by different properties, separately control the two activity components. Also *D. melanogaster* locomotor activity shows two components, a morning activity bout (M-peak) and an evening activity bout (E-peak). These two components free-run independently under certain conditions (Yoshii et al., 2004; Rieger et al.,

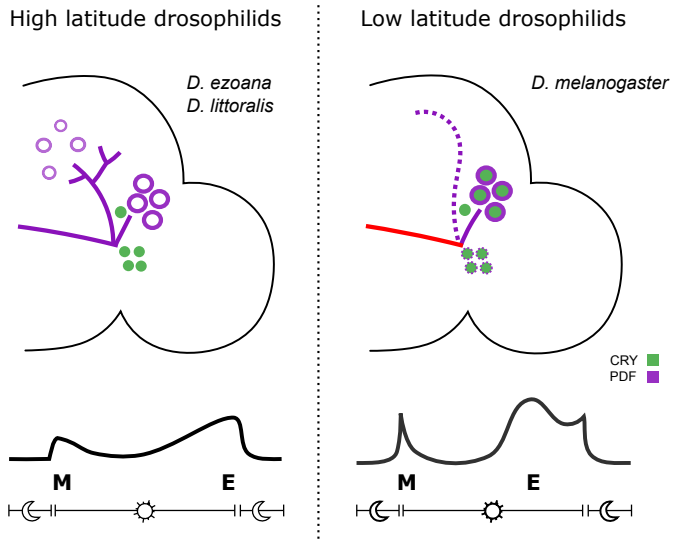

Figure 9: Schematic representation of the model proposed by Menegazzi et al., 2017. *Adapted from Menegazzi et al., 2017.*

2006; Doleželova et al., 2007), suggesting that they are controlled indeed by two distinct but mutually coupled functional units (Helfrich-Förster, 2000; Yoshii et al., 2012). The powerful genetic mosaic tools available in *D. melanogaster* have allowed to unravel the anatomical basis of such M and E oscillators. The *Drosophila* M oscillator is housed in the PDF^+ s-LN_v and in the CRY^+ DN1p (M-cells, Grima et al., 2004; Stoleru et al., 2004; Zhang et al., 2010). The *Drosophila* E oscillator is housed in the the 5^{th} s-LN_v and the CRY^+ LN_d (E-cells, Picot et al., 2007; Stoleru et al., 2007; Zhang et al., 2010).

Importantly, the proposed dual-oscillator model predicts that light accelerates the speed of the M oscillator and decelerates that of the E oscillator (Pittendrigh and Daan, 1976). Indeed, the *Drosophila* M-cells were shown to speed up by light, whereas the E cells are slowed down by light (Yoshii et al., 2004; Rieger et al., 2006). This allows the two activity bouts to track dawn and dusk under increasing or decreasing photoperiods. Most interestingly, a PDF increase in the dorsal brain splits the free-running activity rhythm into two components, as it does under LL in cry^0 background, suggesting that the two oscillators respond differently to PDF release (Helfrich-Förster et al., 2000; Wülbeck et al., 2008). Therefore, PDF might be responsible for speeding up the M cells and slowing down the E cells in response to seasonal variation in photoperiod.

Morning and evening circadian oscillators were also described within the mammalian SCN, where two distinct peaks in electrical activity are generated by different cell populations that change activity in response to photoperiod (Jagota et al., 2000).

Although the concept of only two distinct functional units is most likely oversimplified also for *D. melanogaster* (Dissel et al., 2014), it is clear that the master clock consists of multiple independent oscillators which compose a flexible network, and it is thought that the M and E oscillators of *Drosophila* may be crucial in photoperiodism.

1.9 Photoperiodic clocks

Photoperiod is the dominant environmental signal used to predict seasonality. Compared to other environmental signals (i.e. thermoperiod) photoperiodic changes are clear and noise-free markers of local time. It is now known that a photoperiodic timer is used by animals and plants to track and predict the advent of seasonal adversities. Photoperiodism is generally assessed by a photoperiodic response curve (PPRC), which relates the incidence of the photoperiodic response (i.e. diapause) with the length of the day/night. The critical photoperiod (CPP) is the photoperiod inducing 50% diapause incidence, and it was shown to be genetically based and to vary geographically (Lankinen, 1986; Bradshaw and Holzapfel, 2007; Mathias et al., 2007; Paolucci et al., 2013). Nevertheless, CPP itself is not sufficient to induce the photoperiodic switch, but it also requires a photoperiodic counter. Therefore, compared to the circadian clock system (Figure 2), the simplest model describing a photoperiodic clock requires instead four elements: 1. input; 2. photoperiodic timer; 3. photoperiodic counter; 4. output.
Already in 1936, Erwin Bünning proposed that the circadian clock is involved in the photoperiodic time measurement (Bünning, 1936). After that, several studies questioned or supported this view, and several theoretical models have been proposed (Vaz Nunes and Saunders, 1999; Tauber and Kyriacou, 2001; Saunders, 2014). The two main theoretical models which imply the involvement of the circadian clock in the photoperiodic timing system are the following:

1. the external coincidence model proposes that photoperiodic induction occurs when a defined photoinducible phase of the circadian clock (φi) coincides with darkness (or light). In this model, light has the dual role of entraining the circadian clock and of inducing the response.

2. the internal coincidence model proposes that photoperiodic induction occurs because of a defined internal phase relationship between two (or more) oscillators. In this model, light has the only role of entraining the oscillators.

The dual-oscillator characterized in the brain of *D. melanogaster* might support adaptations to seasonal changes in photoperiod according to the dual-oscillator model (Pittendrigh and Daan, 1976; Yoshii et al., 2012).

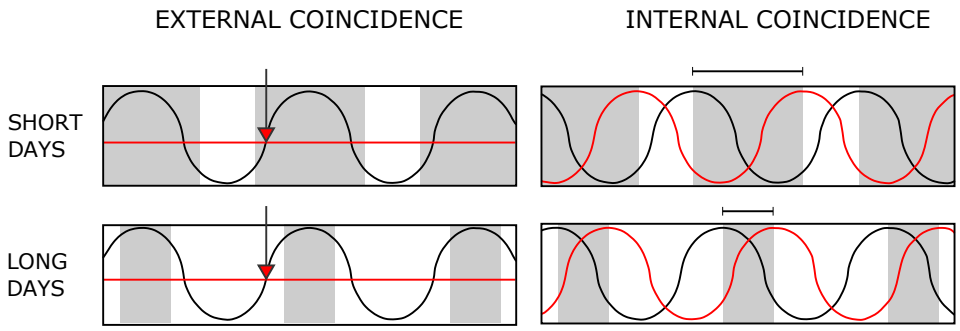

Figure 10: Simplified representation of the external and internal coincidence models, which propose the involvement of the circadian clock in the photoperiodic time measurement *Adapted from Davis, 2002.*

1.10 Insect diapause

As poikilothermic animals, insect are highly vulnerable to seasonal changes. Diapause is the developmental, physiological, and behavioural state which animals undergo to overcome seasonal harsh conditions and enhance survival (Denlinger, 2002; Hahn and Denlinger, 2011). During this stage metabolic and physiologic activities are reduced and mating behaviour is suppressed in order to store energy. Compared to quiescence, which is another kind of dormancy, diapause is a programmed response and not a mere reaction to a direct stimulus. Indeed, diapause is an anticipatory response that must be induced prior to the upcoming unfavourable environmental change.

There are several different types of diapause described for insects. Generally, diapause occurs only in a single defined stage during the life cycle (Doležel, 2015). For example, diapause can be elicited at the larval stage (larval diapause) or at the adult stage (adult reproductive diapause). Larval diapause often occurs at the final instar and is characterized by arrest in larval development. Adult reproductive diapause is characterized by arrest in reproduction, which results in marked reduction of ovarian development. To be induced, diapause requires environmental information. Both temperature and photoperiod contain information on the yearly course of the seasons. In nature, temperature is the principal clue that allows or excludes development or reproduction (Saunders, 2014). On the other side, changes in photoperiod are more regular than temperature throughout the year, and a pho-

toperiodic timer is long time thought to be involved in diapause induction (Bünning, 1936; Adkisson, 1966). Whether the circadian clock is involved in the induction of photoperiodic diapause in insects is highly controversial (Bradshaw and Holzapfel, 2010; Saunders, 2010). This is partly due to the fact that the common insect model used in laboratory, the fruit fly *D. melanogaster*, is not a suited model to study insect photoperiodism because of its mild photoperiodic diapause (Emerson et al., 2009). Photoperiodic diapause was described for *D. melanogaster* (Saunders et al., 1989), but its photoperiodic component has been widely questioned in the past years (Emerson et al., 2009; Bradshaw and Holzapfel, 2010; Koštál, 2011). Viceversa, other insect species that exhibit strong photoperiodic diapause lack the genetic tools necessary to discern the molecular cogs of the photoperiodic timer (Doležel, 2015). Therefore, new insect models need to be established in order to investigate the possible interactions between circadian and photoperiodic timing.

1.11 Insect species presented in this work

1.11.1 *Chymomyza costata*

The malt fly *Chymomyza costata* (Zetterstedt)(Diptera: Drosophilidae) is an Holarctic drosophilid which occurs in Central and Western Europe, Northern Canada, Iceland, Northern Japan (Okada, 1956; Lankinen and Riihimaa, 1992), and probably in the Swiss Alps (Dr. Shu Kondo, *personal communication*). In the wild, adults of *C. costata* are collected in summer in lumber-yards forests, often where logs are piled up. In contrast to most other drosophilids that inhabits Northern regions and overwinter as adults, *C. costata* overwinters as larvae (Lankinen and Riihimaa, 1997). In winter, the larva undergoes a physiological state called diapause, thanks to which it becomes extremely resistant to unfavourable conditions, like for example to low temperatures in winters (Enomoto, 1981a). As an example, diapausing larvae of *C. costata* have been shown to survive submergence in liquid nitrogen (−196°C) (Moon et al., 1996; Koštál et al., 2011).

Photoperiod and temperature work synergistically to determine the developmental fate in *C. costata* (Enomoto, 1981a). The third larval instar is the stage during which the environmental information is translated by the animal in favour of two alternative ways: 1. the progress in development, which leads to metamorphosis and the reproductive adult stage; 2. the arrest of development into the quiescent diapausing state. *Chymomyza* larval diapause is induced by short photoperiods and low temperatures (Enomoto, 1981a; Koštál et al., 2000). The critical photoperiod

(CPP) of *C. costata* has been determined for several strains collected at different latitudes, and it exhibits latitudinal variation (Riihimaa and Kimura, 1989; Yoshida and Kimura, 1995; Koštál et al., 2000). Initially, the involvement of the circadian clock in the photoperiodic response of *C. costata* has been investigated by using classical protocols, like the T-cycles, the night-interruption, and the Nanda-Hamner experiments (Vaz Nunes and Saunders, 1999). These approaches highlighted that *Chymomyza* enters diapause in response to dark-phases longer than 9 hours, and it was proposed that a "weak" circadian clock contributes to photoperiodic time measurement (Yoshida and Kimura, 1995). Afterwards, the isolation of a non-photoperiodic diapause strain (*NP* or *npd*) allowed to investigate the genetic bases of such a response (Riihimaa and Kimura, 1988). The *npd* mutant carries a deletion in the 5'-UTR of the clock gene *timeless* (Stehlík et al., 2008). This mutation causes indeed impaired eclosion rhythm (Lankinen and Riihimaa, 1997) and loss of mRNA cycling of several clock genes (Koštál and Shimada, 2001; Kobelková et al., 2010), suggesting the importance of the *timeless* locus in both circadian and photoperiodic time measurement.

Early studies have measured circadian rhythms of *C. costata* eclosion. Under light-dark or temperature cycles, most flies emerge at the beginning of the day whereas emergence is repressed at night, and this emergence pattern is modulated by the photoperiod (Lankinen and Riihimaa, 1992; Lankinen and Riihimaa, 1997). Under constant darkness (DD) or constant light (LL) the eclosion rhythm of *C. costata* is very weak or absent (Lankinen and Riihimaa, 1992). Only when temperature cycles are applied, a weak free-running rhythm of eclosion was observed under DD (Lankinen and Riihimaa, 1997). Nevertheless, such rhythm was always damped relatively quickly, and taken together with previous studies (Yoshida and Kimura, 1995) authors suggested that the circadian oscillator of *C. costata* may lose its oscillation after the first 24 hours cycle (Lankinen and Riihimaa, 1997). The loss of rhythm under DD was shown to happen also to the oscillation of *per* mRNA in fly adult heads (Koštál and Shimada, 2001). Diel activity of *C. costata* adults is reported only in old studies (Nuorteva and Hackman, 1970).

1.11.2 *Bactrocera oleae*

The olive fly *Bactrocera oleae* (Gmelin)(Diptera: Tephritidae), classified in the past as *Dacus oleae*, is a major pest of cultivated olives. *B. oleae* presence has been historically reported in Mediterranean and African countries and, more recently, it has spread to Central America and California (Daane and Johnson, 2010). The

adult female flies lay eggs through the skin of olive fruits, where the larva feeds. Tunnelling in the fruit mesocarp lead to crop damage and premature drop. Multiple and overlapping generations every year cause enormous loss to olive agriculture and the economy of olive products (Tzanakakis, 2003).
Current control methods against *B. oleae* rely overwhelmingly on the use of chemical insecticides (Vontas et al., 2001; Vontas et al., 2002). On the other side, modern approaches, unified under the concept of *Integrated Pest Management* (IPM, or *Integrated Pest Control* (IPC)), are aiming at avoiding the over-use of chemicals in modern agriculture. Among these, the *Sterile Insect Technique* (SIT, also known as Nuclear Sterile Insect Technique) has been historically proposed as a valid option (Knipling, 1955).

Sterile Insect Technique (SIT) and *OX3097D-Bol*

The SIT is a targeted method of pest control. It is based on laboratory breeding of very large numbers (10s of millions) of sterilized insects (traditionally by irradiation) which are subsequently released into the wild. The introduction of an excess of sterile insects reduces the reproductive potential of a target population through infertile matings, leading to suppression (Alphey, 2007; Hendrichs, 2000). Repeated SIT applications allow to keep insect pests under control in an eco-friendly and species-specific manner (Dyck et al., 2005).

The olive fly *B. oleae* was among the first insect species to be considered for SIT. However, SIT against *B. oleae* has achieved very limited success, due to the poor quality of the irradiated insects and mating asynchrony between the laboratory-reared and wild olive flies (Economopoulos, 1972; Economopoulos et al., 1976; Zervas and Economopoulos, 1982; Economopoulos and Zervas, 1982). In particular, it was reported that γ-ray irradiated males differed from wild females in their time of mating, with laboratory-reared flies mating several hours earlier than wild flies (Economopoulos and Zervas, 1982; Zervas and Economopoulos, 1982). This is, not surprisingly, a considerable obstacle to the application of SIT against *B. oleae*.

An alternative to irradiation to sterilize flies for SIT is the use of genetic engineering (Morrison et al., 2010; Black et al., 2011). The English biotechnology company *Oxitec ltd* (www.oxitec.com) uses molecular techniques to generate stable lines that carry a self-limiting trait (i.e. dominant lethal transgene) under the control of a genetic switch. This method (in the past reported as RIDL®, Release of Insects with Dominant Lethality) is in use to control several key insect pest species, such as the diamondback moth *Plutella xylostella*, the pink ballworm *Pectinophora gossyp-*

iella, the medfly *Ceratitis capitata*, the mexfly *Anastrepha ludens*, the yellow fever mosquito *Aedes aegypti*, and the Asian tiger mosquito *Aedes albopictus*. *OX3097D-Bol* is the current Oxitec's self-limiting olive fly technology. This genotype incorporates a tetracycline-off system (Tet-Off) designed to produce a conditional female-specific self-limiting trait when reared in the absence of a sufficient concentration of tetracycline (Ant et al., 2012).

The success of SIT, whether traditional or using novel technologies, is largely driven by the mating behaviour of the species of concern. The mass-released males must exhibit the same mating behaviour as wild males, or they will be unsuccessful in gaining females (Shelly et al., 1994). In most Bactrocera species mating occurs at a species-specific time window during the day, most commonly associated with dusk and less frequently midday (Fletcher, 1987). In *Bactrocera* species this is known to be modulated by the circadian clock (Tychsen and Fletcher, 1971; Smith, 1979), as it is in *Drosophila* (Sakai and Kitamoto, 2006). Circadian rhythms in *B. oleae* were previously described for several behaviours, for example mating (Loher and Zervas, 1979), exodus of larvae from diet (Tsitsipis and Loher, 1989), and pheromone emission (Levi-Zada et al., 2012). Yet, very little is known about the mechanisms of the underlying circadian clock.

1.11.3 *Pyrrhocoris apterus*

The linden bug or firebug *Pyrrhocoris apterus* (Linnaeus)(Hemiptera: Pyrrhocoridae) has been historically used as a model insect in physiology (Slama and Williams, 1965; Socha and Hodková, 1993). These bugs inhabit temperate regions, and were collected in Europe up to 59°N (Pivarciova et al., 2016). Adult reproductive diapause is adopted by *P. apterus* to overwinter and it is known to be induced by temperature and photoperiodic cues (Hodek, 1971a; Numata et al., 1993; Socha and Hodková, 1993). Diapausing bugs (males and females) show decreased behavioural activity and arrest in reproduction (Socha and Hodková, 1993; Urbanová et al., 2016). The sensitive stage at which photoperiodic information is translated into developmental fate spans from the third nymph instar to adulthood (Hodek, 1971b; Hodková, 2015). In artificial laboratory conditions, *P. apterus* is reproductive when exposed to long photoperiods whereas it undergoes diapause under short photoperiods (Saunders, 1987; Numata et al., 1993). The decision between diapause or reproduction has been shown to involve the juvenile hormone (JH) released from the *corpora allata* glands (Hodková, 1976; Hodková et al., 2001), and the clock genes *Clock*, *cycle* (*Bmal1*-type), *Pdp1*, and *cry2* (Bajgar et al., 2013; Urbanová et al.,

2016). The mechanism upon which the photoperiodic information is processed in favour of the two remains elusive, neverthless it is thought that the circadian clock plays a role in it (Doležel, 2015).

P. apterus offers also a convenient model to study circadian rhythms. Under artificial laboratory conditions, adult linden bugs show robust circadian rhythm in locomotor activity (Hodková et al., 2003). This was reported for an extensive collection of strains (Pivarciova et al., 2016), as well as for nymphs (Kotwica-Rolinska et al., 2017). Both adults and nymphs can adjust their behaviour in response to long or short photoperiods, and adults show robust self-sustained rhythm under constant darkness (DD, Pivarciova et al., 2016; Kotwica-Rolinska et al., 2017). At the molecular level, *per* and *cyc* (*Bmal1*-type) mRNA levels oscillate under short and long days in nymph heads (Kotwica-Rolinska et al., 2017). In adults, oscillation of clock genes at the mRNA level has not been observed yet, but difference in the level of expression is found between bugs raised under short or long photoperiod (Hodková et al., 2003; Syrová et al., 2003; Doležel et al., 2008; Bajgar et al., 2013). The possibility to perform RNAi in *P. apterus* allows to test whether putative clock components have a function in both circadian and photoperiodic mechanism. It has been shown that knocking down *cyc* and *Clk* in nymphs induces adult diapause phenotype (Kotwica-Rolinska et al., 2017). Nonetheless, since these core clock genes are transcriptional factors, it is challenging to exclude clock from non-clock functions (Doležel et al., 2005; Emerson et al., 2009).

1.11.4 *Megaselia scalaris*

The scuttle fly or coffin fly *Megaselia scalaris* (Loew)(Diptera: Phoridae) is named for the "short, rapid bursts of running motion characteristic of adults" (Varney and Noor, 2010). It is a species which inhabit predominantly warm regions, but it has been also reported in Northern regions of America, Europe, and Asia. Larvae of this species are known to feed on an incredible variety of organic material. Feeding on carrions, including human carcasses, this species is of particular importance in forensic entomology (Disney, 2008). *M. scalaris* has been adopted as a laboratory model to study the evolution of sex determination in flies (Traut, 1994). Circadian rhythms of *M. scalaris* locomotor activity, oviposition, and eclosion have been recently published (Bostock et al., 2017), nevertheless nothing is known about the underlying molecular and neuronal system in this family (Phoridae).

1.11.5 *Megaselia abdita*

The scuttle fly or hump-backed fly *Megaselia abdita* (Schmitz)(Diptera: Phoridae) is a cosmopolitan species (Rafiqi et al., 2011e). As for its sibling species *M. scalaris* (see section 1.11.4), also *M. abdita* shows largely subterranean lifestyle and it is involved in the colonization of dead corpses (Manlove and Disney, 2008). *M. abdita* has been adopted as a laboratory model for studying developmental biology in flies (Rafiqi et al., 2011a; Rafiqi et al., 2011b; Rafiqi et al., 2011c; Rafiqi et al., 2011d; Rafiqi et al., 2011e; Wotton et al., 2014).

2 Aims of this study

This thesis aims at expanding the view on circadian clocks toward new insect models. Applying behavioural, molecular, anatomical, and evolutionary approaches I have characterized the circadian biology in the following insect species and for the following reasons:

- *Pyrrhocoris apterus* and *Chymomyza costata* are emerging models in chronobiology because of their robust photoperiodic diapause. The characterization of their circadian clock is a primary goal in order to test whether the circadian clock is involved in their photoperiodic response.

- *Bactrocera oleae* is the single most important pest for the majority of olive plantations. The characterization of its circadian clock is the basis to understand its daily behaviour and might become fundamental for new pest management approaches.

- *Megaselia scalaris* and *Megaselia abdita* belong to an evolutionary lineage where the circadian clock is poorly described (Diptera: Phoridae). These flies offer new models to understand how the clock neuronal network controls circadian behaviour.

3 Materials and methods

3.1 Insects strains and husbandry

The insect species reported in this thesis have been reared and maintained in the laboratory. The origin of the strains used and the rearing protocols are described hereafter.

Chymomyza costata

Two isofemale lines of *C. costata* have been used in this work:

- *Sapporo*: this strain was originally collected in Sapporo (Japan, 43°N, Figure 11) in 1983 (Riihimaa and Kimura, 1988). I received this line from Vladimír Koštál (Biology Centre CAS, České Budějovice, CZ) in 2014. Because of an unfortunate crossing of this wild-type strain with the mutant strain *npd* in 2012 (*non-photoperiodic-diapause*, Riihimaa and Kimura, 1988; Koštál and Shimada, 2001), its genetic background has been back-crossed several times to get rid of the mutant *tim* allele (Vladimír Koštál, *personal communication*). I verified the presence of the wild-type *tim* allele in this population performing PCR amplification of the *timeless* locus using the following primers (F: AATAGACAAAATGTCGCTGGCTGG; R: TTCCAACTGAAATTGTGCTAGCGG, data not shown), and inducing larval diapause at short photoperiods.

- *Oulu*: this strain was established from multiple females collections in Oulu (Finland, 65°N, Figure 11) in August 2012 by Dr. Ari Riihimaa (University of Oulu, Finland). We have obtained these flies from Dr. Kondo Shu (National Institute of Genetics, Shizuoka, Japan) in 2016.

Adult *C. costata* were kept at 18°C under LD 12:12 on standard *Drosophila* medium (see recipe at page 33). Because of the tendency of flies to stick on the food, autoclaved playground sand was sprinkled on the surface of the food. Newly hatched adult flies were weekly transferred into new vials. Vials with eggs were moved at 18°C or 20°C under long photoperiod (LD 18:6) or constant illumination (LL), in order to prevent the induction of larval diapause (Enomoto, 1981a; Koštál et al., 2000). Because of *Chymomyza* relatively long life cycle (∼30 days from eggs to adults at 20°C) and the tendency of our diet to dry, water was sprayed weekly into the vials. Wood wool was inserted onto the food to prevent larvae to pupate inside the diet.

Bactrocera oleae

Three strains of *B. oleae* are reported in this work:

- *Demokritos*: this is a wild-type strain originally collected in Demokritos (Greece, 37°N, Figure 11) and maintained in laboratory from the ´60s (Tzanakakis et al., 1966).

- *Argov*: this is a wild-type strain collected in Israel (Biofly, 32°N, Figure 11) in 2007 (Argov et al., 2011).

- *OX3097D-Bol*: this is a self-limiting strain which incorporates a tetracycline-off system (TetOFF) designed to produce a conditional female-specific self-limiting trait when reared in the absence of a sufficient concentration of tetracycline (Ant et al., 2012). It also expresses a fluorescent marker gene to enable detection and monitoring in operational deployment.

Adult flies were kept under LD 12:12 at 23°C or 25°C in plastic cages and fed on diet consisting of: - 100 g yeast autolysate; - 400 g icing sugar; - 30 g egg yolk powder from chicken. A ceresin wax cone was placed inside the cage in order to collect eggs, which were rescued from the cone using distilled water containing 0.05% propionic acid. The recovered eggs were pipetted onto dishes containing larval diet. This diet consists of: - 550 ml distilled water; - 30 g soy hydrolysate; - 0.5 g potassium sorbate; - 2 g nipagin; - 20 g sugar; - 75 g brewer's yeast; - 30 ml HCl; - 275 g cellulose powder; - 20 ml olive oil; - 7.5 ml Tween-80.
Dishes were closed with the plastic lid, to retain humidity, and placed at 25°C inside a box. Once larvae started to emerge from diet (around 7 days after seeding), the lid was removed and larvae dropped onto the sand (autoclaved playground sand) placed on the floor of the box. Pupae were collected by sieving and moved into new plastic cages with a wax cone, where they emerged and a new cycle of eggs collection was restarted.

The transgenic strain *OX3097D-Bol* had to be raised in the presence of tetracycline (on-tet). Only for this genotype the solution to rinse the eggs from the cone was added with 100 µg/mL tetracycline.

Pyrrhocoris apterus

Two strains of *P. apterus* are reported in this work:

- *Oldřichovec*: this strain was collected in Oldřichovec (Czech Republic, 49°N, Figure 11), and it is part of the extensive collection done by the group of David Doležel (Pivarciova et al., 2016). These bugs were used to perform immunocytochemistry (ICC).

- *Lyon-red*: this strain was collected in Lyon (France, 45°N, Figure 11) in 1992 (Socha and Hodková, 1993). These bugs were used for the RNAi injections and the subsequent recording of locomotor activity.

Bugs were maintained under long photoperiod (LD 18:6) at 25°C in glass or plastic jars, where water was available *ab libitum* and food supplied as linden seeds. Rolled pieces of paper were introduced into the jars to promote social and mating behaviour. Eggs were collected by sieving and moved into new jars where larvae grew until becoming reproductive.

Megaselia scalaris

The isofemale strain of *M. scalaris* reported in this thesis was generated by a single female fly collection done by Dr. Christa Kistenpfennig in summer 2010 in Würzburg (49°N, Figure 11). The identification of the species was confirmed by Henry Disney (Department of Zoology, University of Cambridge, UK). This strain was maintained throughout on standard *Drosophila* food at 25°C under LD 12:12.

Megaselia abdita

The isofemale strain of *M. abdita* was collceted in Freiburg (47°N, Figure 11) in 1991 (Rafiqi et al., 2011e). We received these flies from Steffen Lemke (Centre for organismal studies, University of Heidelberg, Germany) in 2017. These flies were maintained throughout on standard *Drosophila* food or on fish food (Rafiqi et al., 2011a) at 25°C under LD 16:8.

Drosophila melanogaster

The strains of *D. melanogaster* were maintained under LD 12:12 at 18°C or 25°C on standard *Drosophila* medium prepared in our laboratory. This consists of : - 8.0% malt extract; - 8.0% corn flour; - 2.2% sugar beet molasses; - 1.8% yeast; - 1.0% soy flour; - 0.8% agar; - 0.3% Hydroxybenzoic acid; - tap water.
The origins of these strains are the following: - *Canton-S* (Lindsley and Grell, 1968); - w^{1118} (Lindsley and Grell, 1968); - per^{01} (Konopka and Benzer, 1971); - tim^{01} (Sehgal et al., 1992; Myers et al., 1995); - Clk^{Jrk} (Allada et al., 1998); - $pdp1^{3135}$

(Zheng et al., 2009); - cry^{01} (Doleželova et al., 2007); - pdf^{01} (Renn et al., 1999): - han^{5304} (Hyun et al., 2005).

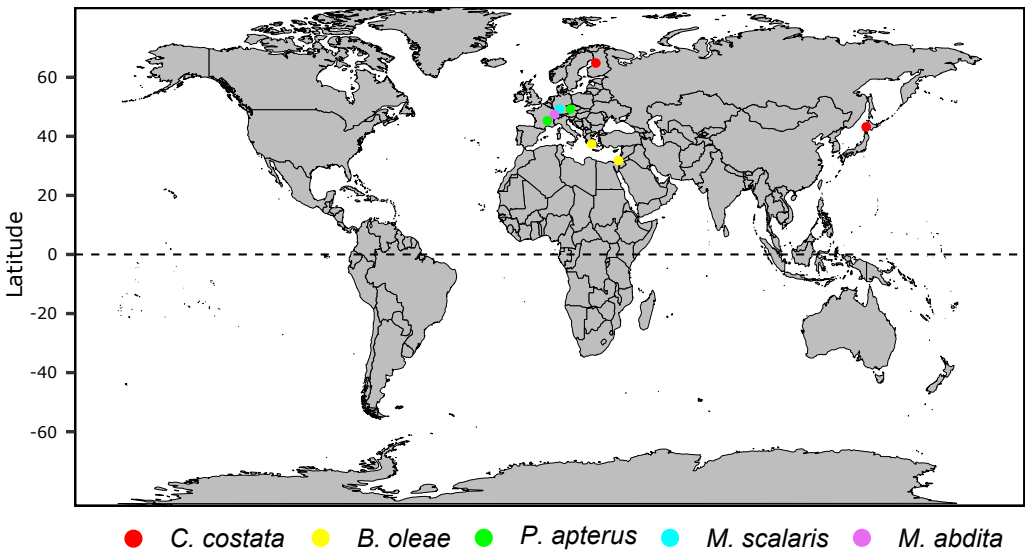

Figure 11: Geographical sites of collection of the insect strains used in this work. *C. costata* (*Sapporo*, Japan (43°N, 141°E); *Oulu*, Finland (65°N, 25°E)); *B. oleae* (*Demokritos*, Greece (37°N, 23°E); *Argov*, Israel (32°N, 35°E)); *P. apterus* (*Oldřichovec*, Czech Republic (49°N, 14°E); *Lyon*, France (45°N, 4°E)); *M. scalaris* (*Würzburg*, Germany (49°N, 9°E)); *M. abdita* (*Freiburg*, Germany (47°N, 7°E)).

3.2 Locomotor activity recording and analysis

I recorded locomotor activity of insects using custom monitoring system (*Drosophila* Activity Monitoring, DAM) by Trikinetics (Trikinetics, Waltham MA, USA). This apparatus counts the times that a single individual, placed into a glass tube in the presence of diet on one side, interrupts an infra-red laser beam which crosses the middle of the tube (Pfeiffenberger et al., 2010). Crossing beam data were recorded and saved from the DAM software with the resolution of 1 minute. Generally, 32 individuals were loaded into one single monitor. The diameter of the tubes used for these kind of experiment depends on the size of the insect species of concern. Too small diameters do not allow the comfortable movement and the turning of animals, too big diameters might miscount the passage of the animal across the laser beam detection range. In this work, the tubes used to record locomotor activity of the different species are the following: - 5 mm diameter tubes were used for *D. melanogaster*, *M. scalaris*, and *M. abdita*; - 7 mm diameter tubes were used for *C.*

costata; - 10 mm diameter tubes were used for *B. oleae*; - 15 mm diameter tubes were used for *P. apterus*. Food was provided as 2% sucrose and 4% agarose for flies, or linden seeds and cotton soaked in water for bugs.

Locomotor activity data representation and analysis

Activity data generated by the DAM system have been visualized and analysed using the ImageJ pluging actogramJ (Schmid et al., 2011). In this thesis, double plotted actograms, which are a standard way to visualize circadian activity, were generated using a self-made script in R (R Core Team, 2013) and *ggplot2* (Wickham, 2009). Activity was summed into 15 minutes bins and represented as vertical black bar on a double-plotted 48 hours scale. Activity that exceeded 15 crosses/15 minutes was normalized to the maximum. The first day of the actograms is not double-plotted.
The activity profile under cycles of light and darkness (LD) was calculated on the last days of the entrainment phase, normally the last three of seven. The activity profile of single flies was averaged among the population from the same experiment. Plots were normalized to the daily maximum activity.
The timing of the activity bouts (morning and evening peaks) was calculated manually for single flies and averaged among flies from the same experiment.
Free-running rhythm under constant condition (DD or LL) was firstly inspected by eye looking at the actograms, and afterwards rhythms were analysed using different tools. General principles of rhythm detection algorithms for biological data are described and compared in Refinetti et al., 2007. The χ^2 periodogram analysis (Enright, 1965) was performed using the ImageJ pluging actogramJ (Schmid et al., 2011). The Lomb-Scargle analysis (Lomb, 1976; Scargle, 1982) was performed either using actogramJ or using a self-made script in R[1]. The CLEAN analysis (Roberts et al., 1987; Rosato and Kyriacou, 2006) was performed using BeFLY v5.3 by Ed Green[2].
In order to blindly assess rhythmicity in locomotor activity of *C. costata* under constant darkness (DD), I combined the use of Lomb-Scargle and CLEAN. Analysing the outcomes of the two procedures, I considered rhythmic only flies where both periodograms gave significant peaks that are not more than 30 minutes far apart. I excluded from this analysis flies which showed period outside the circadian range ($20<\tau<28$). This procedure helps to get rid of significant peaks generated most likely by artefacts in the analysis. I report the estimated period detected by both

[1]thanks to Bulah Chia-hsiang Wu for help.
[2]thanks to Giorgio Fedele for help.

Lomb-Scargle and CLEAN periodogram, and the average power of the rhythms as an indication of the rhythm strength.
The percentage of rhythmic flies (% of significant periodograms) was calculated as the number of rhythmic flies divided by the total number of flies that survived for at least 10 days under constant condition.

Light and temperature conditions

Due to the different size of the monitors used to record locomotor activity for the different species, experiments were carried out into either environmental chambers or incubators. Large monitors (cm30x20x5), built to record locomotor activity with 10 mm or 15 mm diameters tubes (therefore used for *B. oleae* and *P. apterus*), were placed into incubators (Panasonic, MIR-154-PE) where temperature was kept constant and light cycles were produced using the neon tubes furnished by the manufacturer. The light intensity produced from these tubes was in the range of 200-500 lux, depending on the distance from the light source. The light emission spectra of this apparatus (white fluorescent light incubator) is shown in Figure 12. Small monitors (cm13x6x5), built to record locomotor activity with 5 mm or 7 mm diameters tubes (therefore used for *C. costata*, *M. scalaris*, *M. abdita*, *D. melanogaster*), were placed into light boxes manufactured by our workshop. Light was supplied as ∼100 lux and the emission spectra of this apparatus (multi-color LED light box) is shown in Figure 12. Small oscillation in temperature between lights-on and lights-off, often unavoidable because of the active electric circuit, were all within the range of ± 1°C (Figure 12). Temperature cycles experiments were carried out into incubators (MIR-154-PE (Panasonic) or Intellus environmental controller (Percival)) and the oscillation was monitored using temperature logger (MSR Data loggers). When light and temperature cycles were co-applied, light was provided by the multi-color LED light box (Figure 12).

