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The *Drosophila* circadian clock going wild:

PERIOD and TIMELESS oscillations under natural conditions

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<u>Abstract</u>

The circadian clock of the fruit fly *Drosophila melanogaster* relies on 7 groups of clock neurons per brain hemisphere which are bilaterally clustered in dorsal (DN1s, DN2s and DN3s) and lateral (s-LNvs, l-LNvs, LNds and LPNs) according to their positions in the brain.

In these neurons, clock genes such as *period* (*per*) and *timeless* (*tim*) operate in interlocked feedback loops. Under rectangular 12:12 light:dark (LD) regimes and constant temperature PER and TIM proteins start to accumulate in the cytoplasm of all clock neurons in the middle of the night and reach their maximum levels at the end of the dark phase. At lights-on TIM is degraded in a light dependent manner; in the absence of TIM, PER is also degraded.

To date, almost all behavioural and molecular analyses of fly circadian rhythmicity have been carried out in the laboratory. Nevertheless laboratory conditions do not reflect the complexity of the stimuli that are present in the natural environment.

In 2006 our lab started a research project (granted by the European Commission, 6th Framework Programme; Project EUCLOCK N° 018741), in collaboration with the group of Prof. C. P: Kyriacou at the Department of Genetics, University of Leicester (UK), dealing with the characterization of the circadian clock of *D. melanogaster* under real natural conditions.

To investigate the functioning of the circadian clock in a real natural environment we studied PER and TIM expression in the circadian clock neurons of flies exposed to natural conditions throughout 2008 and 2009. The flies analysed belong to the WT-ALA (Wild Type ALto Adige) strain of *D. melanogaster* which has been established in 2006 from a natural population sampled in the North of Italy.

Upon analysis of PER and TIM expression profiles within the clock neurons of fruit flies experiencing real natural environment we found that, unexpectedly, PER and TIM oscillations appear to be decoupled in certain circumstances. In fact, under long and hot days as in Summer conditions (Natural LD~ 15:9; Tmax~35°C; Tmin~25°C) the peak of PER is advanced and the peak of TIM delayed, leading to an oscillation almost in antiphase. Moreover, we hypothesize that the decoupling in PER and TIM oscillation profiles is linked to the decoupling in the phase of the morning and evening burst of activity also observed under natural conditions (Bhutani *et al.*, submitted).

In addition, we observed that, irrespectively of the season, the peak of PER within the DN1s is always advanced compared to that within the lateral cells and this phase advance do not depends on PDF signaling.

Once the molecular oscillation profiles of PER and TIM under natural conditions were revealed, we attempted to reproduce our findings under laboratory conditions. This part of the project was achieved thanks to the collaboration with Prof. Charlotte Helfrich-Förster's laboratory, at the Department of Neurobiology and Genetics, University of Würzburg (DE). In particular, wild type flies were entrained to laboratory LD 16:8 regimes in which the slow increasing and decreasing in light intensity typical of natural dawns and dusks was simulated, at two different constant temperatures (20°C and 30°C). We confirmed that the huge phase shift between PER and TIM oscillations which characterizes natural Summer days is caused, at least in part, by high temperatures, albeit natural thermocycles appear to be stronger environmental cues than constant high temperatures. Moreover, we observed that the phase advance in PER cycling within the DN1s hold true in the lab under the particular conditions we used for the entrainment. Therefore, we hypothesize that this phase advance is mainly a response to a specific environmental conditions, namely the ramping in light intensities we used to simulate sunrise and sunset.

Riassunto

La sede anatomica del *core* dell'orologio circadiano di *D. melanogaster* è costituita da circa 100 neuroni per emisfero cerebrale, suddivisi in 7 gruppi: 3 gruppi di neuroni dorsali (DN1s, DN2s e DN3s) e 4 gruppi di neuroni laterali (s-LNvs, l-LNvs, LNds, e LPNs). A livello molecolare, l'orologio circadiano consta di un sistema di *loop* a retroazione negativa interconnessi tra loro. In condizioni standard di laboratorio, ovvero in regimi di LD 12:12 e a temperatura costante, i geni *period* (*per*) e *timeless* (*tim*) vengono trascritti ad opera dei fattori di trascrizione CLOCK (CLK) e CYCLE (CYC). Le proteine PERIOD (PER) e TIMELESS (TIM) si accumulano durante la notte e raggiungono un picco alla fine della notte/inizio del giorno, in maniera sincrona in tutti i neuroni orologio. Verso la fine della notte, PER e TIM entrano nel nucleo, dove inibiscono la trascrizione degli stessi geni che li codificano (*per* e *tim*). All'accensione della luce i livelli di TIM scendono rapidamente a causa della degradazione luce dipendente mediata dal fotorecettore per la luce blu CRYPTOCHROME (CRY). In assenza di TIM, anche PER va incontro a degradazione.

Ad oggi, gli studi sull'orologio circadiano di *Drosophila* sono stati condotti esclusivamente in condizioni di laboratorio, anche se in qualche caso si è cercato di riprodurre le caratteristiche dell'ambiente naturale. Tuttavia, gli stimoli ambientali a cui i moscerini sono esposti in laboratorio sono di gran lunga meno complessi degli stimoli realmente presenti in natura. Nell'ambiente naturale la luce cambia continuamente, sia per quanto riguarda la sua intensità che la composizione del suo spettro ed anche la temperatura è soggetta a variazioni continue più o meno accentuate a seconda della stagione e della latitudine.

Nel 2006 il nostro laboratorio ha avviato un progetto di ricerca (EU Project EUCLOCK N° 018741, 6t[h] Framework Programme) in collaborazione con il gruppo del Prof. C.P. Kyriacou (Department of Genetics, University of Leicester, UK) con l'obiettivo di studiare e caratterizzare il funzionamento dell'orologio circadiano di *Drosophila* in condizioni naturali. In questo lavoro sono riportati i risultati della sperimentazione condotta nell'ambito della mia tesi di dottorato che si è concentrato sull'analisi dei profili di oscillazione delle proteine PER e TIM nei diversi neuroni orologio di moscerini esposti a condizioni naturali. Il ceppo di moscerini selvatici utilizzato negli esperimenti (WT-ALA) è stato stabilizzato a partire da una collezione di linee isofemminili campionate nel 2006 nel Nord Italia (Val Venosta, BZ).

L'analisi dei dati ottenuti nelle diverse condizioni ambientali analizzate, (rappresentative delle quattro stagioni) ci ha permesso di rilevare che i profili di oscillazione di PER e TIM appaiono disaccoppiati in certe condizioni ambientali. Infatti, abbiamo riscontrato che quando le giornate sono lunghe e calde (Primavera/Estate) il picco di PER è ritardato rispetto a quello che si osserva in giornate più corte e fresche (Autunno) mentre quello di TIM sembra anticipare.

Questi spostamenti dei picchi delle due proteine fanno si che, in particolare durante l'Estate, PER e TIM mostrino il loro massimo di espressione in alcuni gruppi di neuroni orologio con quasi 12 ore di differenza; PER e TIM sembrano quindi oscillare in antifase.

Abbiamo osservato inoltre che, indipendentemente dalle condizioni ambientali cui sono esposti i moscerini, PER raggiunge il picco di espressione prima nei DN1s e successivamente nei neuroni orologio laterali. Abbiamo inoltre dimostrato che questa anticipazione dei DN1s è indipendente dal neuropeptide PDF, prodotto da s-LNvs e l-LNvs e implicato nell'output dell'orologio circadiano.

Dopo aver descritto in dettaglio i profili di oscillazione di PER e TIM in condizioni naturali, abbiamo ritenuto opportuno, al fine di identificare le componenti ambientali responsabili dei fenomeni osservati, cercare di riprodurre in laboratorio i risultati ottenuti, apportando alcune modificazioni mirate ai tradizionali profili (rettangolari) di LD e alle temperature utilizzati negli esperimenti di laboratorio.

Questi esperimenti sono stati condotti in collaborazione con il gruppo della Prof.ssa C. Helfrich-Förster (Department of Neurobiology and Genetics, University of Würzburg, DE).

Abbiamo esposto i moscerini a cicli di LD 16:8 e simulato albe e tramonti mediante variazioni controllate dell'intensità luminosa, a due differenti temperature costanti (20°C e 30°C).

Con questi esperimenti abbiamo potuto confermare quanto avevamo ipotizzato analizzando i risultati ottenuti in condizioni naturali, ovvero che lo spostamento delle fasi dei picchi di PER e TIM sia non solo determinato dal fotoperiodo ma, almeno in parte, anche dalla temperatura. Inoltre, tali esperimenti ci hanno permesso di collegare l'anticipazione del picco di PER nei DN1s al graduale aumento o diminuzione dell'intensità luminosa che si verifica normalmente durante l'alba e il tramonto.

<u>Abbreviations</u>

DD   Constant Darkness

LL   Constant light

LD   Light: Dark cycle

LDR   Light:Dark cycle with Ramping in light intensity to simulate dawn and dusk

LM   Light:Moonlight cycle

NLD   Natural Light:Dark cycle

TC   Thermophase:Cryophase cycle

NTC   Natural Thermophase:Cryophase cycle

ZT   *Zeitgeber* Time

DN1s   Dorsal Neurons 1

DN2s   Dorsal Neurons 2

DN3s   Dorsal Neurons 3

s-LNvs   small ventral Lateral Neurons

l-LNvs   large ventral Lateral neurons

LNds   dorsal Lateral Neurons

LPNs   Lateral Posterior Neurons

WT-ALA   Wild-Type ALto Adige

Chapter 1:

Introduction

These days our lives totally depend on cell phones, watches and alarm clocks that allow us to respect the appointments of our social life. At the same time, our bodies are under the control of an internal timer that is an evolutionary very old biological clock: it can be found in almost all living beings on our planet, from bacteria to plants and animals. This internal clock influences the majority of our behaviour and physiology (e.g. sleep-wakefulness cycle, feeding, reproduction, learning and memory).

For centuries it was believed that biological periodicities were caused by the environmental rhythms with which they were synchronized. The French astronomer Jacques d' Ortous de Mairan noticed that the daily leaf movements of the plant *Mimosa pudica,* that normally opens its leaf during the day and closes them at night, persisted for several days, according to the day-night cycle, also after placing it in constant darkness (de Mairan, 1729). We now know that biological rhythms are guided by a molecular mechanism internally generated.

Internal timekeeping systems can be classified depending on the length of their period: *ultradian* when their period is less than 24 hours, *infradian* when the rhythmicity is characterized by an interval longer than a day and *circadian* when the repeating interval is close to 24 h.

Circadian (from Latin *circa diem*) rhythms are kept in synchrony with the environment through various external cues, like light and temperature that are the most important *Zeitgeber* (from German: "time giver"). In the presence of these environmental stimuli the circadian clock follows a *Zeitgeber* Time (ZT, with ZT0=lights-on). The synchronization of the clock is termed "entrainment" and is an integral property of all circadian rhythms. However, these rhythms also persist in the absence of external cues or rather under constant conditions. Circadian clocks are also capable to maintain their rhythm within a range of viable temperatures: this is named temperature compensation. Circadian clocks can be dissected into three components: an input pathway through which external information reaches the clock; the clock itself that generates a self-sustained circadian oscillation; output pathways through which the clock regulates specific physiological processes. At the molecular level circadian clocks consist of feedback loops in which clock genes act inhibiting their own transcription.

A huge variety of living beings, very different from each other, posses a circadian clock likely because of the evolutionary selection pressure induced by the rotation of the earth around its own axis and consequent successions of day and night and seasons. A lot of examples of the adaptive significance of daily rhythms come from insects. Adults of *D. pseudoobscura* emerge from their puparium close to dawn when the relative humidity in the air is at its maximum so that the wings

of just hatched flies do not desiccate (Pittendrigh *et al.*, 1958). Bees can be trained to visit a food source at a particular time of the day so that they visit nectar sources at the same time every day. This is really important considering the observation that not only flowers open and close everyday at particular hours but also nectar availability depends on the time of the day.

The main advantage that comes from the fact that an event is controlled by the circadian clock is that in this ways it happens simultaneously among individuals of a population.

The fruit-fly *Drosophila melanogaster* has been extensively used as a model organism to understand the molecular mechanism of the circadian clocks, the location of the circadian pacemaker neurons, as well as input and output pathways that reach or come from the body oscillators.

An important feature of fruit fly circadian timekeeping is that most aspects of its molecular clockwork are conserved in mammals, including humans. For example, individuals affected with familial advanced sleep phase syndrome (FASP) exhibit an advanced phase of sleep-wake rhythms and shortened circadian period that is inherited in Mendelian dominant manner. Mutations in the human *Per2* and *Ck1δ* genes, orthologs of the flies circadian genes *per* and *Dbt*, respectively, are responsible for this advanced sleep phase (Toh *et al.*, 2001; Xu *et al.*, 2005).

1.1 Circadian behaviours in *Drosophila*

In *D. melanogaster* circadian rhythms are usually assayed by monitoring the locomotor activity.

Locomotor activity shows a bimodal distribution during the 24 hours light-dark cycle, which is characterized by morning and evening activity bouts respectively during the lights-on and lights-off transitions.

The activity of wild-type flies gradually increases before both lights-on and lights-off transitions: this phenomenon is called "anticipation". There is also a burst of activity right after light transitions: this is a response, evoked by changing environment, called "masking".

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). A bimodal activity is present in most living organisms, diurnal or nocturnal (**Fig 1.1**).

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 such as sunrise and sunset.
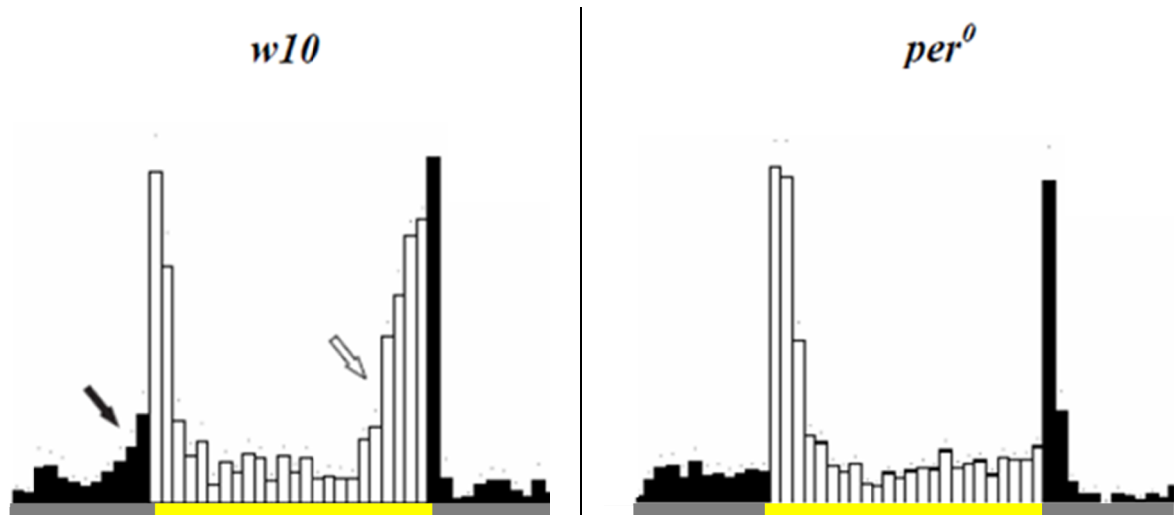
Fig 1.1: Locomotor activity in light-dark cycles. Histograms represent the distribution of the activity through the 24 hours, averaged for n flies over three days of LD. Each figures show activity in 30 mins interval. Dots indicate the s.e.m. of the activity for each 0.5 hour interval. Filled and open arrows point to lights-on and lights-off anticipation respectively. Yellow bars represent the light phase, the gray ones the dark period. Notably, anticipation of light transitions is lost in *per^0* mutants (Grima *et al.*, 2004)

In *Drosophila* many other behaviours are under circadian regulation, e.g. sleep, learning and memory, courtship and mating.

When a fruit fly sleeps, it exhibits reduced activity movements and a reduced responsiveness to light-sensory stimuli. Moreover sleep deprivation leads to death both in fruit flies and mammals (Shaw *et al.*, 2002). Since flies carrying dominant negative or null alleles for the clock transcription factors *Clk* and *cyc* sleep less than wild-type flies, it has been inferred that sleep is under circadian control, as well as in mammals (Hendricks *et al.*, 2003).

Fruit flies exhibit many sorts of learning and memory and it has been shown by Lyons and Roman, 2009, that the ability to form short term memory is much higher in fruit flies during early night while this modulation is abolished in clock mutants.

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

Also mating and courtship are under circadian control in *D. melanogaster*: this occur via circadian regulation of genes involved in determination of these behaviours such as the cytochrome P450 family member *sxe1*, necessary for an high mating success in males, that shows oscillation at the protein level with a peak during the night (Fujii *et al.*, 2008)

1.2 The *Drosophila* molecular clock

The study of the clock mechanism in *Drosophila* started at the end of the sixties with Konopka's screening for mutants showing an altered pattern of pupal eclosion in light-dark (LD) conditions and daily rest activity cycles, which are the main phenotypes influenced by the circadian clock.

Thanks to this study, in 1971 the first clock gene was genetically identified (Konopka and Benzer, 1971): since mutants for this gene showed an altered periodicity in their activity rhythms under constant conditions, it was called *period* (*per*).

Afterwards, other clock genes have been identified and a wide genetic analysis has uncovered the transcriptional feedback loops that constitute the molecular core of the circadian clock in *Drosophila*. These feedback circuits consist of sequence-specific DNA binding proteins that stimulate transcription of their own repressors (**Fig. 1.2**).

The first feedback loop

The genes *Clock* (*Clk*) and *cycle* (*cyc*) are involved in the loop that controls daily oscillation of *per* and *timeless* (*tim*). *Clk* and *cyc* encode for transcription factors containing a PAS protein-dimerization domain and a basic helix-loop-helix (bHLH) domain.

cyc is constitutively transcribed while *Clk* expression is circadianly regulated. *Clk* mRNA and protein show a similar temporal profile, reaching peak levels around subjective dawn (the time when in a LD cycle the light would go on) and their lowest levels in the early subjective night (Lee *et al.*, 1998).

CYC and CLK heterodimerize and bind to E-box sequences (an E-box constitutes of six consensus nucleotides that are target for bHLH transcription factors) in the promoters of *per* and *tim* genes activating their transcription. Since CLK levels vary during the day and CYC is

constitutively present, the number of CLK/CYC heterodimers which can be formed depends on CLK.
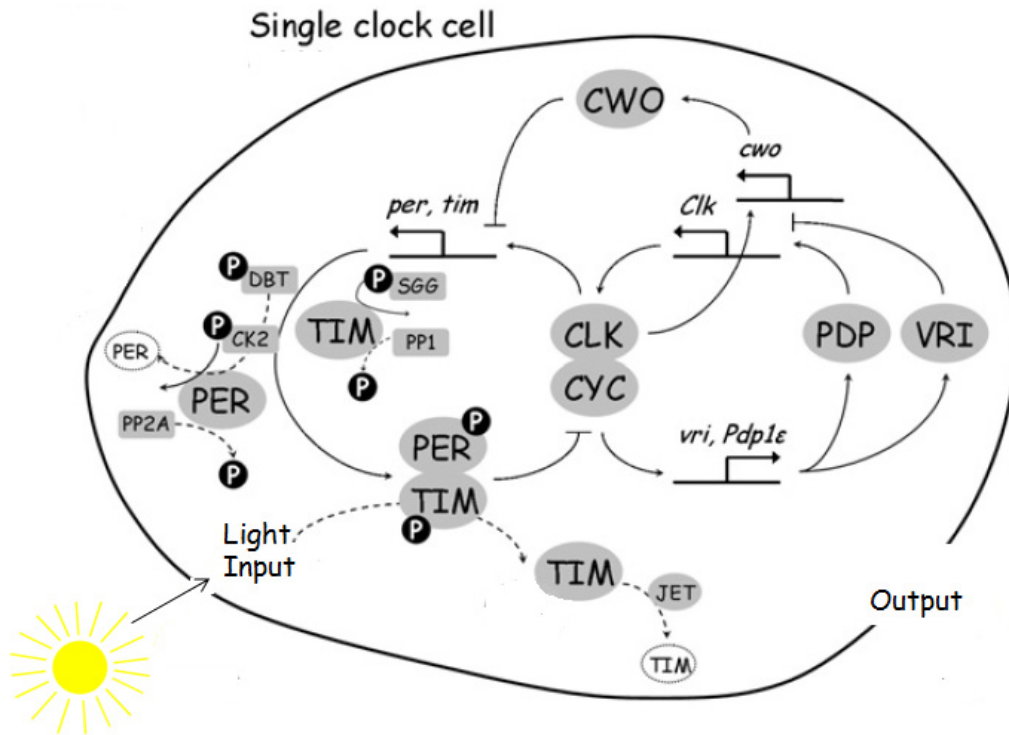
Fig. 1.2: Schematic representation of the *Drosophila* molecular clock. Modified from Kostál, 2010. Acronyms can be found in the text, **Section 1.2**.

PER is a component of the PAS-family and lacks a bHLH binding domain. The PAS domain functions as a binding site for TIM (Gekakis *et al.*, 1995). TIM contains three ARMADILLO-like dimerization domains, two of which overlap with the region that can bind to PER in cultured *Drosophila* cells (Kyriacou and Hastings, 2001).

CLK- and CYC-mediated transcription of *per* and *tim* starts in the middle of the day and peak levels of each mRNA are reached in the early evening. PER and TIM proteins accumulate with a ca. 4- to 6-hours delay, reaching peak levels late at night. Formation of PER/TIM heterodimers in the cytoplasm of clock cells promotes the nuclear entry of both proteins (Curtin *et al.*, 1995; Gekakis *et al.*, 1995): newly synthesized monomeric PER is destabilized by the *doubletime* (*dbt*)-encoded kinase (Price *et al.*, 1998). Therefore DBT function delays accumulation of PER-TIM dimers until sufficient amounts of TIM are available in the cytoplasm. Once in the nucleus, PER and TIM repress transcription of their own genes by interfering with their activators CLK and

CYC. Most likely this occurs via a direct binding of the PER-TIM dimer to the CLK-CYC dimer in the late night/early morning (Bae *et al.*, 2000). Notably, PER and TIM may dissociate prior to entering the nucleus (Meyer *et al.*, 2006).

PER, TIM and CLK exhibit rhythms of phosphorylation which peak during the late night/early day.

PER is phosphorylated by CASEIN KINASE Iε (CK1ε)/ DBT and CASEIN KINASE 2 (CK2). Phosphorylation by DBT and CK2 enhances PER repressor activity (Kivimae *et al.*, 2008).

TIM is phosphorylated by GLYCOGEN SYNTHASE KINASE (GSK3)/ SHAGGY and CK2. The length of the delay before nuclear entry of PER/TIM is, at least in part depending on the activity of the SGG kinase: SGG overexpression leads to hyperphosphorylated TIM, in this situation PER/TIM heterodimer translocates earlier into the nucleus (Blau, 2003).

Phosphorylated PER and TIM are substrates for the phosphatases PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1), respectively.

A nuclear PER/DBT complex also associates with and phosphorylates CLK, triggering its degradation (Kim and Edery, 2006).

A high level of phosphorylation precedes proteins disappearance. The E3 ubiquitin ligase SUPERNUMERARY LIMBS (SLIMB) selectively associates with and ubiquitinates at least DBT phosphorylated PER (Chiu *et al.*, 2008; Grima *et al.*, 2002; Ko *et al.*, 2002), triggering its degradation through the ubiquitin proteasome pathway during the early day. The loss of PER stops repression of CLK/CYC permitting a new cycle of transcriptional activation.

The second feedback loop

In the second interlocked-feedback loop (**Fig. 1.2**), CLK/CYC heterodimer activates the transcription of the genes *vrille* (*vri*) and *PAR-domain protein1ε (pdp1ε)* at about noon. *vri* and *pdp1ε* mRNAs and proteins accumulate with different kinetics such that VRI protein accumulates first and represses *Clk* expression. PDP1ε protein then accumulates and activates *Clk* transcription after VRI-mediated repression ends in the middle of the night.

The newly produced CLOCK protein is inactive because of the presence of PER repressor. Once PER is degraded, CLK/CYC reactivates *per, tim, vri* and *pdp1ε* genes transcription to start a new cycle (Cyran *et al.*, 2003).

CLK/CYC also activates a bHLH repressor encoded by *clockwork orange* (*cwo*). CWO specifically binds CLK/CYC E-box targets and represses CLK/CYC mediated transcription (Kadener *et al.*, 2007).

1.3 *period* and *timeless* in detail

The rhythmic expression of clock genes in specific brain neurons constitutes the molecular basis of the clockwork that controls daily behavioural rhythms and other rhythmic outputs of the brain. The *per* and *tim* genes are cardinal components of the circadian clock in *Drosophila*:

- mutations in both genes lead to arrhythmicity or alteration of the periodicity (Konopka and Benzer, 1971; Rutila *et al.*, 1996; Sehgal *et al.*, 1994);
- both their mRNAs and protein products show a well pronounced cycling and the molecular oscillations are dependent on the function of both genes (Hardin *et al.*, 1990; Price *et al.*, 1995; Sehgal *et al.*, 1994; Sehgal *et al.*, 1995; Zeng *et al.*, 1994; Zerr *et al.*, 1990).

per was cloned and functionally assayed in 1984 (Bargiello *et al.*, 1984; Reddy *et al.*, 1984; Zehring *et al.*, 1984). whereas *tim* was identified and characterized more than ten years later (Gekakis *et al.*, 1995; Sehgal *et al.*, 1994).

period

In 1990 it was shown by Hardin *et al.* (1990) that the level of PER oscillates out of phase compared to that of its mRNA and this finding suggested that PER is a negative regulator of its own transcription: in a 12:12 LD regime, *per* mRNA presents a peak at the beginning of the night (after sunset) and a trough at the end of the night whereas PER protein reaches the maximum level at the end of the night and starts to be degraded after lights-on.

In *D. melanogaster per* is located on the X chromosome and is constituted by 8 exons and 7 introns. The 4.5 Kb mRNA is translated in a protein composed by 1218 amino acids (aa).

