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Are the heterogeneous nuclear ribonucleoproteins SQUID and Hrb87F involved in the regulation of circadian rhythmicity in *Drosophila melanogaster*?

Thesis written with the financial contribution of Fondazione *CARIPARO*

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4. MATERIALS and METHODS

ABSTRACT

The search for new genes involved in circadian rhythmicity in *Drosophila melanogaster* led to the identification of the RNA binding proteins Hrb87F and SQUID, as partners of Cryptochrome, the photoreceptor responsible for light-synchronization of the circadian clock. In the clock machinery, different post-transcriptional mechanisms have evolved to adjust and consolidate the oscillation of clock genes and proteins in interlocked negative feedback loops. The aim of my PhD project was to study the possible involvement of Hrb87F and SQUID in the post-transcriptional control of the circadian clock in *Drosophila*.

These preliminary data were confirmed by Co-Immunoprecipitation and western-blot experiments using transgenic flies expressing a tagged version of Cry (*HACRY*) under the control of the driver *tim*GAL4, for SQUID, while for Hrb87F the interaction was validated by yeast two-hybrid assay, in which dCRY was challenged to Hrb87F as prey.

To achieve more information about a possible role of the clock in the expression of *Squid* and *Hrb87F* in Drosophila head, the analysis of the expression profile of the two hnRNPs (mRNAs and proteins) was performed during the 24 hours in wild type (*wt*) and clock mutant per^0 flies. As for *Hrb87F,* mRNA levels showed an oscillatory trend in LD (Light-Dark) and DD (Dark-Dark) either in wt and per^0 flies; the protein levels oscillate in LD and DD in *wt* flies, but not in per^0, suggesting a potential role for the circadian clock in the translational/posttranslational control of the protein. As for *Squid*, neither the mRNA nor the protein showed rhythmic expression in LD and DD.

Subsequently, the involvement of SQUID and Hrb87F in the generation of the circadian rhythmicity was studied analyzing the locomotor activity pattern of flies' mutant for each of the two genes. Mutants for both genes showed an impairment of the daily rhythmicity, with a loss of the morning anticipation in LD and low levels of rhythmicity in DD at 29°C, 23°C, 18°C and 15°C, suggesting that *Squid* and *Hrb87F* could play a role either in the generation and in the light-synchronization of the circadian behaviour. However, the analysis of locomotor activity in constant light, performed in order to further dissect the possible role of *Squid* in the light synchronization of the clock, revealed that the light-synchronization of the clock is not impaired in these flies.

The expression of *period* and *timeless* and the alternative splicing variants in relation to the temperature were also analyzed in *wt* and *Squid* mutant brains by using multiplex real time PCR. The results suggest that the expression of *period* and *timeless* mRNA is altered in *Squid* mutant compared to *wt* both in LD and DD at each temperature. Moreover, the quantities of *per* unspliced at high temperature and *tim* unspliced at low temperature are lower both in LD and DD in the

mutant. This suggests an increase of the splicing events and thus an involvement of SQUID in the post-transcriptional control of clock genes.

In collaboration with Dr Milena Damulewicz (Jagiellonian University of Krakow - Poland), the expression of PERIOD was analyzed in the clock neurons by immunocytochemistry in flies reared at both 18°C and 23°C in LD and DD. At 23°C and 18°C the oscillation of PER in l-LN_vs clock neurons is lost, while, in s-LN_vs, the accumulation of protein is delayed by 3 hours with a broader peak that reflects a low kinetic of degradation. The analysis of PDF projections from clock neurons shows a profound disorganization of PDF release, suggesting that it can, at least partially, account for the locomotor activity defect observed in the *Squid* mutant.

Taken together, these results highlight the possible involvement of the hnRNPs Hrb87F and SQUID in the generation and maintenance of circadian rhythmicity in *Drosophila melanogaster.*

ABSTRACT ITALIANO

La ricerca di nuovi geni coinvolti nel controllo della ritmicità circadiana in *Drosophila melanogaster* ha permesso di identificare una possibile interazione delle due ribonucleoproteine SQUID e HRB87F con CRYPTOCROME. Nella regolazione molecolare dell'orologio circadiano, si sono evoluti diversi eventi di regolazione post trascrizionale che aggiustano e consolidano l'espressione ritmica dei geni orologio e delle corrispettive proteine, secondo un meccanismo a feedback negativo. Per tale motivo lo scopo di questo lavoro è stato studiare e analizzare il possibile coinvolgimento delle hnRNPs nel controllo post-trascrizionale dell'orologio circadiano in *Drosophila.*

In una fase iniziale, per confermare i risultati ottenuti in precedenza, sono stati condotti esperimenti di Co-Immunoprecipitazione e western-blot in mosche transgeniche in grado di esprimere una versione di CRY associata all'epitopo HA (HACRY) sotto il controllo del driver *tim*Gal4. L'anticorpo anti-SQUID ha identificato una proteina nel campione co-immunoprecipitato, confermando l'interazione, mentre il legame con HRB87F è stato osservato mediante l'utilizzo del sistema del doppio ibrido di lievito.

In seguito è stato valutato il possibile coinvolgimento dell'orologio circadiano nell'espressione delle hnRNPs nell'intera testa del moscerino. I livelli di espressione del mRNA di *Hrb87F* mostrano un trend oscillatorio in LD (luce-buio) e DD (buio costante), sia nel *wt* che nel mutante per^0, mentre la proteina oscilla in LD e DD nelle mosche *wt*, ma non nelle per^0, suggerendo un possibile ruolo dell'orologio circadiano nel controllo traduzionale o post-traduzionale della proteina. L'analisi dell'espressione del gene *Squid* ha dimostrato, invece, che né mRNA né proteina vengono espressi in maniera ritmica, né in LD né DD.

Successivamente, è stato studiato l'intervento delle due ribonucleoproteine nella generazione della ritmicità circadiana, analizzando l'attività locomotoria di mosche mutanti per entrambi i geni. Tutti i mutanti hanno riportato un'alterazione della ritmicità circadiana, con una perdita dell'anticipazione dell'attività mattutina in LD e bassi livelli di ritmicità in DD a 29°C, 23°C, 18°C e 15°C. I risultati ottenuti suggeriscono che *Squid* e *Hrb87F* possano partecipare alla generazione di un fenotipo ritmico nell'attività locomotoria. Questa analisi è stata completata studiando, inoltre, l'attività locomotoria del mutante *squid* in condizione di luce costante (LL), poiché esibiva un fenotipo associabile a quello in cui è presente un'alterazione del meccanismo di sincronizzazione della luce; si è visto che, le mosche mutanti presentano un risposta in LL paragonabile al *wt*, sottolineando un corretto funzionamento del meccanismo di sincronizzazione della luce.

Abbiamo analizzato i profili di espressione dei geni *period* e *timeless* e delle rispettive varianti dovute a splicing alternativo legato alla temperatura. I risultati ottenuti mostrano che l'espressione è alterata nel mutante *Squid*, rispetto al controllo wt, sia in LD sia in DD ad ogni temperatura considerata, evidenziando che è presente un elevata attività di splicing.

In fine, in collaborazione con la Dott. Milena Damulewicz (Università di Jagiellonian di Kracovia - Polonia), è stata condotta un'analisi dell'espressione di PERIOD nei neuroni orologio in mosche mutanti *Squid* e *wt*, mediante immunocitochimica a 18°C e 23°C in LD e DD. Si è visto che sia a 23°C sia a 18°C, l'oscillazione di PER nei neuroni orologio l-LN_v, viene persa sia in LD sia in DD, mentre nei neuroni s-LN_v sia a 23°C sia a 18°C si assiste a un ritardo nell'accumulo della proteina seguito da una cinetica di degradazione più lenta rispetto ai controlli. L'analisi delle proiezioni del neuropeptide PDF ha evidenziato una profonda disorganizzate nel mutante *squid*.

Nel complesso, tutti questi risultati suggeriscono un coinvolgimento di HRB87F e SQUID nella generazione e nel mantenimento della ritmicità circadiana in *Drosophila melanogaster.*

Abbreviations

ZT	Zeitgeber Time
CT	Circadian Time
LD	Light-Dark
DD	Constant Dark
CLK	Clock
CYC	Cycle
PER	Period
TIM	Timeless
VRI	Vrille
DBT	Doubletime
CRY	Cryptochrome
norpA	No receptor potential A
PDF	Pigment Dispersing Factor
DN	Dorsal Neurons
LPN	Lateral Posterior Neurons
LN_v	Ventral Lateral Neurons
LN_d	Dorsal Lateral Neurons
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
Sqd	Squid
Hrb87F	Heterogeneous Nuclear Ribonucleoprotein at 87 F

Chapter 1

INTRODUCTION

1.1. Circadian rhythms in nature

Living organisms are constantly exposed to cyclical variations in their environment. The ability to anticipate these variations has an important evolutionary role and relies on the presence of endogenous mechanisms that allow the temporal organization of several metabolic, physiological and behavioural activities. The cyclical variations can be classified as circadian, from the latin *circa-diem,* with a period of 24 hours, ultradian, with a period shorter than 24h, and infradian, with a period longer of 24 h. Examples of rhythms in nature are the daily, tidal, lunar and seasonal rhythms (Kalsbeek et al., 2012; Numata and Helm, 2014).

A cyclic biological activity was first described by Carl Nilsson Linnaeus, who analysed the rhythmic opening and closing of petals in different varieties of flowers. Subsequently, many other scientists described biological processes with a rhythmic activity in different organisms, from bacteria to humans. Endogenous clock that give the rhythm to every behavioural, biochemical and physiological process have been now described in every organism (Harmer et al., 2001; Brunner and Schafmeier, 2006; Zhang et al., 2009; Lowrey and Takahashi, 2011).

Endogenous circadian clocks have common features:

- The rhythm is an innate property of the organism and it is maintained also in absence of external cues, with a period of about 24 hours.
- Biological rhythms respond and are synchronised to environmental stimuli. Light and temperature play an important role in the synchronization of the molecular clock (defined as *Zeitgeber*¸ German, *Zeit* = time, *Geber* = giver).
- The endogenous oscillation is “temperature compensated”, meaning that the period is maintained constant within a physiological range of temperature, preventing the alterations of the physiological responses as function of temperature.

Studying the circadian clock requires the discrimination between Zeitgeber time (ZT) and Circadian time (CT) (Zhang and Emery, 2012). The fundamental difference is the presence or absence of external environmental cues entraining the core of the clock. ZT relies on the presence of a stimulus that synchronises the endogenous clock to the environmental changes. CT, instead, is characterized by the absence of external stimuli and refers to constant light or temperature. This condition is also called “free running”.

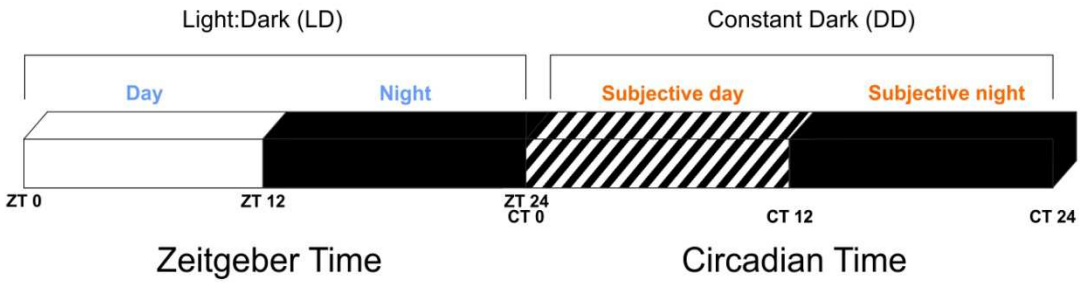


Figure 1. Schematic representation of the timeline in which Light:Dark (LD) and constant darkness (DD) conditions are represented. During the Light:Dark (LD) cycle the white bar represents the light phase, while the black bar the dark phase. Conventionally, the beginning of light phase is defined as ZT 0. In the right part, the *Circadian Time* is depicted: this time is characterized by constant Darkness and subjective days and night can be identified.

In experimental conditions of ZT, individuals are for example reared in 12:12 Light-Dark (LD) cycles (12 hours of light and 12 hours of darkness) at constant temperature. In this case light represents the Zeitgeber. In a LD 12:12 cycle, ZT0 correspond to the beginning of the light phase, while ZT12 correspond to the light switch off. It is also possible to entrain organisms to temperature cycles: in this case, the light is maintained constant while the temperature oscillates during the 24 hours (Pittendringh, 1960; Liu et al., 1997).

In experimental conditions of CT, individuals experience constant regimes of light (or dark) and temperature. CT can be additionally described in terms of subjective day and subjective night. The canonical free-running conditions are constant darkness and constant temperature.

In a schematic view, in the circadian circuit is characterised by three important components:

1. INPUT: It is the sequence of events via which information from the environment, such as changes in light and temperature, are transduced to the oscillator.
2. OSCILLATOR: It is the cell-autonomous timekeeper responsible for generating self-sustained rhythmicity. This comprises the molecular elements that are expressed rhythmically with a period of 24 hours.
3. OUTPUT: It is represented by all the circadian phenotypes. Most outputs are generated by the cyclic transcription of output genes (clock controlled genes, CCGs), whose products, in turn, act on other genes and system to generate rhythmic cellular, physiological and behavioural processes.

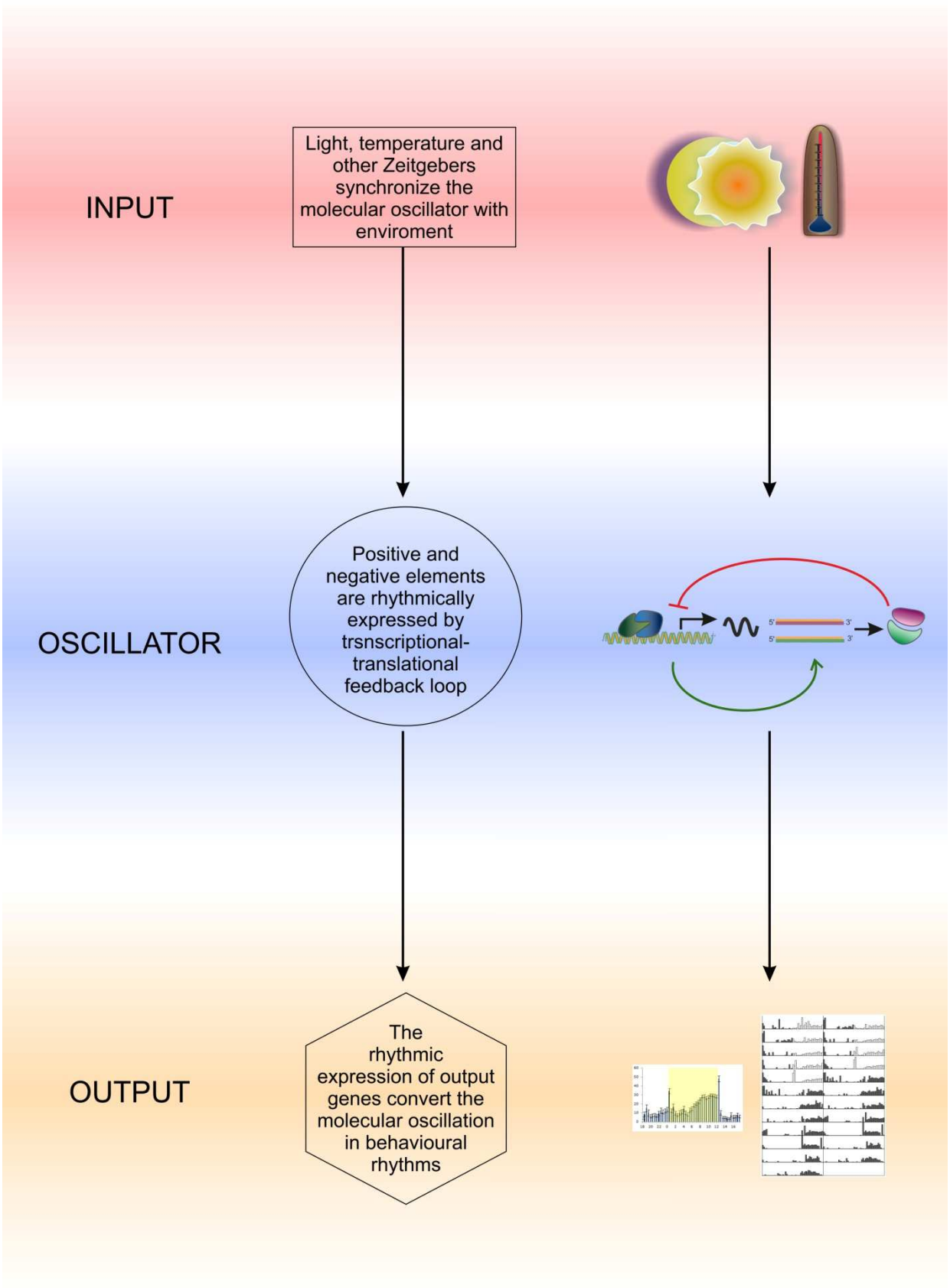


Figure 2. Schematic representation of the basic components of a circadian system. Light and temperature (INPUT) synchronize the molecular OSCILLATOR, that sustains biological rhythms through OUTPUT pathways.

1.2. INPUT

One the most important characteristic of the circadian clock is the possibility to synchronise its rhythms with the external world, and different input pathways connect the circadian pacemaker to the environment.

1.2.1. Light

Light plays a predominant role in the synchronization of the molecular endogenous mechanisms.

Drosophila melanogaster perceives the light inputs through three different pathways: retinal photoreceptors (compound eyes and ocelli), extra-retinal photoreceptors (the Hofbauer-Buchner (HB) eyelet) and by the CHRYPTOCHROME (CRY) inner photoreceptor.

The compound eyes consist of about 800 ommatidia, identical exagon-shaped units each containing 6 “outer photoreceptors” (R1-6) and 2 “inner photoreceptors” (R7-8) cells, able to catch photons. The R1-6 photoreceptors are involved in motion detection and image formation and project into the lamina, one of the four optic ganglia comprised in the fly’s visual system. They have large rhabdomers (highly packed microvillar structures formed by the plasma membrane) and mostly express rhodopsin 1 (Rh1), a blue-sensitivity opsin with a broad absorption spectrum. The inner photoreceptors, R7 and R8 are involved in colour vision and project in the medulla. They have smaller rhabdomeres and express a complex pattern of four different rhodopsins (Rh3, Rh4, Rh5 or Rh6 (Behnia and Desplan, 2015; Yoshii et al., 2016). The phototransduction cascade is initiated by the isomerisation of the chromophore linked to the rhodopsin from the 11- cis to all-trans retinal. This leads to the formation of an active metarhodopsin, which in turn activates G-protein (Gq). A subsequent cascade of events involving Phospholipase Cβ (PLC), allows the opening of the Transient Receptor Potential (TRP) and the Transient Receptor Potential-Like (TRPL) cation channels (Hardie 2001; Tian et al 2011; Montell 2012). This leads to the depolarization of the photoreceptor membrane and the consequent release of neurotransmitters. Ocelli are located in the vertex of the fly’s head: the three ocelli. They consist of about 70-90 photoreceptor neurons, which contain a thin layer of pigment cells expressing the Rh2 opsins, a violet light absorbing pigments (Pollock and Benzer, 1988). They have been shown to play a minor role in the circadian light entrainment (Rieger et al., 2003) The HB eyelet is an extra-retinal circadian photoreceptor located between the retina and the lamina in the fly’s optic lobe (Hofbauer and Buchner, 1989). This particular light-perceiving organ derives from the larval visual system or Bolwig Organ, which is composed of twelve photoreceptors: eight green sensitive (expressing Rh6)

and four blue-sensitive (expressing Rh5) ones (Sprecher et al., 2007). During metamorphosis, all the Rh6-expressing cells die and the four Rh5-expressing larval photoreceptors switch from the blue to the green chromophore absorbing pigment, thus becoming the four photoreceptor of the adult H-B eyelet, expressing Rh6 (Sprecher and Desplan, 2008). In both larval and adult brains, the projections of Bolwig´s organs and HB eyelets directly contact some of the main clock neurons (Helfrich-Förster et al., 2002; Malpel et al., 2002; Yoshii et al., 2016).

In addition to external photoreceptors, a subset of clock neurons expresses the protein CRYPTOCROME (CRY), a photopigment sensitive to UV and blue light, which enables these neurons to directly perceive light (Benito et al., 2008 Aug; Yoshii et al., 2008). Upon light exposure, CRY binds to the TIMELESS (TIM) protein, an important clock component, promoting its degradation (Koh et al., 2006; Peschel et al., 2009). The light-induced degradation of TIM destabilizes PERIOD (PER), an essential core clock component and a partner of TIM, ultimately regulating the circadian oscillation of PER and TIM levels (Ishida et al., 1999; Helfrich-Forster, 2005).

Very recently, a new discovered rhodopsin (Rh7) has found to be expressed in some clock neurons and in R8 photoreceptor cells of the compound eyes and in the Hofbauer-Buchner eyelets and proven to be involved in the circadian photoentrainment and the timing of locomotor activity (Kistenpfennig et al., 2017; Ni et al., 2017).

1.2.2. Temperature

Drosophila can use daily changes in the environmental temperature to adjust its endogenous period and sleep-activity cycle to the 24-hour light-dark cycle, therefore temperature can act as Zeitgeber for adult circadian behaviour (Busza et al., 2007). In fact, temperature cycles are able to synchronise behavioural rhythms and oscillations of the clock proteins PERIOD and TIMELESS in constant light, a situation in which molecular and behavioural rhythms are abolished (Glaser and Stanewsky, 2005). When light conditions are maintained constant, the phase of the endogenous molecular clock and therefore its output rhythms entrain either to heat pulses (Sidote, 1998) or temperature cycles (Glaser and Stanewsky, 2005).

Two genes involved in circadian temperature reception have been identified: *nocte* (*no circadian temperature entrainment*) and *norpA* (*no receptor potential A*). *nocte* mutant flies exhibit normal behaviour in LD and DD and normal temperature compensation, but defects in synchronization to temperature cycles. These behavioural defects were mirrored by very low molecular oscillation form *per* promoter in temperature cycles (in LL and DD), but robust and entrained oscillation in LD

(Glaser and Stanewsky, 2005). *norpA* encodes enzyme phospholipase C-β (PLC- β) with a well established role as crucial component in the phototransduction cascade and in the light entrainment of the circadian clock mediated by the compound eyes (Stanewsky et al, 1998; Emery et al., 2000; Helfrich-Förster et al., 2001; Mealey-Ferrara et al., 2003). *norpA* has also been shown to be involved in the seasonal adaptation of the Drosophila clock, mediated by light and temperature changes. In particular it seems to be important for the thermal regulation of the splicing of *per* transcript, the molecular mechanism at the basis of the fly's seasonal behaviour, that is more activity during the day in early spring/late fall (shorter days and colder temperature) or in the early to late night in summer (long days, warm nights) (Majercaket al., 1999, 2004; Collins et al., 2004).

1.3. OSCILLATOR

Circadian clocks are generated by an evolutionary conserved transcriptional-translational feedback system (TTFL) (Figure 3).

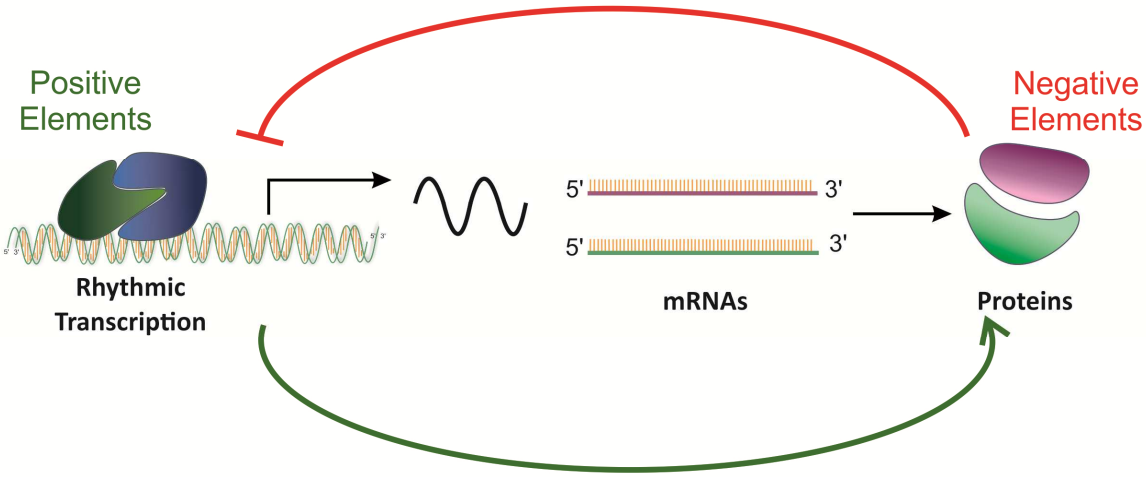


Figure 3. Transcriptional-Translational feedback loop. In this regulatory mechanism, positive elements (blue and dark green) promote the rhythmic transcription of the negative elements (green and purple), which, in turn, inhibit the activity of the positive elements, in a negative feedback loop.

In *Drosophila*, in the first negative feedback loop, the heterodimer formed by the transcriptional factors CYCLE (CYC) and CLOCK (CLK) acts positively directly activating the transcription of the negative factors *period* (*per*) and *timeless* (*tim*) (Hardin, 2011) (Figure 4).

CLK and CYC contain the basic-helix-loop-helix (bHLH) DNA-binding domain and PER-ARNT-SIMS (PAS) protein-protein interaction domains (Huang et al., 1993; Allada et al., 1998; Rutila et al.,

1998). From midday to early night, CLK/CYC heterodimers activate the transcription of *per* and *tim* by binding to specific binding sites for the bHLH domains (CACGTG), called E-boxes (Darlington et al., 1998). *per* and *tim* mRNA start to accumulate with a peak early in the evening (Hao et al., 1997; McDonald et al., 2001; Wang et al., 2001). However, the peak of the PER and TIM proteins do not happen until late in the night, about 4-6 h after *per* and *tim* mRNA peaks. This temporal delay is due to post-translational regulation mechanisms (Hardin et al, 2011). Soon after translation, PER is phosphorylated and degraded by the action of DOUBLETIME (DBT) kinase (Price et al., 1998), counterbalanced by dephosphorylation mediated by the protein phosphatase 2A (PP2A) (Fang et al., 2007). During the night, TIM accumulates and binds the PER/DBT complex, preventing the degradation of PER and therefore promoting its accumulation. PER/TIM/DBT complex enters into the nucleus, favoured by the action of other kinases that act either on PER, such as Casein kinase 2 (CK2) (Akten et al., 2003; Lin et al., 2002 and 2005), or on TIM, such as SHAGGY (SGG), encoded by *shaggy* gene, homologous to the mammalian *Glycogen synthase kinase 3 (Gsk3)* (Martinek et al., 2001). TIM is dephosphorylated by protein phosphatase 1 (PP1) (Fang et al., 2007).

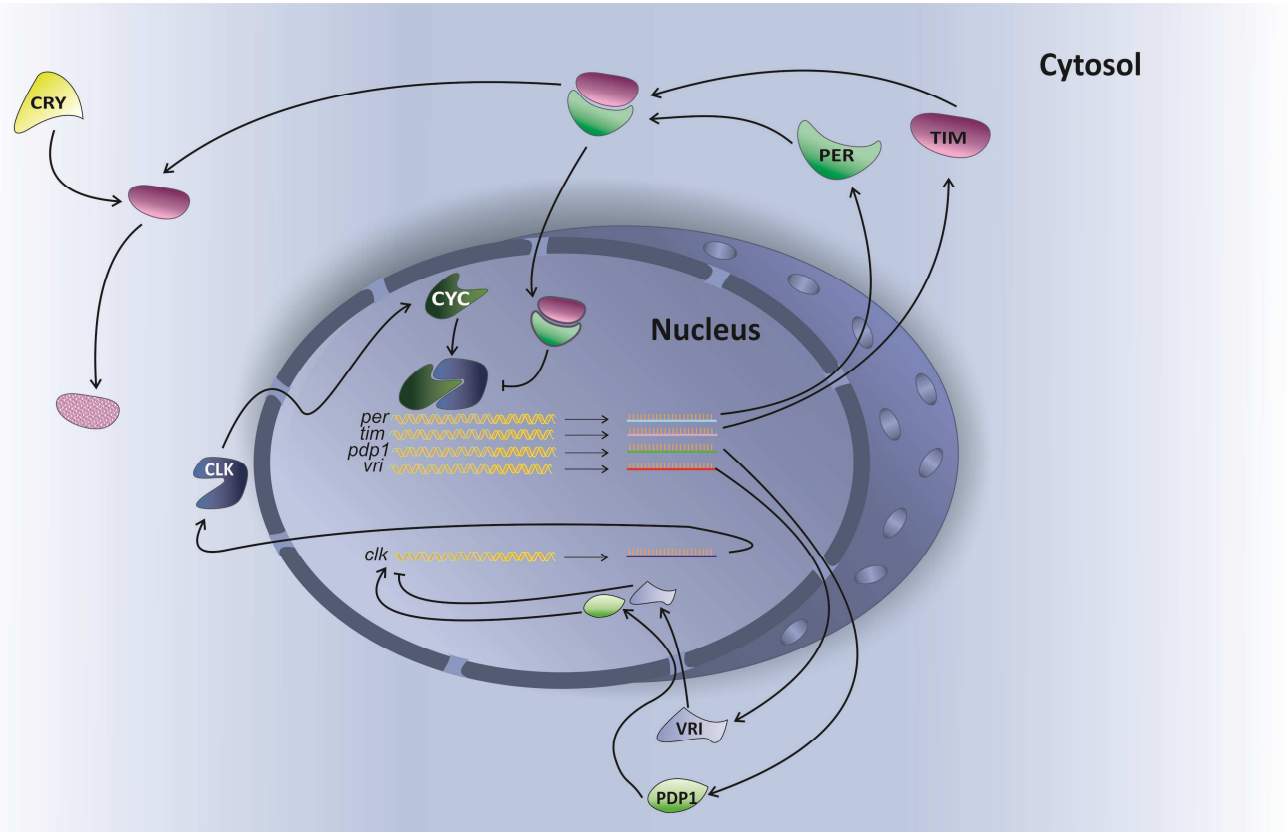


Figure 4. Interlocked molecular feedback loops in *Drosophila melanogaster*. CLK/CYC heterodimer acts as transcriptional activator (positive element) for *per* and *tim* genes. The heterodimer PER/TIM acts as inhibitor for its own transcription (negative element). The VRI and PDP1 proteins, whose expression is regulated by CLK/CYC, control the rhythmic expression of *clk* gene, ultimately regulating the levels of CLK/CYC complex. The protein Cryptochrome (CRY) is implicated in the light entrainment pathways of the *Drosophila* molecular clock.

It has been shown that the complex PER/TIM/DBT dissociates and the proteins enter into the nucleus separately (Meyer et al., 2006); this seems to be supported by the conformational structure of PER, where the PAS domain seem to allow the PER dimerization with itself, replacing to PER/TIM heterodimer in the regulation of the circadian cycle (Landskron et al., 2009). During the first part of the day, PER and TIM get into the nucleus and act on CLK, causing a progressive reduction of the activity of the complex CLK/CYC on the *tim* and *per* promoters (Darlington et al., 1998). This inhibition leads to a reduction of *per* transcription and therefore PER accumulation (Rutila et al., 1998).

A second, interconnected, feedback loop both positively and negatively regulates *Clk* transcription through the actions of PAR domain protein 1 epsilon (PDP1ε) and VRILLE (VRI), respectively, both of which are regulated via E-box elements (Blau and Young, 1999; Cyran et al., 2003). The transcription of *vri* and *pdp1ε* is activated by CLK/CYC during the first part of the day and reaches the maximum during the beginning/middle of the night (Blau and Young, 1999; Cyran et al., 2003). VRI accumulates quickly into the cytosol and translocates to the nucleus where it binds some specific sequences within the *clk* and *Pdp1ε genes* (called V/P-box), blocking their transcription. When VRI levels are low, PDP1 levels increase and the protein replaces VRI on V-P box sequences and reactivates the transcription of *clock* gene, restarting the cycle. As a result, the *clk* gene is expressed rhythmically (Glossop et al., 2003).

Finally, a third feedback loop in flies involves the E-box-regulated gene *clockwork orange* (*cwo*), encoding a transcriptional repressor that competes with CLK:CYC for binding to E-box elements of clock-controlled genes (CCGs) and therefore represses both the basal E-box-containing promoter activity and CLK-mediated transactivation [Kadener et al., 2007; Matsumoto et al., 2007).

As already mentioned, the light synchronization of the circadian oscillation relies on the degradation of TIM in response to light, in a mechanism that is initiated by the binding of CRY and involves the JETLAG protein (Koh et al., 2006). In absence of TIM, PER fails to accumulate and, as a consequence, a new cycle starts with CLK and CYC that bind the specific sequences of *per* and *tim*. (Yu and Hardin, 2006).

The main components of the circadian machinery in *Drosophila* are listed in Table 1.

Table 1. Summary of the main components of the circadian clock in Drosophila melanogaster

Componet	Protein domains	Expression	Function	References
period (per)	PAS domain	mRNA and protein expressed cyclically	Interacts with TIM Negative regulation of CLK and CYC	Konopka and Benzer, 1971; Bargiello and Young, 1984; Jackson et al., 1986; Citri et al., 1987
timeless (tim)	ARM/HEAT domains	mRNA and protein expressed cyclically	Interacts and stabilizes PER; Negative regulation of CLK and CYC	Sehgal et al., 1994; Vosshal et al., 1994; Myers, 1995; Rosato et al., 1997
clock (clk)	bHLH e PAS domains	mRNA expressed cyclically	Interactions with CYC. Activates the transcription of *per, tim vri* and pdp1ε	Allada et al., 1998 Darlington et al., 1998
cycle (cyc)	bHLH e PAS domains	Constitutive	Interactions with CLK. Activates the transcription of *per, tim vri* and pdp1ε	Rutila et al., 1998 Darlington et al., 1998 Blau, 2001
Cryptocrome (cry)	Photoreceptor of blue light	mRNA expressed cyclically The protein has a cyclic profile in LD, while it accumulates in DD	Mediation between the light signal and the "core" of the clock. Interactions with TIM light-dependent Involved in the correct operation of the peripheral clocks	Emery et al., 1998 Stanewsky et al., 1998 Dissel et al., 2004 Rosato et al., 2001
Doubletime (dbt)	Casein kinase 1ε	Constitutive	Phosphorylation of PER	Price et al., 1998 Kloss et al., 1998
Shaggy (sgg)	Kinase	Constitutive	Phosphorylation of TIM	Martinek et al., 2001;
Slimb (slmb)	Part of the protein family Fbox/WD40	Constitutive	Degradation of PER and TIM	Grima et al., 2002 Ko et al., 2002
Casein kinase 2 (CK2)	Kinase	Constitutive	Phosphorylation of TIM and PER	Lin et al., 2002
PAR domain protein 1ε (PDP1)	PAR and b-ZIP domains	mRNA and protein expressed cyclically	*clk* activator	Cyran et al., 2003
Vrille (vri)	b-ZIP domains	mRNA and protein expressed cyclically	*clk* repressor	Blau and Young, 1999

1.4. OUTPUT

Circadian clocks drive rhythms of complex fly behaviours such as eclosion (i.e., the adult emergence from pupal case) and locomotor activity (Konopka et Benzer, 1971). In nature, *Drosophila* eclosion is restricted to the hours of dawn (Myers, 2003) while flies are active predominantly in the morning and evening (Hamblen-Coyle et al., 1992). These rhythms persist under constant darkness conditions with ~ 24-h periodicity.

1.4.1. Locomotor Activity

It is by far the rhythmic behaviour most studied. The activity of wild-type flies

gradually increases before both lights-on and lights-off transitions: this phenomenon is called "anticipation" (reviewed in Rosato and Kyriacou, 2006). However, in LD conditions, there is a burst of activity right after light transitions: this is a response, evoked by changing environment, called

"masking" (Rosato and Kyriacou, 2006) (Figure 5A). These activities are controlled by the circadian clock, as inferred from the loss of morning and evening anticipations in *per*^{0} mutants (mutant flies which carry a mutation leading to a non-functional, truncated PER protein) (Chen et al., 1998). The ability of wild-type flies to anticipate environmental transitions denotes the main function of a circadian clock adjust behaviours in advance with respect to environmental changes. In constant dark conditions (DD), the morning peak disappears and only the evening peak persists, reoccurring with a period of ~23.8 hr (Figure 5B).

In *Drosophila* many other behaviours are under circadian regulations, for instance, sleep, learning and memory, courtship and mating.

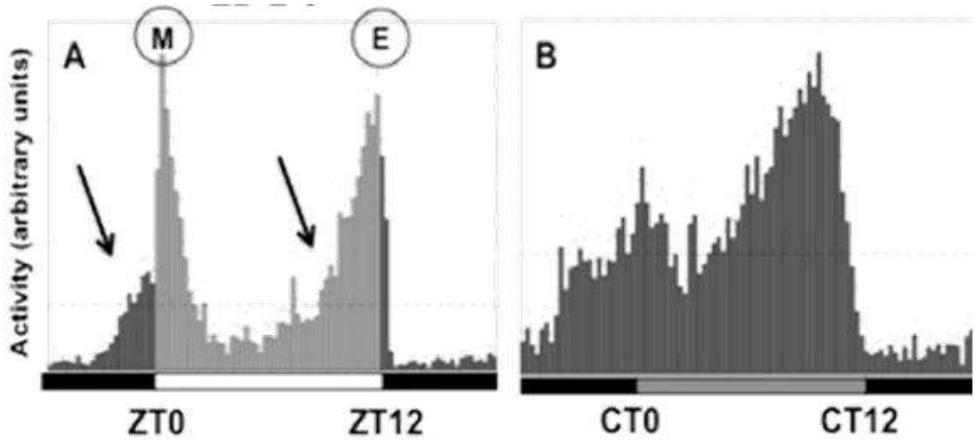


Figure 5. *Drosophila* circadian locomotor behaviour. A) Average locomotor activity plot for wild-type flies. Light and dark bars indicate normalized activity levels during 12 h of light and 12 h of dark, respectively. Increases in activity are evident in advance of light-to-dark and dark-to-light transitions. **B)** Average locomotor activity plot of wild-type flies in DD. Horizontal black bars denote the subjective night and grey bars the subjective day under DD conditions (modified from Chiu et al. 2010).