3.3 Immunocytochemistry (ICC)

To investigate spatial and temporal expression of clock proteins in the central nervous system of insects, I performed immunocytochemistry (ICC) on whole mounts or agarose sections. General principles and techniques of immunofluorescence and immunocytochemistry are described in Helfrich-Förster, 2007 and Selcho and Wegener, 2015. Primary and secondary antibodies used in this work are reported in Table 1. Primary antibodies were diluted at the reported concentration (Table 1, the concentration varies depending on the species of concern) in PBST (Phosphate

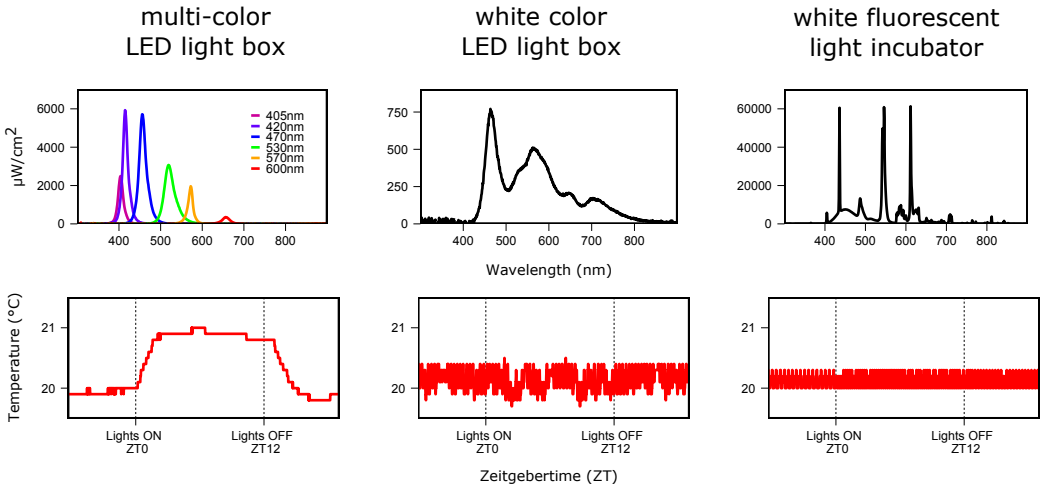

Figure 12: Light boxes used to produce artificial light-dark cycles in this work. Top: Emission spectra of the three light sources used, measured with an *Ocean Optics QE65000* spectrometer. The apparatuses are the following: - multi-color LED light box (∼100 lux: 0.05 Watt/m^2 405 nm; 0.1 Watt/m^2 420 nm; 0.12 Watt/m^2 470 nm; 0.11 Watt/m^2 530 nm; 0.03 Watt/m^2 570 nm; 0.01 Watt/m^2 600 nm); - white color LED light box (∼100 lux); - white fluorescent light incubator (∼200-1000 lux). Bottom: Temperature oscillation within the boxes generated by the LD cycle.

Buffered Saline (PBS) added with 0.3% Triton-X100, Sigma-Aldrich), with 5% NGS (Normal Goat Serum, Invitrogen) and 0.002% NaN_3. The primary antibodies working solution was rescued and reused several times to perform different experiments. Secondary antibodies were used at the concentration of 1:400, in PBST with 5% NGS. Fluorophores with distant emission spectra were chosen, if possible, in order to avoid bleeding-through (i.e. Alexa Fluor 488 and Alexa Fluor 635).

ICC standard protocol

To perform ICC on insects nervous systems, I generally followed the following standard protocol:

1. flies are sampled by fixation in PFA (paraformaldehyde) 5% in PBST (generally at selected timepoints);
2. fixative is removed by several washes of PBS (at least 4 x 10 minutes);
3. brains are dissected manually under stereoscopic microscope and kept in PBS at 4°C;
4. tissues are blocked with NGS 5% in PBST, for 3 hours at ambient temperature;

PRIMARY ANTIBODIES

antibody	immunogen	donor animal	standard dilution	source
anti-PDF C7	PDF *D. melanogaster*	mouse monoclonal	1:500 - 1:5000	DHSB, J. Blau
anti-PDF cricket	PDF *G. bimaculatus*	rabbit polyclonal	1:1500	Abdelsalam et al., 2008
anti-PDH	β-PDH *U. pugilator*	rabbit polyclonal	1:2000	Dircksen et al., 1987
anti-ITP	ITP *D. melanogaster*	rabbit polyclonal	1:1000 - 1:10000	Ring et al., 1998
anti-NPF	NPF *D. melanogaster*	rabbit polyclonal	1:300	Shen and Cai, 2001
anti-sNPF	sNPF *D. melanogaster*	rabbit polyclonal	1:2000 - 1:4000	Johard et al., 2009
anti-CRY	CRY *D. melanogaster*	rabbit polyclonal	1:1000	Yoshii et al., 2008
anti-PDP1	PDP1 *D. melanogaster*	rabbit polyclonal	1:1000	Reddy et al., 2000
anti-SYN 3C11	SYNORF1 *D. melanogaster*	mouse monoclonal	1:10 - 1:50	DSHB, Klagges et al., 1996

SECONDARY ANTIBODIES

fluorophore conjugated	immunogen	donor animal	standard dilution	source
Alexa Fluor 488 Alexa Fluor 555 Alexa Fluor 635	rabbit IgG	goat polyclonal	1:400	ThermoFisher, Invitrogen
Alexa Fluor 488 Alexa Fluor 555 Alexa Fluor 635	mouse IgG	goat polyclonal	1:400	ThermoFisher, Invitrogen

Table 1: Primary and secondary antibodies used in this work to perform ICC on insects nervous system. Primary antibodies were diluted in PBST, with 5% NGS and 0.002% NaN_3. Secondary antibodies were used in PBST with 5% NGS.

5. brains are incubated in primary antibodies working solution;

6. tissues are washed 6 times with PBS/PBST (3 x 10 minutes PBS and 3 x 10 minutes PBST);

7. brains are incubated in secondary antibodies working solution (O.N. at 4°C);

8. brains are washed 6 times with PBST/PBS (3 x 10 minutes PBST and 3 x 10 minutes PBS).

The time of fixation in PFA highly depends on the size of the species and the permeability of the tissue of concern. This can be very variable and it represents a key step in the ICC protocol. The fixation time for the different species, optimized at ambient temperature, is: - *C. costata* 2.5 hours; - *B. oleae* 4 hours; - *P. apterus* 4 hours (the head cuticle must be punctured with a scalpel); - *M. scalaris* 4 hours; - *M. abdita* 3 hours. Good results were also obtained fixing samples overnight at 4°C.
The time of incubation in primary antibodies solution depends on the antibodies specificity to each single species. Generally, overnight incubation at 4°C was a good compromise, but longer incubation helps.
After brains are dissected they are delicately moved into small collection baskets and all the steps afterwards are carried out in 96 well plates. At the end of the staining procedure brains are delicately moved onto SuperFrost glass slides where PBS is removed and tissues are mounted in Vectashield (Vector Laboratories). Preparations were sealed using transparent nail polish and stored at 4°C and preferably scanned in the few days after.

For the double labelling that used antibodies anti-PDP1 (Reddy et al., 2000) and anti-PDF cricket (Abdelsalam et al., 2008) together (Figure 24), which are both raised in rabbit, the full staining procedure was performed twice. The first anti-PDP1 staining (labelled with anti-rabbit Alexa Fluor 488) was fixed a second time and the protocol was repeated using anti-PDF cricket antibody (labelled with anti-rabbit Alexa Fluor 635).

Agarose sections of *P. apterus* brains

In order to increase tissues permeability to antibodies in performing ICC on *P. apterus* brain, agarose sections were made. Heads were punctured with holes in the cuticle using a scalpel, and fixed in PFA for 4 hours at ambient temperature. Subsequently, PFA was removed by several washes of PBS and heads were embedded in 7% agarose (low EEO (electroendosmosis), Sigma-Aldrich). Sections of 80-90 µm were cut using a vibratome (Leica VT1000S). The brain slices were delicately transferred into collection baskets, and the ICC protocol was performed as described at page 37.

Time-course analysis of protein levels performing ICC

In this work, ICC was used to investigate changes in protein amount over a temporal window, generally referred as timecourse. Flies were entrained under LD cycles (white colour LED boxes, Figure 12) and sampled in PFA at selected timepoints. At least 10 brains for timepoint are generally required to obtain a solid dataset. In order to achieve the best outcome from this kind of experiments, flies were collected from different light-boxes, with shifted time regimes, at the same time of the day and all the steps of the ICC protocol (see page 37) were carried out in parallel.

Microscopy and image analysis

Preparations were scanned using confocal microscopy (*Leica TCS SPE* or *Leica TCS SP8* (Leica, Wetzlar, Germany)) using 20x objective (ACS APO 20.0x0.60 IMM). The acquisition parameters were set and tested to avoid bleeding-through and saturation of the signal and they were maintained constant throughout. When the experiment required the quantification and the subsequent comparison of the staining intensity among timepoints (timecourse), the scanning was performed preferably within a single or consecutive days, in order to maintain the hardware configuration as much constant as possible. Images files (.lif) were visualized and analyzed in Fiji (Schindelin et al., 2012). To produce the figures shown in this thesis, images were cropped, maximum projections were compiled, brightness and contrast were adjusted, but no other manipulations on the images have been applied.
When the staining level was quantified and compared among conditions, the analysis was performed on the original image file without applying any sort of manipulation. The level of the signal was measured within manually selected ROIs (for PDP1 the nuclei of clock neurons) as the "mean gray value" of the area. This value was subtracted with the background intensity (measured in the same way and averaged among three ROIs in background regions), and averaged first between hemispheres, and subsequently between samples.

3.4 RNAi in *P. apterus*

In order to perform RNAi knock-down of *Pdp1* in *P. apterus*, *Pdp1* transcript fragments (318 bp) were amplified by PCR (F: AAAAAGATGAAGCAGAGC; R: CAGCAGGTGAAGGTGGTG) and cloned into plasmids under the control of a T7 promoter. The dsRNA was synthesized using the MEGAscript® RNAi Kit (Ambion). 2 µL of dsRNA (4 µg/µL) were injected in the hemolymph of adult bugs

from the dorsal side and the following day bugs were placed into locomotor activity tubes for recording. As a control, bugs were either not injected or injected with dsRNA against the bacterial *lacZ* gene (Kotwica-Rolinska et al., 2017).

3.5 Phylogenetic analysis

The predicted protein sequences of *B. oleae* PER, CYC, and CRY were aligned using ClustalOmega (Sievers et al., 2011) with the corresponding reference sequences obtained from GenBank from other insect taxa (Benson et al., 2017). Alignments were filtered using Gblocks (Castresana, 2000) and only homologous parts present in at least 50% of the sequences were kept. The resulted clean alignment was then used for phylogenetic reconstruction using RAxML with 100 bootstraps and the BLOSUM62 substituition matrix (Stamatakis et al., 2008). Phylogenetic trees were produced using FigTree v1.8.4 by Andrew Rambaut.

3.6 Statistic analysis

Statistical analysis was performed using R (R Core Team, 2013). Data descriptives were generated using the package *psych* (Revelle, 2017). Samples were tested for normality distribution applying the Shapiro-Wilk test. Homogeneity of variance was tested applying Levene´s test (*car* package, Fox and Weisberg, 2011). When the assumptions of normal distribution and homogeneity of variance were respected, I applied ANOVA or 2-way ANOVA in order to compare means. When significant difference was revealed, pairwise comparisons between groups were performed adopting Bonferroni corrections for multiple testing. When only the assumption of normal distribution was respected, differences in means were tested with Welch's t-test. When both assumptions were not respected, I applied the non-parametric Mann-Whitney-Wilcoxon and Kruskal–Wallis tests by ranks. In order to test circadian oscillation in protein levels, I additionally applied harmonic regression using the software CircWave (Dr. Roelof Hut, Oster et al., 2006).

4 Results

4.1 The circadian clock of *Chymomyza costata*

The malt fly *C. costata* is a promising model organism for studying the interactions between circadian and photoperiodic timers in insects, primarily because of its robust photoperiodic larval diapause. Nevertheless, the mechanism and the localization of the circadian clock in *Chymomyza* adults have not been described yet.

4.1.1 The clock network of *C. costata*

In order to unravel the neural organization of the master clock network in *C. costata* adults, I performed ICC on brains applying several primary antibodies generated from previous works (Table 1).

All available antibodies against the clock neuropeptide PDF (or the crustacean ortholog, PDH) highlighted overlapping expression pattern, indicating antibodies specificity. As for *D. melanogaster* (Figure 4), *C. costata* (*Sapporo*) expresses PDF in the lateral ventral cluster of clock cells (specifically, 4 s-LN_v and 4 l-LN_v). The somata of the 4 s-LN_v are located in a region corresponding to the accessory medulla (AME) of dipterans, and they send projections to the dorsal protocerebrum (Figure 13), as it is known for *D. melanogaster*. The 4 l-LN_v instead densely innervate the medulla and project towards the controlateral hemisphere via the posterior optic commissure (POC) (Figure 13).

Also the antibody against *Drosophila* ITP highlights in *Chymomyza* the same pattern of cells that are described in *D. melanogaster* (Figure 4). ITP is expressed in the putative 5^{th} of the s-LN_v (which is PDF^-), and in one cell of the LN_d cluster (Figure 13). In addition, two groups of neurosecretory cells in the protocerebrum, named *ipc-1* and *ipc-2* in *D. melanogaster* (ITP-immunoreactive protocerebral neurons, Dircksen, 2009; Kahsai et al., 2010), also react to anti-ITP antibody. In *Drosophila* these cells are not clock neurons and most likely they do not express the clock proteins in the circadian system of *Chymomyza* either.

In order to confirm the homology with the *Drosophila* clock network, I performed ICC using a combination of anti-PDF and anti-PDP1 antibodies. The anti-PDP1 antibody (Reddy et al., 2000) stains strongly and reliably the core clock protein PDP1ε in the nuclei of the clock cells in *D. melanogaster* (Nitabach et al., 2006; Dissel et al., 2014; Kistenpfennig et al., 2018), and it has been widely adopted in this work to compare the circadian clock network of different insect species. Nevertheless, most probably because of the several isoforms generated at the *Pdp1* locus (Reddy

et al., 2000), anti-PDP1 polyclonal antibody signal is found not only within clock neurons but also in many other neurons and glia cells in the protocerebrum of *C. costata*, as it is known for several other *Drosophilidae* species (Hermann et al., 2013). In *C. costata*, PDP1 co-localizes with PDF in the nuclei of the LN_v (Figure 13). In addition, the staining reveals the presence of the other putative clock clusters homologous to those in *Drosophila* (LN_d and DN_s), which can be identified by their location in the protocerebrum (Figure 13). The cycling of PDP1 staining within the

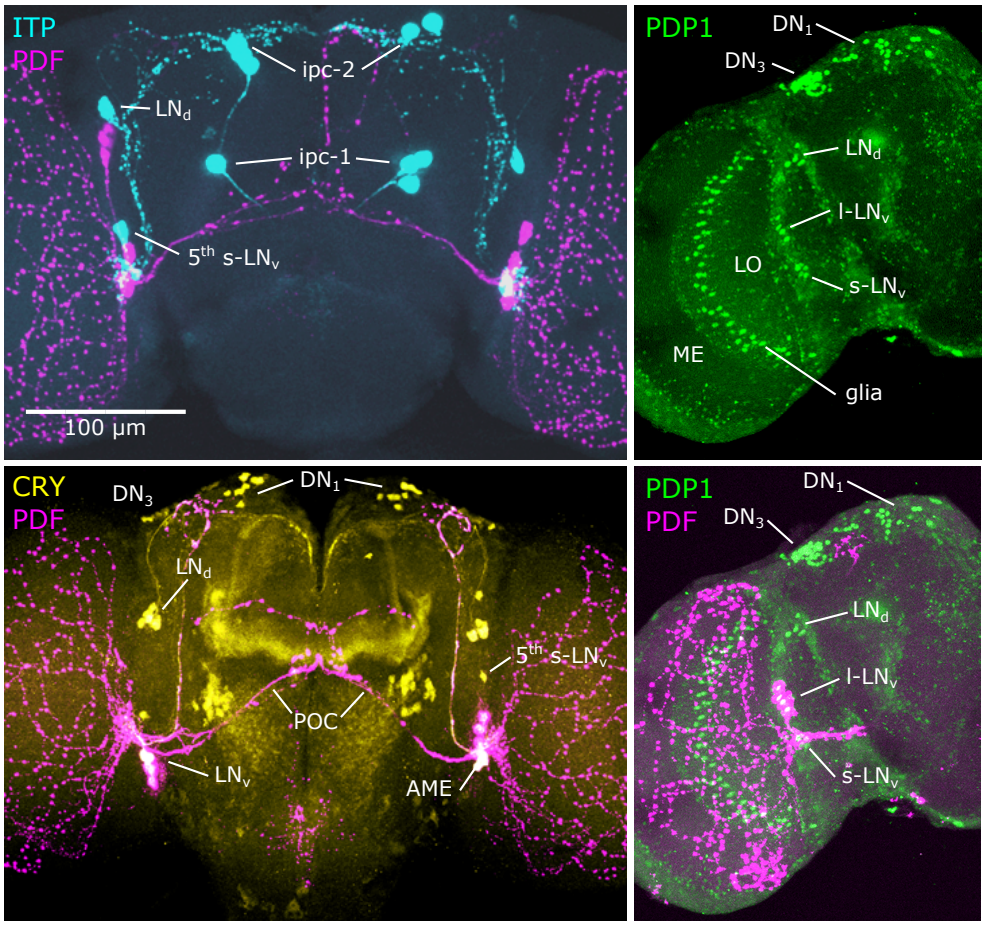

Figure 13: The clock neuronal network of *C. costata* (*Sapporo*) expresses PDF (magenta) in 4 s-LN_v and 4 l-LN_v. ITP (cyan) is expressed in the putative 5th s-LN_v and in one cell of the LN_d cluster, as well as in the *ipc-1* and *ipc-2* cells that are not clock neurons. CRY (yellow) is expressed in all the LN_v (s-LN_v, 5th s-LN_v, l-LN_v), in about half of the LN_d, and in the dorsal cluster (DNs,). PDP1 (green) co-localize in the nuclei of the PDF$^+$ LN_v, in the 5th s-LN_v, in the LN_d, and in the DNs, as well as in several non-clock cells (neurons and glia). (POC: posterior optic commissure; AME: accessory medulla; ME: medulla; LO: lobula; antibodies used: anti-PDF (DHSB); anti-ITP (Ring et al., 1998); anti-CRY (Yoshii et al., 2008); anti-PDP1 (Reddy et al., 2000)).

clock neurons under LD cycles shown in the next section (Figure 22) strengthens the homology of the clock network of *C. costata* with that of *D. melanogaster*.
The photoreceptor CRY is the main clock resetter in *D. melanogaster* and it is known to be expressed in about half of the clock neurons (Figure 4). In *Chymomyza*, anti-CRY antibody (Table 1) strongly stains the lateral ventral cluster of clock neurons (LN_v), which co-express PDF in the s-LN_v and in the l-LN_v, but not the 5^{th} s-LN_v (Figure 13). In addition, also about half of the LN_d (3-4 out of 6) express CRY, as it is known for *D. melanogaster*. Anti-CRY immunostaining is also present in the putative dorsal cluster of clock neurons ($DN_{1\text{-}2}$, DN_3).
Overall, the clock network of *C. costata* shows high homology with that of *D. melanogaster*, but it differs in terms of neurochemistry from those of other drosophilids like *D. ezoana* and *D. littoralis* which are actually known to co-habit the same high latitudes (Hermann-Luibl et al., 2014; Menegazzi et al., 2017). Specifically, the clock neuropeptide PDF and the photoreceptor CRY are co-expressed in the s-LN_v (except the 5^{th} s-LN_v) and in the l-LN_v.

4.1.2 Locomotor activity rhythm of *C. costata*

In order to investigate the properties of the circadian clock of *C. costata*, I recorded locomotor activity as a readout of the master circadian oscillator activity.
Under light-dark cycle (LD cycle), the two strains *Sapporo* and *Oulu* exhibit diurnal activity for both males and females (Figure 14 and Figure 16). Compared to *D. melanogaster* which is a crepuscolar animal and concentrate its activity at dawn and dusk, *C. costata* does not exhibit a clear bimodal activity pattern. Under LD 12:12, its activity level constantly increases from the lights-on to the lights-off reaching its peak (E-peak) around the lights-off transition (Figure 14 and Figure 15). In contrast to *D. melanogaster*, the morning peak of activity (M-peak) of *C. costata* is not as prominent and it is difficult to distinguish this clock controlled component from the startle response that flies exhibit when the light turns on.
I additionally recorded *C. costata* locomotor activity under short and long photoperiods, therefore simulating summer- or winter-like photoperiodic conditions in non-equatorial regions (Figure 14). Under short winter photoperiods (LD 4:20 or LD 8:16) the activity of the two strains seems to differ slightly. The strain *Sapporo* is able to concentrate its activity within the light phase better than *Oulu*, which instead delays its E-peak into the first half of the night under extremely short photoperiod (LD 4:20). The same shift of activity is known to happen also for *D. melanogaster* under the same condition (Figure 14). Under long summer photoperiods (LD 16:8

or LD 20:4) the E-peak of both strains clearly anticipate of few hours the lights-off transition (Figure 14), as it is very well known also for *D. melanogaster*. Importantly, this result indicates that the locomotor activity pattern of *C. costata* under LD cycles is not a mere response to the LD cycle itself, but rather a clock controlled behaviour which is modulated in response to increasing or decreasing periods of light. For a comparison, *D. melanogaster* strains that carry an impaired circadian clock (per^{01}, tim^{01}, Clk^{Jrk}) are unable to modulate their activity under increasing or decreasing LD cycles (Figure 37).

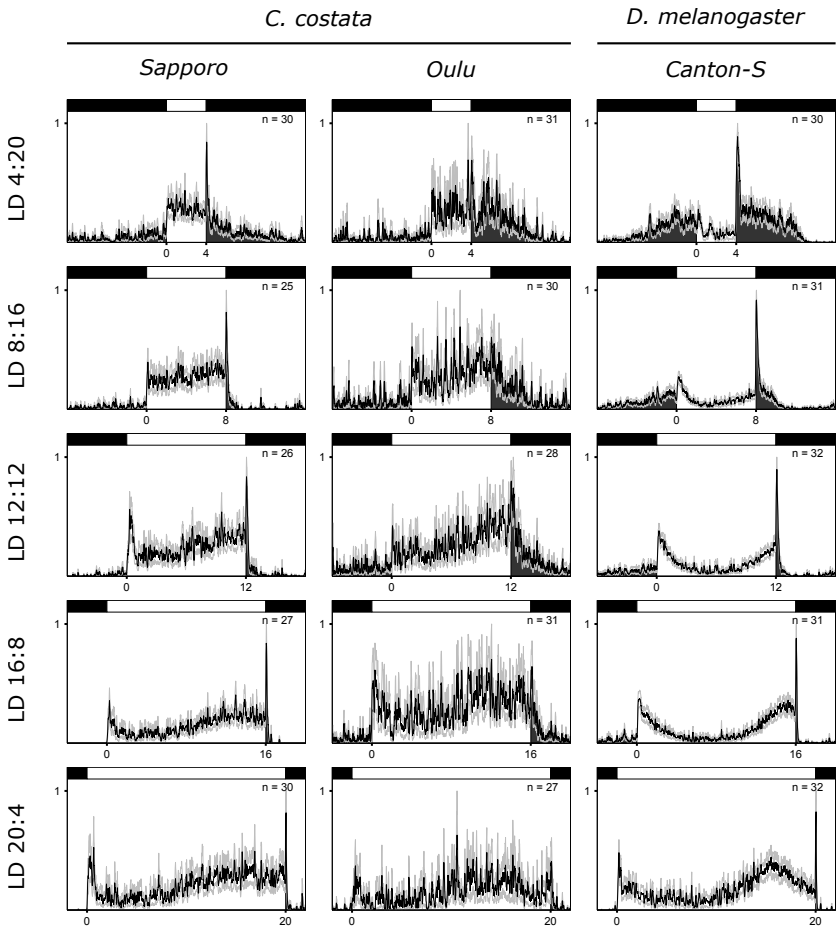

Figure 14: Average activity profile of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) under LD 4:20, LD 8:16, LD 12:12, LD 16:8, and LD 20:4 at 20°C. Black bars represent light-off and white bars represent light-on. The total number of flies averaged is indicated at the top right corner of each plot. Data are normalized to the maximum value of each condition.

The timing of the E-peak of *C. costata* could be manually quantified for single flies under LD 12:12, LD 16:8, and LD 20:4 (Figure 15). This quantification was not possible for short photoperiods (LD 8:16 and LD 4:20) because in these cases the activity peak under is not so pronounced. The E-peak of both *Chymomyza*

strains significantly delays with increasing photoperiods (Sapporo: $F(2,58) = 70.17$, $p < 0.001$; Oulu: $F(2,48) = 43.78$, $p < 0.001$). Although the two strains analysed are originating from distinct latitudes (*Sapporo* 43°N, *Oulu* 65°N), they do not differ in timing their E-peak under increasing photoperiods (LD 12:12: $t(36) = -1.44$, $p=0.16$; LD 16:8: $t(41) = -1.73$, $p=0.09$; LD 20:4: $t(29) = -0.3$, $p=0.76$). This contrasts the behavior of other drosophilids (i.e. *D. melanogaster*) collected over a latitudinal gradient, where flies originating from high latitudes are able to delay their evening activity under long photoperiods more than flies from equatorial regions (Rieger et al., 2012; Menegazzi et al., 2017).

Taken together with the previous reported expression of PDF and CRY in the clock network, *C. costata* shows *D. melanogaster*-like locomotor activity pattern under increasing photoperiods (Figure 15). This activity pattern is supported by the *D. melanogaster*-like co-expression of PDF and CRY in the LN_v (Figure 13). This expression differs from other Drosophilids species that co-habit the same Northern regions, like *D. littoralis*, *D. virilis*, and *D. ezoana*, which are instead able to extend more their E-peak towards dusk under extremely long photoperiods (LD 20:4, Menegazzi et al., 2017).

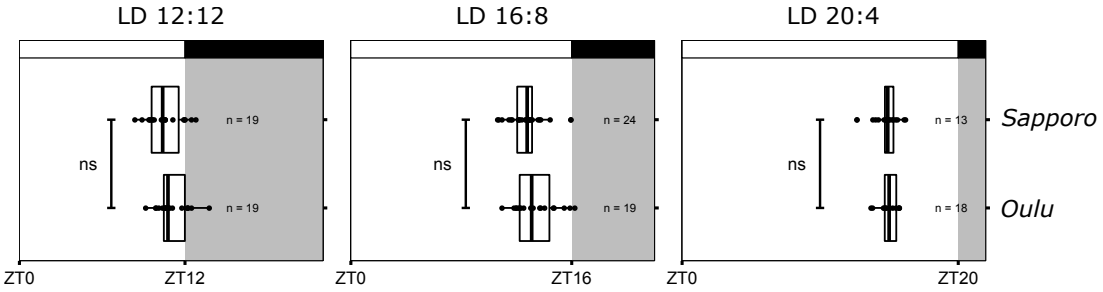

Figure 15: Quantification of the E-peak timing of *C. costata* (*Sapporo* and *Oulu*) under LD 12:12, LD 16:8, and LD 20:4 at 20°C. The two strains do not differ in timing their E-peak under increasing photoperiods (LD 12:12: $t(36) = -1.44$, $p=0.16$; LD 16:8: $t(41) = -1.73$, $p=0.09$; LD 20:4: $t(29) = -0.3$, $p=0.76$), although they originate from different latitudes.

Surprisingly, when *C. costata* is released into DD after LD 12:12 entrainment at 20°C, the rhythm in locomotor activity is lost (Figure 16). This result does not support the very well known model of the *D. melanogaster* circadian clock, as well as those of many other insect models (i.e. cockroaches (Page and Block, 1980), bees (Moore and Rankin, 1985), bugs (Pivarciova et al., 2016), and many others). In all these other well studied models, circadian rhythms persist under DD and free-runs with a defined period (τ) which is a specific readout of the TTFL activity. On the

other side, under LL *Chymomzya* becomes arrhythmic (Figure 16) as it is known to happen in *D. melanogaster* (Konopka et al., 1989). In *Drosophila* this is known to be caused by constitutive CRY activation by light (Ceriani et al., 1999). Since *C. costata* expresses CRY within the same clock neurons as *D. melanogaster* (Figure 13), arrhythmicity under LL is most likely caused by the same phenomena.

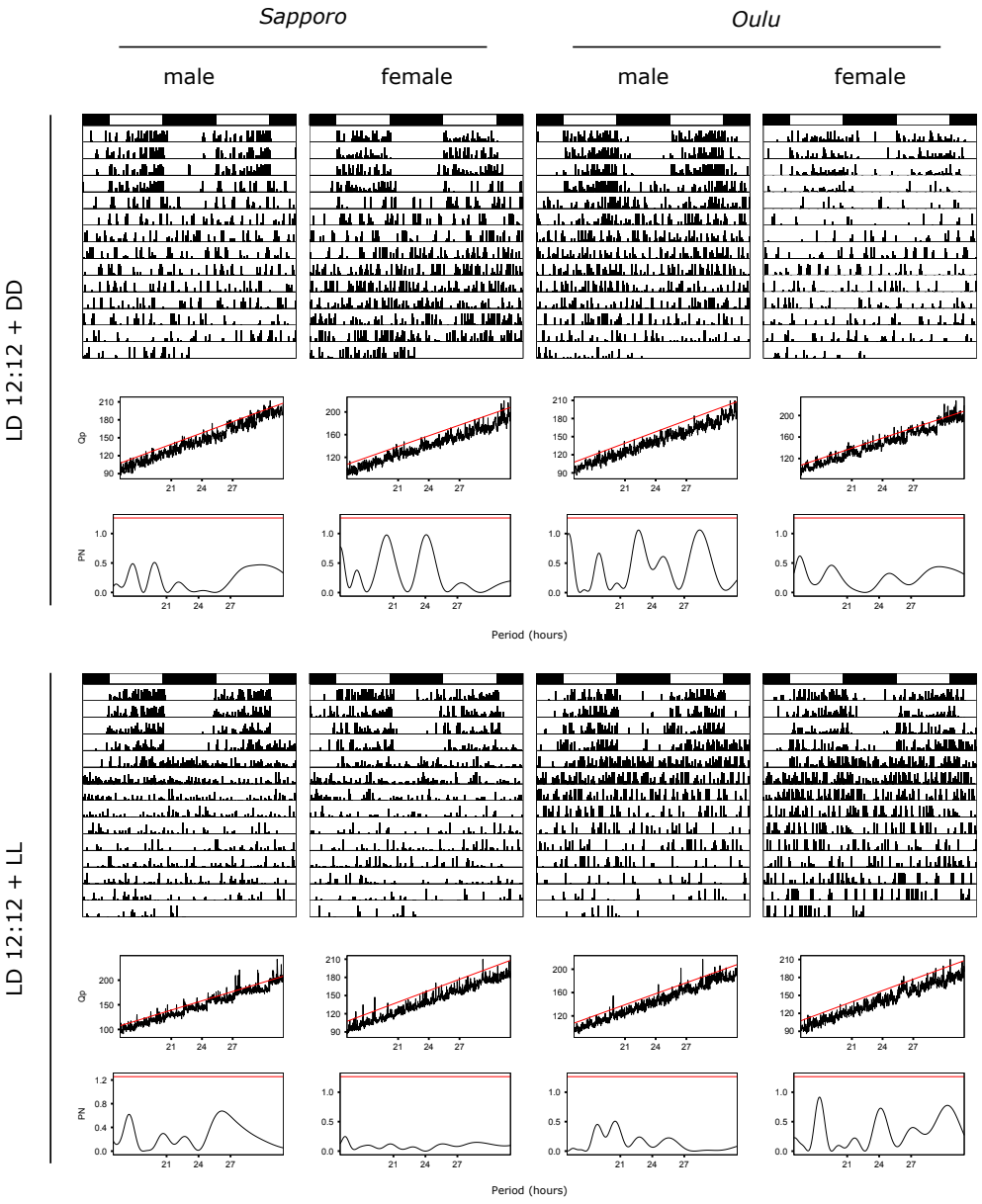

Figure 16: Representative actograms of *C. costata* (*Sapporo* and *Oulu*, males and females) entrained under at least seven days of LD 12:12 (4 days are shown) followed by 10 days of DD (top) or LL (bottom) at 20°C. χ^2 (Qp) and Lomb-Scargle (PN) periodogram analyses for the respective actograms are shown. The red line in the periodograms indicates the significance threshold ($p<0.05$).

In order to test whether *Chymomyza* possibly exhibits weak rhythm under constant

darkness (DD), that is undetectable by bare eye, I applied a number of analysis. The use of such algorithms to detect biological rhythms has both strengths and weaknesses (Refinetti et al., 2007) and this issue is further discussed at page 81. I combined the use of the the two powerful algorithms, Lomb-Scargle (Lomb, 1976; Scargle, 1982) and CLEAN (Roberts et al., 1987; Rosato and Kyriacou, 2006). The percentage of rhythmic flies, obtained combining these two periodograms, is summarized in Figure 17. Importantly, Figure 17 shows that such an analysis works well when applied to detect rhythms in wild-type *D. melanogaster*, which, as it is well known, shows self-sustained rhythm in the totality of the flies (29/29). On the contrary, *D. melanogaster* mutants, which carry and impaired clock (per^{01}, tim^{01}, Clk^{Jrk}, $pdp1^{3135}$), show extremely low percentage of rhythmicity. This low levels might be caused by artefacts in the analysis or by residual rhythmicity (Helfrich and Engelmann, 1987). Most importantly, the percentage of rhythmic *C. costata* detected by this method is extremely low (*Sapporo*: 4/24; *Oulu*: 2/28). When these levels are compared to the rhythmicity of the clock mutants or to wild-type *D. melanogaster* subjected to LL (Figure 17), it is clear that the small percentage of rhythmic *Chymomyza* is not representative of an underling self-sustained circadian clock. To support this hypothesis, the estimated period (τ) and the power of the rhythms detected by this analysis are summarized in Figure 44 and show that, the few flies detected as rhythmic by this analysis display anyway very low power (that is an indication of rhythm strength) and inconsistent free-running periods (large standard deviation) among flies.

Rhythms in locomotor activity might be not detectable also because of the low amount of activity. This seems to be not the case for *C. costata* since the levels of activity recorded by the DAM system are comparable to those of *D. melanogaster* under LD 12:12, DD, or LL for both strains (Figure 38). Only the strain *Sapporo* seems to decrease the level of activity in the switch from LD cycles to constant condition (both DD or LL).

In nature *C. costata* adults are most likely subjected only to long photoperiods, since they overwinter as diapausing larvae and they are reproductive during the long summer days (Enomoto, 1981a; Koštál et al., 2000). I recorded locomotor activity of *C. costata* under constant darkness also after entraining flies under long (LD 16:8, LD20:4) or short (LD 4:20, LD 8:16) photoperiods. Clear-cut circadian rhythm in locomotor activity is not detectable under DD also after these entrainment conditions (Figure 18). Nevertheless, when these data were analysed with the same procedure described above (Lomb-Scargle + CLEAN) the proportion of

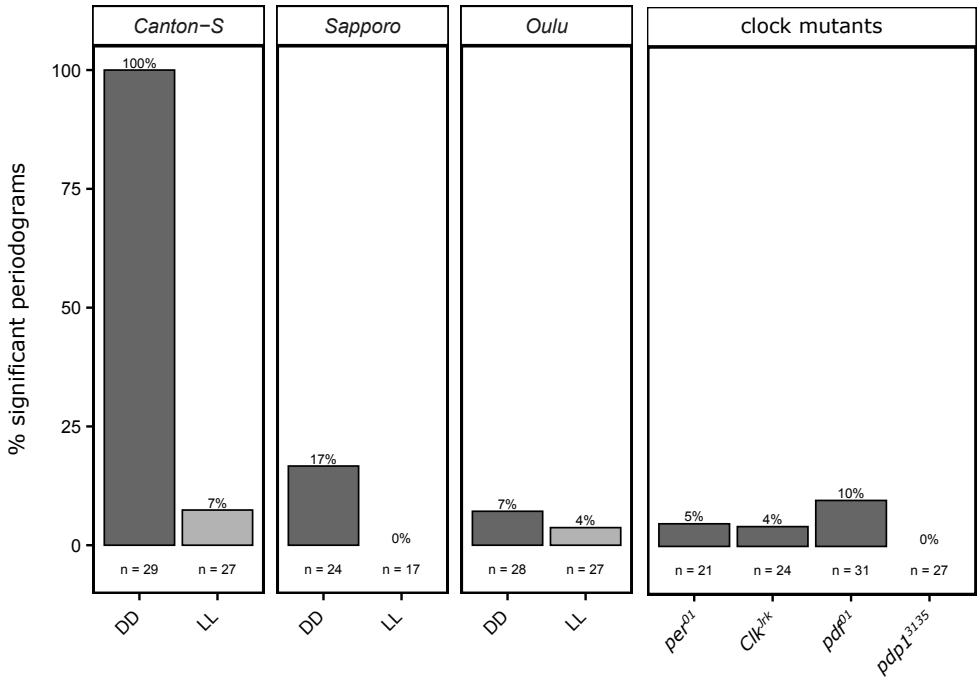

Figure 17: Rhythmicity of *C. costata* (*Sapporo* and *Oulu*), *D. melanogaster* (*Canton-S*) under DD or LL, and *Drosophila* clock mutants under DD (per^{01}, Clk^{Jrk}, pdf^{01}, $pdp1^{3135}$) analysed over 10 days of constant condition at 20°C after entrainment under LD 12:12.

flies that resulted in significant periodograms was slightly higher, especially after LD 16:8 entrainment (*Sapporo*: 5/21, *Oulu*: 10/29, Figure 21). Despite the higher percentage of rhythmic flies, both period and power of these detected rhythms are very inconsistent, as discussed before, when compared to other models (Figure 44).