PER protein contains several domains (**Fig. 1.3a**):

- NLS (Nuclear Localization Signal) domain that allows the nuclear translocation of PER to downregulate its own transcription;
- PAS domain subdivided in two regions which both have important roles in protein-protein interactions;
- CLD (Cytoplasmatic Localization Domain) that maintains PER in the cytoplasm as long as the NLS is inactive;
- TG domain that contains a run of threonine and glycine amino acids doublets that is polymorphic in the number of repeats with natural variants ranging between 14 to 24 pairs (Costa and Kyriacou, 1998). The Thr-Gly natural variation represents, at population level, an example of balancing selection (Sawyer *et al.*, 1997) which structurates the distribution of the polymorphism and provides *per* with an important role in geographic adaptations.
- CCID (CLK and CYC inhibition domain) that is essential for the regulation of PER through the disruption of the circadian transcription factors CLK and CYC. Moreover, in this region a second NLS domain has been described, which seems to be more important than the one mentioned above (Chang and Reppert, 2003).

per mRNA cycling is affected both by light and temperature. In 1998 two alternatively spliced transcripts of the *period* gene were described by Cheng and collaborators (Cheng *et al.*, 1998).: the two transcript types differ only by the presence (type A) or absence (type B') of an alternative intron (*dmpi8*) in the 3' untranslated region (UTR).

At low temperature and short photoperiods mimicking winter conditions, the splicing of the *per* 3'UTR is enhanced, leading to an early accumulation of PER protein and a subsequent advance in the evening peak of locomotor activity (Majercak *et al.*, 1999). This is presumably ecologically advantageous because it would ensure that the majority of the fly's activity would occur during the middle of the day in winter, when the temperatures are relatively warm. At high temperatures and long photoperiods, representative of summer, *per* splicing is inhibited and the peaks of activity are shifted towards dawn and dusk, leading to a sort of 'siesta', localized during the hotter parts of the day and ensuring in this way that the majority of the activity occurs during the cooler parts of the day.
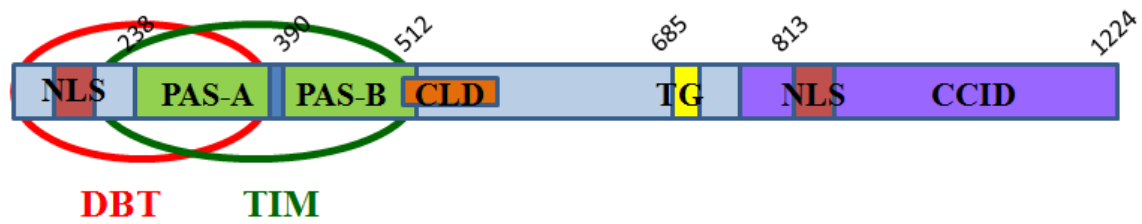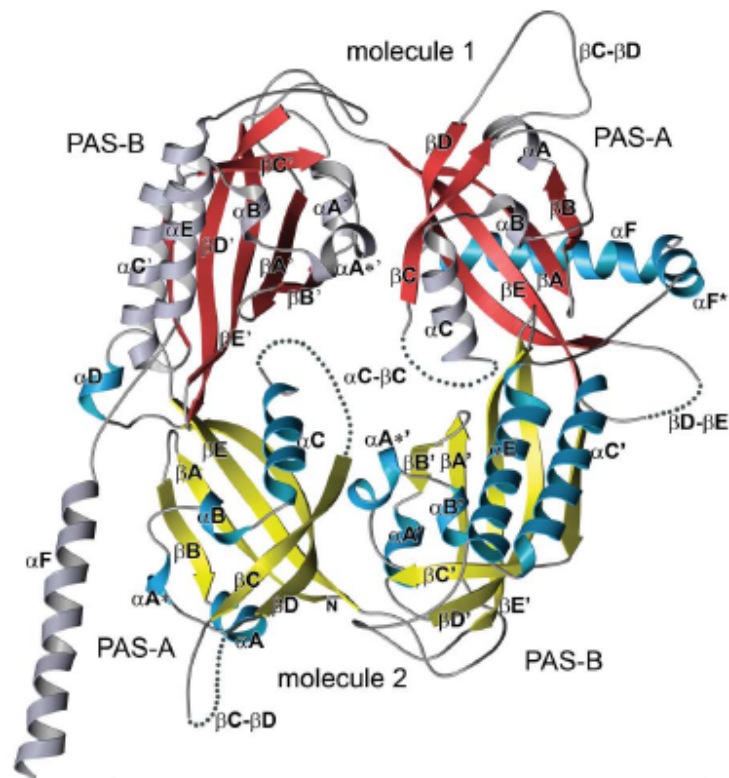
Fig 1.3:
a) Functional domain of the *Drosophila* PERIOD. NLS: Nuclear Localization Signal; PAS-A and PAS-B represent the two region of the PAS domain; CLD: Cytoplasmatic Localization Domain; TG: Thr-Gly domain; CCID: CLK and CYC Inhibition Domain. The red circle represents the DBT binding site, the green circle the TIM binding site. Figure modified from Yildiz *et al.*, 2005.
b) Ribbon presentation of the PER:PER homodimer. Molecule one is shown in red and gray, molecule 2 in yellow and blue. (Yildiz *et al.*, 2005).

norpA (no-receptor-potential-A) is a gene which encodes Phospholipase C (PLC) (Bloomquist *et al.*, 1988), which is essential for fly vision. Mutants for *norpA* show an earlier upswing of PER at both low and high temperatures, via enhanced *per* splicing. Thus *norpA* provides an important

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signalling component for temperature information into the circadian clock (Collins *et al.*, 2004; Majercak *et al.*, 2004). Surprisingly even under constant darkness (dark-dark, DD) *norpA* flies show elevated *dmpi8* splicing that is not reduced by acute photic inhibitions.

PER protein can form both PER:TIM heterodimers and PER:PER homodimers (**Fig. 1.3b**). Only very recently a function for the PER:PER homodimer was proposed; it seems to be involved both in PER repressor activity and in regulating its translocation into the nucleus (Landskron *et al.*, 2009).

timeless

timeless is located on the chromosome 2. In 1995 the *timeless* gene was cloned and sequenced by positional cloning (Myers *et al.*, 1995) and its mRNA is translated into a protein composed by 1398 aa. At the same time, Gekakis and collaborators showed that PER PAS domain is able to bind TIM (Gekakis *et al.*, 1995).

tim cyclic expression is abolished both by TIM over expression (Zeng *et al.*, 1994) and by tim^{01} mutations (Sehgal *et al.*, 1994).

Differently from PER, TIM lacks a PAS domain. A comparative analysis of *tim* sequences from a number of *Drosophila* species allowed the identification of conserved regions:

- a 32 aa region at the N terminus which is necessary for TIM function;
- a region necessary for PER/TIM interaction in which is also located a NLS domain;
- a second region involved in PER/TIM interaction;
- a CLD domain at the C terminus.

At the transcriptional level, it is known that a light pulses stimulates *tim* mRNA expression at cold (18°C) but not at warmer (25°C) temperatures (Chen *et al.*, 2006).

Even temperature exerts a transcriptional effect on *tim* through the alternative splicing of the last intron of the gene. Unspliced *tim* encodes a protein approximately 3.5 kD smaller than that from spliced *tim*. The spliced *tim* transcript is referred to as tim^{cold} but the biological function of its product has yet to be unravelled (Boothroyd *et al.*, 2007).

Moreover *timeless* presents a natural length polymorphism in its 5' sequence that contains two start codons (*ATG*) (Rosato *et al.*, 1997). When in frame, (as in the *ls*-tim allelic variant), these

can generate a long and a short isoform of the TIM protein which differ by 23 amino acids. In the *s-tim* allelic variant the two *ATG*s are not in frame because of the presence of a *G* nucleotide insertion in between and only the second maintains its information. Therefore the translation of this mRNA results only in the short isoform of TIM due to a stop codon located 19 residues after the first methionine which prevents the formation of the longer TIM isoform. The distribution of the two alleles follows a latitudinal cline with *ls-tim* prevalent in southern Italy while *s-tim* predominates in northern Europe (Sandrelli *et al.*, 2007; Tauber *et al.*, 2007). The ancestral allele is the *s-tim* while the *ls-tim* originated about 8000-10000 years ago due to a single nucleotide insertion between the two *ATG*s.

1.4 The central pacemaker of *D. melanogaster*

The analysis of the expression patterns of clock genes in the brain allowed the understanding of the anatomical and functional organization of the pacemaker that controls rhythmic behaviour. In *Drosophila* the master circadian clock is located in the brain and relies on about 100 clock neurons per hemisphere, bilaterally clustered in 7 groups: 3 groups of dorsal neurons (DNs) and 4 groups of lateral neurons (LNs) (**Fig. 1.4**).
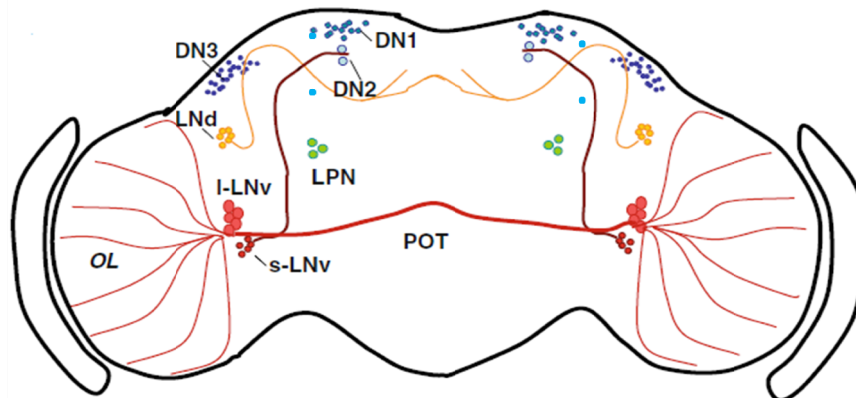

Fig 1.4: Schematic representation of the different clusters of clock neurons within the brain of *D. melanogaster*. In red the LNvs, yellow the LNds, green the LPNs and blue the DNs. The PDF positive projections of the s-LNvs and l-LNvs are also shown. OL: Optic Lobe; POT: Posterior Optic Tract (Tomioka and Matsumoto, 2010).

The lateral neurons 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 in turn classified, based on their relative size, into small and large ventral lateral neurons (s-LNvs and l-LNvs respectively). The dorsal neurons are divided into three groups based on their relative positions and are called the DN1s (approximately 17 in number), the 2 DN2s and the DN3s (a large group with more than 40 neurons). Amongst the DN1s, two are anterior in location (DN1a) and the remaining are posterior (DN1p). The two anterior DN1s are the adult counterparts of the larval circadian neurons.

So far many sophisticated attempts have been carried out in order to understand the function of each group of neurons and it is now quite clear that they are organized in a complex network and that neurons of a specific group can accomplish different function.

ventral Lateral Neurons

Among the different clusters of clock neurons only 4 s-LNvs and the l-LNvs express the neuropetide PDF (PIGMENT DISPERSING FACTOR) involved in the output of the clock (see **Section 1.8**).

In wild-type flies l-LNvs send PDF positive projections to the accessory medulla (aMe) and arborize on the surface of the medulla (Helfrich-Forster, 1997). Additionally, they project via the posterior optic tract (POT) to the other hemisphere.

Albeit the function of l-LNvs is still not fully understood, recent studies proposed that 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 appears to be clearer: it has been shown that these cells are responsible for driving the anticipation of lights-on transition in the morning and are sufficient for maintaining behavioural rhythmicity under constant darkness (Grima *et al.*, 2004; Stoleru *et al.*, 2004). These cells send PDF projections towards the dorsal brain.

The fifth PDF negative s-LNv was first discovered in larvae in close proximity to the four PDF positive cells (Kaneko *et al.*, 1997) whereas in adults it is located in close proximity to the l-LNvs (Rieger *et al.*, 2006). Its function it's not clarified yet; it has been implicated in control of the evening anticipation in addition to three out of six LNds (Grima *et al.*, 2004). It also seems to

perceive light differently from the PDF positive s-LNvs: under constant light (light-light, LL) the period of its clock gene molecular oscillation is shortened whereas the one of PDF positive s-LNvs is lengthened (Rieger *et al.*, 2006).

dorsal Lateral Neurons

The LNds can be further divided in subgroups: the somata of two cells are larger than the other and at least one of these shows a rather high-amplitude cycling for the clock proteins PER and TIM (Rieger *et al.*, 2006). In adult flies LNds cells project into the dorsal protocerebrum, where their neurites largely overlap with the fibers coming from the DNs (Kaneko and Hall, 2000). In 2004 it was shown that LNds are responsible for controlling the evening anticipation of lights-off transition (Grima *et al.*, 2004; Stoleru *et al.*, 2004). These cells are unable to sustain rhythmicity under DD conditions but they drive rhythmic behaviour under LL; indeed it has been proven by Picot and collaborators (2007) that 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).

Lateral Posterior Neurons

This cluster of neurons was first described in 2000 by Kaneko and co-workers as pacemaker neurons expressing TIM but not PER and they were referred to as LPNs for the first time in 2005 (Helfrich-Forster, 2005). In more recent studies, LPNs have been involved in temperature entrainment. Yoshii and co-workers showed that LPNs are among the clock neurons that still present PER oscillation under constant light and temperature cycles (Yoshii *et al.*, 2005). Two years later it was demonstrated that when flies are subjected to a light cycle combined with a temperature cycle advanced by 6 h relative to the light cycle, the dorsally located neurons (DNs) and lateral posterior neurons (LPNs) shift their phase of TIM expression, but the laterally located protocerebral neurons (LNs) basically maintain their original phase (Miyasako *et al.*, 2007).

Dorsal neurons 1

DN1s 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).

In 2004 it was shown by Klarsfeld and co-workers that GLASS positive DN1s contribute to the light sensitivity of the pacemaker network. More recently they have been involved in driving oscillatory behaviour under constant light (Murad *et al.*, 2007; Stoleru *et al.*, 2007).

Only very recently it has been discovered a particular genetic tool that permits to ablate or maintain a functional clock specifically within DN1ps (Zhang *et al.*, 2010): by expressing PER protein only in DN1ps, it was found that these cells promote only morning activity under standard (high light intensity) light:dark cycles and that 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.

Dorsal Neurons 2

The cell bodies of the two DN2s are in close proximity to the dorsal PDF positive projections of the s-LNvs. It has been proposed by (Miyasako *et al.*, 2007) that they are involved in temperature sensitivity as well as LPNs.

Dorsal neurons 3

Genetic analysis in which PER expression is driven only in these neurons showed that these pacemaker cells are not sufficient to maintain locomotor rhythms under constant conditions but they are able to elicit the *per*-dependent evening peak of activity under LD conditions (Veleri *et al.*, 2003).

1.5 Peripheral oscillators

The use of antibodies recognizing clock proteins has indicated their wide distribution throughout the fly body. Apart from the central nervous system (CNS), these tissues include: gut, salivary glands, gonads, compound eyes, fat bodies, malpighian tubules and epidermis. While the expression of clock genes in the brain may be clearly associated with rhythmic phenomena such as locomotion and eclosion, that in peripheral tissues suggests clock-like functions in numerous sites outside the CNS (Giebultowicz, 1999). Of particular interest is clock gene expression in endocrine organs such as the prothoracic glands, or Dipteran ring glands (Emery *et al.*, 1997), which suggests clock regulation of developmental and metamorphic hormones.

In 1997 it was demonstrated that PER protein cycles in the malpighian tubules of *Drosophila* and that its cytoplasmic accumulation was followed by translocation into the nucleus (Hege *et al.*, 1997). The authors showed that this pattern of expression was maintained in decapitated flies proving that malpighian tubules contain a circadian pacemaker that function independently from the brain.

More recent studies allowed to understand some functions of peripheral pacemakers.

In 2008 Numata's group demonstrated that the rhythm of deposition of the two chitin layers with different orientation found in *Drosophila* endocuticle is driven by a peripheral pacemaker located in the epidermis (Ito *et al.*, 2008). Ito and co-workers also showed that this rhythm is independent from the brain clock while it is lost in clock mutants.

Fascinatingly it has been proven by Chatterjee and collaborators that even the responsiveness of flies to tastants is under control of a peripheral circadian oscillator located in the proboscis (Chatterjee and Hardin, 2010). More precisely they showed that tastant-evoked responses are suppressed in the middle of the night and flies carrying a disrupted clock in the proboscis show a typical behaviour of starvation induced stress such as hyperphagicity and hyperactivity.

1.6 Similarities with the mammalian clock

The Mammalian central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Like in *Drosophila*, it is based on transcriptional feedback loops involving

heterodimer PAS-containing proteins that act as transcriptional activators, and other proteins of the same family of the *Drosophila* PER and CRY, as negative elements (**Fig. 1.5**).

In addition to these conserved principles, the mammalian molecular clock reveals duplications of several clock factors that can play different roles, while other factors (notably TIM) appear to have been lost. This is one of the reasons that make the vertebrate's clock more complex than the *Drosophila* one.

The genes *mClk* and *Bmal1* (orthologs of the *Drosophila cyc* gene), (Gekakis *et al.*, 1998), activate the expression of negative regulatory elements like *mPer1, mPer2* and *mPer3* (three orthologs of *dper*) and also the genes *mcry1* and *mcry2* (Kume *et al.*, 1999) and *Rev-erbα* (Preitner *et al.*, 2002) which encode proteins that result to feedback and block their own expression. The REV-ERB protein binds then to the *Bmal1* promoter to repress its expression, which is activated by ROR (Emery and Reppert, 2004)

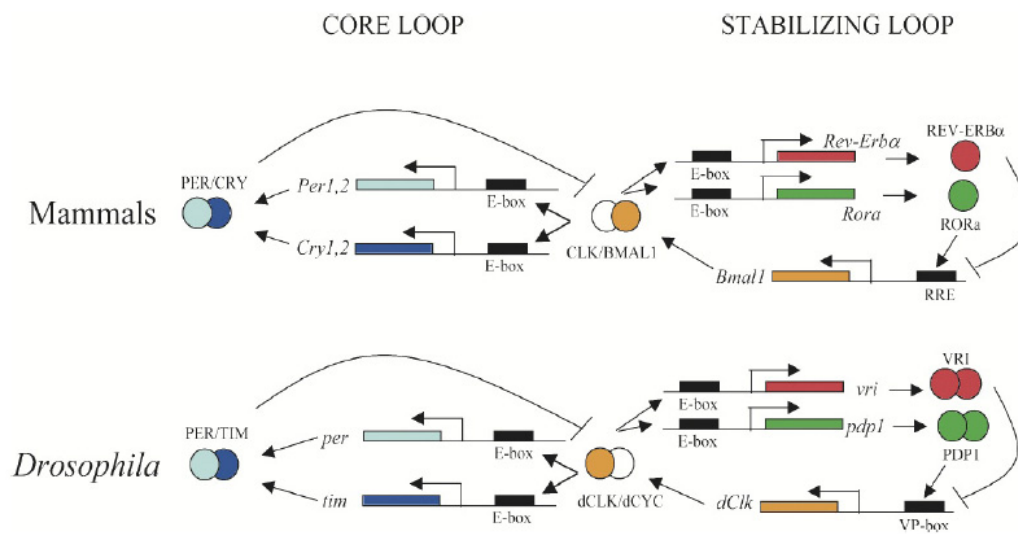**Fig. 1.5**: Schematic representation of the clock machineries of mammal and *Drosophila* (Emery and Reppert, 2004). See text for acronyms.

PER and CRY form a heterodimer able to block the BMAL1-CLOCK complex (BCC): this results in the decreasing of *per*, *cry* and *Rev-erbα* mRNAs. Moreover, PER proteins are phosphorylated by the Casein-kinase1 (homolog of CK1-*dbt*), and their degradation is controlled by TrCP (dSLIMB homolog).

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A protein similar to *Drosophila* TIM, that is a paralog of dTIM, is present in the SCN, but no clear role can be ascribed to it yet. In fact CRY and CRY2 seem to be the functional analogous of dTIM protein; meanwhile these proteins seem to be not involved in light sensing as in *Drosophila* as well as in most other species.

These similarities indicate that a basic clock mechanism was present in a common ancestor, before the separation of insect and mammals more than 500 million years ago.

1.7 Entrainment of the *Drosophila* molecular clock

One of the main properties of the circadian clock is represented by its ability to synchronize in response to environmental stimuli, such as light and temperature. When a circadian system is entrained to a certain *Zeitgeber* its overt period is on average identical to the one of the *Zeitgeber* and the phase relationship between internal and external rhythms is stable.

Entrainment by light

Circadian clocks are entrained by light to follow the daily-solar cycle. *Drosophila* uses at least three light input pathways for this entrainment: (1) CRYPTOCHROME (CRY), a blue-light photoreceptor related to the family of photolyase and involved in the synchronisation of central and peripheral clocks, (2) the compound eyes, and (3) extraocular photoreception, involving an internal structure known as the Hofbauer-Buchner eyelet, which is located underneath the compound eye and projects to the pacemaker center in the brain.

The consequence of light entrainment is that the phase of the internal clock is modified until the internal subjective day corresponds to the external phase of the day.

CRY belongs to a family of blue-light sensitive protein, which includes pholtolyases and plant blue light photoreceptors. CRY uses a vitamin B2-based flavin chromophore and shows a maximum sensitivity to blue light at 400-500nm.

Flies over-expressing CRY are hypersensitive to light pulses (Emery *et al.*, 1998). On the other hand, flies carrying a mutation on the *cry* gene (*cry^b*: amino acid substitution in the flavin binding domain that renders the protein hypomorph with little, if any, functionality) show an altered ability to respond to light both at molecular and behavioural level: e.g. disruption of PER and

TIM molecular cycle in peripheral tissue under LD conditions and inability of mutant flies to reset their clock after short light pulses (Stanewsky *et al.*, 1998). Moreover it has been shown that *cry*[b] flies are rhythmic in intense constant light, a condition that renders wild-type flies arrhythmic (Emery *et al.*, 2000).

CRY expression pattern has been unambiguously revealed only recently by C. Helfrich-Förster group (Yoshii *et al.*, 2008). Yoshii and co-workers generated a mutant lacking most of the CRY protein (CRY[OUT]: deletion of the DNA encoding the N-terminal 96 amino acids of the total of 542 residues constituting the protein) and raised an antiserum against the entire CRY protein to clarify its localization. They observed specific CRY staining in all s-LNvs and l-LNvs, in three out of six LNds, in the two DN1as and in six DN1ps. No staining was observed within the DN3s and LPNs. Moreover, they found that the staining intensity in the s-LNvs (the 4 PDF positive cells as well as the 5th PDF negative) and in the three LNds was stronger than in the other CRY positive pacemaker neurons. Furthermore several cells were labelled in the anterior central brain; these cells projected to the ellipsoid body of the central brain complex. Staining was also present in putative glial cells, in terminal photoreceptor cells and in compound eyes.

CRY levels oscillated within the clock neurons, with a through during the day and a peak during the night, confirming a previously reported light dependent CRY degradation (Lin *et al.*, 2001).

The role of CRY within the molecular clock is to induce light-dependent TIM degradation. Yeast two-hybrid essays showed that CRY interacts with TIM and PER-TIM complex in the presence of light but not in the dark (Ceriani *et al.*, 1999). So the CRY-TIM interaction leads to the degradation of TIM coupled with molecular re-setting of the clock-gene cycling (**Fig 1.6**).

The CRY-TIM interaction involves another protein called JETLAG (Koh *et al.*, 2006) which is also involved in the light-dependent degradation of CRY (Peschel *et al.*, 2009). It has been demonstrated that this interaction is mediated by the carboxy-terminus of the CRY protein. This has been demonstrated using CRYΔ which lacks the C-terminal end (the last 20 amino acids). CRYΔ is constitutively active and binds PER and TIM in both light and dark (Dissel *et al.*, 2004). This C-terminal region of CRY is highly variable among species while the N-terminal region is more conserved.
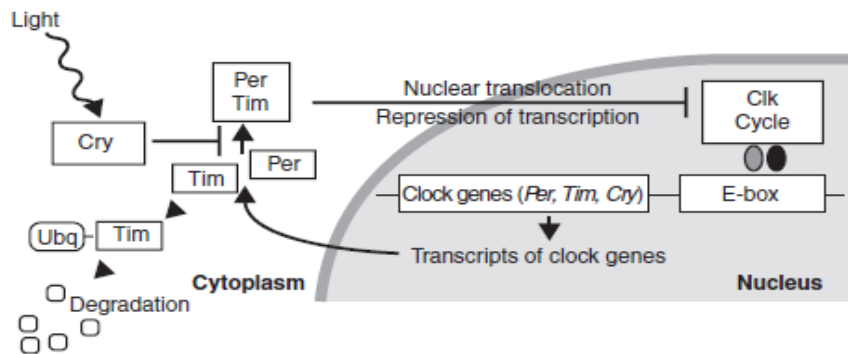
Fig 1.6: Schematic representation of CRY mediated resetting of the molecular circadian clock in *Drosophila*. See text for acronyms (Lin and Todo, 2005)

Since *cry*[b] flies can still entrain to LD cycles, it has been proposed by Helfrich-Förster and collaborators the possible existence of a CRY-independent light input pathway (Helfrich-Forster *et al.*, 2001).

Possible candidates were the compound eyes, the ocelli and the Hofbauer-Buchner (H-B) eyelet, a pair of putative extra-retinal eyes underneath the retina that projects directly towards the LNvs. All these types of structures express opsin-based photo pigments that are implicated in behavioural entrainment, since eyes and vitamin A depleted flies show a reduced circadian light sensitivity. The authors used mutants lacking all known retinal eye structures as well as the extra retinal H-B eyelet in addition to being CRY depleted. These flies were totally unable to adjust the circadian system to LD cycles. Thus, fruit flies exploit at least three photoreceptors for light entrainment: CRY, compound eyes and H-B eyelet.