1.4.2. Sleep

Drosophila periodically enters a state of quiescence that can be considered as sleep. Circadian clock ensures appropriate timing of sleep, although amount and quality of sleep are the results of a homeostatic balance between sleep and wake (Dubowy and Sehgal, 2017). However circadian clock and timing of sleep are two processes that can be separated; in fact, *per*^{01} flies have fragmented sleep across the day, but can have normal overall sleep amounts (Hendricks et al. 2003) and, at the same time, flies with mutations that result in very low total sleep amounts can still show robust circadian activity rhythms (Dubowy and Sehgal, 2017). Several studied have suggested that circadian regulation of sleep does not depend on the oscillation of a single sleep- or wake-promoting factor, but is rather driven by time-of-day specific modulation of distinct sleep- and wake-promoting mechanisms. (Kunst et al., 2014; Liu et al., 2014).

1.4.3. Memory

Several studies have demonstrated a circadian regulation of memory that can occur at multiple stages including the formation and recall of memory. *per* gene is critically involved in LTM (Long Term Memory) formation induced by courtship conditioning; in fact, mutations in *per* gene inhibited formation of LTM, a mutant phenotype that could be rescued by a wild-type per– transgene in a *per*-null background. Moreover, overexpression of *per* in the wild-type background enhanced formation of LTM (Sakai et al., 2004). Also, dCREB2 transcription factor shows circadian regulated oscillations in its activity, and has been shown to be important for both circadian biology and memory formation (Fropf et al., 2014). A circadian modulation has been found also in short-term memory (STM) formation, with a peak in performance occurring in the early night. This rhythm is abolished in *per* and *tim* mutants and is absent in constant light conditions (Lyons and Roman, 2009).

1.4.4. Immunity

A complex physiological processes such as immunity appear to be under circadian control in flies: wild type flies are less protected from bacterial infection during the early day whereas loss of function clock mutants appear to be always susceptible to infection regardless the time of the day (Lee and Edery, 2008).

1.4.5. Mating-Courtship

Also mating and courtship are under circadian control in *Drosophila*: this occurs via circadian regulation of genes such as the cytochrome P450 family members *sxe1*, necessary for high mating success in males that shows oscillation at the protein level with a peak during the night (Fujii et al., 2008).

The molecular clocks present in the circadian neurons drive rhythmic gene expression and set up 24-h rhythms in neuronal activity. Moreover communication between clock neurons synchronizes their molecular clocks and adds robustness to the system.

Rhythms in pacemaker neurons can propagate to downstream cells by different mechanisms: rhythmic hormonal signals that entrain and synchronize molecular clocks in peripheral tissues, by imposing rhythmic activity on downstream neurons via direct neuronal communication, or by

propagating neuron electrical rhythms through downstream neuronal circuits that control specific components of circadian behaviour (Cavey et al., 2016).

One the most important circadian messengers in *Drosophila* is represented by Pigment dispersing factor (PDF), an 18-amino acid neuropeptide rhythmically released from the core pacemaker clock cells (Renn et al. 1999). The important regulatory role of this peptide in the circadian network derives from experiments using *Pdf* null mutant (Pdf^{01}) flies or selective ablation of PDF containing neurons: while these mutated flies could entrain to light-dark cycles (LD), the majority of them showed arrhythmicity under constant darkness (Renn et al. 1999). In case they remained rhythmic in DD, they displayed a shorter free-running period compared to wild type flies. Furthermore, locomotory activity records of Pdf^{01} mutants and PDF cell-ablated flies revealed that they lack lights-on anticipatory activity and display advanced evening activity peak in LD cycles (Renn et al 1999 Peng et al., 2003; Lin et al., 2004).

Other important proteins, involved in the output of the endogenous clock in *Drosophila*, are shown in Table 2.

Table 2. Main genes involve in the output of the circadian endogenous clock of *Drosophila melanogaster.*

Componet	Protein domains	Expression	Function	References
Pigment dispersing factor (PDF)	Peptide secreted	mRNA constitutive The protein accumulates cyclically in the axonal terminations of ventral lateral neurons	The main output of the endogenous circadian system	Park e Hall, 1998; Park et al., 2000; Helfrich-Forster et al., 2000;
Pigment dispersing factor receptor (PDFR)	Transmembrane protein	mRNA constitutive	PDF Receptor	Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005;
Lark	RNA binding protein	mRNA constitutive Protein cyclically	Control of the pupal hatch	Newby and Jackson, 1993; Newby and Jackson, 1996; McNeil et al., 1998;
ebony (eb)	B-alanyl dopamine synthetase	mRNA and protein cyclically	Locomotor activity regulator	Suh e Jackson, 2007;
dunce (dnc)	CAMP phosphodiesterase	Unkonwn the rhythmic expression	Mutations in this locus cause alterations in the entrainment mechanism and reduction of the locomotor activity period	Levine et al., 1994; Majercak et al., 1997;
Protein Kinase A (PKA)	Kinase dependent by cAMP	No cyclic expression was detected	Regulator of the locomot activity	Levine et al., 1994; Majercak et al., 1997;
dCREB2	Transcription factor	mRNA and protein cyclically	The mutants have a shorter period in the locomoto activity	Belvin et al., 1999
Drosophila fragile X-related gene (dfmr1)	RNA binding protein	Protein constitutive	The mutans are arrhythmic and they have a reduced courtship activities	Dockendorff et al., 2002; Inoue et al., 2002;
Neurofibromatosis1 (Nf1) Ras/MAPK pathway	Ras-GTPasi	Unkonwn the rhythmic expression	The null mutants have defects in the rhythm of locomotor activity and an increase of the activity of MAPK pathway	The et al., 1997; Williams et al., 2001;
Takeout (to)	Signal peptide	mRNA and protein cyclically	The null mutants have defects in the rhythm of locomotor activity; They die fast in response to fasting	Sarov-Blat et al., 2000; So al., 2000;
Slowpoke (slo)	Potassium channel calcium dependent	mRNA and protein cyclically	The mutant flies are arrhythmic with a reduced courtship activities	Peixoto e Hall, 1998; Atkinson et al., 2000; Ceriani et al., 2002;
Numb	Phosphotyrosine binding domain	mRNA cyclically	Shorter period of locomotor activity	Zwalen et al., 2000; Santolini et al., 2000; Stempfl et al., 2002;
Regular (rgr)	C2H2 zinc-finger domain	mRNA cyclically	Unknown	Scully et al., 2002;
Clock regulated gene-1 (Crg-1)	HNF3/fork DNA binding domain	mRNA cyclically	Unknown	Rouyer et al., 1997;

1.5. The molecular components of the "*CORE*"

Among the main components of the circadian machinery (listed in Table 1), *period* and *timeless*, the cardinal negative elements of the primary transcriptional/translational loop, and *cryptochrome* (*cry*), the main circadian photoreceptor, will be described in details.

1.5.1. *period (per)*

The *period* gene was isolated in 1971 in an EMS-mutagenesis designed to screen for mutants in the rhythmic eclosion (Konopka and Benzer 1971). Three mutant lines were isolated in which the rhythmicity of eclosion was altered: their period was shortened to 19h, speeded up to 29h or completely abolished. Further analyses showed that the same effect was discernible on locomotor activity. Genetic mapping of the mutations showed that they were indeed three alleles of the same X-linked locus; the locus was named *period* and the three alleles per^{S} (S=Short), per^{L} (L= Long) and per^{0} respectively (Reddy et al., 1984; Bargiello et al., 1984).

The primary transcript contains 8 exons and gives rise to a 4,5 kb mRNA (Citri et al., 1987). The conceptual translation product is a 1218 amino-acid chain that shows some salient features:

- a PAS domain (after Per, Arnt and Sim, the proteins in which the domain was first recognised, consisting in a 270 amino-acid long stretch containing two degenerate repeats, named PAS-A and PAS-B, involved in the mechanism of dimerization (Huang et al., 1993;
- a cytoplasmic localization domain (CLD aa 453-511), representing another binding site for TIM and a motif required for cytoplasmic localization in cultured cells (Saez and Young, 1996).
- a Thr-Gly (TG) repeat, consisting of about 20 Thr-Gly pairs. This region is highly polymorphic in length, with at least eight different length variants found in natural populations (Costa et al., 1991; Peixoto et al., 1992). The mutability of this region is important for the thermal compensation of the circadian behaviour (Costa et al., 1991; Sawyer et al., 1997);
- a dCLK:CYC inhibition domain (CCID), mapped in the C-terminus at aa position 764-1034, identified as the region necessary for inhibiting dCLK:CYC-mediated transcription and containing a novel NLS important fo PER's nuclear transport in S2 cells (Chang, et al., 2003)

Two variants of mRNA have been described which differ for the presence ($per^{UNSPLICED}$) or absence ($per^{SPLICED}$) of a 89 bp intron (dmpi8) in the 3' UTR, due to a temperature-dependent alternative splicing (Cheng et al., 1998) (Figure 6). This alternative splicing event, not altering the protein length, confers to the clock the dynamic flexibility to respond to daily or seasonal changes in

temperature, and is particularly important for advancing the phase of locomotor activity on short, cold days temperatures (Majercak et al., 1999, 2004; Collins et al., 2004; Low et al., 2012).

The splicing of *per* results in earlier accumulation of mRNA and protein, which correlates with earlier evening activity at colder temperatures (Majercak et al., 1999), while at higher temperatures the non-spliced transcript results in later evening activity.

Also the light regulates *per* alternative splicing; indeed, during a short photoperiod in 6:18 LD condition, with constant temperature, the $per^{SPLICED}$ isoform increases (Majercak et al., 2004). Consequently, the advanced accumulation of PER promotes the anticipation of the evening activity of the flies during the last hours of light (Majercak et al., 2004). Changes in photoperiod and temperature could be associated to seasonal variations; therefore the temperature-dependent regulation of the alternative splicing promotes the adaptation of the flies to the natural condition (Collins et al., 2004; Majercak et al., 2004; Vanin et al., 2012).

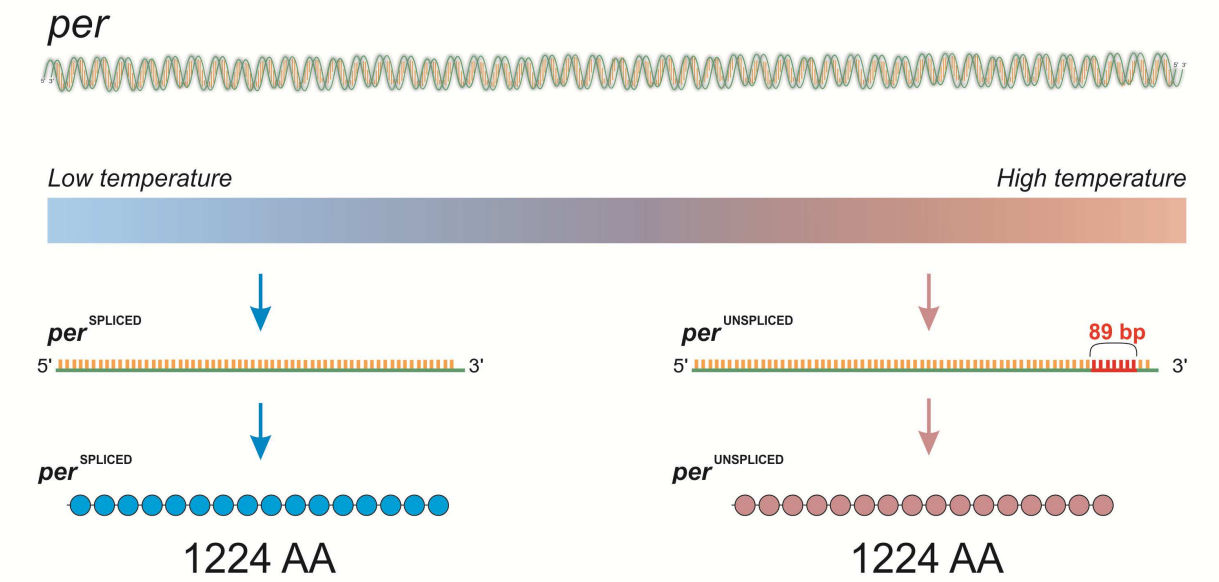


Figure 6. Post-transcriptional regulation of the *period* gene in *Drosophila*. The temperature-dependent alternative splicing of an intron of 89 bp in the 3'UTR generates two mRNA isoforms ($per^{spliced}$ and $per^{unspliced}$). At low temperatures the $per^{spliced}$ form is accumulated more rapidly.

1.5.2. *Timeless (tim)*

Timeless (tim) is an autosomal gene located on chromosome 2. It encodes for a 1398 aa protein, TIMELESS (TIM), (Myers et al., 1995) that possesses several domains:

- a functional domain (505–906), harbouring two regions for interaction with PER (Gekakis et al., 1995);
- a functional *Nuclear Localization Signal* (NLS), 595-538, in the last part of N-Terminal region (Ousley et al., 1998);

- a region at the C-terminus (160 amino acids) responsible for its cytoplasmic localization in the absence of PER (Saez and Young, 1996);
- ARMADILLO (ALD) domains, related to the interactions with the cytoskeletal proteins (Hatzfeld, 1999).

The sequence of *tim* contains two open reading frames (ORFs), resulting in two different TIMELESS isoforms: a long isoform protein consisting of 1421 amino acids (L-TIM$_{1421}$) a short one contains 1398 amino acids (S-TIM$_{1398}$) (Rosato et al. 1997). The *ls-tim* allele generates both long L-TIM$_{1421}$ and short S-TIM$_{1398}$ products, while in case of *s-tim*, deletion of the G nucleotide at position 294 interrupts the upstream open reading frame with a stop codon, therefore only S-TIM$_{1398}$ can be generated from the downstream open reading frame (Rosato et al. 1997). Interestingly, there is a latitudinal gradient of *ls-tim* frequency, with higher incidence of *ls-tim* in southern Europe. It was suggested that *ls-tim* allele is derived from the *s-tim*, arose in southern Italy about 10,000 years ago, and it has recently spread in all directions due to directional selection (Tauber et al. 2007).

A thermosensitive splicing is also observed in *tim* where at colder temperatures, the last 858 bp intron is retained and the corresponding transcript, *tim*^{COLD} or *tim*^{UNSPLICED}, gives rise to a TIM protein 33 amino acids shorter than the full length *tim*^{NORMAL} or *tim*^{SPLICED} isoform, due to a premature STOP codon (Boothroyd et al., 2007) (Figure 7). The function of the TIM^{UNSPLICED} protein remains still unknown, although its high affinity for the circadian photoreceptor CRY, may suggest that it could guarantee a stronger light response under colder conditions (Montelli et al., 2015).

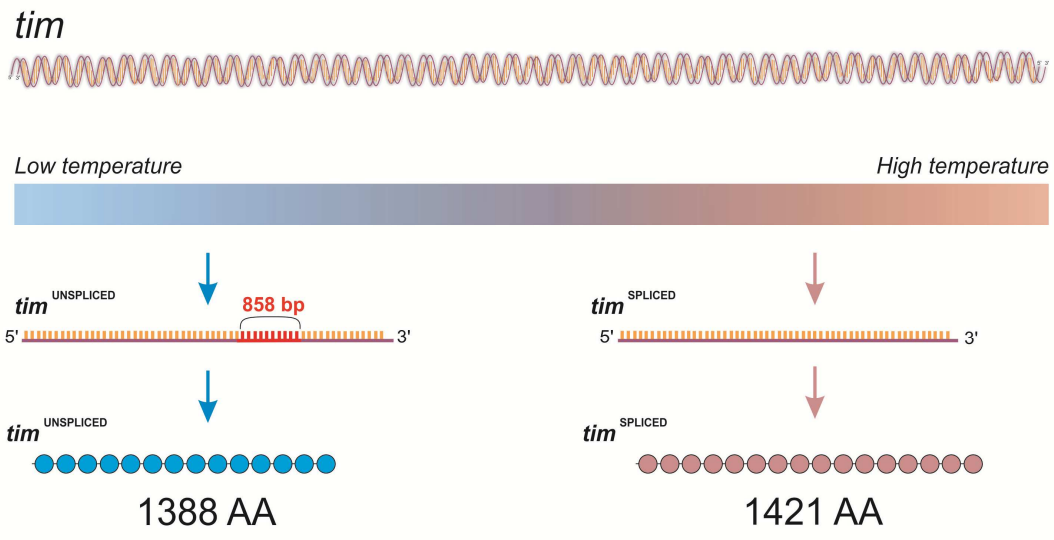


Figure 7. Post-transcriptional regulation of the *timeless* gene in *Drosophila*. The temperature-dependent alternative splicing of an intron of 858 bp generates two splicing variants (*tim*^{norma} *or tim*^{spliced} *and tim*^{cold} *or tim*^{unspliced}). At low temperature, a premature STOP codon in the retained intron gives rise to a shorter protein.

1.5.3. *Cryptochrome (cry)*

Drosophila CRY is a predominant nuclear protein that mediates regulation of the circadian clock by light (Emery et al., 1998; Stanewsky et al., 1998), although it can also be found in the cytosol (Ceriani et al., 1999). The *cryptochrome* gene (*cry*), located on the third chromosome, is rhythmically expressed, with a maximum at the beginning of the day (ZT1-6). This oscillation is completely abolished in clock mutants (Emery et al., 1998). CRY protein levels oscillate only in LD cycles while in constant light the protein is rapidly degraded and in constant dark it accumulates continuously (Emery et al., 1998). These data suggest a double regulation for *cryptochrome*: a clock controlled gene expression and a post-transcriptional and post-translational control on the protein exerted by light.

cry encodes a protein of 541 amino acids, with a high homology to photolyases, involved in the light signal pathway and in the regulation of the phases of the circadian clock (Stanewsky et al., 1998). It is a blue light photoreceptor, able to bind FAD (flavin) and MTHF (methyltetrahydrofolate), with an absorbance of 420 nm (Thompson and Sancar, 2002). CRY is rapidly degraded in presence of light through proteasome (Lin et al., 2001; Sathyanarayanan et al., 2008): after light exposure, however, it binds TIM (Ceriani et al., 1999), promoting its degradation through the proteasome, with the help of the F-box protein JETLAG (Koh et al., 2006), thus guaranteeing the light-dependent synchronization of the clock (figure 8).

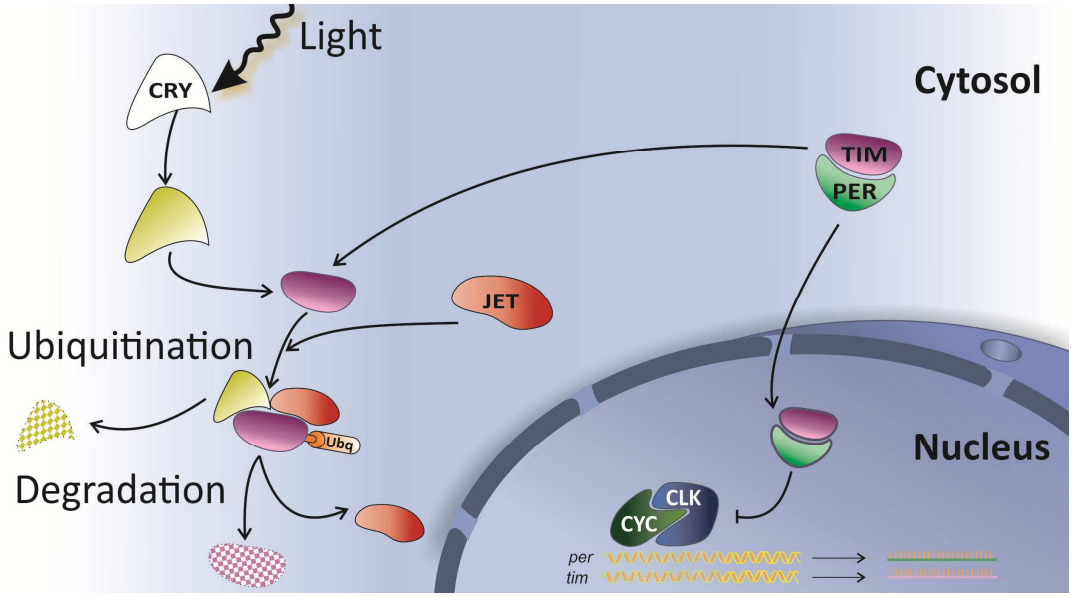


Figure 8. CRY mediated light synchronization of the clock. Following light-activation, CRY is able to bind to TIM and JET. This binding leads to ubiquitination of both TIM and CRY and subsequent degradation of the ubiquitinated proteins to reset the clock.

CRY is also able to bind PER (Rosato et al., 2001). Light induces a conformational change in the C-terminal of CRY; this is a hot-spot for several protein-protein interaction domains and possesses linear motifs evolutionarily conserved in different species (Hemsley et al., 2007; Mazzotta et al., 2013). The absence of C-terminus results in a particular CRY isoform able to bind TIM both in light and dark (Dissel et al., 2004). Several experiments have confirmed the essential role of CRY in the clockwork functioning and resetting (Ceriani et al., 1999; Emery et al., 2000; Rieger et al., 2003; Dolezelova et al., 2007; Dissel et al., 2004; Ozturk et al., 2011). Flies maintained in LL condition become arrhythmic due to the constant degradation of TIM, while *cry* null mutants maintain either a rhythmic profile or the capacity of entrainment (Emery et al., 2000; Dolezelova et al., 2007).

1.6. The central pacemaker and the clock neurons

In *Drosophila melanogaster* the master clock (*pacemaker*) operates within a circadian circuitry comprised of a network of 150 clock neurons, divided, for each cerebral hemisphere, in 7 groups: 3 groups of Dorsal Neurons (DN1, DN2, DN3) and 4 groups of Lateral Neurons. These are further divided into three sub-groups, the lateral posterior neurons (LPNs), the ventral lateral neurons (LNvs) and the dorsal lateral neurons (LNds). The ventral lateral neurons are classified, based on their relative size, into small and large ventral lateral neurons (s-LNvs and l-LNvs respectively). In addition, the dorsal neurons are divided into three groups based on their relative positions, such as DN1s, DN2s and DN3s, DN1a (anterior) and DN1p (posterior). There is also a new group of clock neurons, lateral posterior neurons (LNPs), that express *per* and *tim,* but their role in the circadian clock hasn't been specified yet (review in Yoshii et al 2005; Miyasako et al., 2007).

In detail (figure 9):

- The dorsal neurons 1 (DN1): This group comprises 17 neurons with different function. They can be further divided into two subgroups: 2 DN1s anterior (DN1as) and ~15 DN1s posterior (DN1ps). DN1as express the neuropeptide IPNamide (Shafer *et al.*, 2006) and they do not express the transcriptional factor GLASS as all the other DN1s do (Veleri *et al.*, 2003). The DN1ps cells promote only morning activity under standard (high light intensity) light:dark cycles and they are able to generate a robust evening peak of activity under a temperature cycle in constant darkness. Most likely the DN1ps behavioural output is under both photic and thermal regulation (Zhang et al., 2010).

- The dorsal neurons 2 (DN2): The cell bodies of the two DN2s are in close proximity to the dorsal PDF positive projections of the s-LNvs.
- The dorsal neurons 3 (DN3): This group comprises 40 cells, and only 2 are CRY-positive (Helfrich-Forster, 2007); a genetic analysis of PER expression in DN3 neurons showed that these pacemaker cells are not sufficient to maintain locomotor rhythms under constant conditions but they are able to elicit the evening peak of activity under LD conditions (Veleri et al., 2003).
- The posterior lateral neurons (LPNs): This group is made of 3 cells involved in temperature entrainment (Miyasako et al., 2007). This cluster was the first described pacemaker neurons expressing TIM but not PER (Helfrich-Forster, 2005). However, following studies showed that these neurons express also PER, albeit at lower levels (Shafer et al., 2006)
- The ventral lateral neurons (LNvs): Among the different clusters of clock neurons only 4 s-LNvs and the l-LNvs express the neuropeptide PDF (Helfrich-Forster 2005). They modulate arousal and wakefulness as well as sleep stability: when the excitability of these cells is altered, the normal pattern of light-driven activity during the day is reversed and activity during the night is preferred (Sheeba et al., 2008). The function of the PDF positive s-LNvs is to drive the anticipation of lights-on transition in the morning and to maintain behavioural rhythmicity under constant darkness (Grima et al., 2004). These cells send PDF projections towards the dorsal brain. The s-LNv expresses also small neuropeptide F (sNPF) (Johard et al., 2009), an orthologue of mammalian neuropeptide Y (NPY), which is produced in the hypothalamus and controls food consumption (Gehlert 1999).
- The dorsal lateral neurons (LNds): This group comprises 6 neurons, expressing the neuropeptide F (NPF) and three of them also CRY, involved in both the control of the free-running period in constant darkness (DD) and the phasing and amplitude of the evening activity in light-dark (LD) cycles (Hermann et al., 2012). It has been shown that LNds are responsible for controlling the anticipation of lights-off transition (Grima et al., 2004). These cells are unable to sustain rhythmicity under DD conditions but they drive rhythmic behaviour under LL; indeed, light inhibits the output from the morning oscillator composed by the s-LNvs whereas it activates the output from the LNds (evening oscillator) (Picot et al., 2007).

The different clock neurons are connected by projections forming a complex network that starts from the optic lobes and goes through the dorsal region of the brain (Helfrich-Forster, 2005). This structure controls independently the morning activity phase from the evening, especially due to the presence of morning (M-cells) and evening (E-cells) oscillator (see below). In presence of a

different length of photoperiod, the action of these two oscillators can increase or decrease in response to light period, promoting an advancement or decreasing phase of morning of evening peak (Pietterdigh e Daan, 1976).

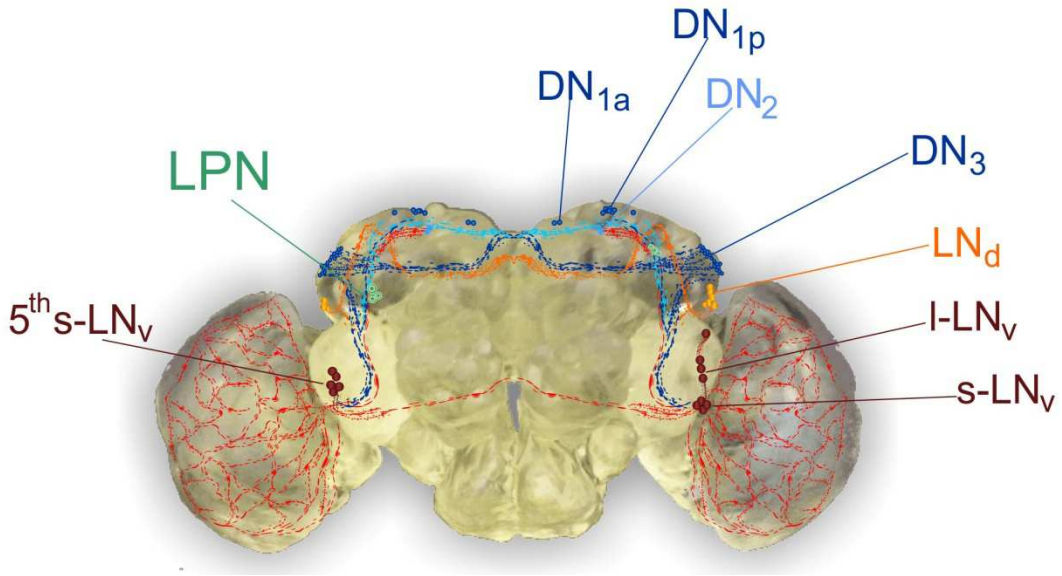


Figure 9. Central nervous system (SNC) of *Drosophila melanogaster*. The main neuronal clusters are represented (blue DN, green LPN, orange LN_d, dark reed LN_v). The complex network of connections between the clusters is also highlighted.

In detail, in *Drosophila* two neurons subsets are specifically associated with the control of the morning and evening activity: four PDF positive s-LNvs are responsible of the morning activity (morning oscillators), while the PDF negative LNd and s-LNv regulates the evening activity (evening oscillators) (Grima et al., 2004; Stoleru et al., 2004). Both these groups, *Morning clock* (M-Cells) and *Evening cells* (E-cells), have a functional molecular clock that regulates independently the morning and evening activity (Grima et al., 2004). Furthermore, the M-cells represent the *master clock* that maintains the circadian rhythmicity in constant dark condition (*free-running*), also when the molecular clock in E-Cells is impaired (Helfrich-Forster, 2001; Helfrich-Forster et al., 2007).

1.7. Peripheral clocks

In addition to the central pacemaker, in *Drosophila* there are many peripheral clocks located in other organs and tissues (Plautz et al., 1997); these are composed by cell-autonomous molecular oscillator and their activity can be grouped in 3 different categories: independent, “slave” and driven by the central clock (Ito and Tomioka, 2016). This creates a great heterogeneity in circadian organization in periphery. For instance, Giebultowicz et al., (2000) have shown that the original phase of TIM oscillation in Malpighian tubules (MT) is maintained in DD when the tissue is

transplanted into the abdomen of flies previously entrained to antiphase LD cycle, confirming that clock machinery in MT is independent of central clock. A peripheral clock "slave" of central clock was found in oenocytes, which regulate pheromone production (Krupp et al., 2008); indeed, in transgenic flies which have PER expression only in clock neurons but not in peripheral, the cycle expression of clock genes were abolished in oenocytes (Krupp et al., 2008).

Recent studies focused on the eclosion timing shown that protoracic glands (PG) are drive by LN_vs of the central pacemaker (Morioka et al., 2012); indeed, to dissect this relation, the gene transcriptional and post transcriptional rhythm was investigated in PG cultured *in vitro*. They found that PER oscillation of these cells receives light information from LN_vs, while TIM maintains its oscillation under DD (Morioka et al., 2012). In the peripheral clocks, CRY can have a double role as photoreceptor and clock component: in fact, in *cry*^{b} mutant eyes, CLK/CYC target genes were derepressed, similarly to per null mutants. Moreover, overexpression of PER and CRY together were able to repress CLK/CYC mediated transcription (Collins et al., 2006).

1.8. The circadian clock in mammals

In mammals, the central pacemaker controlling behavioural and physiological rhythms is located in the suprachiasmatic nuleus (SCN), a bilateral structure in the anterior hypothalamus comprising about 20.000 clock neurons, each containing the molecular clock machinery (reviewed in Honma, 2018). The mammalian primary transcriptional/translational feedback loop involves interaction between the transcriptional activators CLOCK and BMAL1 and the negative elements formed by PERIOD and CRY The CLOCK/BMAL1 heterodimer binds to E-box elements present in the promoter of *Per1/2* and *Cry1/2* genes, triggering their expression. PER/CRY complexes translocate to the nucleus where they inhibit their own transcription by direct association with CLOCK/BMAL1 (reviewed in Honma, 2018). As in *Drosophila*, interlocked multiple feedback loops are in place to fine tune circadian periodicity also in mammals. For example, the rhythmic expression of *Bmal1* is controlled by retinoic acid receptor-related orphan receptors (ROR) and REV-ERB□/□, through ROR enhancer site located upstream of the *Bmal1* gene; in particular ROR binding activates gene expression, while REV-ERB binding inhibits transcription (Sato et al., 2004). ROR and REV-ERV are themselves controlled by the circadian clock through E-box elements present in their promoters (Sato et al., 2004). In another loop, the dimer CLOK/BMAL1 controls the expression of *Dec1* and *Dec2*, encoding two basic helix–loop–helix transcription factors that act as negative elements as

they repressed CLOCK/BMAL1-induced transcription of *Per1* through direct protein–protein interactions with Bmal1 and/or competition for E-box elements (Honma et al., 2002).

It is important to highlight that, despite a substantial similarity between the mammalian and *Drosophila* clock, that has allowed the quick identification of the key members of the transcriptional/translational feedback loop in mammals (and vice-versa), there are also a few differences between mammals and *Drosophila*. For example, in mammals there are three *Per* homologs, *Per1, Per2,* and *Per3*, displaying different regulation, pattern of expression and different roles in circadian rhythmicity and entrainment (Takumiet al., 1998).

Moreover, while in *Drosophila* TIMELESS protein is degraded in presence of light and plays a fundamental role in regulating the circadian rhythmicity, mammalian TIMELESS is involved in processes as cell cycle and carcinogenesis (Unsal-Kaçmaz et al., 2005), and its role in the circadian rhythm is still not well defined. Furthermore, unlike the *Drosophila* counterpart, mammalian cryptochromes have no photoreceptive functions but are integral component of the transcriptional translational feedback loop (Griffin et al., 1999).

A schematic representation of the molecular clockwork in mammals is presented in figure 10.

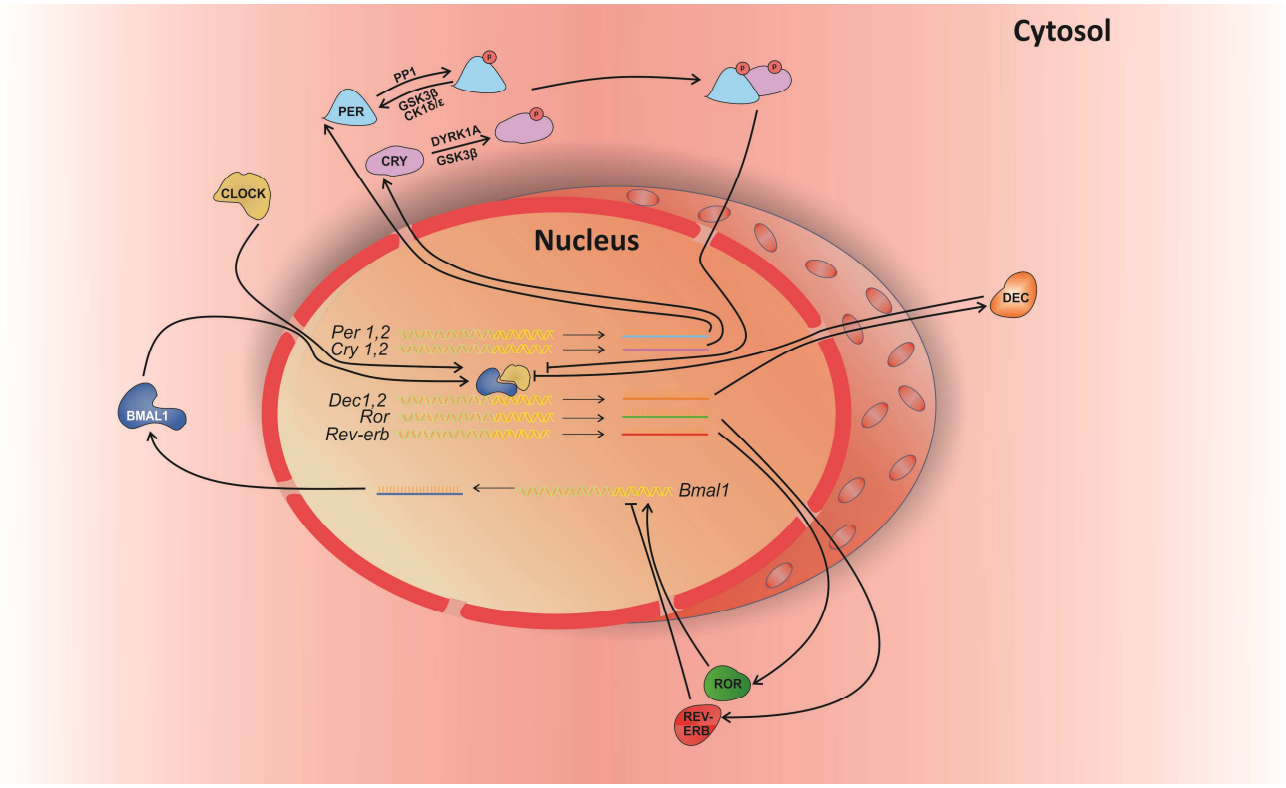


Figure 10. Transcription–translation feedback loops in the mammalian circadian clock. The complex CLOCK/BMAL1 binds to target E-boxes in the promoters of *Per*, *Cry*, *Dec*, *Rev-erb*, and *Ror*. PER (cyan) and CRY (purple) are phosphorylated (P) by various kinases including CK1δ/ε, GSK3β, and DYRK1A. PER:CRY heterodimers translocate to the nucleus where they repress their own transcription. A second feedback loop regulate the rhythmic expression of *Bmal1* through the actions of REV-ERB (red) or ROR (green). In a third feedback loop DEC proteins (orange) displace BMAL1:CLOCK from promoter E-boxes (modified by Honma, 2017).

1.9. Post-transcriptional and post-translational control of circadian rhythm

Rhythmic clock-gene expression is important for rhythmic protein accumulation; however, cycling protein levels do not depend only on cycling mRNA levels but post-transcriptional and post-translational mechanisms have also evolved to adjust and consolidate the proper pace of the clock (reviewed in Mendoza-Viveros et al., 2017).

Among these, alternative splicing, mRNA nuclear export, polyadenilation, regulation of translation and degradation, play fundamental roles in the post-transcriptional regulation of circadian gene expression (Sanchez et al., 2010; Kojima et al., 2012; Huang et al., 2013; MacGregor et al., 2013; Robles et al., 2014, Mendoza-Viveros et al., 2017). A schematic representation of the post-trancriptional control is shown in figure 11.