Some works suggested that flies from the North are more rhythmic under LL rather than under DD, in particular when constant light is given at low intensity (Kauranen et al., 2012; Menegazzi et al., 2017). I tested *C. costata* under LD 12:12 cycles and subsequent release into constant light (LL) given at different intensities (from ∼10 lux down to ∼0.01 lux). For a comparison, all the other experiments in this work are carried out at ∼100 lux (Figure 12). Figure 39 shows that *C. costata* is able to entrain also with very low light intensity, although *D. melanogaster* seems to be more sensitive. Nonetheless, under LL of any intensity *C. costata* does not exhibit self-sustained rhythm in locomotor activity. In contrast, the *D. melanogaster* clock exposed to constant low light (∼0.1 and ∼0.01 lux) is able to support rhythmicity (Figure 39).

Also light composition has a significant impact onto the circadian clock, since daily and seasonal light spectrum changes predictably in nature. I recorded locomotor

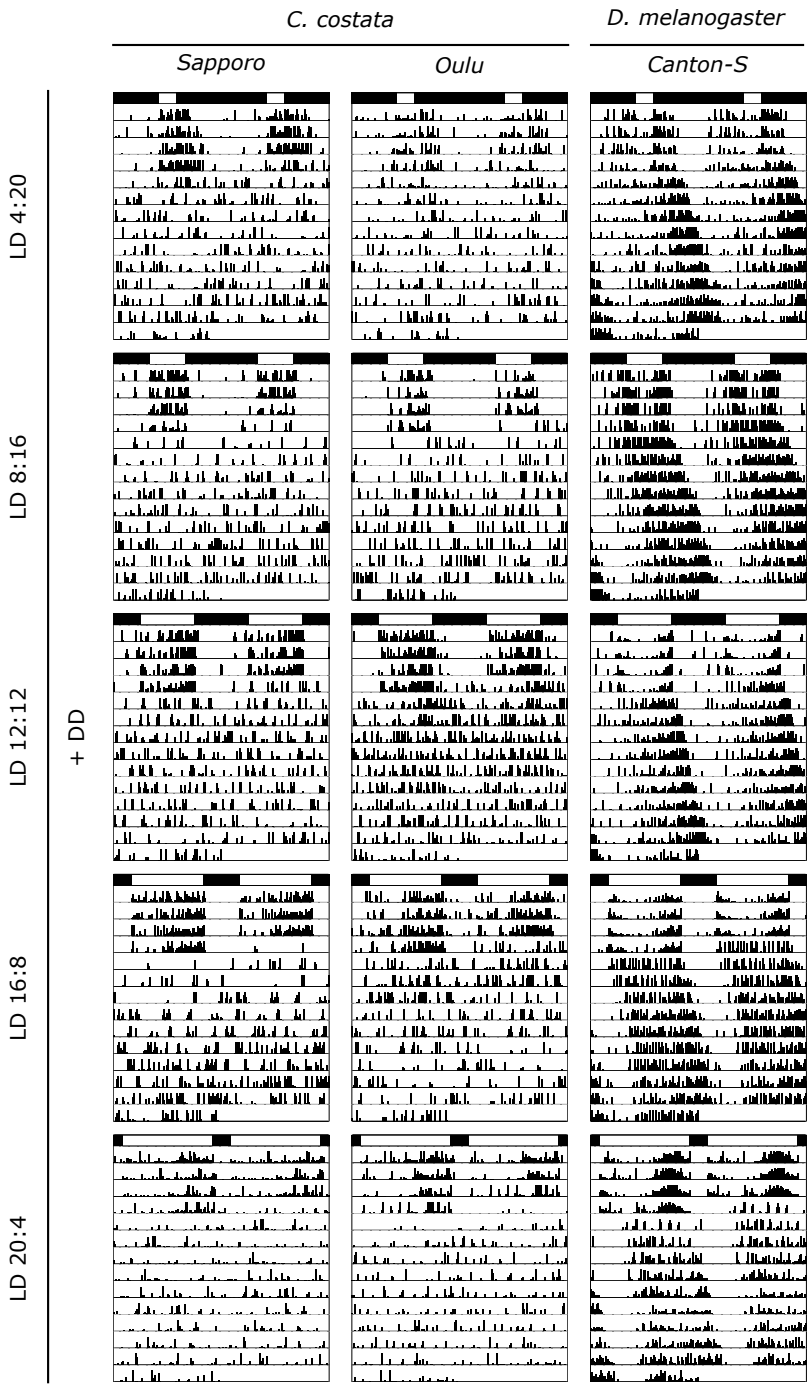

Figure 18: Representative actograms of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) entrained under at least seven days (4 days are shown) of LD cycles of different photoperiods (LD 4:20, LD 8:16, LD 12:12, LD 16:8, LD 20:4) followed by 10 days of DD at constant 20°C.

activity of flies at 20°C subjected to LD 12:12 cycles, produced using blue (470 nm) or red light (600 nm) supplied at different intensity (Figure 12). *C. costata* can entrain to LD cycles of both wavelength also at very low intensity (down to ∼0.01

Watt/cm^2). When released into constant coloured light (constant blue or constant red light) the circadian rhythm in locomotor activity is lost (Figure 40).

Arrythmicity in constant condition could be caused also by a clockwork which is not sufficiently entrained. In order to increase the entrainment strength I combined light-dark cycles with temperature cycles (LD + TC 12:12, square cycles of 12.5°C-17.5°C or 17.5°C-22.5°C) before the release of flies into DD at 15°C or 20°C, respectively (Figure 19). The percentage of rhythmic flies, detected after such enhanced entrainment, slightly increased, especially for the *Oulu* strain (13/30) and less for *Sapporo* (4/27, Figure 21). Nonetheless, the same inconsistency, already discussed for the other conditions regarding the period and the power of the detected rhythms, is also present (Figure 44). Figure 19 also highlights that *C. costata* is able to synchronize its activity only to temperature cycles under DD.

Interestingly, the percentage of rhythmic flies increased also when experiments have been carried out at constant lower temperature (15°C or 17.5°C, Figure 20). In particular, a couple of flies under DD at 15°C condition showed a weak, but traceable by eye, free running period which was also significant after the periodogram analysis (Figure 43). These two examples are nevertheless not representative for the condition. A more emblematic example is shown in Figure 20. In spite of that, also the periodogram analysis (Lomb-Scargle + CLEAN) detected higher percentage of rhythmic flies under DD at 15°C or 17.5°C (Figure 21). At lower temperature (10°C), the activity levels of *C. costata* (and *D. melanogaster* as well) are very repressed and flies are barely rhythmic also under LD cycles (Figure 20).

Overall, it is clear that the locomotor activity rhythm of *C. costata* is supported by a functional circadian clock when *Zeitgebers* are present, those provided as light or temperature cycles or both. I showed that the master clock of *C. costata* modulates the locomotor activity pattern of the fly in response to increasing or decreasing photoperiods, and that *Chymomyza* exhibits a *D. melanogaster*-like behaviour under extremely long days. Unexpectedly, the circadian rhythm in locomotor activity of *C. costata* is lost when *Zeitgebers* are removed and flies are subjected to constant conditions (DD, LL, and several others). This does not support the well established clock model known for *D. melanogaster*. This evidence is corroborated also by the oscillation of PDP1 within the clock neurons (see next section, 4.1.3). Interestingly, there are few evidences that a weak self-sustained rhythm in locomotor activity is enhanced under DD when temperature is decreased.

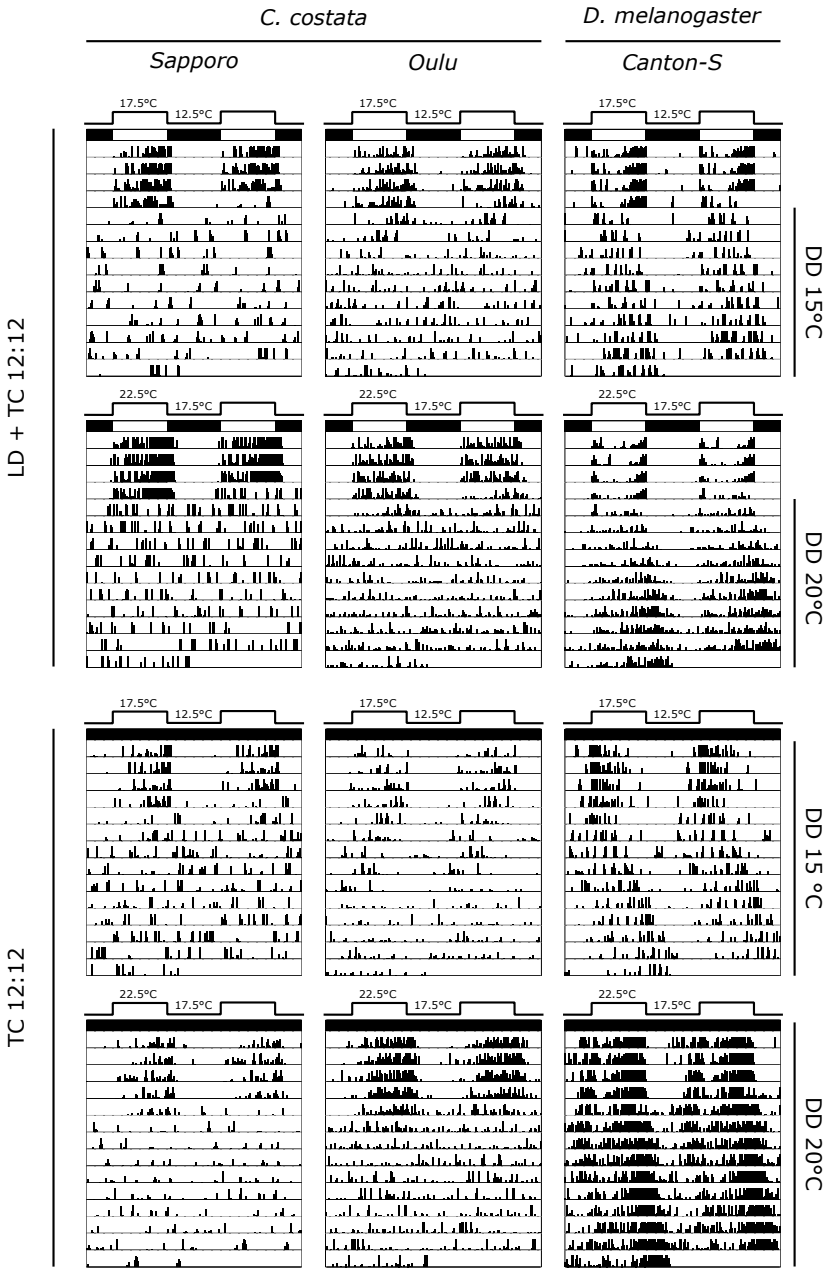

Figure 19: Representative actograms of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) entrained under: (top) light-dark (LD) and temperature cycles (TC 12.5-17.5°C and TC 17.5-22.5°C, LD + TC 12:12), followed by 10 days of DD at constant 20°C or 15°C; (bottom) temperature cycles (TC 12.5-17.5°C and TC 17.5-22.5°C) in DD, followed by 10 days of DD at constant 20°C or 15°C.

4.1.3 PDP1ε oscillation in *C. costata* clock neurons

I reasoned that there might be at least two explanations for the arrhythmicity observed in *C. costata* behaviour under constant darkness: 1. arrhythmicity is generated downstream of the master clock, so the clock machinery is functional under

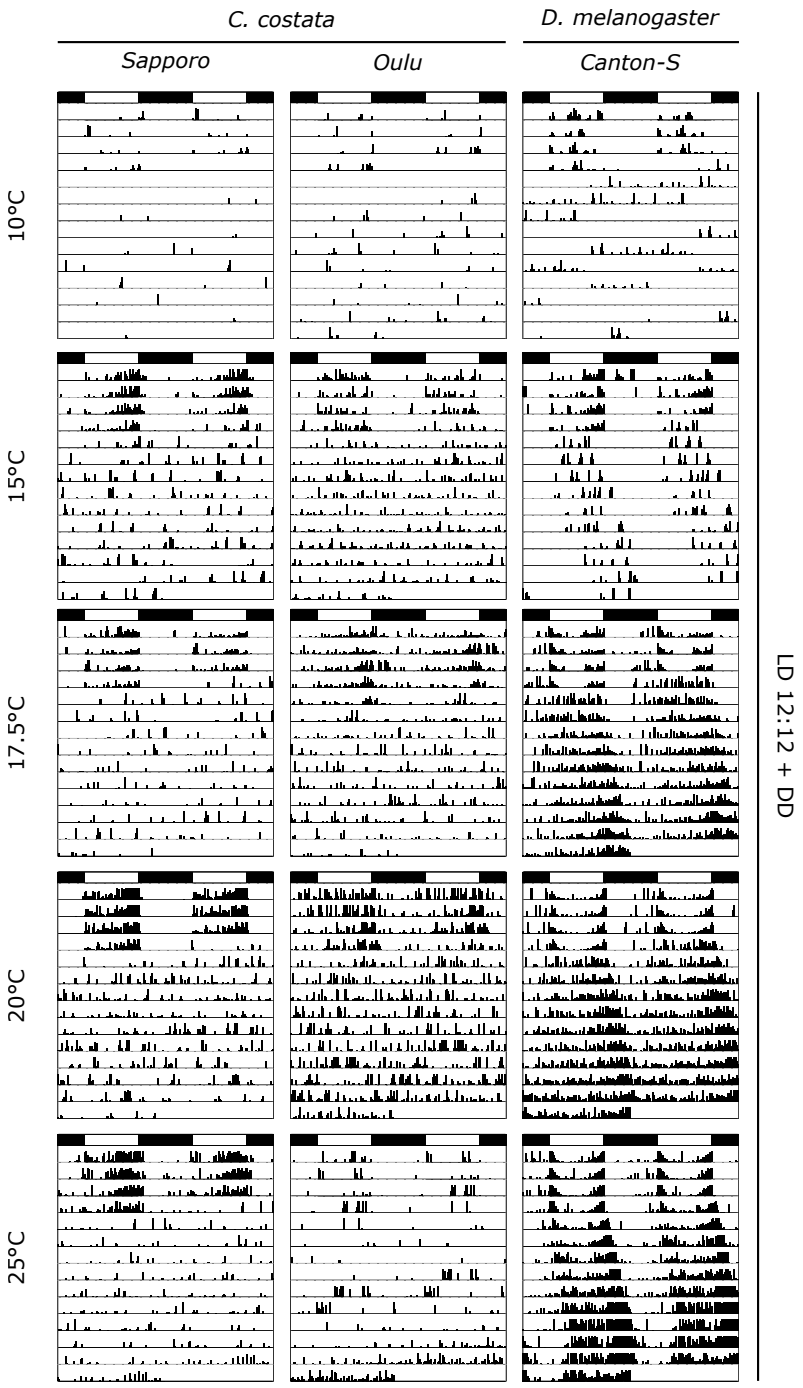

Figure 20: Representative actograms of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) entrained under at least seven days of LD 12:12 (4 days are shown) followed by 10 days of DD at different constant temperature (10°C, 15°C, 17.5°C, 20°C, 25°C).

DD but it is uncoupled from its output; 2. arrhythmicity is caused by a non-functional master clock which does not self-sustain under DD. In order to answer to these points, I investigated the molecular oscillation of the clock machinery under

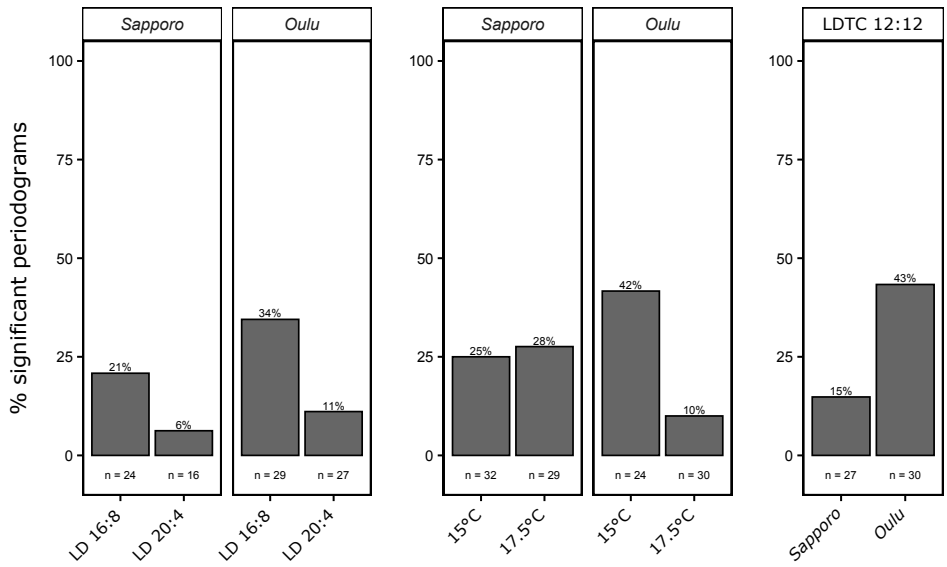

Figure 21: Rhythmicity of *C. costata* (*Sapporo* and *Oulu*) under: (left) DD after LD 16:8 and LD 20:4 at 20°C (see Figure 18); (middle) DD after LD 12:12 at 15°C and 17.5°C (see Figure 20); (right) DD after LDTC 12:12 (light + temperature cycles) at 20°C (see Figure 19). Data were analysed using the combination of Lomb-Scargle and CLEAN over 10 days of constant condition.

the first day of DD within the clock neurons of *Chymomyza*. In order to do so, I took the advantage of staining the core clock protein PDP1ε in *C. costata* clock network (Figure 13). This analysis does not consider the signal of PDP1ε within the dorsal neurons (DNs) because of the lack of co-localization with a marker, like for example anti-PDF staining. The 5th s-LN$_v$ and the LN$_d$ were instead easily distinguishable from the other PDP1$^+$ cells because of their strong signal and the clear position in the protocerebrum (Figure 13).

In *D. melanogaster*, the core clock protein PDP1ε takes part in the generation of the second feedback loop (Figure 3), and it is known to oscillate in abundance during the day with a peak at ZT18-21 and a trough at ZT6 under LD 12:12 (Cyran et al., 2003; Nitabach et al., 2006; Dissel et al., 2014). Figure 22 shows that such an oscillation in *D. melanogaster* is shifted towards the end of the night (ZT24=CT0), when flies are exposed to longer photoperiod (LD 16:8), and that it is highly synchronized among all neuronal clusters. Most importantly, the oscillation of PDP1 in *D. melanogaster* persists when flies are subsequently exposed to constant darkness (DD). This correlates with the self-sustained rhythm observed under DD in locomotor activity (Figure 18). Only the l-LN$_v$ lose the oscillation of PDP1 in DD (Figure 22). This is known to happen also at the oscillation of PER in the l-LN$_v$ (Veleri et al., 2003; Roberts et al., 2015).

Both strains of *C. costata* (*Sapporo* and *Oulu*) shown rhythmic oscillation in PDP1 level under LD 12:12 or LD 16:8, respectively (Figure 22). This oscillation is significant and robust in all the clusters (Table 4), and its phase resembles the oscillation of PDP1 in *D. melanogaster*. Nevertheless, the peak of PDP1 accumulation is broader, and its phase is more variable among clusters than in *D. melanogaster*. It seems that the s-LN_v and the l-LN_v show advanced PDP1 oscillation compared to the 5^{th} s-LN_v and the LN_d (Figure 22), both under short (LD 12:12, *Sapporo*) or long photoperiod (LD 16:8, *Oulu*). Importantly, this oscillation at molecular level correlates with the rhythmic activity pattern that these flies show under LD cycles (Figure 14 and Figure 15), and supports the evidence that *C. costata* has a functional circadian clock which highly resembles the circadian clock of *D. melanogaster*.

Interestingly, the oscillation of PDP1ε signal is quickly dampened when *Chymomyza* is released into the first day of DD. The two strains, *Sapporo* and *Oulu*, need to be treated separately, also because of the different entrainment condition (LD 12:12 or LD 16:8, see Figure 22).

The strain *Sapporo* shows decreased level of PDP1 under the first day of DD, and the circadian oscillation which is exhibited under LD 12:12 is highly dampened (or absent) under DD in all clock neuronal clusters (Table 4). This correlates with the arrhythmicity in behaviour reported in the previous section (Figure 16 and Figure 17), and suggests that *C. costata* circadian clock does not self-sustain under DD.

The strain *Oulu* shows instead differences in the PDP1 levels between the different clusters. The s-LN_v are highly dampened under DD, in contrast to the l-LN_v where PDP1 levels remain higher. Whether these two clusters show circadian oscillation in PDP1 levels is controversial (Table 4). Differently, the 5^{th} s-LN_v and the LN_d show a more stable oscillation in the levels of PDP1, with a trough in the middle of the subjective day (CT9-CT12) and an increase towards the end of the subjective night (CT24). Such oscillation is highly significant (Table 4), and in this case it does not correlate with the arrhythmicity in behaviour recorded for this strain under DD (Figure 16 and Figure 17). This suggest that for the strain *Oulu*, contrary to the *Sapporo*, the circadian clock of *C. costata* might be dampened but still self-sustained, at least under the first day of DD. Notably, I also detected higher percentage of rhythmic flies under DD when flies were entrained under longer photoperiods (Figure 21).

Overall, it is clear that the molecular clock of *C. costata* is ticking under light-dark cycles entrainment, and supports circadian control of locomotor activity under rhythmic environmental conditions, suggesting high similarity with the clock net-

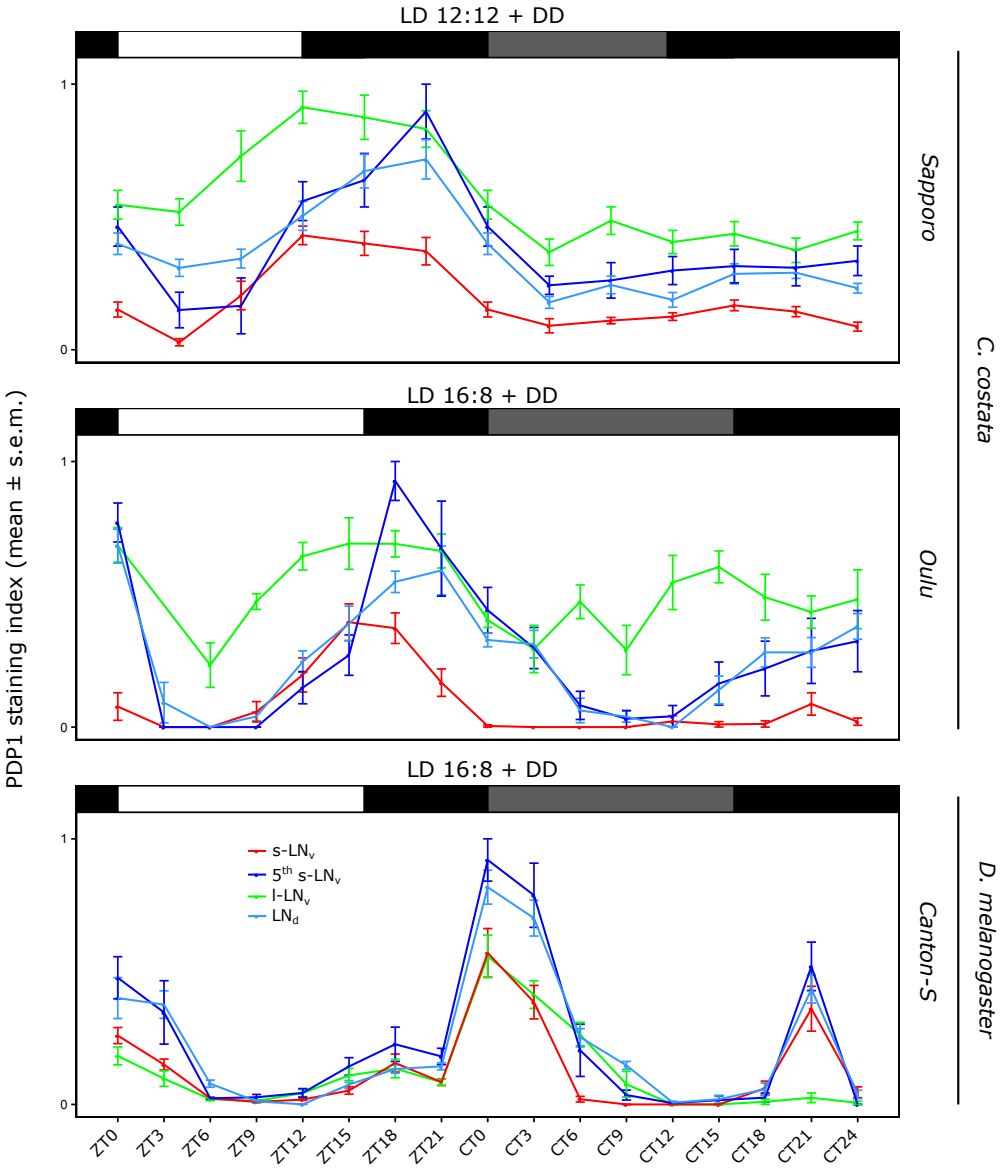

Figure 22: Quantificantion of the PDP1 staining within the clock neurons (s-LN$_v$, l-LN$_v$, 5th s-LN$_v$, LNd) of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) under LD 12:12 followed by DD (*Sapporo*) or LD 16:8 followed by DD (*Oulu* and *Canton-S*) at 20°C.

work of *D. melanogaster*. On the other hand, although it carries a *D. melanogaster*-like clock network, *C. costata* clock machinery does not self-sustain under constant darkness, as it shows quickly dampened molecular oscillation within the clock neurons. Such dampening in the molecular oscillation most likely generates the arrhythmic behaviour observed for these flies under constant conditions.

4.2 The circadian clock of *Bactrocera oleae*

The olive fly *B. oleae* is a major pest of olive plantation, and current methods for pest control (i.e. *OX3097D-Bol*) require the understanding of its circadian biology. This chapter reports the characterization of the circadian clock of the olive fly at behavioural and anatomical level. The reader is invited to refer to Bertolini et al., 2018 to have a better understanding of the molecular clock component and the temporal expression of clock genes in *B. oleae*.

4.2.1 Phylogenetics of PER and CYC of *B. oleae*

Thanks to the identification of the olive fly clock genes performed by our collaborators (Bertolini et al., 2018), I analysed the phylogenetic relationship of the putative protein sequences for PER and CYC, representative of the negative and the positive regulators of the *Drosophila* TTFL, respectively (Figure 3). It is well known that the presence of PER is highly conserved among taxa, from flies to humans (Glossop et al., 1999; Brown et al., 2012). In contrast, *Drosophila* CYC has a quite unique role in the *D. melanogaster* TTFL, since it seems that most of the other insects carry the mammalian form, called *Bmal1* (Figure 8). In collaboration with Dr. Alexander Keller (CCTB - University of Würzburg), I reconstructed the phylogeny of PER and CYC families, aligning the obtained predicted sequences for *B. oleae* with those available from selected species retrieved from Genbank (see Figure 23 for details). This analysis shows that *B. oleae* PER and CYC fit nicely within the Brachycera suborder (higher Diptera), which comprises Drosophildae, Muscidae, and other Tephritidae species (Figure 23). These taxa cluster separately from other species, for example mosquitoes and sand flies, that belong instead to the Nematocera suborder (lower Diptera). Indeed, the lower Diptera have been shown to carry the mammalian form of the gene *cyc* (Meireles-Filho et al., 2006).
Like for *cyc*, also the gene *cryptochrome* is found in two distinct variants in Diptera: *cry1* and *cry2*. During the course of this PhD thesis, the genome of the olive fly has been sequenced by the i5k consortium (Evans et al., 2013), and processes of automatic annotation have reported the gene *cry2* in *B. oleae* (XP_ 014103186.1 PREDICTED: cryptochrome-2). In contrast, we identified only the *cry1* form in *B. oleae* using our approach. Performing phylogenetic analysis, I showed that the *B. oleae* gene annotated as *cry2* in Genbank clusters together with photoloyases (6-4 photolyase) characterized in other insect species, and clearly differs from the insect CRY2 form that is present in the genome of Lepidoptera and Hymenoptera, but also in the genome of Diptera of the suborder Nematocera (Figure 45).

These results, together with the structural analysis and the temporal expression of the olive fly clock genes (Bertolini et al., 2018), strongly indicate that the composition and the mechanism of the TTFL of *B. oleae* highly match with that of *D. melanogaster*, and fit nicely within the Brachycera-type clock (Chahad-Ehlers et al., 2017).

4.2.2 The clock network of *B. oleae*

I characterized the neuroarchitecture of the *B. oleae* clock network performing ICC against the clock neuropeptides PDF and ITP (Figure 24). I identified four small (s-LN_v) and four large (l-LN_v) ventro-lateral neurons that express PDF, as well as four neurosecretory cells in the *Pars Lateralis* (PL, I refer to them as ipc-2 according to the naming in *D. melanogaster* (Dircksen et al., 2008)). The 4 s-LN_v project to the dorsal protocerebrum, whereas the 4 l-LN_v innervate the medulla (ME) and project to the controlateral hemisphere via the posterior optic commissure (POC). ITP is expressed in the 5^{th} s-LN_v and in one cell of the dorsal-lateral neurons (LN_d), as well as in three out of four PDF positive ipc-2 (Figure 24). It is additionally stained in three other putative IPC cells (ipc-1, as they are named in *D. melanogaster*) in the central brain (Figure 24).

In order to strengthen the homology with the *Drosophila* clock network, I performed ICC using the antibody against the core clock protein PDP1. When I performed double staining using anti-PDF and anti-PDP1 antibodies together, cytoplasmic PDF signal co-localized with nuclear PDP1 signal in both s-LN_v and l-LN_v (Figure 24), confirming the homology with *Drosophila* lateral ventral neurons. In addition, anti-PDP1 antibody labelled also the other putative clock clusters (DN_1, DN_3, LN_d) that are located in similar positions as those ones in *D. melanogaster* (Figure 4) and in other *Drosophila* species (Hermann et al., 2013). In order to test whether PDP1 oscillates in its abundance, I analysed the intensity of the anti-PDP1 staining in the nucleus of the LN_v (colabelled with PDF) at several timepoints across the LD 12:12 cycle. I found synchronous oscillation in the levels of PDP1 in both s-LN_v ($H(7) = 34.78$, $p < 0.001$) and l-LN_v ($H(7) = 19.26$, $p < 0.05$) (Figure 22), reaching their maxima and minima at ZT21 and ZT6, respectively. These oscillations are consistent with the oscillations of PDP1ε described in *D. melanogaster* (Cyran et al., 2003).

Overall, the clock network of the olive fly *B. oleae* highly resembles the organization of the master clock in *D. melanogaster*, in particular concerning the expression of the clock neuropeptides PDF and ITP, and the oscillation of the core clock protein

PDP1ε within the lateral ventral cluster (LN_v).

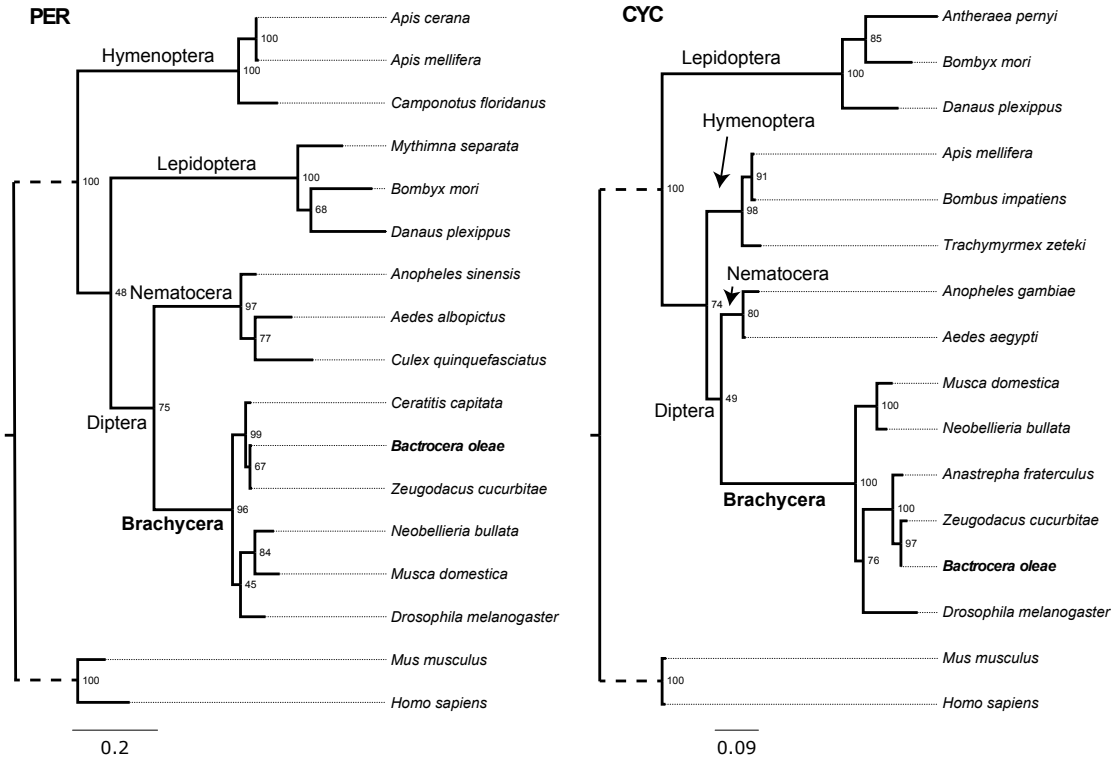

Figure 23: Gene trees of PER and CYC reconstructed from amino acids with Maximum Likelihood using RaxML. Values at the nodes represent bootstrap values, determined with 1000 replicates. GenBank ID/reference: CYC (*Antheraea pernyi*, AAR14937.1; *Bombyx mori*, NP 001036982.1; *Danaus plexippus*, EHJ64590.1; *Apis mellifera*, XP 016770595.1; *Bombus impatiens*, XM 012382960.1; *Trachymyrmex zeteki*, XM 018454278.1; *Aedes aegypti*, JN573265.1; *Anopheles gambiae*, Meireles-Filho et al., 2006; *Anastrepha fraterculus*, AQV08515.1; *Zeugodacus cucurbitae*, NP 001291680.1; *Drosophila melanogaster*, AAC39124.1; *Musca domestica*, XM 005180588.3; *Neobellieria bullata*, ACJ08742.1; *Mus musculus*, AAH25973.1; *Homo sapiens*, D89722.1); PER (*Apis cerana*, NP 001315410.1; *Apis mellifera*, ARB43935.1; *Camponotus floridanus*, XP 011264661.1; *Mythimna separata*, AQY60264.1; *Bombyx mori*, NP 001036975.1; *Danaus plexippus*, AAO48719.1; *Anopheles sinensis*, KFB40661.1; *Aedes albopictus*, AEX14535.1; *Culex quinquefasciatus*, XP 001849299.1; *Ceratitis capitata*, ABB20914.1; *Zeugodacus cucurbitae*, NP 001291681.1; *Neobellieria bullata*, ACJ08740.1; *Musca domestica*, AAD39163.1; *Drosophila melanogaster*, NP 001259194.1; *Mus musculus*, AAC53592.1; *Homo sapiens*, NP 073728.1). The major insect orders and suborders are indicated (black arrows).