The transduction of light information by the eyes to the clock occurs as follows: rhodopsins, when activated by light, are converted into metarhodopsins which in turn bind to G proteins; this complex then activate PLC (Phospholipase C) encoded by the gene *norpA* and opens two calcium permeable channels called Transient Receptor potential (TRP) and TRP-like (TRPL) channel (Hardie and Raghu, 2001; Pak and Leung, 2003)

Temperature entrainment

The fly circadian clock can be synchronized daily by both LD cycles and temperature cycles (thermopase-cryophase, TC). TC cycles can synchronize the behaviour with an amplitude as

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small as 3°C (Wheeler *et al.*, 1993) and also induce behavioural rhythmicity even under constant light conditions (Yoshii *et al.*, 2005). Adult flies can synchronize to TC and once released in constant conditions (in this case constant temperature) they keep the rhythm of the previous TC cycle (Busza *et al.*, 2007).

In *Drosophila*, many tissues/structures (e.g. legs, wings, heads) are able to entrain to TC cycles in an autonomous manner (Glaser and Stanewsky, 2005) and it was shown that the antennae contain peripheral thermosensors responsible for moderate temperature sensing (Sayeed and Benzer, 1996).

Glaser and Stanewsky (2005) also found that, under TC, isolated brains show a 12 hours phase advance in the bioluminescence peak of a *per-luciferase* reporter they used compared to all other tissues. This phase advance was not noticed under LD regimes at constant temperature meaning that light can be the predominant entrainment stimulus for the brain.

Only recently, Sehadova and co-workers demonstrated that the master clock in the brain receive temperature information by peripheral thermosensors located in the chordotonal organs and that mutants for the gene *nocte*, that encodes for a glutamine rich protein, are defective in temperature entrainment (Sehadova *et al.*, 2009).

Other mutants that fail to entrain to TC under constant light conditions are *norpA* mutants (Glaser and Stanewsky, 2005): this occurs most probably because *norpA* is involved in the signalling pathway that regulates the thermosensitive splicing of *per* mRNA (Collins *et al.*, 2004; Majercak *et al.*, 2004). Thus *norpA* provides an important component for the temperature entrainment of the circadian clock, in addition to its well established function in the phototrasduction pathway.

Wild type flies respond to high-temperature pulses as they do to light pulses: it has been demonstrated that the blue-light photoreceptor CRY is also involved in this response. Kaushik and colleagues described a high temperature dependent interaction between CRY and PER:TIM during the early night and showed that *cry^b* flies are unable to phase shift their locomotor activity rhythms in response to heat pulses (Kaushik *et al.*, 2007). Moreover the authors found that the arrhythmic behaviour of wild type flies in LL conditions was clearly reduced at low temperature (15°C): the interaction between CRY and PER:TIM occurs prevalently at higher temperature.

1.8 Outputs

The internal circadian clock is essential to the organisms because it allows the rhythmicity of important physiological and behavioural processes: the circadian pacemaker is connected to many effector systems and organs through output pathways.

Glutamate and four other transmitter have been identified as candidate signalling molecules in the fly circadian system: Pigment Dispersing Factor (PDF), short neuropeptide F (sNPF), Neuropeptide Precursor-Like Protein 1 (NPLP1) and Ion Transport Peptide (ITP).

The *Drosophila* vesicular Glutamate Transporter (DvGluT) is expressed within the DN1s in the larval brain and in some DN1s and DN3s in the adult brain (Hamasaka *et al.*, 2007). Moreover it was found that the Glutamate receptor DmGluRA is expressed within the LNvs of both larvae and adults. Since DmGluRA knockdown flies showed altered LD activity patterns and a lengthened period under constant darkness, it was inferred by Hamasaka and collaborators that a glutamate signal from some of the DNs to the LNvs modulates the rhythmic behaviour pattern via DmGluRA.

sNPF is expressed within a subset of the LNds and in the four PDF-positive s-LNvs in the male adult brain (Johard *et al.*, 2009). In the same work the authors found another neuropetide called Ion Transport Peptide (ITP) to be expressed among the clock neurons, more precisely in one NPF positive LNd and in the fifth PDF negative s-LNvs.

NPLP1 was first described following a peptidomics analysis of larval nervous system (Baggerman *et al.*, 2002). The precursor contains multiple predicted peptides, two of which have been identified: IPNamide and MTYamide. NPLP1 is expressed in the 2 DN1as: the cells of the DN1s cluster that do not express the transcription factor GLASS and derives from the larval DNs (Shafer *et al.*, 2006).

Among the neuropeptides expressed by the circadian clock neurons the most studied and best characterized so far is for sure PDF. It is constitutively expressed by the l-LNvs and by four out of five s-LNvs (**Fig 1.7**).
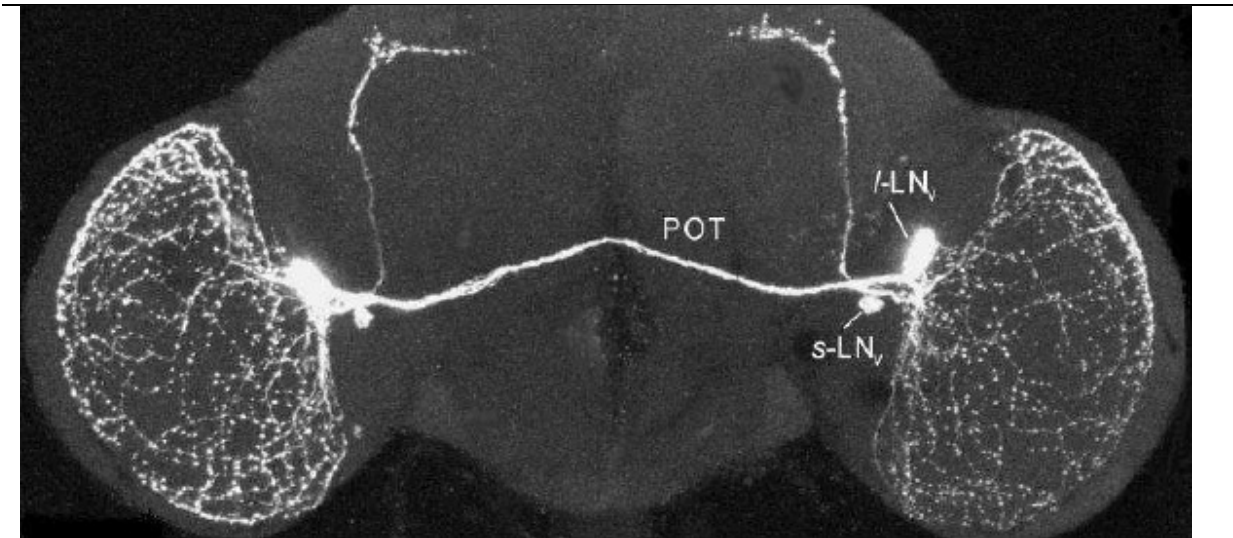
Fig 1.7: PDF immunoreactivity of *D. melanogaster* brain. (Helfrich-Forster, 2003)

PDF is rhythmically secreted by the dorsal projections of the s-LNvs with a peak at ZT2-3 and a trough at ZT13-15. Ectopic expression of the neuropeptide in the same brain region in which are the terminal axons of the LNvs, strongly affects the locomotor activity and the eclosion rhythms (Helfrich-Forster *et al.*, 2000). These findings strongly suggest that that the rhythmic release of the PDF neuropeptide allows the temporal information to the other nervous cells and to the locomotors centres.

pdf^0 flies (*pdf*-null mutants) show almost normal locomotor activity under LD, but they are unable to anticipate lights-on transitions and their evening peak is advanced (Renn *et al.*, 1999). It has been also shown by Renn *et al.* (1999) that PDF is required to maintain behavioural rhythmicity in constant darkness : a large fraction of pdf^0 flies becomes arrhythmic when released in DD.

In 2004, it has been shown by Lin and collaborators that the lack of PDF affects in a different manner the circadian pacemaker in s-LNvs (normally PDF-positive) and LNds (normally PDF negative), (Lin *et al.*, 2004). In pdf^0 flies kept in constant darkness, they observed that the phase of the s-LNvs gradually dispersed, contrary to wild type flies. The phase of LNds did not disperse, but instead appeared permanently advanced.

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In some respects, the phenotype of *pdf* mutant flies resembles the phenotype of knockout mice lacking the function of VIP orVPAC2 receptor genes (Aton *et al.*, 2005).

The receptor for PDF (PDFR) is a G-protein-coupled seven transmembrane receptor (Hyun *et al.*, 2005). PDFR mutants phenocopy *pdf⁰* flies: they show arrhythmicity or short period rhythms under constant darkness.

In 2008 Shafer and co-workers measured receptivity to PDF using transgenic flies expressing Epac1-camps (a genetically encoded fluorescence resonance energy transfer, FRET, based cAMP reporter) under GAL4 control: they reported that all groups of clock neurons but l-LNvs respond to PDF with an increase in cyclic nucleotide levels (Shafer *et al.*, 2008). Although many works tried to elucidate PDFR expression pattern, only recently it was unambiguously detected: PDFR is expressed within some of the clock neurons, some non-clock brain cells and in the visual system (Im and Taghert, 2010). More precisely among the clock neurons six DN1s, the 5th s-LNv and three LNds strongly express PDFR whereas the four PDF positive s-LNvs, two l-LNvs, four DN1s, the DN2s and the DN3s express it at lower level.

1.9 The circadian clock of D. *melanogaster* under natural conditions

So far, the majority of the studies on the circadian clock, either in *Drosophila melanogaster* or other animals, have been carried out under controlled laboratory conditions.

Nevertheless some attempts to understand how the clock entrains to the real natural environment have been made mimicking natural-like conditions in the lab.

At the behavioural level it was shown that environmental conditions that simulate seasonal changes can alter clock genes expression to adjust the behaviour to the mutated conditions (Majercak *et al.*, 1999).

In 2004 Shafer and colleagues evaluated the effect of changing day length on nuclear accumulation of PER and TIM within a subgroup of pacemaker neurons: the LNvs (Shafer *et al.*, 2004).

The authors found that the nuclear accumulations of PER and TIM respond differently to changing day length:

- under short photoperiod (LD 8:16) TIM was detectable in the nucleus 4-6 hours after lights-off and the nuclear signal was predominant 8 hours after light-off; under long

photoperiod TIM was never found to be predominantly nuclear, it was as strong as the cytoplasmic one 8 hours after lights-off but then it dropped right after lights-on.

- PER was evident in the nucleus 6 hours after lights-off both under long and short photoperiods, however only under a long photoperiod PER staining appeared to be higher than the cytoplasmic staining. Moreover under short night conditions, PER staining was detectable within the clock neurons until 8-10 hours after lights-on.

It is clear that light is a key *Zeitgeber* for the *Drosophila* circadian clock but also temperature plays an important role: in fact either light or temperature are able to quickly reset the circadian clock. Numerous studies focused on the analysis of the role of one of the two environmental stimuli on the clock, nevertheless in nature light and temperature are tightly linked together. Only in 2009, Yoshii and colleagues measured natural cycles of light and temperature in the field and tried to reproduce these cycles in the lab to dissect the role of the two parameters in entraining the circadian rhythm of locomotor activity as well as the molecular clock (Yoshii *et al.*, 2009). Upon analysis of locomotor activity under LD, TC or combined LD and TC, the authors suggested that probably the phase of the peak of activity is set by light whereas temperature may support the entrainment by modulating the temporal extension of activity. To test whether these diverse environmental conditions can differently affect the molecular oscillation of the clock proteins within the pacemaker neurons the authors looked at TIM profile under LD, TC or a combination of the two. It was shown that the combination of LD and TC induces higher amplitude of TIM cycling in DNs and LPNs and, in general, causes a delay both in TIM accumulation and degradation.

The main function of the circadian clock is to synchronize the internal processes of the organism with the changing environment. In nature a stable synchronization to the environment is more difficult than in the lab since, for example, light intensity and light spectrum compositions during dawn and dusk can vary considerably from day to day depending on weather conditions.

In 1969, Bünning measured irradiances during day and night finding that the smallest fluctuations from one day to the other were recorded during early dawn and late dusk (irradiances < 10 lux). He also demonstrated that bean plants synchronize to light of moonlight intensities (Bunning and Moser, 1969). Whether animals could be so light sensitive as plants is still under investigation.

In *Drosophila melanogaster* the effect of dim light on the circadian clock has been studied in detail by Helfrich-Förster's group. In 2007 Bachleitner and co-workers studied the effect of

cycles of light-moonlight (LM) on both locomotor activity and PERIOD and TIMELESS oscillation, therefore mimicking moonlight in the lab (Bachleitner *et al.*, 2007). The authors showed that LM cycles produce an increase of locomotor activity during the night compared to LD conditions. Moreover they found that the phase of the 5th s-LNv is advanced compared to the other clusters of clock neurons under LD conditions whereas it is delayed under LM conditions and the phase of the s-LNv is shifted towards opposite directions under the two different conditions. Thus, the phase of the 5th s-LNvs shifts as the evening activity peak does comparing the activity under LD or LM conditions, whereas the shift in the phase of the s-LNvs could explain the advance of the morning peak under LM. In any case, a couple of year later the same group demonstrated that more likely the increased nocturnal activity under LM is induced by the direct effect of light since it is present also in clock mutants (Kempinger *et al.*, 2009).

Until very recently, the laboratory studies on the circadian clock of *D. melanogaster* used individuals entrained in rectangular cycling conditions, usually at constant temperature. Despite the attempts made to mimic natural conditions in the lab, laboratory conditions cannot reproduce properly what happens in the real natural environment. In nature not only light intensity changes continuously during the day (and night) but also the composition of the light spectrum varies throughout the 24 hours.

Apart from the differences in the light characteristics between natural and laboratory environment, also natural temperature changes cannot be exactly reproduced in the lab: ambient temperature changes continuously and even when this changes were mimicked in the lab, it was not considered that outside temperature is never the same from day to day.

In 2006 our lab started a project (granted by the European Commission, 6th Framework Programme; Project EUCLOCK N° 018741) in which a collaboration with the group of Prof. C. P: Kyriacou's at the Department of Genetics, University of Leicester (UK), was aimed to the characterization of the circadian clock of *D. melanogaster* under real natural conditions. This has been done by studying the entrainment of flies exposed to the natural environment by placing the standard equipment used in the lab studies outside (both in Leicester, UK, and in Villorba, TV, Italy). Prior to the work reported in this thesis, locomotor activity and oscillations of *per* and *tim* mRNA levels have been analyzed in detail in the studies carried out by S. Bhutani (University of Leicester) and S. Montelli (University of Padova) respectively. It is important to mention that a natural strain of wild type flies (WT-ALA) was used throughout these works.

The results obtained by these studies are summarized in the next paragraph.

Locomotor activity of wild-type flies under natural conditions

Wild-type flies show a bimodal activity at high temperatures whereas they exhibit a unimodal pattern when temperature is low. This turned out to be true for both natural and lab mimicked natural conditions. Under natural conditions, during the hottest summer days it was evident a third peak of activity, named the "afternoon peak", occurring in the middle of the day, when flies are supposed to rest because of the hot temperature (siesta). It has also been shown that under the same summer conditions the activity during the night is increased. Furthermore it was proven that in nature moonlight do not affect the activity of the fruit flies has hypothesized under lab conditions (Bachleitner *et al.*, 2007). More in detail, it has been observed that the onset of the morning activity is very sensitive to ambient temperature and it occurs earlier during long and hot summer days than in spring or autumn (**Fig. 1.8a**). In addition, during cold days the onset of morning activity occurs far after lights-on, most likely there is no anticipation of dawn, whereas in summer the morning activity can be initiated by light intensities that are really low (nautical twilight, occurring when the center of the sun is between 6° and 12° below the horizon). Also the evening peak of activity has been shown to be temperature sensitive since it occurs earlier when temperature is lower (**Fig1.8b**). Moreover comparing the behaviour of flies exposed to full natural conditions to the behaviour of flies experiencing the natural T cycles in artificially induced constant darkness it was proposed that light delays the onset of the evening peak: flies experiencing natural T cycles under constant darkness starts to move earlier in the evening than flies exposed to full natural conditions.

Fig 1.8a: Change in the position of the onset of morning activity with seasons in Leicester (blue) and Padova (orange). The position of the morning onset is represented as the number of minutes before or after nautical twilight across the seasons. Negative values indicate anticipation, positive values mean that the onset of the morning activity occurred after nautical twilight. (S. Bhutani, PhD Thesis, 2009).
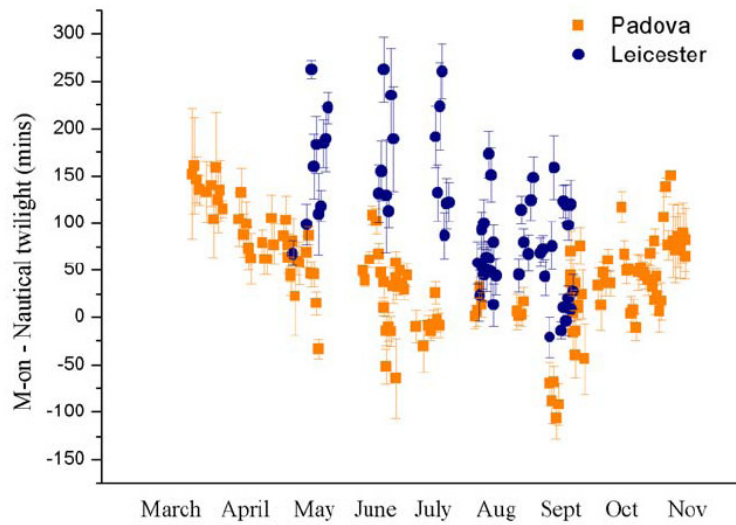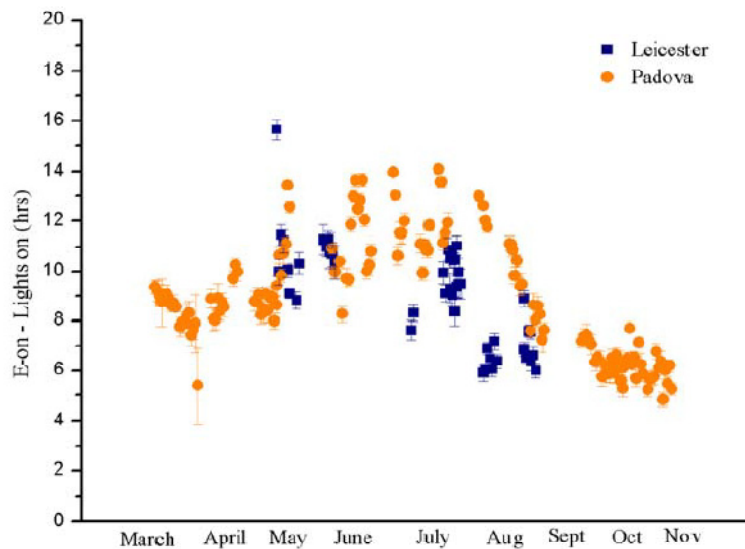

Fig 1.8b: Change in the position of the onset of evening activity with seasons in Leicester (blue) and Padova (orange). The position of the evening onset is represented with respect to lights-on, i.e., the time when light intensity starts to increase (lux ≥1). (S. Bhutani, PhD thesis, 2009)

per and *tim* mRNA profiles under natural conditions

per and *tim* mRNA cycling was analyzed via RT-PCR. Flies were sampled both under natural conditions and, as a control, laboratory conditions at three different constant temperatures.

Under lab conditions (LD12:12, at constant 23, 18 or 10°C) it was evident that both *per* and *tim* mRNA were cycling at 23 and 18°C but no messenger oscillation was detected at 10°C. Moreover the result obtained by Majercak and co-workers in 1999 was confirmed. The amount of *per* mRNA is higher at lower temperature whereas the quantity of *tim* mRNA increases with increasing temperature.

Upon analysis of the results obtained under natural conditions it was evident that *tim* mRNA is always cycling, both under long and warm days and under short and cold days. Besides, *per* mRNA showed a statistically significant cycle only under long and hot days; its profile appeared to be virtually flat at the beginning of Spring and at the end of Autumn and it only showed a maximum at the end of the night and a minimum at the end of the day in all other conditions. Notably, peak of *per* and *tim* mRNAs, when present was occurring almost in synchrony.

1.10 Aims

In the last decade many studies have been carried out with the aim of a better understanding of mechanisms through which the different clusters of clock neurons perceive environmental stimuli, communicate with each other and control the different aspects of physiology and behaviour. Nowadays we do know that the s-LNvs are able to drive circadian rhythmicity in DD (Grima *et al.*, 2004; Stoleru *et al.*, 2004) or that LPNs and DN2s can be involved in sensing temperature (Miyasako *et al.*, 2007; Yoshii *et al.*, 2005). Obviously, in order to understand this in detail, the circadian clock system of *Drosophila* has been simplified concerning both the environmental conditions in which flies were entrained and the complexity of the neural network that underlies circadian rhythmicity.

If knowing in detail how the circadian clock works in a simplified environment is of extreme importance, it also true that at the moment we do not have any documented idea of what is happening in the real life, we cannot be certain yet that what we are observing in the lab is also true outside. As already mentioned in the previous paragraph the natural environmental

conditions are really different to what flies experience in the lab where parameters such as light and temperature are artificially controlled. In nature light is changing continuously both in intensity and spectrum, temperature is never the same, a sunny day can be followed by a cloudy or rainy one.

The work reported in this thesis is part of the project jointly undertaken by our laboratory at the Department of Biology of the University of Padova and Prof. Kyriacou's lab at the Department of Genetics of the University of Leicester (UK); the project was granted by the European Commission (6th Framework Programme; Project EUCLOCK N° 018741).

After the analysis of locomotor activity and *per* and *tim* mRNA oscillations under natural conditions we wanted to describe in detail the molecular clockwork within the neurons that control circadian rhythmicity. More precisely we decided to look at the oscillations, within the clock neurons, of two cardinal components of the first feedback loop of the *Drosophila* molecular clock: PERIOD and TIMELESS, whose mRNA oscillation had already been studied under such conditions in the work performed by S. Montelli at the Department of Biology of the University of Padova. Here also a natural wild-type strain of flies (WT-ALA) has been used.

Once we revealed the oscillation profiles of PER and TIM under natural conditions we tried to recreate it in the lab: this part of the project was achieved thanks to the collaboration with Prof. Charlotte Helfrich-Förster's laboratory, at the Department of Neurobiology and Genetics, University of Würzburg (DE).

Chapter 2:

Materials and Methods

2.1 GAL4-UAS system

The GAL4-UAS binary system represents an important tool to target gene expression in specific tissues or cells in *Drosophila melanogaster* (**Fig. 1.1**).

GAL4 encodes a protein of 881 amino acids, identified in the yeast *Saccharomyces cerevisiae* as a transcriptional factor able to bind DNA sites, defined Upstream Activation Sequences (UAS).

By using this system, the yeast GAL4 protein can be expressed in specific cells or tissues by using selected enhancers or promoters. GAL4, upon binding to upstream activating sequences (UAS), activates a gene of choice which has been cloned under the control of UAS motif.

To generate transgenic lines expressing GAL4 in different cell- and tissue-specific patterns, an enhancer detection vector carrying the GAL4 gene is inserted randomly into the genome by P-element-mediated transposition.

Fig. 1.1: Schematic representation of the GAL4-UAS system
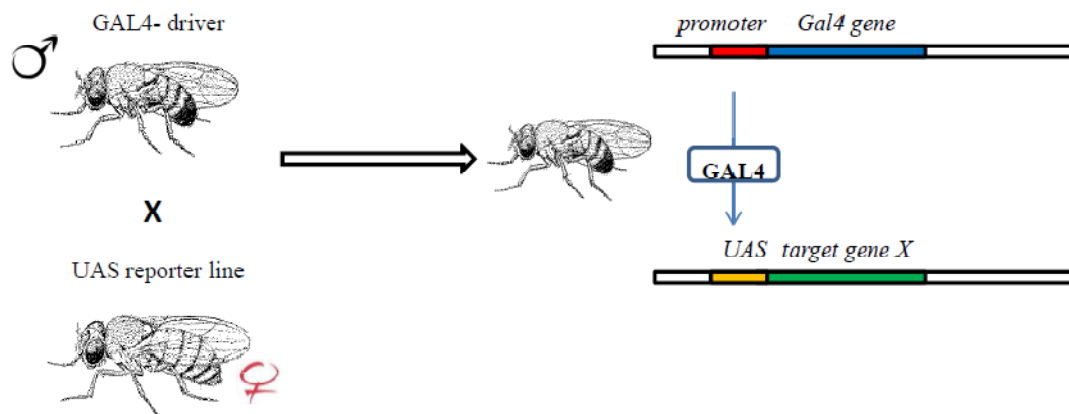
When the vector integrates near a genomic enhancer, that enhancer can activate GAL4 expression. A GAL4-dependent target gene is then constructed by linking GAL4 binding sites to a sequence of interest (Gene X). The target gene is silent in the absence of GAL4. To activate the target gene in a cell- or tissue-specific or temporal pattern, flies carrying the target gene (UAS-Gene *X*) are crossed to flies expressing GAL4. In the progeny of this cross, it is possible to activate *UAS-Gene X* in the cells where and when GAL4 is expressed.

2.2 Fly stocks, media and growth conditions

Fly stocks

The various wild-type and mutant strains of *Drosophila melanogaster* used for locomotor activity and immunocytochemistry experiments are listed below.

Wild-type

- WT-ALA (Wild-Type ALto Adige) is a natural strain obtained from *D. melanogaster* females collected during September/October 2004 in different contiguous localities in the North of Italy (46°N, 250-590 m a.s.l., **Tab. 2.1**). The original isofemales lines are also maintained in the lab.