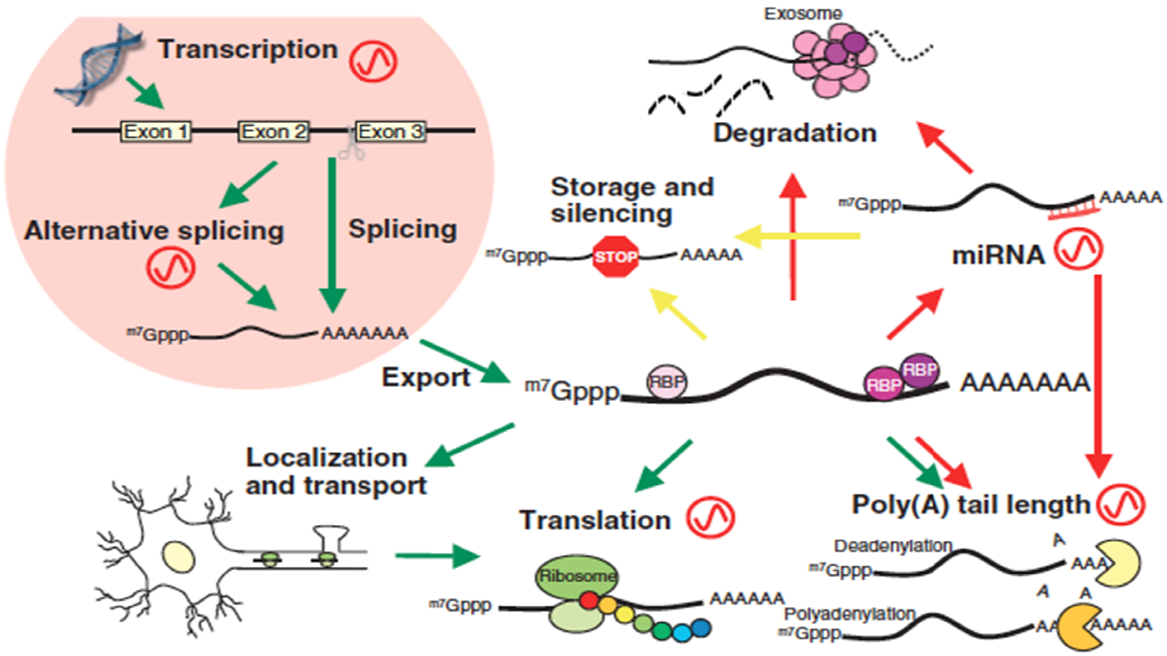


Figure 11. Circadian clock and post-transcriptional regulation. The red oscillators indicate post-transcriptional mechanisms known to be controlled by circadian clock. Translational, translational silencing and mRNA degradation pathways are indicated, by yellow, red and green arrows, respectively. The ^{m7}Gppp means 7-methylguanosine cap (taken from Kojima et al., 2011).

As previously mentioned, alternative splicing (AS) has been revealed as an important regulatory step in supporting circadian rhythms in gene expression in Drosophila. In fact, *per* and *tim* undergo thermosensitive alternative splicing: as for *per*, several evidences suggest that it influences the phase of the clock according to temperature and photoperiod (Majercak et al., 1999; Collins et al., 2004; Majercak et al., 2004), while for *tim* it might have functional implications relevant to circadian photoreception and perhaps to seasonal responses (Boothroyd et al., 2007; Montelli et al., 2015). Moreover PRMT5 (Protein Arginine Methyl Transferase 5) has been shown to regulate circadian rhythms and splicing of *per* and several clock-associated genes (Sanchez et al., 2010).

Very recently, a pivotal role in the fly's pacemaker neurons has been reported for the alternative splicing regulator SRm160 (SR-related matrix protein of 160 kDa): it is important for proper per expression, rhythmic accumulation of the neuropeptide PDF and ultimately circadian rhythms in locomotor behaviour (Beckwith et al., 2017). Several *trans*-factors (mostly RBPs) regulate the expression of clock or clock-controlled genes in *Drosophila*. *Lark* (homolog to mammalian RBM4) encodes for an RNA-binding protein (Newby and Jackson, 1993; Lai et al., 2003; Kojima et al., 2007) that affects the rhythms in eclosion and locomotor activity (Newby and Jackson, 1996; Huang et al., 2009). At molecular level, LARK promotes the translation of *Eip74EF*, an ecdyson-induced protein (Huang et al., 2007). In *lark* mutants, the eclosion is abnormally advanced in comparison to wild-type (Newby and Jackson, 1993), suggesting that the post-transcriptional control of *Eip74EF,* mediated by LARK, is essential to maintain the correct rhythm. Interestingly, the expression of LARK protein is rhythmic, due to the post-transcriptional regulation of its mRNA that, instead, maintains a linear expression profile (McNeil et al., 1998; Kojima et al., 2007). Another group of RBPs play an important role in the post-transcriptional regulation in the circadian circuit: the heterogeneous nuclear ribonucleoproteins (hnRNPs) (Piccolo et al., 2014). Every single hnRNPs has more than one function in the post-transcriptional activities and they are highly conserved among the various species (more details will be provided in a dedicate paragraph).

In recent years, a role for small, non-coding RNAs (miRNA) as post-transcriptional regulators of circadian rhythmicity in Drosophila has been also established. They regulate the proper development and maintenance of circadian rhythms acting either directly on the expression of clock genes or indirectly on signalling outputs. Among the former, bantam has been shown to regulate the translation of Clock and stabilize circadian transcription (Kadener et al., 2009; Lerner et al., 2015), let-7 influences molecular and behavioural rhythms by repressing CLOCKWORK ORANGE (CWO) (Chen et al., 2014) and miR-276a inhibits *tim* expression in the clock-neurons (Chen & Rosbash, 2016); among the latter, miR-279 regulates fly's rhythmic behaviour acting on the JAK/STAT pathway (Luo & Sehgal, 2012) while miR-124 regulates locomotor activity both in LD and DD acting on BMP signalling (Garaulet et al., 2016; Zhang et al., 2016). Recently, a role for the clock- and light-controlled miR-92a in the modulation of neuronal excitability has been described : it targets the NAD-dependent histone deacetylase Sirt2, ultimately affecting either sleep and phase shift response of the clock (Chen and Rosbash, 2017). A cluster of six miRNAs, miR-959-964,

is rhythmically expressed in the adult head fat bodies, and regulates processes as innate immunity, metabolism and feeding behaviour in a circadian fashion (Vodala et al., 2012).

Examples of post-transcriptional regulation of the circadian clock in *Drosophila* are listed in Table 3.

Table 3. Summary of the post-transcriptional mechanisms that regulate the circadian-related genes expression.

Post-transcriptional mechanism	Gene name	Regulation	References
Translation	*Period*	3'-UTR-mediated rhytmic translation	Chen et al., 1998
	Eip74EF	Upregulated translation through interactions with LARK	Huang et al., 2007
mRNA stability	*Period*	Rhythmic changes in mRNA stability	So and Rosbash, 1997
	Clock	Rhythmic changes in mRNA stability	Kim et al., 2002
Alternative splicing	*Timeless*	Thermosensitive splicing of last intron	Boothroyd et al., 2007
	Period	Thermosensitive splicing of an intron in the 3'UTR	Majercak et al., 1999 Majercak et al., 2004
miRNA regulation	*bantam*	Regulation of period length possibly through *clk* expression	Kadener et al., 2009
	miR-263a/b	Rhythmically expressed miRNAs	Yang et al., 2008

1.10. Post-translational regulation

Post-translational modification of clock proteins plays a crucial role in determining their stability and the timing and extent of their nuclear trafficking. A predominant role is exerted by phosphorylation. As previously mentioned, soon after translation PER undergoes a phosphorylation by the kinase DOUBLETIME (DBT), homolog to mammalian casein kinase 1e and this event leads PER to degradation (reviewed in Hardin, 2011 and in Mendoza-Viveros, 2016). However the binding of TIM results in the stabilization of the complex PER/DBT and the accumulation or a ternary complex PER/DBT/TIM in the cytoplasm (reviewed in Hardin, 2011 and in Mendoza-Viveros, 2016). The phosphorylation of PER exerted by Casein Kinase 2 (CK2) and that of TIM by SHAGGY (SGG), homolog to mammalian Glycogen Synthase Kinase 3 (GSK3), times the translocation of the complex in the nucleus, where PER/DBT complex binds CLK, promotes its phosphorylation and inhibit CLK/CYC mediated transcription by releasing the complex from E-boxes (reviewed in Hardin, 2011 and in Mendoza-Viveros, 2016). Hyperphosphorylated PER is ubiquitinated by the E3 ubiquitin ligase SLIMB and targeted for proteasome-mediated degradation (reviewed in Hardin, 2011 and in Mendoza-Viveros, 2016). The stability of PER is regulated by a

balance between the action of the described kinases and protein phosphatases: in fact, Protein Phospatase 1 (PP1) and Protein Phospatase 2A (PP2A) are shown to target PER, affect its phosphorylation levels and stabilize the protein in vitro and in vivo (reviewed in Hardin, 2011 and in Mendoza-Viveros, 2016).

Beside the previously mentioned effect on PER, the ubiquitin mediated proteasome degradation plays an important role in the photic entrainment pathway. In fact, upon light activation, the photoreceptor CRY binds TIM that is ubiquitinated by the E3 ubiquitin ligase JETLAG (JET) and subsequently targeted towards proteasome mediated degradation (Koh et al., 2006). Moreover, following light stimulation, JET interacts also with CRY, triggering its degradation (Peschel et al 2009). A schematic representation of the post-translational control is shown in figure 12.

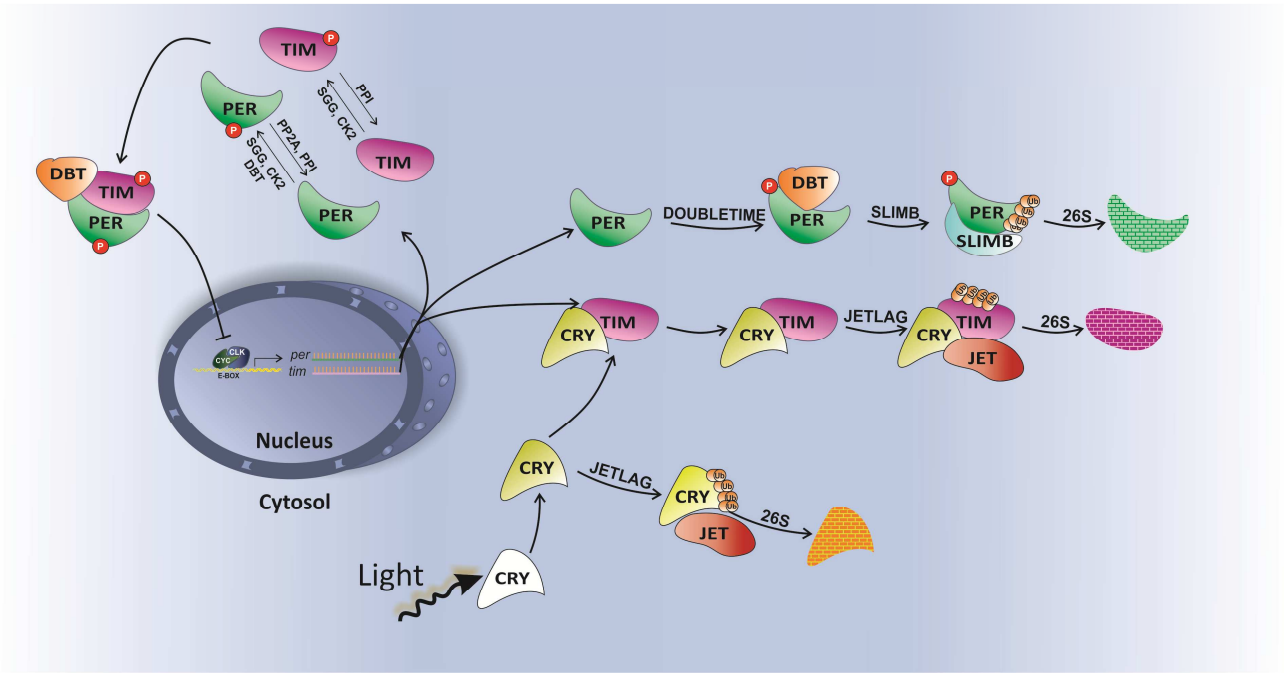


Figure 12. Post-translational regulation in Drosophila circadian clock. Soon after translation, PER is phosphorylated by DBT; the binding induces PER ubiquitination and degradation. PER and TIM are phosphorylated by SGG and CK2, which promote PER:TIM nuclear translocation. JETLAG-dependent ubiquitination of CRY and TIM, followed by proteasomal degradation is an important signal that triggers the light resetting of the clock. TIM ubiquitination depends on the formation of a tripartite complex involving TIM, CRY and JETLAG (modified from Dubruille and Emery, 2008).

1.11. Heterogeneous nuclear ribonucleoproteins hnRNPs and their roles of post-transcriptional regulation

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA-binding proteins with important roles in gene regulatory processes at a post-transcriptional level including packaging of nascent RNA, regulation of constitutive and alternative splicing, RNA trafficking, regulation of RNA stability,

mRNA translation (Borah et al., 2009; Han et al.,2010; Piccolo et al., 2014). Disrupted regulation of hnRNPs is correlated with a variety of human diseases including cancer (Carpenter et al., 2006; Gao et al., 2013; Dery et al., 2011), diabetes, hypertension (Abdo et al., 2013) and neurodegenerative diseases (Hanson et al., 2012).

Ever since, the hnRNPs are classified as proteins involved in several mechanisms of pre-mRNA processing and maturation. Among other functions, they can participate in mRNA export from nucleus to cytoplasm, site-specific localization, stability and translational regulation of mRNAs (Dreyfuss et al, 1993; Dreyfuss et al., 2002; Chaudhury et al., 2010; Han et al., 2010; Bush and Hertel, 2012). Recent studies have showed that hnRNPs are involved also in mechanisms of DNA repair, telomere biogenesis and cell signalling (Krecic and Swanson, 1999; Singh, 2001; He and Smith, 2009).

Generally, RNPs present a RNA-binding domain (RBD) or RNA recognition motif (RRM), located in the N terminal of the amino acid sequence; moreover, the interaction with RNA is facilitated by other structural motifs such as: RGG repeats, KH domains, Zinc-binding domains, double stranded RNA binding motif (dsRBM) and Pumilio homology domain (PUF) (Chang and Ramos, 2005; Auweter et al., 2006).

In *Drosophila melanogaster,* 14 hnRNPs are the most representative members of this RNA binding protein family, with functional and structural characteristics similar to mammalian (listed in Table 4, Lo Piccolo et al., 2014; Haynes et al., 1991; Matunis et al., 1992a; Matunis et al., 1992b; Buchenau et al., 1997; Reim et al., 1999; Hovemann et al., 2000; Blanchette et al., 2009). Usually, hnRNPs form large complexes containing a great number of proteins and RNAs. In the fruit fly, in fact, some nucleus-localized hnRNPs are associated with non-coding RNAs (hsr-omega RNA) to form a novel class of nuclear speckles called omega speckles figure 13 (Lakhotia et al. 1999; Prasanth et al. 2000; Lakhotia et al. 2001; Jolly and Lakhotia 2006; Ji and Tulin 2009; Onorati et al. 2011). They are localized in the nucleoplasm close to chromatin edge and mainly function as storage sites for unengaged hnRNPs and other related RNA-processing proteins (Piccolo et al., 2014). In addition, since RNPs are involved in several cellular processes, the organization in omega-speckles allows a fine control of their availability and function (Lakhotia et al. 1999; Prasanth et al. 2000; Jolly and Lakhotia 2006; Onorati et al. 2011; Lakhotia 2011).

Table 4. List of *Drosophila's* hnRNPs functions and homologies with *H.sapiens*.

Gene	Name	Synonyms	Process	*H. sapiens* homolog	References
caz	cabeza	SARFH, P19	Neuromuscular development	FUS (or HNRNPP2), EWSR1, TAF15	(Zinszner et al. 1997; Wang et al. 2011)
glo	glorund	p67	Cell polarity	HNRNPH2, GRSF1, HNRNPH3, RBM12, HNRNPH1, HNRNPF	(Kalifa et al. 2006)
heph	hephaestus	PTB, CG2094, l(3)03429, dmPTB, ema	Cell polarity	RBM20, PTBP1, PTBP3, PTBP2	(Davis et al. 2002)
Hrb57A	Heterogeneous nuclear ribonucleoprotein at 57A	bancal, Q18	Nucleoplasmic compartment organization	HNRNPK	(Hovemann et al. 2000)
Hrb87F	Heterogeneous nuclear ribonucleoprotein at 87 F	hrp36, P11	Nucleoplasmic compartment organization, interaction with chromatin component, regulated by covalent modifications and small metabolites, neuromuscular development	HNRNPA0, HNRNPA3; HNRNPA1; HNRNPA2B1; HNRNPA1L2	(Haynes et al. 1991)
Hrb98DE	Heterogeneous nuclear ribonucleoprotein at 98DE	Hrp38	Regulated by covalent modifications and small metabolites, neuromuscular development	HNRNPA0	(Kim et al 2013)
Hrb27C	Heterogeneous nuclear ribonucleoprotein at 27C	Hrp48, p50, RRM7, Hrb27-C	Nucleoplasmic compartment organization, cell polarity	DAZAP1	(Goodrich et al. 2004)
nonA	no on or off transient A	diss, nonA, Bj6	Nucleoplasmic compartment organization, neuromuscular development	SFPQ, PSPC1, NONO	(Starewsky et al. 1996; Reim et al. 1999)
Pep	Protein on ecdysone puffs	PEP/X4	Nucleoplasmic compartment organization, interaction with chromatin component	CIZ1	(Reim et al. 1999)
rump	rumpelstiltskin	hrp59, hnRNP M	Nucleoplasmic compartment organization, interaction with chromatin component	MYEF2, HNRNPM	(Sinsimer et al. 2011)
sm	smooth	smo	Neuromuscular development	HNRNPL, HNRPLL	(Mackay 1985)
Syp	Syncrip	Syp	Cell polarity, neuromuscular development	RBM47, RBMY4G, DND1, SYNCRIP, A1C, EHNRNPR	(McDermott et al. 2012)
sqd	squid	hrp40, l(3)j4B4, Squid, RRM3	Nucleoplasmic compartment organization, cell polarity, regulated by covalent modifications and small metabolites, neuromuscular development	HNRPDL, HNRNPAB,	(Krecic and Swanson 1999)
TBPH	TDP-43	TAR DNA-binding protein, TDP-43	Neuromuscular development	TDP-43 or TARDBP	(Buratti and Baralle 2009; Strong 2010; Buratti and Baralle 2010)

In fact, a loss of function of the omega-speckles causes a widespread distribution of the hnRNPs in the nucleoplasm with consequent alteration of the cellular processes (Prasanth et al. 2000; Mallik and Lakhotia 2011; Lakhotia et al. 2012). Recent studies have demonstrated that hnRNPs play a central role in the regulation of gene expression by interacting directly or indirectly with the chromatin remodelling factors and participating with nucleosomal molecular dynamic (Piccolo et al., 2014). For instance, ISWI, the catalytic subunit of several ATP-dependent chromatin remodelling proteins, is essential in the formation of nucleoplasmatic components as the omega speckles: *ISWI* mutant flies have highly disorganized hnRNPs due to destabilization of omega speckles, with consequent altered chromatin condensation, post transcriptional disorder and uncontrolled activity of RNPs (Onorati et al., 2011). Another interesting study shows the interaction between Hrb87F and RUMP (two *Drosophila's* hnRNPs) with Brahma (Brm), the catalytic subunit of the chromatin remodelling complex SWI/SNF (Tyagi al., 2009). Brm promotes the recruitment of the RNPs on pre-mRNA and regulates the alternative splicing mechanism (Kwok et al., 2015); in this way, the SWI/SNF complex regulates both the amount of synthesized mRNA and the production of the specific protein as result of alternative splicing (Tyagi et al., 2009). Piacentini et al., (2009) have demonstrated that the Heterochromatin Protein 1 (HP1a) contribute to the maintaining and modulation of chromatin condensation, by interacting with several hnRNPs; this interaction between HP1a, hnRNPs and also histonic variants induces formation of heterochromatin causing gene silencing (Piacentini et al., 2009).

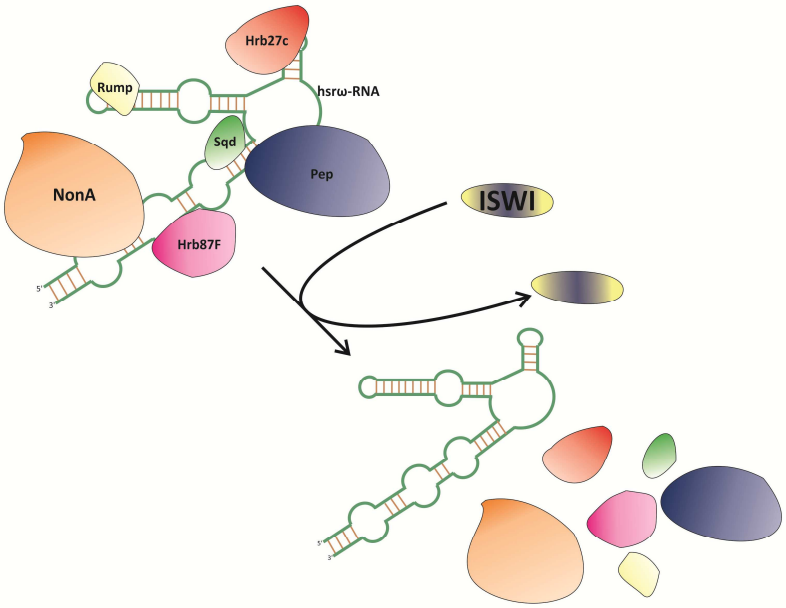


Figure 13. Structure of omega speckle; ncRNA hsr-omega (green line) binds the hnRNPs, limiting the correct release of specific RNPs (other colours). When the hnRNPs are required the ISWI factor acts on omega speckles, disengaging the proteins (modified from Lo Piccolo et al., 2014).

Recent studies have suggested a correlation between alternative splicing and the abundance of RNPs in the cells, highlighting a potential role for hnRNPs in the regulation of this process (Borah et al. 2009; Nichols et al. 2000; Olson et al. 2007; Nilsen and Graveley 2010). In addition, hnRNPs activity in the alternative splicing is modulated by post-translational modifications: for instance, in mammals, the methylation of hnRNP A1, homolog of *Drosophila's Hrb87F/Hrb98DE* (Borah et al., 2009), blocks the protein activity, in a functional standby, preventing the formation of hydrogen bonds arginine-dependent mediated by specific interactions with RNA and certain proteins (Calnan et al., 1991; Najbauer et al., 1993). Whether *Drosophila's* hnRNPs are subject to other post-translational changes is still unknown, however, mammals hnRNPs such as A1, A3, F, H and K undergo to sumoylation (Li et al., 2004); other post-translational changes, such as phosphorylation and ubiquitination, promote the activity of ribonucleoproteins in mammals (Li et al., 2004F). Interestingly, for instance, the heat shock events increase the phosphorylation activity of the hnRNPs, leading to a decrease their binding with the RNA with consequent disorganized localization between the chromatin and nucleoplasm (Ji and Tulin, 2012; 2013).

hnRNPs are involved in the establishment of embryonic polarity during oogenesis, interacting and regulating *gurken (grk)*, *oskar (osk)* and *nanos (nos)* mRNAs (McDermott et al., 2012; Huynh et al., 2004; Kalifa et al., 2006). Moreover, recent studies have showed that hnRNPs, in particular nonA, are also involved in the neuromuscular growth, creating a mRNA-RNP complex together with nuclear RNA export factor (NXF1) to facilitate the intranuclear mobility of mRNA-RNP particles (Kozlova et al., 2006).

An important role of hnRNPs in the neural development has also been elucidated; in particular, the ribonucleoprotein Glorund (GLO) expressed in the central nervous system (CNS) during the late phase of embryogenesis, regulates the translation of neuronal mRNAs (Piper and Holt, 2004; Martin, 2005; Kalifa et al., 2006; Olson et al, 2007). Other hnRNPs have important neuronal and neuromuscular roles: <87F (see below), Smooth (sm) and TBPH, homologous of human TAR-DNA binding protein (TARDBP), involved in the transcriptional pathway of HIV virus (Ou et al., 1995), in amyotrophic lateral sclerosis (ALS) (Neumann et al., 2006), in front-temporal lobar degeneration (FTLD) (Arai et al., 2006), in Alzheimer's, Parkinson's and Huntington's diseases (Forman et al., 2007; Chen-Plotkin et al., 2010).

hnRNPs have been implicated also in circadian regulation of clock genes. For example, in mice, hnRNP Q (the homolog of Drosophila Syncrip) controls the rhythmic translation of mPER1 and mPER3 : knockdown of mhnRNP Q results in a decrease in mPER1 levels and a slight delay in

expression (Lee et al., 2012; Kim et al., 2011), while deletion of the hnRNP Q binding region in mPer1 5'UTR disrupted rhythmicity (Lee et al., 2012). hnRNP Q and polypyrimidine tract-binding protein (PTB) modulate rhythmic translation of Rev-erbα, a transcriptional repressor in the mouse circadian clock (Kim et al., 2010). In rats, hnRNP Q is involved in the rhythmic control of serotonin arylalkylamine N-acetyltransferase (AANAT) required for maintaining the rhythmic release of pineal melatonin, the primary marker of circadian rhythm in vertebrates. Silencing of hnRNP Q resulted in severe decrease of AANAT protein levels accompanied with reduced melatonin production in pinealocytes (Kim et al., 2007). In mice, hnRNP D shows circadian expression and is a fine regulator of mcry1 turnover and rhythmic translation with cytoplasmic levels showing a pattern in antiphase compared to that of mcry1. Knocking down hnRNP D enhanced oscillation amplitude by stabilizing mcry1 while causing a slight phase delay (Woo et al., 2010; Lee et al., 2014). In Arabidopsis, the hnRNP-like protein AtGRP7 is part of a negative auto-regulatory circuit that influences circadian oscillations of its own and the AtGRP8 transcript through alternative splicing linked to nonsense-mediated decay (NMD) (Schöning et al.,2008). Thus, evidences of hnRNPs involvement in the circadian system are accumulating.

1.12. *Squid*

Squid is located on 3rd chromosome in 87F5-F6 position and encodes 5 isoforms of SQUID protein originated by alternative splicing. As the other members of RNA binding proteins (RBP) family, it harbours two RRM domains and one DNA binding domain in the N terminal region (Krecic and Swanson, 1999). SQUID is a component of ribonucleosomes involved in several cellular processes such as mRNA localization, alternative splicing, mRNA stability and chromatin remodelling (Hartmann et al., 2011). The protein SQUID is subjected to post translational modification such as methylation mediated by 9 arginine methyltransferase (PRMTs), named DART1-9 (*Drosophila A*rginine Transferase 1-9) and expressed during *Drosophila'*s development (Boulanger et al., 2004). SQUID play an important role during oogenesis: it is involved in the restriction of *grk* RNA and proteins at the dorsal anterior region of oocytes (Kelley, 1993; Neuman Silberberg and Schupbach, 1993), necessary for the localized activation of epidermal growth factor receptors (EGFR) (Neuman-Silberberg and Schupbach, 1993). In *squid* null flies, an altered pattern of *grk* RNA localization and translation causes ectopic EGFR activation and induction of dorsal cell fates, which ultimately result in impaired oogenesis (Kelly 1993; Neuman-Silberberg and Schupbach, 1993). In addition, SQUID, together with ribonucleoprotein Hrb27C, the germ line-specific ovarian tumor

(Otu) and the alternative splicing factor Poly U-binding factor 68KDa (pUF68), participates to the correct location of *grk* RNA in the dorsal anterior region of oocytes (Norvell et al., 2005; Goodrich et al., 2004). SQUID is also involved in formation of the complex *osk* RNA-ribonucleoparticle (RNP) that regulates the transport of the *osk* RNA inside the nucleosol (Norvell et al., 2005).

1.13. *Hrb87F*

The ribonucleoprotein Hrb87F is encoded by a gene located on the 3rd chromosome in position 87F7-87F7. Similarly to SQUID, it possesses two RRM at the N terminal that allow RNAs binding; moreover, Hrb87F has a putative domain, pADP-binding domain that, similarly to its mammalian ortholog is subjected to phosphorylation (Ji and Tulin, 2013). ADP-ribose regulates, in non-covalent manner, the ability of the hnRNP to bind the RNAs; in fact, the ADP-ribose induced hyperphosphorylation of Hrb87F results in the inhibition of its activity, with a consequent reduced RNAs binding efficiency (Ji and Tulin, 2013). Hrb87F participates in several biological processes, such as omega speckle formation, development of the organism, response to stress events and neuronal development (Singh and Lakhotia, 2012). For instance, Sengupta and Lakhotia (2006) have demonstrated that Hrb87F is involved in the modulation of the poly-glutamine (PolyQ) toxicity disease; the data show that RNAi-mediated down regulation of nuclear hsrω-n leads to an increase of Hrb87F and transcriptional regulator cAMP response element-binding (CREB) protein (CBP) availability, thus decreasing the PolyQ toxicity (Malik and Lakhotia, 2010).

It has also been demonstrated that in flies models for fragile X-associated tremor/ataxia syndrome (FXTAS), Hrb87F is able to bind the CGG trinucleotide repeat in the 5'UTR of fragile X mental retardation 1 (FMR1) gene (Jin et al., 2003), whose several expansion causes the onset of fragile X syndrome, reducing the hnRNP bioavailability (Sofola et al., 2007). Down-regulation of *hrb87f* leads to the activation of the retrotransposon *gypsy* transcription, increasing the neurodegenerative effects (Tan et al., 2012). Moreover, low level of Hrb87F fail to block the *mir277* transcription, a smallRNA responsible for the neurodegeneration modulation that causes the fragile X syndrome, by promoting the fragile X pre-mutation CGG repeats (Jin et al., 2003).

State of the art

In a pilot experiment, a co-immunoprecipitation assay on transgenic flies overexpressing dCRY and dissection of the complexes by mass spectrometry revealed that dCRY interacts with Hrb87F and Squid, two proteins of the hnRNPs family. These findings support the hypothesis that hnRNPs

could be involved in post-transcriptional control mechanisms of the circadian clock machinery. In particular, as Hrb87f has been linked to alternative splice-site selection (Zu et al., 1996) it can be hypothesized a functional role for this interaction in the circadian regulation of alternative splicing in Drosophila.

Chapter 2

RESULTS

2.1. The hnRNPs SQUID and HRB87F are partner of CRY in the *Drosophila* head

A preliminary study was initiated in our laboratory in order to find new molecular component of the clock machinery in Drosophila, by searching for interactors of CRY. In particular, protein extracts from head of transgenic flies expressing HACRY in TIM positive cells were subjected to Co-Immunoprecipitation with an anti-HA antibody; the immunocomplexes were then separated by SDS-PAGE and gel bands corresponding to the putative interactors analyzed by mass spectrometry (Mazzotta et al., 2013). This approach, performed on individuals maintained in the dark and after a 30 min light exposure, led to the identification of the hnRNPs SQUID and HRB87F (Figure 14).

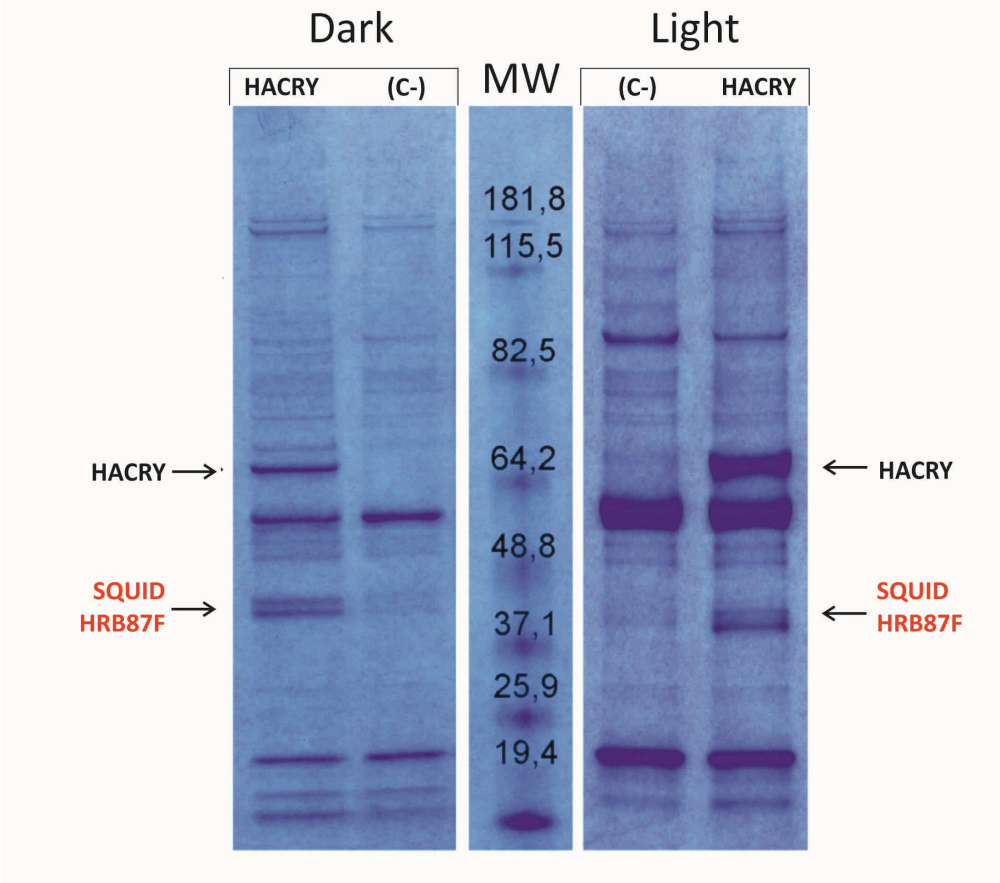


Figure 14. Co-Immunoprecipitation of proteins extracted for heads of HACRY over-expressing flies. Coomassie blue-stained gel of heads of protein extracts coimmunoprecipitated with an anti-HA antibody. HACRY-overexpressing flies (HACRY*, yw;tim-GAL4/+; UAS-HAcry/+)* and relative controls (C, *yw;tim-GAL4)* were reared in 12:12 light:dark and collected in the dark (ZT24) and in the light (ZT24 + 30-min light pulse). MW, molecular weight. Bands corresponding to HACRY are indicated in black, while stained proteins excised and characterized by mass spectrometry are indicated in red.

In order to confirm the previous results, a Co-Immunoprecipitation followed by western blot was performed on flies overexpressing a HA tagged form of CRY (HACRY) in the TIM expressing cells. These flies were obtained by using the GAL4-UAS binary system, in which flies bearing the *UAS-HAcry* construct (Dissel et al., 2004) were crossed with the *timGal4* driver: the progeny was entrained in LD 12:12 cycles and collected at ZT24, before lights on, and after 30 minutes of light

pulse (ZT0 and ZT12 define the beginning and the end of the light phase, respectively). Native protein extracts from about 200 heads were subjected to immunoprecipitation with an Anti-HA Affinity Matrix and the HACRY immunocomplex subjected to western-blot and probed with specific anti-SQUID and anti-Hrb87F antibodies. The same blot was hybridized also with an anti-HA antibody, in order to assess the specificity of the interaction. The results are reported in Figure 15.

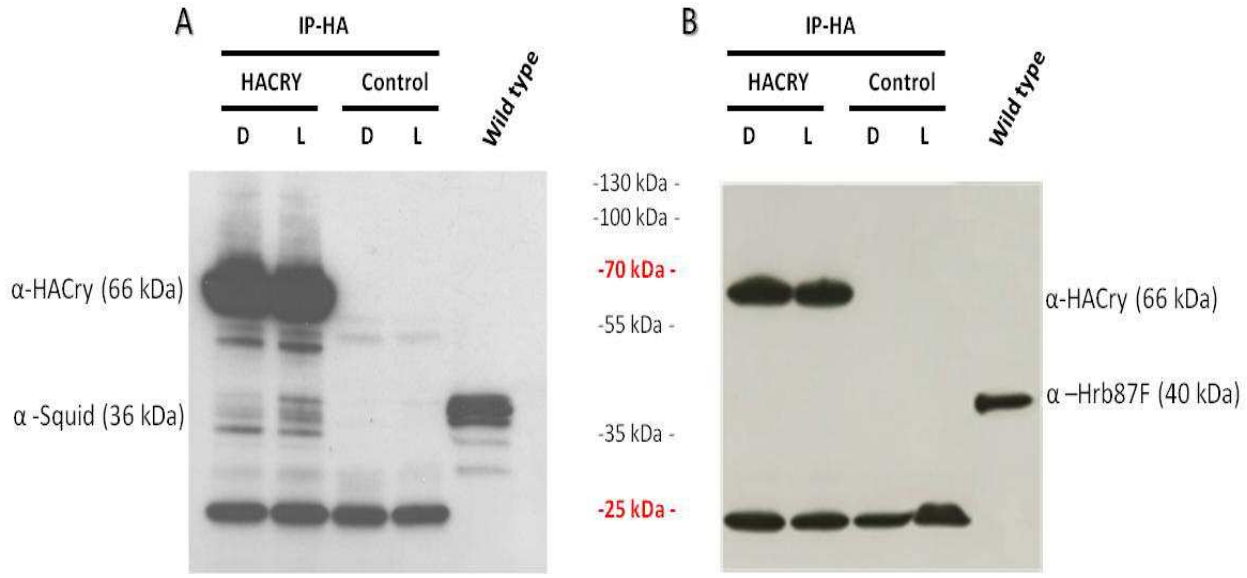


Figure 15. Co-Immunoprecipitation and Western blot. HACRY-overexpressing flies (*yw*;*tim*-GAL4/+; UAS-*HAcry*/+) and relative control (t*im*-GAL4) were reared in 12:12 light:dark and collected in the dark (ZT24) and in the light (ZT24+30-min light pulse). Membranes were probed with anti-SQUID (A), anti-HRB87F (B) and anti-HA antibodies. w^{1118} flies collected at ZT1 were used as positive control of the antibody.