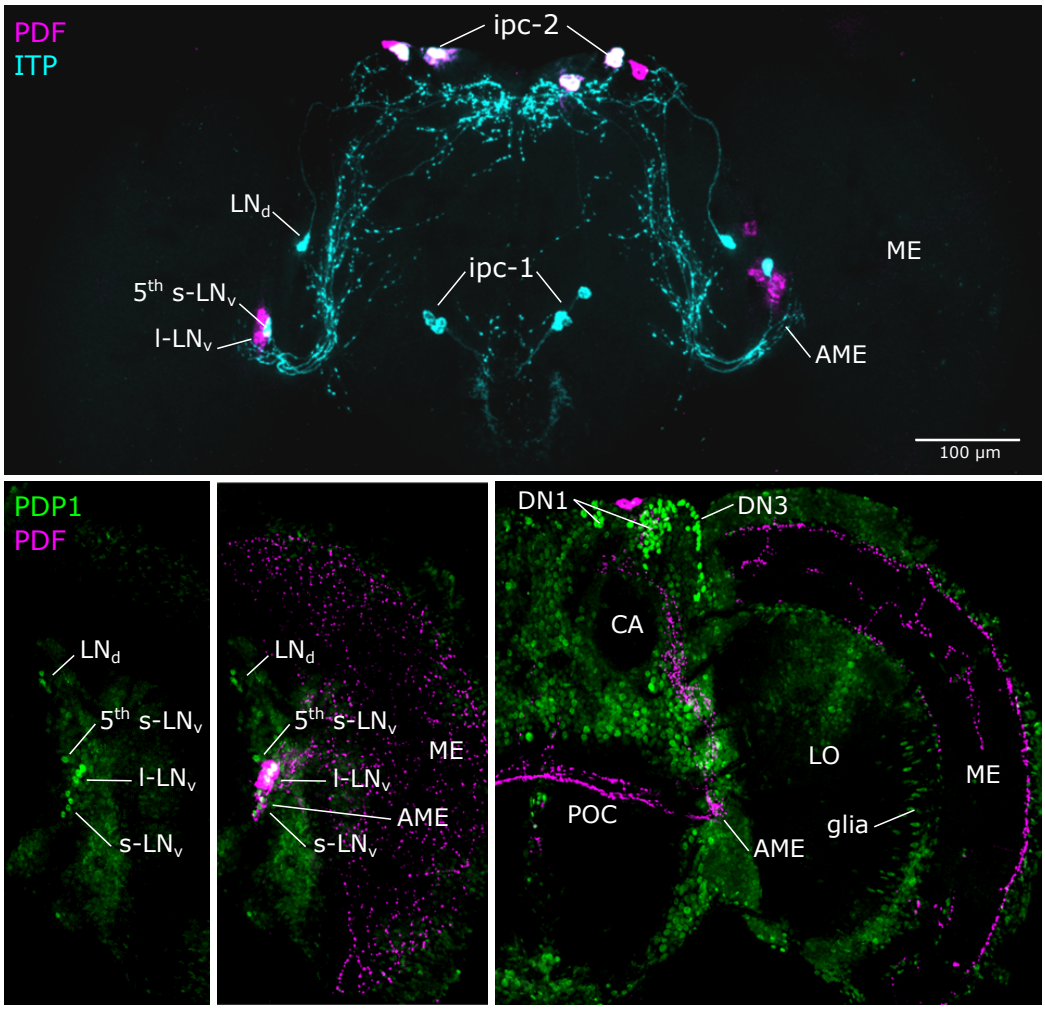

Figure 24: The clock neuronal network of *B. oleae*. The clock neuropeptide PDF (magenta) is expressed in 4 l-LN$_v$ and 4 s-LN$_v$, as well as in 4 putative ITP producing cells (ipc-2) in the PL. ITP (cyan) is expressed in the 5th s-LN$_v$ and in one cell of the LN$_d$ group, as well as in putative ipc-1 and ipc-2. anti-PDP1 (green) co-localize in the nuclei of PDF positive cells (magenta) in the LN$_v$ cluster. Antibody reveals also other putative clock clusters (LNd, DN) and stains many other non-clock cells. (AME: accessory medulla; ME: medulla; CA: calyx; LO: lobula; antibodies used: anti-PDF (DHSB, Abdelsalam et al., 2008); anti-ITP (Ring et al., 1998); anti-PDP1 (Reddy et al., 2000)).

4.2.3 Locomotor activity rhythm of *B. oleae*

To study the properties of the circadian clock of *B. oleae*, I recorded its locomotor activity as a readout of the master circadian oscillator activity. In general, activity recording worked well in olive flies, although they showed a high mortality rate when isolated into the glass tubes (∼50 % of the flies died within the first week of recording). Furthermore, olive flies showed quite low activity levels (on average

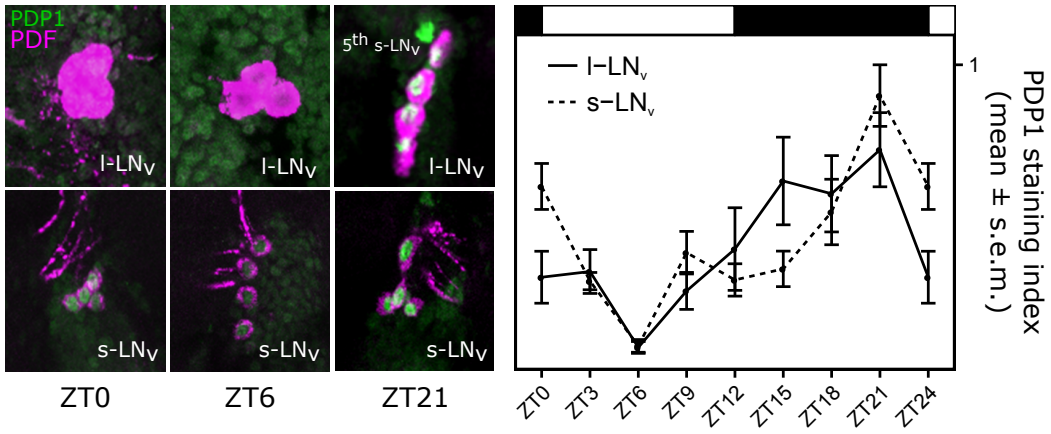

Figure 25: PDP1 oscillation in the LN_v of *B. oleae* entrained under LD 12:12 at 20°C. PDP1 protein level in the nuclei oscillates in the s-LN_v ($H(7) = 34.78$, $p < 0.001$) and l-LN_v ($H(7) = 19.259$, $p < 0.05$) with the peak at ZT21 and the trough at ZT6.

17 beam crosses/hour, Figure 46) but this did not interfere with the detection of circadian rhythms using our system.

Under LD 12:12 cycles, all flies (males and females) are diurnal and more or less uniformly active throughout the day (Figure 27 and Figure 26). After being transferred in DD, the majority of olive flies show self-sustained rhythmicity in their locomotor activity, with a free-running period shorter than 24 hours in all tested genotypes (Figure 28 and Table 2). When *B. oleae* was instead released in constant light (LL) its locomotor activity became arrhythmic (Figure 26), as it is known to happen in *D. melanogaster* (Konopka et al., 1989; Emery et al., 2000).

Taken together, these results demonstrate that the locomotor activity of *B. oleae* is clock-controlled, and that the fundamental properties of the olive fly clock (rhythmic clock under DD and arrhythmic clock under LL) are common with *D. melanogaster*.

4.2.4 Characterization of *OX3097D-Bol* clock

OX3097D-Bol is the self-limiting olive fly technology offered by the company Oxitec ltd and it is potentially an alternative management approach to this insect pest (Ant et al., 2012). As stated in the introduction, asynchrony in the time of mating between wild and laboratory strains has obstructed previous attempts of performing SIT against *B. oleae* (Economopoulos and Zervas, 1982; Zervas and Economopoulos, 1982). Since it is known that mating timing is controlled by the circadian clock in flies, I characterized the behavioural basis of the circadian rhythms of *OX3097D-Bol*. In order to exclude differences at the level of the clock between the transgenic strain

(*OX3097D-Bol*) and wild-type genotypes (*Demokritos* and *Argov*), I recorded *B. oleae* locomotor activity under light-dark cycles (LD) and constant darkness (DD) at 20°C. In general, *OX3097D-Bol* males were significantly more active than wild-type (on average 39 beam crosses/hours; $H(5) = 56.17$, $p < 0.001$; Figure 46) and exhibit stronger bimodal activity pattern, with higher activity associated to the morning and evening as described for *D. melanogaster* (Figure 27). The amount of activity associated with the evening was significantly different among strains ($F(5) = 6.8$, $p < 0.001$), with *OX3097D-Bol* males showing the highest level (Figure 46). When *OX3097D-Bol* is released into constant darkness (DD), the rhythm in locomotor activity persists in the majority of flies (Table 2). No significant differences were

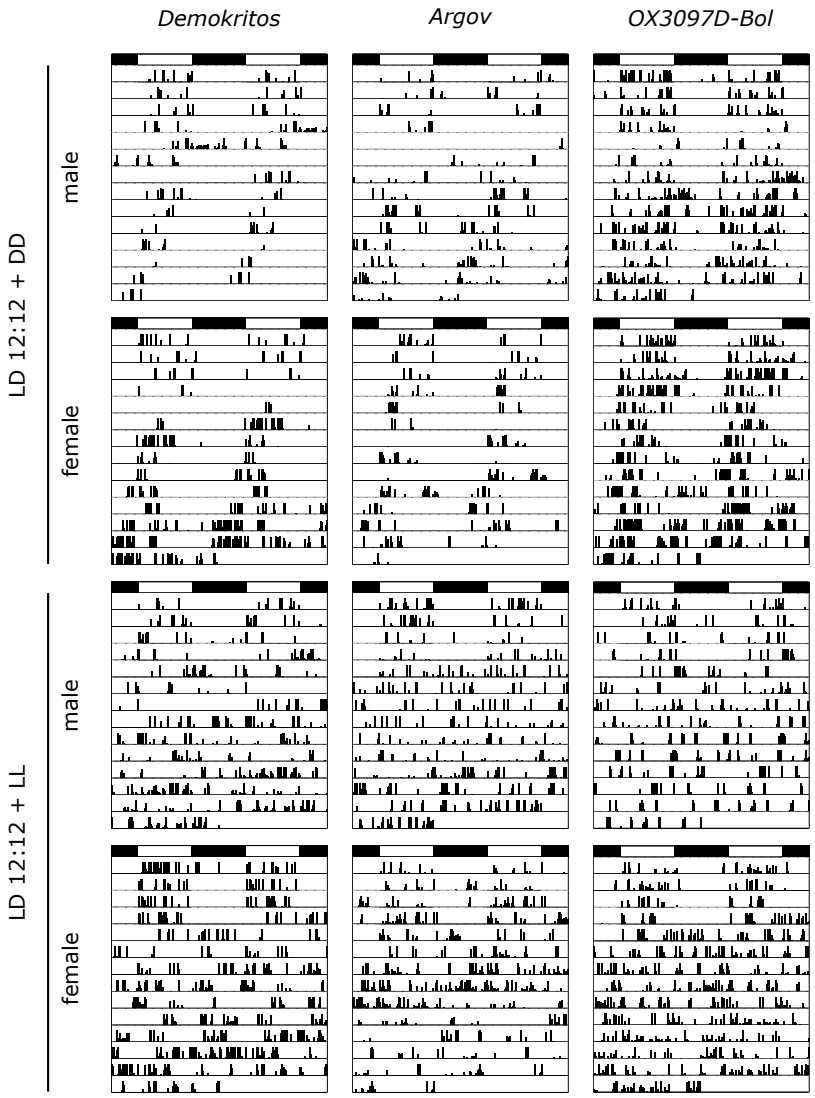

Figure 26: Representative actograms of *B. oleae* (*Demokritos*, *Argov*, and *OX3097D-Bol*, males and females) entrained under at least seven days of LD 12:12 (4 days are shown) followed by 10 days of DD or LL at 20°C.

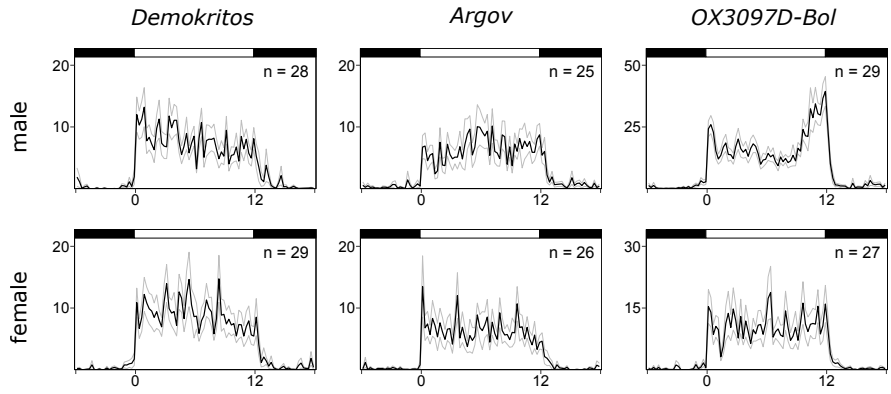

Figure 27: Average activity profile of *B. oleae* (*Demokritos*, *Argov*, and *OX3097D-Bol*, males and females) under LD 12:12 at 20°C. Black bars represent light-off and white bars represent light-on. The total number of flies averaged is indicated at the top right corner of each plot.

found in the percentage of rhythmic flies (Table 2; $\chi 2 = 4.8$, df = 5, p = 0.44) and in the free-running period in DD (Figure 28, H(5) = 10.92, p = 0.053) between the different strains and sexes. Under LL, *OX3097D-Bol* showed arrhythmic behaviour like it is know for the wild-type genotypes (Figure 27 and Table 2).

		DD		LL
genotype	gender	% rhythmic flies (N)	period ± s.e.m. (h)	% rhythmic flies (N)
Demokritos	M	72.7 (22)	23.24 ± 0.19	0 (16)
	F	91.7 (12)	23.24 ± 0.19	0 (26)
Argov	M	73.7 (19)	23.01 ± 0.17	0 (15)
	F	93.3 (15)	23.49 ± 0.1	0 (12)
OX3097D-Bol	M	78.6 (28)	23.36 ± 0.08	0 (14)
	F	68.8 (16)	23.54 ± 0.1	0 (20)

Table 2: Rhythmicity percentage of *B. oleae* under DD and LL, and estimated free-running period under DD of several strains (*Demokritos*, *Argov*, *OX3097D-Bol*, males and females) after LD 12:12 entrainment at 20°C.

Taken together with the analysis of the temporal expression of the clock genes *per*, *cyc*, *Clk*, and *cry* in *Demokritos*, *Argov*, and *OX3097D-Bol* (Bertolini et al., 2018), there is no evidence that the circadian clock of the transgenic strain *OX3097D-Bol* differs from the clock of wild-type genotypes, confirming its suitability for operational use.

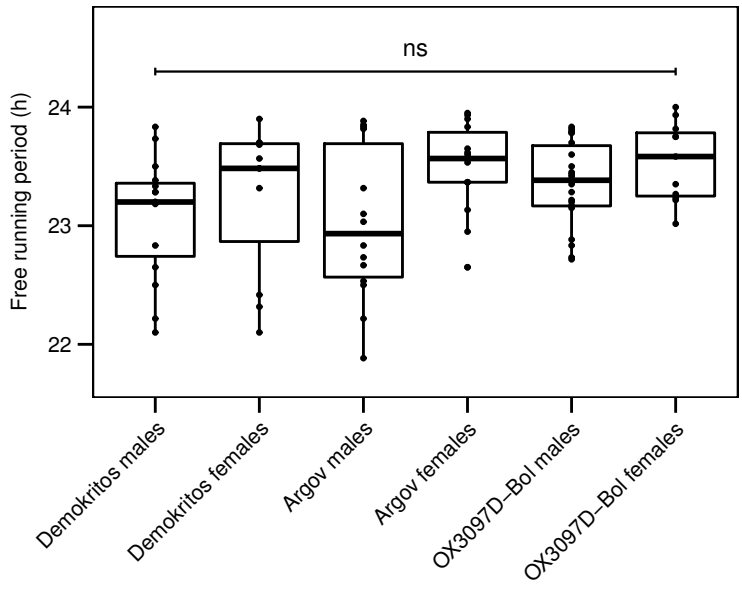

Figure 28: Free-running period of the clock of *B. oleae* (*Demokritos*, *Argov*, *OX3097D-Bol*, males and females) under DD after entrainment under LD 12:12 at 20°C. There is no significant differences in the free-running period in DD among genotypes ($H(5) = 10.92$, $p = 0.053$).

4.3 The circadian clock of *Pyrrhocoris apterus*

The firebug *P. apterus* is an emerging model organism for studying the interactions between circadian and photoperiodic timers in insects, because of its strong and robust circadian (locomotor activity) and photoperiodic (diapause) rhythms, which can be easily assessed in laboratory. Although its putative molecular clock mechanism and its circadian behaviour have been recently described (Pivarciova et al., 2016; Kotwica-Rolinska et al., 2017), the localization of the master circadian clock in *Pyrrhocoris* has not been demonstrated yet.

4.3.1 The clock network of *P. apterus*

I investigated the expression of central clock components in *P. apterus* performing ICC on brains, using several primary antibodies generated from previous works (Table 1). Both antibodies anti-PDF C7 (DHSB) and anti-PDH (Dircksen et al., 1987) give an overlapping expression pattern in *Pyrrhocoris* protocerebrum, when used together, and this indicates signal specificity (Figure 29 and Figure 30, co-labelling not shown). Figure 29 shows the overall expression of PDF in the brain of *P. apterus*. For technical reason, the brain of the bug could not be scanned from the anterior-posterior axis, as it is normally done for *Drosophila* and other Diptera, but it was instead scanned from the dorsal-ventral axis. The reader is invited to pay attention to the comparison of the PDF networks of *P. apterus* and *D. melanogaster*, and if necessary, to refer to Helfrich-Förster and Homberg, 1993, where the dorsal view of the *Drosophila* PDF network is illustrated. Overall, the expression of PDF in *P. apterus* brain highly resembles the PDF network of *D. melanogaster* (Figure 4). Several PDF^+ cells cluster in a region in the proximity of the optic lobe (OL), which is named PDFMe in other studies of non-drosophild species (Ikeno et al., 2014). This cluster sends fibres towards the lobula (LO) and innervates the medulla (ME), as it is known in *D. melanogaster*. To strengthen the homology, PDF^+ projections are also sent to the dorsal protocerebrum and to the controlateral hemisphere via the posterior optic commissure (POC).

In *Drosophila*, these PDF^+ lateral ventral clock neurons are classified in small (s-LN_v) and large (l-LN_v) based on the size of their somata. These two groups also differ in their projection pattern and neurochemistry (Figure 4). In *Pyrrhocoris*, it was possible to cluster the PDF^+ cells into two subgroups, which indeed are characterized by large somata at the dorsal part and small somata at the ventral part (Figure 29). These data possibly suggest a very high degree of homology between the PDF network of Hemiptera (*P. apterus*) and Diptera (*D. melanogaster*).

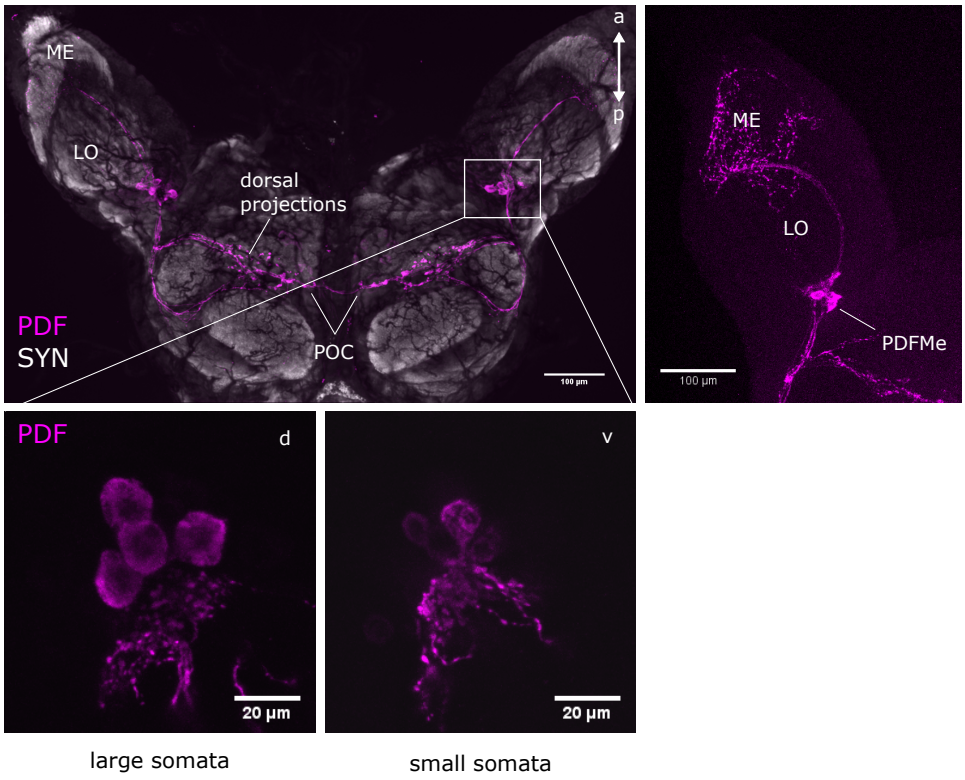

Figure 29: The PDF network in the brain of *P. apterus* shows high homology with that of *D. melanogaster*. Several PDF$^+$ cells (magenta, PDFMe) cluster in a region in the proximity of the optic lobe (OL) and innervate the medulla (ME), the dorsal protocerebrum, and the controlateral hemisphere. PDF$^+$ somata differ in size also according to ventral (v) or dorsal (d) localization. anti-SYN staining (gray) highlights the morphology of *P. apterus* brain (a: anterior; p: posterior). (POC: posterior optic commissure; LO: lobula; antibodies used: anti-PDH (Dircksen et al., 1987); anti-SYN (Klagges et al., 1996)).

In order to strengthen this homology, I performed double staining combining anti-PDF (DHSB) and anti-PDP1 (Reddy et al., 2000) antibodies. The latter strongly stains the nuclei of the clock cells in *D. melanogaster* and several other species (Hermann-Luibl et al., 2014; Bertolini et al., 2018), and it has been extensively used in this work to make inter-specific clock comparisons. In *P. apterus*, the anti-PDP1 antibody produced strong nuclear signal in several groups of cells across the brain, distributed laterally and dorsally (Figure 30). This is known also in *D. melanogaster*, where anti-PDP1 staining is restricted not only to clock neurons but also to several other cells (neurons and glia), all across the protocerebrum.

Despite permeability problems, it was possible to detect strong PDP1 signal in close vicinity to the PDFMe (Figure 30). Surprisingly, PDP1 seems to be not expressed by the PDF$^+$ cells in *P. apterus*. In order to increase tissues permeability to antibodies,

I performed ICC on agarose brain sections. As a result, in some preparations PDP1 staining shows weak localization in the nuclei of the PDF cells in *P. apterus* (Figure 30). The signal, although bugs were sampled at different timepoints across the day (ZT0, ZT6, ZT12, ZT18), seems to be constantly weak and localized only in few of the PDF$^+$ cells, but not in all.

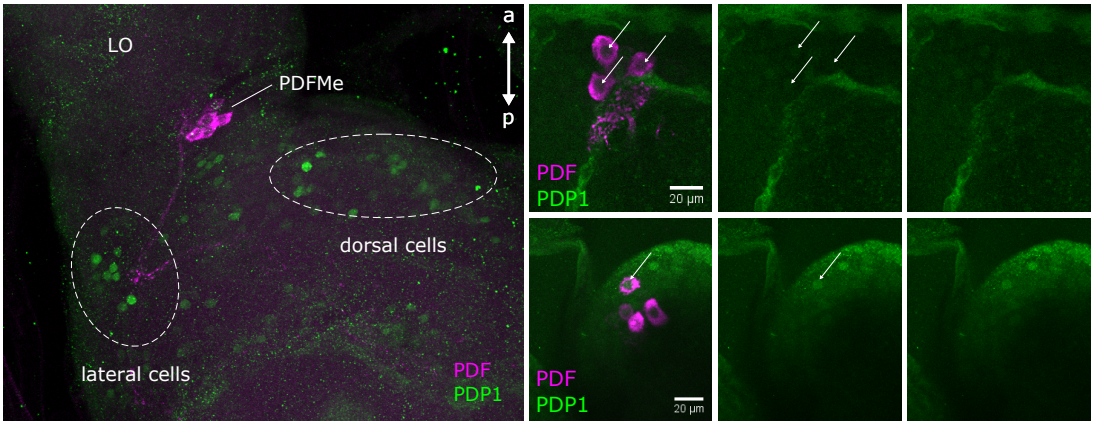

Figure 30: Anti-PDP1 staining (green) in *P. apterus* gives nuclear signal in several cells across the protocerebrum, highlighting lateral and dorsal clusters, also in the vicinity of the PDF$^+$ cells (magenta, PDFMe). Weak anti-PDP1 signal is found in the nucleus of some (but not all) PDF$^+$ cells (white arrows) when the ICC protocol is performed on agarose brain slices. (LO: lobula; a: anterior; p: posterior; antibodies used: anti-PDP1 (Reddy et al., 2000); anti-PDF (DHSB)).

4.3.2 Locomotor activity rhythm of *P. apterus*

In order to test anti-PDP1 (Reddy et al., 2000) antibody specificity to *P. apterus*, I performed knock-down of PDP1 via RNAi in *P. apterus*. As a readout of the efficacy of the RNAi knock-down, I recorded locomotor activity of bugs under light-dark cycles (LD 16:8) and subsequent release into constant darkness (DD, Figure 31). Not injected *P. apterus* (*Lyon-red*) exhibits diurnal locomotor activity under LD cycles, as reported in another study (Pivarciova et al., 2016). Such rhythm persists under constant darkness (DD) and it is easily detectable by periodogram analysis (Figure 31). The knock-down of *Pdp1* expression using RNAi results in impaired locomotor activity rhythms under DD, suggesting that the it is most likely targeting a core clock component (Figure 31).

Overall, even though the PDF network of *P. apterus* highly resembles the clock neuronal organization of *D. melanogaster*, it is not yet clear if these cells express the molecular mechanisms of the circadian clock.

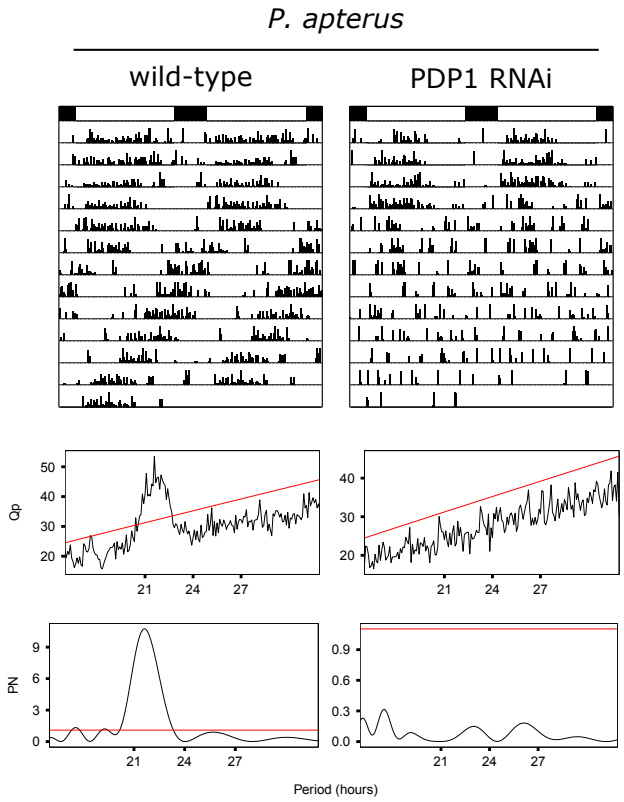

Figure 31: Representative actograms of *P. apterus* males (*Lyon-red*) non-injected (wild-type) or injected with dsRNA against PDP1 (PDP1 RNAi), entrained under LD 16:8 (4 days are shown) followed by 10 days of DD at 25°C. χ^2 (Qp) and Lomb-Scargle (PN) periodogram analyses for the respective actograms are shown. The red line in the periodograms indicates the significance threshold ($p<0.05$).

4.4 The circadian clock of the insect genus *Megaselia*

Although circadian behaviour of *M. scalaris* has been characterized (Bostock et al., 2017), the molecular and anatomical basis of such endogenous rhythmicity remains unknown. This chapter reports the behavioural and anatomical characterization of the circadian clock of two *Megaselia* species, *M. scalaris* and *M. abdita*.

4.4.1 The clock network of *M. scalaris* and *M. abdita*

I performed ICC on brains of *M. scalaris* and *M. abdita* using several antibodies raised against the clock neuropeptide PDF (anti-PDF C7 (DHSB); anti-PDF cricket (Abdelsalam et al., 2008); anti-PDH (Dircksen et al., 1987)). Surprisingly, none of these antibodies were functional in staining putative PDF cells, nor any of their putative projections, in the brain of both *Megaselia* species (Figure 32). In particular, the anti-PDH antibody is generally an excellent tool, and it specifically recognizes and stains PDF in a wide range of species, from insects to crustaceans (Stengl and Homberg, 1994; Strauss et al., 2011; Kay et al., 2018). Also the anti-PDF C7 and the anti-PDF cricket antibodies have been used to describe the PDF network in insect species that are pyhlogenetically distant from *D. melanogaster* (Abdelsalam et al., 2008; and *P. apterus* in this thesis, Figure 30). Because none of these antibodies highlights PDF expression, this strongly suggests that the circadian network of *Megaselia* lacks PDF expression in the brain.

In *Drosophila*, PDF expressing cells have been described also in the abdominal ganglion (Helfrich-Förster and Homberg, 1993; Shafer and Taghert, 2009). In this region eight non-clock cells (4 with large (l-Ab) and 4 with small (s-Ab) somata) strongly express PDF and project posteriorly to the abdomen. Immunostaining, making use of the anti-PDH antibody, in the abdominal ganglion of *M. scalaris* and *M. abdita* revealed the presence of these PDF^+ cells, which are bilaterally distributed at the very posterior side, and resemble the putative PDF^+ l-Ab in *D. melanogaster* (Figure 32). The quality of the immunostaining in *Megaselia* was not fine enough to classify this group of cells in large (l-Ab) or small (s-Ab), but the high specificity of the signal only within these few cells in the whole *Megaselia* central nervous system strongly supports the evidence that the clock networks of *M. scalaris* and *M. abdita* express PDF in the abdominal ganglion, but most surprisingly they lack PDF expression in the brain.

4.4.2 Locomotor activity rhythms of *M. scalaris* and *M. abdita*

D. melanogaster engineered flies that lack PDF signalling exhibit altered circadian locomotor activity. More precisely, flies that lack PDF (pdf^{01}) or its receptor PDFR (han^{5304}) generate advanced E-peak and they lose (or decrease) the M-peak under LD cylces (Renn et al., 1999; Hyun et al., 2005; Schlichting et al., 2016).

I recorded locomotor activity of *M. scalaris* and *M. abdita* under LD cycles of different photoperiods at 20°C (LD 4:20, LD 8:16, LD 12:12, LD 16:8, LD 20:4). Under all photoperiods, locomotor activity of both *Megaselia* species appears to be bimodal, characterized by a morning (M) and an evening (E) component (Figure 33), as it is known for *D. melanogaster* (Figure 14). *M. abdita* exhibits a very low amount of activity, compared to other models (∼86 ± 7 beam crossings/24 h, compared to *M. scalaris* (∼490 ± 35 beam crossings/24h) or *D. melanogaster* (∼350 ± 19 beam crossings/24h)). Nonetheless, such a low amount of activity seems to not interfere with the analysis of circadian rhythms in *M. abdita*. For *M. scalaris*, M and E activity peaks in locomotor activity were previously described under LD 12:12 (Bostock et al., 2017). Here, subjecting flies to long or short photoperiods, I could clearly demonstrate that the two activity components are not a mere response to the light-dark cycle, but a clear anticipatory behaviour modulated by a functional circadian mechanism. As a comparison, this modulation of the timing of the M and E peak

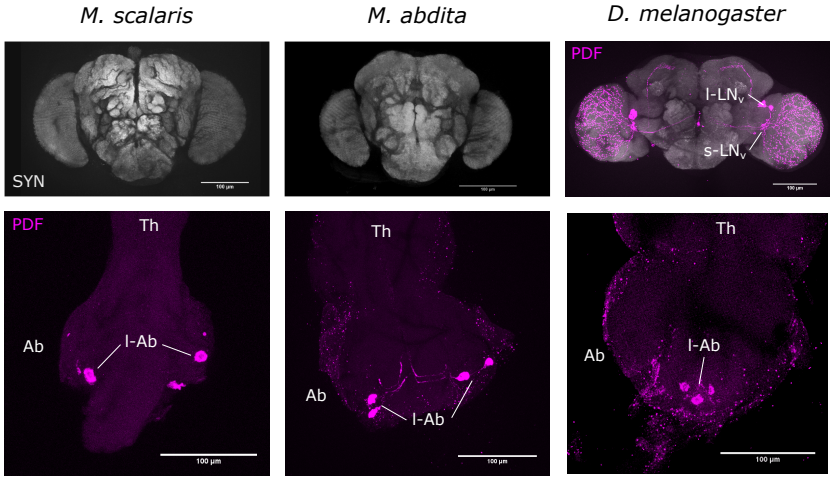

Figure 32: Immunocytochemistry using anti-PDH antibody in the brain (top) and abdominal ganglion (bottom) of *M. scalaris*, *M. abdita*, and *D. melanogaster* (*Canton-S*). anti-SYN staining is used to highlight the neuropils in the brain of the flies. (Th: thoractic ganglion; Ab: abdominal ganglion; antibodies used: anti-PDH (Dircksen et al., 1987); anti-SYN (Klagges et al., 1996).

is absent when *D. melanogaster* carries an impaired circadian clock (Figure 37). Nevertheless, the activity of the two *Megaselia* species is not concentrated around the twilight, as for *D. melanogaster*, and, although they belong to the same genus and share a similar life style, also *M. scalaris* and *M. abdita* slightly differ in their activity pattern.

M. scalaris shows a strong E-peak and a shallow M-peak; the latter is more evident when photoperiod is decreased (LD 4:20, LD 8:16), as it clearly anticipates the lights-on transition. Also the E-peak spans in the dark phase under short photoperiod, whereas under all the other conditions it clearly anticipates the light-off transition.

M. abdita shows a very robust and precisely timed M-peak, which is very easily detectable despite the very low amount of activity. The E-peak of *M. abdita* is less prominent than the peak during the morning activity, but it clearly anticipates the lights-off transition also under very short photoperiods (LD 4:20).

Thanks to the very precisely timed activity peaks of these two *Megaselia* species, it was possible to quantify the timing of the peaks for single individuals, as reported in Figure 34. This analysis strengthens what shown in the average activity profiles (Figure 33), and most importantly it highlights how the two peaks are strongly coupled to each other for both *M. scalaris* and *M. abdita*, under short and long photoperiods. In contrast, *D. melanogaster* Pdf^{0} do not generate prmoninent M-peak of activity, therefore the latter cannot be quantified (Renn et al., 1999).

D. melanogaster that lack PDF signalling (pdf^{01} or han^{5304}) shows high levels of arrhythmicity under DD when compared to wild-type flies (Renn et al., 1999; Hyun et al., 2005; Im and Taghert, 2010). Surprisingly, when *M. scalaris* and *M. abdita* are released into constant darkness (DD), they exhibit a strong and clear self-sustained rhythm (Figure 35). The period (τ) of this rhythm is slightly shorter than 24 hours for both species (Figure 35 and Table 3). This rhythm is detectable in the totality of *M. scalaris*, whereas in *M. abdita* is detectable in ∼60% of the flies (Table 3). Because *M. abdita* exhibit very low amount of activity when isolated into locomotor activity tubes (∼86 ± 7 beam crossings/24 h), it is likely that this relatively low amount of detected rhythmic flies is caused by low sensitivity of the periodogram analysis.

When *M. scalaris* is released into constant light (LL) it exhibits arrhythmic behaviour, as it is known to happen also for *D. melanogaster* because of the constitutive activation of CRY (Emery et al., 1998). When *M. abdita* is subjected to LL ∼15 % of flies are found rhythmic. As already discussed, the low amount of activity of *M. abdita* causes issues in the detection of biological rhythms in the dataset.