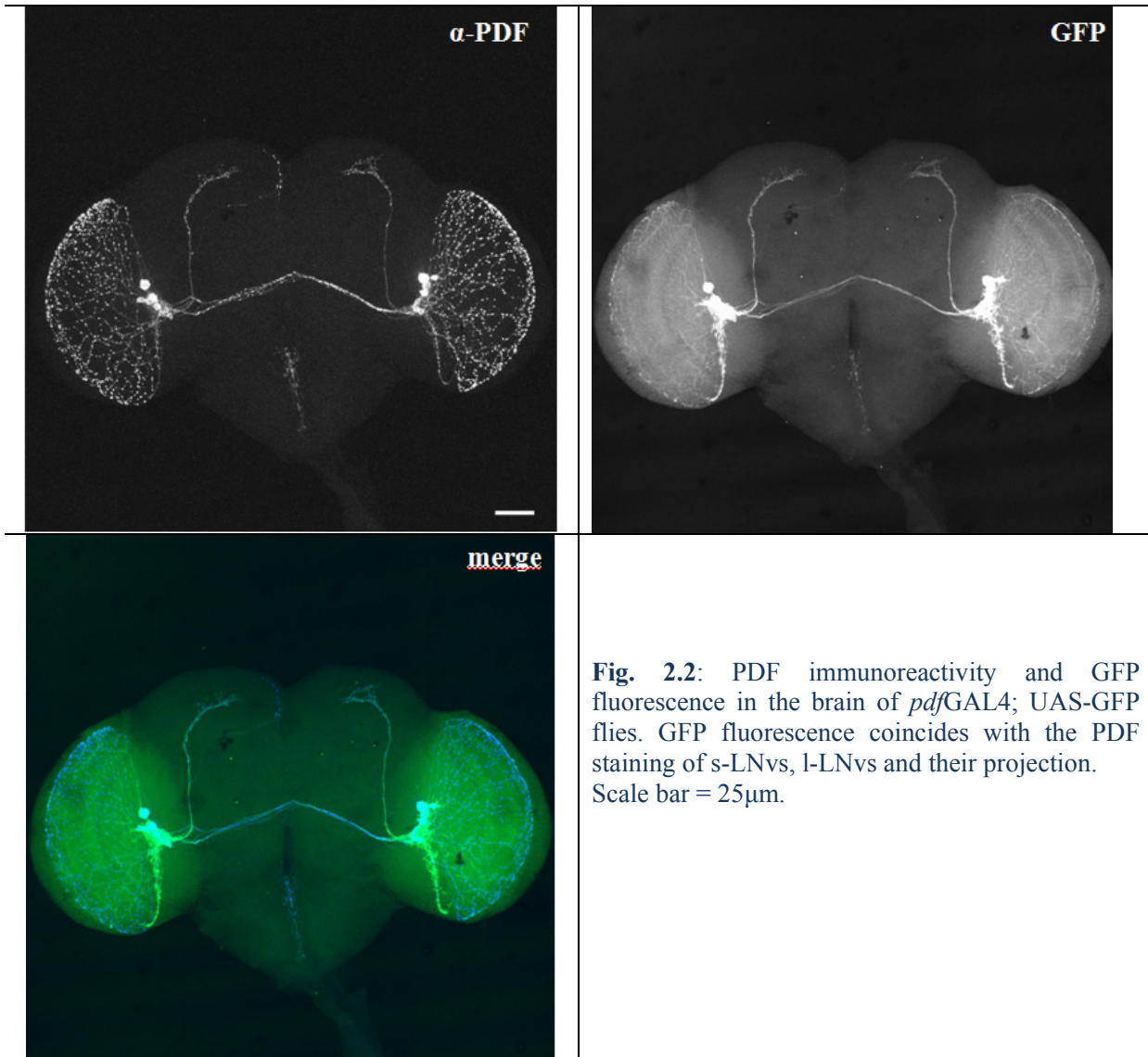| Area of Sampling	Location (Altitude a.s.l.)	Number of Isofemale lines	Sub-Total
Val Venosta, Merano (BZ)	Naturno (590 m)	7	20
	Foresta- Lagundo (380 m)	3	
	Foresta (460 m)	2	
	Postal (280 m)	8	
Bassa Val Venosta	Bolzano (250 m)	17	17

Tab. 2.1: Isofemale lines used to establish the wild-type strain WT-ALA.

In order to maintain stable an high genetic variability in the WT-ALA population established from the natural lines, individuals coming from all 37 isofemale lines are routinely (monthly) added to the strain in use.

Mutant strains

- $per^0 w$;; UASper16: recessive null mutation at the *per* gene (nucleotide substitution at position 464 that results in a premature stop codon). These flies lack both morning and evening activity peaks in LD conditions and they are completely arrhythmic in constant darkness (DD conditions). They still have lights-on and lights-off responses. These flies also carry the *per* gene under the control of an UAS sequence. The UAS*per16* construct contains a *per* cDNA starting 46 bp upstream the ATG and ending at position 4230 in the mRNA; therefore, this construct lacks part of the 3' UTR of the *per* mRNA. (Blanchardon *et al.*, 2001). This strain was kindly provided by F. Rouyer, INAF, CNRS Gif sur Yvette, FR.

- tim^{01}: loss of function mutation due to a frame-shift caused by a deletion of 64 nucleotide that leads to a premature stop codon (Myers *et al.*, 1995). Mutants exhibit a non functional clock, thus, as in per^{01} flies, anticipations of lights transitions are lost as well as rhythmic locomotor activity under constant conditions.

- *yw*-UAS*hid*-UAS*reaper*: P-element insertion on the X chromosome which contains the sequence encoding *hid* and *reaper* genes. *head involution defective* (*hid*) and *reaper* are pro-apoptotic genes in *Drosophila* which induce cell death where they are expressed.

- *yw; pdf*GAL4: GAL4 transgene insertion under control of *pdf* promoter. The GAL4 expression in the brain is therefore driven only in the PDF-positive ventral lateral neurons (LN_vs; see **Fig. 2.2**).

- *w;* UAS-GFP: P-element insertion carrying the *gfp* gene under control of UAS sequences. The Green Fluorescent Protein (GFP) is a jellyfish protein that emits green light when

excited. GFP expression driven by different GAL4 drivers permits to visualize exactly the cells in which they are active.

Fig. 2.2: PDF immunoreactivity and GFP fluorescence in the brain of *pdf*GAL4; UAS-GFP flies. GFP fluorescence coincides with the PDF staining of s-LNvs, l-LNvs and their projection. Scale bar = 25μm.

Media and growth conditions

Flies were raised to adulthood in incubators with alternative cycle of 12 hours of light and 12 hours of dark in vials containing sugar food (231.5g sugar, 50gagar, 41g dried yeast, 5l water, 50ml 20% Nipagen in ethanol).

2.3 Locomotor activity experiments

Different types of devices recording *Drosophila* activity were developed in the past 20 years. The commercial TriKinetics (www.trikinetics.com) *Drosophila activity monitors* are currently the most widely used.

Individual flies are introduced in small glass tube half filled with the usual growth medium. Each tube is monitored by an infrared emitter-detector that will record how many times the walking fly has interrupted the light beam during a defined period of time (bin). Each bin of the experiment will be associated with an activity value and the resulting file will be analyzed to detect rhythmic patterns.

The data were recorded every 5 mins and transferred to a computer and then grouped into half hour bins for further analysis.

Since experiments were performed under natural conditions, the TriKinetics locomotor activity monitors were kept outside and connected to a computer. Even though this environment could be not fully representative of the real habitat of fruit-flies, nevertheless it enables us to monitor their activity in 'natural' light and temperature cycles. For this purpose, it was ensured that no artificial light reached the location where the flies were kept. Also, the monitors were protected from rain, and, something important, the chosen location was not under direct sunlight and the monitors were placed in a homogenously shadowed area.

More precisely the natural station for our field studies was located in Villorba, Treviso (Italy; 45° 43' 19" N, 12°15'14" E, 22 m a.s.l.).

An environment monitor (DEnM, TriKinetics Inc) was used to record the ambient temperature in degrees C, light intensity in lux and humidity of the conditions throughout all the experiments conducted in the wild. The conversion of light intensity from Lux to the more appropriate μEinstein/m2/sec depends on the wavelength of light and thus on the light source itself. An approximate measure of conversion is 1000lux = 16- 20μEinstein/m2/sec.

Data collection and Analysis

The locomotor activity monitors were connected to a computer using a Power Supply Interface Unit (TriKinetics Inc) and the data were collected using the DAMSystem2.1.3 software

(TriKinetics Inc). The data for the days of interest were then extracted using the DAMFileScan102 software (TriKinetics Inc). Only flies that survived till the end of the experiment were used for all subsequent analysis.

The average activity histogram (actogram) of entrained flies was plotted using Microsoft Excel® 2007. The actograms were then used to determine the parameters studied for all the natural activity data that had been collected. In **Fig. 2.3** a diagram representing locomotor activity and environmental parameters recorded or estimated under natural conditions is shown.
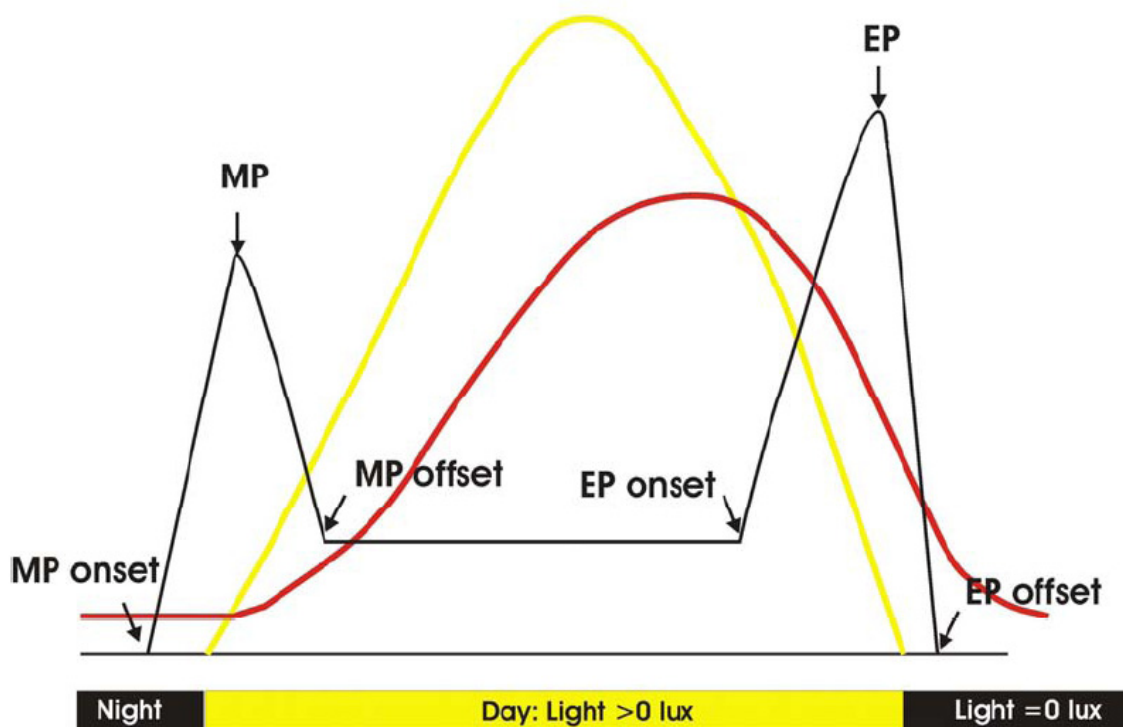

Fig. 2.3: Diagrammatic representation of locomotor activity and environmental parameters (yellow=light and red=temperature) recorded under natural conditions. For both morning and evening burst of activity the onset, peak and offset are represented.

2.4 Immunocytochemistry (ICC)

For the immunocytochemistry experiments flies were either entrained under natural conditions (as explained in **Section 2.3**) or in the lab.

For lab entrainment, the vials containing the flies were housed in light boxes equipped with 15 light emitting diodes (LEDs) programmed to turn on and off using timers according to the experimental light regime. The light boxes were placed into incubators (SANYO Biomedical) that maintained the experimental temperature. The LEDs of each light box were electrically wired such that they could be turned on as 3 sets of 5 LEDs each if so required.

Buffers composition

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH of 7.4

PFA: Paraformaldehyde 4% in PBS

0.3% PBS T: 1% triton X-100 in PBS

1% PBS T: 1% triton X-100 in PBS

BSA: Bovine serum albumin

Primary antibodies

	Protein detected	*Origin*	*Working concentration*	*Provenience*
α-PER	PERIOD	Rabbit	1:2500	R. Stanewsky (London)
α-TIM	TIMELESS	Rat	1:2500	L. Saez (New York)
α-PDF	PIGMENT DISPERSING FACTOR	Mouse	1:5000	Hybridoma Bank

Tab. 2.2: Different primary antibodies used.

Secondary antibodies

	IgG detected	Origin	Working concentration	Provenience
Alexa Fluor 488	Mouse	Goat	1:250	Invitrogen
Alexa Fluor 568				
Alexa Fluor 647				
Alexa Fluor 568	Rat			
Alexa Fluor 488	Rabbit			

Tab. 2.3: Different secondary antibodies used.

Sample preparation

After entrainment, at least 10 flies were collected and dissected per time point.

Flies were collected at the appropriate time points and fixed in 4% Paraformaldehyde (PFA) by incubating for approximately 2 h on a rotating wheel. They were then washed three times for 15 mins each in PBS and then dissected in PBS. Afterwards, brains were treated as follow:

Fixation: PFA 4% 1 hour at room temperature (RT);

Washing: 0.3% PBS-T, 6 times 10 min, RT;

For GFP observation brains were prepared following the step **mounting**; for the immunoassay, brains were processed following the subsequent steps:

Permeabilization: 1% PBS-T, 10 min, RT;

Blockage: 1% BSA in PBS-T, 2 hours RT;

Primary antibody: primary antibody diluted in 0,1% BSA, 0.3% PBS-T, 3 days at 4C° (or overnight, RT);

Washing: 0.3% PBS-T, 6 times 10 min, RT;

Blockage: 0.3% PBS-T + 1% BSA, 1 hour, RT;

Secondary antibody: secondary antibody diluted in 0,1% BSA, 0.3% PBS-T, overnight at 4C°(or three hours, RT);

Washing: PBS, 6 times 10 min, RT;

Mounting step: between slide and coverslide with Vectashield, (Vector Laboratories, Inc.);

Observation (or conservation at –20C°).

Visualization

The obtained slides have been visualized with one (or more than one) of the microscope listed below:

- Semiconfocal microscope: Nikon Eclipse 80i equipped with a QiCAM Fast Camera using the Image ProPlus software (Department of Biology, University of Padova, Italy);
- Confocal microscope Nikon Eclipse E600 running the LaserSharp 2000 software (Department of Biology; University of Padova, Italy);
- Confocal Microscope Leica DM5500 Q running the Leica LAS AF software (Department of Neurobiology and Genetics, University of Würzburg, Germany).

For each brain, individual images were taken at different depths in order to form a Z-series. The size of each section, with either one of the microscope used, was approximately $1\mu m \pm 0,2$.

Quantification of staining intensity

Staining intensity has been quantified as reported in **Tab. 2.4**:

Chapter	*Type of Quantification*	*Software*	*Formula*
3 to 5	Nucleus + Cytoplasm	ImageJ 1.43	(S-B)/B
6	Nucleus + Cytoplasm	Fiji	(S-B)
6	Nucleus	ImageJ 1.43	(S-Bavg)/Bavg

Tab. 2.4: Schematic summary of the methods used to quantifying the staining intensity. A specific method was chosen depending on the aim of the quantification and on the microscope used. S= fluorescence signal of a specific clock cells; B= background adjacent to the positive clock cell; Bavg= average background of three areas adjacent to the positive cell.

The values obtained by the formula (S-B)/B or (S-Bavg)/Bavg were normalized using the higher value as 100%. All data were analyzed and plotted using Microsoft Excel 2007, whereas the statistical analyses were done using Statistica 8 (StatSoft).

The ImageJ software is freely available at http://rsbweb.nih.gov/ij/.

Chapter 3:

WT-ALA under standard laboratory conditions

In order to understand how the circadian clock of *Drosophila* works under natural conditions a wild-type strain of flies was collected in the North of Italy in 2004 (See **Section 2.2**, **Tab. 2.1**). The strain adopted in the experiments (named WT-ALA) was established in our lab starting from individuals belonging to 37 isofemale lines immediately established after sampling and then kept in the lab. Moreover, every month the WT-ALA strain is integrated with the isofemale lines to keep a high genetic variability.

Before the work reported here and in the PhD Thesis by S. Bhutani (Department of Genetics, University of Leicester) and S. Montelli (Department of Biology, University of Padova), WT-ALA flies had never been tested in the lab or in the wild.

So far, only "historical" fly strains kept in the lab for many decades have been used in all the works carried out to study the circadian clock of *Drosophila*: for example Canton-S (Canton-Special) flies have been studied as a wild-type strain for more than sixty years (Stern, 1943).

Since locomotor activity of WT-ALA flies under lab conditions was assayed in detail by S. Bhutani, whereas *per* and *tim* mRNA oscillations were studied by S. Montelli (see **Section 1.9**), the work reported here is aimed to describe PERIOD and TIMELESS oscillations under LD 12:12 at constant temperature (standard laboratory conditions). The locomotor activity of WT-ALA flies exposed to these conditions is also described here.

3.1 The locomotor activity of WT-ALA under lab conditions

Under standard laboratory conditions, namely LD 12:12 at constant temperature (generally ranging between 20 and 25°C), wild-type flies show a bimodal activity profile characterized by morning and evening activity bouts respectively during the lights-on and lights-off transitions. During the day flies are less active, this period of inactivity is called "siesta" (See also **Fig. 1.1**).

To check whether WT-ALA showed the same behaviour compared to the traditional "laboratory" wild-type strains, flies were exposed to rectangular light dark cycles (LD 12:12) at constant temperature and their activity was recorded. In **Fig. 3.1** the locomotor activity profile of WT-ALA flies under such conditions is represented. WT-ALA under LD 12:12 and at constant 23°C in this case, behave as any other strain of wild-type flies does: individuals show a bimodal activity with a morning and an evening peak and a siesta in between. It is also clear that flies are able to anticipate lights-on and -off transitions. Remarkably, this result confirms what already

found by S. Bhutani: the behaviour of flies recently collected from the wild natural strains resembles that of a wt "lab" strain under comparable environmental conditions.
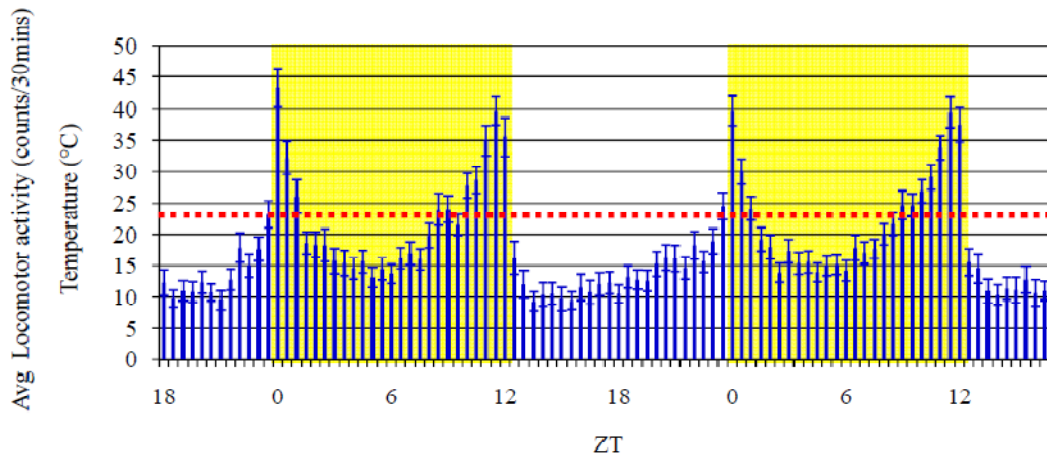

Fig. 3.1: Average locomotor activity (blue, n=26) of WT-ALA flies entrained under LD 12:12 at constant 23°C. Data have been recorded during two subsequent days. Light is represented in yellow, whereas temperature is shown by the dotted red line. The red and blue arrows indicate morning and evening anticipation, respectively. During the day ("siesta") and during the night flies are less active.

3.2 PERIOD and TIMELESS oscillations under standard laboratory conditions

A large amount of information about the molecular basis of the clock has been accumulated over the years. Various clock genes have been identified and their function have been elucidated (**Section 1.2** and **1.3**). Most of the genes that constitute the core of the clock cycle in the abundance of their products (RNA and protein) over the course of a day. This cycling of core clock gene products is the molecular basis for the circadian activity profile of flies in the laboratory.

Under LD 12:12 and at constant temperature PER and TIM oscillate in a typical manner in wild-type flies: they start to accumulate in the cytoplasm of all clock neurons in the middle of the night to reach their maximum levels at the end of the dark phase (See **Section 1.3**). At lights-on TIM is degraded in a light dependent manner; in the absence of TIM, PER is also degraded (See **Section 1.7**).

Flies were entrained for three days in the incubator before collection and dissected brains were stained either with α-PER or α-TIM antibody. Flies were sampled every 3 hours for 24 hours and at least ten brains per timepoint were considered and analyzed.

In **Fig. 3.2** (**a** and **b**, respectively) an example of PER and TIM staining at their maximum and minimum level is reported. In every ICC experiment also the α-PDF antibody was used in order to recognize the different cluster of neurons.
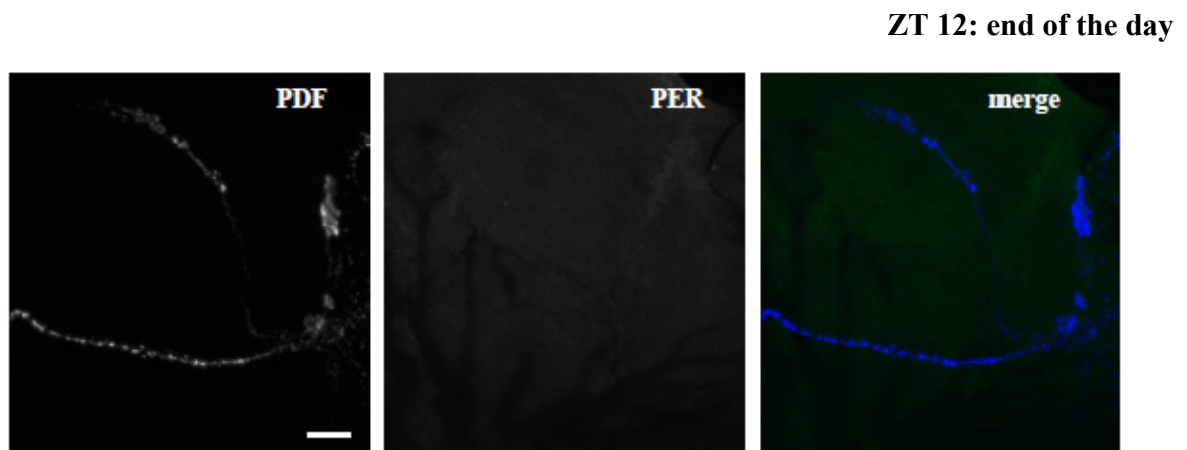
ZT 0: end of the night
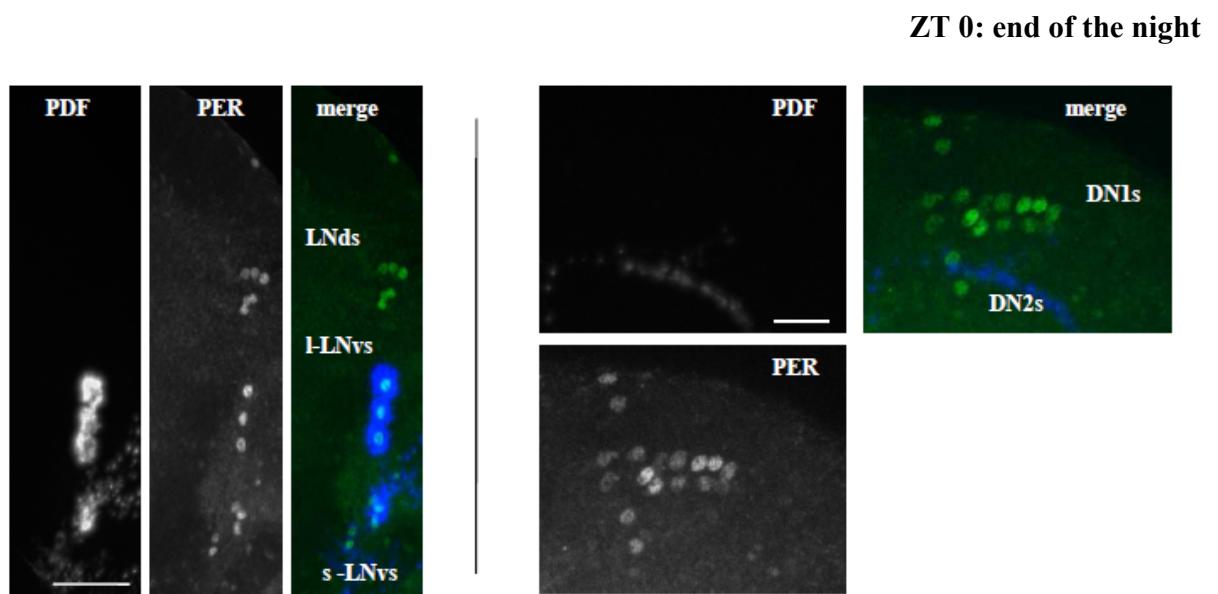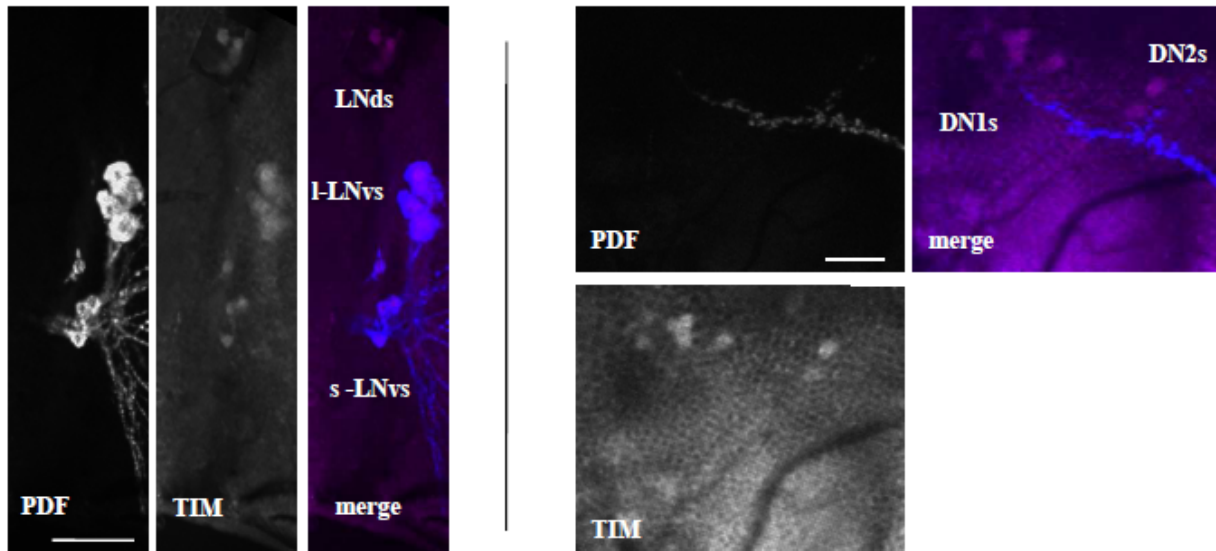

ZT 12: end of the day

Fig. 3.2a: PER and PDF immunoreactivity within the clock neurons of WT-ALA flies entrained under rectangular LD 12:12 and constant 23°C. (Scale bar= 25μm). PER staining is high in all clock neurons at ZT0 whereas the protein is almost undetectable at the end of the day.
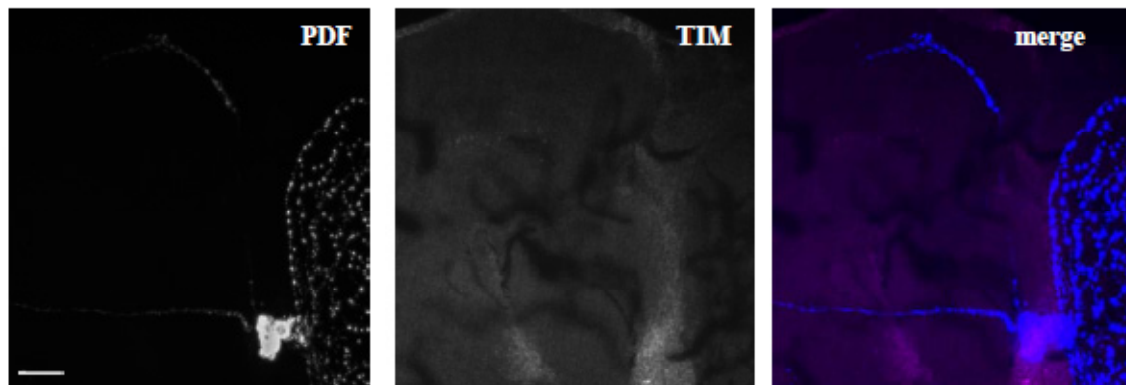
Fig. 3.2b: TIM and PDF immunoreactivity. Flies were entrained as explained in **Fig. 3.2a**. (Scale bar 25= 25μm). As well as PER, also TIM showed a stronger staining at the end of the night ant it appeared to be undetectable at the end of the day.