As shown in Fig.15 A, the anti-SQUID antibody recognizes several bands of the expected molecular weight (around 36 kDa) that can be ascribed to the different SQUID isoforms, specifically in the samples where HACRY is present, and not in the controls, demonstrating an interaction between the two proteins either in the dark and after 30 minutes of light exposure. Flies from the *timGal4* driver collected at the same time-points were used as negative control of the interaction, while wild type flies w^{1118} were used as control for the antibody.

This approach didn't allow us to confirm the interaction between HACRY and HRB87F (Fig.15 B). A possible explanation could be a low affinity or an unsteady interaction between the two proteins, which cannot be revealed with this technique. We therefore decided to exploit the yeast two-hybrid system, in which CRY was challenged as bait with HRB87F as prey. Indeed, using this technique, the two proteins displayed an interaction either in light or in dark, with light enhancing the affinity between them (Fig. 16)

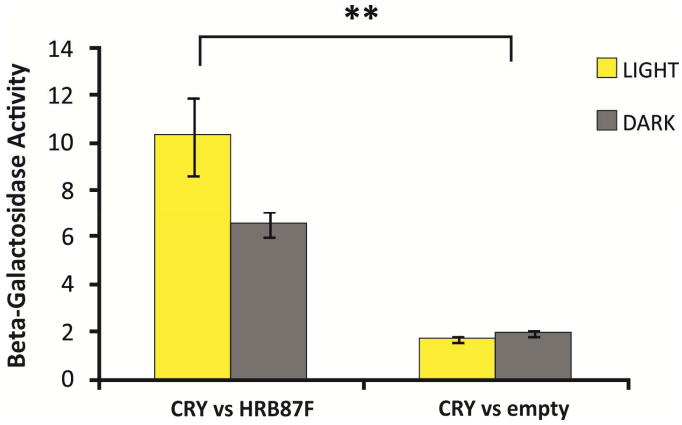


Figure 16. Yeast two-hybrid assay showing the interaction between CRY and HRB87F. Relative β-galactosidase activity (Miller units) is reported. Mean ± SEM of at least six independent clones, analyzed in triplicates, is shown (CRY *vs* HRB87F and CRY *vs* empty plasmid). The Student's t-test was used for the statistical analysis (** p-value < 0.01).

2.2. Characterization of *Squid* and *Hrb87F* expression

In order to characterize the temporal expression of these ribonucleoproteins and to reveal a possible control by the circadian clock, mRNA and protein levels were analyzed through a circadian cycle, in both wild type (w^{1118}) and clock mutant (per^{0}) flies. Flies were reared in 12:12 LD condition and subsequently moved to constant darkness (DD) and collected at regular intervals, every 3 hours, both in LD and DD.

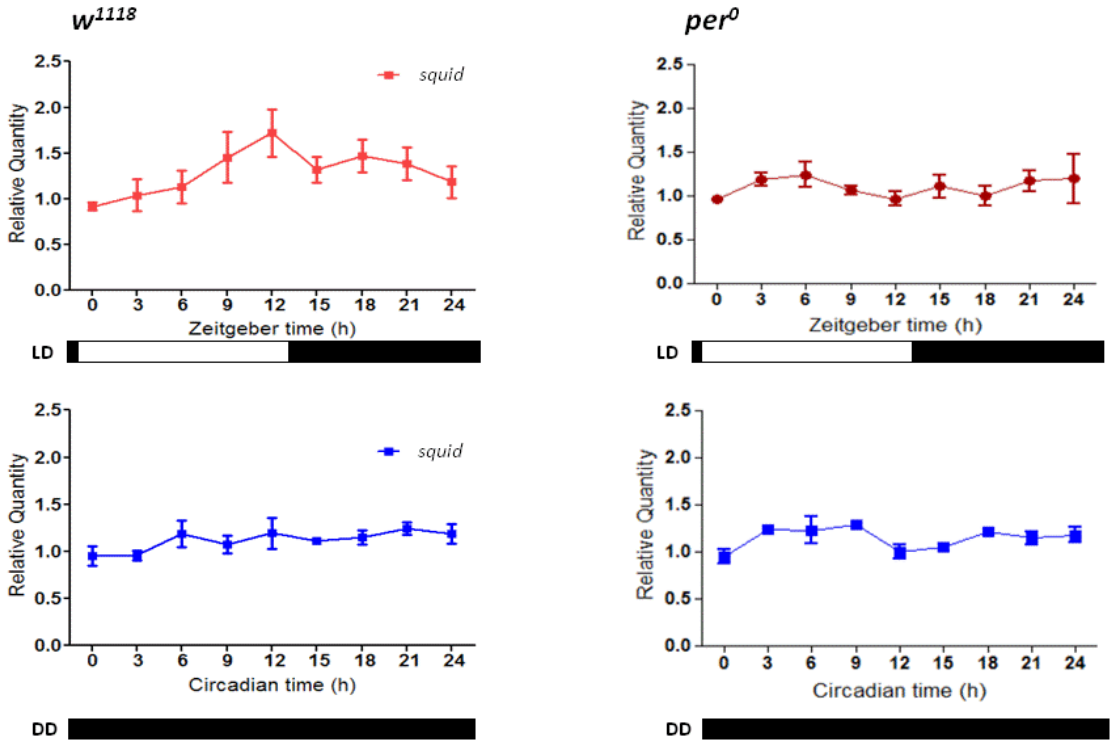


Figure 17. *Squid* expression analysis on heads from w^{1118} (left) and per^{0} (right) flies reared in 12.12 LD (upper panels) or over the first day in DD (lower panels). Relative abundance of *Squid* mRNA was defined as a ratio with *rp49*. Values represent mean levels ± SEM of three biological replicates. The analysis with RAIN and CircWave did not reveal oscillatory trend.

For mRNA analysis, total RNA was extracted from heads, retrotranscribed and subjected to Real-Time PCR. As reported in Fig.17, *Squid* mRNA does not show rhythmic expression, both in LD and DD, and no differences were observed between *wt* and *per*^{0} flies. A statistical analysis performed using the algorithms RAIN and CircWave confirmed the absence of any oscillatory trend.

The analysis of protein expression performed on heads extracts by western blot (Fig. 18), revealed that, similarly to what observed for mRNA, SQUID protein does not show a rhythmic profile in its expression in any of the tested strains and conditions. Nevertheless, we cannot completely exclude a rhythmic expression for both mRNA and protein in the clock neurons, as these cells represent only a small portion of the whole head.

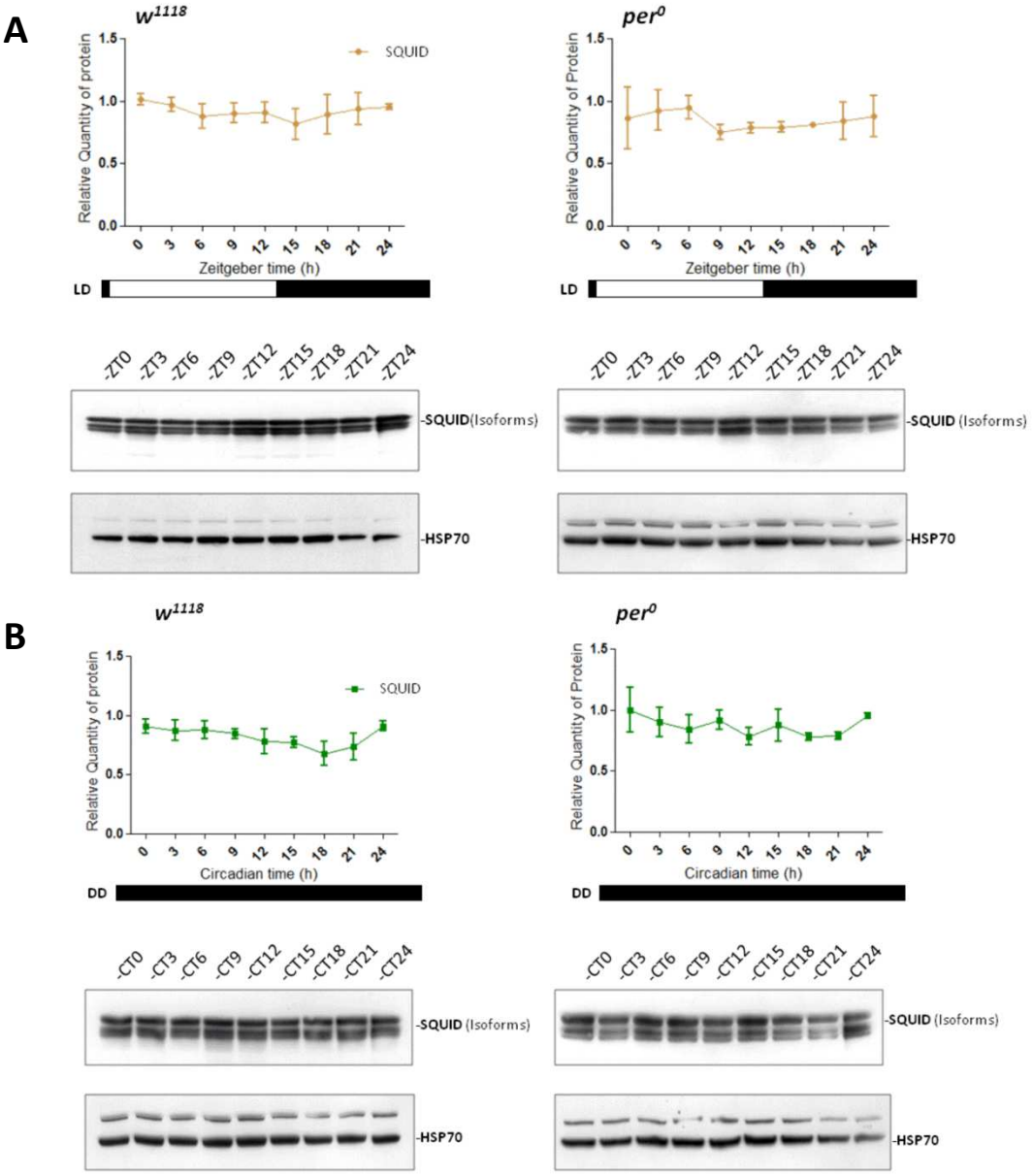


Figure 18. SQUID expression analysis in *w*^{1118} (left) and *per*^{0} (right) flies, collected every 3 hours under (A) LD or (B) DD. SQUID signals were normalized to HSP70 (loading control). Three independent experiments were performed and means were plotted ± SEM. The trend of expressions was evaluated by using of RAIN and CircWave, resulting not oscillatory.

The same expression analysis, performed for *Hrb87F*, revealed that the mRNA levels of this gene show an oscillatory trend in LD and DD with maximum values at the end of the night, both in *wt* and per^0 flies (Fig.19) although the p-value obtained by RAIN and CircWave is close to the limit of significance (0.486 and 0.493 in LD, 0.473 and 0.482 in DD, respectively for wild type and per^0).

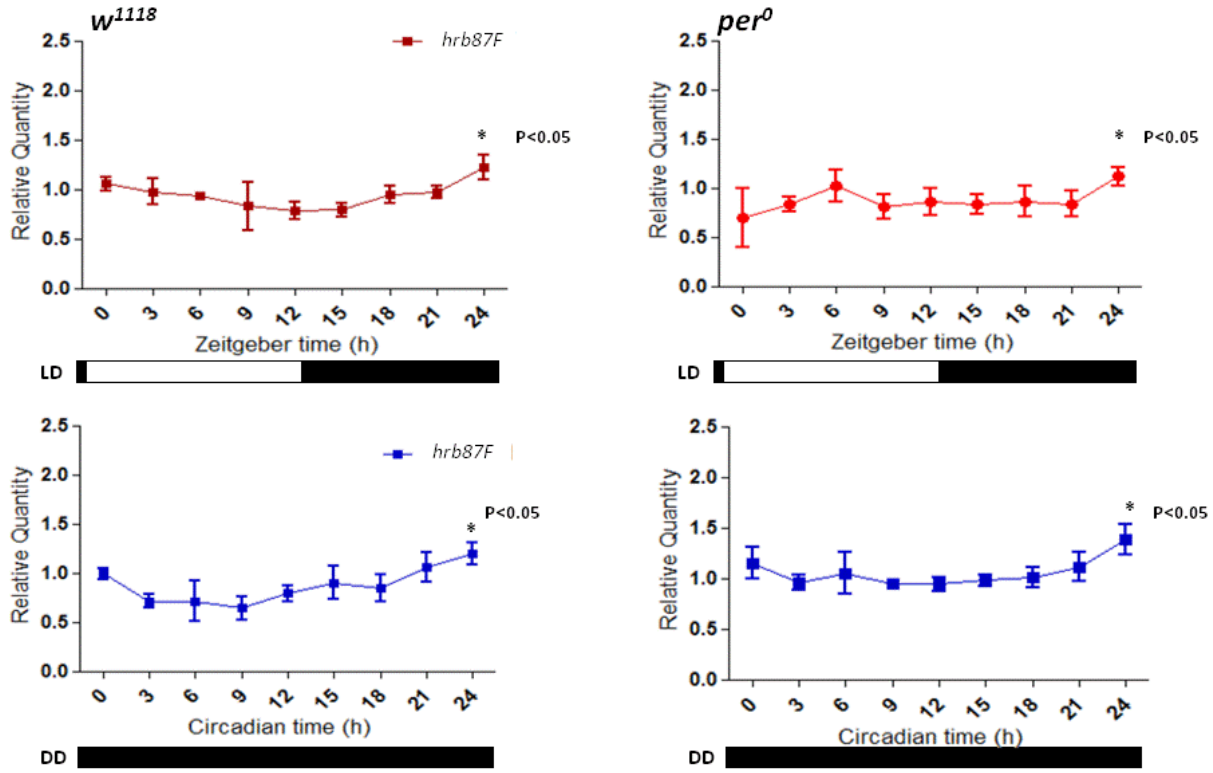


Figure 19. *Hrb87F*expression analysis on heads from w^{1118} (left) and per^0 (right) flies reared in 12.12 LD (upper panels) or over the first day in DD (lower panels). Relative abundance of *Hrb87F* mRNA was defined as a ratio with *rp49*. Values represent mean levels ± SEM of three biological replicates. The analysis with RAIN and CircWave revealed oscillatory trend (*p-value < 0.05).

In wild type flies, HRB87F protein expression profile shows a statistically significant oscillatory trend either in LD or in DD with maximum levels a ZT3 (in LD) CT9 (in DD) (Fig.20). This oscillation is lost in the per^0 mutant (Fig. 20).

These results suggest a possible role for the circadian clock in the translational/posttranslational control of HRB87F protein; moreover the six hours shift of peak levels when the flies are moved in DD suggests that light is also involved in this regulation.

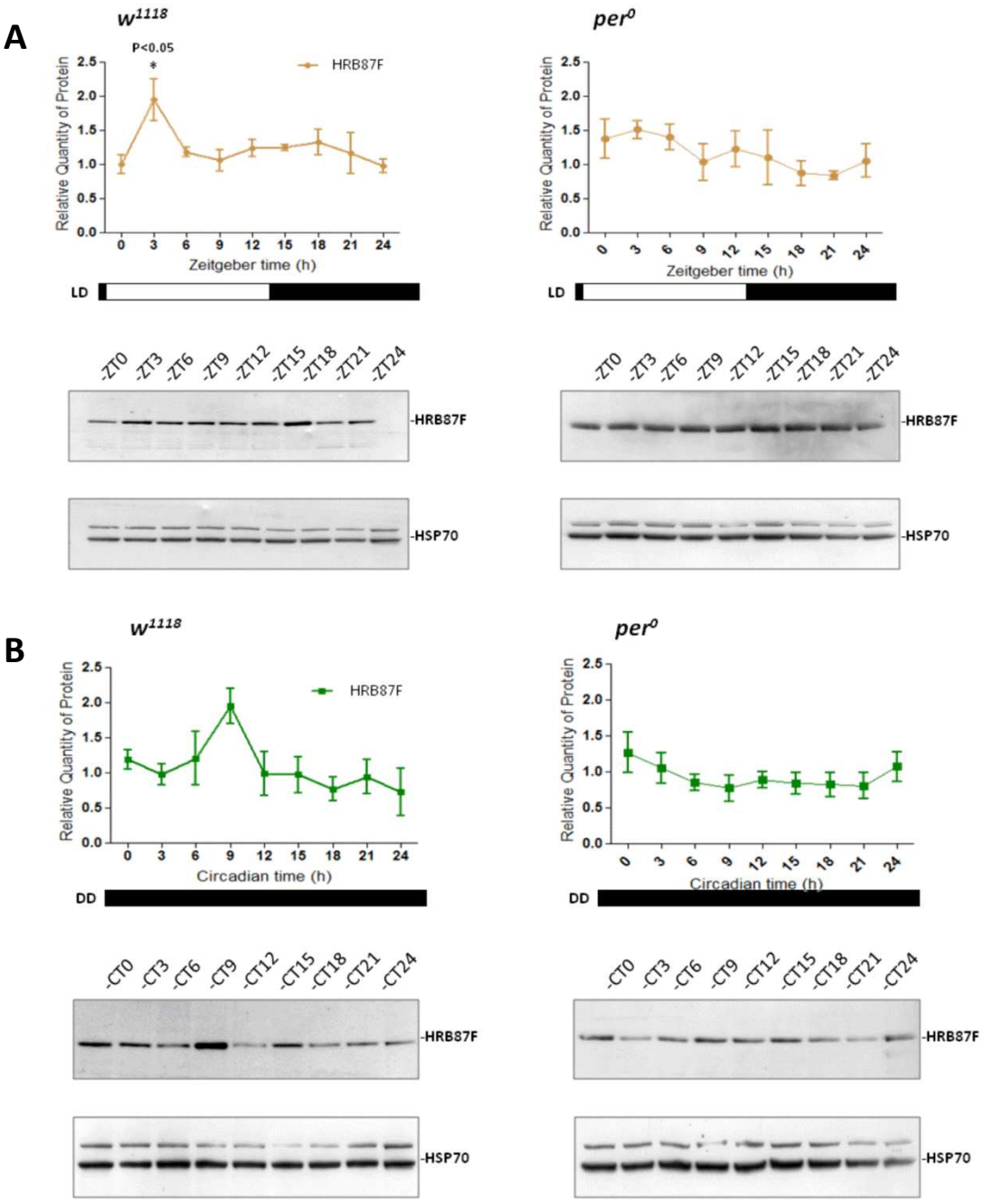


Figure 20. HRB87F expression analysis in w^{1118} (left) and per^0 (right) flies, collected every 3 hours under (A) LD or (B) DD. HRB87F signals were normalized to HSP70 (loading control). Three independent experiments were performed and means were plotted ± SEM. The trend of expressions was evaluated by using of RAIN and CircWave. In wild type flies both in LD and DD an oscillatory trend was identified with a peak respectively at ZT 3 and CT 9 (*p-value < 0.05).

2.3. Analysis of rhythmicity in *Squid* and *Hrb87F* mutants

In order to test whether these two hnRNPs might have a role in the circadian rhythmicity in *Drosophila*, we have decided to analyze the rhythmicity of *Squid* and *Hrb87F* mutants at behavioural and molecular levels.

Locomotor activity is the most studied circadian behaviour in *Drosophila*. In standard LD conditions, wild type flies exhibit two bouts of activity: one in the morning and one in the evening, that are controlled by the endogenous clock in response to the light/dark transitions (Chiu et al., 2010). In fact, an indicative property of a functional clock is indeed the ability to anticipate the light-dark and dark-light transitions, and not passively respond to them (Rosato and Kyriacou, 2006). This bimodal activity becomes unimodal when flies are moved to constant darkness (free-running).

To test for a possible role of hnRNPs in *D. melanogaster* circadian rhythmicity, the locomotor activity profiles were studied in order to understand the rhythmic behaviours. Male flies (3 days old) were first entrained at 12:12 LD for five days and subsequently moved to constant darkness (DD) or constant light (LL). In order to study also the role of temperature, the analyses were performed at four different temperatures, 15°C, 18°C, 23°C and 29°C.

2.3.1. Locomotor response of *Hrb87f* mutant

The mutant ry^{506}*; Df(3R)Hrb87F*, from now on called (*def.Hrb87F*) was produced and described by Haynes et al. (1997). This mutant has a deletion of the entire locus of *Hrb87F* gene and is characterized by reduced fertility in males, reduction in adult life span, decreased female fecundity, high sensitivity to starvation and lower survival rate at high temperature (above 25°C) (Haynes et al., 1997; Singh and Lakhotia, 2012). A western-blot analysis, performed by using an antibody against the entire HRB87F protein, confirmed this deletion (Fig.21).

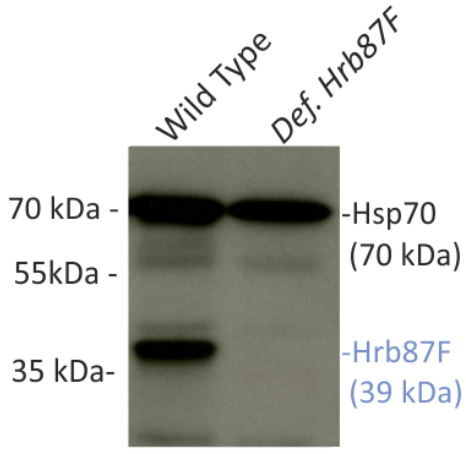


Figure 21. *def.Hrb87F* characterization by western blot. A ≈39 kDa signal, ascribed to HRB87F, is present only in wild type control. The membrane was also probed with an anti-HSP70 antibody as control.

The locomotor activity profile of *def.Hrb87F* was analyzed for each temperature in 12:12 LD and DD, and the results were compared with those from ry^{506} control flies (Fig.22-23-24-25).

The activity profiles recorded in 3 days of LD showed the canonical bimodal activity at each of the tested temperatures with activity phases more nocturnal at higher temperatures (Fig.22-23-24-25). This result is in accordance to previous observation showing that the peak of the evening activity is anticipated during the daytime at low temperature (Majercak et al, 1999). However, while ry^{506} control flies show the canonical anticipation of the morning activity, whose phase is anticipated before the dark-light transition at high temperature (Fig.22-23-24-25), *def.Hrb87F* flies showed a severe loss of morning anticipation, at 18°, 23° and 29° (Fig.23-24-25 B and Table 5), but it was maintained at 15°C (Fig.22 A and Table 5). When moved to constant darkness, *def.Hrb87F* flies showed high levels of arrhythmicity at any of the tested temperatures (Fig.22-23-24-25 A and Table 5), while ry^{506} flies show an unimodal response with a rhythmic profile (Fig.22-23-24-25 A and Table 5). No defects were monitored concerning the period, which was comparable to wild type (Fig.22-23-24-25 A and Table 5).

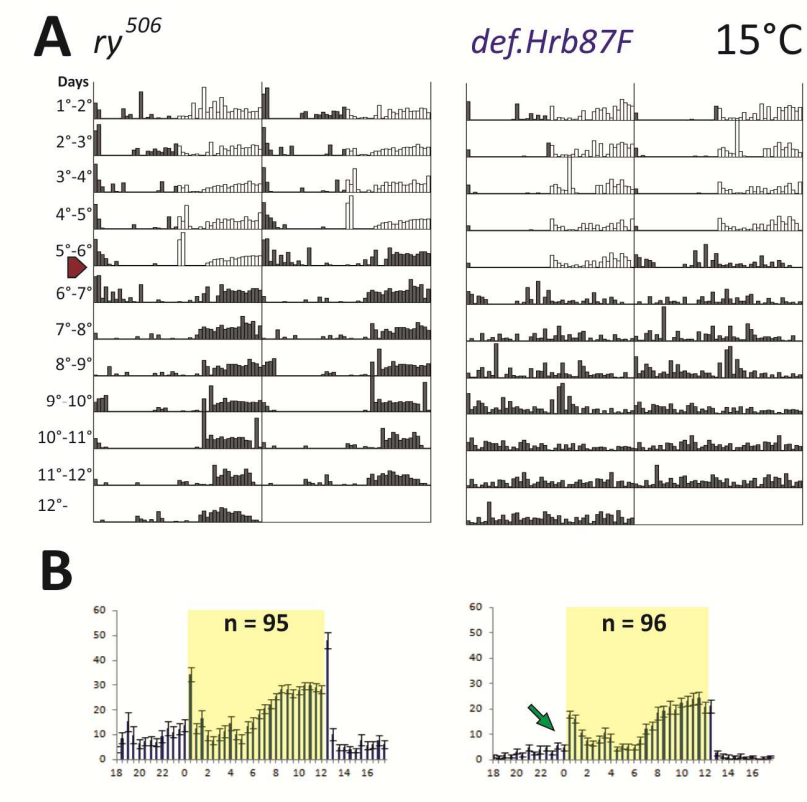


Figure 22. Locomotor activity of ry^{506} control and *def.Hrb87F* mutant flies at 15°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively**. (B)** Activity means ± SEM of flies over three days of LD cycles. n =number of flies. Green arrow indicates the lack of morning anticipation in *Hrb87F* mutant. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

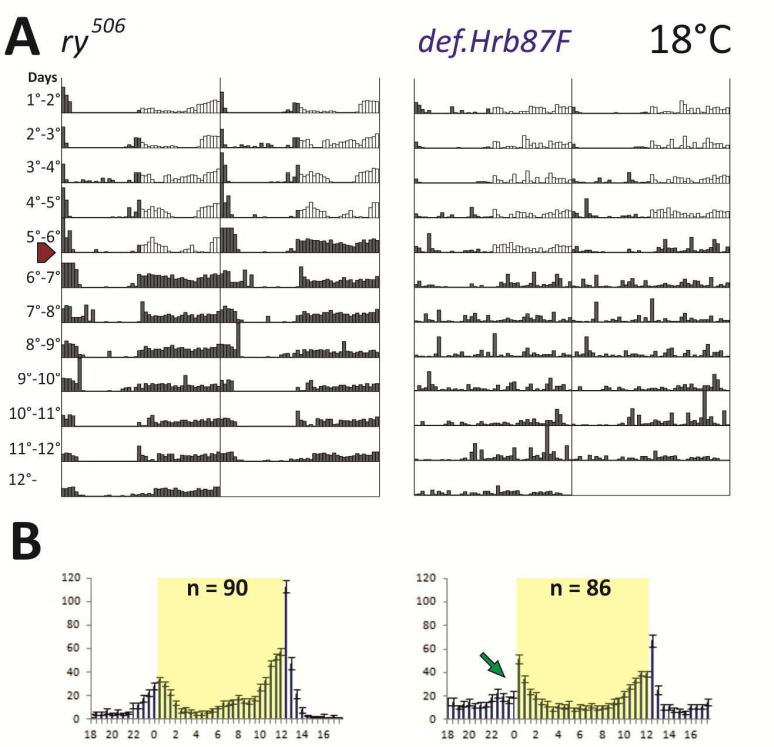


Figure 23. Locomotor activity of *ry*^{506} control and *def.Hrb87F* mutant flies at 18°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. **(B)** Activity means ± SEM of flies over three days of LD cycles. n = number of flies. Green arrow indicates the lack of morning anticipation in *Hrb87F* mutant. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

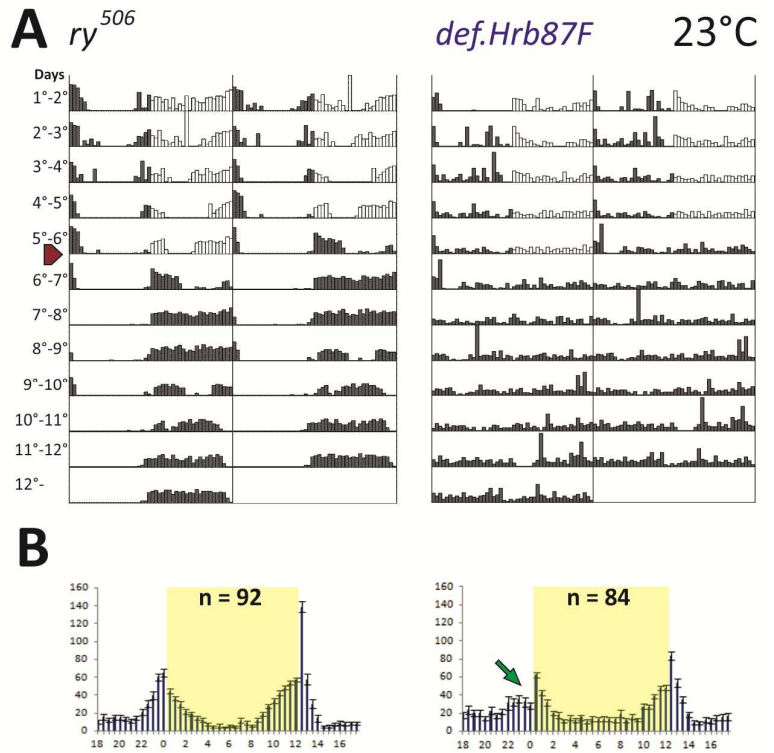


Figure 24. Locomotor activity of *ry*^{506} control and *def.Hrb87F* mutant flies at 23°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. **(B)** Activity means ± SEM of flies over three days of LD cycles. n =number of flies. Green arrow indicates the lack of morning anticipation in *Hrb87F* mutant. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

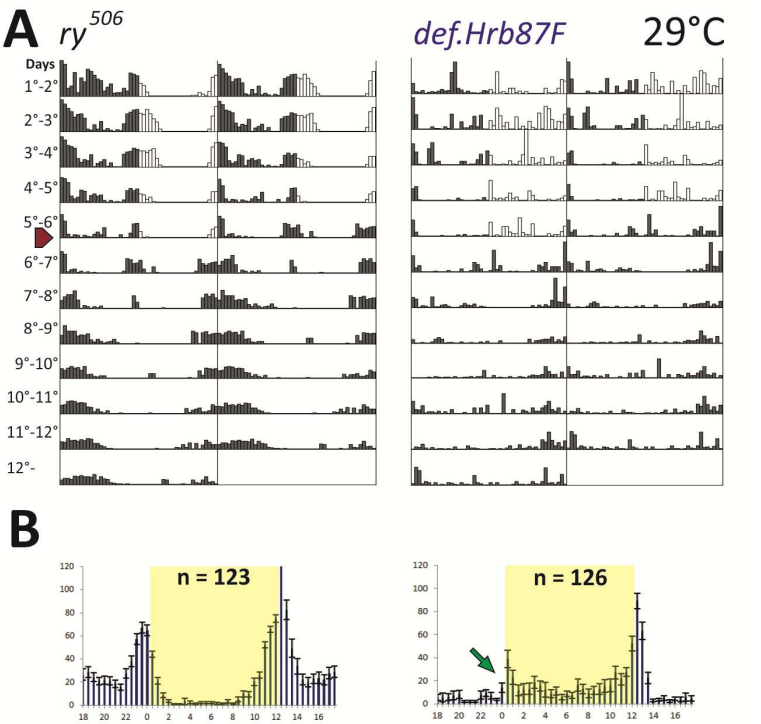


Figure 25. Locomotor activity of *ry*^{506} control and *def.Hrb87F* mutant flies at 29°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. **(B)** Activity means ± SEM of flies over three days of LD cycles. n =number of flies. Green arrow indicates the lack of morning anticipation in *Hrb87F* mutant. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

Table 5. Summary of locomotor activity data; ***P < 0.001; **P < 0.01 (*def.Hrb87F vs ry*^{506} control, two-way ANOVA, Bonferroni post *hoc* test.

Genotype	Temperature (°C)	Number of flies	Alive	Rhythmic		Period τ (h)	SEM	Morning Onset	Evening Onset	Morning Index	SEM
			n°	n°	%			%	%		
ry^{506}	**29°**	123	83	80	96,4	24,4	0,05	90,4	96,4	0,19	0,02
def.Hrb87F		125	42	18	42,9**	23,9	0,04	35,7***	81	0,1***	0,03
ry^{506}	**23°**	92	82	79	96,3	23,8	0,03	97,6	100	0,24	0,01
def.Hrb87F		84	74	22	29,7**	23,7	0,05	40,5**	77	0,09***	0,02
ry^{506}	**18°**	94	82	70	85,4	23.7	0,04	85,4	95,1	0,18	0,06
def.Hrb87F		92	86	23	26,7**	23,8	0,05	33,7**	90,7	0,04***	0,09
ry^{506}	**15°**	95	92	21	22,8	23,8	0,08	56,5	85,9	0,12	0,14
def.Hrb87F		96	84	9	10,7	22,8	0,09	32,4	88,1	0,09	0,08

In Table 5 the results of statistical analyses are also reported. The percentage of rhythmicity is statistically lower in mutants in comparison to controls at 18°C, 23°C and 29°C (p-values < 0.01); indeed, at 29°C only 42,9% of mutant flies are rhythmic compared to 96,4 % of control flies. This difference is much higher at 23°C and 18°C where the percentage of rhythmicity is of 29,7 % and 26,7 % respectively for mutants, and 96,3 % and 85,4 % of control.

Also the percentage of mutant flies showing morning anticipation is statistically lower in mutants compared to control (0.001 < p-values < 0.01). At 23°C, 18°C e 15°C the percentages of *def.Hrb87F* flies with morning anticipation oscillates between 33 and 41% while in the control flies is over 85%. The morning index (M-Index) was calculated (Seluzicki et al., 2014), confirming the absence of the morning anticipation in *def.Hrb87F* mutants at 29°C, 23°C and 18°C.

No statistically significant differences between mutant and control flies were observed, either for the rhythmicity and for the morning anticipation, at 15°C: indeed, at this temperature the circadian behaviour was impaired in both lines, probably because the clock ticking is affected in this conditions (Menegazzi et al., 2013).

In Table 5 the number of flies that survive the entire experiment is also reported: it is worthy to notice that at 29°C the number of living flies at the end of the experiment is much lower in *def.Hrb87F* flies compared to control. This observation is in agreement with previous results showing an increased sensitivity of the mutants at higher temperatures (over 25°C) (Singh and Lakhotia, 2012).

2.3.2. Locomotor response of *Squid* mutants

The locomotor activity response was also analyzed in two different mutant of the *Squid* gene: *w; P{wHy}sqd^{DG09709}* (from now on named *sqd^{DG}*) and *w; P{EP}sqd^{EP3631}* (from now on named *sqd^{EP}*), generated by transposable element insertion, in the first intron and in the 5' UTR respectively (Rorth, 1996; Huet et al., 2002). Both mutants are not viable when the insertion is in homozygosity, as other mutant alleles of *Squid* (Kelley, 1993; Norvell et al., 1999), therefore balanced lines were used for the analyses. These mutants show highly reduced expression of the gene (Piccolo et al., 2017).

Sqd^{DG} flies showed a phenotype very similar to that displayed by *def.Hrb87F*: they lacked of morning anticipation in entrainment conditions and displayed very high level of arrhythmicity in constant dark, at every tested temperature (Fig.26-27-28-29 and Table 6).

In entrainment conditions, *sqd*^{EP} flies showed a loss of morning anticipation, regardless of temperature (Figures 26-27-28-29 and Table 6). As for the evening activity, we observed a delay in the early night at 29°, 23° and 18° (Figures 27-28-29), while at 15° it was anticipated in the light phase, similarly to *w*^{1118} control (Fig.26), and in accordance to reported observations (Majercak et al., 1999).

In constant conditions, *sqd*^{EP} flies showed high percentages of arrhythmicity; however, those flies that remained rhythmic showed longer periods of endogenous locomotor activity compared to control (Tab.6). At 29°C, 67,3% of mutant flies displayed a rhythmic phenotype, with a period of 26,9 ± 0,03 hours; at 23°C the percentage of rhythmic flies decreased to 38,8% and the period slightly lengthened (27,7± 0,1 h) (Tab.6). A similar response was present at 18°C where *sqd*^{EP} flies showed a lower percentage of rhythmic flies and a longer period (28,1± 0.06 h) compared to control (Tab.6). At lower temperature (15°C) the number of rhythmic flies was further reduced (30,3 %) and the period remained long (27 h ± 0,1 h).