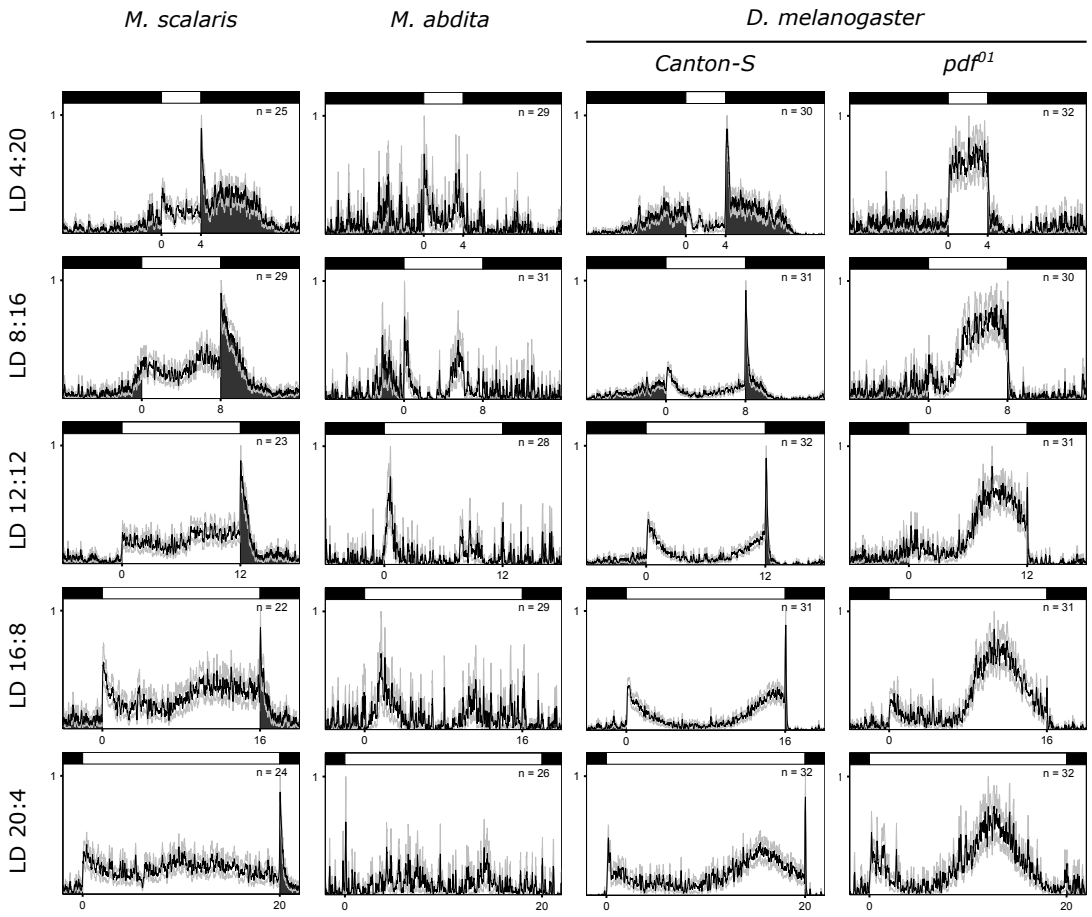

Figure 33: Average activity profile of *M. scalaris*, *M. abdita*, and *D. melanogaster* (*Canton-S* and pdf^{01}) under LD 4:20, LD 8:16, LD 12:12, LD 16:8, and LD 20:4 at 20°C. Black bars represent light-off and white bars represent light-on. The total number of flies averaged is indicated at the top right corner of each plot. Data are normalized to the maximum value of each condition.

	DD		LL	
	% rhythmic flies (N)	period ± s.e.m. (hours)	% rhythmic flies (N)	period ± s.e.m. (hours)
M. scalaris	100 (26)	23.82 ± 0.05	0	-
M. abdita	59.3 (27)	23.63 ± 0.15	14.3 (21)	23.77 ± 1.07

Table 3: Rhythmicity percentage of *M. scalaris* and *M. abdita* and estimated free-running period under DD and LL after LD 12:12 entrainment at 20°C.

As shown in Figure 35, the representative reported example of *M. abdita* under LL shows almost significant rhythmicity using the Lomb-Scargle analysis under LL, and, therefore, it is likely to be that the percentage of rhythmic flies under LL could

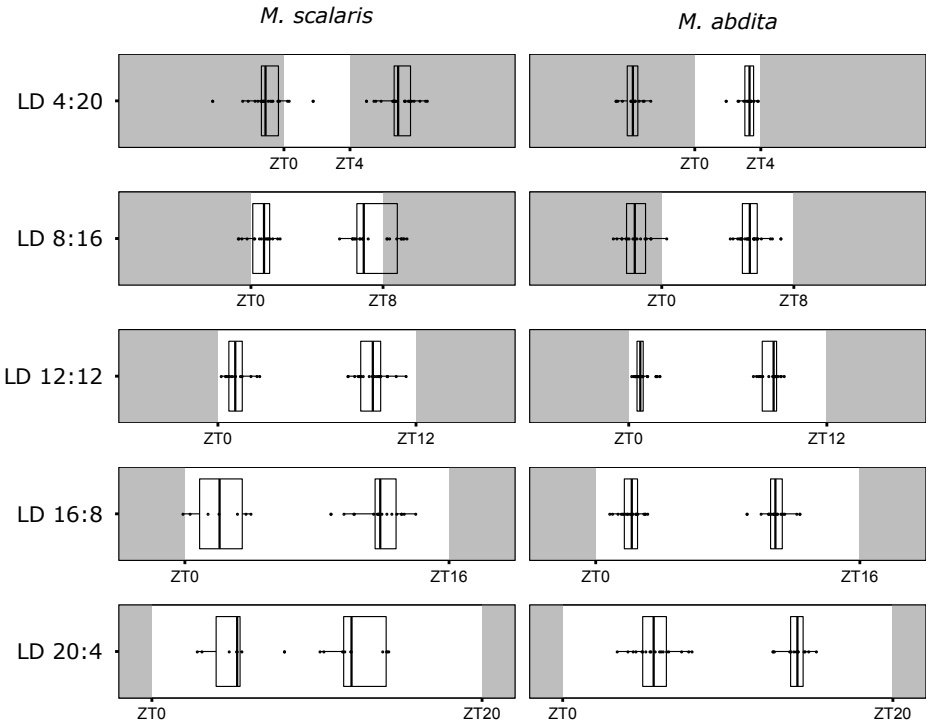

Figure 34: Quantification of M- and E-peak timing in *M. scalaris* and *M. abdita* under LD 4:20, LD 8:16, LD 12:12, LD 16:8, and LD 20:4 at 20°C.

be higher.

Overall, flies from the genus *Megaselia* seem to lack PDF expression in the brain but, contrary to *D. melanogaster*, exhibit strongly coupled M- and E-peaks of activity under light-dark cycles, and strong self-sustained rhythm under DD. The fly *M. abdita* also exhibits self-sustained rhythmicity under LL.

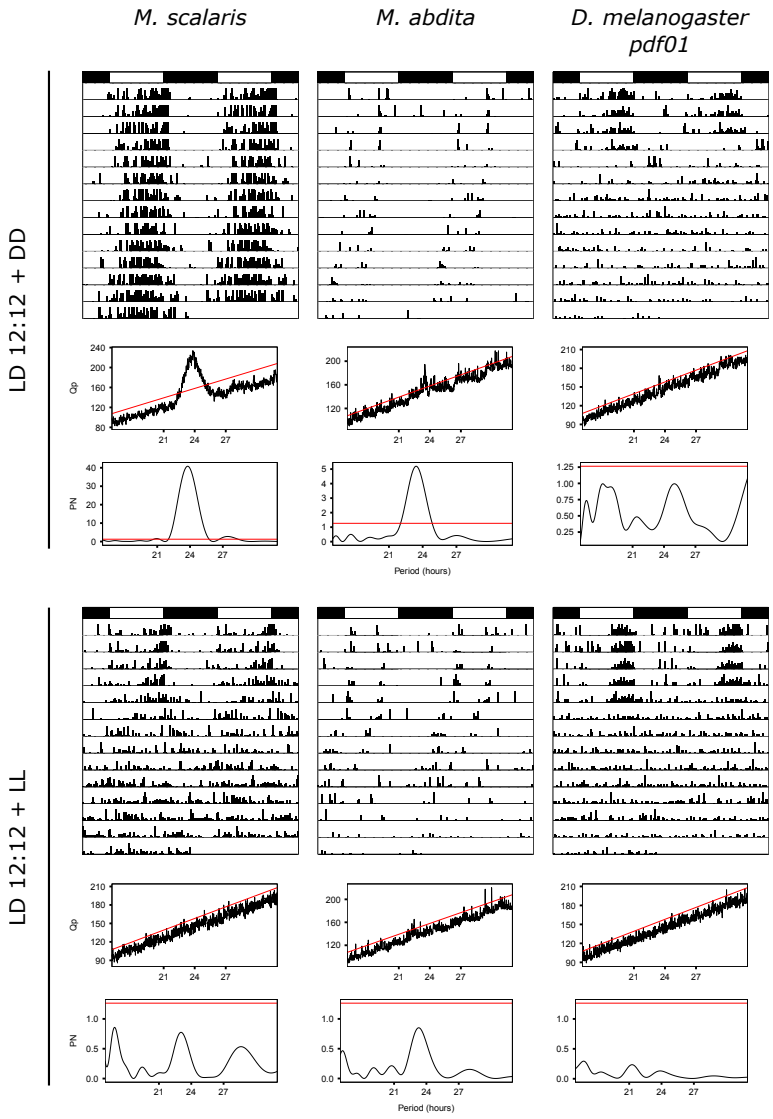

Figure 35: Representative actograms of *M. scalaris*, *M. abdita*, and *D. melanogaster* lacking PDF (pdf^{01}), entrained under at least 7 days of LD 12:12 (4 days are shown) followed by 10 days of DD at 20°C. χ^2 (Qp) and Lomb-Scargle (PN) periodogram analyses for the respective actograms are shown. The red line in the periodograms indicates the significance threshold ($p<0.05$).

5 Discussion

This thesis reports the characterization of the circadian biology of five insect species. Hereafter, my results are discussed in the context of each single genus.

5.1 A *D. melanogaster*-like clock in *C. costata* does not self-sustain under constant condition

The malt fly *Chymomyza costata* is characterized by strong photoperiodic larval diapause, which allows this species to survive to extremely severe winter conditions (Enomoto, 1981b; Koštál et al., 2011). In particular, the photoperiodic information plays a major role in the induction of such diapause (Enomoto, 1981a; Koštál et al., 2016), and *C. costata* is currently an emerging model in chronobilogy to tackle the so-called Bünning hypothesis, which predicts the involvement of the circadian clock in the photoperiodic time measurement (Bünning, 1936). My work characterized the clock of adult *C. costata* at behavioural, anatomical, and molecular level. This was performed in parallel to the investigation of the clock at the larval stage performed by our collaborators (Vladimír Koštál group, Biology Centre CAS, České Budějovice, CZ).

Like other Drosophilidae species that have been recently studied in our group, *Chymomyza* adult flies offered us another genus to investigate the organization of the clock network in flies, and in particular its adaptation to high latitudes. It was reported that some Drosophilidae species (*D. pseudoobscura*, *D. virilis*, *D. ezoana*, *D. littoralis*) differ from *D. melanogaster* in the expression of PDF and CRY in the LN_v (Hermann et al., 2013). In these species, PDF is not expressed (or show very diminished expression) in the s-LN_v, but it is expressed in the l-LN_v. Viceversa, CRY is not expressed in the l-LN_v, but it is expressed in all s-LN_v (5^{th} s-LN_v included). The expression of CRY and PDF within the LN_v was recently correlated with the ability of these flies (*D. ezoana* and *D. littoralis*) to adapt their locomotor activity to long photoperiods (Menegazzi et al., 2017). Indeed, these strains were collected in Northern Europe (Oulu, Finland, 65°N) and are therefore naturally exposed to the long summer days characteristic of high latitudes. Differently, *D. melanogaster* has tropical origin and it was shown that it cannot adapt its activity pattern to extreme summer photoperiods (i.e. LD 20:4). Here I could demonstrate that the clock network of *Chymomyza* highly resembles the clock network of the laboratory fruit fly *D. melangaster*. In particular, performing immunocytochemistry on the

central nervous system, I could show that the clock neuropeptides PDF, ITP, and the photoreceptor CRY are expressed in the same subsets of clock neurons like in *D. melanogaster* (Figure 13). In details, PDF is expressed in all LN_v (s-LN_v and l-LN_v) except the 5^{th} s-LN_v. ITP is expressed in the 5^{th} s-LN_v and in one LN_d. CRY is expressed in all the LN_v, in half of the LN_d, and in several dorsal neurons (DNs). Overall, although *C. costata* also co-habit Northern regions (one of the two strains investigated here was also collected in Oulu, Finland), it carries a *D. melanogaster*-like clock network. In order to test the model proposed by Menegazzi et al., 2017, I recorded locomotor activity of *C. costata* as a readout of the clock activity to understand how these flies cope with Northern long days.

So far, the circadian behaviour of *C. costata* has been only studied at the pre-adult stage, which is the stage at which the photoperiodic response is induced (Lankinen and Riihimaa, 1992; Lankinen and Riihimaa, 1997). Working with adult flies has several advantages in studying circadian clocks, and one of these is the possibility to easily record locomotor activity rhythms of single individuals. In contrast, at pre-adult stages the circadian clock of flies is often studied as rhythm in pupal eclosion, which is a population phenomena and it is driven by the effect called "circadian gating" that restrict eclosion to specific times of the day (Pittendrigh, 1954; Konopka and Benzer, 1971; Sheeba et al., 1999; Selcho et al., 2017). Early studies on eclosion rhythms of *C. costata* investigated the role of light and temperature in the entrainment of the circadian clock of *C. costata*, and showed that *C. costata* pre-adult clock is only weakly entrainable by light-dark cycles (LD), and rather more sensitive to temperature cycles (TC), showing higher rhythmicity under TC cycles given at decreased temperature (Lankinen and Riihimaa, 1992; Lankinen and Riihimaa, 1997). In particular, the free-running rhythm of *Chymomyza* could scarcely be observed at all under constant darkness, or constant light, in the eclosion of these flies. Only at low temperature authors observed weak free-running circadian gating of eclosion which damped relatively quickly, within 2-3 days (Lankinen and Riihimaa, 1997). Because of the rapid damping of the free-running rhythm in *C. costata*, the analysis of the underlying clock system was highly restricted.

I recorded locomotor activity of single adult *C. costata* under several conditions. Compared to measuring eclosion rhythm, individual adult behaviour can be recorded for long time (up to three weeks using my system at 20°C; longer when temperature is decreased because food preserves better). Another important aspect of my work was the possibility to work with a new strain of *C. costata*, collected in Oulu (Finland, 65°N) in 2012. Together with this new collected strain, I studied another

wild-type strain, *Sapporo*, which is used as a laboratory model from the ´80s (Riihimaa and Kimura, 1988). Importantly, *Sapporo* is the genetic background of the *npd* mutation (Riihimaa and Kimura, 1988), and the genome of this strain is now sequenced and under annotation thanks to the work of Vladimír Koštál and collaborators. Most importantly here, the *Oulu* strain did not go through excessive generations in artificial laboratory conditions, and this allows to exclude the often encountered undesirable effect of inbreeding. To be mentioned, the *Oulu* strain in my hands appeared to be much easier to maintain and handle in laboratory, and therefore it could become a convenient alternative to the classic *Sapporo* genotype.

In general, *Chymomyza* survived well in isolation in locomotor activity tubes, and the amount of activity measured under LD cycles or constant conditions (DD or LL) is comparable to the amount of locomotor activity of *D. melanogaster* strains which are well established models in our group (*Canton-S* or w^{1118}, Figure 38). My data clearly show that under LD cycles *C. costata* adults concentrate their activity within the light phase, both under short (LD 4:20, LD 8:16, LD 12:12) or long (LD 16:8, LD 20:4) photoperiods. The activity of *C. costata* increases constantly from the moment when light is turned on, without showing an evident siesta in the middle of the day as it is well know for *D. melanogaster* (Figure 14). Even though the activity peak of *C. costata* associated to dusk is not as sharp as in other species, the activity reaches its maximum (E-peak) before the light-off transition, and the timing of this peak could be quantified for single flies under long photoperiods (Figure 15). In contrast, the activity peak associated to dawn is not as prominent. The middle and last part of the day are the warmest hours in Northern summers, and consequently the most favourable time to support reproduction at high latitudes. Different to *C. costata*, *D. melanogaster* is a species which originated from equatorial regions, it is a corpuscular animal, and it decreases activity in the middle part of the day which is too warm at the tropics. It is to be noted that *Chymomyza* survives winters at the larval stage, therefore adult activity has probably not been shaped by short winter photoperiods. Nevertheless, *C. costata*, exposed to increasing or decreasing photoperiods, is able to modulate its activity pattern in response to these (Figure 14). When these results are compared to *D. melanogaster* mutant strains which carry an impaired circadian clock (per^{01}, tim^{01}, Clk^{Jrk}, Figure 37), it is clear that such an anticipatory behaviour under LD cycles is a clear circadian clock response and demonstrates that *C. costata* adults possess a functional circadian clock.

Despite originating from high latitudes, under very long photoperiod (LD 20:4, Figure 14) *C. costata* cannot stretch its activity profile towards dusk, but its E-peak

clearly anticipates the light-off transition by few hours (Figure 15). This behaviour is very similar to that of *D. melanogaster*. Viceversa, it was shown from our group that other drosophilids, collected at the same latitude, extend their locomotor actvity profile more towards dusk under long days (Kauranen et al., 2016; Menegazzi et al., 2017). These flies differ from *D. melanogaster* in the expression of CRY and PDF in the clock network. Taken together with the anatomical characterization of the clock discussed previously (Figure 13), *C. costata* shows a *D. melanogaster*-like clock network, which correlates with a *D. melanogaster*-like behaviour under LD conditions. This fits nicely with the model proposed by Menegazzi et al., 2017, and demonstrates that at high latitudes flies adopt different ways to respond to photoperiodic changes.

In this work I show that the clock of *C. costata* can entrain both to light and to temperature cycles. In the majority of the experiments, except where indicated, light was supplied at ∼100 lux, and this intensity was clearly enough to entrain the clock of *C. costata*, as demonstrated also by the molecular oscillation of PDP1ε within the clock neurons (Figure 22). Interestingly, early studies showed that eclosion rhythm of *C. costata* synchronizes better to temperature cycles rather than light cycles, and that this is enhanced at low temperature (Lankinen and Riihimaa, 1992; Lankinen and Riihimaa, 1997). Speculatively, the clock of *C. costata* might gain light sensitivity after the pre-adult/adult transition, but this has not been investigated further here.

More surprising was that the circadian rhythm in locomotor activity of *C. costata* does not persist when *Zeitgebers* are removed. In other words, when these flies are exposed to constant conditions (for example DD, LL, or several others that I tested), the circadian rhythm in their locomotor activity is lost. These results challenge the standard model known for *D. melanogaster*, where circadian rhythm in locomotor activity persists in DD (but not in LL) and free-runs with a defined period (τ) for several days (Helfrich-Förster, 2002). The same model is true indeed for the circadian biology of several other insect species reported in literature (Moore and Rankin, 1985; Fuchikawa and Shimizu, 2007; Pivarciova et al., 2016; Beer et al., 2016; Kay et al., 2018), as well as for the other main model organisms in chronobiology (*M. musculus*, *Homo sapiens*, etc.). The self-sustaining property of a circadian oscillator is one of the three main features generally reported to introduce circadian biology (see page 2).

In order to prove this unexpected feature of the circadian biology of *C. costata*, I recorded its locomotor activity under many conditions of light and temperature.

These are the following:

1. entraining flies under LD cycles of short or long photoperiods, and afterwards releasing them into DD at constant temperature (Figure 18);

2. entraining flies under LD cycles, and afterwards releasing them into constant light given at low light intensities at constant temperature (Figure 39);

3. entraining flies under LD cycles using single wavelength light (blue or red), and afterwards releasing them into constant wavelength light at different intensities at constant temperature (Figure 40);

4. entraining flies under TC cycles in constant darkness, and afterwards releasing them into constant temperature and constant darkness (Figure 19);

5. entraining flies combining LD and TC cycles, and afterwards releasing them into constant temperature and constant darkness (Figure 19).

Despite the efforts, no clear self-sustained rhythm under any of such conditions was found, as instead it is the case for *D. melanogaster* that has always been used as a positive control in my experiments. Of course, the few actograms presented in this thesis are only representative examples of *C. costata* behaviour for each shown condition. Nonetheless, experiments were carried out using several flies (usually one DAM monitor records 32 flies), but the single behaviour of all the flies could not be shown here because of obvious space limitation. Figure 41 is the effort to show the activity of all the flies from the same condition averaged together during the last days of entrainment and the first days in constant condition.
Although a first important analysis of actograms requires an expert visual inspection, the significance of biological rhythms has to be assessed by periodogram analyses (Refinetti et al., 2007). In order to detect circadian rhythmicity in *C. costata*, I have applied several powerful algorithms for the detection of rhythms. The periodograms χ^2 (Chi-Square, Enright, 1965) and Lomb-Scargle (Ruf, 1999) are generally used to determine the significance of rhythms and to calculate the free-running period. The χ^2 analysis did not detect rhythms in *C. costata* (Figure 16) The Lomb-Scargle periodogram is more sensitive in detecting rhythmicity in noisy activity patterns, but on the other hand, it is known to be susceptible to artifacts, and it often generates significant peaks which are not coherent (Refinetti et al., 2007). In order to render this analysis more robust, but at the same time also more strict, I have combined the outcome of the Lomb-Scargle analysis with the outcome of a third analysis named CLEAN. The CLEAN algorithm is used by the group of Bambos Kyriacou

(University of Leicester, UK) and it is known to be able to track also weak rhythms in *D. melanogaster* behaviours (Kyriacou and Hall, 1989; Rosato and Kyriacou, 2006). Combining the two peridogram analyses I have obtained a strict but unbiased way to detect putative significant rhythmicity in *C. costata* locomotor activity, that is not traceable in other ways. The results summarized in Figure 17 and Figure 44 show that such an analysis works very well since it detects rhythms in the totality of *D. melanogaster* under DD.

In *C. costata* the percentage of rhythmic flies under DD at 20°C detected by this procedure (Lomb-Scargle + CLEAN) is extremely low (Figure 17). These levels of rhythmicity are actually comparable with those detected by the same analysis in *Drosophila* clock mutants (per^{01}, Clk^{Jrk}, pdf^{01}, $pdp1^{3135}$, Figure 17), subjected to the same condition, or to wild-type *D. melanogaster* exposed to constant light (LL, Figure 17), suggesting that significant peaks might still be generated by artefacts. Indeed, the period (τ) and the power (PN) of the rhythms detected are very inconsistent or very low, respectively (Figure 44), for all conditions tested. This indicates that the detected rhythmicity in *Chymomyza* is most likely not derived from a functional underling circadian oscillator for all the experiments. On the contrary, the rhythms detected by this method in *D. melanogaster* are of high power, and the estimated periods are all within a circadian range ($24<\tau<26$ for *Canton-S*, Figure 44). As a comparison, the reader is invited to look at the results obtained performing this analysis for the scuttle fly *M. abdita*, for which, although these flies exhibit extremely low amount of activity under DD, the Lomb-Scargle analysis clearly identifies a significant peak corresponding to the free-running period (Figure 35).

When this same procedure was applied to detect rhythmicity under DD of *C. costata* previously entrained under longer photoperiods (LD 16:8 and LD 20:4), the percentage of detected rhythmic flies slightly increased (Figure 21). As already mentioned, *Chymomyza* survives winters into a diapausing larval stage, therefore adult activity has not been shaped by short photoperiods (i.e. the standard LD 12:12). Nonetheless, the same low power and inconsistency of the detected rhythms, discussed previously, is also arguable for these conditions (Figure 44). A similar outcome is obtained when flies are entrained combining light and temperature (LD + TC), and subsequently released into DD at constant temperature (Figure 21). Altogether, the behavioural data that I have accumulated so far, seem to point out that, at 20°C, *C. costata* rhythm in locomotor activity does not self-sustain under constant darkness.

Interestingly, when experiments were carried out at lower temperature (17.5°C or

15°C, Figure 20), the same analysis showed that the percentage of flies with significant rhythm, detected by the periodogram analysis (Lomb-Scargle + CLEAN), increases (Figure 21). Most importantly, at 15°C, I could visually spot out the only two cases of free-running behaviour observed for *C. costata* under constant darkness (Figure 43), both for the *Sapporo* and *Oulu* strains. These two actograms are not reported in the main text (Figure 20) because they are definitely not representative for the condition. The two flies also show completely opposite free-running periods, with *Sapporo* showing a very slow endogenous clock, and *Oulu* showing a fast clock. Although these are very rare examples, it is a matter of fact that this is the first and only time that the free-running period of *C. costata* is observed. I reckon that *C. costata* activity under lower temperature is characterized by several rhythmic weak components, which can be visually tracked only over few cycles in few flies but their analysis is very complex. In other studies, individuals that show such multiple components in their activity, and therefore cannot be clearly classified as rhythmic or arrhythmic, are classified as "*complex*" (Helfrich et al., 1985; Nitabach et al., 2006; Choi et al., 2009; Dissel et al., 2014; Beckwith and Ceriani, 2015; Pivarciova et al., 2016). Nevertheless, the "complex rhythmicity" that is described in these studies is way more "rhythmic" that any of those I could observe for *C. costata*. The molecular and neuronal basis of such complex rhythms are yet not completely understood also for *D. melanogaster*, but they are most likely generated by desynchronized oscillators. Based on my current results, it is not possible to claim that *C. costata* shows complex rhythmicity under constant darkness, but overall the circadian clock of *C. costata* seems to become more stable when temperature are decreased.

Locomotor activity is definitely the most adopted way to record the clock activity in flies and other insects, but for *C. costata* this might not be a suitable readout of the circadian clock activity. I have therefore tested other possibilities to record the output of the master clock activity, for example measuring feeding activity. Feeding is indeed known to be controlled by the circadian clock in *D. melanogaster* (Xu et al., 2008). I tried to adapt the automated FLIC assay (Fly Liquid-Food Interaction Counter, Ro et al., 2014) to record feeding activity of *C. costata*. Unfortunately, this system is not yet well optimized for long term recording also for *D. melanogaster*, and I could not generate a satisfactory dataset because of the short lifespan of *C. costata* in the arenas and the high noise-signal ratio obtained. Therefore, I could not find alternative measurements, other than locomotor activity, to record long term individual behaviour of *C. costata*.

I reasoned that *C. costata* arrhythmic behaviour observed under constant darkness

might be explained by at least two models:

1. the circadian clock of *C. costata* is functional (self-sustained) under DD, but it is uncoupled from its output (locomotor activity);

2. the circadian clock of *C. costata* is not functional (not self-sustained) under DD at all.

In order to answer to this important point I took advantage of using flies (Diptera), since it is well known that their master clock is localized in a limited number of brain neurons (Helfrich-Förster, 2005b). Performing immunocytochemistry against the core clock protein PDP1ε, I could observe the molecular oscillation of the clock within single clock clusters also in *C. costata*. The antibody against PDP1 (Reddy et al., 2000), which recognizes the core clock protein PDP1ε in the nuclei of the clock cells, has revealed to be a very reliable tool to perform inter-specific studies (Figure 13). By performing ICC at several timepoints across the LD cycle, I demonstrate that the level of PDP1ε oscillates within the lateral clock neurons (s-LN_v, 5^{th} s-LN_v, l-LN_v, LN_d, Figure 22), and such an oscillation is consistent in phase with the oscillation of PDP1ε known for *Drosophila* (Cyran et al., 2003). It is also well known that PDP1ε in *D. melanogaster* oscillates under DD up to several days (Nitabach et al., 2006; Dissel et al., 2014; Figure 22). In contrast, the PDP1 oscillation dampens during the first day in constant darkness in *C. costata* (Figure 22). The two strains show differences in their response: *Oulu* (entrained under LD 16:8) seems to be more rhythmic that *Sapporo* (entrained under LD 12:12). It has to be noticed that higher percentage of rhythmicity under DD is detected when flies are previously entrained to longer photoperiods (Figure 21). We are currently investigating the molecular oscillation of PDP1ε in *Oulu* after 5 days of constant darkness. Most importantly, the reduced or lost PDP1 oscillation that we find under constant darkness is correlated with the arrhythmic behaviour observed in these flies under the same condition. We are currently expanding this view also to another drosophilid collected at high latitude, *Drosophila ezoana* (Menegazzi et al., 2017). Taken together, my work demonstrates that the molecular clock of *C. costata* oscillates under light-dark cycles, but that this oscillation dampens in constant darkness. This explains why the flies are rhythmic under light-dark cycles, but arrhythmic under constant darkness. Future studies have to reveal the reasons why the molecular clock dampens under constant conditions.

Polar and subpolar regions are exposed to relatively constant light (LL) during polar summers or constant darkness (DD) during polar winters (Figure 1). One

would speculate that organisms that inhabit such high latitudes would possess a strong clock to keep measuring time during these months. Nevertheless, an highly dampening clock for the Holarctic malt fly *C. costata* was suggested for eclosion rhythms in early studies (Lankinen and Riihimaa, 1992; Lankinen and Riihimaa, 1997; Yoshida and Kimura, 1995). With my work I am expanding this concept also to the adult stage.

As well as for *Chymomyza*, arrhythmicity under constant conditions is also reported for several other Holarctic flies, like *Drosophila littoralis* (Lankinen and Forsman, 2006; Menegazzi et al., 2017), *Drosophila montana* (Kauranen et al., 2012), and *Drosophila ezoana* (Vaze and Helfrich-Förster, 2016; Menegazzi et al., 2017). We are currently recording locomotor activity of additional *Chymomyza* species (*C. procnemis*, *C. pararufithorax*, *C. amoena*) which were collected at low latitudes. Our preliminary results clearly indicate that these species are, in contrast to *C. costata*, strongly rhythmic under DD (data not shown). Interestingly, there are several cases reported in literature of animals, not only for flies, that show arrhythmic behaviour under Northern constant condition. This is true for arctic mammals like the ground squirrel (*Spermophilus parryii* or *Urocitellus parryii*) and the European hamster (*Cricetus cricetus*) that undergo underground hibernation during the harsh winter season (Hut et al., 2002). Also non-hibernating reindeers (*Rangifer tarandus*) are virtually arrhythmic during the times of the year at which they are naturally exposed to relatively constant conditions (Van Oort et al., 2007). This happens during November to January, when the sun remains constantly below the horizon, and during April to September, when it remains above the horizon. Highly decreased rhythmicity under DD for these species have been shown for rhythms in body temperature (Hut et al., 2002; Williams et al., 2012b; Williams et al., 2012a), activity (Van Oort et al., 2007), melatonin secretion (Stokkan et al., 2007; Lu et al., 2010), and gene expression (Revel et al., 2007; Lu et al., 2010). Taken together, all these data point out that there is correlation between adaptation of life at high latitudes with the loss of self-sustained rhythm under constant darkness.

This aspect, actually, could be turned into the following question: why does a biological clock have to persist under constant condition? Or the following: why species like *D. melanogaster*, which originate from equatorial regions, have a clock which self-sustain in DD? There is no doubt that the endogenous rhythm that many animals (*D. melanogaster*, *M. musculus*, and many others) exhibit when *Zeitgebers* are removed has been crucial for discovering and understanding the mechanism of endogenous oscillators (Konopka and Benzer, 1971). Nonetheless, it is clear that

constant environments seldom occur in nature, and that constant darkness (DD) or constant light (LL) at constant temperature are merely artificial environments that are recreated in laboratory. There are probably only few natural habitats where organisms are exposed to such strict constant conditions; one might think for example at deep-sea, subterranean cave, or indeed polar habitats (Beale et al., 2016). Most likely, the clock did not evolve in order to support rhythmicity under such unnatural conditions, but it rather evolved to organize the time-structure of life in a rhythmically changing environment. I speculate that a clock that is functional under rhythmic environment (i.e. LD cycles), but does not self-sustain under DD (or LL), would be equally well fulfilling its coordinating and anticipatory function as good as a clock which keeps ticking for weeks in constant condition. Although Subarctic habitats are periodically characterized by relatively constant light or darkness conditions during summers and winters, one cannot exclude that factors other than light (small temperature oscillations, changes in light intensity and composition during the day, moon cycles, and probably other parameters) are used by flies, and probably other animals, to understand the time of the day.

5.2 The Brachycera clock of *B. oleae* for improving future pest management strategies

Among Diptera, the insect family Tephritidae includes several species that are of major economic importance in agriculture, for example the olive fly *Bactrocera oleae* (Rossi). Modern pest management approaches require the understanding of the life-history traits of these flies, yet very little is known about the mechanisms of the circadian clock of *Bactrocera* species. In contrast, the fruit fly *D. melanogaster* (Diptera: Drosophilidae) has been widely adopted as the primary insect model. While both *Bactrocera* and *Drosophila* are classified as fruit flies, they actually belong to quite separate evolutionary lineages (Figure 7). I characterized the circadian clock of *B. oleae* in collaboration with Dr. Christa Kistenpfennig and Dr. Martha Koukidou from the English biotechnology company Oxitec ltd (www.oxitec.com). One relevant aspect of our study was the investigation of Oxitec self-limiting strain, *OX3097D-Bol*, which offers an alternative control strategy to chemical interventions (Ant et al., 2012).

Based on the homology of known clock genes sequences from other closely related species reported in Genbank (*D. melanogaster*, *C. capitata*, *B. tryioni*, *B. curcubitae*, see Bertolini et al., 2018), we identified the clock genes *per*, *Clk*, *cyc*, and *cry* of the olive fly *B. oleae* using the RACE protocol (RACE, Rapid Amplification of cDNA Ends, Yeku and Frohman, 2011). When aligned with their respective *Drosophila* and *Bactrocera* counterparts, the predicted amino acid sequences of *B. oleae* show high identity levels, particularly within the characterized functional domains (Bertolini et al., 2018). These four genes take all part in the central core mechanism and in the light entrainment of the circadian clock in *D. melanogaster* (higher Diptera, Brachycera). However, other Diptera species, such as mosquitoes and sandflies (lower Diptera, Nematocera), have been shown to carry the mammalian form of the gene *cyc* (Meireles-Filho et al., 2006), also called *Bmal1*. This alternative form encodes a peptide that carries an extra domain at the C-terminus (BCTR, BMAL C-terminus region) working as transactivation domain required for binding and activating transcription of the *per* gene. Most probably because of this, *cyc* is rhythmically expressed in mosquitoes (Meireles-Filho et al., 2006; Meuti et al., 2015), whereas in *Drosophila* and other Brachycera it is constantly expressed (Bae et al., 2000; Codd et al., 2007; Chahad-Ehlers et al., 2017). In contrast, the CLK protein of Brachycera possesses a transactivation domain (multiple poly-Q sites at the C-terminus) and it is responsible for activating *per* transcription and oscillates

in abundance (Lee et al., 1998). The CLK protein of Nematocera is considerably shorter than in higher Diptera and neither oscillates in abundance nor binds and activates *per* (Meireles-Filho et al., 2006; Meuti et al., 2015). Most likely, the CYC and CLK found in lower Diptera are the ancestral forms that are conserved in most insects and mammals. As proposed by Chahad-Ehlers et al., 2017, during evolution, the higher Diptera (Brachycera) seem to have lost the BCTR activation domains at the C-terminus of CYC but gained the poly-Q repeats at the C-terminus of CLK.
Like *cyc* and *Clk*, even the gene *cryptochrome* is found in two distinct variants in Diptera: *cry1* and *cry2*. CRY1 is the only cryptochrome present in *Drosophila*, it is light sensitive and acts as the main circadian photoreceptor in the master clock of *Drosophila* (Emery et al., 1998; Stanewsky et al., 1998). In contrast, CRY2 is the mammalian form of cryptochrome, it is not light-sensitive, and it is instead involved in the core feedback loop (Sandrelli et al., 2008; Yuan et al., 2007). So far, CRY2 is described only in non-Drosophilidae species (Zhu et al., 2005), such as mosquitoes (Nematocera) that express both types of cryptochromes (Gentile et al., 2009; Meuti et al., 2015). In other insect orders, for example Lepidoptera and Hymenoptera, the clock scenario gets more complex. Nevertheless, all so far investigated species express the mammalian form of CYC, CLK, and CRY2 (Sandrelli et al., 2008; Chang et al., 2003; Rubin et al., 2006; Tomioka and Matsumoto, 2015).
Merely because of limitations of our methodology (RACE), we did not identify all the core components of the Brachycera circadian clock, like for example *tim*, *vri*, and *Pdp1*. However, in the meantime the genome of the olive fly has been made available by the i5k consortium (Evans et al., 2013), and it is currently under annotation. In particular, processes of automatic annotation (prediction method: Gnomon) did predict the presence of the *cry2* locus in *B. oleae* (XP_014103186), and also in other *Bactrocera* species (*B. dorsalis*: XM_011211743; *B. latifrons*: XM_018933656). Based on our results, it was very unlikely that this genus carries also the vertebrate form of CRY2. Indeed, my phylogenetic analysis showed that the genes annotated as *cry2* in *Bactrocera* clusters together with photoloyases characterized in other insects (flies, mosquitoes, butterflies, Figure 45). These sequences clearly cluster separately from the CRY2 forms, which are instead annotated in the genome of Lepidoptera and Hymenoptera (Figure 45). This analysis allows to exclude the presence of CRY2 in *B. oleae*, confirming that the olive fly carries clock components that belong to the *Drosophila*-type (perhaps better described as Brachycera-type). Our phylogenetic analysis also supports the notion of dramatic changes in the evolutionary history of the clock genes in Brachycera in comparison to other insects (Bertolini et al., 2018).