Upon quantification of the staining intensity revealed by the immunoassays, both PER and TIM oscillations did not show any unusual or unexpected feature. Within the clock neurons of WT-ALA flies entrained under LD 12:12 at constant temperature (23°C) PER starts to accumulate in the middle of the night and it reaches the maximum level around ZT0-3 whereas TIM is

increasing faster and the level of the protein is maximum at ZT21, 3 hours before lights-on (**Fig. 3.3**).

Most importantly each protein peak at the same time in all cluster of clock neurons. The peak of PER occurs synchronously at ZT0-3 in all neurons whereas the peak of TIM at ZT21. The only exception is found for PER oscillation within the 5th s-LNv: the peak is slightly delayed in this cell compared to the others (i.e. ZT3 instead than ZT0-3).

Notably, neither PER or TIM staining was found within the LPNs under this rectangular light dark cycles and at constant 23°C.

DN1s
DN2s
s-LNvs
5th s-LNv
l-LNvs
LNds

Fig. 3.3: PER (**a**) and TIM (**b**) oscillation profiles within the clock neurons of WT-ALA flies entrained as explained in **Fig. 3.2**.
Both PER and TIM fluctuations were statistically significant (ANOVA analysis, p< 0.05). The yellow area represents the light phase.
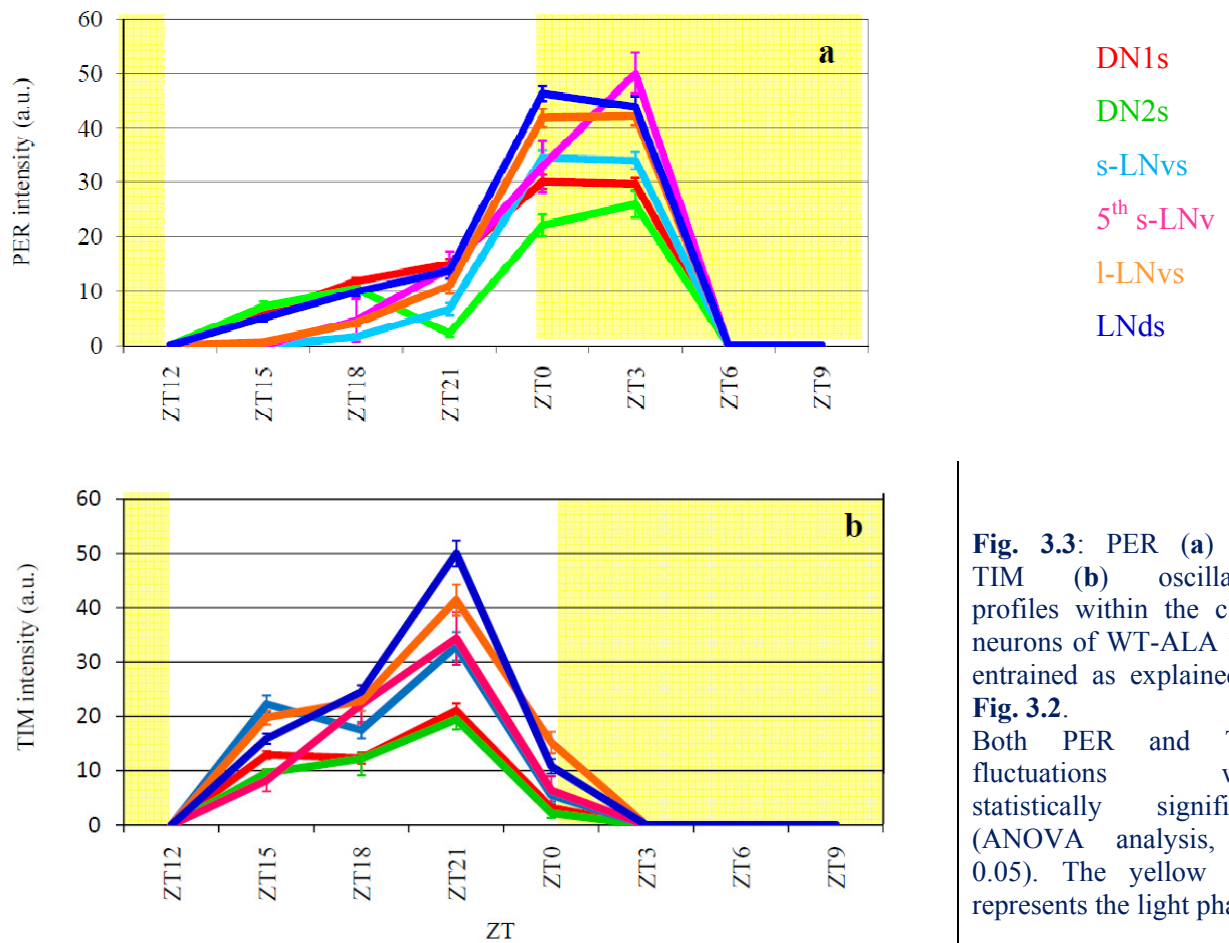
3.3 Discussion

Wild type ALA flies were collected in the wild in 2004 and the derived strain we used to perform the experiments was immediately established in the lab from the isofemale lines sampled in nature. The WT-ALA strain was then regularly enriched with flies coming from the original isofemale lines in order to maintain high levels of genetic variability and counteract genetic drift phenomena.

The WT-ALA natural strain has already been adopted and analyzed in two previous works: the PhD Thesis of S. Bhutani (Department of Genetics, University o Leicester) and S. Montelli (Department of Biology, University of Padova). More precisely these studies were dealing with WT-ALA locomotor activity and *per* and *tim* mRNA profiles under both laboratory and natural conditions.

In this Chapter the oscillation profiles of PER and TIM under rectangular LD 12:12 and constant temperature are reported. The locomotor activity recorded under such conditions is also shown.

At the behavioural level, data previously obtained by S. Bhutani were confirmed: WT-ALA locomotor activity largely resembles the locomotor activity pattern of the other wild-type strains usually utilized for this kind of studies by other laboratories.

Upon analysis of protein oscillation we can also confirm that PER and TIM accumulations appear to be normal under standard laboratory conditions: both protein peak before lights-on to reach a trough at the end of the day (Hunter-Ensor *et al.*, 1996; Yoshii *et al.*, 2009; Zerr *et al.*, 1990). Moreover, each protein appears to be accumulating synchronously in all clusters of clock neurons. It is also reported here that the peak of PER is slightly delayed in the 5th s-LNv relative to the other clock cells: the maximum level of the protein is clearly reached in this cell at ZT3 whereas the other cells show a stable high level between ZT0 and ZT3.

Interestingly, we observed that the peak of PER is slightly delayed relative to the peak of TIM under LD 12:12 and constant temperature: i.e. PER peaks at about ZT3, whereas TIM at ZT 21. This little phase shift between the two clock proteins has also been found in laboratory strains carrying a wild-type clock (e.g. *white[1118]*; P. Cusumano, Department of Biology, University of Padova, personal communication). Moreover, a phase shift between TIM and PER accumulation was already reported by Shafer and colleagues, even if only within the small and large LNvs of wild type Canton S flies (Shafer *et al.*, 2002).

These preliminary findings corroborate previous results reported in the Ph.D. thesis of S. Bhutani and S. Montelli: WT-ALA flies represent a suitable wild type strain to study the effect of the natural environment on the circadian clock; indeed they respond to standard laboratory conditions as other laboratory wild-type strains do.

Chapter 4:

WT-ALA flies under natural conditions

The environmental conditions that are usually imposed on flies in laboratory experiments (rectangular light-dark cycles and constant temperatures) are very different from what they really experience in nature. The environment in the wild is dynamic and multiple parameters such as light intensity and temperature are changing continuously.

As previously mentioned, the project EUCLOCK N° 018741 (EU 6th Framework Programme), carried out jointly by our lab and Prof. Kyriacou's lab at the University of Leicester, was aimed to understand how the circadian clock of *Drosophila* works the real natural environment. Natural locomotor activity and *per* and *tim* mRNA oscillations have been analyzed in detail by S. Bhutani and S. Montelli (S. Bhutani, PhD Thesis, 2009; S. Montelli, PhD Thesis, 2010) and by our own laboratory (Bhutani *et al.*, submitted).

In this work the effects of natural environmental parameters on the molecular circadian clock residing within the clock neurons of *Drosophila* have been analyzed.

We exposed WT-ALA flies to natural conditions throughout 2008 and 2009. In each experiment, flies experienced 3 dawns in the wild before collection. Sampling occurred every 3 hours around the 24 hours. Additional time points were sampled if needed. At least ten brains were analyzed for each time point. In parallel, in each experiment, locomotor activity of WT-ALA flies exposed to the same natural conditions was also recorded.

In this chapter results obtained from both activity recordings and ICC experiments are reported.

4.1 Behaviour of WT-ALA flies throughout the seasons

It has been shown in the laboratory that the activity profile of wild-type flies in LD conditions depends on the ambient temperature. In particular, the activity is bimodal at medium/high constant temperatures such as 25°C and 29°C with a morning peak around the time of lights-on and an evening peak which occurs around lights-off. The "siesta" in-between these two peaks is believed to occur in order to enable the animals to avoid the hottest parts of the day. This is because of the observation that, at colder constant temperatures of 18°C, this "siesta" is significantly reduced and the evening peak shifts towards the middle of the day (Blau and Rothenfluh, 1999). This phenomenon can also be observed under cycles of temperature.

In **Fig. 4.1** locomotor activity data collected during Winter, Spring, Summer and Autumn 2008 are represented. These locomotor patterns were selected as representative of the season.

The environmental conditions recorded on the days of the experiments are reported in **Tab. 4.1**:

| Season	NLD	T max (°C)	T min (°C)	Moonlight
Winter	13:11	12,6	1,7	89%
Spring	15:9	25,7	15,5	No moon
Summer	15:9	35,1	25,2	No moon
Autumn	13:11	22,7	13,7	99%

Tab 4.1: Environmental conditions characterizing four different experiments performed in different seasons. A representative day per season was chosen. The calculation of the NLD (Natural LD cycle) is based on the recordings of the environmental monitors. The moonlight is reported as the percentage of the moon illuminated by the sunlight.

From the comparison of the activity profiles recorded in different season it is evident that during Winter (short days and cold temperatures) flies are less active. WT-ALA flies start to move after lights-on and their activity is unimodal. When days get longer and temperature warmer (Spring) flies start to move earlier and show bimodal activity. During Summer, when the photoperiod is similar to that recorded in Spring but temperature is much higher flies show a morning and evening activity bouts. Interestingly, under these conditions a third peak of activity is also detectable. In fact, in the hottest part of the day, after the offset of morning activity, flies seem to steadily increase their activity and manifest an additional peak in the afternoon: this peak has been referred to as "afternoon peak" (S. Bhutani, PhD Thesis, 2009). The afternoon peak of activity disappears when temperature decreases and days are shorter again (Autumn). In Autumn the activity profiles of WT-ALA flies are very similar to those observed in Spring, when the photoperiod was longer but temperature was comparable.

Since the results concerning the locomotor behaviour are confirming those obtained and already discussed in detail by Bhutani *et al.*, (submitted), (See **Section 1.9**), the locomotor activity of WT-ALA under natural conditions will not be further analyzed herein.

Fig. 4.1: Average locomotor activity of WT-ALA flies throughout the 4 seasons. Locomotor activity is represented in blue, light intensity in yellow and temperature is shown by the dotted red line. N= 26 in Winter, 24 in Spring, 23 in Summer; 26 in Autumn.
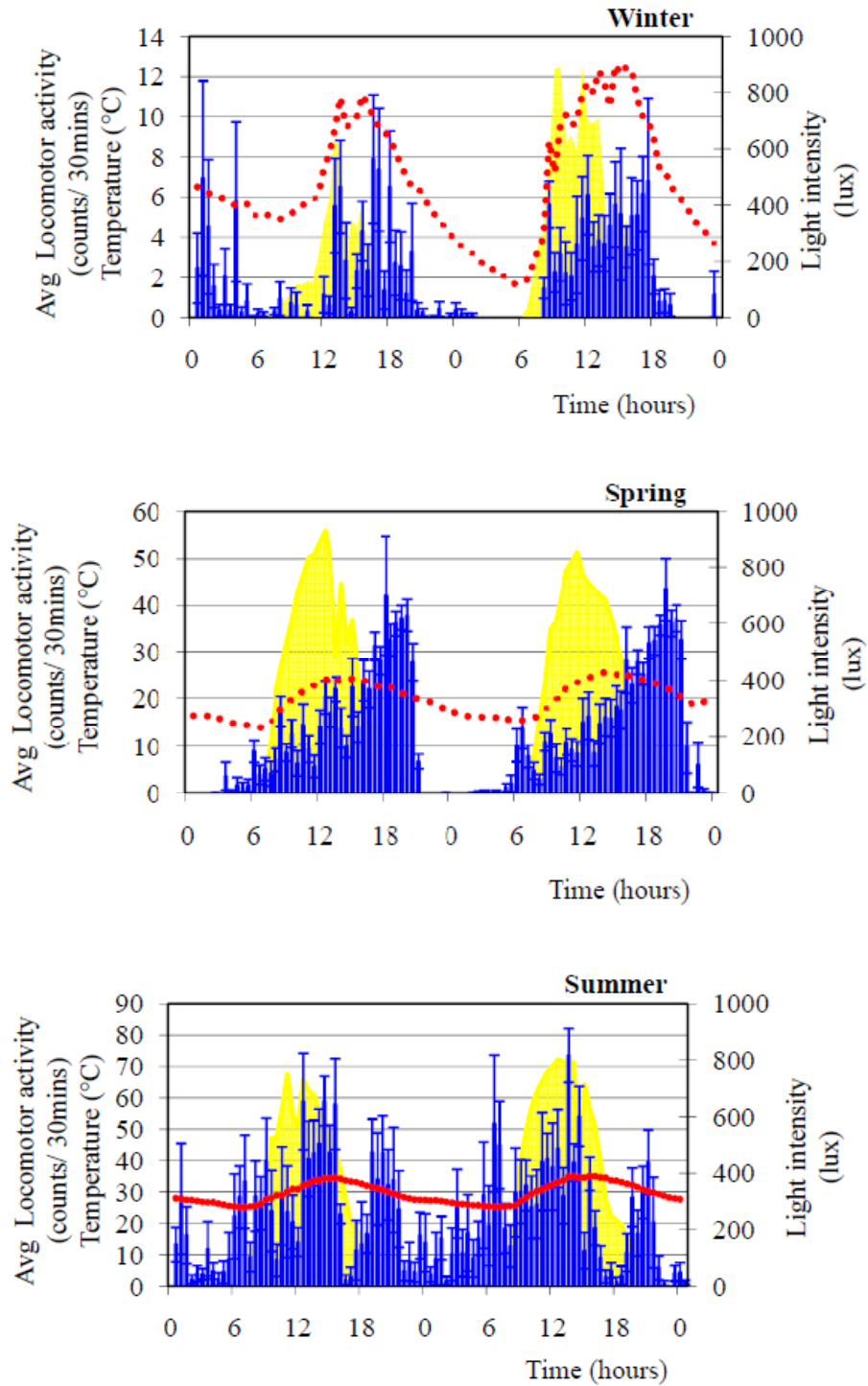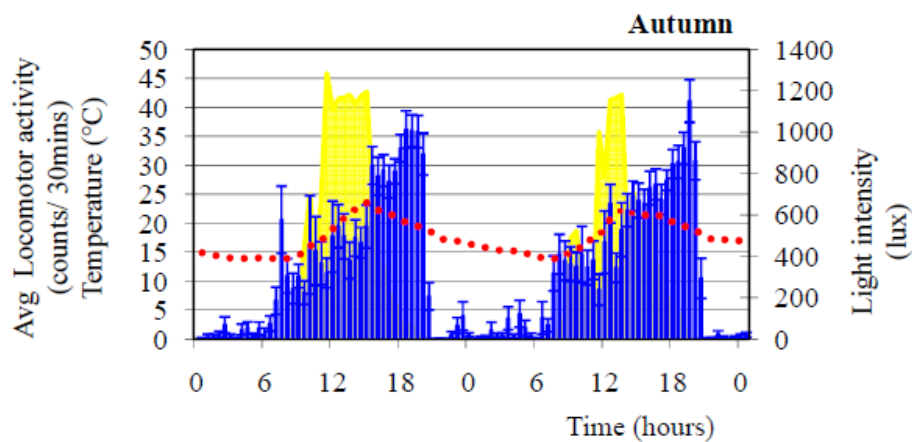
4.2 PERIOD and TIMELESS oscillations under natural conditions

The accumulation of the clock proteins PER and TIM has been widely studied under laboratory conditions and despite some attempts of mimicking in the lab the natural environment have been made in the last few years, almost nothing is known yet about the functioning of fly's molecular clock when working in real natural conditions.

In order to describe some features of the circadian molecular machinery in the natural environment, WT-ALA flies were placed outside and sampled at different ZTs. The plastic vials containing the flies were shielded from direct sun light, artificial light and from rain. Flies were entrained for three days under natural conditions and then collected every three hours (or more often in some cases, see below) in order to perform PER and TIM immunoassays. It was necessary to sample more often to perform informative TIM immunoassays (i.e. every 3 hours during the day and 1,5 hours during the night) as TIM is degraded in a light dependent manner and therefore it is present only during the night. Moreover, if needed, an additional time point was collected right before dawn. In all experiments PDF staining was used to better identify the clusters of specific clock cells. It is worth noticing that ICC experiments performed to detect PER accumulation were carried out in 2008 whereas those for TIM in 2009. PER and TIM results are anyway comparable. In fact, albeit not characterized exactly by the same environmental

conditions, the experiments in 2009 were performed under environmental conditions very similar to those recorded in 2008.

In **Tab. 4.2** and **4.3** the environmental parameters recorded during the days in which experiments have been performed are shown. As mentioned before describing patterns of natural locomotor activity, runs of days that could be considered representative for the season were chosen. Notably, the locomotor activity shown in **Fig. 4.1**, was recorder exactly during the days in which flies were collected to perform the α-PER immunoassays.

The results concerning the ICC experiment are reported below: PER and TIM oscillations are compared in the different seasons. In each figure environmental parameters recorded during the day of the experiments are also shown: light is represented by the yellow area whereas temperature by the dotted red line.

Tab. 4.2: Environmental parameters recorded in 2008, on the days in which PER immunoassays were carried out.

Season	NLD	T max (°C)	T min (°C)	Moonlight
Winter	13:11	12,6	1,7	89%
Spring	15:9	25,7	15,5	No moon
Summer	15:9	35,1	25,2	No moon
Autumn	13:11	22,7	13,7	99%

Tab. 4.3: Environmental parameters recorded in 2009, on the days in which TIM immunoassays were carried out.

Season	NLD	T max (°C)	T min (°C)	Moonlight
Winter	12:12	13,8	2,4	No moon
Spring	16:8	27,6	18,3	24%
Summer	15:9	35,6	25,6	No moon
Autumn	13:11	28,4	17,2	No moon

Winter

During Winter experiments temperature was barely above 10°C during the day and always below that temperature during the night. Either for PER or TIM experiments, flies were collected at each planned time point, brains dissected and stained. After images acquisition and analysis it

appeared that PDF staining was clear in all time points analyzed whereas both TIM and PER were undetectable under this conditions (**Fig. 4.2a**). To test whether these finding was due to a temperature effect we entrained flies at constant 10°C, LD 12:12, under lab conditions. Brains were the dissected only at ZT0. The analysis of the images acquired revealed again that both PER and TIM were not detectable (**Fig. 4.2b**).

The same antibody solutions used to incubate the brains of the flies entrained under natural conditions and lab conditions at constant 10°C were used to perform a control ICC experiment on brains dissected from flies entrained under lab LD 12:12 at constant 23°C and 15°C and in both cases PER and TIM staining was now clearly detectable (data not shown).

In order to exclude that the absence of both PER and TIM staining was due to a limited sensitivity of the microscope used (Nikon Eclipse 80i), we performed another lab experiment using a different apparatus to acquire the images (Leica DM 5500 Q, provided by Prof. Charlotte Helfrich-Förster, Department of Neurobiology and Genetics, University of Würzburg, DE).

The new apparatus allowed the detection of PER and TIM signal, at ZT0, within the clock neurons of flies entrained under LD 12:12 and at constant 10°C. Nevertheless, both PER and TIM staining appeared to be predominantly cytoplasmic at ZT0. Pictures are shown in **Fig. 4.3a** and **b**.
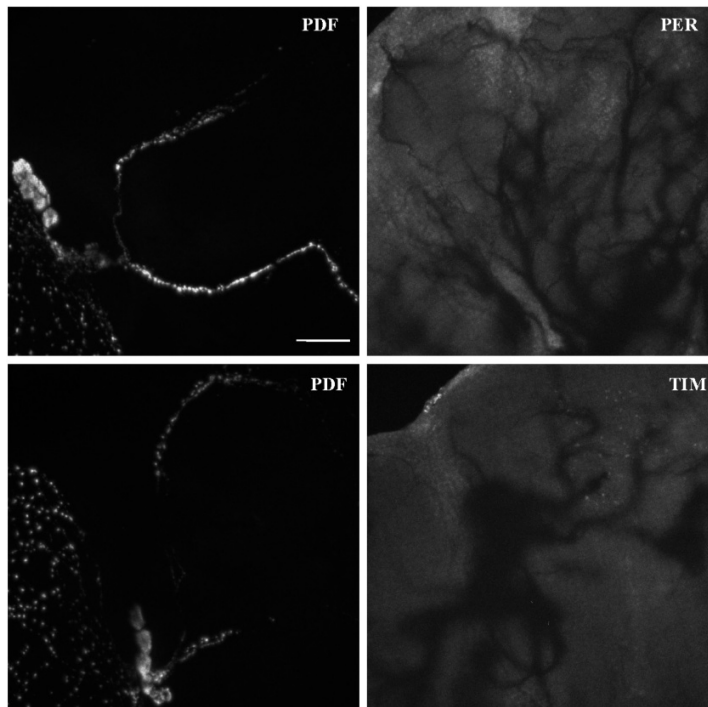
Fig. 4.2a: PDF, PER and TIM immunoreactivity on brains of WT-ALA flies entrained under natural Winter conditions (See **Tab. 4.1** and **4.2**). These images were acquired from brains dissected right before dawn, when the maximum level of PER and TIM were expected. PDF staining was evident at all time points whereas PER and TIM were always undetectable. Scale bar= 20 μm.
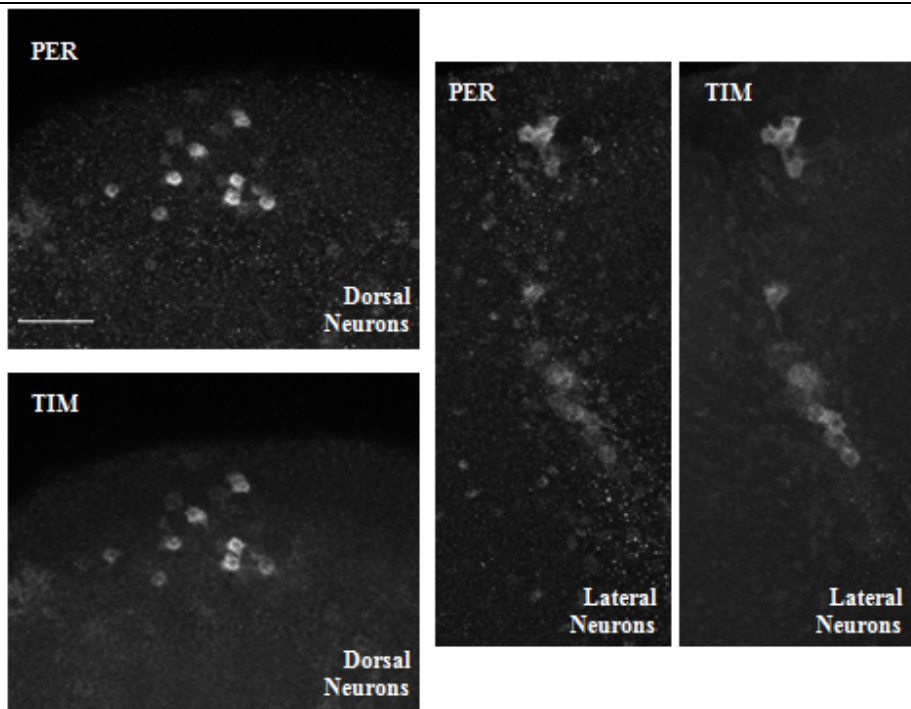
Fig. 4.2b: PDF, PER and TIM immunoreactivity on brains of WT-ALA flies entrained under laboratory rectangular LD 12:12 at constant 10°C.