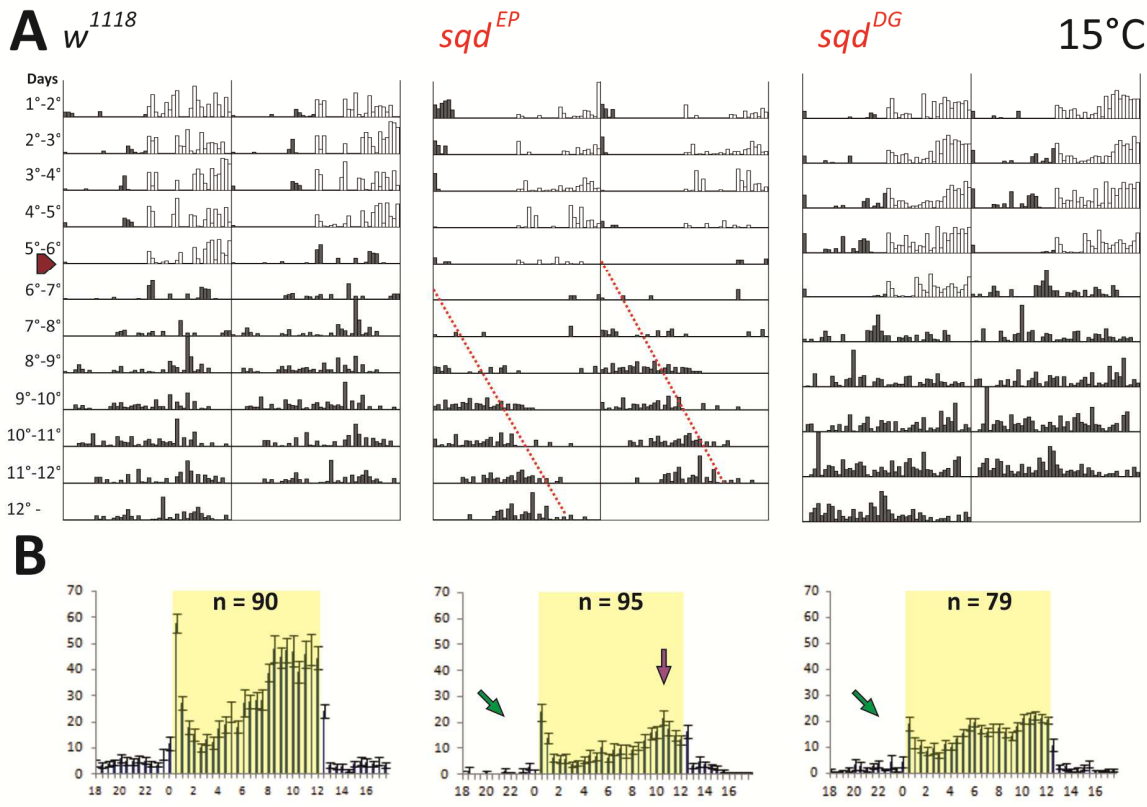


Figure 26. Locomotor activity *w*^{1118} control and *sqd*^{EP} and *sqd*^{DG} flies at 15°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted vertical line indicates a >24 hours eriodicity in *sqd*^{EP} flies. **(B)** Activity means ± SEM of flies over three days of LD cycles. n: number of flies. Green arrows indicate the lack of morning anticipation in *squid* mutants. Red arrow indicates the phase of evening activity. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

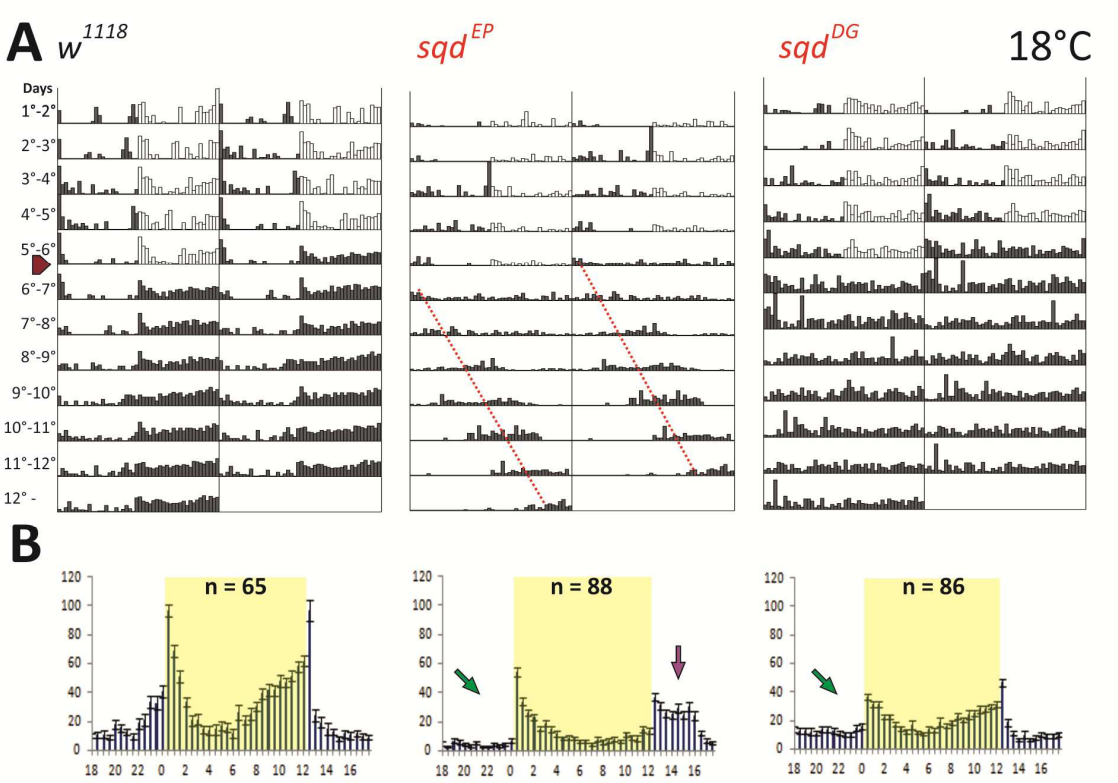


Figure 27. Locomotor activity w^{1118} control and sqd^{EP} and sqd^{DG} flies at 18°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted vertical line indicates a >24 hours periodicity in sqd^{EP} flies. **(B)** Activity means ± SEM of flies over three days of LD cycles. n: number of flies. Green arrows indicate the lack of morning anticipation in *squid* mutants. Red arrow indicates the phase of evening activity. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins**.**

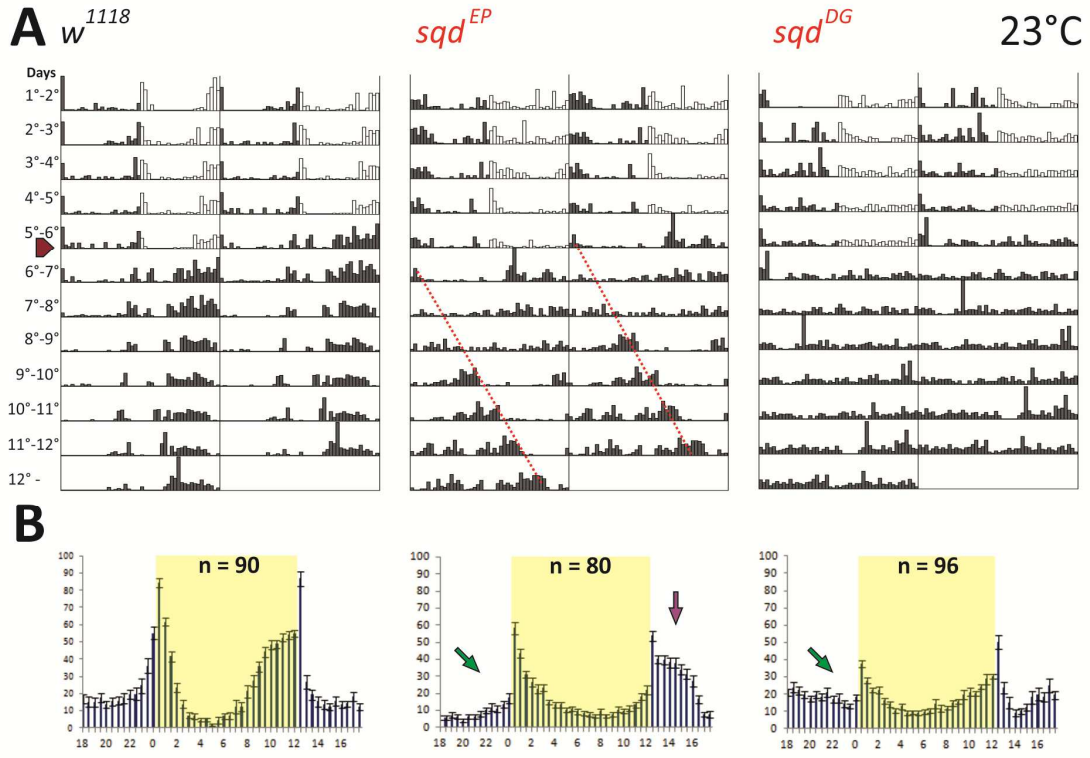


Figure 28. Locomotor activity w^{1118} control and sqd^{EP} and sqd^{DG} flies at 23°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted vertical line indicates a >24 hours periodicity in sqd^{EP} flies**. (B)** Activity means ± SEM of flies over three days of LD cycles. n: number of flies. Green arrows indicate the lack of morning anticipation in *squid* mutants. Red arrow indicates the phase of evening activity. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

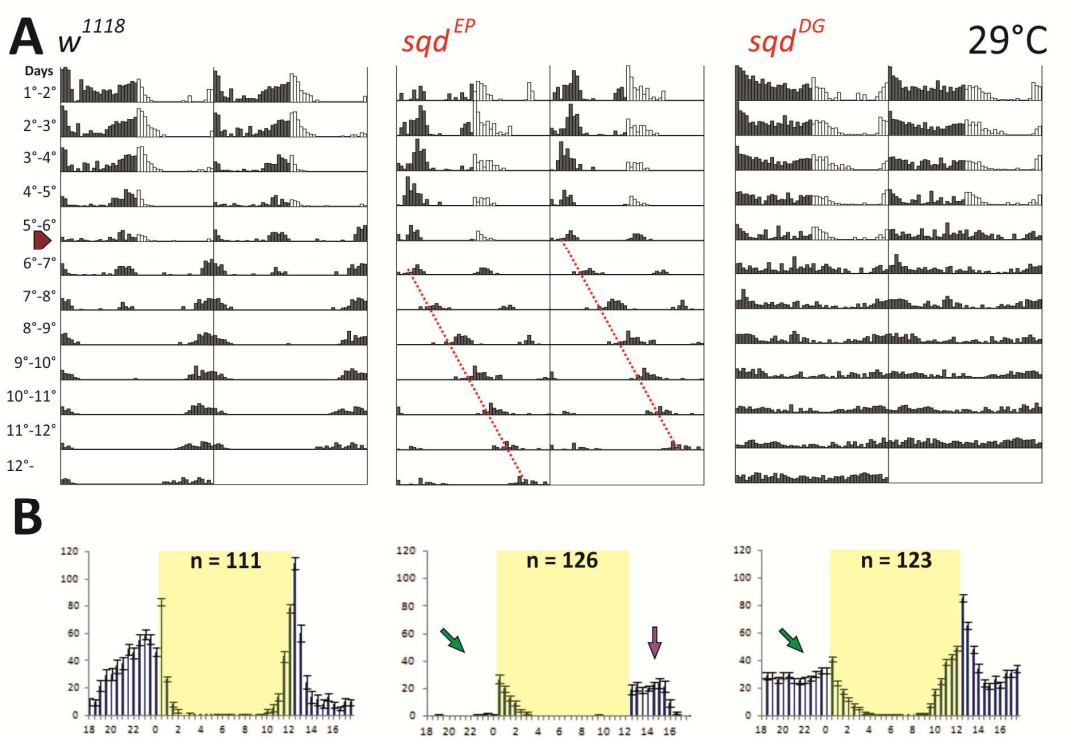


Figure 29. Locomotor activity w^{1118} control and sqd^{EP} and sqd^{DG} flies at 29°C. (A) Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted vertical line indicates a >24 hours periodicity in sqd^{EP} flies. **(B)** Activity means ± SEM of flies over three days of LD cycles. n: number of flies. Green arrows indicate the lack of morning anticipation in *squid* mutants. Red arrow indicates the phase of evening activity. On the x axis are reported the hours of day and on the y axis the number of recorded events in 30 min bins.

Table 6. Summary of locomotor activity data; ***P < 0.001; **P < 0.01; *P < 0.05 (sqd^{EP} *vs* w^{1118} control flies / sqd^{DG} *vs* w^{1118} control flies), two-way ANOVA, Bonferroni post *hoc* test.

Genotype	Temperature (°C)	Number of flies	Alive	Rhythmic		Period τ (h)	SEM	Morning Onset	Evening Onset	Morning Index	SEM
			n°	n°	%			%	%		
w^{1118}		111	69	68	98,6	23,2	0,06	92,8	94,2	0,26	0,03
sqd^{EP}	**29°**	123	52	35	67,3*	26,9*	0,03	19,2***	82,7	0,01***	0,04
sqd^{DG}		126	95	37	38,9**	23,4	0,05	34,7***	90,5	0,04***	0,02
w^{1118}		90	77	64	83,1	23,8	0,05	72,7	98,7	0,14	0,02
sqd^{EP}	**23°**	93	80	31	38,8**	27,7*	0,1	26,3***	90	0,03***	0,03
sqd^{DG}		96	80	3	3,8***	23,9	0,04	21,3***	72,5	0,02***	0,02
w^{1118}		91	65	52	80	23,8	0,05	61,5	93,8	0,1	0,06
sqd^{EP}	**18°**	91	88	30	34,1**	28,1*	0,06	19,3**	77,3	0,00***	0,14
sqd^{DG}		93	86	10	11,6***	24,3	0,06	26,7**	69,8	0,01***	0,09

w^{1118}		90	75	32	42,7	24,1	0,05	30,7	94,7	0,05	0,07
sqd^{EP}	**15°**	79	66	20	30,3	27*	0,1	12,1	95,5	0,02	0,07
sqd^{DG}		95	93	20	21,5	23,4	0,06	22,6	82,8	0,01	0,22

The analyses of locomotor activity of sqd^{EP} flies revealed two features that we think are worth to note. First of all, the percentage of rhythmic flies decreases with temperature, in a manner more pronounced compared to control (Table 6).

Secondly, those flies that remain rhythmic display a peculiar temperature-dependent behaviour during the first three days of DD: at 29°C the rhythmicity is exhibited already from the first day of DD, at 23°C and 18°C flies are arrhythmic for two days and then became rhythmic, and at 15°C flies do not show any activity until the fourth day of DD (Fig.30). Control flies show a typical locomotor response at each of the tested temperatures (Fig.30). The behaviour displayed by sqd^{EP} mutant could suggest that these flies are, somehow, unable to be properly entrained by the light-dark cycle.

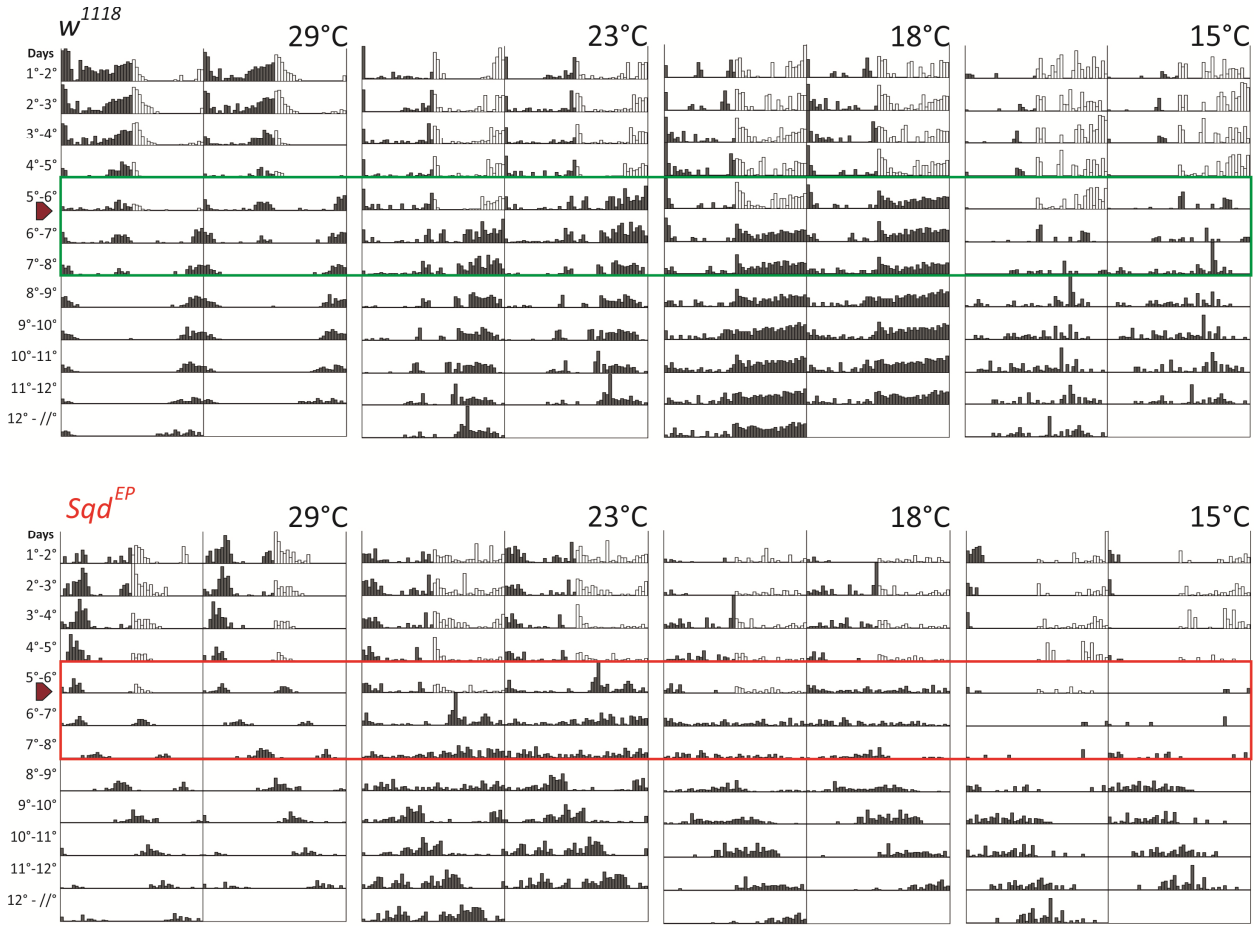


Figure 30. Locomotor activity w^{1118} control and sqd^{EP} flies at different temperatures. Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Green and red boxes include the first three days of DD in activity w^{1118} control and sqd^{EP} flies, respectively.

2.3.3. Locomotor activity of *Squid* mutant under constant light

Given the role of CRY as main circadian photoreceptor involved in the phototransduction pathway (Yoshii et al., 2004; Rieger et al., 2006), we have decided to verify whether the interaction CRY/SQUID might be somehow implicated in the light synchronization of the fly clock. For this purpose we have analyzed the locomotor response of sqd^{EP} flies in constant light. Continuous light severely compromises the light synchronization pathway in wild-type flies, that become arrhythmic, while *cry* mutants in LL display a rhythmic behaviour in which two components with a longer and a shorter period can be observed (Dolezelova et al., 2007).

Sqd^{EP} flies were entrained for 5 days at 12:12 LD and then released in LL. The locomotor behaviour was analyzed at different temperatures (15°C, 18°C, 23°C and 29°C), in order to reveal/exclude any possible effect of temperature in the flies' locomotor behaviour in constant light (Fig.31-32-33-34).

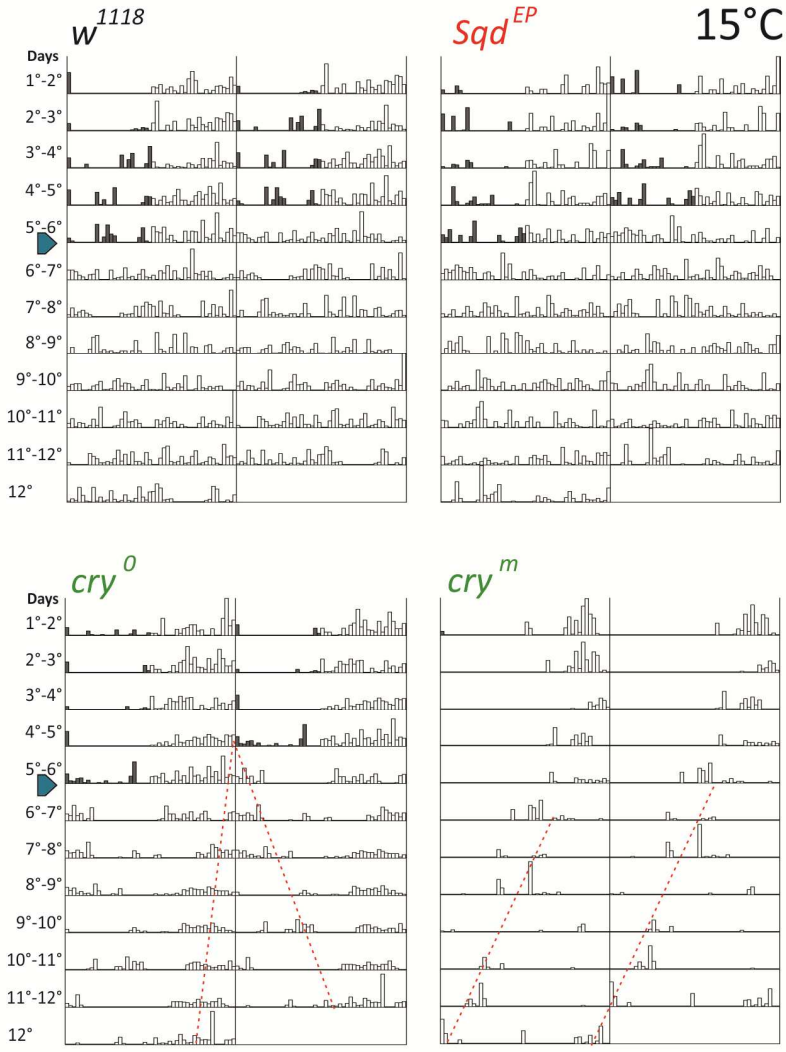


Figure 31. Locomotor activity w^{1118} control and sqd^{EP}, cry^{0} and cry^{m} flies at 15°C. Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted lines highlight the periodicity in *cry* mutants.

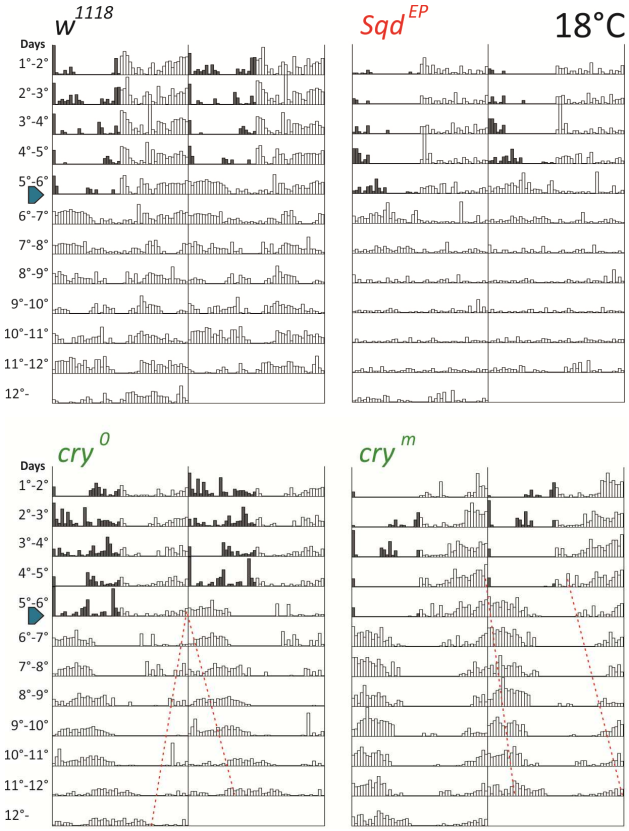


Figure 32. Locomotor activity w^{1118} control and sqd^{EP}, cry^{0} and cry^{m} flies at 18°C. Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted lines highlight the periodicity in *cry* mutants.

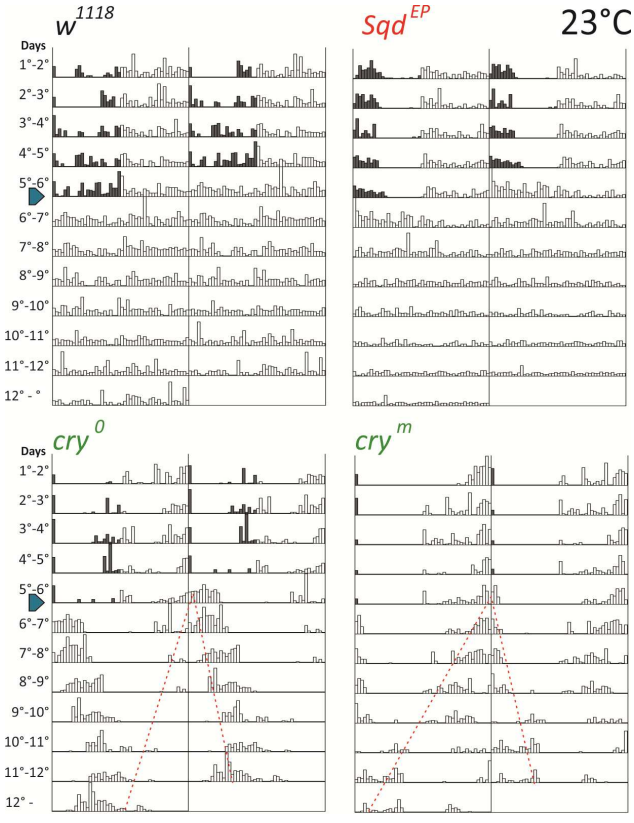


Figure 33. Locomotor activity w^{1118} control and sqd^{EP}, cry^{0} and cry^{m} flies at 23°C. Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted lines highlight the periodicity in *cry* mutants.

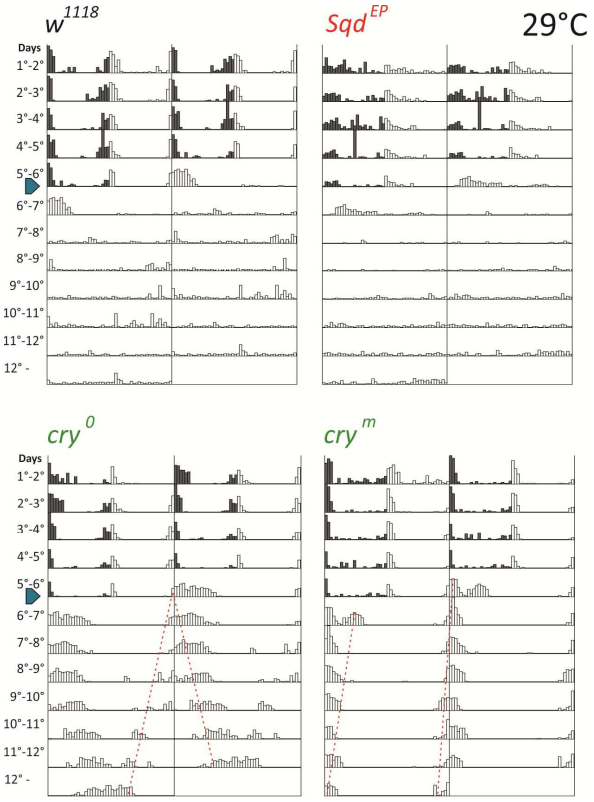


Figure 34. Locomotor activity w^{1118} control and sqd^{EP}, cry^{0} and cry^{m} flies at 29°C. Representative double-plotted actogram of a single fly during 12 days of experiment. Activity in light and dark hours is represented by white and black bars respectively. Red dotted lines highlight the periodicity in *cry* mutants.

Both cry^{0} and cry^{b} mutant exhibit the previous mentioned locomotor rhythm in LL, while both the w^{1118} control and sqd^{EP} mutant displayed an arrhythmic phenotype in any of the tested temperatures (Fig.31-32-33-34 and Table 7), underlining that the deep brain photoreception pathway is not compromised in sqd^{EP} mutant.

Table 7. Summary of locomotor activity data; ***P < 0.001; **P < 0.01; *P < 0.05 (sqd^{EP} versus cry^{0} mutant flies / w^{1118} versus cry^{0} mutant flies), two-way ANOVA, Bonferroni post *hoc* test.

Genotype	Temperature (°C)	Number of flies	Alive	Rhythmic		Period τ (h)	SEM
			n°	n°	%		
w^{1118}	29°	91	55	3	5,5***	23,2	0,04
sqd^{EP}		93	49	2	4,1***	27,5	0,07
cry^{0}		95	43	42	97,7	25,0	0,08
cry^{m}		92	48	46	95,8	23,6	0,03
w^{11188}	23°	93	81	6	7,4***	23,0	0,04
sqd^{EP}		92	86	6	7,0***	27,3	0,16
cry^{0}		94	91	70	76,9	25,3	0,18
cry^{m}		95	83	59	71,1	23,6	0,11

w^{1118}	**18°**	90	71	8	11,3***	22,8	0,16
sqd^{EP}		96	92	5	5,4***	27,9	0,21
cry^{0}		94	94	59	62,8	25,6	0,18
cry^{m}		96	86	59	68,6	23,7	0,12
w^{1118}	**15°**	95	73	0	0,0	-	-
sqd^{EP}		93	82	0	0,0	-	-
cry^{0}		95	84	29	34,5	24,7	0,12
cry^{m}		95	77	29	37,7	23,3	0,07

2.4. Analysis of *period* and *timeless* splicing variants

In the Drosophila molecular clockwork, the mRNAs of *period* and *timeless* undergo a temperature-dependent alternative splicing (Montelli et al., 2015). This post-transcriptional control regulates clock genes expression, in order to adjust and consolidate the proper phase of circadian clock at different temperatures. The thermal regulated alternative splicing represents in *Drosophila* an adaptive response to thermal and photoperiodic seasonal changes (Rosato et al., 1997; Sandrelli et al., 2007; Tauber et al., 2007). In *per,* the thermo-sensitive alternative splicing of an 89 bp intron in 3'UTR originates two isoforms, $per^{SPLICED}$ and $per^{UNSPLICED}$, the latter at warmer temperature (Majercak et al., 2004; Collins et al., 2004; Low et al., 2012). The splicing of *per* results in an earlier accumulation of the correspondent mRNA and leads to an anticipated evening activity at cold temperature (Collins et al., 2004). The phenomenon of thermal alternative splicing was also observed in *tim*, where at cold temperature, the least 858 bp intron is retained and the transcript, $tim^{UNSPLICED}$, gives a TIM protein 33 amino acids shorter than the full length $tim^{SPLICED}$, due to a premature STOP codon (Boothroyd et al., 2007).

2.4.1. Multiplex real-time PCR

A multiplex Taqman real-time PCR assay was performed to analyse the abundance of *per* and *tim* splicing variants. This method has several advantages, including reduced reagents and sample usage, cost-effectiveness and increased accuracy.

As spliced and unspliced isoforms for *period* and *timeless* differ in intron retention, it was not possible to design primers and probes specific to the spliced isoforms (Fig.35).

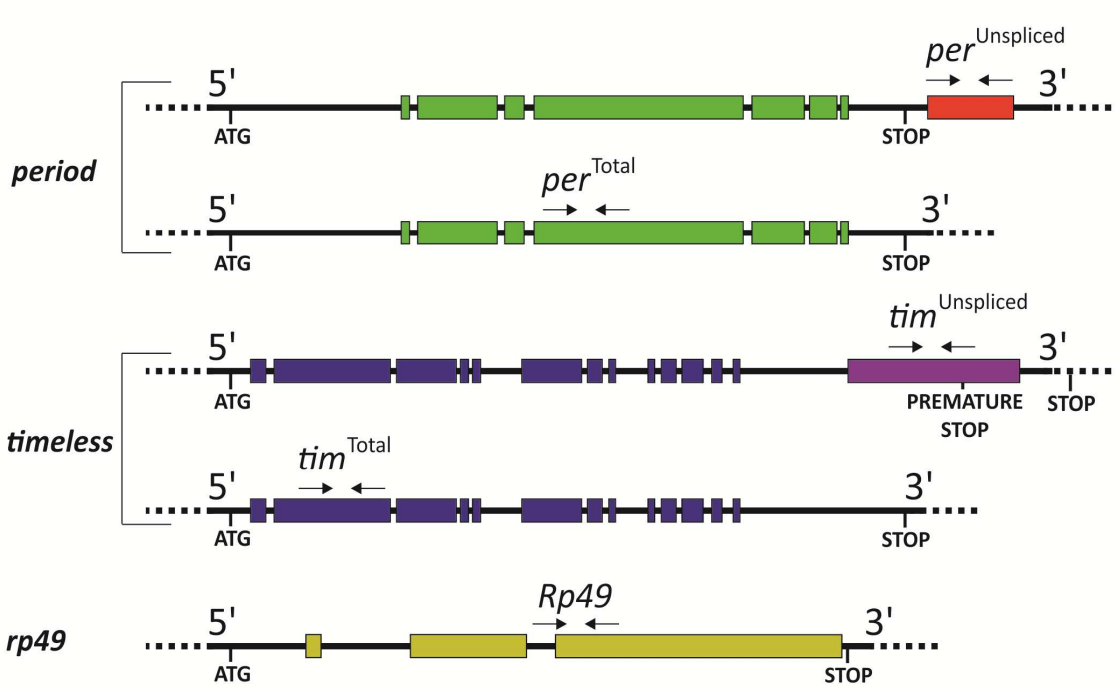


Figure 35. Overview of multiplex real-time target genes and specific primer pairs positions; transcribed regions of each target genes are represented by coloured boxes: *period* exons (green), *timeless* exons (dark blue), *rp49* exons (yellow) *period* retained intron (red) and *timeless* retained intron (violet). Black arrows identify primer pairs position.

Therefore, primers for the unspliced isoforms were designed within the retained introns; while spliced isoform quantification was calculated by difference between the total transcript amount and the unspliced isoform, as described by Vandenbroucke *et al*. (2001).

The main factor that can affect the reliability of a multiplex PCR is represented by competition or inhibition between assays due to interactions between different primer pairs, probes and amplicons. In an attempt to prevent such problems, primers for total transcript quantification were designed several hundred bases away from the retained intron's position, in order to avoid interactions with the unspliced isoform primer pair. Then, to test whether primers for different isoforms could work together in a multiplex experiment without cross-reactivity between different primer pairs, a preliminary experiment was performed using classic SYBR green real-time PCR. Dissociation curve analysis confirmed primer pairs specificity.

In the second phase of multiplex real-time PCR optimization, each Taqman assay was independently tested. Since all the five assays showed high quality amplification curves, a multiplex real-time PCR was performed in order to verify primer pairs efficiencies and whether relative expression levels of targets were comparable to one another (Fig.36).

Lack of the amplification curves of the unspliced isoforms of *period* and *timeless* that is observable in Figure 36 A could be explained by a lower level of expression or a lower efficiency of the

respective primers pairs. In order to promote the amplification of unspliced isoforms and to ensure the simultaneous amplification of all targets, primers concentrations of unspliced isoforms were increased up to 900 nM (from 250 nM). Moreover, since the amount of the housekeeping gene is higher than the splicing variants, its primers concentration was decreased to 150 nM to promote the amplification of the other targets and to limit depletion of reaction mix reagents. Primers concentrations optimization resulted in a proper multiplex amplification of all the five targets (Fig.36 B)

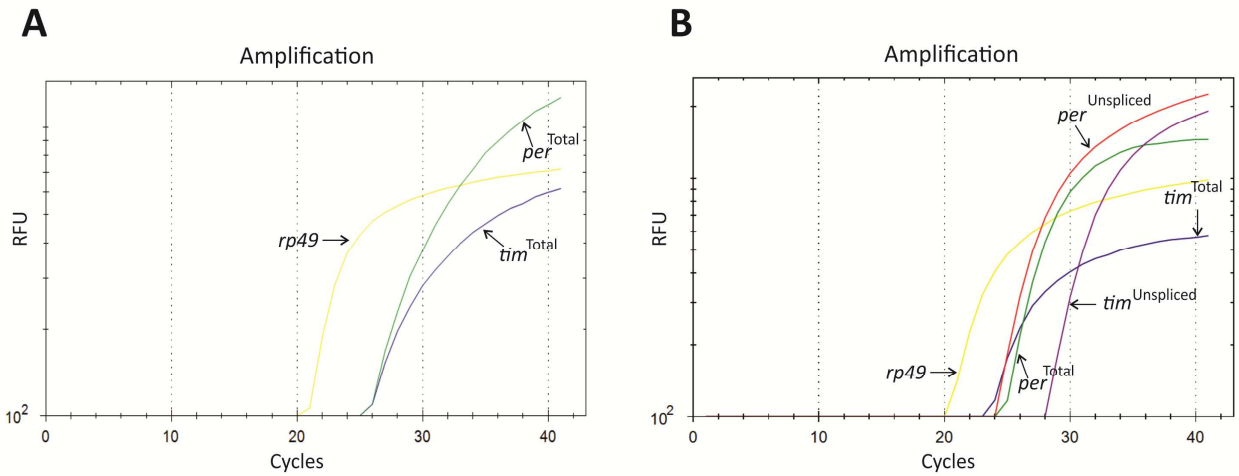


Figure 36. Optimization of primer pairs concentrations. (A) Amplification curves obtained with an equal concentration of all primer pairs; the unspliced isoforms of *period* and *timeless* were not amplified. **(B)** Amplification cures obtained after the optimization of primer pairs concentration; all the five target were successfully amplified.

Preliminary multiplex experiments highlighted the expected differences in fluorophores intensities, which make the comparison between relative expression levels of the isoforms impossible. In order to perform an absolute quantification five standard curves were made, one for each target genes (per^{total}, $per^{unspliced}$, tim^{total} , $tim^{unspliced}$ and *RP49*), with six serial dilutions (0,05 pg/µL, 5 fg/µL, 0,5 fg/µL, 0,05 fg/µL, 5 ag/µL and 0,5 ag/µL) to cover the entire range of C_t observed in the experiments (Appendix 1).

To evaluate the validity and feasibility of this assay, preliminary results have been compared with those obtained in previously published studies, in which a semi-quantitative PCR approach was used. As shown in Figure 37 (A and B), in w^{1118} control the amount of the unspliced variant of *period* increased with temperature (from 18°C to 29°C) from about the 25% to the 73% of the total *period* transcript amount. This result reflects what has been previously observed by Majercak *et al.* (1999) and Collins *et al.* (2004), which reported a temperature-induced increase in the amount of the *period*'s unspliced variant of about 25% under the same experimental conditions. Also the abundance of the spliced variant of *timeless* increases with the temperature (18°C to 23°C) from

about the 56% to the 81% of the total transcript amount (Figure 37 C and D). This result is in accordance with what has been reported by *Montelli* et al. (2015) where a 30% increase in the spliced variant of *timeless* was observed with increasing temperature, to the detriment of the unspliced isoform.