At the anatomical level, several studies have shown that the distribution of certain neuropeptides in the clock network are quite different within closely related fly species. For example, drosophild species that inhabit cold habitats (high latitudes) differ in their PDF expression pattern from *D. melanogaster*, as they lack PDF expression in the s-LN_v (Bahn et al., 2009; Hermann et al., 2013; Menegazzi et al., 2017). Here, I show that the olive fly, like *D. melanogaster*, expresses PDF in both s-LN_v and l-LN_v. Additionally, I demonstrate that the expression of the other central clock neuropeptide ITP is highly conserved (Figure 24). In conclusion, the clock network in the brain of *B. oleae* shows high homology to that of *D. melanogaster*. Importantly, it is even more similar to *D. melanogaster* than the clock network of Drosophilidae from higher latitudes, like *D. ezoana* and *D. littoralis*. Since *B. oleae* prevalently inhabits warm habitats, mostly around the Mediterranean area, this underscores the hypothesis that changes in the clock network preferentially happened in species that radiated to the North, but not in flies that remained in subtropical regions (Menegazzi et al., 2017).

Despite the high mortality rate and the low activity of isolated olive flies, I could show that *B. oleae* locomotor activity is under the control of the circadian clock. The rhythmic locomotor activity pattern of *B. oleae* persists in the absence of *Zeitgebers* under constant darkness (DD) at constant 20°C (Figure 26). The relative low activity level of olive flies might be due to their natural life style. In the wild, Bactrocera flies (especially males) spend much of the day resting in the canopy of trees (Ero et al., 2011), with resource foraging and mating behaviours performed at temporally distinct times (Raghu and Clarke, 2003). It is also known that laboratory mass rearing further decreases the tendency of flies to move (Weldon et al., 2010). The lack of natural environmental stimuli in the glass tubes may also lead to the observed low activity. Nonetheless, the activity level of the majority of flies was high enough to reveal circadian rhythms in locomotor activity under LD cycles and DD conditions. Importantly, rhythmicity under DD is consistent with the finding that PDF is expressed in the s-LN_v in *B. oleae* (Figure 24). These neurons have been shown to be essential for robust activity rhythms under constant darkness in *D. melanogaster* (Shafer and Taghert, 2009; Menegazzi et al., 2017). On the other side, *B. oleae* is arrhythmic under constant light (LL, Figure 26). The same is observed in *D. melanogaster* and can be explained by the permanent degradation of the clock protein TIM via the light-sensitive CRY1 (Ceriani et al., 1999).

Taken together, our results clearly depict from three distinct points of view (molecular, anatomical, and behavioural), that the general organization of the olive fly

circadian clock highly matches the well known clock of *D. melanogaster*.

Despite the continued broad use of pesticides to keep insect pest populations under control, reduced or eliminated pesticide use is getting the consensus of growers and consumers as the more sustainable way to manage pest species in agriculture. Among these, the Sterile Insect Technique (SIT) is already applied in modern agriculture to fight several Tephritidae species such as the Mediterranean fruit fly (*Ceratitis capitata*), the Oriental fruit fly (*Bactrocera dorsalis*), the Melon fly (*Bactrocera cucurbitae*) and the Mexican fruit fly (*Anastrepha ludens*) (Aketarawong et al., 2014; Ito et al., 2003; Hendrichs et al., 2012). The olive fly *B. oleae* was among the first species where SIT has been tested in the field. Unexpectedly, this method has proven to be inefficient in the control of *B. oleae* in the past, and one of the suggested reasons for the low success is the mating asynchrony reported between wild and laboratory-reared individuals (Zervas and Economopoulos, 1982; Economopoulos, 1972). In these studies, nevertheless, the potential role of the circadian clock was not taken into account.

Our collaborators from Oxitec ltd (www.oxitec.com) generated the first olive fly self-limiting strain, *OX3097D-Bol*, which could become a key agricultural strategy to control olive fly infestations (Ant et al., 2012). In order to test *OX3097D-Bol* suitability for operational use, we characterized the circadian behaviour of this olive fly genotypes. My results show that the endogenous period of *OX3097D-Bol* does not differ from the wild-type genotypes (Figure 28). In addition, we characterized the temporal expression of the clock genes *per*, *Clk*, *cyc*, and *cry* in *OX3097D-Bol*, and compared their expression with the wild-type strains. We did not detect any significant difference in the cycling of these clock genes among wild-type and transgenic strains (Bertolini et al., 2018). This supports the fact that the circadian clock of this transgenic strain is functional and unaltered from the wild-type strains, excluding any major clock alteration in this genotype.

Interestingly, *OX3097D-Bol* showed higher overall locomotor activity, especially associated with the evening, when compared to the other strains (Figure 46) . This increased evening activity overlaps with the timing of mating that is known to occur in the hours prior to scotophase under both natural and artificial conditions in Bactrocera (Loher and Zervas, 1979). Whether this feature may be advantageous to future operational applications was not investigated here. However, increased male activity at the time of mating has been directly linked to increased male mating success in other Bactrocera species (Kumaran et al., 2014). It is reasonable therefore to speculate that increased locomotor activity of *OX3097D-Bol* males during

the time window of female receptivity could increase mating success.
In conclusion, our results highlight that the clock structure remains intact in the self-limiting olive fly strain *OX3097D-Bol*, and together with previously published data (Ant et al., 2012) we conclude that sustained release of *OX3097D-Bol* males is a viable strategy for olive fly pest control.

5.3 The PDF brain network of *P. apterus* highly resembles the *D. melanogaster* organization

The firebug *P. apterus* is a convenient insect model for laboratory studies, because of its easy husbandry and the availability of several genetic and molecular tools (Syrová et al., 2003; Bajgar et al., 2013; Kotwica-Rolinska et al., 2017). Relating to chronobiology, *P. apterus* is becoming an important model to study the interactions between the circadian and the photoperiodic timing system, because it exhibits robust and reliable rhythms in locomotor activity and robust photoperiodic adult diapause (Doležel, 2015). Although the investigation of the circadian clock of *P. apterus* has been addressed already at behavioural and molecular levels (Pivarciova et al., 2016; Kotwica-Rolinska et al., 2017), the anatomical localization of its master clock has not been demonstrated yet.

"In order to localize the clock of *P. apterus*, I performed immunocytochemistry against central clock components on its brain. In this thesis, the bug (Hemiptera) is the species phylogenetically most distant from our central insect model *D. melanogaster* (Figure 7). Not surprisingly, most of the antibodies generally used to highlight the clock network of *D. melanogaster* were not functional when tested in *Pyrrhocoris* (Table 1). Nonetheless, because neuropeptides are highly conserved among Hexapoda, I was successful in describing the distribution of the clock neuropeptide PDF in the brain of *Pyrrhocoris*.

The organization of the PDF network of *P. apterus* strongly resembles that of *D. melanogaster*. Several PDF$^+$ cell somata cluster in the proximity of the optic lobe (I refer to them as PDFMe, or LN_v, Ikeno et al., 2014), and send projections to the dorsal protocerebrum, to the medulla, and to the controlateral hemisphere (Figure 29). Surprisingly, it was also possible to classify these PDF$^+$ neurons in two separate clusters based on their size and their location in the brain. I could identify a more dorsal cluster of cells, characterized by large somata, and a more ventral cluster of cells, characterized by small somata (Figure 29). This classification, although based only on morphological criteria, defines high homology with the small and large lateral-ventral-neurons (s-LN_v and l-LN_v) of *D. melanogaster* (Figure 4). Differences in the size of PDF cells are reported also in other Hemiptera, like the bean bug *Riptortus pedestris* (Ikeno et al., 2014) and the kissing bug *Rhodnius prolixus* (Vafopoulou and Steel, 2012). In order to strengthen the homology with the *Drosophila* lateral ventral cluster (LN_v), the co-localization of PDF with other clock neuropeptides might help. For example, *D. melanogaster* co-expresses PDF with

sNPF only in the s-LN_v, and PDF with NPF only in the l-LN_v (Figure 4). Unfortunately, both anti-sNPF (Johard et al., 2009) and anti-NPF (Shen and Cai, 2001) antibodies were not functional to *P. apterus* in my hands and I could not confirm this homology with neurochemistry.

Even though these PDF^+ cells highly resemble the lateral-ventral clock neurons of *D. melanogaster*, it has to be proven that such cells express indeed the clock in the bug. In order to prove this, I tested several antibodies raised against central core clock proteins that are known to play a role in the generation of the TTFL in insects (Figure 8). These are the following: - anti-PER *D. melanogaster* (Stanewsky et al., 1997); - anti-PER *Eurydice pulchra* (Zhang et al., 2013); - anti-PER *Apis mellifera* (Beer et al., 2018); - anti-VRI *D. melanogaster* (Glossop et al., 2003); - anti-PDP1 *D. melanogaster* (Reddy et al., 2000). Eventually, only the antibody raised against *Drosophila* PDP1 stained the nuclei of several cells across the protocerebrum, distributed laterally and dorsally (Figure 30). Since I have gained high confidence in using this antibody for inter-specific studies (I have used it to highlight the clock network of *C. costata*, *C. pararufithorax*, *C. procnemis*, *D. ezoana*, *B. oleae*, *C. capitata*, *M. scalaris*, *M. abdita*, *Nasonia vitripennis*) I personally believe that the staining obtained for *Pyrrhocoris* suggests specificity for the PDP1 protein, although comparison among species phylogenetically so distant is complex. As in *D. melanogaster* and in the other species listed above, PDP1 nuclear staining is not limited only to the clock cells (where only the PDP1ε is expressed), but it is widely spread in presumably all brain regions (where all the other isoforms are most likely expressed).

One main problem that I faced, in performing ICC on *P. apterus*, was the low permeability of some antibodies to inner brain regions. This was clear from the analysis of several image stacks, and I confirmed this also by performing ICC with the anti-SYN antibody (Klagges et al., 1996), as it could mark very well the neuropiles in the outer parts of the brain but not those in the inner parts (Figure 29). In order to increase the permeability of brain tissues, I tested some alternative protocols, for example adding Zinc in the fixative (ZnFA, Ott, 2008). Unfortunately, I could not develop a satisfactory protocol to perform high quality ICC in the large brain of *P. apterus*, therefore I am not able to describe the distribution of PDP1 in the whole protocerebrum. The only improvement of the quality of the anti-PDP1 staining was achieved when I performed ICC on brain agarose slices (Figure 30).

Since the PDF^+ cells of the bug are located in proximity to the optic lobe, the signal in this outer region seemed to be not severely affected by the permeability issue.

Despite the high variability of the staining among samples, in several preparations it was very clear that strong PDP1 staining is found in cell clusters in close vicinity of the PDFMe (Figure 30). Surprisingly, I could not detect PDP1 signal within the PDF cells in whole-mount staining, contrary to many other insects (Hermann et al., 2013; Bertolini et al., 2018). On the other hand, when I performed ICC on brain agarose slices, I could highlight a very weak but clear PDP1 signal in some PDF^{+} neurons, not in all, of *P. apterus*. Taken together, these results show that PDP1 is strongly expressed in the proximity of the PDFMe, but only weakly expressed by the PDF cells. As the PDP1 level is well known to oscillate under LD cycles (Cyran et al., 2003), I performed ICC at several timepoints across the day (ZT0, ZT6, ZT12, ZT18). Nevertheless, no increase of PDP1 signals within the PDF cells was found at any of the collected timepoints.

Although several models of the composition of the TTFL in insects have been proposed (Figure 8), very little is known about the putative second feedback loop in the clock of Hemiptera. The core clock protein PDP1 plays a major role in the second feedback loop of the *Drosophila* TTFL (Figure 3), but its role in other species is poorly described. My immunocytochemistry results suggest that, PDP1 expression in *Pyrrhocoris* differs from that of *Drosophila* model, but for *P. apterus* the specificity of the antibody could be argued because of the lack of a negative control. In order to test whether the anti-PDP1 antibody is specific for PDP1 also in *P. apterus*, I performed RNAi against *Pdp1*. This knock-down should result in subsequent decreased staining intensity when performing ICC. Unfortunately, I was not able to prove decreased intensity of anti-PDP1 staining after dsRNA injections because of technical problems.

Nevertheless, the efficacy of *Pdp1* knock-down was tested recording locomotor activity of bugs under LD cycles and constant darkness (DD). The knock-down of *Pdp1* expression using RNAi results in impaired locomotor activity rhythms under DD (Figure 31). This suggests that *Pdp1* could be involved in the generation of the TTFL also in Hemiptera, like it does in the second feedback loop of *D. melanogaster*. Nevertheless, it has to be observed that the *Pdp1* gene in *D. melanogaster* generates several isoforms (13 transcripts in *D. melanogaster*), but only the ε isoform seems to be involved in the generation of circadian rhythms (Cyran et al., 2003). Since clock genes of *P. apterus* were not completely characterized at the time I performed the knock-down, it was not possible to specifically target the putative *Pdp1*ε isoform. Most likely the functions of other non-clock PDP1 isoforms were also manipulated. Although the arrhythmic locomotor activity in DD is a clear sign of an impaired

circadian clock, it cannot be excluded that the arrhythmicity is caused by other factors.

Overall, the anti-PDP1 antibody seems to be a promising tool to unravel the localization of clock cells in *P. apterus* (and perhaps in other Hemiptera species), but additional investigations are required to test the specificity of this antibody, the co-localization of PDP1 within the PDF cells, and the anatomical characterization of the other putative clock clusters.

5.4 PDF expression in the brain is not required for circadian rhythms in *Megaselia*

The genus *Megaselia* is among the largest groups within the insect family Phoridae, which includes flies that resemble fruit flies, but belongs to a quite separate evolutionary lineage (Figure 7). The circadian clock of this family was so far investigated only for the scuttle fly *M. scalaris* so far, characterized as rhythms in locomotor activity, oviposition, and eclosion (Bostock et al., 2017). Although it seems clear that *M. scalaris* carries *D. melanogaster*-like circadian behaviour, the molecular and the anatomical bases of such rhythmicity have not been investigated yet. In order to gain a wider view over circadian clocks in this family, I included in my analysis an additional species, the humpback fly *Megaselia abdita*.

Surprisingly, ICC performed against the clock neuropeptide PDF, using three different primary antibodies (Table 1), did not reveal PDF expression in the brain of both *Megaselia* species (Figure 32). Generally, PDF is a highly conserved neuropeptide among Hexapoda, and its expression in the brain is described for several arthropods: flies (Hermann-Luibl et al., 2014; Codd et al., 2007), butterflies and moths (Homberg et al., 1991; Závodská et al., 2012), bees (Beer et al., 2018), ants (Kay et al., 2018), bugs (Vafopoulou et al., 2010), crustaceans (Strauss et al., 2011). In particular, the antibody raised against PDH (the PDF ortholog, Dircksen et al., 1987) is the best tool available to highlight PDF expression across species (Menegazzi et al., 2017; Beer et al., 2018; Bertolini et al., 2018; Kay et al., 2018). In order to test the specificity of anti-PDH antibody for *Megaselia*, I performed ICC on the abdominal ganglion of both species. It is known indeed that *D. melanogaster* expresses PDF also in a small group of cells in the ventral nerve cord (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1997; Shafer and Taghert, 2009). Importantly, in both *Megaselia* species I could detect strong PDF signal limited to few specific cells, symmetrically distributed across the anterior-posterior axis, which resemble the PDF^+ abdominal cells of *Drosophila* (Figure 32). These PDF^+ cells are most likely not clock cells, as it is known for *D. melanogaster*, and they have not been further investigated here. Most importantly, this outcome acts as a positive control in claiming that PDF is indeed not expressed in the brain in *M. scalaris* and *M. abdita*, and at the same time it indicates the presence of the *pdf* locus in this genus genomes.

To my knowledge, only the pea aphid *Acyrthosiphon pisum* (Homoptera: Aphididae) among insects seems to lack PDF expression in the brain, but in this case the *pdf*

locus seems to be absent from its genome (Barberà et al., 2017). Also ancient species such as the firebrat *Thermobia domestica* (Thysanura: Lepismatidae) (Závodská et al., 2003) and the crustacean *Daphnia pulex* (Cladocera: Daphniidae) (Strauss et al., 2011) express PDF in the brain. Therefore, the *Megaselia* clock network, perhaps the one of the whole Phoridae family, seems to be a fascinating exception among arthropods.

Astonishingly, the absence of PDF expression in the brain of these two *Megaselia* species does not impair the circadian clock. When I recorded locomotor activity of *M. scalaris* and *M. abdita* under several conditions, the behaviour of both species was rhythmic under both LD and DD (Figure 35). On the contrary, in *D. melanogaster* PDF expression in the LN_v is essential for the coordination of the molecular oscillations between the different clock clusters and for the control of its output (Renn et al., 1999; Lin et al., 2004).

Under LD cycles, both *Megaselia* show a peculiar daily locomotor activity pattern, characterized by a morning (M-) and an evening (E-) peak (Figure 33). The occurrence of these two activity peaks is easily traceable, especially in *M. abdita*, where the daily amount of activity is very low, and the peaks are very prominent, sharp, and consistent among flies (Figure 34). In contrast, *D. melanogaster* strains that lack PDF (pdf^{01}) or PDF receptor (han^{5304}), do not exhibit the M-peak, or, at least, the M-peak of activity is very low and it cannot be quantified (Figure 35 and Figure 37). Additionally, the E-peak of *Megaselia* is significantly advanced, as it anticipates the light-off under all photoperiods (except under LD 4:20 in *M. scalaris*, Figure 33). Such an advance in the timing of the E-peak is known to happen also in pdf^{01} and han^{5304} flies (Figure 33 and Figure 37). Therefore, if on one hand the absence of PDF in the clock network of *Megaselia* does not influence the ability to generate the M-peak, on the other it causes advanced timing of the E-peak that phenocopies the behaviour of *Drosophila* which lacks PDF. Interestingly, it is clear that these two peaks in both *Megaselia* are strongly coupled. When the timing of M- and E-peak is quantified, the phase angle ($\Psi_{M,E}$) of the two oscillators maintains constant under increasing or decreasing photoperiods (Figure 34). In *D. melanogaster* PDF is required to coordinate the phase and amplitude of circadian rhythms among the diverse pacemakers (Lin et al., 2004). As both *Megaselia* lack PDF expression in the brain, these species have evolved an alternative system which compensate for the lack of PDF signalling to maintain coordination among clock clusters.

Under constant darkness (DD), the rhythm in locomotor activity of *Megaselia* self-sustains and free-runs with a clear circadian period (Figure 35). As already men-

tioned, *M. abdita* shows a very low amount of activity when isolated into locomotor activity tubes, and probably because of this lower percentage of rhythmicity is detected (Table 3). Despite this, there is no doubt that both *Megaselia* species clearly sustain rhythmicity under DD without expressing PDF in the brain. Even *D. melanogaster* strains that lacks PDF signalling (pdf^{01} or han^{5304}), retain weaker but significant circadian rhythmicity under DD (Figure 36), as it is already reported in literature (Renn et al., 1999; Lin et al., 2004; Mertens et al., 2005; Lear et al., 2005; Hyun et al., 2005; Im and Taghert, 2010). My results on *M. scalaris* and *M. abdita* supports the notion that PDF is not the only responsible factor for maintaining circadian oscillations under constant darkness in diptera.

Under constant light (LL), the circadian clock of *D. melanogaster* is impaired because of the constant degradation of TIM by CRY (Ceriani et al., 1999). Whereas *M. scalaris* shows arrhythmic behaviour in the totality of flies, some *M. abdita* individuals show clear free running rhythm under LL, at least traceable during the first few days in constant condition (Figure 35). This rhythmicity is also detectable by the Lomb-Scargle periodogram analysis (Figure 35 and Table 3). Speculatively, differences in the expression of the circadian photoreceptor CRY in the clock network could be responsible for this difference in behaviour, as it was suggested for drosophilids (Kauranen et al., 2012; Menegazzi et al., 2017). We are currently performing anti-CRY staining in *M. scalaris* and *M. abdita* in order to understand the putative neuronal basis of these behavioural differences in response to light.

The scuttle flies *M. scalaris* and *M. abdita* offer new fascinating models to study how the clock network controls circadian behaviour. Contrary to *D. melanogaster* flies where PDF expression in the brain is manipulated, *M. scalaris* and *M. abdita* are naturally evolved circadian systems, where the lack of a central clock component like PDF might have an ecological significance. This ecological meaning is obscure to me at the moment. It has to be minded that, although PDF is the main clock neuropeptide in *Drosophila*, it is not the only one; among others, ITP, sNPF, and NPF are known to be expressed in the clock network (Figure 4).

Appendices

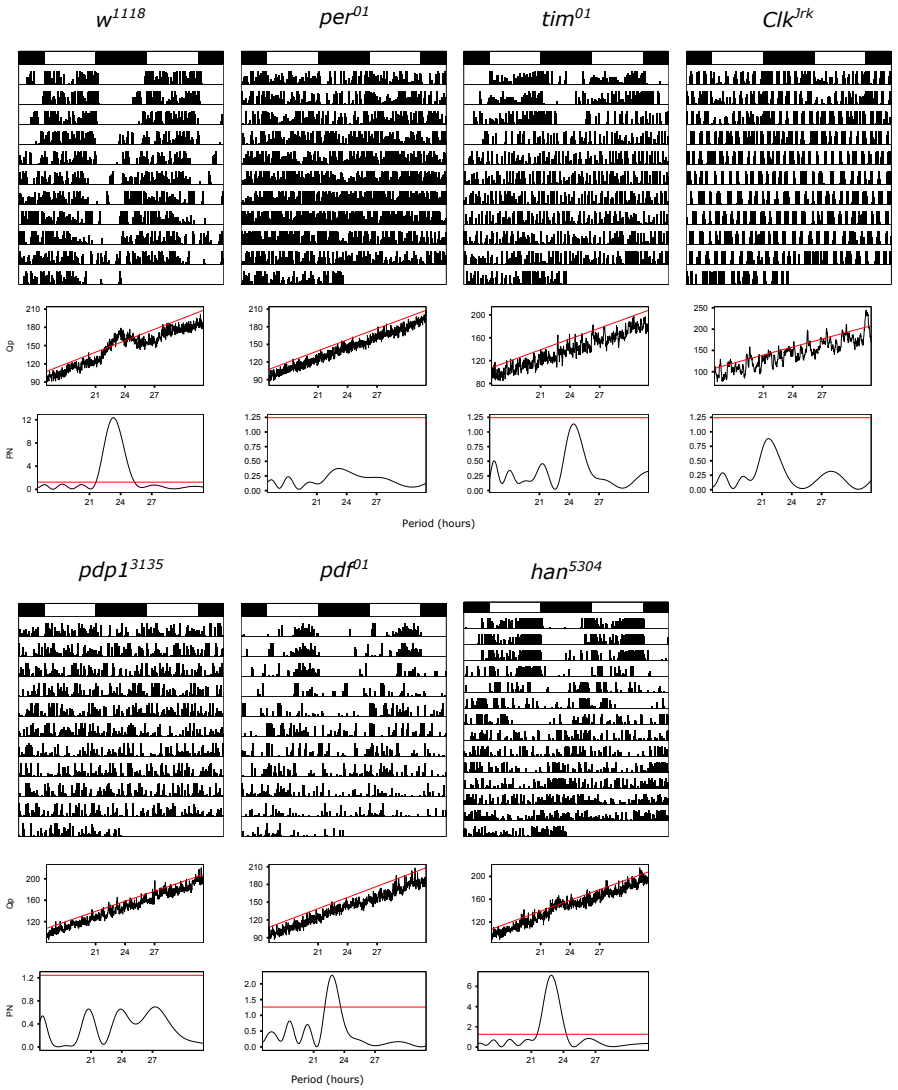

Figure 36: Representative actograms of *D. melanogaster* wild-type (w^{1118}) and clock mutants (per^{01}, tim^{01}, Clk^{Jrk}, $pdp1^{3135}$, pdf^{01}, han^{5304}) entrained under at least seven days of LD 12:12 (3 days are shown) followed by 8 days of DD at 20°C. χ^2 (Qp) and Lomb-Scargle (PN) periodogram analyses for the respective actograms are shown. The red line in the periodograms indicates the significance threshold ($p<0.05$).

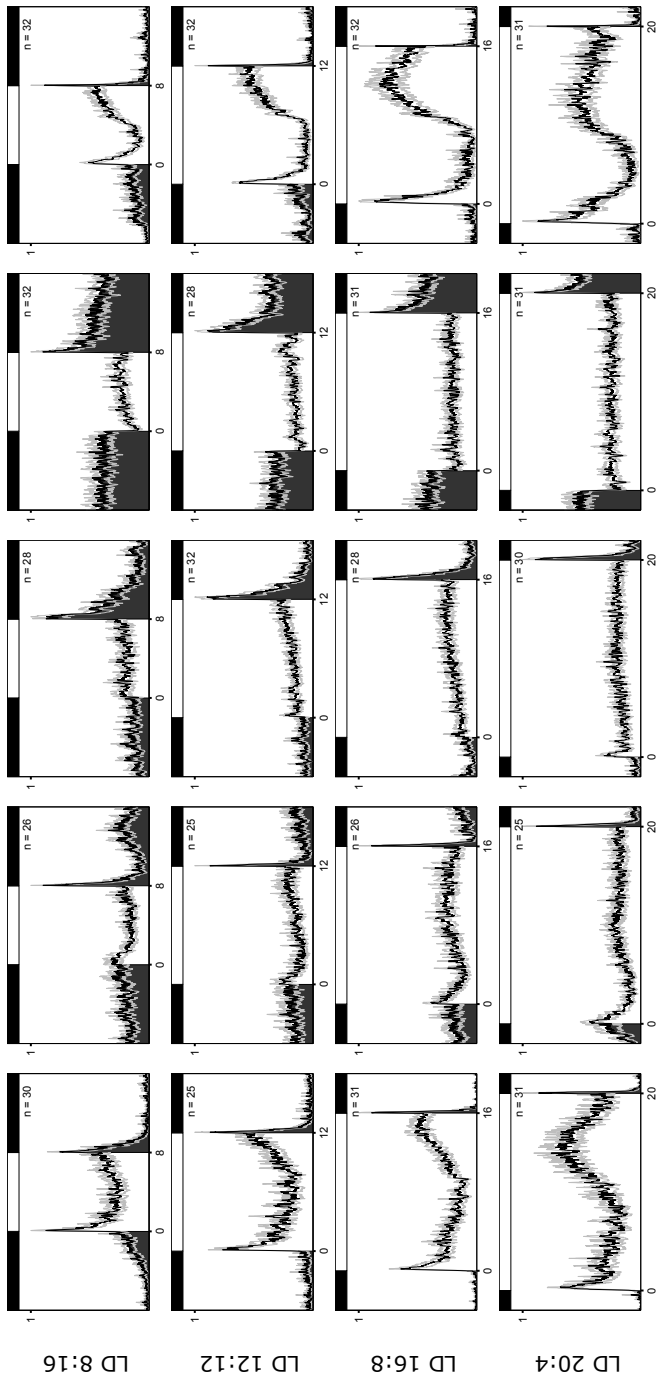

Figure 37: Average activity profile of *D. melanogaster* wild-type (w^{1118}) and clock mutants (per^{01}, tim^{01}, Clk^{Jrk}, $pdp1^{3135}$, han^{5304}) under LD 8:16, LD 12:12, LD 16:8, and LD 20:4 at 20°C. Black bars represent light-off and white bars represent light-on. The total number of flies averaged is indicated at the top right corner of each plot. Data are normalized to the maximum value of each condition.

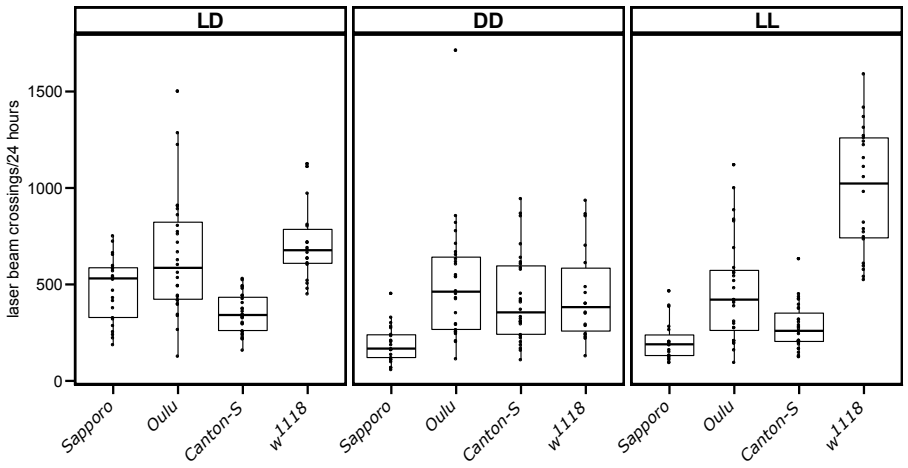

Figure 38: Daily amount of locomotor activity of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S* and w^{1118}) under LD 12:12, DD, or LL at 20°C recorded using the DAM system.

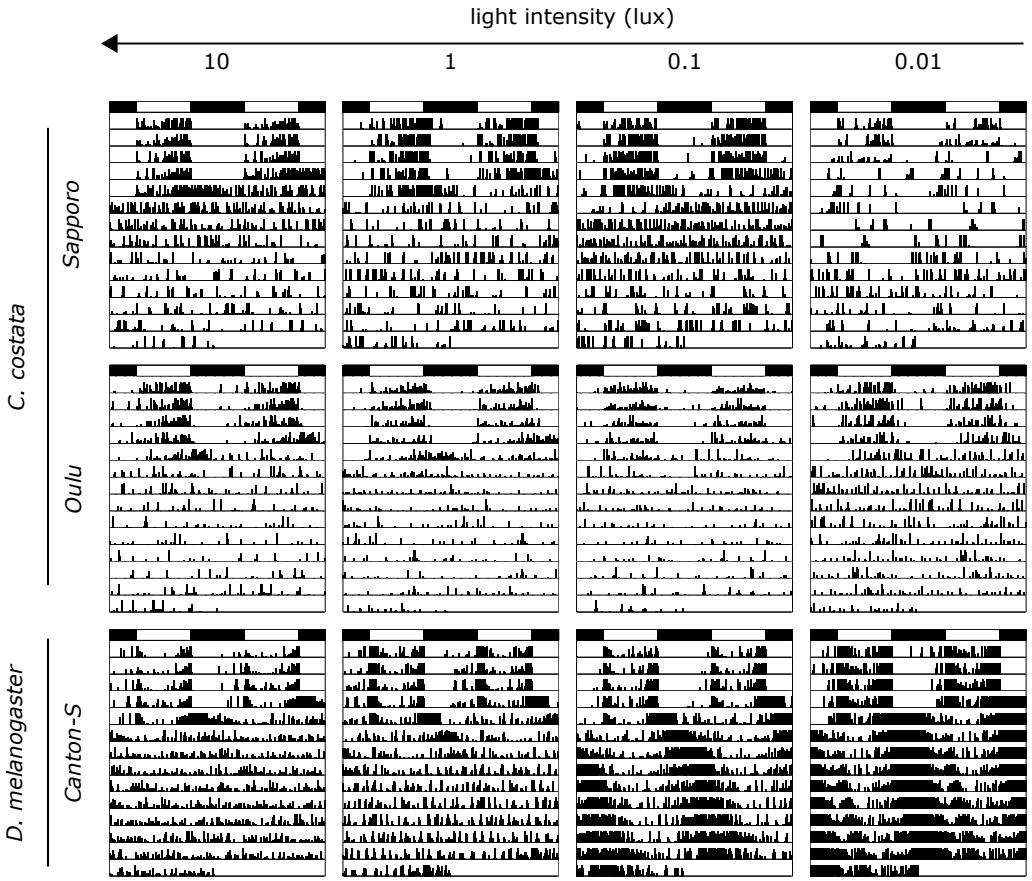

Figure 39: Representative actograms of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) entrained under at least seven days of LD 12:12 (4 days are shown) given at different light intensity (10, 1, 0.1, 0.01 lux) followed by 10 days of LL (of the same intensity of the entrainment) at constant 20°C.

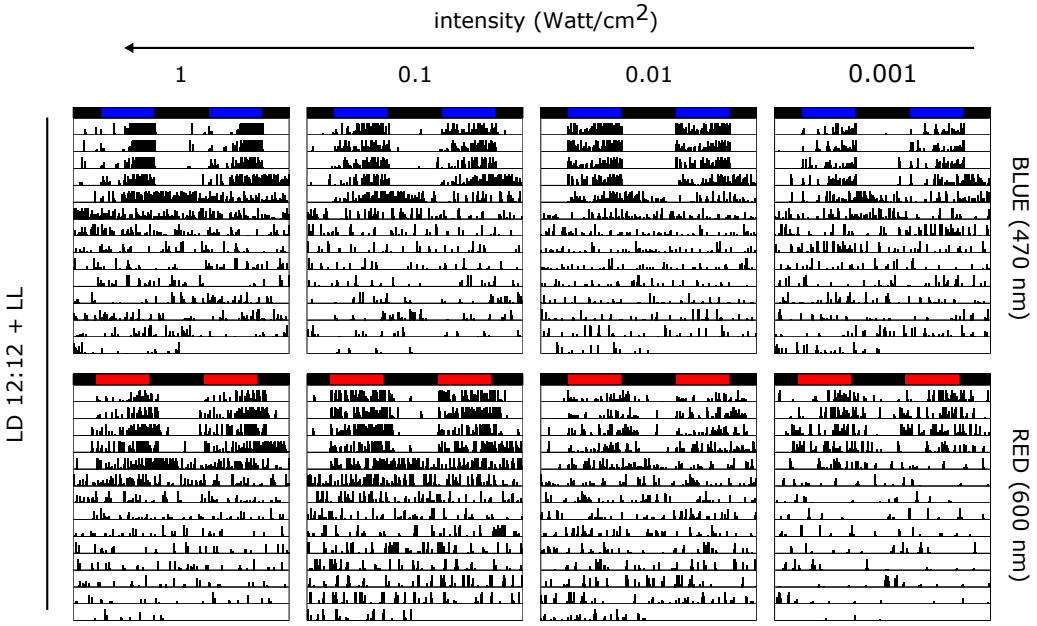

Figure 40: Representative actograms of *C. costata* (*Sapporo*) entrained under at least seven days of LD 12:12 (4 days are shown) given with coloured light (blue: 470 nm; red: 600 nm) at different light intensity (1, 0.1, 0.01 Watt/cm^2) followed by 10 days of LL (of the same wavelength and intensity of the entrainment) at constant 20°C.