At least 10 brains were dissected at the ZT at which the maximum staining was expected (ZT0 for PER and ZT21 for TIM). As under Winter natural conditions, neither PER or TIM staining was detected, whereas PDF staining is evident.
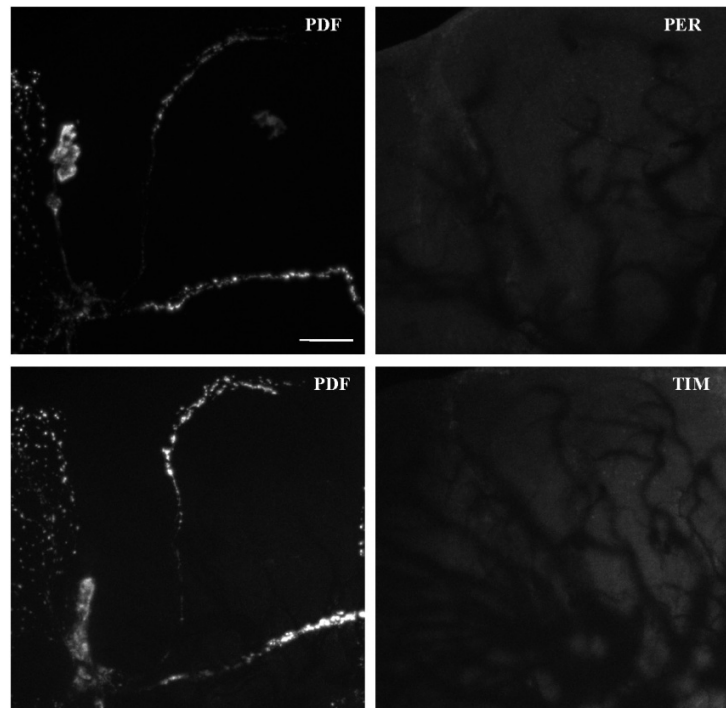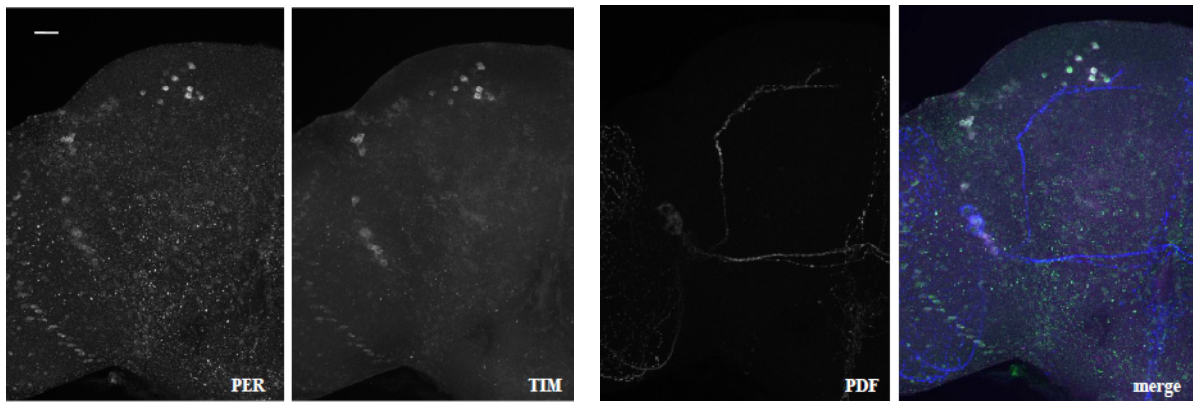
Fig. 4.3 a): PER, TIM and PDF immunoreactivity within the dorsal neurons of flies entrained under LD 12:12 at constant 10°C. Images were acquired with the confocal microscope Leica DM 5500 Q. **b)** TIM and PER immunoreactivity within the DNs and LNs. Same brain as in **a** but higher magnification. Scale bar= 20μm.

<u>Spring</u>

In Spring, the climate in the North of Italy is mostly characterized by a long photoperiod (NLD~16:8) and mild temperature (Tmax ~25°C, Tmin ~15°C). These were the conditions experienced by our WT-ALA flies. As in all other experiments, flies were entrained outside for three full days and then collected to perform the ICC. The results obtained with the Spring experiments are reported in **Fig. 4.4 a** and **b**.

Under a long photoperiod and relatively warm temperature PER starts to accumulate within the clock neurons about three hours after sunset (**Fig. 4.4a**). The level of PER increases to reach a peak at dawn within the DN1s and DN2s. PER level remains then high in these cells until 9 a.m. (about three hours after dawn). Within the LNs the accumulation of the protein is delayed: PER reaches the peak later, at 9 a.m., when the level of the protein within the dorsal cells is still high. After 9 a.m. the level of PER in both lateral and dorsal cells decreases, until the protein becomes completely undetectable at 3 p.m..

TIM shows a different pattern of oscillation under these environmental conditions (**Fig 4.4b**). The main difference is that TIM is present only during the night and it becomes totally undetectable right after dawn. Moreover TIM is expressed also in an additional cluster of clock neurons, the LPNs.

TIM level peaks right before dawn (4:30) in all the anterior cells: s-LNvs, 5th s-LNv, l-LNvs and LN$_d$s. In the posterior clock neurons, DN1s, DN2s and LPNs, levels of TIM increase more rapidly. Within the LPNs the peak is reached right after sunset (22.30) and then the level of the protein slowly decreases. In DN2s the oscillation profile is not so clear; more precisely the level of TIM is already high at 22:30 and then decreases and increases again twice, at 1:30 and 4:30. Within the DN1s the level of TIM again increases rapidly after sunset and keeps increasing until 4:30, to peak at the same time as in all the lateral anterior cells.

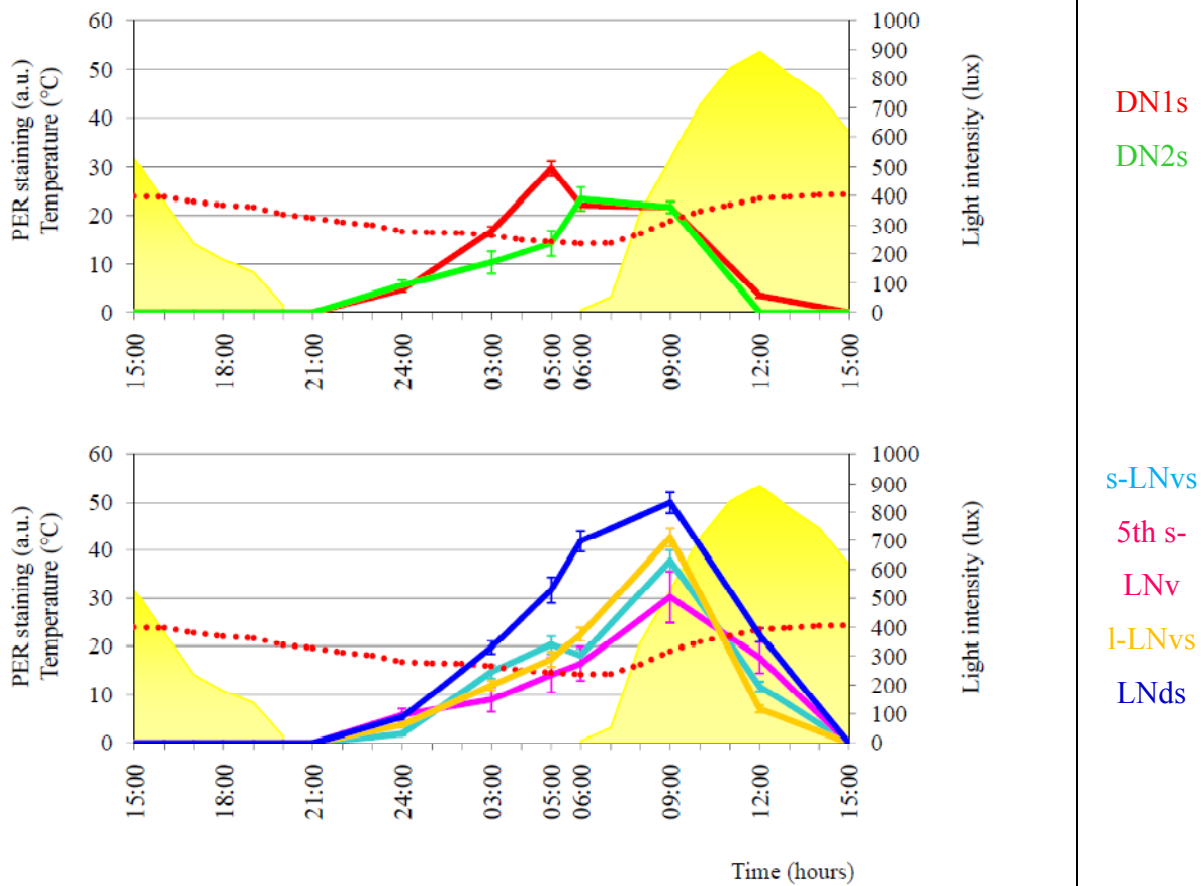**Fig. 4.4a**: PER oscillation profiles under natural conditions. NLD~ 15:9; Tmax ~25°C; Tmin ~15°C. Temperature is represented by the red dotted line, light intensity by the yellow area. PER fluctuation are statistically significant (ANOVA, p< 0.05).

DN1s
DN2s

s-LNvs
5th s-LNv
l-LNvs
LNds
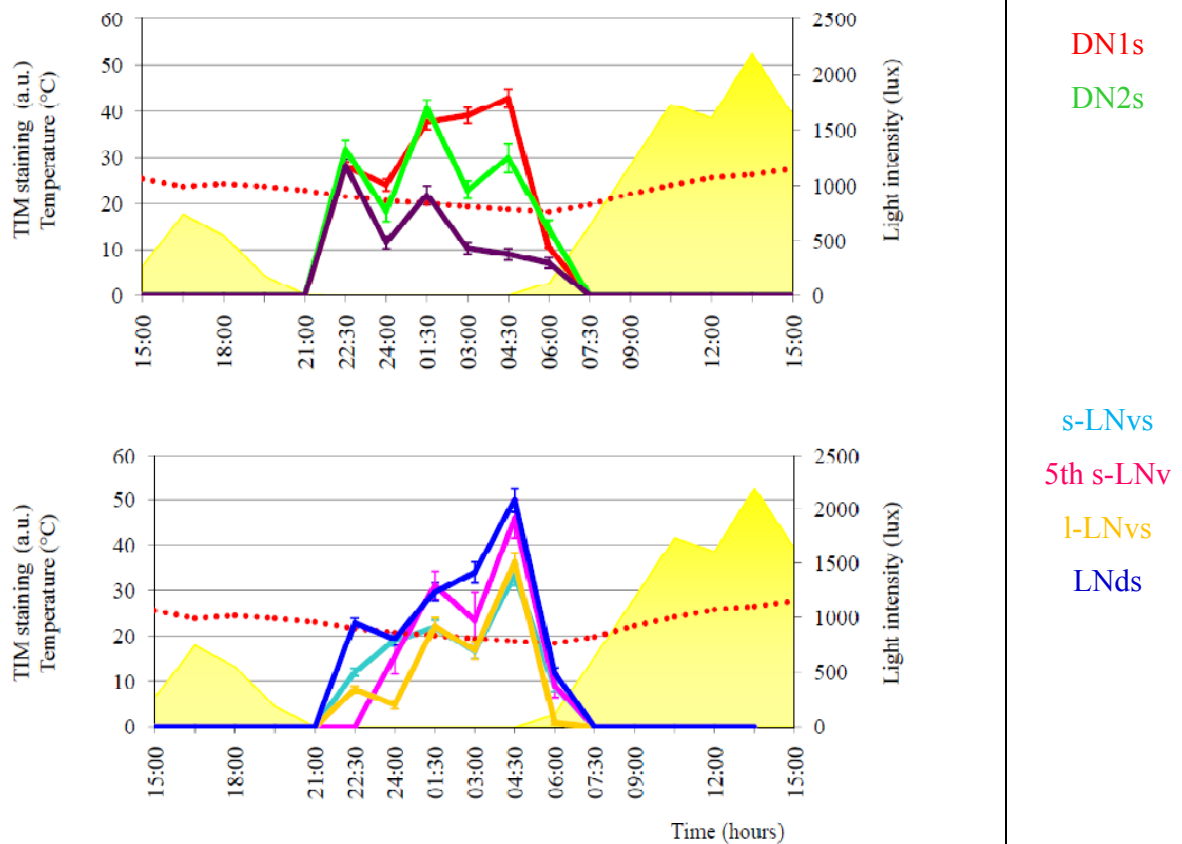
Fig. 4.4b: TIM oscillation profile under natural conditions. NLD~ 16:8; Tmax ~25°C; Tmin ~15°C. Temperature is represented by the red dotted line, light intensity by the yellow area. TIM fluctuations are statistically significant (ANOVA, p< 0.05).

Summer

In Summer in Italy the photoperiod is still long as during Spring (NLD ~15:9) but temperatures are much higher. During our experiment temperature was ranging from 25°C to 35°C; this means that the average temperature in Summer (Tavg: 30°C) is about 10°C higher than the average temperature in Spring (Tavg: 20°C) albeit the photoperiod is very similar. The oscillatory patterns of PER and TIM show really important differences under summer months (**Fig 4.5 a** and **b**).

Fig. 4.5a: PER oscillation profile under natural conditions. NLD~ 15:9; Tmax ~35°C; Tmin ~25°C. Temperature is represented by the red dotted line, light intensity by the yellow area. PER fluctuation are statistically significant (ANOVA, p< 0.05).
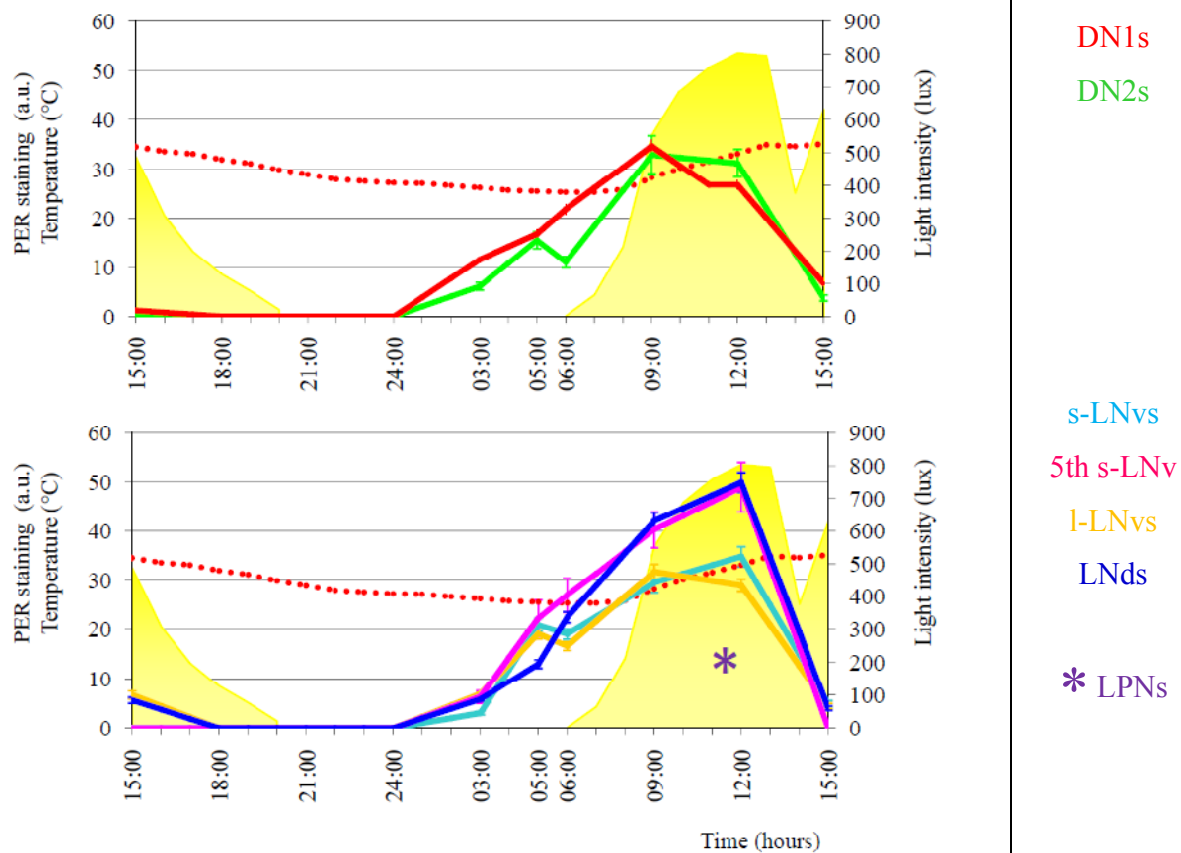
PER (**Fig. 4.5a**) starts to be detectable within all clock neurons late at night, at 3 a.m. The accumulation profile differs between lateral cells and dorsal cells. In DN1s and DN2s, PER peaks at 9 a.m. which is 3 hours after dawn and then starts to decrease and becomes undetectable late during the day (after 3 p.m.). Within the LNs the level of the protein continues to increase and peaks at midday. As for Spring, the peak of PER within the LNs occurs about three hours later than that in DNs. Also within the LNs the signal is almost undetectable at 3 p.m., this means that the level of PER in these cells decreases more quickly than in the dorsal cells. It is worth mentioning that the peak of PER within the lateral cells occurs more than six hours after dawn. Moreover, we found that under Summer conditions, PER is expressed also within LPNs albeit only at one single time point corresponding to the peak of temperature (35°C).
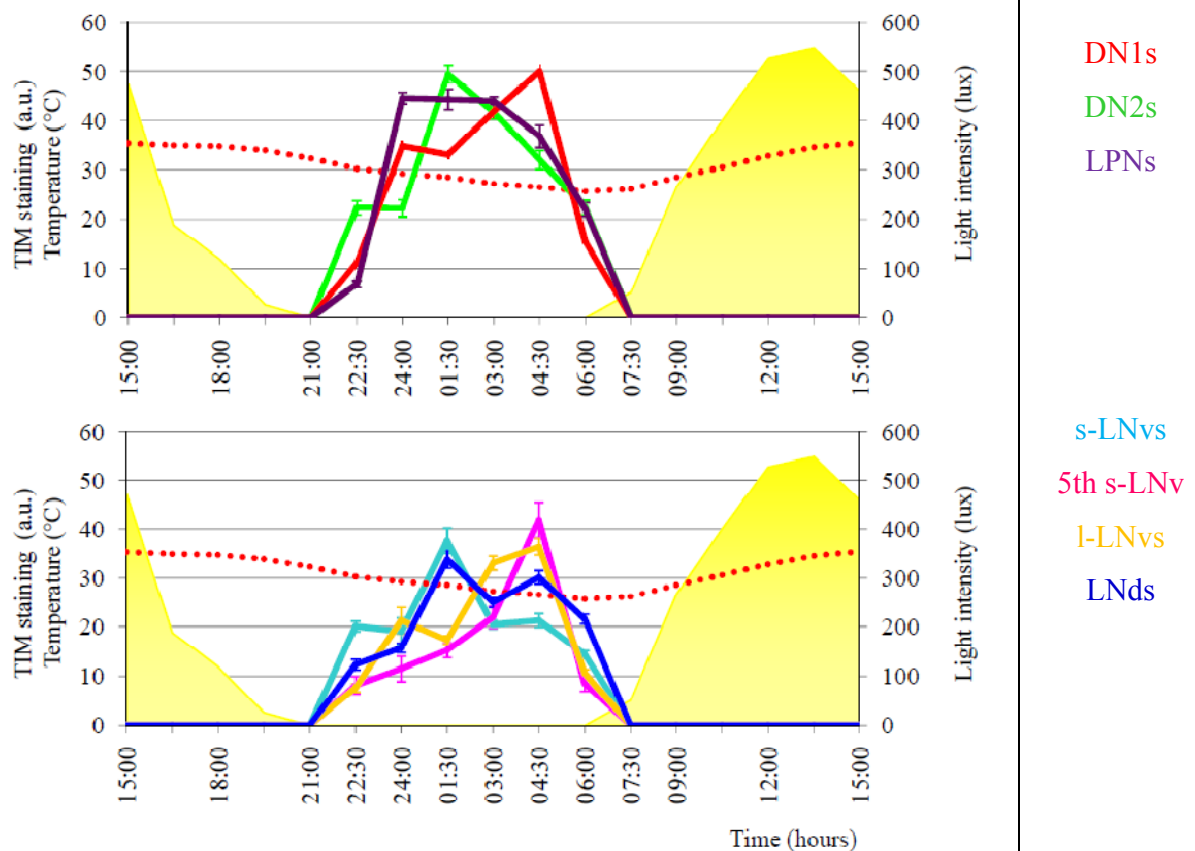
Fig. 4.5b: TIM oscillation profile under natural conditions. NLD~ 15:9; Tmax ~35°C; Tmin ~25°C. Temperature is represented by the red dotted line, light intensity by the yellow area. TIM fluctuations are statistically significant (ANOVA, p< 0.05).

TIM (**Fig. 4.5b**) is present only during the night as it was in Spring and again it was detectable also within the LPNs. Moreover, as in Spring, the oscillation of TIM is not well synchronized among the different clusters of clock neurons:

- LPNs: TIM reaches the maximum level at midnight, stays high until 3 a.m. and then decrease to become undetectable right after dawn (7:30).
- DN1s: TIM increases slowly and peaks right before dawn (6 a.m.). It becomes undetectable 1 hour and a half later (7:30).
- DN2s: the peak of TIM occurs in the middle of the night, TIM becomes undetectable at 7:30.
- s-LNvs and LNds: as in DN2s TIM peaks at 1:30 and it is undetectable at 7:30.

- 5th s-LNv and l-LNvs: the level of TIM increases slowly and peaks at 4:30. TIM is undetectable 3 hours later, again right after dawn as it was in all other groups of clock cells.

Comparing PER and TIM oscillations under Summer conditions it is clear that at least in some of the clock neurons, more precisely s-LNvs and LNds, the two proteins are peaking with more than 10 hours of difference, almost in antiphase. Moreover even in DN2s, the peak of PER is delayed of about 8 hours if compared to that of TIM.

Autumn

The photoperiod length recorded in our "Autumn" experiments resembles the one of the Winter photoperiod (NLD ~13:11) whereas temperatures are really similar to those observed in Spring (Tmax ~25°C and Tmin ~15°C).

In **Fig. 4.6a** and **b** the oscillation profiles of PER and TIM are reported.

PER staining starts to be detectable at midnight within the dorsal cells and at 3 a.m. in the lateral cells. Within DN1s and DN2s the peak is reached at 3 a.m., but the level of the protein remains high until 6 a.m.. The signal is then barely detectable in DN1s at midday while earlier, at 9 a.m., in DN2s.

As already found under Spring and Summer conditions, in Autumn TIM is detectable within the clock neurons only during night period and again it is present within the LPNs. The level of the protein shows a broad peak within all the clusters of posteriors cells (DN1s, DN2s and LPNs) and only within the DN1s the level shows a clear maximum only at one time point (3 a.m.). Within all the lateral anterior cells the level of TIM is increasing slowly until 6 a.m., when the peak occurs.