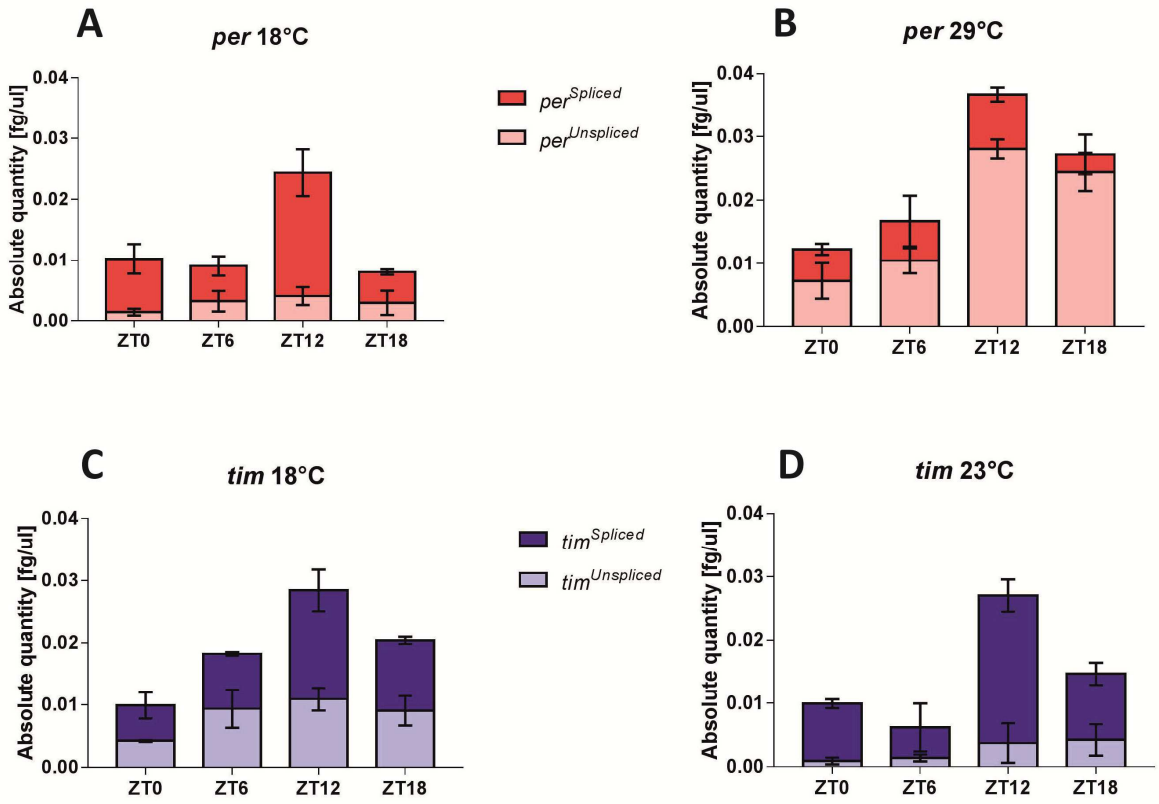


Figure 37. Preliminary quantification of expression levels of the splicing isoforms of *period* (A and **B) and *timeless* (C** and **D)** in brains of w^{1118} wild type flies at different temperatures in LD. For each time point, absolute quantifications of spliced and unspliced variants are reported. Each values is reported as mean ± SEM (N=3).

Although the actual values of relative expression were not exactly the same, the most significant information which emerges comparing the data obtained by the two different technologies is represented by the general overlapping of the expression profiles obtained, that attests the reliability and the applicability of the assay that we have developed. Furthermore, the proven higher accuracy of the Taqman real-time PCR assay, with respect to a semi-quantitative PCR, assured us more precise and reliable results.

In order to investigate whether hnRNPs are involved in the circadian regulation of the alternative splicing in Drosophila, we decided to analyze *period* and *timeless* splicing variants at different temperatures.

Given the stronger phenotype of sqd^{EP} flies, we decided to focus our attention on this mutant, to analyze whether the impairment of the locomotor behaviour could be mirrored by altered splicing

pattern of *per* and *tim*. Flies from both sqd^{EP} mutant and w^{1118} control were first entrained in 12:12 LD, moved to constant darkness and collected every 3 hours both in LD and in the first day of DD. The analysis was performed at 15°C, 18°C, 23°C and 29°C. Brains were dissected from 30 individuals, total RNA was extracted and the expression of *per* and *tim* was analyzed by Multiplex qPCR.

The absolute quantities of *per* (Fig.38-39) and *tim* (Fig.40-41) transcript variants through 24 hours, measured at 29°, 23°C, 18°C and 15°C in LD and DD are represented.

As for *per*, the oscillation of total mRNA was maintained in LD in both strains at all temperatures (Fig.38); however in the sqd^{EP} brains the maximum level was observed at ZT15, that is 3 hours delayed compared to w^{1118}. Indeed, an analysis performed by RAIN and CircWave confirmed the altered oscillatory *per* mRNA pattern in the sqd^{EP} brains (w^{1118} vs sqd^{EP} $p<0.001$ both in LD and DD at each temperature). In constant darkness, the oscillation of *per* was maintained with the same phase (peak at CT12) in w^{1118} at each temperature (Fig.39); the levels of mRNA were more flat in sqd^{EP} brains at lower temperatures (15 and 18°C) where, nevertheless, a maximum of expression was present at CT15 (3 hours delayed compared to w^{1118}). At higher temperature (29°C), instead, *per* mRNA showed a nice oscillation with a peak at CT15 (Fig.39).

Also for *tim* we observed a nice oscillation of total mRNA in LD in either strain at all temperatures and, similarly to *per*, the peak of expression was 3 hours delayed in sqd^{EP} compared to w^{1118} brains (Fig.40). At 15°C the amplitude of the oscillation was more pronounced in sqd^{EP} brain, in which the mRNA levels during the night were double compared to w^{1118}, despite similar levels during the light phase (Fig.40). In constant darkness *tim* total mRNA exhibited a nice oscillation in w^{1118} brains, that was more or less maintained in sqd^{EP} flies at all but 23°C temperatures, at which the amplitude was very low (Fig.41). Also in this case the peak of expression was delayed in sqd^{EP} compared to w^{1118} brains, and the delay seemed to be influenced by the temperature: indeed, we observed a 3 hours delay at 15 and 18°C, while at 29°C it was more pronounced, as we observed a peak of expression at CT21 (Fig.41).

Looking at the splicing isoforms, a general decrease of the unspliced isoform was observed for both *per* and *tim* in sqd^{EP} compared to w^{1118} brains at each temperature both in LD (Fig.38-40-42-44) and in DD (Fig.26-28-30-32), likely as result of an increased splicing activity.

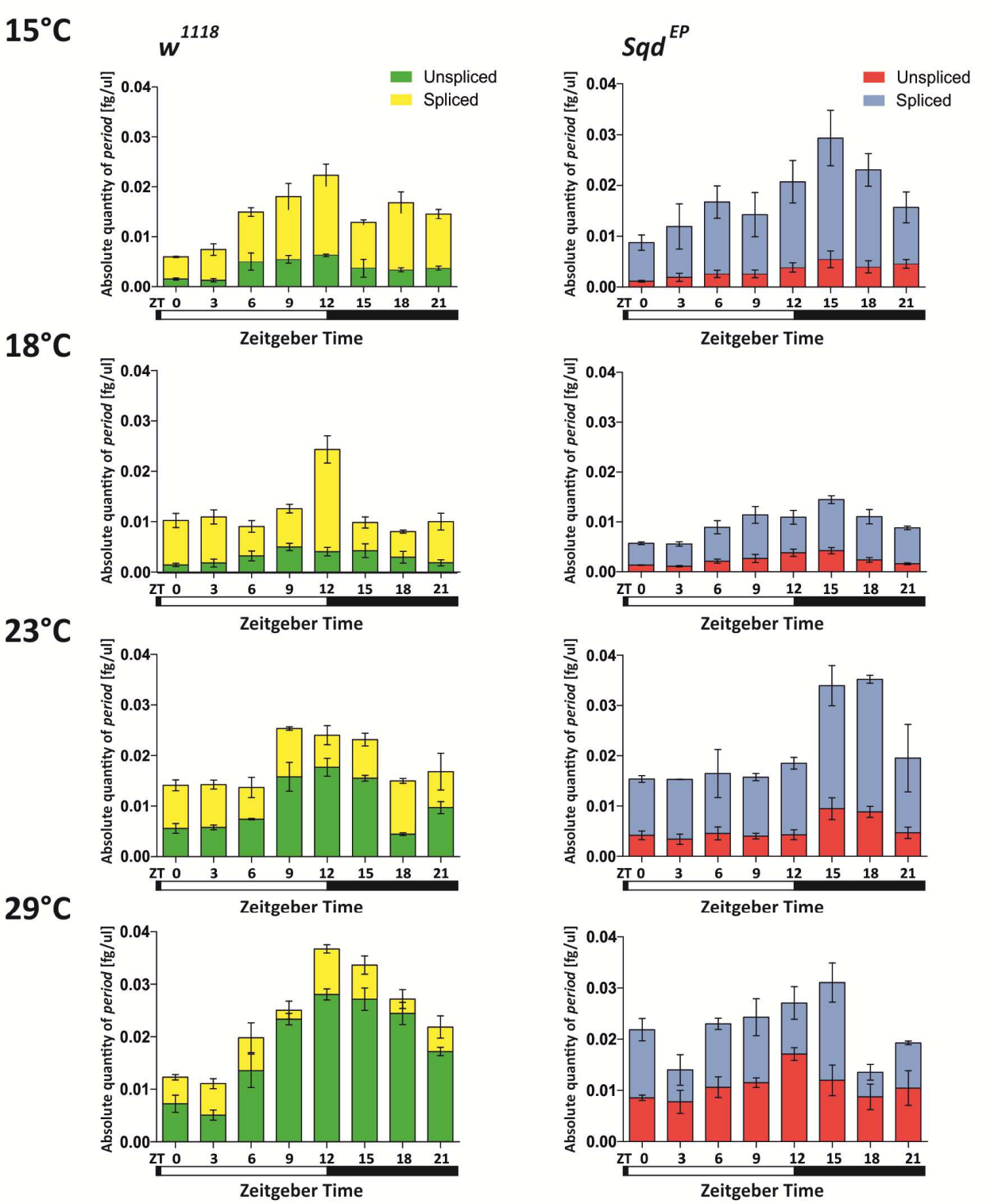


Figure 38. Quantification of *period* expression levels in brains from w^{1118} and sqd^{EP} flies at different temperatures (15°C, 18°C, 23°C, and 29°C) in LD. For each time point, abundance of spliced and unspliced variants are reported. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3).

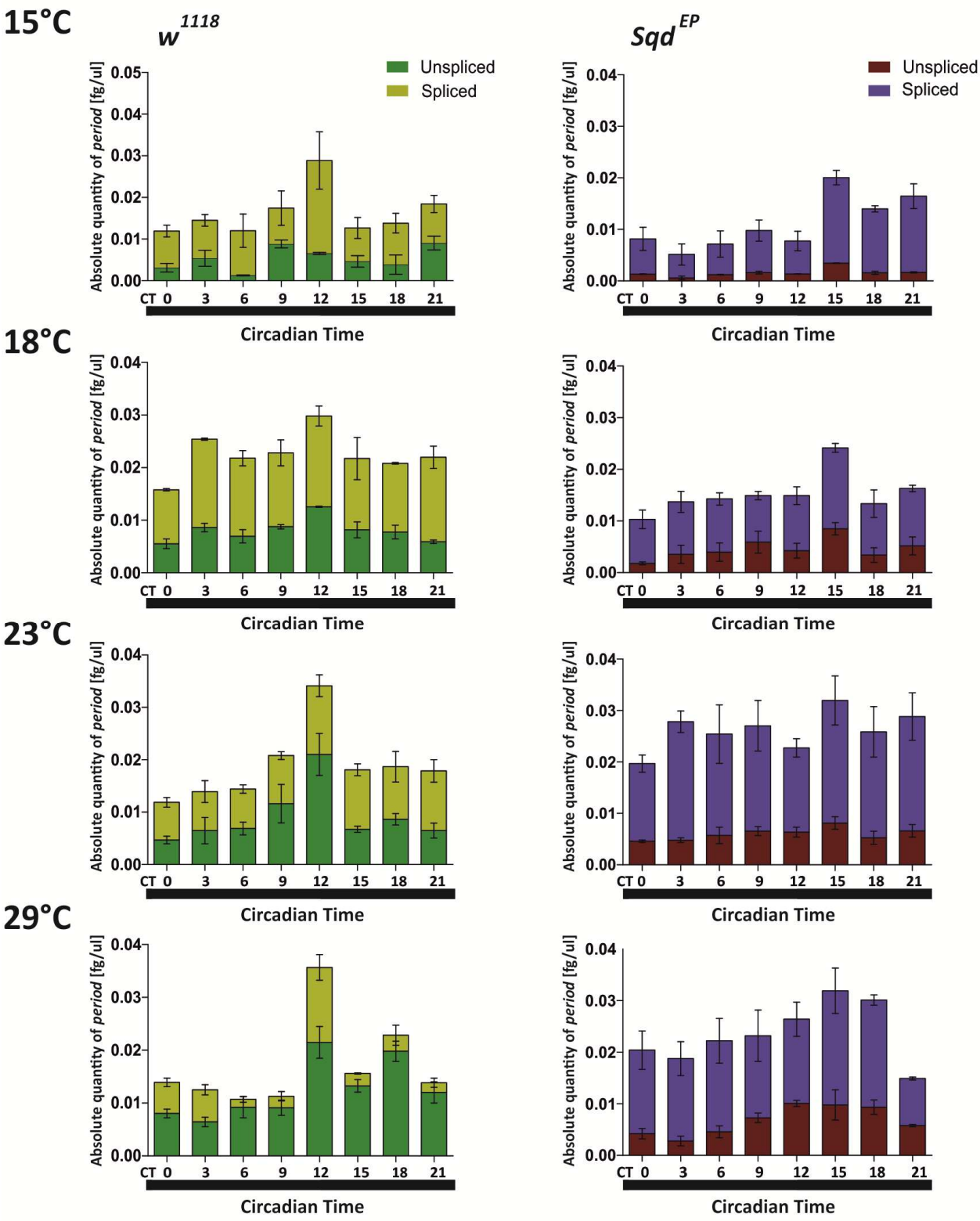


Figure 39. Quantification of *period* expression levels in brains from w^{1118} and sqd^{EP} flies at different temperatures (15°C, 18°C, 23°C, and 29°C) in DD . For each time point, abundance of spliced and unspliced variants are reported. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3).

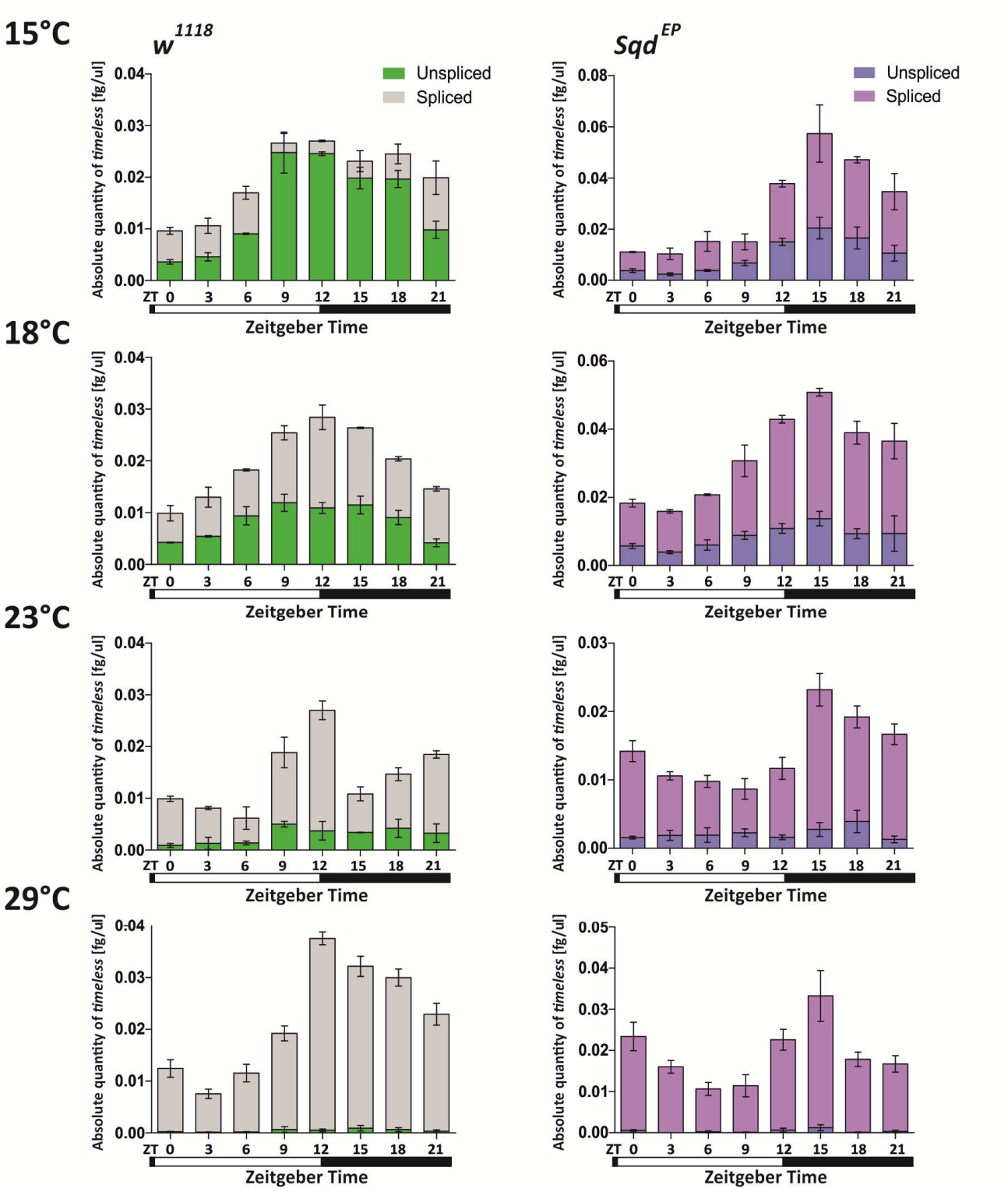


Figure 40 Quantification of *timeless* expression levels in brains from w^{1118} and sqd^{EP} flies at different temperatures (15°C, 18°C, 23°C, and 29°C) in LD. For each time point, abundance of spliced and unspliced variants are reported. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3).

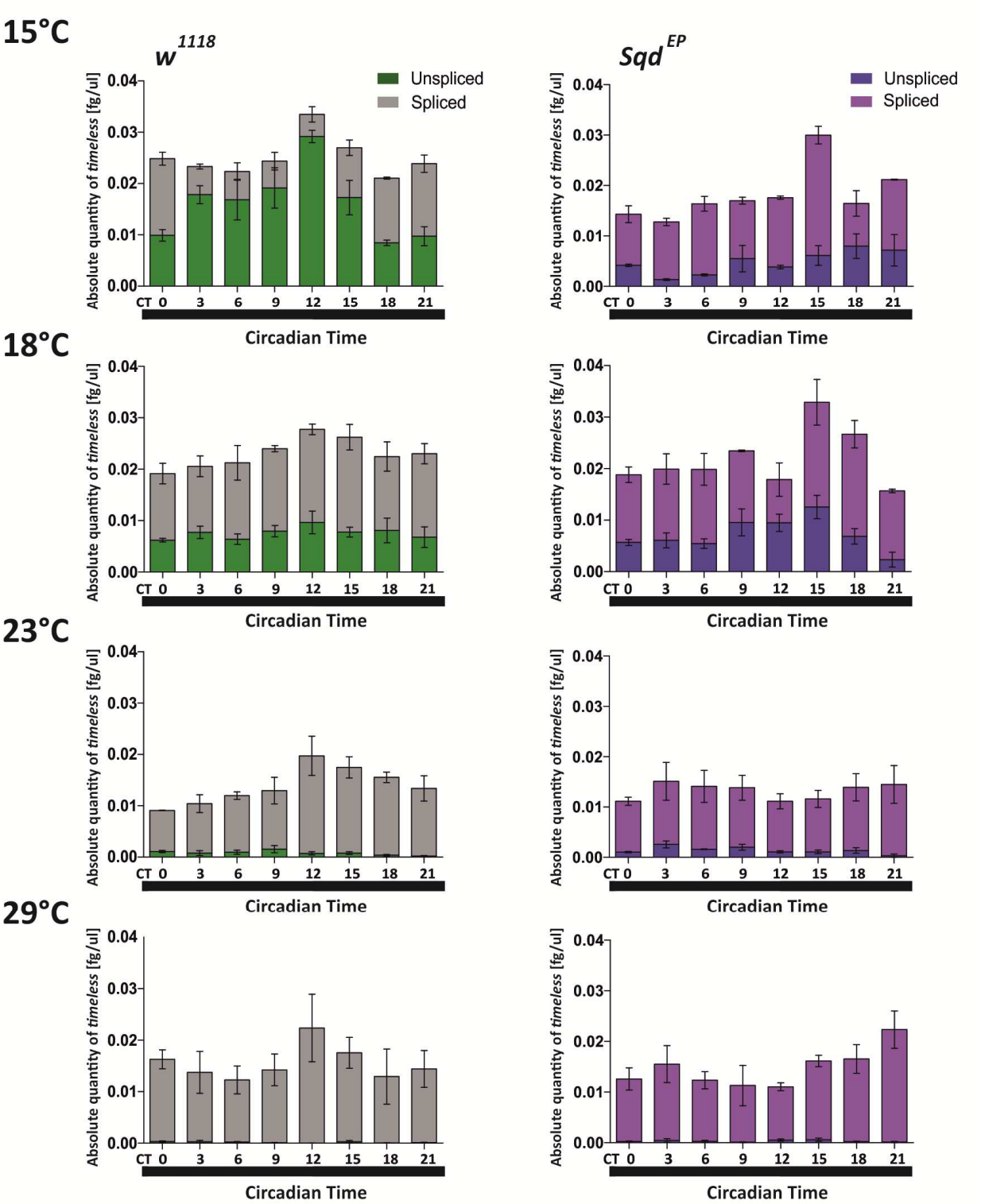


Figure 41. Quantification of *timeless* expression levels in brains from w^{1118} and sqd^{EP} flies at different temperatures (15°C, 18°C, 23°C, and 29°C) in DD . For each time point, abundance of spliced and unspliced variants are reported. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3).

In more details, as for *per*, the decrease of the unspliced isoform in sqd^{EP} somehow positively correlated with temperature. The analysis of the percentage proportion of *per* unspliced (Fig.42 and 44) performed through the 24 hours in LD revealed not statistically significant differences between sqd^{EP} and w^{1118} at 15°C, while at 29°C the differences were significant through the all day;

at intermediate temperatures (18 and 23°C) we observed significance only at specific time points (Fig.42). The same trend was observed in DD, where the differences between *sqd*^{EP} and *w*^{1118} started to be significant at 18°C (Fig.44).

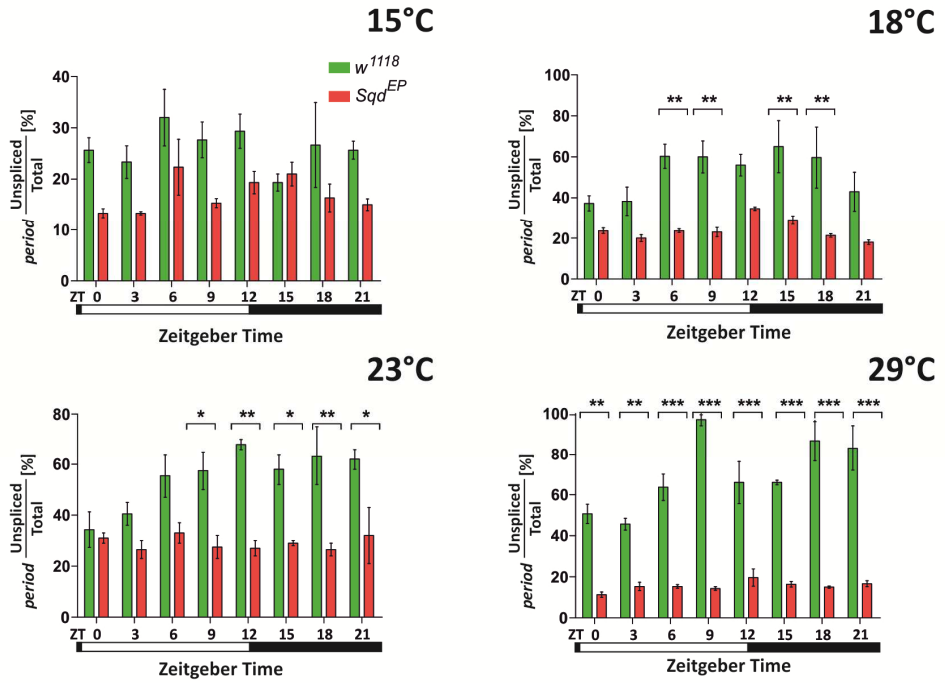


Figure 42. Percentage proportion of *per* unspliced isoform in LD. For each time-point the proportion of per unspliced isoform relative to total mRNA in LD at different temperatures (15°C, 18°C, 23°C, and 29°C) is reported, comparing *w*^{1118} and *sqd*^{EP} brains. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3); ***P < 0.001; **P < 0.01; *P < 0.05 (*sqd*^{EP} *vs w*^{1118}), two-way ANOVA, Bonferroni's post hoc test.

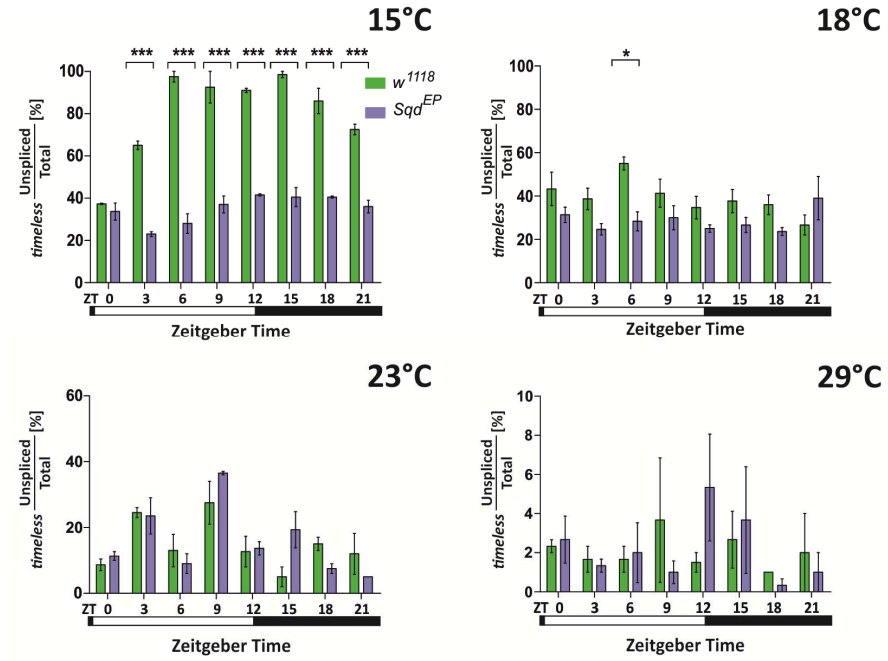


Figure 43. Percentage proportion of *tim* unspliced isoform in LD. For each time-point the proportion of tim unspliced isoform relative to total mRNA in LD at different temperatures (15°C, 18°C, 23°C, and 29°C) is reported, comparing *w*^{1118} and *sqd*^{EP} brains. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3); ***P < 0.001; **P < 0.01; *P < 0.05 (*sqd*^{EP} *vs w*^{1118}), two-way ANOVA, Bonferroni's post hoc test.

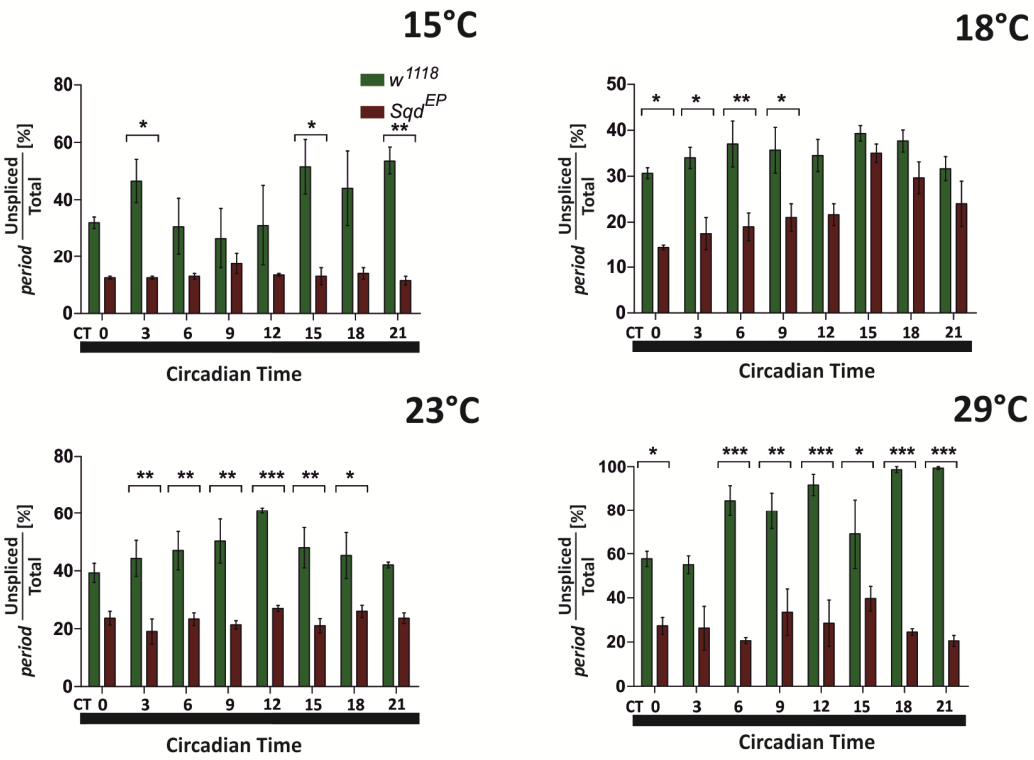


Figure 44. Percentage proportion of *per* unspliced isoform in DD. For each time-point the proportion of per unspliced isoform relative to total mRNA in DD at different temperatures (15°C, 18°C, 23°C, and 29°C) is reported, comparing w^{1118} and sqd^{EP} brains. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3); ***P < 0.001; **P < 0.01; *P < 0.05 (sqd^{EP} *vs* w^{1118}), two-way ANOVA, Bonferroni's post hoc test.

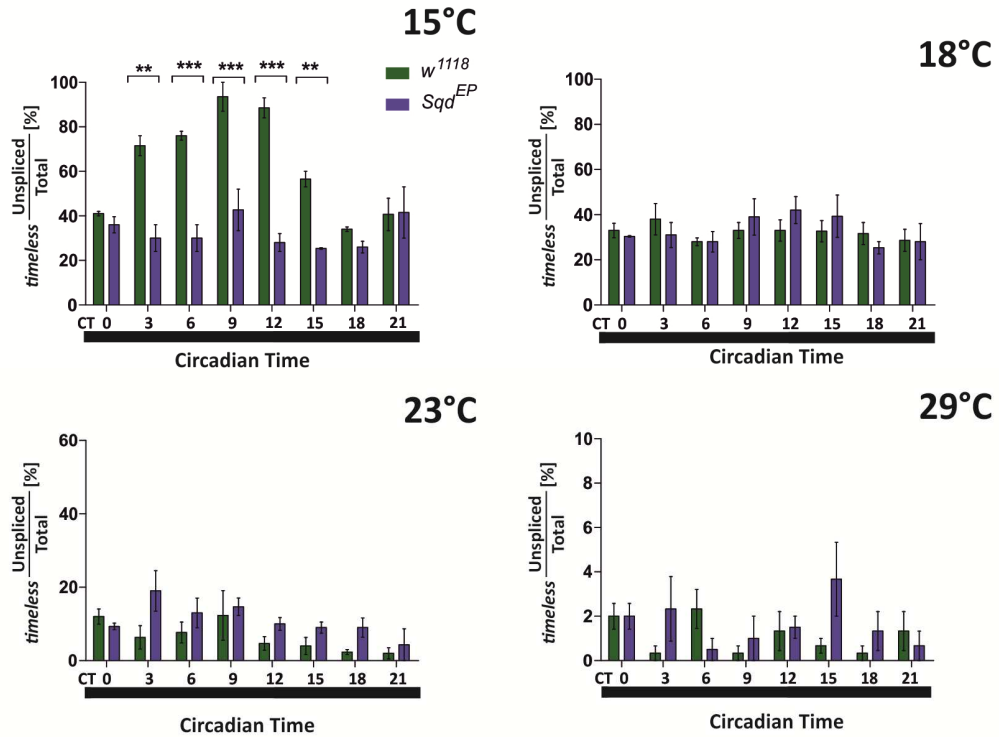


Figure 45. **Percentage proportion of *tim* unspliced isoform in DD**. For each time-point the proportion of per unspliced isoform relative to total mRNA in DD at different temperatures (15°C, 18°C, 23°C, and 29°C) is reported, comparing w^{1118} and sqd^{EP} brains. Under each graph white and black bars indicate the light and dark period respectively. Each values is reported as mean ± SEM (N=3); ***P < 0.001; **P < 0.01; *P < 0.05 (sqd^{EP} *vs* w^{1118}), two-way ANOVA, Bonferroni's post hoc test.

We observed an influence of temperature also on the decrease of the unspliced isoform of *tim* in *sqd*^{EP} brains, but with an opposite trend compared to *per* (Fig.43 and 45). Indeed, the analysis of the percentage proportion of *tim* unspliced performed through the 24 hours in LD revealed that the differences between *sqd*^{EP} and *w*^{1118} were statistically significant only at 15°C, while at higher temperatures the significance was lost (Fig.43). This pattern is perfectly conserved in DD (Fig.45).
In order to better quantify these differences and to unravel the correlation between splicing event and temperature, we calculated the average proportion of the spliced isoform for each mRNA (calculated on the 24 hours) in relation to temperature, either in LD or in DD (Fig.46).

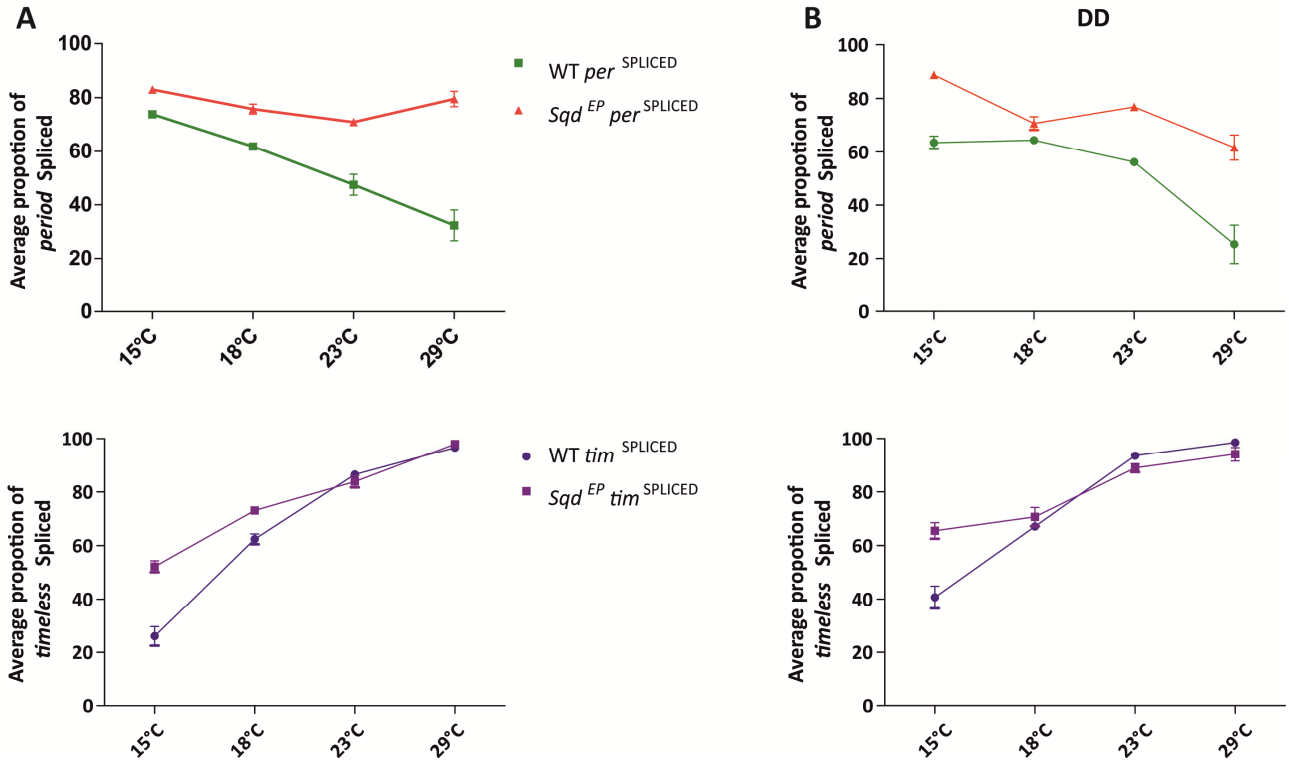


Figure 46. Average proportion of *per*^{SPLICED} and *tim*^{SPLICED} in relation to temperature. (A) Expression of *period* (top) and *timeless* (bottom) SPLICED in LD in wild type and *squid* mutant flies. **(B)** Same representative average proportion in DD condition. Each values is reported with ± SEM.