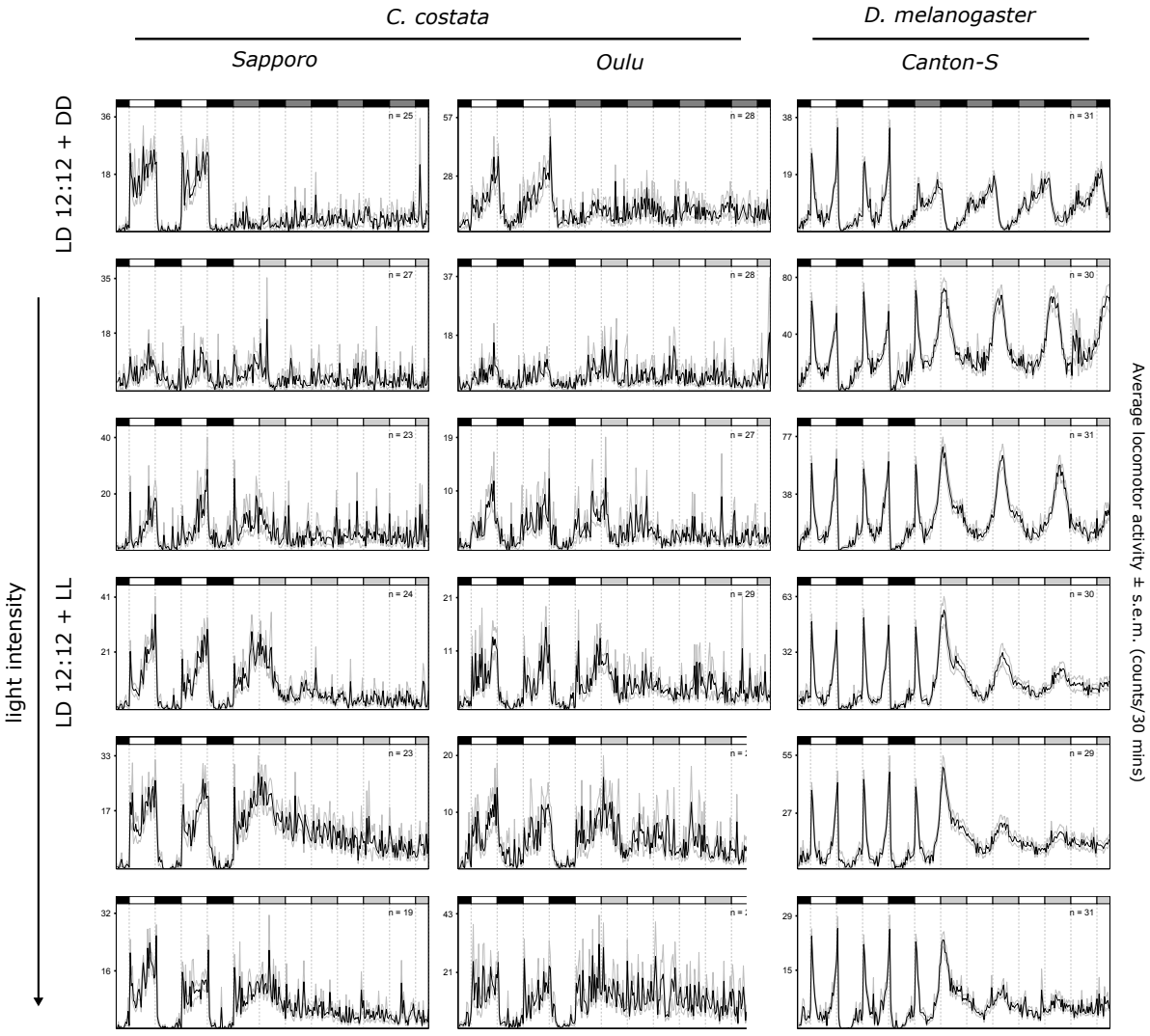

Figure 41: Average activity profile of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) under LD 12:12 followed by DD or LL at different light intensities (0.01, 0.1, 1, 10, 100 lux) at 20°C. Black bars represent light-off, white bars represent light-on, gray bars represent subjective days or nights. The total number of flies averaged is indicated at the top right corner of each plot.

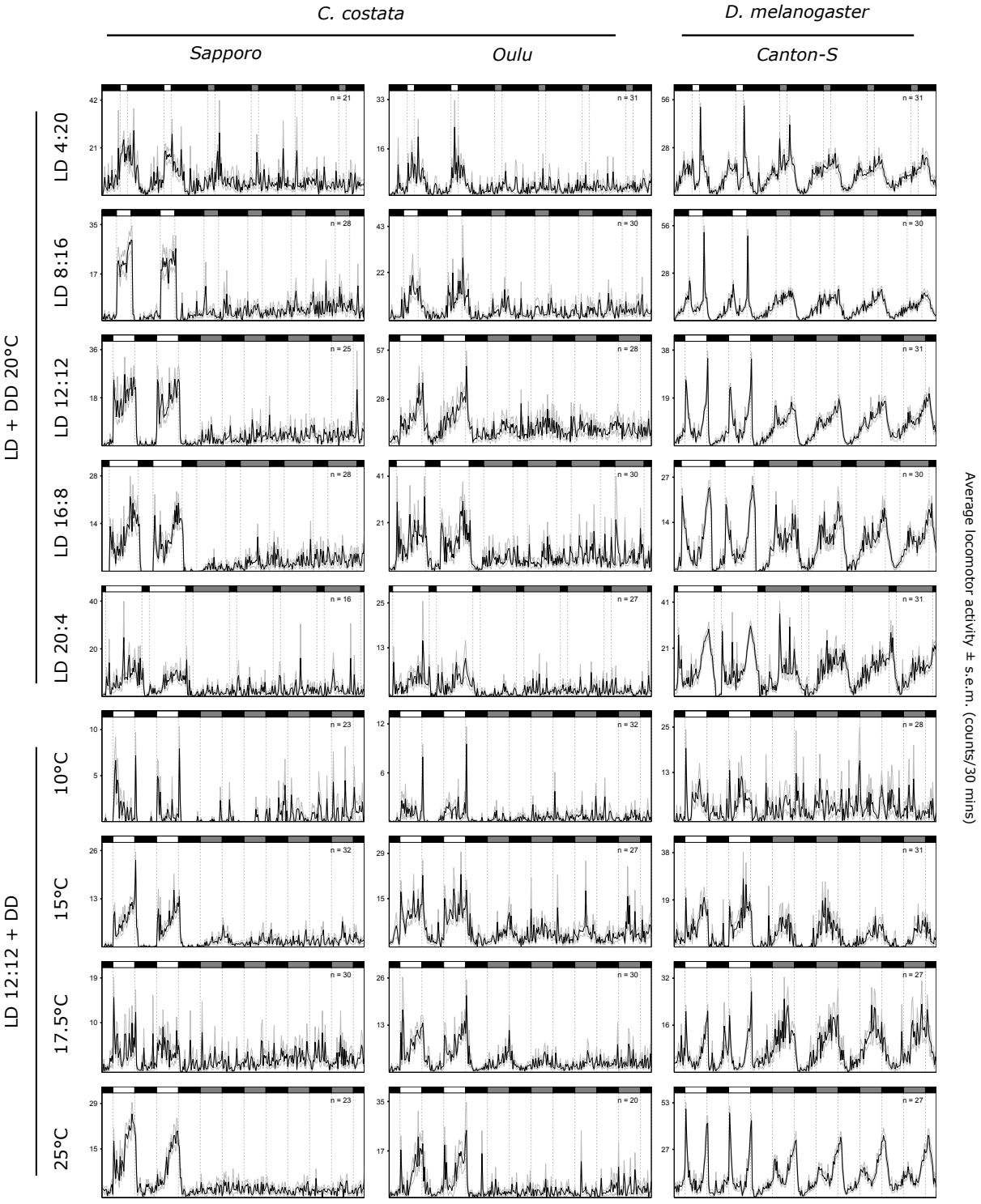

Figure 42: Average activity profile of *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) under LD 4:20, LD 8:16, LD 12:12, LD 16:8, and LD 20:4 followed by DD at 20°C, and under LD 12:12 followed by DD at 10°C, 15°C, 17.5°C, 25°C. Black bars represent light-off, white bars represent light-on, gray bars represent subjective days or nights. The total number of flies averaged is indicated at the top right corner of each plot.

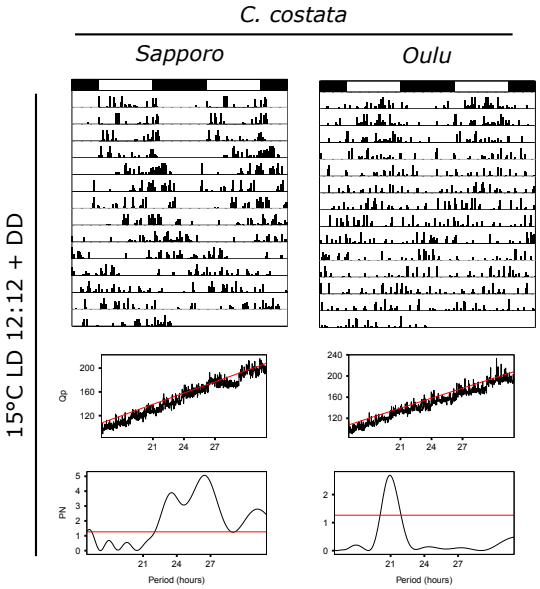

Figure 43: Actograms of *C. costata* (*Sapporo* and *Oulu*) entrained under at least seven days of LD 12:12 (4 days are shown) followed by 10 days of DD at constant 15°C, which exhibit free-running rhythm in DD. χ^2 (Qp) and Lomb-Scargle (PN) periodogram analyses for the respective actograms are shown. The red line in the periodograms indicates the significance threshold ($p<0.05$).

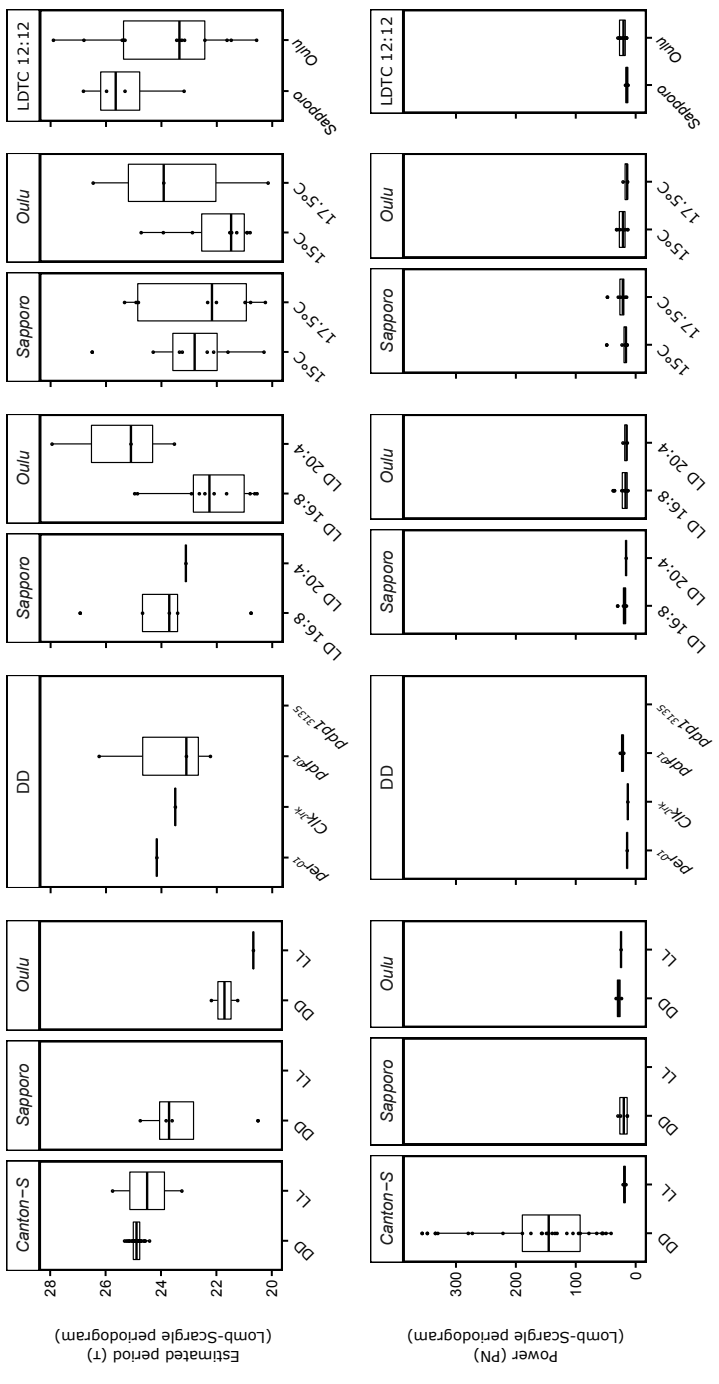

Figure 44: Estimated period (τ) and power (PN) of the significant periodogram analysis shown in Figure 17 and Figure 21.

		CircWave			ANOVA		
		C.costata		*D. melanogaster*	*C.costata*		*D. melanogaster*
		Sapporo	*Oulu*	*Canton-S*	*Sapporo*	*Oulu*	*Canton-S*
LD	s-LN_v	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	5^{th} s-LN_v	p<0.001	p<0.001	p<0.05	p<0.001	p<0.001	p<0.001
	l-LN_v	p<0.001	p<0.001	p<0.05	p<0.001	p<0.001	p<0.001
	LN_d	p<0.001	p<0.001	p<0.05	p<0.001	p<0.001	p<0.001
DD	s-LN_v	p<0.05	p<0.05	p<0.001	p<0.05	p<0.05	p<0.001
	5^{th} s-LN_v	**p>0.05**	p<0.05	p<0.001	**0.0872**	**0.0668**	p<0.001
	l-LN_v	**p>0.05**	**p>0.05**	p<0.001	**0.4231**	**0.1551**	p<0.001
	LN_d	p<0.05	p<0.001	p<0.001	p<0.05	p<0.001	p<0.001

Table 4: Analysis of PDP1 oscillation in *C. costata* (*Sapporo* and *Oulu*) and *D. melanogaster* (*Canton-S*) using CircWave (Oster et al., 2006) and ANOVA. CircWave outcome: NaN = p>0.05 = no cycling; 0 = p<0.001 = cycling. Highlighted in bold are the samples that display no significant cycling.

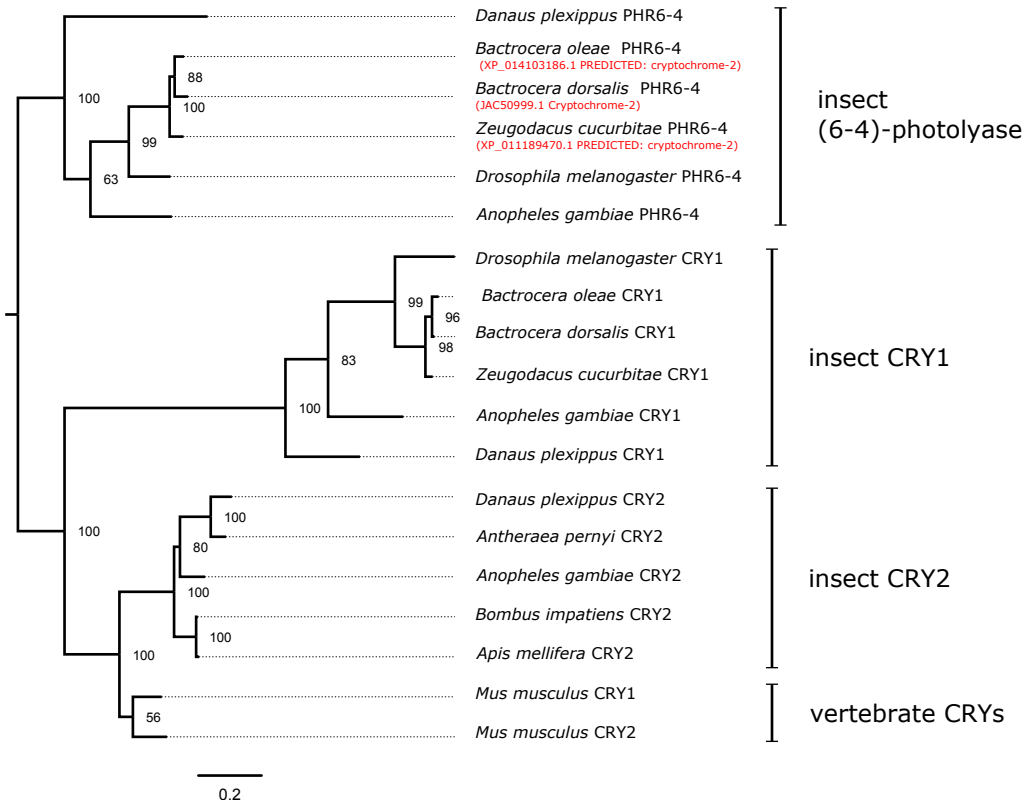

Figure 45: Gene tree of insect CRY1, CRY2, and (6-4)-photolyases reconstructed from amino acids with Maximum Likelihood using RaxML. Vertebrate CRYs are used as outgroup. Values at the nodes represent bootstrap values determined with 100 replicates. Highlighted in red are the sequences wrongly annotated in Genbank as "cryptochrome 2" of *B. oleae* (X_014103186.1), *B. dorsalis* (JAC50999.1), and *Z. curcubitae* (XP_011189470.1). GenbankID: *A. gambiae* CRY1 (ABB29886.1), *A. gambiae* CRY2 (ABB29887.1), *A. gambiae* PHR6-4 (EAA10141.3), *A. mellifera* CRY2 (NP_001077099.1), *A. pernyi* CRY2 (ABO38435.1), *B. dorsalis* CRY1 (XP_011206676.1), *B. impatiens* CRY2 (ABO31112.1), *B. oleae* CRY1 (XP_014096643.1), *D. melanogaster* CRY1 (NP_732407.1), *D. melanogaster* PHR6-4 (NP_732407.1), *D. plexippus* CRY1 (AAX58599.1), *D. plexippus* CRY2 (ABA62409.1), *D. plexippus* PHR6-4 (ABO38436.1), *M. musculus* CRY1 (NP_031797.1), *M. musculus* CRY2 (AAD46561.1), *Z. curcubitae* CRY1 (NP_001291679.1)

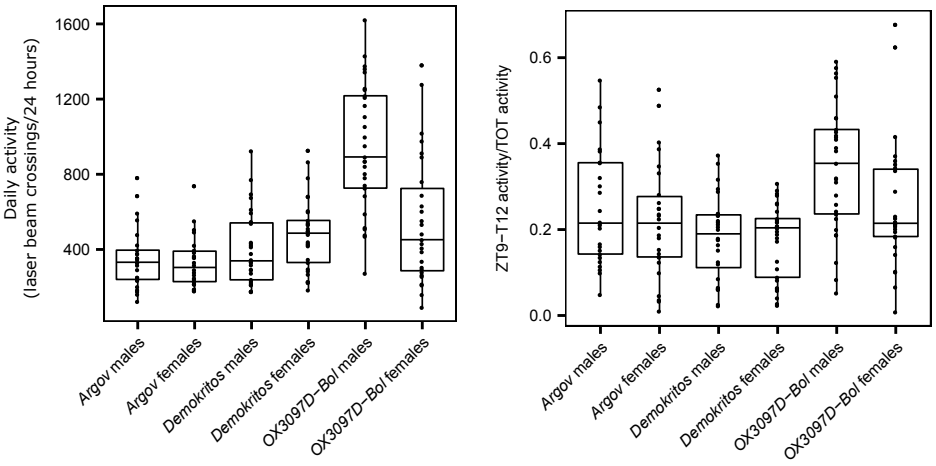

Figure 46: Left: Comparison of the activity level of *B. oleae* males and females from different strains (*Demokritos*, *Argov*, *OX3097D-Bol*) under LD 12:12 at 20°C. Right: Comparison of the evening activity (activity between ZT9-ZT12) of *B. oleae* males and females from different strains (*Demokritos*, *Argov*, *OX3097D-Bol*) under LD 12:12 at 20°C.

Bibliography

Abdelsalam, S., Uemura, H., Umezaki, Y., Saifullah, A. S. M., Shimohigashi, M., & Tomioka, K. (2008). Characterization of PDF-immunoreactive neurons in the optic lobe and cerebral lobe of the cricket, *Gryllus bimaculatus*. *J. Insect Physiol. 54*, 1205–1212.

Adkisson, P. L. (1966). Internal clocks and insect diapause. *Science*, *154*, 234–241.

Aketarawong, N., Guglielmino, C. R., Karam, N., Falchetto, M., Manni, M., Scolari, F., ... Malacrida, A. R. (2014). The oriental fruitfly *Bactrocera dorsalis* s.s. in East Asia: disentangling the different forces promoting the invasion and shaping the genetic make-up of populations. *Genetica*, *142*(3), 201–213.

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

Alphey, L. S. (2007). Engineering insects for the sterile insect technique. *Area-Wide Control Insect Pests, IAEA*, 51–60.

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Economopoulos, A., Vontas, J. G., & Alphey, L. S. (2012). Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biol. 10*(51).

Argov, Y., Kuslutzky, W., & Hoelmer, K. (2011). Biological control of olive fruit fly, Bactrocera oleae, in Israel. In *Proc. meet. jerusalem, 15 - 20 may, 2011.*

Aschoff, J. & Wever, R. (1962). Spontanperiodik des Menschen bei Ausschluss aller Zeitgeber. *Naturwissenschaften*, *49*(15), 337–342.

Bae, K., Lee, C., Hardin, P. E., & Edery, I. (2000). dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. *J. Neurosci. 20*(5), 1746–1753.

Bahn, J. H., Lee, G., & Park, J. H. (2009). Comparative analysis of pdf-mediated circadian behaviors between *Drosophila melanogaster* and *D. virilis*. *Genetics*, *181*, 965–975.

Bajgar, A., Jindra, M., & Doležel, D. (2013). Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. *Proc. Natl. Acad. Sci. 110*(11), 4416–4421.

Barberà, M., Collantes-Alegre, J. M., & Martínez-Torres, D. (2017). Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. *Insect Biochem. Mol. Biol. 83*, 54–67.

Beale, A. D., Whitmore, D., & Moran, D. (2016). Life in a dark biosphere: a review of circadian physiology in "arrhythmic" environments. *J. Comp. Physiol. B*, *186*(8), 947–968.

Beckwith, E. J. & Ceriani, M. F. (2015). Experimental assessment of the network properties of the *Drosophila* circadian clock. *J. Comp. Neurol. 523*, 982–996.

Beer, K., Steffan-Fewenter, I., Härtel, S., & Helfrich-Förster, C. (2016). A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior. *J. Comp. Physiol. A*.

Beer, K., Kolbe, E., Kahana, N. B., Yayon, N., Weiss, R., Menegazzi, P., ... Helfrich-Förster, C. (2018). Pigment-Dispersing Factor-expressing neurons convey circadian information in the honey bee brain. *Open Biol. 8*(170224).

Benna, C., Scannapieco, P., Piccin, A., Sandrelli, F., Zordan, M., Rosato, E., ... Costa, R. (2000). A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. *Curr. Biol. 10*(14), 512–513.

Benito, J., Houl, J. H., Roman, G. W., & Hardin, P. E. (2008). The blue-light photoreceptor cryptochrome is expressed in a subset of circadian oscillator neurons in the *Drosophila* CNS. *J. Biol. Rhythms*, *23*(4), 296–307.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2017). GenBank. *Nucleic Acids Res. 45*, 37–42.

Bertolini, E., Kistenpfennig, C. R., Menegazzi, P., Keller, A., Koukidou, M., & Helfrich-Förster, C. (2018). The characterization of the circadian clock in the olive fly *Bactrocera oleae* (Diptera: Tephritidae) reveals a *Drosophila*-like organization. *Sci. Rep. 8*(816).

Black, W. C., Alphey, L. S., & James, A. a. (2011). Why RIDL is not SIT. *Trends Parasitol. 27*(8), 362–370.

Bostock, E., Green, E., Kyriacou, C. P., & Vanin, S. (2017). Chronobiological studies on body search, oviposition and emergence of *Megaselia scalaris* (Diptera, Phoridae) in controlled conditions. *Forensic Sci. Int. 275*, 155–159.

Bradshaw, W. E. & Holzapfel, C. M. (2007). Evolution of animal photoperiodism. *Annu. Rev. Ecol. Evol. Syst. 38*, 1–25.

Bradshaw, W. E. & Holzapfel, C. M. (2010). Circadian clock genes, ovarian development and diapause. *BMC Biol. 8*(115).

Brand, A. H. & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*, 401–415.

Brown, S. A., Kowalska, E., & Dallmann, R. (2012). (Re)inventing the circadian feedback loop. *Dev. Cell*, *22*(3), 477–487.

Bünning, E. (1936). Die endonome Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Ber. Dtsch. Bot. Ges. 54*, 590–607.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol. 17*(4), 540–552.

Cashmore, A. R. (2003). Cryptochromes: enabling plants and animals to determine circadian time. *Cell*, *114*, 537–543.

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

Chang, D. C., McWatters, H. G., Williams, J. A., Gotter, A. L., Levine, J. D., & Reppert, S. M. (2003). Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. *J. Biol. Chem. 278*(40), 38149–38158.

Chahad-Ehlers, S., Arthur, L. P., Lima, A. L., Gesto, J. S., Torres, F. R., Peixoto, A. A., & de Brito, R. A. (2017). Expanding the view of *Clock* and *cycle* gene evolution in Diptera. *Insect Mol. Biol. 26*(3), 317–331.

Choi, C., Fortin, J. P., McCarthy, E. v., Oksman, L., Kopin, A. S., & Nitabach, M. N. (2009). Cellular dissection of circadian peptide signals with genetically encoded membrane-tethered ligands. *Curr. Biol. 19*, 1167–1175.

Codd, V., Doležel, D., Stehlík, J., Piccin, A., Garner, K. J., Racey, S. N., ... Rosato, E. (2007). Circadian rhythm gene regulation in

the housefly *Musca domestica*. *Genetics*, *177*, 1539–1551.

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

Daane, K. M. & Johnson, M. W. (2010). Olive fruit fly: managing an ancient pest in modern times. *Annu. Rev. Entomol. 55*, 151–169.

Davis, S. J. (2002). Photoperiodism: the coincidental perception of the season. *Curr. Biol. 12*, R841–R843.

Denlinger, D. L. (2002). Regulation of diapause. *Annu. Rev. Entomol. 47*, 93–122.

Dircksen, H., Tesfai, L. K., Albus, C., & Nässel, D. R. (2008). Ion transport peptide splice forms in central and peripheral neurons throughout postembryogenesis of *Drosophila melanogaster*. *J. Comp. Neurol. 509*, 23–41.

Dircksen, H., Zahnow, C. C. A. C., Gaus, G., Keller, R., Rao, K. R., & Riehm, J. P. (1987). The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: identification by an antiserum against a synthetic PDH. *Cell Tissue Res. 250*, 377–387.

Dircksen, H. (2009). Insect ion transport peptides are derived from alternatively spliced genes and differentially expressed in the central and peripheral nervous system. *J. Exp. Biol. 212*, 401–412.

Dissel, S., Hansen, C. N., Özkaya, Ö., Hemsley, M., Kyriacou, C. P., & Rosato, E. (2014). The logic of circadian organization in Drosophila. *Curr. Biol. 24*(19), 2257–2266.

Disney, R. H. L. (2008). Natural history of the scuttle fly, *Megaselia scalaris*. *Annu. Rev. Entomol. 53*, 39–60.

Doležel, D., Vanecková, H., Sauman, I., & Hodková, M. (2005). Is *period* gene causally involved in the photoperiodic regulation of reproductive diapause in the linden bug, *Pyrrhocoris apterus*? *J. Insect Physiol. 51*, 655–659.

Doleželova, E., Doležel, D., & Hall, J. C. (2007). Rhythm defects caused by newly engineered null mutations in drosophila's *cryptochrome* gene. *Genetics*, *177*, 329–345.

Doležel, D., Zdechovanova, L., Sauman, I., & Hodková, M. (2008). Endocrine-dependent expression of circadian clock genes in insects. *Cell. Mol. Life Sci. 65*, 964–969.

Doležel, D. (2015). Photoperiodic time measurement in insects. *Curr. Opin. Insect Sci. 7*, 98–103.

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell*, *96*, 271–290.

Dyck, V. A., Hendrichs, J. P., & Robinson, A. S. (2005). *Sterile Insect Technique - Principles and practice in area-wide integrated pest management* (V. A. Dyck, J. P. Hendrichs, & A. S. Robinson, Eds.).

Economopoulos, A., Voyadjoglou, A., & Giannakakis, A. (1976). Reproductive behavior and physiology of *Dacus oleae*: fecundity as affected by mating, adult diet and artificial rearing. *Ann. Entomol. Soc. Am. 69*(4), 725–729.

Economopoulos, A. & Zervas, G. A. (1982). The quality problem in olive flies produced for SIT experiments.

Economopoulos, A. (1972). Sexual competitiveness of gamma-ray sterilized males of *Dacus oleae*. Mating frequency of artificially reared and wild females. *Environ. Entomol. 1*, 490–497.

Emery, P., Stanewsky, R., Helfrich-Förster, C., Emery-Le, M., Hall, J. C., & Rosbash, M.

(2000). D*rosophila* CRY is a deep brain circadian photoreceptor. *Neuron*, *26*, 493–504.

Emery, P. & Reppert, S. M. (2004). A rhythmic Ror. *Neuron*, *43*, 443–446.

Emerson, K. J., Bradshaw, W. E., & Holzapfel, C. M. (2009). Complications of complexity: integrating environmental, genetic and hormonal control of insect diapause. *Trends Genet. 25*(5), 217–225.

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

Enomoto, O. (1981a). Larval diapause in *Chymomyza costata* (Diptera: Drosophilidae) I. Effects of temperature and photoperiod on the development. *Low Temp. Sci. 39*, 21–29.

Enomoto, O. (1981b). Larval diapause in *Chymomyza costata* (Diptera: Drosophilidae) II. Frost avoidance. *Low Temp. Sci. 39*, 31–39.

Enright, J. T. (1965). The search of rhythmicity in biological time-series. *J. theor. Biol. 8, 426-468.*

Enright, J. T. (1970). Ecological aspects of endogenous rhythmicity. *Annu. Rev. Ecol. Syst. 1*, 221–238.

Ero, M. M., Hamacek, E., & Clarke, A. R. (2011). Foraging behaviours of *Diachasmimorpha kraussii* (Fullaway) (Hymenoptera: Braconidae) and its host *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) in a nectarine (*Prunus persica* (L.) Batsch var. *nectarina* (Aiton) Maxim) orch. *Aust. J. Entomol. 50*, 234–240.

Evans, J. D., Brown, S. J., Hackett, K. J. J., Robinson, G., Richards, S., Lawson, D., ... Zhou, X. (2013). The i5K initiative: advancing arthropod genomics for knowledge, human health, agriculture, and the environment. *J. Hered. 104*(5), 595–600.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., & Hall, J. C. (1992). Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci. 12*(9), 3321–3349.

Fletcher, B. S. (1987). The biology of dacine fruit flies. *Annu. Rev. Entomol. 32*, 115–144.

Fox, J. & Weisberg, S. (2011). *An R companion to applied regression* (Second). Thousand Oaks CA: Sage.

Fuchikawa, T. & Shimizu, I. (2007). Circadian rhythm of locomotor activity in the Japanese honeybee, *Apis cerana japonica*. *Physiol. Entomol. 32*, 73–80.

Gentile, C., Rivas, G. B. S., Meireles-Filho, A. C. A., Lima, J. B. P., & Peixoto, A. A. (2009). Circadian expression of clock genes in two mosquito disease vectors: *cry2* is different. *J. Biol. Rhythms*, *24*(6), 444–51.

Glaser, F. T. & Stanewsky, R. (2005). Temperature synchronization of the *Drosophila* circadian clock. *Curr. Biol. 15*, 1352–1363.

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

Glossop, N. R. J., Lyons, L. C., & Hardin, P. E. (1999). Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science*, *286*, 766–768.

Gorostiza, E. A., Depetris-Chauvin, A., Frenkel, L., Pírez, N., & Ceriani, M. F. (2014). Circadian pacemaker neurons change synaptic contacts across the day. *Curr. Biol. 24*(18), 2161–2167.

Grima, B., Chélot, E., Xia, R., & Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature*, *431*, 869–73.

Hahn, D. a. & Denlinger, D. L. (2011). Energetics of insect diapause. *Annu. Rev. Entomol. 56*, 103–121.

Hardin, P. E. & Panda, S. (2013). Circadian timekeeping and output mechanisms in animals. *Curr. Opin. Neurobiol. 23*, 1–8.

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

Hardin, P. E. (2005). The circadian timekeeping system of *Drosophila*. *Curr. Biol. 15*, R714–R722.

Hardin, P. E. (2011). *Molecular genetic analysis of circadian timekeeping in Drosophila* (1st ed.). Elsevier Inc.

Helfrich-Förster, C., Täuber, M., Park, J. H., Mühlig-Versen, M., Schneuwly, S., & Hofbauer, A. (2000). Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci. 20*(9), 3339–3353.

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

Helfrich-Förster, C., Edwards, T., Yasuyama, K., Wisotzki, B., Schneuwly, S., Stanewsky, R., ... Hofbauer, A. (2002). The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. *J. Neurosci. 22*(21), 9255–9266.

Helfrich-Förster, C., Shafer, O. T., Wülbeck, C., Grieshaber, E., Rieger, D., & Taghert, P. H. (2007a). Development and Morphology of the Clock-Gene-Expressing Lateral Neurons of Drosophila melanogaster. *J. Comp. Neurol. 504*(3), 287–297.

Helfrich-Förster, C., Yoshii, T., Wülbeck, C., Grieshaber, E., Rieger, D., Bachleitner, W., ... Rouyer, F. (2007b). The lateral and dorsal neurons of *Drosophila melanogaster*: new insights about their morphology and function. *Cold Spring Harb. Symp. Quant. Biol. 72*, 517–525.

Helfrich, C., Cymborowski, B., & Engelmann, W. (1985). Circadian activity rhythm of the house fly continues after optic tract severance and lobectomy. *Chronobiol. Int. 2*(1), 19–32.

Helfrich, C. & Engelmann, W. (1987). Evidences for circadian rhythmicity in the per^0 mutant of *Drosophila melanogaster*. *Z. Naturforsch*, *42*(100), 1335–1338.

Helfrich-Förster, C. & Homberg, U. (1993). Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. *J. Comp. Neurol. 337*, 177–190.

Helfrich-Förster, C. (2000). Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster* - sex-specific differences suggest a different quality of activity. *J. Biol. Rhythms*, *15*(2), 135–154.

Helfrich-Förster, C. (2002). The circadian system of *Drosophila melanogaster* and its light input pathways. *Zoology*, *105*, 297–312.

Helfrich-Förster, C. (2003). The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. *Microsc. Res. Tech. 62*, 94–102.

Helfrich-Förster, C. (2005a). Neurobiology of the fruit fly's circadian clock. *Genes. Brain. Behav. 4*, 65–76.

Helfrich-Förster, C. (2005b). Organization of endogenous clocks in insects. *Biochem. Soc. Trans. 33*, 957–961.

Helfrich-Förster, C. (2007). Immunohistochemistry in *Drosophila*. In *Met. mol. biol. - circadian rhythm.* (Vol. 362, pp. 533–547).

Helfrich-Förster, C. (2017). The *Drosophila* clock system. In *Biol. timekeep. clocks, rhythm. behav.* (Chap. 6, pp. 133–176).

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

Helfrich-Förster, C. (1997). Development of Pigment-Dispersing Hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. *J. Comp. Neurol. 380*, 335–354.

Hendrichs, J. P., Robinson, A. S., Cayol, J. P., & Enkerlin, W. (2012). Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies. *Florida Entomol. 85*(1), 1–13.

Hendrichs, J. P. (2000). Use of the sterile insect technique against key insect pests. *Sustain. Dev. Int.* 75–79.

Hermann, C., Yoshii, T., Dusik, V., & Helfrich-Förster, C. (2012). Neuropeptide F immunoreactive clock neurons modify evening locomotor activity and free-running period in *Drosophila melanogaster*. *J. Comp. Neurol. 520*, 970–87.

Hermann, C., Saccon, R., Senthilan, P. R., Domnik, L., Dircksen, H., Yoshii, T., & Helfrich-Förster, C. (2013). The circadian clock network in the brain of different *Drosophila* species. *J. Comp. Neurol. 521*, 367–388.

Hermann-Luibl, C., Yoshii, T., Senthilan, P. R., Dircksen, H., & Helfrich-Förster, C. (2014). The ion transport peptide is a new functional clock neuropeptide in the fruit fly *Drosophila melanogaster*. *J. Neurosci. 34*(29), 9522–9536.

Hermann-Luibl, C. & Helfrich-Förster, C. (2015). Clock network in *Drosophila*. *Curr. Opin. Insect Sci. 7*, 1–6.

Hodková, M., Okuda, T., & Wagner, R. M. (2001). Regulation of corpora allata in females of *Pyrrhocoris apterus* (Heteroptera) (a mini-review). *Vitr. Cell. Dev. Biol. - Anim. 37*, 560–563.

Hodková, M., Syrová, Z., Doležel, D., & Šauman, I. (2003). Period gene expression in relation to seasonality and circadian rhythms in the linden bug, *Pyrrhocoris apterus* (Heteroptera). *Eur. J. Entomol. 100*, 267–273.

Hodková, M. (2015). Why is the number of days required for induction of adult diapause in the linden bug *Pyrrhocoris apterus* fewer in the larval than in the adult stage? *J. Insect Physiol. 77*, 39–44.

Hodek, I. (1971a). Induction of adult diapause in *Pyrrhocoris apterus* L. by short cold exposure. *Oecologia*, *6*, 109–117.

Hodek, I. (1971b). Sensitivity of larvae to photoperiods controlling the adult diapause of two insects. *J. Insect Physiol. 17*, 205–216.

Hodková, M. (1976). Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. *Nature*, *263*, 619–621.