DN1s

DN2s

s-LNvs

5th s-LNv

l-LNvs

LNds

Fig. 4.6a: PER oscillation profile under natural conditions. NLD~ 13:11; Tmax ~25°C; Tmin ~15°C. Temperature is represented by the red dotted line, light intensity by the yellow area. PER fluctuation are statistically significant (ANOVA, $p < 0.05$).
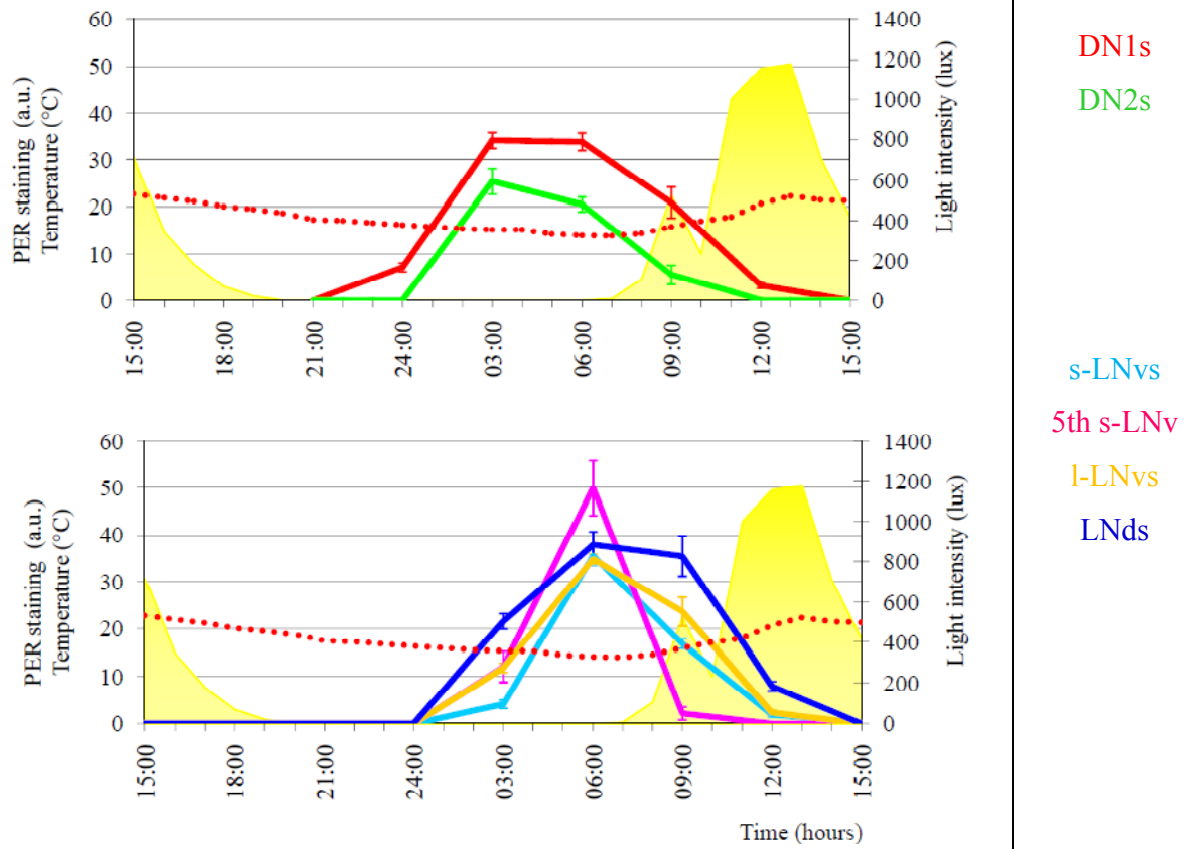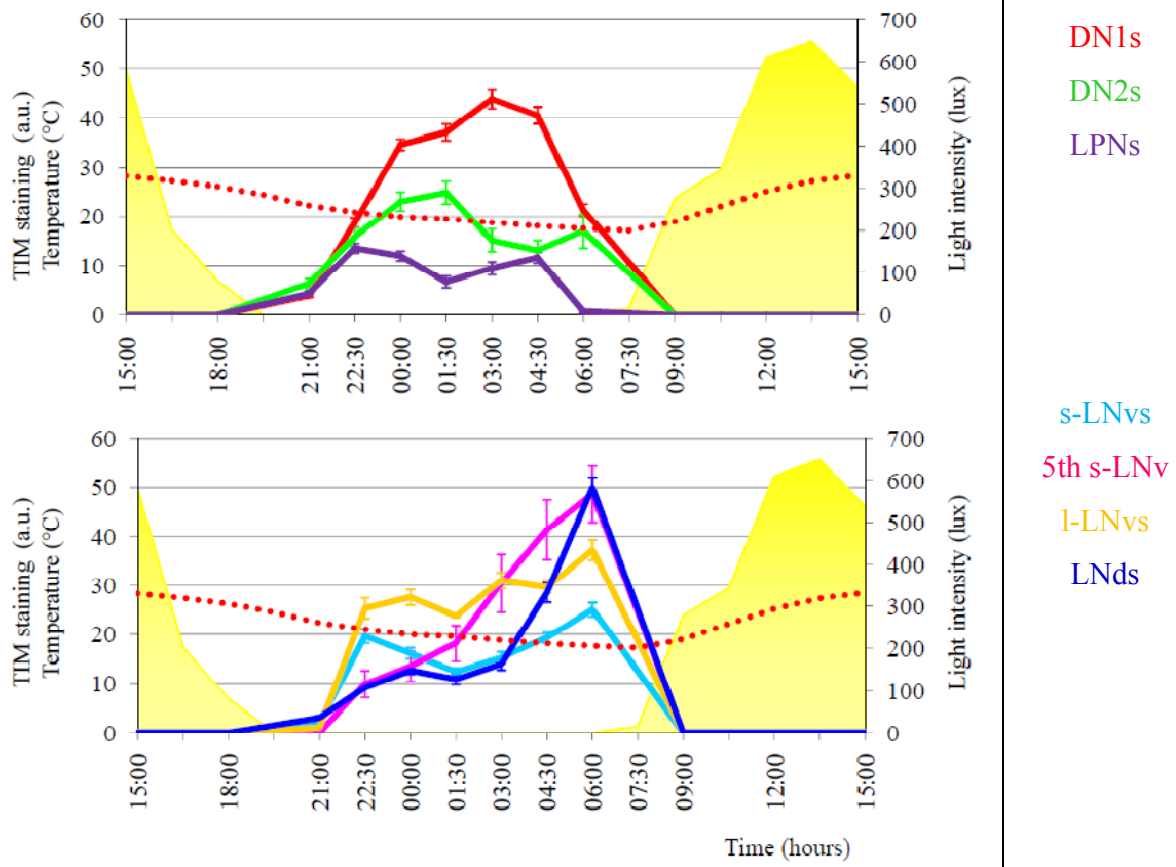
Fig. 4.6b: TIM oscillation profile under natural conditions. NLD~ 13:11; Tmax ~25°C; Tmin ~15°C. Temperature is represented by the red dotted line, light intensity by the yellow area. TIM fluctuations are statistically significant (ANOVA, $p < 0.05$).

4.3 Discussion

WT-ALA locomotor activity under natural conditions

The locomotor activity of WT-ALA in natural conditions has been already analyzed in detail by S. Bhutani in her PhD Thesis, conducted at the Department of Genetics, University of Leicester (UK) and by our own laboratory (Bhutani *et al.*, submitted).

In this Chapter the natural locomotor activity recorded in parallel to ICC experiments is shown as an example and two representative days per season have been chosen.

It is well known that locomotor patterns of flies are bimodal at high and unimodal at low temperatures: the way temperature can influence flies locomotor activity has been investigated in the last years under laboratory conditions (Chen *et al.*, 2007; Low *et al.*, 2008). From our study, it appears that the temperature-dependent modulation of the distribution of the locomotor activity during the 24 hours is retained under natural temperature cycle (**Fig. 4.1**).

The positions of morning and the evening peaks of activity appear to be highly temperature dependent. The morning peak is delayed at low temperatures and advanced at higher temperatures. Moreover, in Winter, when temperatures are below 12°C, flies are not moving until late morning, most likely until when temperature becomes permissive. On the contrary the evening peak is delayed in Summer, when days are long and hot, and advanced during the bad seasons.

On hot days in the wild, flies show an additional third peak of activity (named "afternoon peak", Bhutani *et al.*, submitted). The "afternoon peak" occurs in the middle of the day, between morning and evening peaks; the number of flies that show the afternoon peak increases with increasing temperature. This peak occurs during the hottest period of the day which is usually associated, from laboratory observations, with a "siesta". This "afternoon peak" of activity we have observed could be the result of the stress produced by extremely hot temperatures resulting in attempts of the flies (which are confines in relatively small glass tubes) to escape from this heat which causes a temporary increase in activity. Nevertheless, it was shown by Bhutani *et al.*, submitted, that the position of the afternoon peak during the day is different between wild type flies and mutant flies such as per^s and per^L (flies that under standard conditions show a shorter -per^s- or longer -per^L- period of activity relative to wild type flies). The possibility that the

circadian clock is involved in controlling/modulating the "afternoon peak" of activity similarly to its regulation of the morning and evening ones is currently under investigation in our laboratory.

PER and TIM in Winter

ICC experiments were performed under natural conditions during Winter, when the environmental temperature was below 12°C during the whole day and photoperiod was short (NLD ~12:12). Looking at the brains collected under these conditions we did not found neither PER or TIM staining at any of the time points considered whereas PDF staining was always present. This absence of signal could be due to the low temperature itself. In the real life flies enter diapause to overwinter (Allen, 2007; Emerson *et al.*, 2009) and it may be possible that the circadian clock stops ticking when environmental conditions are not permitting normal life. To further investigate this possibility we entrained flies at constant 10°C under lab conditions and we confirmed the result obtained under natural conditions: neither PER or TIM were detectable; only PDF staining could be seen.

To exclude that the absence of PER or TIM signals was due to a limit in sensitivity of the semiconfocal microscope used throughout the experiments (Nikon Eclipse 80i, see **Section 2.4**), we repeated the experiment at constant 10°C in the lab using another apparatus to acquire the images, namely, the confocal microscope Leica DM 5500 Q provided by Prof. C. Helfrich-Förster, Department of Neurobiology and Genetics, University of Würzburg (DE) where I went to perform the analysis. With this more sensitive apparatus it was actually possible to detect both PER and TIM staining within the clock neurons of flies entrained under low temperature conditions. and at ZT0. Nevertheless the staining for both proteins appeared to be cytoplasmic suggesting that maybe under these conditions PER and TIM translocation into the nucleus is compromised.

In any case, the absence of PER and TIM staining in images acquired with the semiconfocal microscope and the need to use a more sensitive apparatus to be able to observe a fluorescence signal at 10°C could be explained by a significant reduction of both PER and TIM expression in cold environments. However, the presence of PER and TIM at constant 10°C in laboratory conditions cannot exclude a stronger effect of the natural Winter cycles of temperature on the molecular clock and actually on the day in which the natural Winter experiments have been

performed, temperature went far below 10°C. Moreover, we already know that during cold days flies locomotor activity become unimodal and flies prefer to move in the middle of day, when temperature is milder. This particular distribution of locomotor activity during the day could also be explained as a response to the temperature cycles.

In addition, in a work carried out by S. Montelli (Department of Biology, University of Padova) it was found that at low temperature the abundance of PER mRNA increases whereas the one of TIM decreases and that at constant 10°C both PER and TIM mRNA are hardly cycling.

Lateral Posterior Neurons

TIM and PER staining within LPNs has never been reported under rectangular LD conditions and at constant temperature. Recently these cells have been described as temperature entrainable cells (Miyasako *et al.*, 2007; Yoshii *et al.*, 2005).

Accordingly, in our study neither PER nor TIM staining has been found within LPNs under standard lab conditions of LD 12:12 and at constant 23°C (See **Chapter 3**).

Under natural conditions LPNs appear to be TIM positive in all experiments carried out. Independently of the environmental conditions TIM level increases within LPNs after sunset and high levels persist until about dawn (**Fig. 4.7**).

PER has been found within the LPNs only at one time point throughout all the experiments carried out: at midday during Summer, when temperature reached a peak of 35°C (**Fig. 4.7**).

It seems that the Lateral Posterior Neurons need temperature cycling to express TIM and really very high temperatures to express PER.
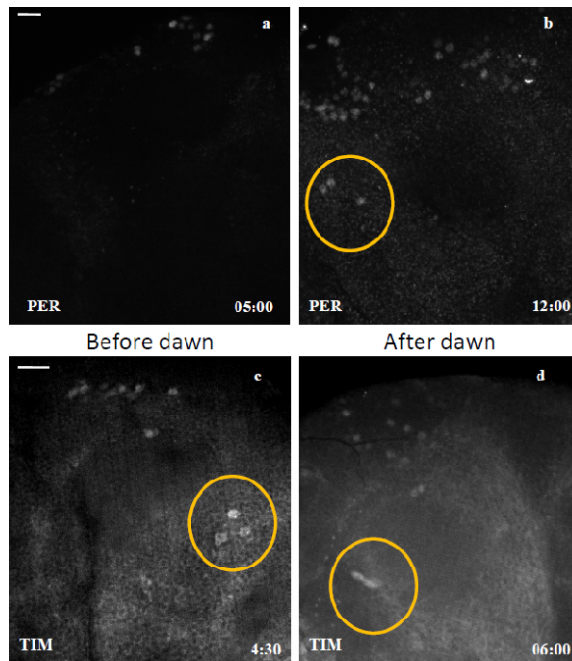
Fig. 4.7 PER and TIM immunoreactive LPNs under Summer conditions.
a, **b**: PER appear to be detectable only at very high temperature (35°C).
c, **d**: under natural conditions TIM is always detectable within the LPNs, during the night period until about dawn.

Moonlight effects on PERIOD and TIMELESS oscillations?

In the last years, the effects of dim light during the night on both fly's locomotor activity and PER and TIM oscillations were analyzed by C. Helfrich-Förster's group. They found that exposing flies to artificial light of moonlight intensity during the night causes a shift of the locomotor activity into the night period and also affects the oscillation profile of clock proteins; in fact, the peak of PER and TIM within the 5th s-LNv is also shifted compared to the other clock cells under artificial moonlight nights (Bachleitner *et al.*, 2007; Kempinger *et al.*, 2009).

It was already shown by S. Bhutani in her PhD Thesis that there are not significant differences in the locomotor activity of flies recorded under natural dark nights or moonlight nights.

The kind of data collected in my work allows the analysis of the putative effects of moonlight at the molecular level only for PER oscillation; indeed, two out of four PER experiments under natural conditions were carried out during full moon nights whereas the remaining two during dark nights. TIM oscillation cannot be analyzed under this point of view since three out of four experiments were carried out under new moon conditions and in the fourth only the 24% of the moon was illuminated by sunlight.

In our results, it is evident that the peak of PER within the 5th s-LNV is always in phase with the peak within the other lateral cells, both under new moon of full moon conditions. We can then affirm that natural moonlight does not affect the phase of PER oscillation.

In any case, it is worth to mention that Kempinger and co-workers already pointed out the possibility that the higher level of nocturnal locomotor activity they have observed under artificial moonlight could be partially caused by direct exposition to low light intensity. The authors found that both wild-type flies and clock less mutants shift their activity into the night when exposed to artificial moonlight; thus, it is likely the clock is not involved in determining this phenotype (Kempinger *et al.*, 2009). It may be that this shift of locomotor activity into the night was not observed under natural moonlight because flies were shielded from direct light either during the day or night (see **Section 2.3**).

Moreover, in the lab experiments the environment was over-simplified. Temperature was not cycling, light was artificial and constant in its spectrum, LD cycles were rectangular; under natural conditions all these parameters are much more complex and continuously changing. We cannot exclude that also natural moonlight could have a role in influencing PER oscillation but most probably natural temperature cycles and daylight patterns are stronger cues. Nevertheless, as in Bachleinter *et al.*, (2007), flies were sampled every two hours during the night we cannot rule out the possibility and that the three hours interval we used in our experiment is not sufficient to reveal, if any, the shift among the different neurons. In order to investigate further on this possibly we are now analyzing a new set of experiments in which flies were entrained in natural conditions under full moon/new moon nights and collected every two hours to perform the ICC experiments.

PER peak is advanced within the DN1s whereas TIM within the LPNs

Previous work described desynchronization among different clusters of clock neurons in response to particular environmental conditions:

- in 2004, Bachleitner and colleagues observed that the peak of both PER and TIM is advanced in the 5th s-LNv of flies exposed to artificial dim light during the night (Bachleitner *et al.*, 2007);

- in 2007 Miyasako and co-workers showed that when LD and temperature cycles are applied 6 hrs out of phase (with the thermocycle preceding the light-dark cycle) the dorsal neurons and LPNs peak earlier than the lateral neurons in TIM abundance (Miyasako *et al.*, 2007).

In our study we found that independently of the environmental conditions DN1s always show an earlier peak of PER compared to the lateral clock cells (**Fig 4.8**).

The observation that the DN1s peak earlier in PER accumulation than the lateral cells could be due to their positions in the fly brain. Since they are situated close to the ocelli, it is possible that they directly receive environmental information and then transmit this to the other groups of neurons in the brain. It is already known that neuronal projections from the lateral to the dorsal neurons exist (Lin *et al.*, 2004; Mertens *et al.*, 2005). It is possible that there are some as-yet unidentified neuronal connections in the other direction which allow the feedback of the dorsal neurons on the lateral ones. We attempted to further investigate this hypothesis (See **Chapter 5**).
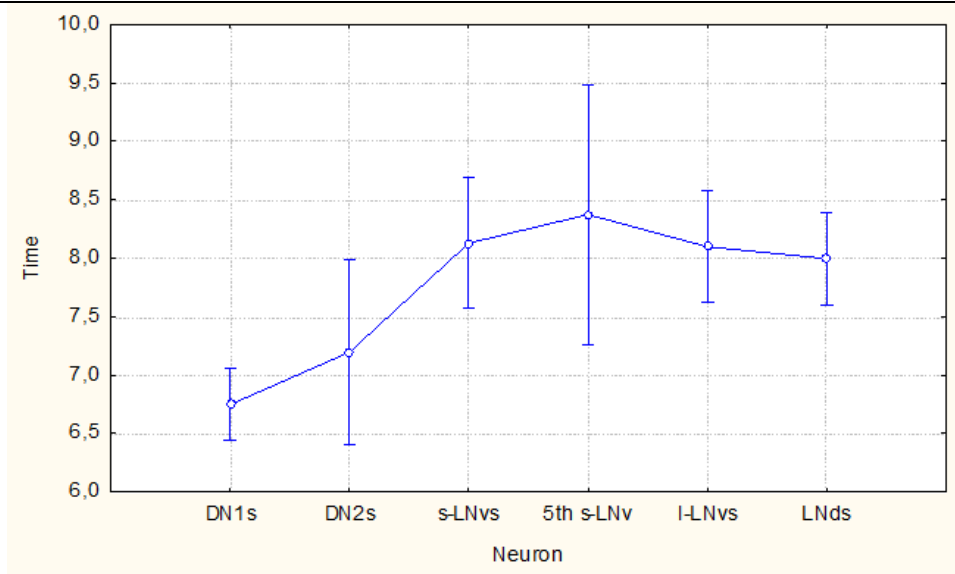

Fig. 4.8a Mean time (±s.e.m.) for PER antigenicity peak intensity measured in Spring, Summer and Autumn 2008. Y axis is local time (a.m.). Two way ANOVA gave a significant effect for Neurons ($F_{5,638}=8,6271$; p~0). The effect of Season is represented in **Fig. 4.9**. No Season X Neuron interaction was found. It is evident that the DN1s peak earlier than the lateral cells irrespectively of seasons.

Another possibility to explain the discrepancy between the peak of PER accumulation in DN1s and lateral neurons could be that the molecular clock runs faster within the DN1s. This possibility is enforced by the observation that eliminating all PDF-expressing cells (s-LNvs and l-LNvs)

results in 35-55% behavioural rhythmicity under natural conditions and that these flies have a short period compared to wild-type (S. Bhutani, PhD thesis).

Similarly, when the apoptotic gene *bax* was expressed in these cells, the rhythmic flies (17%) had a short period of 22.5 h compared to wild-type (Blanchardon *et al.*, 2001). Several other papers have referred to the short period behavioural rhythms that are manifested in flies lacking the PDF expressing ventral lateral neurons. For example, removal of these cells from the circadian network by the lack of PDF in *pdf^{01}* flies results in 24% rhythmicity in constant darkness and these flies have a short period (22.9 h) (Renn *et al.*, 1999). *disco* mutants, which lack the lateral neurons, manifest ultradian or short circadian rhythms (Dushay *et al.*, 1989; Dowse *et al.*, 1989). All these works suggest that clock neurons that do not express PDF have an endogenously shorter circadian rhythm than those that do express the neuropeptide.

A third hypothesis to explain the phase shift between dorsal and lateral cells could be a signalling coming from the Lateral neurons to the dorsal ones. It has been recently shown (Hee *et al.*, 2009) that 17 DN1s express the receptor for the neuropeptide pigment dispersing factor. PDF could be the signal through which s-LNvs, that send PDF positive projections towards the dorsal brains, affect the phase of the oscillation of clock protein within the dorsal neurons. This possibility has been further investigated (See **Chapter 5**).

Concerning TIM oscillation, it is clear that the highest level is reached earlier within the LPNs than in all other clock neurons, with the exception of the DN2s. Since the LPNs have been recognized only recently as clock neurons and almost anything is known about these cells, it is hard to hypothesize the possible reasons for this phase shift. It is now clear that temperature is affecting clock proteins expression within these cells, as previously proposed by Miyasako and Yoshii (Miyasako *et al.*, 2007; Yoshii *et al.*, 2005), and it may be that the peculiar pattern of oscillation is a mere response to decreasing temperature after dusk.
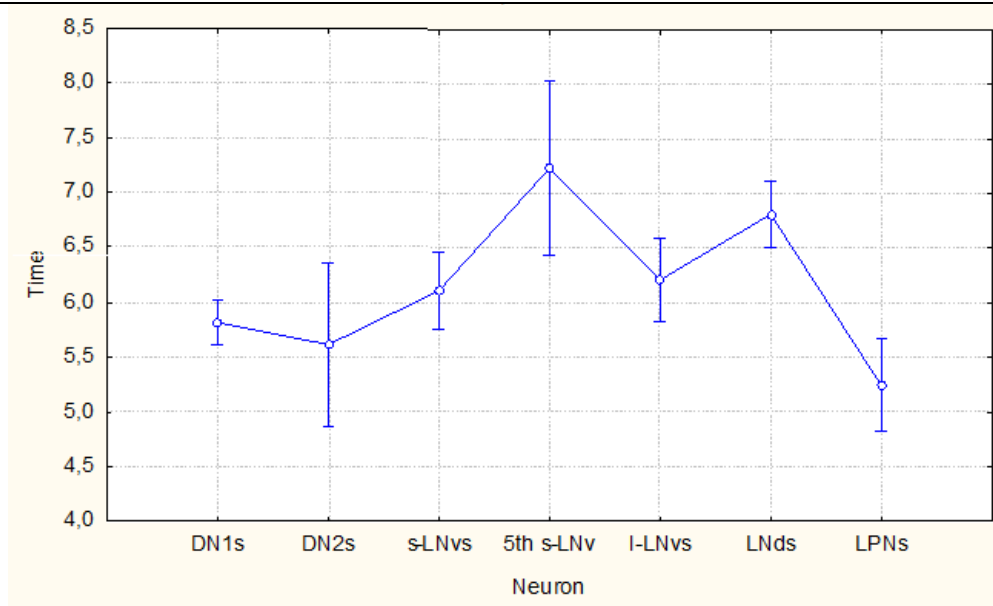
Fig. 4.8b Mean time (±s.e.m.) for TIM antigenicity peak intensity measured in Spring, Summer and Autumn 2009. Y axis represents the number of hours after the first night time point considered (9 p.m.=0). Two way ANOVA gave a significant effect for Neurons ($F_{6,870}$=8,9197; p~0). Also an effect of Season and Season X Neuron interaction was found (See **Fig. 4.9**). The DN1s peak earlier than the other clock cells (except DN2s and s-LNvs) irrespectively of seasons. Also the LPNs appear to peak earlier than the other cells (with the only exception of DN2s).

In Summer PER delays and TIM advances

Shafer and colleagues demonstrated that PER and TIM oscillations within s-LNvs and l-LNvs adjust to the photoperiod (Shafer *et al.*, 2004). More precisely they showed that the peak of TIM is advanced under long days and that in these conditions also its abundance is lowered compared to that characterizing short day conditions. On the contrary, they observed that levels of PER are lowered under short days. Moreover, the peak of PER in the cytoplasm of s-LNvs and l-LNvs was found to be long before lights-on under short days and right after lights-on under long days. Our analysis revealed that the peak of PER within the clock neurons is delayed when days are long (Spring and Summer) while occurs earlier when days are short (Autumn) (**Fig. 4.4**, **4.5** and **4.6 a, Fig. 4.9a** and **b**).

In summer and spring the photoperiod was really similar but in summer temperatures were considerably higher. Under summer conditions the peak of PER occurs later than in spring and this may be due to the higher temperature:

- during Spring the peak of PER was observed at 6 a.m. within the dorsal cells and at 9 a.m. within the lateral cells;

- during Summer the peak of PER was recorded at 9 a.m. within the dorsal neurons and at midday within the lateral cells.

The timing of the peak of TIM throughout the year is also affected by the environmental conditions but in a different way; since TIM is degraded in a light dependent manner it cannot be present after lights-on. When nights are long (and days short, as in Autumn) the peak of TIM is delayed whereas when nights are short (and days long, as in Spring and Summer) the peak of TIM is advanced (**Fig. 4.4**, **4.5** and **4.6 a**, **Fig. 4.10 a** and **b**). We did not find any significant effect of temperature on the time of the peak of TIM.

The overall results clearly indicate that the peaks of PER and TIM are moving in opposite directions throughout the year: they are slightly out of phase in Spring, extremely out of phase in Summer and almost in phase again when Autumn comes.

This peculiar pattern of phase shifting in peaks of PER and TIM could be linked to the seasonal profile of locomotor activity analyzed in detail by Bhutani *et al.*, (submitted). In particular, it has been observed that the morning onset of activity occurs earlier in Summer than in Spring and Autumn (**Fig. 1.8a**). The advance of the morning onset of locomotor activity could be due to the warmer temperatures or longer photoperiods which characterize Summers in Europe. Moreover evening peaks occur later during the day in Summer as compared to Spring and Autumn; this could be attributed to the influence of long photoperiods and hot days (**Fig. 1.8b**).
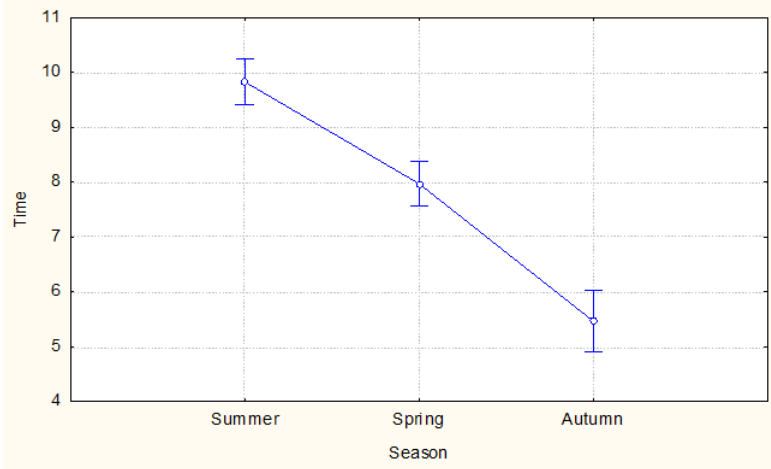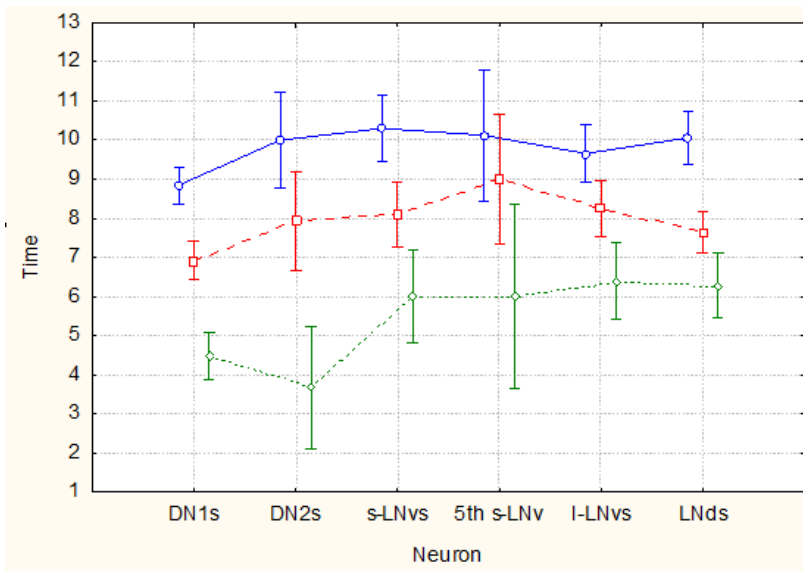
Fig. 4.9

a: Mean (± s.e.m.) time for PER peak level among seasons. Two way ANOVA gave significant effect: $F_{2,638}=75,178$, $p\sim0$.

b: Season x Neuron interaction is not significant: $F_{10,638}=1,1667$, $p=0,31029$.