As for *per*, in LD, at 15°C the proportion of spliced isoform was about 75% in *w*^{1118} and about 85% in *sqd*^{EP} brains, with no significant difference (Fig.46 A, upper panel); with increasing temperature, the amount of *per* spliced significantly decreased in *w*^{1118} brains, reaching an average proportion of 35% (15°C LD vs 29°C LD, $p < 0.0001$) while it remained more or less stable in *sqd*^{EP} and no significant differences were observed comparing the various temperatures (15°C LD vs 29°C LD, $p = 0.7443$) (Fig.46, upper panel). In constant conditions, in *w*^{1118} brains the proportion of *per* spliced remained around 62% at 15, 18 and 23 °C, but significantly decreased with increasing

temperature, from 62% al 23°C to 25% at 29°C (15°C DD vs 29°C DD, $p< 0.0001$) (Fig.46 B, upper panel). In sqd^{EP} brains, the average proportion of *per* spliced significantly decreased from 90% at 15°C to 72% at 18°C (15°C DD vs 18°C DD, $p< 0.0001$), but was not significantly influenced by further increasing of temperature (18°C DD vs 23°C DD, $p< 0.2967$; 18°C DD vs 29°C DD, $p< 0.0511$) (Fig.46 B, upper panel). Taken together, these results are in accordance with what was already observed regarding the thermal splicing of *per* in *wt* flies and revealed that this regulation is significantly impaired in sqd^{EP} brains.

As for *tim*, in LD, although a significant increase of the spliced isoform with increasing temperature was observed for either strain, in w^{1118} brains the proportion of *tim* spliced raised from 22% at 15°C to 98% at 29°C, while in sqd^{EP} brains about 53% of tim was in the spliced isoform already at 15°C (w^{1118} 15°C LD vs 29°C LD, $p< 0.0001$; sqd^{EP} 15°C LD vs 29°C LD, $p< 0.0001$) (Fig. 46 A results, lower panel). A similar trend was observed in constant conditions: in both w^{1118} and sqd^{EP} brains the spliced isoform significantly increased with increasing temperature (w^{1118} 15°C DD vs 29°C DD, $p< 0.0001$; sqd^{EP} 15°C DD vs 29°C DD, $p< 0.0001$) and again the main difference between the two genotypes was the average proportion of *tim* spliced at 15°C, being much higher in the mutant (40% in w^{1118} and 65% in sqd^{EP} brains, $p<0.0001$)(Fig. 46 B, lower panel). As a consequence, while in w^{1118} brains the proportion of *tim* spliced significantly increased from 40% to 60% with temperature raising from 15 to 18°C (15°C DD vs 18°C DD, $p< 0.0001$), in sqd^{EP} brains around 65% of *tim* was spliced at either 15 and 23 °C (15°C DD vs 23°C DD, $p=0.4792$; 15°C DD vs 23°C DD, $p< 0.0001$) (Fig.46 B, lower panel). The complete statistical analysis are reported in appendix 2 and 3.

2.5. PERIOD expression in l-LN$_v$s and s-LN$_v$s neurons.

PER is an essential component of circadian clock machinery in *Drosophila melanogaster* and its oscillation in the circadian clock neurons is fundamental for the generation of circadian rhythmicity (Helfrich-Forster, 1995). In order to understand whether the impaired of rhythmic behaviour could be somehow ascribed to an altered molecular oscillation, we investigated the expression of PER in PDF-expressing cells, in w^{1118} control and sqd^{EP} flies by immunocytochemistry on dissected brains, in collaboration with Dr Milena Damulewicz (Jagiellonian University of Krakow - Poland).

After entrainment at 12:12 LD for at least five days, flies was kept in LD or moved to constant darkness (DD) and collected every 3 hours through 33 hours at 23°C and 18°C. The immunofluorescence signal was quantified in l-LN$_v$s and s-LN$_v$s clock neurons, identified by co-

staining with an ant-PDF antibody, and graphed for both temperatures in LD and DD (Figure 34 and 35 results).

In l-LN$_v$s at 23°C, in LD cycles control flies exhibited a pronounced oscillatory trend that appeared attenuated in mutant: indeed, the peak level at ZT24 was significantly lower in *sqd*^{EP} compared to *w*^{1118} (Fig.47 A left panel). Statistically significant differences were also observed at ZT0, ZT15 and ZT18. Also in constant darkness, the amplitude of PER oscillation was significantly lower in mutant compared to control flies (Fig.47 B left panel).

At 18°C in LD PER in l-LN$_v$s oscillated both in wild type and *sqd*^{EP} mutant and control flies (Fig.47 B left panel), although some significant differences were observed between the two strains at certain some timepoints (Fig.47 B left panel). In DD, the nice oscillation observed in *w*^{1118} was lost in *sqd*^{EP} brains (Fig.47 B right panel).

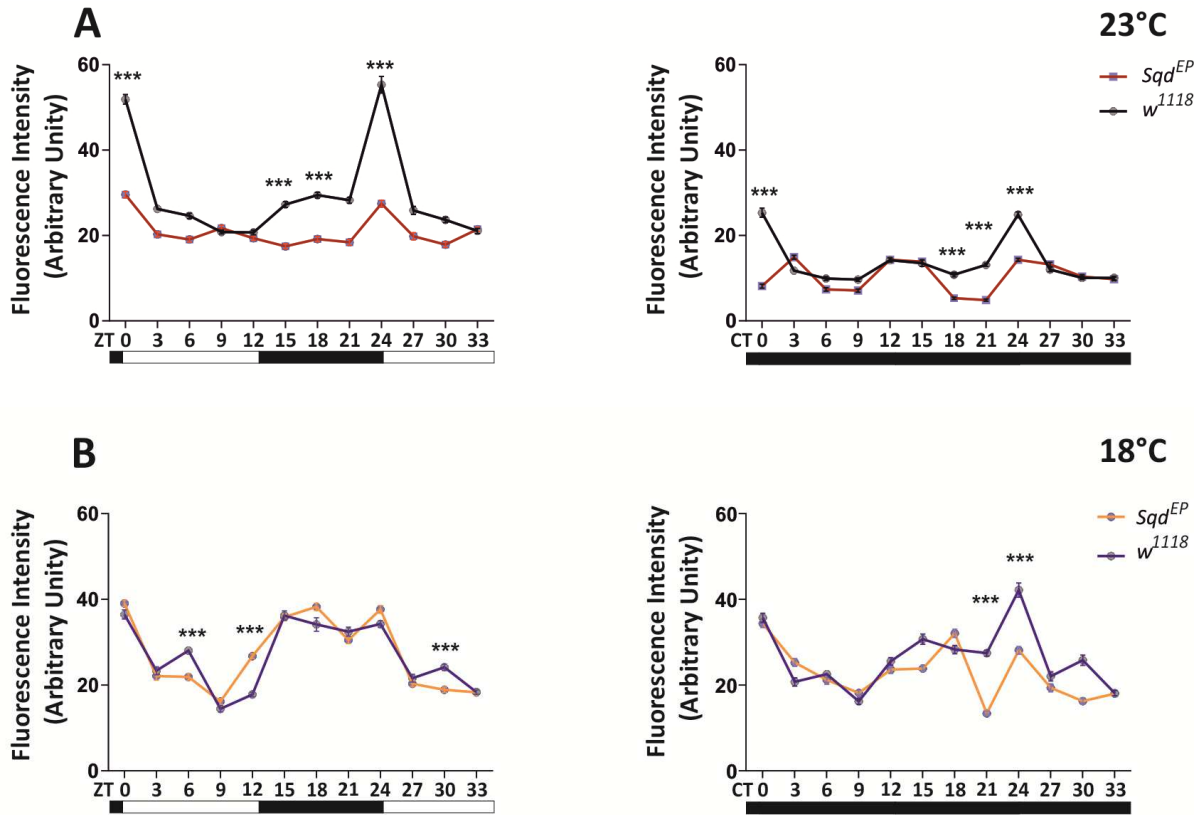


Figure 47. Altered PER expression in large lateral ventral neurons (l-LN$_v$s) of *sqd*^{EP} brains. Flies were first entrained for 5 days in LD and then transferred to constant darkness. They were collected every three hours and stained for PER expression levels during the last day of LD or first day of constant darkness. A) PER quantification in *w*^{1118} (black line) and *sqd*^{EP} (red line) brains at 23°C respectively in LD and DD. (B) PER quantification at 18°C in LD and DD in *w*^{1118} (violet line) and *sqd*^{EP} brains (orange line). Each values is reported with ± SEM; ***P < 0.001 (*sqd*^{EP} mutant *w*^{1118}), one-way ANOVA.

An interesting result was obtained from the analysis of PER expression in s-LN$_v$s (Fig.48). In these clock neurons, at 23°C PER oscillates in either w^{1118} and sqd^{EP} brains, both in LD and in DD, but in the mutant brains the protein reaches the maximum levels at ZT/CT 27, that is 3 hours later than in the control (ZT/CT 24) (Fig.48 A). Moreover, in DD the levels of protein are higher in mutant compared to control, and these differences are statistically significant for several of the time points analyzed (Fig.48 A, right panel). A similar pattern, with higher levels of protein and delayed accumulation, was observed also at 18°C: in LD PER reached the maximum level with 3 hours of delay (ZT27) compared to control, the quantity was significantly higher than control at several time-points (Fig.48 B).

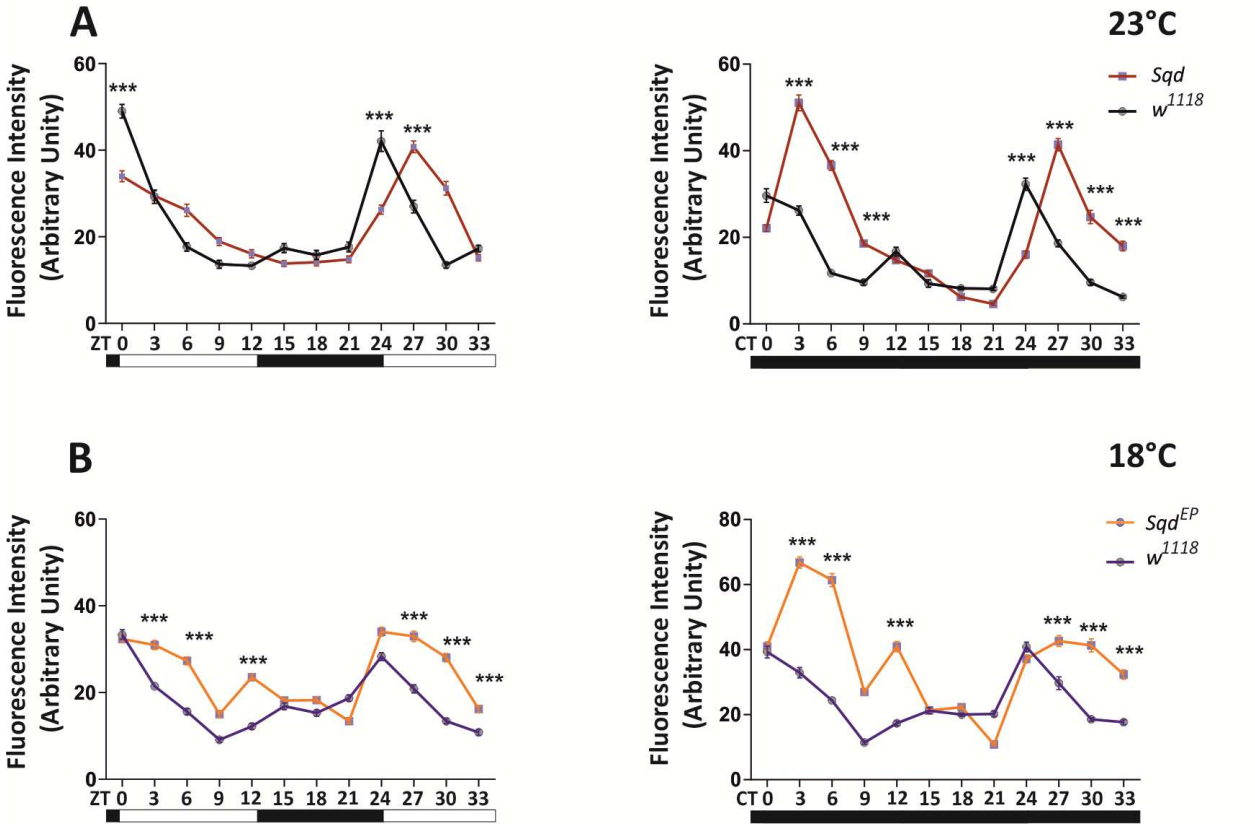


Figure 48. Altered PER expression in small lateral ventral neurons (s-LN$_v$s) of sqd^{EP} brains. Flies were first entrained for 5 days in LD and then transferred to constant darkness. They were collected every three hours and stained for PER expression levels during the last day of LD or first day of constant darkness. A) PER quantification in w^{1118} (black line) and sqd^{EP} (red line) brains at 23°C respectively in LD and DD. (B) PER quantification at 18°C in LD and DD in w^{1118} (violet line) and sqd^{EP} brains (orange line). Each values is reported with ± SEM; ***P < 0.001 (sqd^{EP} mutant w^{1118}), one-way ANOVA.

It is noteworthy that the degradation kinetic of PER seemed slower in sqd^{EP} compared to control, giving rise to a broader peak (Fig.48). This effect was even more evident in DD, where the delayed

accumulation was associated with significantly higher levels of protein at time-points when PER was degraded in control flies (Fig.48 B).

These results, although preliminary, are indicative of an altered pattern of PER expression in important clock neurons in *sqd*^{EP} flies both in LD and DD and in particular at 18°C.

2.6. Analysis of PDF projections

Pigment dispersing factor (PDF) is a neuropeptide functioning as neuromodulator in the circadian clock. In Drosophila it is released by l-LN$_v$ and s-LN$_v$ clock neurons (Renn et al., 1999; Guo et al., 2014).

A preliminary analysis of these PDF-positive cells was performed in order to ascertain whether the behavioural defect could be also due to altered mechanisms downstream PER molecular oscillation. This analysis revealed that PDF projections appear highly disorganized in *sqd*^{EP} mutant compared to control flies (Fig.49), suggesting that the altered pattern of this released neuropeptide could also, at least partially, account for the defects in the locomotor activity observed in the *Squid* mutant.

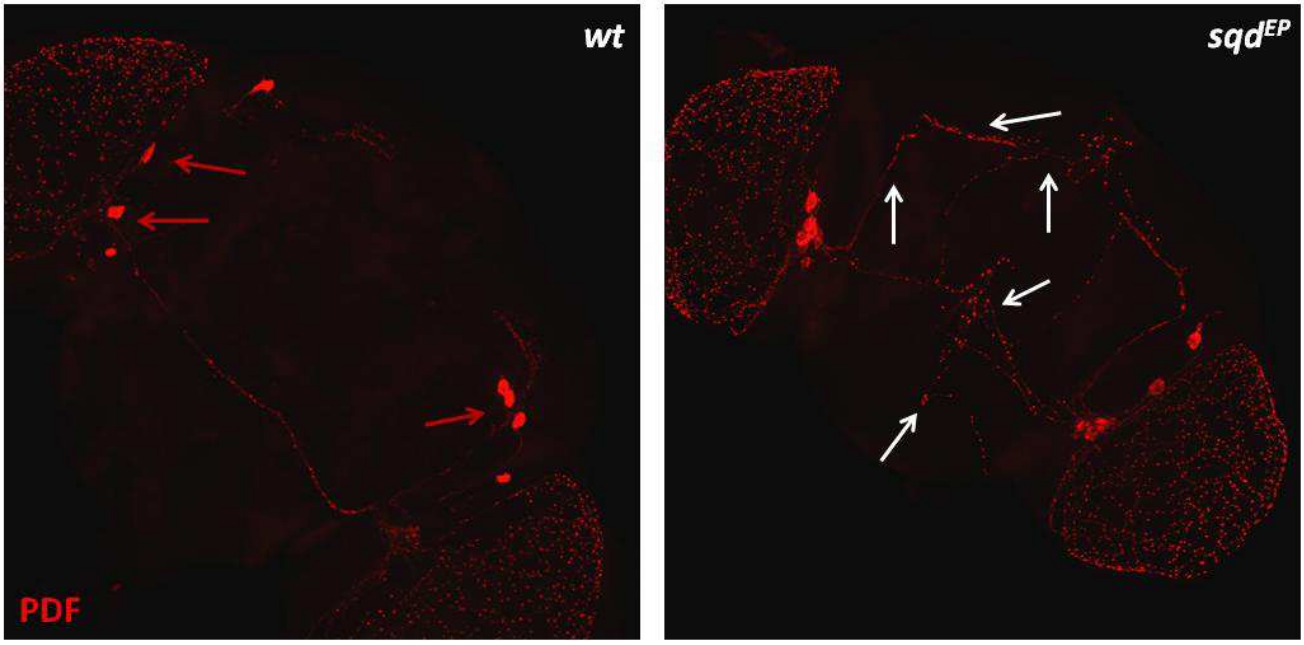


Figure 49. PDF expression pattern in *D. melanogaster* wilt type and *sqd* ^{EP} adult brains. LEFT: In *w*^{1118} control small and large ventrolateral neurons (s-LNv and l-LNv) are indicated by red arrows**. RIGHT:** In *sqd* ^{EP} the projections from LNvs are disorganized. White arrows indicate high levels of disorganization.

Chapter 3

DISCUSSION AND CONCLUSIONS

Biological clocks are self-sustained in the absence of environmental cues. Interlocked transcriptional-translational feedback loops generate rhythmic clock-gene expression and protein accumulation; however, post-transcriptional and post-translational mechanisms have also evolved to finely tune the molecular oscillation that generates the rhythmic phenotypes (Kojima et al., 2011).

In a previous experiment performed in our laboratory, a co-immunoprecipitation (CoIP) assay followed by mass spectrometry, revealed that the circadian photoreceptor CRY interacts with HRB87F and SQUID, two proteins of the hnRNPs family.

In *Drosophila melanogaster* many hnRNPs have been described: at least 14 major proteins with structural and functional homologs in mammals participate in several post-transcriptional regulatory mechanisms (Piccolo et al., 2014). Due to the dynamic nature of hnRNPs and their involvement in many biological processes, we decided to characterize this interaction and to explore a possible involvement of these proteins in the post-transcriptional control mechanisms of the circadian clock machinery.

First of all the preliminary results were validated: by CoIP and western blot it was possible to confirm the interaction between CRY and SQUID, while the binding with HRB87F was validated by the yeast two hybrid system assay.

To characterize the temporal expression of *Hrb87F* and *Squid* and to unravel a possible involvement of the circadian clock in their regulation, a complete analysis of mRNA and proteins in fly heads was performed through a circadian cycle, both in wild-type (w^{1118}) and clock mutant (per^{0}) flies. As for *Hrb87F*, the mRNA showed a weak oscillatory trend in LD and DD, both in wt and per^{0}. On the other end, the levels of the protein oscillate in LD and DD in *wt* flies, but not in per^{0}, indicating a possible role for circadian clock in the translational/post-translational control of the protein. As for *Squid*, neither mRNA nor protein showed rhythmic oscillation in any of the conditions and strains in which the expression was analyzed. This result could be somehow expected, for different reasons. First, for the emerging role of hnRNPs in several essential biological processes (Lo Piccolo et al, 2014): in fact, many proteins with essential cellular functions, as kinases, phosphatases, etc, that have been recruited by the circadian machinery, are often expressed independent of the clock, but is rather their activity that is regulated by clock factors (Partch et al., 2006). Secondly, Squid is present in several isoforms, some of them originated by alternative splicing and recent evidences suggest that different isoforms can contribute to alternative splicing events, for example during sex determination in *Drosophila* (Hartmann et al.,

2011). Given that our qPCR primers and antibody don't enable us to distinguish between isoforms, we cannot exclude that some isoforms can oscillate at mRNA and/or protein level. Third, being our analysis performed in whole head, we cannot completely rule out the possibility that *Squid* (mRNA and/or protein) oscillates in the clock neurons, which represent only a small portion of the fly head.

In the last few years, a role for hnRNPs as global regulators of alternative splicing is emerging (reviewed in Lo Piccolo et al., 2014). As previously described, alternative splicing has been revealed as an important regulatory step in supporting circadian rhythms in gene expression also in *Drosophila*. In fact, *per* and *tim* undergo thermo sensitive alternative splicing: as for *per*, several evidences suggest that it influences the phase of the clock according to temperature and photoperiod (Majercak et al., 1999; Collins et al., 2004; Majercak et al., 2004), while for *tim* it might have functional implications relevant to circadian photoreception and perhaps to seasonal responses (Boothroyd et al., 2007; Montelli et al., 2015). Indeed, at low temperature *tim* mRNA is present in unspliced form with a retained intron that gives a shorter protein, due to the presence of a premature STOP codon (Boothroyd et al, 2007). The temperature-dependent alternative splicing of *period* promotes the spliced variant at low temperature, while at high temperature the mRNA is present in the unspliced form (Majercak et al., 1999; Collins et al., 2004; Low et al., 2012).

We were, then, prompted to undertake an analysis aimed at uncover a possible involvement of hnRNPs in the molecular mechanism that generates the circadian rhythmicity in *Drosophila*, with particularly focusing on the thermal regulated splicing of *per* and *tim*. We performed an analysis of *Squid* and *Hrb87F* mutants at different temperatures, at either molecular or behavioral level, as an altered thermal-splicing regulation has been shown to impinge on the behavioral response in locomotor activity (Collins et al. 2004).

3.1. *Hrb87F*

As for *Hrb87*F, a mutant with a deletion of the entire locus of *Hrb87F* (*def.Hrb87F*), showed a high mortality rate at 29°C, in accordance to the temperature sensitivity and thermal stress previously reported (Singh and Lakhotia, 2012). As for the rhythmic behaviour, it showed a severe loss of the morning anticipation of the locomotor activity in LD cycles. This defect was not significant al lower temperature, where, nevertheless, the percentage of control flies that displayed a morning activity in anticipation of lights-on was quite low (about 56%). This result suggested an impairment of the activity of PDF positive s-LNvs neurons, accounting for this diurnal phenotype (reviewed by Yoshii

et al., 2012). In constant darkness, these flies were largely arrhythmic at each of the tested temperatures, although at 15°C they didn't display significant differences compared to control, in line to previous reports showing a general alteration of rhythmic behaviour at low temperatures (Menegazzi et al., 2013). The high levels of arrhythmicity in constant darkness indicate an impairment in the synchronization between groups of pacemaker neurons in *def.Hrb87F* flies (Peng et al., 2003).

Further investigations are needed to better understand the involvement of *Hrb87F* in the regulation of circadian locomotor activity in *Drosophila*. However, it is known that the eyes of this mutant show slight roughening and that the pseudopupil analysis revealed a loss of photoreceptor neurons (Sengupta and Lakhotia, 2006). This, for example could impair the light transmission from the compound eyes to the clock structures and therefore influence the locomotor response.

3.2. *Squid*

As for *Squid*, we analyzed two different mutants: *w; P{wHy}sqd^{DG09709}* (from now on named *sqd*^{DG}) and *w; P{EP}sqd^{EP3631}* (from now on named *sqd*^{EP}), generated by transposable element insertion, in the first intron and in the 5' UTR respectively (Rorth, 1996; Huet et al., 2002). Similarly to other *Squid* mutant alleles (Kelley, 1993; Norvell et al., 1999), both mutants are not viable when the insertion is in homozygosity, therefore balanced lines were used for the analyses. These mutants show highly reduced expression of the gene (Piccolo et al., 2017).

Sqd^{DG} flies showed lack of morning anticipation in entrainment conditions and displayed very high level of arrhythmicity in constant dark, at every tested temperature.

Sqd^{EP} flies showed a behavior that for certain features was influenced by temperature. In entrainment conditions, flies lacked of morning activity in anticipation of lights-on at each of the tested temperature; as for the evening activity, we observed a shift in the early night at 29°, 23° and 18° whereas at lower temperature (15°C) it was anticipated in the light phase, similarly to *w^{1118}* control and in accordance to previous observations (Majercak et al., 1999). In constant conditions, *Sqd^{EP}* flies showed high percentages of arrhythmicity and those flies that remained rhythmic displayed longer periods of endogenous locomotor activity compared to control. Two features worthy to note were also revealed by this analysis: First of all, the percentage of rhythmic flies decreased with temperature, in a manner more pronounced compared to control. Secondly, those flies that remained rhythmic displayed a peculiar temperature-dependent behavior during

the first three days of DD: with the decreasing of temperature their showed a progressive difficulty in keeping the rhythmicity they had in LD and at 15°C flies were completely inactive.

These defects displayed by Sqd^{EP} flies, that is absence of the morning anticipation and shift of the evening activity in the dark phase in entrainment condition, as well as the difficulty in keeping the rhythmicity at the beginning of the constant conditions, could be indicative of some deficiencies in the light synchronization pathway. Being SQUID a partner of CRY in the fly heads, we decided to make an attempt in investigating whether this interaction could be somehow important for the CRY-mediated light input to the clock. As a first shot, we studied the locomotor behavior of flies in continuous light, which severely compromises the light synchronization pathway in wild-type flies, but not in *cry* mutants (Dolezelova et al., 2007). Sqd^{EP} flies were completely arrhythmic, suggesting that the CRY-mediated light synchronization pathway is not compromised. Nevertheless, we cannot completely rule out the possibility of some defects in the circadian photo-entrainment. In fact, very recently, an additional light-sensing pathway has been described in fly pacemaker neurons, mediated by Rhodopsin 7 (Ni et al., 2017). When *Rh7* mutants are analyzed for their locomotor behavior in LL, most flies are arrhythmic; however, a small percentage of them can keep their rhythmicity and these values very much depend on light intensity (Ni et al., 2017). Further investigations are therefore needed in order to better understand whether SQUID might have a role in the circadian photo-entrainment in *Drosophila*.

We sought to analyze whether the temperature-influenced behavior of sqd^{EP} flies could be explained by altered thermal-splicing regulation of *per* and *tim*, known, at least for *per*, to have some effects on the behavioral response in locomotor activity (Collins et al. 2004).

A multiplex Taqman real-time PCR protocol was optimized in our lab with the aim to analyze the splicing variants of *per* and *tim* in different conditions. It has many advantages compared to the traditional singleplex RT-qPCR: it has a significantly less reagent cost, is less-time consuming, and allows improved data quality and quantity in a single reaction. TaqMan probes specific for both total transcript and unspliced isoform were designed, while the expression of the spliced isoform was inferred by subtraction. An initial effort was made to set-up the technique, starting with testing the optimal concentration of RNA template, primers and probes, and checking the quality of amplification curves, fluorophores brightness, etc. The most important aspect was to have the possibility to perform both reverse transcription and real time PCR in a single step, using a low quantity of mRNA for each reaction, as well as to reduce the experimental errors. This method can

be applied to investigate the expression profile of several genes by using appropriate primers and probes.

First of all we considered the absolute quantities of *per* and *tim* mRNA through the 24 hours, measured at 29°, 23°C, 18°C and 15°C in LD and DD. As for *per*, we observed in sqd^{EP} flies a rhythmic expression in LD, with maximum levels at ZT15, that is 3 hours delayed compared to control, at each temperature. This delay in mRNA accumulation is in accordance with the delay of evening locomotor activity that in these flies is shifted in the dark phase. In the first day of constant conditions, at lower temperature the levels of *per* mRNA are flatter in sqd^{EP} than in control flies, and this somehow accounts for the low levels of rhythmicity exhibited by the mutant and for the difficulty of maintaining the rhythmicity in the first days of constant conditions. In any case, higher *per* mRNA levels could be observed at CT15, in accordance to the long period of locomotor activity displayed by the small percentage of rhythmic flies. At 29°C, *per* mRNA showed a significant oscillatory pattern, with a peak a CT15; this result fits with the observation that the percentage of rhythmic flies display a rhythmic behavior form the first day of DD. As for tim, the oscillation of mRNA in LD was maintained in sqd^{EP} at each temperature, with a 3 hours delay compared to control, although at 15°C the amplitude of this oscillation was much higher in comparison to control, due to high levels of mRNA during the night phase. The oscillation of *tim* was more or less maintained in the first day of constant conditions, and again the peak of expression was delayed in sqd^{EP} compared to control. In this case the delay seemed to be influenced by temperature: at 15 and 18°C the peak of expression was at CT15 (3 hours delay), while at 29°C the maximum level of expression was reached at CT21 (9 hours delay).

When we looked at the splicing isoforms, a general decrease of the unspliced isoform was observed for both *per* and *tim* in sqd^{EP} compared to w^{1118} brains, regardless the conditions, likely as result of an increased splicing activity. Looking more carefully and analyzing the average proportion of spliced isoforms as function of temperature, we observed in sqd^{EP} flies opposite effects on *per* and *tim*. In fact, as for *per* an increase of unspliced isoform in sqd^{EP} somehow positively correlated with temperature: at 15°C no significant difference was observed between the two strains, but with increasing temperature in sqd^{EP} brains the proportion of *per* spliced remained high, while in w^{1118} brains the proportion of *per* spliced gradually decreased, as previous reports (Majercak et al., 1999, 2004).

As for *tim*, we observed that the average proportion of the spliced isoform significantly increased with increasing temperature, in both strains; however, being the average proportion of *tim* spliced

at 15°C significantly higher in *sqd*^{EP} brains, the influence of temperature is less dramatic in these flies compared to *w*^{1118}.

Taken together, these observations suggest that the thermal regulation of *per* and *tim* splicing is severely impaired in *sqd*^{EP} flies.

Our molecular results explain the behavioral data only partially. In fact, the delay of *per* and *tim* accumulation can account for the shift towards the dark phase of the evening locomotor activity of *sqd*^{EP} flies in LD and the long period of the small percentage of rhythmic flies. However, looking at the splicing events, our results are in disagreement to previous observations. In particular, the splicing of *per* was reported to result in an earlier accumulation of the correspondent mRNA and to lead to an anticipated evening activity at cold temperature (Majercak et al., 1999; 2004; Collins et al., 2004). Here we observe a delay of the mRNA accumulation and a shift of the evening activity despite an increased average proportion of *per* spliced isoform. Further investigation will be needed in order to clarify this discrepancy and to better elucidate the molecular mechanisms that regulate the post-transcriptional fate of these RNAs. It is known, in fact, that hnRNPs as Squid and Hrb87F associate with a long non-coding RNA (lncRNA), transcript of the *hrs*□ gene, to form omega speckles (□-speckles), specialized nuclear compartments acting in storage and sequestration of proteins involved in RNA processing and maturation (reviewed in Piccolo et al., 2014). An alteration of these structures in *sqd*^{EP} mutant could be the cause of an altered post-transcriptional regulation of *per* and/or *tim* RNA. This hypothesis is supported by observation that formation of □-speckles with a correct function of Squid and Hrb87F influences the relative abundance of a large part or their RNA targets in the brain (reviewed in Piccolo et al., 2014). It will be therefore interesting to verify whether *per* and *tim* RNAs are, indeed, target of Squid, and whether other factors (proteins and/or ncRNAs) are involved in their post-transcriptional regulation.

The analysis of PER expression in PDF positive clock neurons at two temperatures revealed an altered oscillation of the protein in *sqd*^{EP} flies compared to control: in l-LN$_v$s we observed a general attenuation of the amplitude of the oscillation in LD that is further reduced when flies are exposed to constant conditions. In s-LN$_v$s neurons we observed higher level of protein and a delayed accumulation, associated to a low degradation kinetic, and again this effect is more evident in constant darkness. This altered oscillation phase of the clock neurons and their consequent de-synchronization could explain the locomotor defects of *sqd*^{EP} flies.

The preliminary observation that PDF projections appear highly disorganized in *sqd*^{EP} mutant compared to control, indeed suggests that Squid is somehow involved in controlling the pattern of

this released neuropeptide, whose alteration can, at least partially, account for the defects in the locomotor activity observed in the *sqd*^{EP} flies. This observation is in accordance to the results of a recent study, in which a pivotal role in the fly's pacemaker neurons has been reported for the alternative splicing regulator SRm160 (SR-related matrix protein of 160 kDa): it is important for proper *per* expression, rhythmic accumulation of PDF and ultimately circadian rhythms in locomotor behaviour (Beckwith et al., 2017).

Take together the results of this work fully support the hypothesis that the hnRNPs Hrb87F and Squid are involved in the generation and maintaining of circadian rhythmicity in *Drosophila melanogaster.*

Chapter 4

MATERIALS AND METHODS

4.1. Fly Strains and rearing conditions

The following *Drosophila* strains were used in this work:

- ***w*^{1118}** : It is characterized by: white (w, 1-1.5), recessive allele caused by a large deletion in the *white* locus, which determines the "white eyes" phenotype. The deleted gene is involved in production and distribution of brown (ommochrome) and red (phteridin) pigments present within adult fly eyes (Zachar and Bingham, 1982);
- ***ry*^{506}** : characterised by an amorphic allele with a loss of function due to a gamma ray mutation of *rosy* gene; there is a deletion of approximately one-third of the coding region inside the first intron. The flies have red eyes (Reaume et al., 1989);
- ***per*^{01}** : line characterized by loss of function of *period* gene. A single nucleotide substitution inside the fourth exon creates a premature stop codon (Yu et al., 1987);
- ***cry*^{01}** : loss of function of the *cryptochrome* gene due to substitution of entire gene with mini-*white*$^{+}$ gene by homologous recombination (Dolezelova et al., 2007);
- ***cry*^{m}** : characterized by a premature STOP codon due to a non-sense mutation inside the *cry* gene that gives a CRY truncated protein lacking the last 19 amino acids (Busza et al., 2004);
- ***yw; timGal4*** : transgenic flies that express the GAL4 protein under the control of the *timeless* gene promoter; the specific expression is localized in all the clock neurons (Emery et al., 1998);
- ***w; UAS-HAcry 16.1*** : transgenic line which express the fusion protein HACRY after activation by the UAS-GAL4 system. The yeast upstream activation sequence (UAS) is the binding site for the GAL4 protein that allows the transcription of the construct. The UAS sequence controls the expression of dCRY full-lenght cDNA fused at the N-terminal with the HA (HemoAgglutinin) coding sequence (Dissel et al., 2004).
- ***w; P{wHy}sqd*^{DG09709}** : mutation of *Squid* gene due to P-element construct P[wHy] insertion in the first intron; the construct carries two visible markers, the mini-*white* marker and *yellow* marker, which flank a transposition-competent *hobo* element. This transgene is balanced with MKRS balancer (Huet et al., 2002);
- ***w; P{EP}sqd*^{EP3631}** : mutation of *Squid* gene due to P-element construct P[EP] insertion inside the 5' UTR; the construct carries a mini-*white* visible marker, *Scer|UAS* binding sites

for the *Scer/Gal4* transcriptional regulator, and bacterial sequences that allow plasmid rescue. This transgene is balanced with TM6b balancer (Rorth, 1996);

- ***ry*^{506}**; ***Df(3R)*Hrb87F** : chromosomal deletion of entire locus (Zu et al., 1996).

Flies were reared in in plastic vials containing standard cornmeal food (72.0 g/l corn flour, 79.3 g/l sugar, 8.5 g/l agar, 50.0 g/l dried yeast powder, 0.27% Methyl parahydroxybenzoate in ethanol, 0.3% propionic alcohol). Unless otherwise stated, all the fly stocks were maintained at 23°C, 70% relative humidity, in 12-hour light/12-hour dark cycles (LD 12:12).

4.2. HACRY over expression

The overexpression of HACRY in *timeless*-positive cells was achieved by the UAS-Gal4 system (Brand & Perrimon 1993). It is a powerful binary system for precise manipulation of gene expression in D. melanogaster. It consists of two independent component initially separated into two distinct transgenic lines. The first component is the yeast transcriptional activator GAL4, which is expressed under the control of a cell- or tissue-specific promoter, while the second element is the upstream activating sequence (UAS), an enhancer to which GAL4 specifically binds to activate gene transcription. The target gene is transcriptionally silent, unless flies carrying it are crossed to those of an activator line containing the GAL4. In the progeny of this cross the transgene will be expressed in the same spatial and temporal patterns as the specific driver (Brand & Perrimon 1993). For HACRY overexpression, virgin females from *timGal4* strain were crossed with males from *UAS-HAcry 16.1*.

4.3. Protein extraction and Co-Immunoprecipitation (CoIP)

Three- to five-day-old flies overexpressing HA-dCRY (yw; tim-GAL4/+; UAS-HAcry/+) were collected at ZT24 and after a 30-min light pulse given at the same time point and heads separated from the body using liquid nitrogen.

The proteins were extracted from 250 heads using the following **lysis buffer**:

Reagents	Concentration
Hepes pH 7.5 (Sigma-Aldrich)	20 mM
KCl (Sigma-Aldrich)	100 mM

EDTA pH 8.0 (Sigma-Aldrich)	2,5 mM
Glycerol (Sigma-Aldrich)	5 % (vol/vol)
Triton-X 100 (Sigma-Aldrich)	0,5 % (vol/vol)
DTT (Sigma-Aldrich)	1 mM
Complete protease inhibitors (Roche)	20 µL/mL

Heads were first homogenized in 200 µL of lysis buffer and then the volume was increased to 800 µL. Samples were incubated on ice for 40 min and mixed by vortex every 10 min. They were then centrifuged at 4°C for 20 min at 13.000 g and the supernatant was collected in new tubes. The amount of extracted proteins was quantified with Bradford protein assay (BIO-RAD).

30 µL of Anti-HA affinity matrix (Roche) was added in the total extract and the solution was incubated at 4°C on a rocker for 3 hours. After the incubation, the samples were centrifuged at full speed for 30 min at 4°C. The supernatant was removed and the matrix was washed for 3 times using the lysis buffer, without disturbing the matrix with the tip. At the end of the last wash, the matrix was resuspended in 2 volumes of **NuPAGE electrophoresis sample buffer** (Invitrogen). The samples were denaturated for 10 min at 70°C, centrifuged at 14.000 g for 2 min and the supernatant, containing the denatured proteins, was subjected to SDS-PAGE (NuPAGE).

4.4. SDS_PAGE and Western-blot

SDS-PAGE was performed using the NuPAGE system (Invitrogen) using MOPS as running buffer.

The following gels were used:

- 4–12 % (wt/vol) NuPAGE Novex Bis-Tris Gels (Invitrogen) for the analysis of the proteins expression;
- 12 % (wt/vol) NuPAGE Bis-Tris (Invitrogen) for the Co-Immunoprecipitation analysis.