Hofbauer, A. & Buchner, E. (1989). Does *Drosophila* have seven eyes? *Naturwissenschaften*, *76*, 335–336.

Hogenesch, J. B., Gu, Y. Z., Jain, S., & Bradfield, C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. 95*, 5474–5479.

Homberg, U., Wiirden, S., Dircksen, H., & Rao, K. R. (1991). Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tissue Res. 266*, 343–357.

Hut, R. A., Barnes, B. M., & Daan, S. (2002). Body temperature patterns before, during, and after semi-natural hibernation in the European ground squirrel. *J. Comp. Physiol. B*, *172*, 47–58.

Hyun, S., Lee, Y., Hong, S.-T., Bang, S., Paik, D., Kang, J., ... Kim, J. (2005). Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. *Neuron*, *48*, 267–278.

Ikeno, T., Numata, H., Goto, S. G., & Shiga, S. (2014). Involvement of the brain region containing pigment-dispersing factor-immunoreactive neurons in the photoperiodic response of the bean bug, *Riptortus pedestris*. *J. Exp. Biol. 217*, 453–462.

Im, S. H. & Taghert, P. H. (2010). PDF receptor expression reveals direct interactions between circadian oscillators in *Drosophila*. *J. Comp. Neurol. 518*, 1925–1945.

Ingram, K. K., Kutowoi, A., Wurm, Y., Shoemaker, D., Meier, R., & Bloch, G. (2012). The molecular clockwork of the fire ant *Solenopsis invicta*. *PLoS One*, *7*(11), 1–11.

Ito, Y., Kakinohana, H., Yamagishi, M., & Kohama, T. (2003). Eradication of the melon fly, *Bactrocera cucurbitae*, from Okinawa, Japan, by means of the sterile insect technique, with special emphasis on the role of basic studies. *J. Asia. Pac. Entomol. 6*(2), 119–129.

Jagota, A., de la Iglesia, H. O., & Schwartz, W. J. (2000). Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro*. *Nat. Neurosci. 3*(4), 372–376.

Johard, H. a. D., Yoishii, T., Dircksen, H., Cusumano, P., Rouyer, F., Helfrich-Förster, C., & Nässel, D. R. (2009). Peptidergic clock neurons in *Drosophila*: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. *J. Comp. Neurol. 516*, 59–73.

Kahsai, L., Kapan, N., Dircksen, H., Winther, Å. M. E., & Nässel, D. R. (2010). Metabolic stress responses in *Drosophila* are modulated by brain neurosecretory cells that produce multiple neuropeptides. *PLoS One*, *5*(7), 1–12.

Kaneko, M. & Hall, J. C. (2000). Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol. 422*, 66–94.

Kauranen, H., Menegazzi, P., Costa, R., Helfrich-Förster, C., Kankainen, A., & Hoikkala, A. (2012). Flies in the north: locomotor behavior and clock neuron organization of *Drosophila montana*. *J. Biol. Rhythms*, *27*(5), 377–387.

Kauranen, H., Ala-Honkola, O., Kankare, M., & Hoikkala, A. (2016). Circadian clock of *Drosophila montana* is adapted to high variation in summer day lengths and temperatures prevailing at high latitudes. *J. Insect Physiol. 89*, 9–18.

Kay, J., Menegazzi, P., Mildner, S., Roces, F., & Helfrich-Förster, C. (2018). The circadian clock of the ant *Camponotus floridanus* is localized in dorsal and lateral neurons of the brain. *J. Biol. Rhythms*, *20*(10), 1–17.

Kistenpfennig, C. R., Nakayama, M., Nihara, R., Tomioka, K., Helfrich-Förster, C., & Yoshii, T. (2018). A tug-of-war between cryptochrome and the visual system allows the adaptation of evening activity to long photoperiods in *Drosophila melanogaster*. *J. Biol. Rhythms*, *33*(1), 24–34.

Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., ... Buchner, E. (1996). Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J. Neurosci. 16*(10), 3154–3165.

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *J. Econ. Entomol. 48*(4), 459–462.

Kobelková, A., Bajgar, A., & Doležel, D. (2010). Functional molecular analysis of a circadian clock gene *timeless* promoter from the Drosophilid fly *Chymomyza costata*. *J. Biol. Rhythms*, *25*(6), 399–409.

Konopka, R. J. & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci. 68*(9), 2112–2116.

Konopka, R. J., Pittendrigh, C. S., & Orr, D. (1989). Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *J. Neurogenet. 6*, 1–10.

Koštál, V., Shimada, K., & Hayakawa, Y. (2000). Induction and development of winter larval diapause in a drosophilid fly, *Chymomyza costata*. *J. Insect Physiol. 46*, 417–428.

Koštál, V. & Shimada, K. (2001). Malfunction of circadian clock in the *non-photoperiodic-diapause* mutants of the drosophilid fly, *Chymomyza costata*. *J. Insect Physiol. 47*(11), 1269–1274.

Koštál, V., Zahradnícková, H., & Šimek, P. (2011). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proc. Natl. Acad. Sci. 108*(32), 13041–13046.

Koštál, V., Mollaei, M., & Schöttner, K. (2016). Diapause induction as an interplay between seasonal token stimuli, and modifying and directly limiting factors: hibernation in *Chymomyza costata*. *Physiol. Entomol.* 1–14.

Koštál, V. (2011). Insect photoperiodic calendar and circadian clock: Independence, cooperation, or unity?

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., & Doležel, D. (2017). The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. *Physiol. Entomol. 42*, 266–273.

Kumaran, N., Prentis, P. J., Mangalam, K. P., Schutze, M. K., & Clarke, A. R. (2014). Sexual selection in true fruit flies (Diptera: Tephritidae): Transcriptome and experimental evidences for phytochemicals increasing male competitive ability. *Mol. Ecol. 23*, 4645–4657.

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

Kyriacou, C. P. & Hall, J. C. (1989). Spectral analysis of *Drosophila* courtship song rhythms. *Anim. Behav. 37*, 850–859.

Lamba, P., Bilodeau-Wentworth, D., Emery, P., & Zhang, Y. (2014). Morning and evening oscillators cooperate to reset circadian behavior in response to light input. *Cell Rep. 7*, 1–8.

Lam, V. H. & Chiu, J. C. (2017). *Evolution and Design of Invertebrate Circadian Clocks.*

Lankinen, P. & Forsman, P. (2006). Independence of genetic geographical variation between photoperiodic diapause, circadian eclosion rhythm, and Thr-Gly repeat region of the *period* gene in *Drosophila littoralis. J. Biol. Rhythms*, *21*(1), 3–12.

Lankinen, P. & Riihimaa, A. J. (1992). Weak circadian eclosion rhythmicity in *Chymomyza costata* (Diptera: Drosophilidae), and its independence of diapause type. *J. Insect Physiol. 38*, 803–811.

Lankinen, P. & Riihimaa, A. J. (1997). Effects of temperature on weak circadian eclosion rhythmicity in *Chymomyza costata* (Diptera: Drosophilidae). *J. Insect Physiol. 43*(3), 251–260.

Lankinen, P. (1986). Genetic correlation between circadian eclosion rhythm and photoperiodic diapause in *Drosophila littoralis. J. Biol. Rhythms*, *1*(2), 101–118.

Lear, B. C., Merrill, C. E., Lin, J.-M., Schroeder, A., Zhang, L., & Allada, R. (2005). A G protein-coupled receptor, *groom-of-PDF*, is required for PDF neuron action in circadian behavior. *Neuron*, *48*, 221–227.

Ledford, H. & Callaway, E. (2017). Circadian clocks scoop Nobel prize. *Nature*, *550*, 18.

Lee, G., Bahn, J. H., & Park, J. H. (2006). Sex- and clock-controlled expression of the *neuropeptide F* gene in *Drosophila. Proc. Natl. Acad. Sci. 103*(33), 12580–12585.

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

Levi-Zada, A., Nestel, D., Fefer, D., Nemni-Lavy, E., Deloya-Kahane, I., & David, M. (2012). Analyzing diurnal and age-related pheromone emission of the olive fruit fly, *Bactrocera oleae* by sequential SPME-GCMS analysis. *J. Chem. Ecol. 38*, 1036–1041.

Lin, Y., Stormo, G. D., & Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. *J. Neurosci. 24*(36), 7951–7957.

Lindsley, D. L. & Grell, E. H. (1968). *Genetic variation of Drosophila melanogaster.* Carnegie Institution of Washington Publication.

Loher, W. & Zervas, G. A. (1979). The mating rhythm of the olive fruitfly, *Dacus oleae* Gmelin. *J. Appl. Entomol. 88*, 425–435.

Lomb, N. R. (1976). Least-squares frequency analysis of unequally spaced data. *Astrophys. Space Sci. 39, 447–462.*

Lowrey, P. L. & Takahashi, J. S. (2011). Genetics of circadian rhythms in mammalian model organisms. *Adv. Genet. 74*, 175–230.

Lu, W., Meng, Q. J., Tyler, N. J. C., Stokkan, K. A., & Loudon, A. S. I. (2010). A circadian clock is not required in an arctic mammal. *Curr. Biol. 20*, 533–537.

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

Manlove, J. D. & Disney, R. H. L. (2008). The use of *Megaselia abdita* (Diptera: Phoridae) in forensic entomology. *Forensic Sci. Int. 175*, 83–84.

Mathias, D., Jacky, L., Bradshaw, W. E., & Holzapfel, C. M. (2007). Quantitative trait loci associated with photoperiodic response and stage of diapause in the pitcher-plant mosquito, *Wyeomyia smithii. Genetics*, *176*, 391–402.

Mayhew, P. J. (2007). Why are there so many insect species? Perspectives from fossils and phylogenies. *Biol. Rev. 82*, 425–454.

Meireles-Filho, A. C. A., Amoretty, P. R., De Souza, N. A., Kyriacou, C. P., & Peixoto, A. A. (2006). Rhythmic expression of the *cycle* gene in a hematophagous insect vector. *BMC Mol. Biol. 7*(38).

Menegazzi, P., Dalla Benetta, E., Beauchamp, M., Schlichting, M., Steffan-Dewenter, I., Helfrich-Förster, C., ... Helfrich-Förster, C. (2017). Adaptation of circadian neuronal network to photoperiod in high-latitude European drosophilids. *Curr. Biol. 27*, 833–839.

Mertens, I., Vandingenen, A., Johnson, E. C., Shafer, O. T., Li, W., Trigg, J. S., ... Taghert, P. H. (2005). PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. *Neuron*, *48*, 213–219.

Meuti, M. E., Stone, M., Ikeno, T., & Denlinger, D. L. (2015). Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*. *J. Exp. Biol. 218*, 412–422.

Miyasako, Y., Umezaki, Y., & Tomioka, K. (2007). Separate sets of cerebral clock neurons are responsible for light and temperature entrainment of *Drosophila* circadian locomotor rhythms. *J. Biol. Rhythms*, *22*(2), 115–126.

Moore, D. & Rankin, M. A. (1985). Circadian locomotor rhythms in individual honeybees. *Physiol. Entomol. 10*, 191–197.

Moon, I., Fujikawa, S., & Shimada, K. (1996). Cryopreservation of *Chymomyza* larvae (diptera: Drosophilidae) at -196°c with extracellular freezing. *CryoLett. 17*, 105–110.

Morrison, N. I., Franz, G., Koukidou, M., Miller, T. A., Saccone, G., Alphey, L. S., ... Polito, L. C. (2010). Genetic improvements to the sterile insect technique for agricultural pests. *Asia-Pacific J. Mol. Biol. Biotechnol. 18*(2), 275–295.

Murad, A., Emery-Le, M., & Emery, P. (2007). A subset of dorsal neurons modulates circadian behavior and light responses in Drosophila. *Neuron*, *53*, 689–701.

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

Nitabach, M. N., Wu, Y., Sheeba, V., Lemon, W. C., Strumbos, J., Zelensky, P. K., ... Holmes, T. C. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *J. Neurosci. 26*(2), 479–489.

Numata, H., Saulich, A. H., & Volkovich, T. A. (1993). Photoperiodic responses of the linden bug, *Pyrrhocoris apterus*, under conditions of constant temperature and under thermoperiodic conditions. *Zoolog. Sci. 10*, 521–527.

Nuorteva, P. & Hackman, W. (1970). Diel periodicity of activity in *Chymomyza costata* (Zett.) (Diptera, Drosophilidae) in the subarctic. In *Ann. zool. fenn.* (pp. 267–269).

Okada, T. (1956). *Systematic study of Drosophilidae and allied families of Japan.*

Oster, H., Damerow, S., Hut, R. A., & Eichele, G. (2006). Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. *J. Biol. Rhythms*, *21*(5), 350–361.

Ott, S. R. (2008). Confocal microscopy in large insect brains: Zinc-formaldehyde fixa-

tion improves synapsin immunostaining and preservation of morphology in whole-mounts. *J. Neurosci. Methods*, *172*, 220–230.

Özkaya, Ö. & Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. In *Adv. genet.* (Vol. 77, pp. 79–123).

Ozturk, N., Selby, C. P., Annayev, Y., Zhong, D., & Sancar, A. (2011). Reaction mechanism of *Drosophila* cryptochrome. *Proc. Natl. Acad. Sci. 108*(2), 516–521.

Page, T. L. & Block, G. D. (1980). Circadian rhythmicity in cockroaches: effects of early post-embryonic development and ageing. *Physiol. Entomol. 5*, 271–281.

Paolucci, S., Van de Zande, L., & Beukeboom, L. W. (2013). Adaptive latitudinal cline of photoperiodic diapause induction in the parasitoid *Nasonia vitripennis* in Europe. *J. Evol. Biol. 26*, 705–718.

Park, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M., & Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. 97*(7), 3608–3613.

Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends Cell Biol. 24*(2), 90–99.

Peschel, N., Chen, K. F., Szabo, G., & Stanewsky, R. (2009). Light-dependent interactions between the *Drosophila* circadian clock factors cryptochrome, Jetlag, and timeless. *Curr. Biol. 19*(3), 241–247.

Pfeiffenberger, C., Lear, B. C., Keegan, K. P., & Allada, R. (2010). Locomotor activity level monitoring using the Drosophila activity monitoring (DAM) system. In *Cold spring harb. protoc.* (pp. 1238–1242).

Picot, M., Cusumano, P., Klarsfeld, A., Ueda, R., & Rouyer, F. (2007). Light activates output from evening neurons and inhibits output from morning neurons in the *Drosophila* circadian clock. *PLoS Biol. 5*(11), e315.

Pittendrigh, C. S. & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents V. Pacemaker structure: a clock for all seasons. *J. Comp. Physiol. A*, *106*(3), 333–355.

Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proc. Natl. Acad. Sci. 40*(10), 1018–1029.

Pivarciova, L., Vaneckova, H., Provaznik, J., Wu, B. C. H., Pivarci, M., Peckova, O., … Dolezel, D. (2016). Unexpected geographic variability of the free running period in the linden bug Pyrrhocoris apterus. *J. Biol. Rhythms*, *31*(6), 568–576.

Prabhakaran, P. M. & Sheeba, V. (2012). Sympatric drosophilid species *melanogaster* and *ananassae* differ in temporal patterns of activity. *J. Biol. Rhythms*, *27*(5), 365–376.

Preitner, N., Damiola, F., Luis-Lopez-Molina, Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*, *110*, 251–260.

Pu, D. Q., Liu, H. L., Gong, Y. Y., Ji, P. C., Li, Y. J., Mou, F. S., & Wei, S. J. (2017). Mitochondrial genomes of the hoverflies *Episyrphus balteatus* and *Eupeodes corollae* (Diptera: Syrphidae), with a phylogenetic analysis of Muscomorpha. *Sci. Rep. 7*, 44300.

R Core Team. (2013). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. Vienna, Austria.

Rafiqi, A. M., Lemke, S., & Schmidt-Ott, U. (2011a). M*egaselia abdita*: culturing and egg collection. In *Cold spring harb. protoc.* (pp. 423–426).

Rafiqi, A. M., Lemke, S., & Schmidt-Ott, U. (2011b). M*egaselia abdita*: cuticle preparation from injected embryos. In *Cold spring harb. protoc.* (pp. 432–435).

Rafiqi, A. M., Lemke, S., & Schmidt-Ott, U. (2011c). M*egaselia abdita*: fixing and devitellinizing embryos. In *Cold spring harb. protoc.* (pp. 429–432).

Rafiqi, A. M., Lemke, S., & Schmidt-Ott, U. (2011d). M*egaselia abdita*: preparing embryos for injection. In *Cold spring harb. protoc.* (pp. 426–428).

Rafiqi, A. M., Lemke, S., & Schmidt-Ott, U. (2011e). The scuttle fly *Megaselia abdita* (Phoridae): a link between *Drosophila* and mosquito development. In *Cold spring harb. protoc.* (pp. 349–354).

Raghu, S. & Clarke, A. R. (2003). Spatial and temporal partitioning of behaviour by adult dacines: direct evidence for methyl eugenol as a mate rendezvous cue for *Bactrocera cacuminata*. *Physiol. Entomol. 28*, 175–184.

Reddy, K. L., Wohlwill, A., Dzitoeva, S., Lin, M. H., Holbrook, S., & Storti, R. V. (2000). The *Drosophila PAR domain protein 1* (*Pdp1*) gene encodes multiple differentially expressed mRNAs and proteins through the use of multiple enhancers and promoters. *Dev. Biol. 224*, 401–414.

Refinetti, R., Cornélissen, G., & Halberg, F. (2007). Procedures for numerical analysis of circadian rhythms. *Biol. Rhythm Res. 38*(4), 275–325.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (1999). A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*, *99*, 791–802.

Reppert, S. M. (2006). A colorful model of the circadian clock. *Cell*, *124*, 233–236.

Reppert, S. M. (1998). A clockwork explosion! *Neuron*, *21*, 1–4.

Revel, F. G., Herwig, A., Garidou, M.-L., Dardente, H., Menet, J. S., Masson-Pevet, M., … Pevet, P. (2007). The circadian clock stops ticking during deep hibernation in the European hamster. *Proc. Natl. Acad. Sci. 104*(34), 13816–13820.

Revelle, W. (2017). *Psych: Procedures for psychological, psychometric, and personality research*. R package version 1.7.8. Northwestern University. Evanston, Illinois.

Rieger, D., Stanewsky, R., & Helfrich-Förster, C. (2003). Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. *J. Biol. Rhythms*, *18*(5), 377–391.

Rieger, D., Shafer, O. T., Tomioka, K., & Helfrich-Förster, C. (2006). Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. *J. Neurosci. 26*(9), 2531–2543.

Rieger, D., Peschel, N., & Dusik, V. (2012). The ability to entrain to long photoperiods differs between 3 *Drosophila melanogaster* wild-type strains and is modified by twilight simulation. *J. Biol. Rhythms*, *27*(1), 37–47.

Riihimaa, A. J. & Kimura, M. T. (1988). A mutant strain of *Chymomyza costata* (Diptera: Drosophilidae) insensitive to diapause-inducing action of photoperiod. *Physiolo*, *13*, 441–445.

Riihimaa, A. J. & Kimura, M. T. (1989). Genetics of the photoperiodic larval diapause in *Chymomyza costata* (Diptera ; Drosophilidae). *Hereditas*, 193–200.

Ring, M., Meredith, J., Wiens, C., Macins, A., Brock, H. W., Phillips, J. E., & Theilmann, D. A. (1998). Expression of *Schistocerca gregaria* ion transport peptide (ITP) and its homologue (ITP-L) in a Baculovirus/Insect Cell System. *Insect Biochem. Mol. Biol. 28*(1), 51–58.

Ro, J., Harvanek, Z. M., & Pletcher, S. D. (2014). FLIC: high-throughput, continuous analysis of feeding behaviors in *Drosophila*. *PLoS One*, *9*(6), e101107.

Roberts, L., Leise, T. L., Noguchi, T., Galschiodt, A. M., Houl, J. H., Welsh, D. K., & Holmes, T. C. (2015). Light evokes rapid circadian network oscillator desynchrony followed by gradual phase retuning of synchrony. *Curr. Biol. 25*, 858–867.

Roberts, D. H., Lehár, J., & Dreher, J. W. (1987). Time series analysis with CLEAN. I. Derivation of a spectrum. *Astron. J. 93*(4), 968–989.

Rosato, E. & Kyriacou, C. P. (2006). Analysis of locomotor activity rhythms in *Drosophila*. *Nat. Protoc. 1*(2), 559–568.

Rubin, E. B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H., & Bloch, G. (2006). Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome Res. 16*, 1352–1365.

Ruf, T. (1999). The Lomb-Scargle periodogram in biological rhythm research: analysis of incomplete and unequally spaced time-series. *Biol. Rhythm Res. 30*(2), 178–201.

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., & Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell*, *93*, 805–814.

Sakai, T. & Kitamoto, T. (2006). Clock, love and memory: circadian and non-circadian regulation of *Drosophila* mating behavior by clock genes. *Sleep Biol. Rhythms*, *4*, 255–262.

Sandrelli, F., Costa, R., Kyriacou, C. P., & Rosato, E. (2008). Comparative analysis of circadian clock genes in insects. *Insect Mol. Biol. 17*(5), 447–463.

Sancar, A. (2004). Regulation of the mammalian circadian clock by cryptochrome. *J. Biol. Chem. 279*(33), 34079–34082.

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., ... Hogenesch, J. B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron*, *43*, 527–537.

Saunders, D. S., Henrich, V. C., & Gilbert, L. I. (1989). Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. 86*, 3748–3752.

Saunders, D. S. (2010). Controversial aspects of photoperiodism in insects and mites. *J. Insect Physiol. 56*, 1491–1502.

Saunders, D. S. (2014). Insect photoperiodism: effects of temperature on the induction of insect diapause and diverse roles for the circadian system in the photoperiodic response. *Entomol. Sci.* 1–16.

Saunders, D. S. (1987). Insect photoperiodism: the linden bug, *Pyrrhocoris apterus*, a

species that measures daylength rather than nightlength. *Experimentia*, *43*, 935–937.

Sayeed, O. & Benzer, S. (1996). Behavioral genetics of thermosensation and hygrosensation in *Drosophila*. *Proc. Natl. Acad. Sci. 93*, 6079–6084.

Scargle, J. D. (1982). Studies in astronomical time series analysis. ii — statistical aspects of spectral analysis of unevenly spaced data. *Astrophys. J. 263, 835–853.*

Schmid, B., Helfrich-Förster, C., & Yoshii, T. (2011). A new ImageJ plug-in "ActogramJ"for chronobiological analyses. *J. Biol. Rhythms*, *26*(5), 464–467.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, *9*(7), 676–682.

Schlichting, M., Menegazzi, P., Lelito, K. R., Yao, Z., Buhl, E., Dalla Benetta, E., ... Shafer, O. T. (2016). A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. *J. Neurosci. 36*(35), 9084–9096.

Schubert, F. K., Hagedorn, N., Yoshii, T., Helfrich-Förster, C., & Rieger, D. (2018). Neuroanatomical details of the lateral neurons of *Drosophila melanogaster* support their functional role in the circadian system. *J. Comp. Neurol. 526*, 1209–1231.

Sehadová, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T., & Stanewsky, R. (2009). Temperature entrainment of *Drosophila*'s circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain. *Neuron*, *64*, 251–266.

Sehgal, A., Price, J. L., & Young, M. W. (1992). Ontogeny of a biological clock in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. 89*, 1423–1427.

Selcho, M. & Wegener, C. (2015). Immunofluorescence and genetic fluorescent labeling techniques in the *Drosophila* nervous system. In A. M. Lossi & Laura (Eds.), *Immunocytochem. relat. tech.* (Vol. 101, pp. 39–62).

Selcho, M., Millán, C., Palacios-Muñoz, A., Ruf, F., Ubillo, L., Chen, J., ... Ewer, J. (2017). Central and peripheral clocks are coupled by a neuropeptide pathway in *Drosophila*. *Nat. Commun. 8*, 15563.

Shafer, O. T. & Taghert, P. H. (2009). RNA-interference knockdown of *Drosophila* pigment dispersing factor in neuronal subsets: the anatomical basis of a neuropeptide's circadian functions. *PLoS One*, *4*(12), e8298.

Shen, P. & Cai, H. N. (2001). Drosophila neuropeptide F mediates integration of chemosensory stimulation and conditioning of the nervous system by food. *J. Neurobiol. 47*, 16–25.

Shelly, T. E., Whittier, T. S., & Kaneshiro, K. Y. (1994). Sterile insect release and the natural mating system of the mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Ann. Entomol .Soc. Am. 87*(4), 470–481.

Sheeba, V., Sharma, V. K., Chandrashekaran, M. K., & Joshi, A. (1999). Persistence of eclosion rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment. *Naturwissenschaften*, *86*, 448–449.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol. 7*(539).

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., & Hall, J. C. (1988). Antibodies to the *period* gene product of drosophila reveal diverse tissue distribution and rhythmic

changes in the visual system. *Neuron*, *1*, 141–50.

Slama, K. & Williams, C. (1965). Juvenile hormone activity for the bug *Pyrrhocoris apterus*. *Proc. Natl. Acad. Sci. 54*, 411–414.

Smith, P. H. (1979). Genetic manipulation of the circadian clock's timing of sexual behaviour in the Queensland fruit flies, *Dacus tryoni* and *Dacus neohumeralis*. *Physiol. Entomol. 4*, 71–78.

Socha, R. & Hodková, M. (1993). Selection for non-diapause in the heteropteran *Pyrrhocoris apterus*. *Hereditas*, *120*, 81–85.

Stamatakis, A., Hoover, P., & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML Web servers. *Syst. Biol. 57*(5), 758–771.

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M. J., Rosbash, M., & Hall, J. C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and a *lacZ* reporter: mapping elements of the PER protein involved in circadian cycling. *J. Neurosci. 17*(2), 676–696.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., ... Hall, J. C. (1998). The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell*, *95*, 681–692.

Stehlík, J., Závodská, R., Shimada, K., Sauman, I., & Koštál, V. (2008). Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. *J. Biol. Rhythms*, *23*(2), 129–139.

Stengl, M. & Homberg, U. (1994). Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol. A. 175*, 203–213.

Stoleru, D., Peng, Y., Agosto, J., & Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. *Nature*, *431*, 862–868.

Stokkan, K. A., Van Oort, B. E., Tyler, N. J., & Loudon, A. S. (2007). Adaptations for life in the Arctic: evidence that melatonin rhythms in reindeer are not driven by a circadian oscillator but remain acutely sensitive to environmental photoperiod. *J. Pineal Res. 43*, 289–293.

Stoleru, D., Nawathean, P., Fernández, M. d. l. P., Menet, J. S., Ceriani, M. F., & Rosbash, M. (2007). The *Drosophila* circadian network is a seasonal timer. *Cell*, *129*, 207–219.

Strauss, J., Zhang, Q., Verleyen, P., Huybrechts, J., Neupert, S., Predel, R., ... Dircksen, H. (2011). Pigment-dispersing hormone in *Daphnia* interneurons, one type homologous to insect clock neurons displaying circadian rhythmicity. *Cell. Mol. Life Sci. 68*, 3403–3423.

Syrová, Z., Doležel, D., Sauman, I., Hodková, M., & Šauman, I. (2003). Photoperiodic regulation of diapause in linden bugs: are *period* and *Clock* genes involved? *Cell. Mol. Life Sci. 60*, 2510–2515.

Tauber, E. & Kyriacou, C. P. (2001). Insect photoperiodism and circadian clocks: models and mechanisms. *J. Biol. Rhythms*, *16*(4), 381–390.

Tomioka, K. & Matsumoto, A. (2015). Circadian molecular clockworks in non-model insects. *Curr. Opin. Insect Sci. 7*, 58–64.

Traut, W. (1994). Sex determination in the fly *Megaselia scalaris*, a model system for primary

steps of sex chromosome evolution. *Genetics*, *136*, 1097–1104.

Tsitsipis, J. A. & Loher, W. (1989). Circadian rhythmical exodus of olive fruit fly larvae from the diet for pupation. In *Fruit flies of economic importance 87 : Proceedings of the cec/iobc international symposium, rome 7-10, april 1987* (pp. 203–208).

Tychsen, P. H. & Fletcher, B. S. (1971). Studies on the rhythm of mating in the Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol. 17*, 2139–2156.

Tzanakakis, M. E., Economopoulos, A., & Tsitsipis, J. A. (1966). Improved artificial food media for larvae of *Dacus oleae* (Gmelin) (Diptera: Tephritidae). In *"Democritus"nucl. res. cent. greek at. energy comm. athens* (Vol. 59, pp. 127–130).

Tzanakakis, M. E. (2003). Seasonal development and dormancy of insects and mites feeding on olive: a review. *Netherlands J. Zool. 52*(2-4), 87–224.

Urbanová, V., Bazalová, O., Vaněčková, H., & Doležel, D. (2016). Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. *Insect Biochem. Mol. Biol. 70*, 184–190.

Vafopoulou, X., Terry, K. L., & Steel, C. G. H. (2010). The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J. Comp. Neurol. 518*, 1264–1282.

Vafopoulou, X. & Steel, C. G. H. (2012). Metamorphosis of a clock: remodeling of the circadian timing system in the brain of Rhodnius prolixus (Hemiptera) during larval-adult development. *J. Comp. Neurol. 520*, 1146–1164.

Van Oort, B. E., Tyler, N. J. C., Gerkema, M. P., Folkow, L., & Stokkan, K. A. (2007). Where clocks are redundant: weak circadian mechanisms in reindeer living under polar photic conditions. *Naturwissenschaften*, *94*, 183–194.

Varney, R. L. & Noor, M. A. F. (2010). The scuttle fly. *Curr. Biol. 20*(11).

Vaze, K. M. & Helfrich-Förster, C. (2016). D*rosophila ezoana* uses an hour-glass or highly damped circadian clock for measuring night length and inducing diapause. *Physiol. Entomol. 41*, 378–389.

Vaz Nunes, M. & Saunders, D. S. (1999). Photoperiodic time measurement in insects: a review of clock models. *J. Biol. Rhythms*, *14*(2), 84–104.

Veleri, S., Brandes, C., Helfrich-Förster, C., Hall, J. C., & Stanewsky, R. (2003). A self-sustaining, light-entrainable circadian oscillator in the *Drosophila* brain. *Curr. Biol. 13*, 1758–1767.

Vontas, J. G., Cosmidis, N., Loukas, M., Tsakas, S., Hejazi, J., Ayoutanti, A., & Hemingway, J. (2001). Altered acetylcholinesterase confers organophosphate resistance in the olive fruit fly *Bactrocera oleae*. *Pestic. Biochem. Physiol. 71*, 124–132.

Vontas, J. G., Hejazi, M. J., Hawkes, N. J., Cosmidis, N., Loukas, M., Janes, R. W., & Hemingway, J. (2002). Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol. Biol. 11*(4), 329–336.

Weiner, J. (1999). *Time, love, memory: A great biologist and his quest for the origins of behavior*. Borzoi book. Knopf.

Weldon, C. W., Prenter, J., & Taylor, P. W. (2010). Activity patterns of Queensland fruit

flies (*Bactrocera tryoni*) are affected by both mass-rearing and sterilization. *Physiol. Entomol. 35*, 148–153.

Wheeler, D. A., Hamblen-Coyle, M. J., Dushay, M. S., & Hall, J. C. (1993). Behavior in light-dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. *J. Biol. Rhythms*, *8*(1), 67–94.

Wickham, H. (2009). *Ggplot2: Elegant graphics for data analysis*. Springer-Verlag New York.

Williams, C. T., Barnes, B. M., & Buck, C. L. (2012a). Daily body temperature rhythms persist under the midnight sun but are absent during hibernation in free-living arctic ground squirrels. *Biol. Lett. 8*, 31–34.

Williams, C. T., Barnes, B. M., Richter, M., & Buck, C. L. (2012b). Hibernation and circadian rhythms of body temperature in free-living Arctic ground squirrels. *Physiol. Biochem. Zool. 85*(4), 397–404.

Wotton, K. R., Jiménez-Guri, E., García Matheu, B., & Jaeger, J. (2014). A staging scheme for the development of the scuttle fly *Megaselia abdita*. *PLoS One*, *9*(1), e84421.

Wülbeck, C., Grieshaber, E., & Helfrich-Förster, C. (2008). Pigment-dispersing factor (PDF) has different effects on *Drosophila*'s circadian clocks in the accessory medulla and in the dorsal brain. *J. Biol. Rhythms*, *23*(5), 409–424.

Xu, K., Zheng, X., & Sehgal, A. (2008). Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab. 8*, 289–300.

Yeku, O. & Frohman, M. A. (2011). Rapid Amplification of cDNA Ends (RACE). In *Rna, methods mol. biol.* (Vol. 703, pp. 107–122).

Yoshii, T., Funada, Y., Ibuki-Ishibashi, T., Matsumoto, A., Tanimura, T., & Tomioka, K. (2004). D*rosophila cry*^{b} mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. *J. Insect Physiol. 50*, 479–488.

Yoshii, T., Heshiki, Y., Ibuki-Ishibashi, T., Matsumoto, A., Tanimura, T., & Tomioka, K. (2005). Temperature cycles drive *Drosophila* circadian oscillation in constant light that otherwise induces behavioural arrhythmicity. *Eur. J. Neurosci. 22*, 1176–1184.

Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R., & Helfrich-Förster, C. (2008). Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. *J. Comp. Neurol. 508*, 952–966.

Yoshii, T., Hermann, C., & Helfrich-Förster, C. (2010). Cryptochrome-positive and -negative clock neurons in *Drosophila* entrain differentially to light and temperature. *J. Biol. Rhythms*, *25*(6), 387–398.

Yoshii, T., Rieger, D., & Helfrich-Förster, C. (2012). Two clocks in the brain: an update of the morning and evening oscillator model in Drosophila. In *Prog. brain res.* (Vol. 199, pp. 59–82).

Yoshida, T. & Kimura, M. T. (1995). The photoperiodic clock in *Chymomyza costata*.

Yu, W. & Hardin, P. E. (2006). Circadian oscillators of *Drosophila* and mammals. *J. Cell Sci. 119*(23), 4793–4795.

Yuan, Q., Metterville, D., Briscoe, A. D., & Reppert, S. M. (2007). Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol. Biol. Evol. 24*(4), 948–955.

Závodská, R., Šauman, I., & Sehnal, F. (2003). Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic

nervous system of insects. *J. Biol. Rhythms*, *18*(2), 106–122.

Závodská, R., Fexova, S., von Wowern, G., Han, G.-B., Doležel, D., & Sauman, I. (2012). Is the sex communication of two pyralid moths, Plodia interpunctella and Ephestia kuehniella, under circadian clock regulation? *J. Biol. Rhythms*, *27*(3), 206–216.

Zervas, G. A. & Economopoulos, A. (1982). Mating frequency in caged populations of wild and artificially reared (normal or gamma-sterilized) olive fruit flies. *Environ. Entomol. 11*, 17–20.

Zerr, D. M., Hall, J. C., Rosbash, M., & Siwicki, K. K. (1990). Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci. 10*(8), 2749–2762.

Zhang, L., Chung, B. Y., Lear, B. C., Kilman, V. L., Liu, Y., Mahesh, G., ... Allada, R. (2010). DN1p circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in *Drosophila*. *Curr. Biol. 20*, 591–599.

Zhang, L., Hastings, M. H., Green, E. W., Tauber, E., Sladek, M., Webster, S. G., ... Wilcockson, D. C. (2013). Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. *Curr. Biol. 23*, 1863–1873.

Zheng, X., Koh, K., Sowcik, M., Smith, C. J., Chen, D., Wu, M. N., & Sehgal, A. (2009). An isoform-specific mutant reveals a role of PDP1e in the circadian oscillator. *J. Neurosci. 29*(35), 10920–10927.

Zhu, H., Yuan, Q., Briscoe, A. D., Froy, O., Casselman, A., & Reppert, S. M. (2005). The two CRYs of the butterfly. *Curr. Biol. 15*(23), R953–R954.

Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P., & Reppert, S. M. (2008). Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biol. 6*(1), e4.

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- Bertolini E., Kistenpfennig C., Menegazzi P., Keller A., Koukidou M., Helfrich-Förster C. (2018). The characterization of the circadian clock in the olive fly *Bactrocera oleae* (Diptera: Tephritidae) reveals a *Drosophila*-like organization. Sci. Rep. 8(1), 816

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Affidavit

I hereby confirm that my thesis entitled *Comparative analysis of insect circadian clocks: a behavioural, anatomical, and molecular study* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *Vergleichende Analyse der zirkadianen Uhr von Insekten: eine verhaltensbezogene, anatomische und molekulare Studie* eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Ort, Datum Unterschrift