For each samples a volume corresponding to 20 µg of proteins was taken and mixed with NuPAGE LDS sample buffer 4X (Invitrogen) and DTT 200mM (Sigma-Aldrich), followed by an incubation at 70 °C for 10 min to promote the denaturation. Gels were blotted on Nitrocellulose using a blotting apparatus (Invitrogen) (1h at 30V) and equal transfer of proteins was controlled by Ponceau staining. Nitrocellulose membrane was then incubated for 1h in 5% milk blocking solution and subsequently incubated in primary antibody overnight at 4°C.

The following primary antibodies were used:

Antibody	Dilution	Producer
Mouse anti-HRB87F	1:1000	(Prof. Onorati, University of Palermo)
Mouse anti-SQUID	1:1000	(DSHB)
Mouse anti-HA	1:10000	(Sigma-Aldrich)
Mouse anti-HSP70	1.1000	(Sigma-Aldrich)

The membrane was washed in TBST solution (0.01M Tris-HCl pH7.5, 0.14M NaCl, 0.05% Tween-20, 0.05% Triton) (3 x 10min) and incubated with secondary antibody, HRP conjugated goat anti-mouse (1:10000, Sigma-Aldrich), for 2 hours at room temperature. Membrane was washed in TBST solution (3 x 10min) and chemiluminescence reaction was performed with fresh made solution (2.25mM Luminol, 0.2mM cumaric acid, 0.1M Tris-Hcl pH8.5, 0.01% H_2O_2). Protein bands were visualized using Amersham Hyperfilm ECL (GE Healthcare). For quantification of the immunodetected signals, each film was analysed with Image J software (available at http://rsb.info.nih.gov/ij).

4.5. Yeast Two-Hybrid Assays

The experiments were performed in the EGY48 yeast strain (MATα, ura3, trp1, his3, 3LexA-operator-LEU). Baits were prepared by cloning the sequence of interest fused to the LexA moiety in the bait vector (pEG202) while full-length Hrb87F was fused to the "acid-blob" portion of the prey vector (pJG4-5) (Golemis and Brent, 1997).

The full length Hrb87F coding sequence was amplified from cDNA extracted from heads of w1118 flies, by using the primers:

- pJG-Hrb87F_F 5'tgtgccagattatgcctctccccgaattcATGGGCGGAACAAAACGATTCC3'
- pJG-Hrb87F_R 5'cgaagaagtccaaagcttctcgagCTAGTAACGTCTATTGCCGCCTC5'

The cloning were performed by using the In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, USA). The constructs were fully sequenced to assess the in-frame insertion of the cDNA and to control for unwanted mutations. Quantification of β-galactosidase activity was performed in liquid culture as in Ausbel FM (1998) and the experiment was repeated three times.

4.6. Locomotor activity analysis

Locomotor rhythms of individual male flies were recorded with Drosophila activity monitors DAMSystem© (*Drosophila* Activity Monitor System ©) (Trikinetics Inc, Waltham, MA, USA). Flies were kept for at least 5 days in 12 h_12 h LD cycles before being transferred to constant-dark conditions (DD) in which they remained for at least 7 days. The experiments were performed at constant temperatures of 15°C, 18°C, 23°C and 29°C.

The light (on/off and lux) and the temperature (°C) during the locomotor activity experiments were strictly monitored through environmental monitor (DEnM, Trikinetics Inc). No relevant temperature oscillations were observed in any of the experiments, however, a variation of ± 0.5 °C was fixed as good.

The locomotor activity data recorded every 5 min over the 24 hours by TriKinetics software (DAMsystem 2.1.3) and converted in:

- excel files through a "parser" program for the LD locomotor events analysis;
- system files through a Python script, CLEAN (Rosato and Kyriacou, 2006), for statistical autocorrelation analysis to investigate the rhythmicity and the period of the locomotor activity in the free-running condition (DD);
- system files through a Python script for the LD and DD to obtain an representative actograms of the locomotor activity events to understand the rhythmicity during the entire experiment.

The bins registered every 5 min were summed to obtain 30 min bins and reported in graph to analyze the LD profile and the presence of morning and evening onset anticipation. Moreover, a Morning Index was calculated for each channel using the following mathematical approach (Seluzicki et al., 2014):

$$M.Index = \frac{\left(\sum activity.3h.before.light.on\right)}{\left(\sum activity.6h.before.light.on\right)} - 0.5$$

When the locomotor activity 3 hours before the light-on is double than the 6 hours, the M.Index value is 0.16, indicating a morning onset anticipation, while when the activity 3 and 6 hours before ligh-on is equivalent, the value is 0, indicating the absence of the morning anticipation (Fig.50).

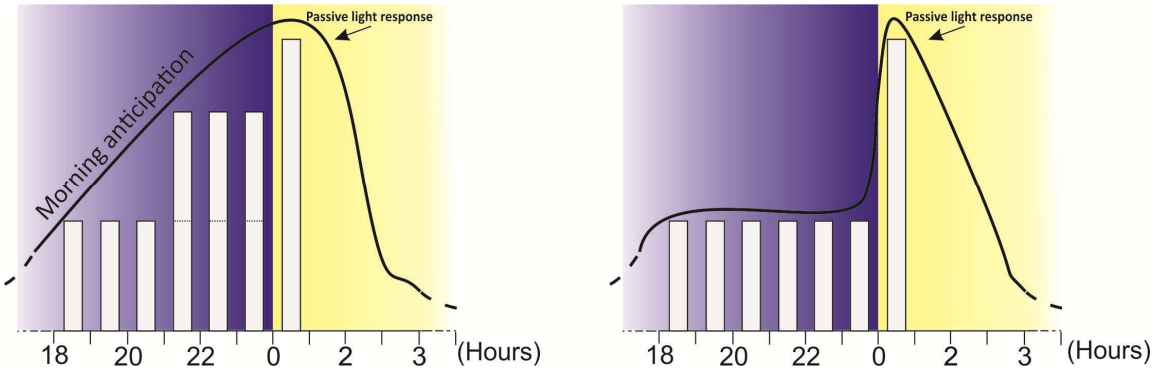


Figure 50. Schematic representation of morning anticipation. White bars indicate the locomotor activity of flies. **(A)** Presence of morning anticipation: the locomotor activity in the 3 hours before light on is higher than the locomotor response in the six hours before. **(B)** Absence of morning anticipation: locomotor activity 6 hours before light-on is constant without an increment (Seluzicki et al., 2014).

The 0.5 was subtracted so flat activity over the six hours analyzed is equal to 0. In cases where no activity counts occurred in the 6 hours before light-on, resulting in an undefined 0/0, the ratio was set to 0.5, indicating no change in activity over that time period.

The circadian rhythmicity during the constant darkness was valuated with the actograms and the autocorrelation analysis. Autocorrelation is based on the relationship among the values of the signal and the same in a range of time, usually later, and gives information about the periodicity of the signal (Fig.51).

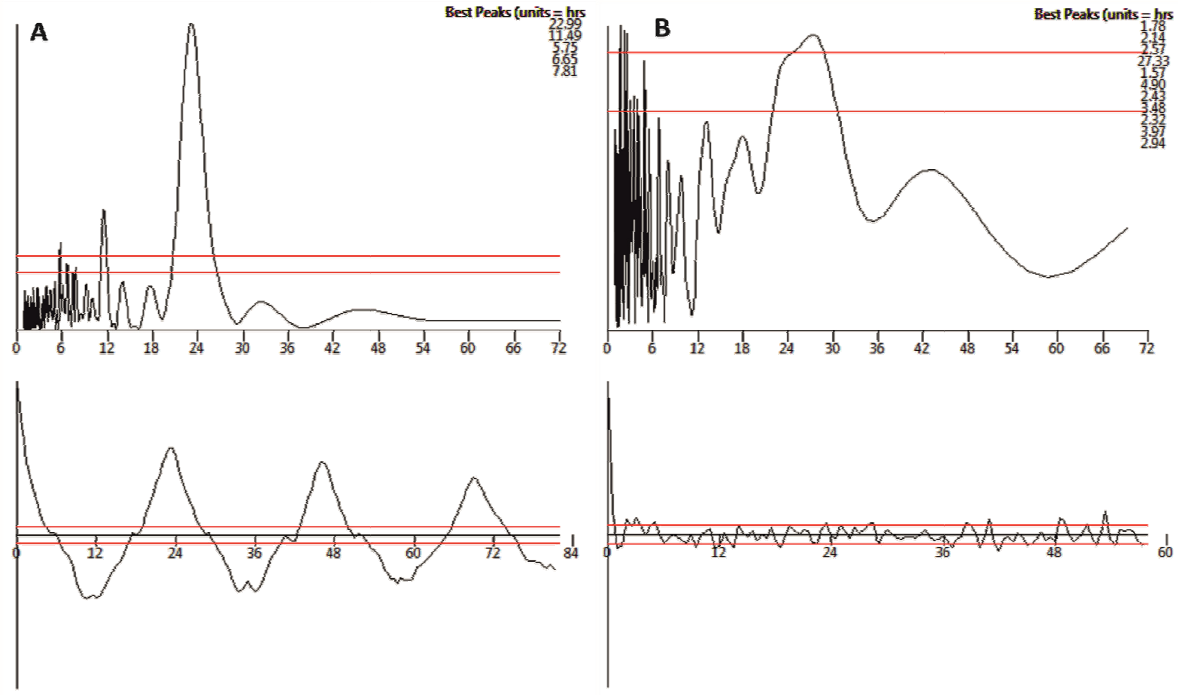


Figure 51. Locomotor activity analysis of period and rhythmicity; (A) CLEAN analysis and autocorrelation spectrum of a rhythmic fly. **(B)** CLEAN analysis and autocorrelation spectrum of an arrhythmic fly. The red lines indicate the significance of the test; when the curves go over the significance, the analysis is genuine.

4.7. RNA extraction

4.7.1. Heads

The flies were collected at specific time points and the heads were separated from the body using liquid nitrogen. First of all the heads were homogenized in 200 µL of TRIzol (Invitrogen); then, 800 µL of TRIzol was added to a final volume of 1 mL. 200 µL of chloroform was added to the solution and the samples were mixed 15 times. Samples were incubated at room temperature for 3 min and were centrifuged at 16.000 g for 15 min at 4°C. The upper aqueous phase was transferred to a new tube. 400 µL of isopropanol was added to promote the RNA precipitation, the samples were mixed 20 times and incubated on ice for 20 min. After the incubation, samples were centrifuge for 20 min at 12.000 at 4°C and the isopropanol was removed carefully without touching the pellet. 500 µL of ethanol at 75% was added to wash the pellet and, after a centrifugation at 12.000 g at 4°C for 10 min, the supernatant was removed. The pellet was air dried and resuspended with 15-20 µL of RNase free water.

4.7.2. Brains

30 brains were dissected (see below) for every timepoints and collected in 700 µL of TRIzol. The tissues were disrupted and homogenized by vortexing the samples for 5 min; the homogenate was then incubates at room temperature (15°-25°C) for 5 min. 140 µL of chloroform were added and the samples were shacked vigorously for 15 sec. After a short incubation, 2-3 min at room temperature, the samples were centrifuged for 15 min at 16.000 g at 4°C. The upper aqueous phase was processed using the miRNeasy Mini kit according to manufacturer instructions. Total RNA was eluted with 20 µL of RNase-free water. RNA was quantified using NanoDrop 1000 spectrophotometer (ThermoScientific). 200 ng of each sample were analysed using RNA 6000 Nano LabChip on a 2100 Bioanalyzer (Agilent) to estimate RNA integrity.

4.8. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was retrotranscribed using the SuperScript™ II Reverse Transcriptase (Invitrogen), following manufacture's instructions.

For each sample, 1 µg of RNA was mixed with 150 ng of random primers and nuclease free water to a final volume of 10 µL. The mixture was incubated at 65°C for 5 min to eliminate any RNA secondary structures. First strand reaction mixture was completed with1x First-Strand Buffer, 10

mM DTT, 1 mM dNTPs mix (Promega), and nuclease free water to a final volume of 19 µL. After a 2 min incubation at 25°C, 200 units of SuperScript™ II Reverse Transcriptase (1 µL) were added to the mix and the RT-PCR was performed with the following program: 25°C for 10 min, 42°C for 50 min and 70°C for 15 min to inactivate the reaction.

4.9. Real-Time polymerase chain reaction (qRT-PCR)

The mRNA amount of the *squid* and *Hrb87f* genes was evaluated by quantitative real-time PCR using the SYBR green chemistry.

Each real-time PCR was performed in quadruplicate with the GoTaq qPCR Master Mix (Promega) in a 10 µl reaction volume. A couple of specific primers for each gene of interest (Table 7) were designed using the on-line tool Primer3 (http://primer3.ut.ee/). RP49 was selected as reference genes to normalize *squid* and *Hrb87f* expression levels. Primers' specificity was assessed by dissociation curves. In order to test the efficiency of each couple of primers, a standard curve was performed starting from 4 serial dilutions of a retro-transcribed pool. Efficiency was calculated as $10^{(-1/x)}$ where x is the slope of the linear regression of the resulting dilution on Ct graph (dilution in logarithmic scale). Primers characterized by less than 90% efficiency were discarded.

Table 7. Primers for *hnRNPs* relative quantification by real time PCR.

Primer (5' to 3')	Amplicon length	Gene
Forward CAG TTC GGA CAA CCA ATC G **Reverse** CGC GGA GTT CCT TCT CAG T	102 bp	*Squid*
Forward AAC GGA AAC TAC GAC GAT GG **Reverse** TCC GCC GAT GAA CAG TTT	79 bp	*Hrb87F*
Forward ATC GGT TAC GGA TCG AAC AA **Reverse** GAC AAT CTC CTT GCG CTT CT	165 bp	*RP49*

The reaction was performed using a 7500 Real Time PCR System (Applied Biosystems) with the following conditions: 10 minutes of denaturation at 95°C, followed by 40 cycles of 15 sec denaturation step at 95°C and 1 minute of annealing/elongation at 60°C.

Differences in gene expression were evaluated by relative quantification (RQ). The $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001) implemented in the 7500 Real Time PCR System software was used.

4.10. Multiplex real time PCR for the expression analysis of clock genes isoforms

Multiplex real time PCR allows quantification of expression of multiple target genes within a single PCR reaction. This method, based on TaqMan technology, enables the simultaneous amplifications of different target by using specific fluorescent reporters whit different emission spectra. Five couples of primers were designed (Primer3 online software, http://primer3.ut.ee/) in order to analyse the expression of the 2 *period* mRNA splicing isoforms (per^{Total}), *period* unspliced ($per^{Unspliced}$) (with primers designed between the retained intron and the upstream exon), the 2 *timeless* splicing isoforms (tim^{Total}), *timeless* unspliced ($tim^{Unspliced}$) (with primers designed inside the retained intron), and the housekeeping gene *RP49* (Tab.8).

Table 8. Primers for *period* and *timeless* isoforms quantification by using multiplex real time PCR.

Primer (5' to 3')	Amplicon Length	Isoforms
Forward CAT GAA GGA GAC CTA CGA GAC G **Reverse** GTT CTG GAT GAG GAA GCG GT	91 bp	per^{Total}
Forward TCC TGG AAA CGA GTG AGC A **Reverse** AGG GAA TGG AAG GGG GAG T	93 bp	$per^{Unspliced}$
Forward TTC TCC GTG GAC GTG ATG TA **Reverse** CTC GGT AAA TGC TTC CTT GC	96 bp	tim^{Total}
Forward ACC AAG GAG GGG AAA AGC T **Reverse** CGC AAA TGG CTG AAA TTG A	95 bp	$tim^{Unspliced}$

TaqMan probes were designed inside the PCR amplicons. Five different 5'-terminal fluorophores have been chosen avoiding the possibility of overlap among their emission spectra (6FAM, HEX, Texas Red, Cy5 and Quasar705). Suitable quenchers have been added to the 3'-end (Tab.9).

Table 9. TaqMan probes designed inside the amplification region of respective primers. Fluorophores: 6-Carboxyfluorescein (6FAM), Texas Red (TxRd), 6-carboxy-hexachlorofluorescein (HEX), Cyanine 5 (Cy5), and Quasar705 (Q705). Quenchers: Black Hole Quencher 1, 2, and 3 (Sigma-Aldrich).

Probes (5' to 3')	Isoforms
[6FAM] AAG AAG GGT CAG ACG GCG GG **[BHQ1]**	*per*^{Total}
[TxRd] CCA GCC AGT CCC ACC AGT TC **[BHQ2]**	*per*^{Unspliced}
[HEX] GGG TCG CCA CAC CAT CTT CG **[BHQ1]**	*tim*^{Total}
[CY5] GGG CAT TGC TGA ACA GTG TCT CA **[BHQ3]**	*tim*^{Unspliced}
[Q705] CGT GCG CGA GCT GGA GGT CC **[BHQ3]**	*RP49*

Reverse transcription and qPCR were performed within the same reaction using the One-step TaqMan RT-qPCR kit (BIO-RAD). Final concentrations of probes and primers were optimized for a 20 µL reaction: 250 nM TaqMan probes, 200 nM *per*^{Total}, 900 nM *per*^{Unspliced}, 200 nM *tim*^{Total}, 900 nM *tim*^{Unspliced}, and 150 nM *RP49*. 10 ng of total RNA were used as template.

Each amplification reaction was performed in quadruplicate with a CFX96 Touch™ qPCR System (BIO-RAD). The amplification conditions were the following: 10 min at 50°C to allow the reverse transcription reaction, 3 min at 95°C to activate the polymerase and to promote DNA denaturation, and 40 cycles of a 10 sec denaturation step at 95°C and a 30 sec amplification step at 60°C.

4.10.1. Standard curves for absolute quantification

In order to quantitatively compare results obtained from different fluorescent reporters, absolute target quantification had to be performed. Reporter-specific standard curves were generated starting from purified and quantified PCR amplicons. Total mRNA was retro transcribed and amplified by PCR (see above) with the specific primer pairs of each target gene (Table 3). Products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega).

PCR products were quantified using Qubit dsDNA HS assay (Thermo Fisher Scientific) and mixed to obtain a pool of targets' templates with known concentrations (0,05 pg/µL each). Starting from this pool, six serial 1:10 dilutions were analysed to obtain five standard curves specific to each fluorescent reporter.

4.10.2. Multiplex real time PCR analysis

Raw Ct were collected using the Bio-Rad CFX Manager software (BIO-RAD). Average C_ts from each temperature, condition and timepoint were normalized to the respective housekeeping C_t and plotted on the reporter-specific standard curve to obtain an absolute quantification of the targets. Absolute quantities of spliced *period* and *timeless* were estimated by subtraction of the unspliced isoform from the respective total amount.

Since each PCR plate enable the analysis of a single time serie at a time, in order to compare results obtained from different time series a calibration plate was performed by analysing at the same time the first time point (time 0) of each time series. The calibration plate allowed to compare C_ts of different time series avoiding possible errors due to variability between independent experiments in term of PCR reaction efficiency and fluorescent yield of the reporters.

4.11. Immunocytochemistry (ICC)

The flies were entrained at 18°C and 23°C in 12:12 LD, collected in LD or DD and fixed for 2 hours at room temperature in 4% paraformaldehyde (PFA) in PBS and subsequently washed in PBS 3 times for 10 min at room temperature on a rotating wheel.

Brains dissected in PBS were fixed in 4% PFA for 50 min at room temperature and permeabilised with 1% TritonX100 (Sigma-Aldrich) for 10 min at room temperature. To avoid non-specific staining, a blocking step was set up by incubation in 1% BSA/0,1% TritonX100 at room temperature for 2 hours.

Brains were then incubated with primary antibody diluted in 1% BSA and 0,3% TritonX100 for 72 hours at 4°C, and subsequently washed for 5 times at room temperature on a shaker for 10 min with PBS. The blocking step was performed again in 1% BSA/0,1% TritonX100 at room temperature for 1 hour, and then the brains were incubated with the secondary antibody diluted in diluted in 1% BSA and 0,3% TritonX100 for 1 hour at room temperature.

After 6 washes of 10 min with PBS at room temperature, the brains were mounted on a slide with Antifade Mounting Medium (Vectashield® mounting medium, Vector). The samples were conserved at -20°C until the visualisation through confocal microscope (Leica TCS SP5).

Table 10. Antibodies used for ICC.

Antibody	Dilution	Producer
Rabbit anti-PER	1 : 2500	R. Stanewsky (London)
Mouse anti-PDF	1 : 2500	Hybridoma Bank
Alexa Fluor 488 (goat anti-mouse)	1 : 250	Invitrogen
Alexa Fluor 568 (goat anti-mouse)	1 : 250	Invitrogen

Several scans were performed for each brain at different depth to form a Z-series. The size of each section was approximately 1 µm.

The images were elaborated and the fluorescence was quantified by using of Image J software.

4.12. Statistical analyses

All the experiments were performed with 3 different biological replicates and the data were elaborated by using Prism (Graphpad) and Excel software. The data in the graphs were reported with ± Standard Error of the Mean (SEM) and the differences were considered significant to p-value < 0.05.

To compare the data, the utilized statistical tests were:

- Two way ANOVA with Bonferroni post-hoc test, for the *tim* and *per* expression;
- Student's t test, for the locomotor activity data;
- Jonckheere-Terpstra test by Rain (Bioconductor) algorithm, for *squid, Hrb87F, period* and *timeless* oscillatory trend;
- F-test in combination with harmonic regression by CircWaveBatch 3.3 tools, for *squid, Hrb87F, period* and *timeless* oscillatory trend.

4.13. Rain (Bioconductor) algorithm

Rain detects rhythms in time-series using non-parametric methods. It uses an extension of the rank test for Umbrella Alternatives, based on the Jonckheere-Terpstra test, which tests whether sets of groups have a trend or not. The Umbrella method extends this to independent rising and falling sets. The method tests whether a time course consists of alternating rising and falling slopes, repeated with a distinct period. The phase is defined as the time point with the peak (Westermark et al., 2014).

4.14. CircWaveBatch 3.3

Circwavebatch is the batch version of the CircWave program. It provides an extremely fast and easy-to-use analyses tool for determining circadian profiles and their significance using harmonic regression in combination with the F-test statistic. CircWaveBatch is designed to handle microarray and circadian data that represent circadian expression patterns of genes. Each microarray data point can be included and no pre-calculation of averages or SD is required. Any number of genes with a maximum of 100 data points per gene (equidistant or non-equidistant) can be analysed for a range of periods without any complicated parameter initialisation procedures or non-linear regression iteration settings.

Data input should be a comma separated text file (*.csv), where the first line contains the column headers while the first column contains a string (<10 characters) indicating the gene code. Subsequent columns represent the timepoints and subsequent rows represent the genes.

The calculation of the shape of the profile wave form is based upon forward linear harmonic regression. This means that a fundamental sinusoidal wave will be fitted through the data and its significance will be tested against a fitted horizontal line through the overall average. This fundamental wave is described by the function:

$$f(t) = a + \sum_{i=1}^{\infty} \left[p_i \sin i2\pi \frac{t}{\tau} + q_i \cos i2\pi \frac{t}{\tau} \right]$$

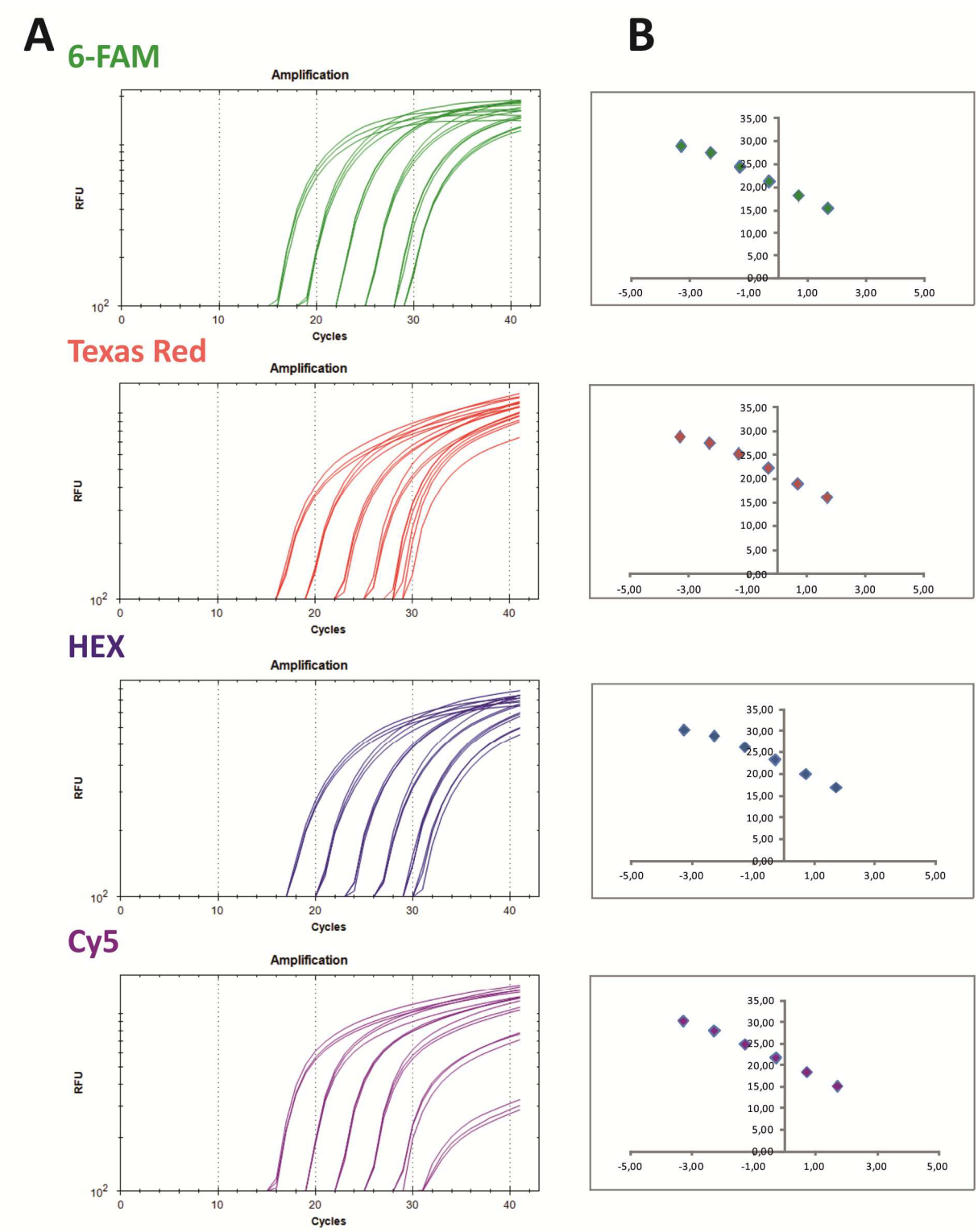
About:

- **a** = average;
- **i** = 1, 2, 3, indicating the fundamental wave, the first harmonic, the second harmonic, ….;
- **pi** = is the sine coefficient of the harmonic;
- **qi** = the coefficient of the harmonic;
- **t** = timepoint value (modulo τ);
- **f(t)** = the calculated function value at time point t.

When the F-test indicates that this function explains the variation in the data points significantly better than just the horizontal line, than p1 will be smaller than alpha (which is set to 0.05 by default). If p1 < alpha, than the program will analyse a more complex wave form by adding the first harmonic

APPENDIX

Appendix 1. Standard curves for the absolute quantification of *period* and *timeless* isoforms. (A) Amplification plots of per^{TOTAL} (green), $per^{UNSPLICED}$ (red), tim^{TOTAL} (blue), $tim^{UNSPLICED}$ (violet) and *rp49* (yellow). Amplification curves of all the six dilutions are represented. Fluorescent intensities (RFU, relative fluorescence units) are plotted against the number of amplification cycles. **(B)** Standard curves used for the absolute quantification; Ct values are plotted against the logarithm of concentration to base 10.



Appendix 2. Summary of the statistical survey of *period* and *timeless* expression: in the table the p-values of the statistical comparisons between w^{1118} and sqd^{EP} are reported for the timepoints in which the expression is statistically significant.

Gene	Temperature	Condition	Comparison *white* ^{1118} vs *squid* ^{EP}	*p-value*
per	15°C	DD	CT 3	0,01543
			CT 15	0,01868
			CT 21	0,00566
		LD	ZT 6	0,00530
			ZT 9	0,00507
			ZT15	0,00582
			ZT 18	0,00422
	18°C	DD	CT 0	0,01394
			CT 3	0,01271
			CT 6	0,00844
			CT 9	0,01307
		LD	ZT 9	0,01192
			ZT 12	0,00245
			ZT 15	0,01386
			ZT 18	0,00424
			ZT 21	0,01192
	23°C	DD	CT 3	0,00616
			CT 6	0,00860
			CT 9	0,00031
			CT 12	0,00385
			CT 15	0,00445
			CT 18	0,02138
		LD	ZT 0	0,00070
			ZT 3	0,00030
			ZT 6	0,00020
			ZT 9	0,00001
			ZT 12	0,00026
			ZT 15	0,00017
			ZT 18	0,00002
	29°C	DD	ZT 21	0,00005
			CT 0	0,01622
			CT 6	0,00030
			CT 9	0,00364
			CT 12	0,00062
			CT 15	0,01877
			CT 18	0,00024
			CT 21	0,00016
tim	15°C	LD	ZT 3	0,00005
			ZT 6	0,00000
			ZT 9	0,00001
			ZT 12	0,00003
			ZT 15	0,00001
			ZT 18	0,00005
			ZT 21	0,00022
		DD	CT 3	0,00021
			CT 6	0,00012
			CT 9	0,00038
			CT 12	0,00023
			CT 15	0,00588
	18°C	LD	ZT 6	0,01936

Appendix 3. Summary of the statistical analysis of average proportion of period SPLICED at different temperatures in w^{1118} and sqd^{EP} both in LD and DD. In the table a Mean difference, Confidence Interval, Significance, p-Value, Mean and Numerosity of the statistical comparisons between different temperature are reported.

Bonferroni's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	p-Value	Mean 1	Mean 2	Mean Diff,	SE of diff,	N1	N2	q	DF
***period* in w^{1118} in LD**													
15°C vs. 18°C	12	2,921 to 21,08	Yes	**	0,0041	73,88	61,88	12	3,505	8	8	4,841	208
15°C vs. 23°C	26,3	17,22 to 35,38	Yes	****	<0,0001	73,88	47,58	26,3	3,505	8	8	10,61	208
15°C vs. 29°C	41,51	32,43 to 50,59	Yes	****	<0,0001	73,88	32,36	41,51	3,505	8	8	16,75	208
18°C vs. 23°C	14,3	5,221 to 23,38	Yes	***	0,0004	61,88	47,58	14,3	3,505	8	8	5,769	208
18°C vs. 29°C	29,51	20,43 to 38,59	Yes	****	<0,0001	61,88	32,36	29,51	3,505	8	8	11,91	208
23°C vs. 29°C	15,21	6,134 to 24,29	Yes	***	0,0001	47,58	32,36	15,21	3,505	8	8	6,137	208
***period* in sqd^{EP} in LD**													
15°C vs. 18°C	5,623	-3,774 to 15,02	No	ns	0,4098	83,04	77,41	5,623	3,628	8	7	2,192	208
15°C vs. 23°C	3,6	-5,479 to 12,68	No	ns	0,7338	83,04	79,44	3,6	3,505	8	8	1,452	208
15°C vs. 29°C	3,537	-5,541 to 12,62	No	ns	0,7443	83,04	79,5	3,537	3,505	8	8	1,427	208
18°C vs. 23°C	-2,023	-11,42 to 7,374	No	ns	0,9444	77,41	79,44	-2,023	3,628	7	8	0,7886	208
18°C vs. 29°C	-2,086	-11,48 to 7,312	No	ns	0,9395	77,41	79,5	-2,086	3,628	7	8	0,8129	208
23°C vs. 29°C	-0,0625	-9,141 to 9,016	No	ns	>0,9999	79,44	79,5	-0,0625	3,505	8	8	0,02522	208
***period* in w^{1118} in DD**													
15°C vs. 18°C	-0,9	-9,979 to 8,179	No	ns	0,9941	63,58	64,48	-0,9	3,505	8	8	0,3631	208
15°C vs. 23°C	7,338	-1,741 to 16,42	No	ns	0,1587	63,58	56,24	7,338	3,505	8	8	2,96	208
15°C vs. 29°C	38,24	28,44 to 48,05	Yes	****	<0,0001	63,58	25,33	38,24	3,786	8	6	14,28	208
18°C vs. 23°C	8,238	-0,8412 to 17,32	No	ns	0,0903	64,48	56,24	8,238	3,505	8	8	3,323	208
18°C vs. 29°C	39,14	29,34 to 48,95	Yes	****	<0,0001	64,48	25,33	39,14	3,786	8	6	14,62	208
23°C vs. 29°C	30,9	21,1 to 40,71	Yes	****	<0,0001	56,24	25,33	30,9	3,786	8	6	11,54	208
***period* in sqd^{EP} in DD**													
15°C vs. 18°C	18,05	8,971 to 27,13	Yes	****	<0,0001	88,78	70,73	18,05	3,505	8	8	7,282	208
15°C vs. 23°C	11,89	2,809 to 20,97	Yes	**	0,0046	88,78	76,89	11,89	3,505	8	8	4,796	208
15°C vs. 29°C	27,1	18,02 to 36,18	Yes	****	<0,0001	88,78	61,68	27,1	3,505	8	8	10,93	208
18°C vs. 23°C	-6,163	-15,24 to 2,916	No	ns	0,2967	70,73	76,89	-6,163	3,505	8	8	2,486	208
18°C vs. 29°C	9,05	-0,02875 to 18,13	No	ns	0,0511	70,73	61,68	9,05	3,505	8	8	3,651	208
23°C vs. 29°C	15,21	6,134 to 24,29	Yes	***	0,0001	76,89	61,68	15,21	3,505	8	8	6,137	208
***timeless* in w^{1118} in LD**													
15°C vs. 18°C	-36,31	-46,66 to -25,96	Yes	****	<0,0001	26,14	62,45	-36,31	3,997	5	8	12,85	208
15°C vs. 23°C	-60,41	-70,76 to -50,06	Yes	****	<0,0001	26,14	86,55	-60,41	3,997	5	8	21,38	208
15°C vs. 29°C	-70,46	-80,81 to -60,11	Yes	****	<0,0001	26,14	96,6	-70,46	3,997	5	8	24,93	208
18°C vs. 23°C	-24,1	-33,18 to -15,02	Yes	****	<0,0001	62,45	86,55	-24,1	3,505	8	8	9,723	208
18°C vs. 29°C	-34,15	-43,23 to -25,07	Yes	****	<0,0001	62,45	96,6	-34,15	3,505	8	8	13,78	208
23°C vs. 29°C	-10,05	-19,13 to -0,9713	Yes	*	0,0235	86,55	96,6	-10,05	3,505	8	8	4,055	208
***timeless* in sqd^{EP} in LD**													
15°C vs. 18°C	-20,86	-31,98 to -9,743	Yes	****	<0,0001	52,23	73,09	-20,86	4,293	4	8	6,872	208
15°C vs. 23°C	-31,69	-42,81 to -20,57	Yes	****	<0,0001	52,23	83,91	-31,69	4,293	4	8	10,44	208
15°C vs. 29°C	-45,76	-56,88 to -34,64	Yes	****	<0,0001	52,23	97,99	-45,76	4,293	4	8	15,07	208
18°C vs. 23°C	-10,83	-19,9 to -1,746	Yes	*	0,0122	73,09	83,91	-10,83	3,505	8	8	4,367	208
18°C vs. 29°C	-24,9	-33,98 to -15,82	Yes	****	<0,0001	73,09	97,99	-24,9	3,505	8	8	10,05	208
23°C vs. 29°C	-14,08	-23,15 to -4,996	Yes	***	0,0005	83,91	97,99	-14,08	3,505	8	8	5,678	208
***timeless* in w^{1118} in DD**													
15°C vs. 18°C	-26,38	-37,01 to -15,75	Yes	****	<0,0001	40,72	67,1	-26,38	4,105	5	7	9,088	208
15°C vs. 23°C	-52,86	-63,21 to -42,5	Yes	****	<0,0001	40,72	93,58	-52,86	3,997	5	8	18,7	208
15°C vs. 29°C	-57,78	-68,13 to -47,43	Yes	****	<0,0001	40,72	98,5	-57,78	3,997	5	8	20,44	208
18°C vs. 23°C	-26,48	-35,87 to -17,08	Yes	****	<0,0001	67,1	93,58	-26,48	3,628	7	8	10,32	208
18°C vs. 29°C	-31,4	-40,8 to -22	Yes	****	<0,0001	67,1	98,5	-31,4	3,628	7	8	12,24	208
23°C vs. 29°C	-4,925	-14 to 4,154	No	ns	0,4977	93,58	98,5	-4,925	3,505	8	8	1,987	208
***timeless* in sqd^{EP} in DD**													
15°C vs. 18°C	-5,205	-14,6 to 4,192	No	ns	0,4792	65,54	70,74	-5,205	3,628	8	7	2,029	208
15°C vs. 23°C	-23,43	-32,5 to -14,35	Yes	****	<0,0001	65,54	88,96	-23,43	3,505	8	8	9,451	208
15°C vs. 29°C	-28,56	-37,96 to -19,17	Yes	****	<0,0001	65,54	94,1	-28,56	3,628	8	7	11,13	208
18°C vs. 23°C	-18,22	-27,62 to -8,822	Yes	****	<0,0001	70,74	88,96	-18,22	3,628	7	8	7,101	208
18°C vs. 29°C	-23,36	-33,06 to -13,65	Yes	****	<0,0001	70,74	94,1	-23,36	3,747	7	7	8,815	208
23°C vs. 29°C	-5,138	-14,53 to 4,26	No	ns	0,4909	88,96	94,1	-5,138	3,628	8	7	2,002	208

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