Y axis is the local time (a.m.).
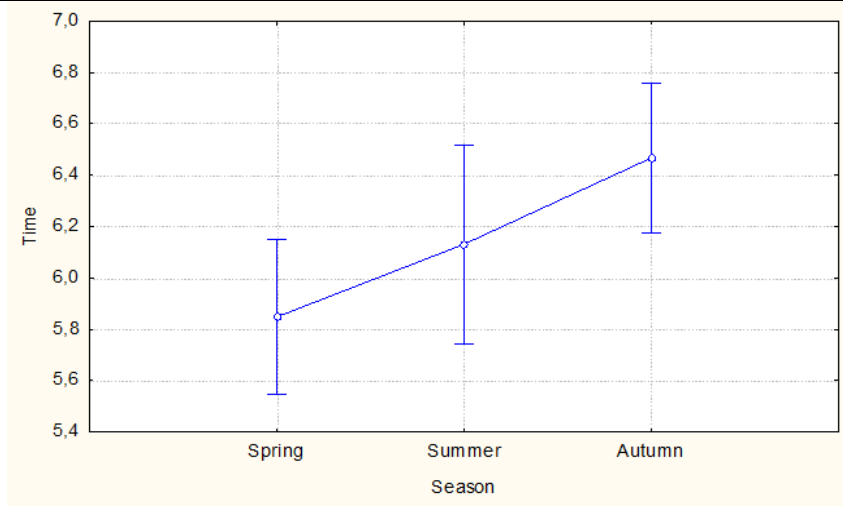
Fig. 4.10

a: Mean (± s.e.m.) time for the peaks of TIM among seasons. Two way ANOVA gave significant effect: $F_{2,870}=4,2123$, p~0.

a
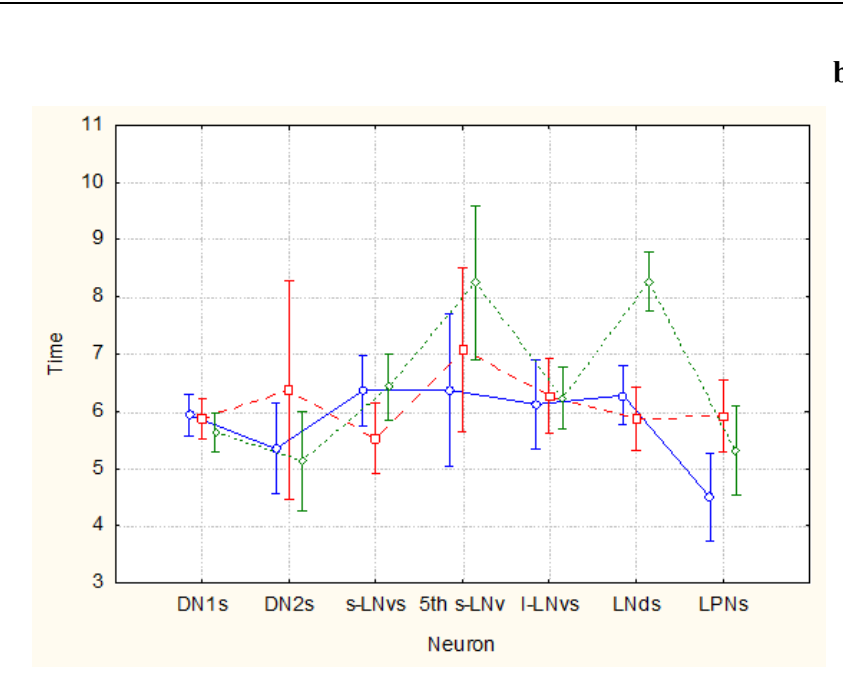
b

b: Also a significant Season x Neuron interaction was found: $F_{12,1705}=3,1361$, p<0. Thus specific environmental conditions differently affect each cluster of neurons.

Spring   Summer   Autumn

Y axis: the first time point in which TIM signal was detectable was considered as "hour =1"; thus higher number represents time points nearer to light-s on.

Results concerning both the seasonal profiles of locomotor activity and PER and TIM oscillations are summarized in **Tab.4.4**. Spring conditions are used as a reference.

	Peak of **TIM** (time, hours)	Peak of **PER** (time, hours)	Onset of **Morning activity** (mins)	Onset of **Evening activity** (hours)
Spring	04:30	09:00	50	8
Summer	advanced	delayed	advanced	delayed
Autumn	delayed	advanced	delayed	advanced

Tab. 4.4: Morning and Evening onsets of locomotor activity as in Bhutani *et al.*, submitted, are reported together with PER and TIM peaking time.
The onset of the morning activity is calculated as the difference between the average time at which flies start to move and the time at which nautical twilight has been recorded (www.usno.navy.mil). Thus, in Spring the onset of morning activity is on average 50 mins after nautical twilight)
The onset of the evening activity is calculated as the difference between the average time at which the evening peak of activity starts and the time at which light started to increase (light intensity>1lux) as recorded with the Trikinetics environmental monitor. Thus in Spring the evening peak of activity starts on average 8 hours after lights-on.

Our data suggest the existence of a relationship between PER and TIM oscillations and the onset of either morning or evening activities. More precisely, the peak of TIM is advanced in Summer and delayed in Autumn as is the onset of morning activity, whereas PER's peak appears to be more linked to the onset of evening activity.

Upon analysis of PER and TIM oscillation profiles under each examined conditions, we observe that in Summer PER and TIM peaks occur with almost 12 hours of difference in at least s-LNvs and LNds (**Fig. 4.5 a** and **b**). An antiphase between the peak of PER and TIM has never been observed so far, neither in lab nor in the wild. The environmental cue that could be driving this phase shift is likely temperature. In fact, similar photoperiods were present during Spring and Summer experiments, nevertheless only in Summer, at higher temperatures, the peaks of PER and TIM occur in antiphase. This hypothesis has been further investigated and results are reported in **Chapter 6**.

Chapter 5:

The unexpected advanced peak of PER within the DN1s

Surprisingly, the analysis of PER oscillation pattern in the brains of flies exposed to the natural environment uncovered a consistent phase advance in PER upswing in the DN1s compared to flies maintained under rectangular LD regimes and at constant temperature (**Chapter 4: Fig. 4.4a**, **4.5a**, **4.6a** and **4.8a**). Since this phase advance in the peak of PER is absent under standard lab conditions, it is likely promoted by the complex natural environment.

We can formulate some putative explanations to account for this phenomenon:

- DN1s may receive specific signals coming from the other clock cells and advance their phase in response to this input;
- since DN1s are located near to the ocelli they may directly receive environmental information and eventually transmit this information to the other clock neurons;
- the molecular clock within the DN1s may run faster than in the lateral clock cells under natural conditions and this faster rhythm could be hidden in the lab in the absence of complex stimuli.

At first we tried to understand if the phase advance was caused by input coming from the lateral clock neurons. The results of this study are reported below in **Section 5.1**. Moreover flies carrying a functional clock only within the LNs were analyzed in order to check whether the absence of stimuli coming from the other impaired clock neurons would alter PER oscillation. In this case, results are reported in **Section 5.2**.

5.1 PDF signalling is not responsible for PER phase advance within the DN1s

It has been recently shown by (Im and Taghert, 2010) that 17 DN1s express the receptor for the neuropeptide Pigment Dispersing Factor (PDFR). PDF is expressed in the s-LNvs: these clock cells send PDF positive projections towards the dorsal brain (Helfrich-Forster, 1997). The phase advance that was found in the peak of PER within DN1s compared to the lateral cells may be due to a response to PDF signalling. To test this hypothesis we used flies in which PDF expressing cells are ablated because of the expression in these cells of the apoptotic genes *hid* and *rpr* (under control of *pdf* promoter; See **Section 2.2** and **Fig. 2.2a**). PER expression in the brain of UAS*hid*-UAS*rpr*; *pdf*GAL4 flies is shown in **Fig. 5.1**. These flies lack both PDF producing cells (namely,

s-LNvs and l-LNvs) and PDF itself and will be referred to as LNvs-/PDF- flies from now on. WT ALA flies were used as a wild type control.
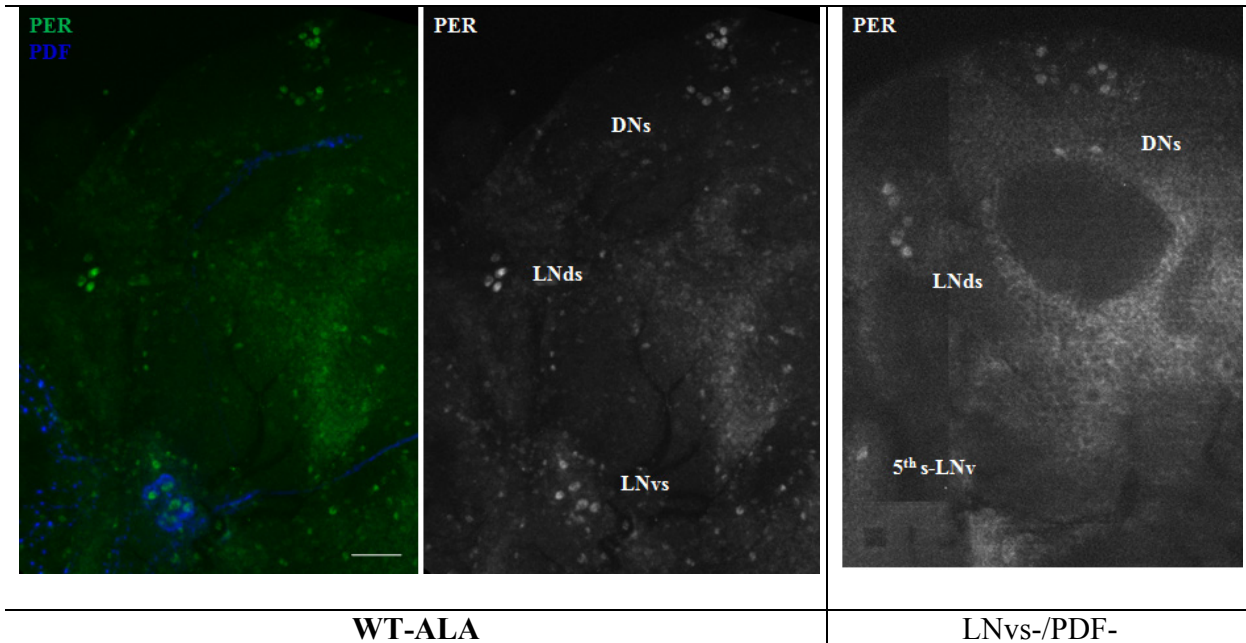

| WT-ALA | LNvs-/PDF- |

Fig. 5.1: PER (and PDF) immunoreactivity within brains of WT-ALA and LNvs-/PDF- flies at 9 a.m.. In WT-ALA brains all clock neurons are present and express PER; the LNvs also express PDF. Since in LNvs-/PDF- flies the s-LNvs and the l-LNvs are missing, PER is expressed only in DNs, LNds and the 5th s-LNv (see **Section 2.1**). Thus, PDF is not produced in the brain of LNvs-/PDF- flies. Scale bar =20μm.

For the experiment, both LNvs-/PDF- and WT-ALA flies were entrained for three days under natural conditions and then collected every three hours in order to dissect the brains and perform the immunoassay with α-PER and α-PDF antibodies.

The overall results obtained by the quantification of the fluorescent signal at the different time points monitored are shown in **Fig.5.2**. The experiment was carried out at the beginning of June 2010, (NLD~16:8, Tmax 31,9°C, Tmin 20,7°C).

Under these environmental conditions the oscillation of PER appears to be very similar to what observed under Spring conditions in 2008 (**Fig. 4.4a**), despite in this case temperature is slightly higher. In WT-ALA flies the peak of PER is at 6 a.m. within the dorsal neurons and at 9 a.m. (3 hours later) within the lateral cells.

In LNvs-/PDF- flies PER staining was quantified only in DN1s, DN2s, 5th s-LNv and LNds, since PDF positive cells were ablated. We found that PER peaks in DN1s and DN2s at the same time it does within the dorsal clock neurons of control wild-type flies. Also the 5th s-LNv shows a

maximum level of PERIOD at 9 a.m. as in WT-ALA. The cycling of PER within the LNds is slightly different compared to the control, in particular the peak here is a bit broader.

In any case, from these data we can exclude that either PDF signalling or PDF producing cells are affecting the phase of the dorsal neurons given that in their absence the peak of PER appear to occur at the same time it does in wild-type flies.
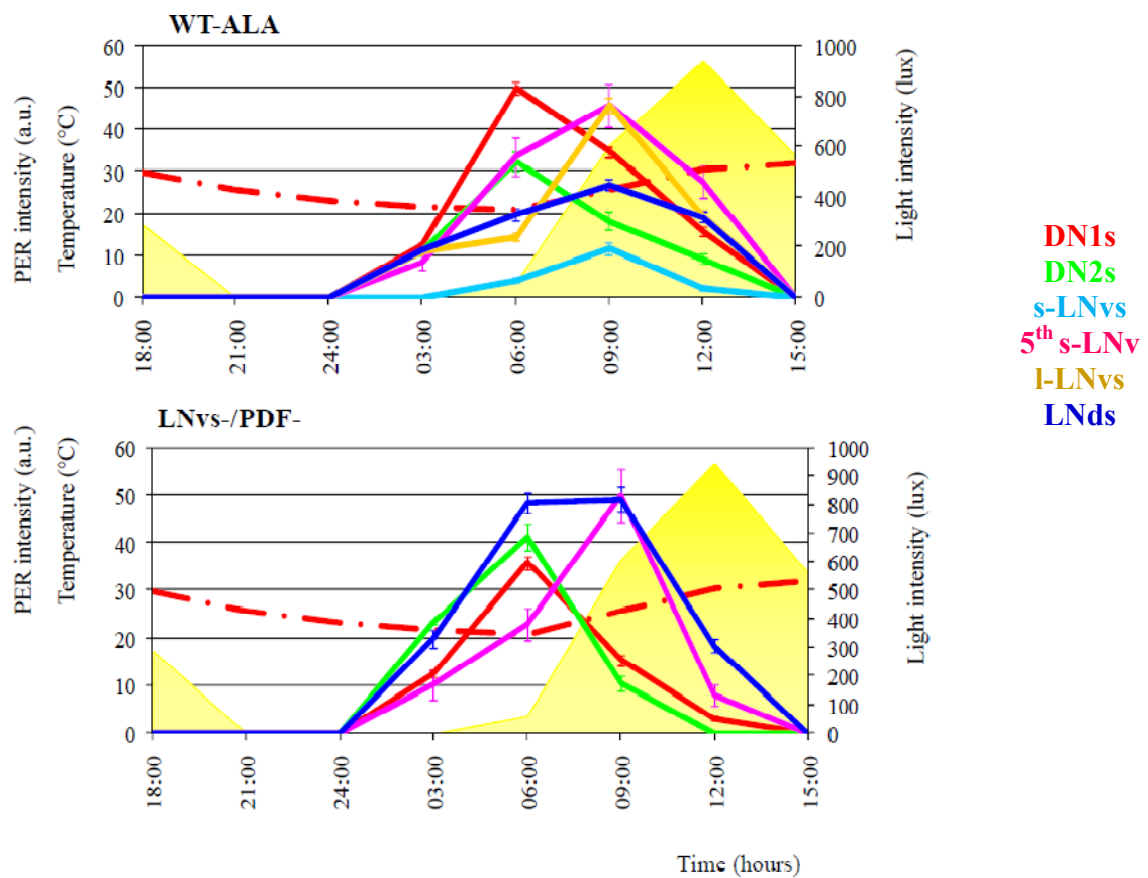
Fig. 5.2: PER accumulation profile within the clock neurons of WT-ALA and LNvs-/PDF- flies. Light intensity is represented by the yellow area, temperature by the dotted red line. The DN1s show a maximum level of staining intensity at the same time in both strains; thus, PDF signalling is not needed for PER phase advance.
The experiment was carried out in June 2010; NLD~16:8, Tmax=30°C, Tmin=20°C.
PER fluctuations are statistically significant (One way ANOVA, $p < 0.05$).

5.2 Do the dorsal neurons affect the phase of the lateral cells?

DN1s are located in the dorsal brain near the ocelli and it may be that these cells directly receive specific stimuli, albeit unknown yet, coming from the environment. If the DN1s are specifically sensing these stimuli, and most likely responding for first, they could then elaborate and transmit this information to the lateral cells.

To test this hypothesis we looked at the oscillation profile of PER within the Lateral neurons of flies carrying a functional clock only in the PDF positive cells (s-LNvs and l-LNvs): per^0w; *pdf*GAL4; UAS*per16* express PER under control of the *pdf* promoter in per^0 background.

Since the PDF positive LNvs have been proven to be responsible for driving the morning peak of activity and therefore indentified as the Morning Oscillator (MO) (Grima *et al.*, 2004), per^0w; *pdf*GAL4; UAS*per16* flies will be refer to as MO+ flies from now on. PER expression within the brain of MO+ flies is shown in **Fig. 5.3**.

Flies were entrained outside for three days before sampling in order to perform an ICC experiment with α-PER and α-PDF antibodies. The experiment was carried out in July 2010 (NLD~16:8, Tmax=36,2°C, Tmin=25,8°C). Under these environmental conditions we expected the peak of PER to be after lights-on in both Lateral and Dorsal neurons; for this reasons we collected flies every three hours starting from late night 3 a.m.) until early afternoon (3 p.m.). Results are reported in **Fig.5.4**. If LNvs need to receive information from the PDF negative cells, then the profile of PER accumulation would have been somehow altered in MO+ flies compared to wild-type ALA.

In WT-ALA flies the peak of PER appeared to be at 9 a.m. within the dorsal neurons and the maximum level of the protein was revealed at midday within the lateral neurons. These data confirmed what we already obtained describing PER oscillation profile during Summer 2008 (See **Fig. 4.5a**, **Section 4.2**).

MO+ flies carry a functional clock only in s-LNvs and l-LNvs, since expression of PER is driven only in PDF positive cells under control of *pdf* promoter. We found that PER oscillation within the clock neurons of these genetically manipulated flies was completely different from what observed in wild-type flies. PER shows a maximum level of expression at 3 a.m., when in wild-type flies its level is still really low. After this time point the level of PER is dampening to be

then undetectable at 3 p.m.. Thus, the oscillation of PER within the clock neurons of MO+ flies is completely out of phase with the oscillation of PER within the s-LNvs and l-LNvs of WT ALA.

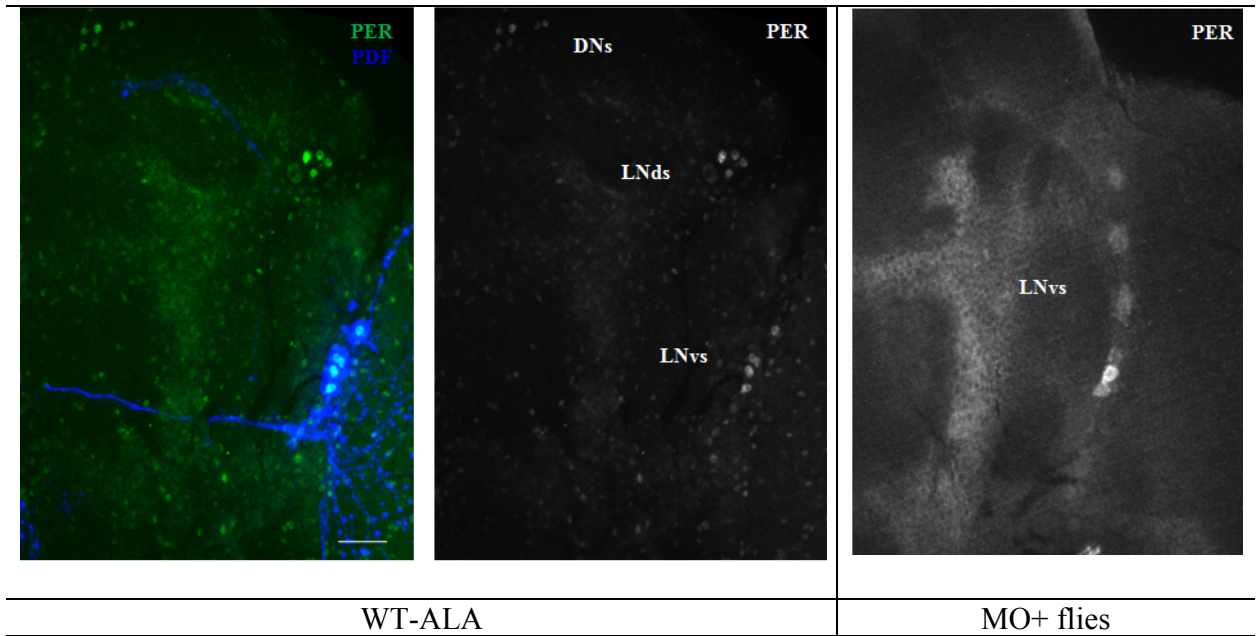| WT-ALA	MO+ flies

Fig. 5.31: PER (and PDF) immunoreactivity within the brains of WT-ALA and MO+ flies at 9 a.m.. In WT-ALA brains all clock neurons are present and express PER; the LNvs also express PDF. In MO+ flies, PER expression is driven only in PDF+ cells under control of *pdf* promoter in per[0] background: thus the only PER positive cells are the s-LNvs and l-LNvs. Scale bar =20μm.
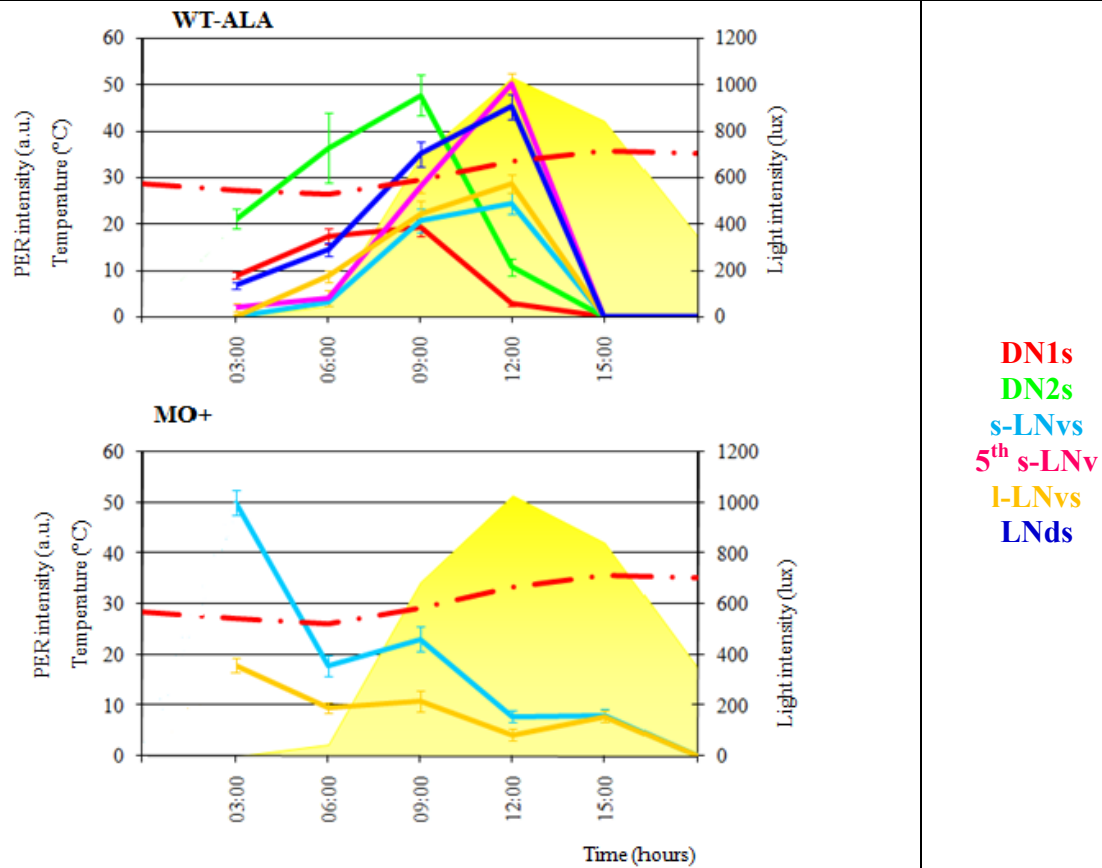
Fig. 5.4: PER accumulation profile within the clock neurons of WT-ALA and MO+ flies. Light intensity is represented by the yellow area whereas temperature by the dotted red line. PER oscillation within MO+ flies clock neurons are totally out of phase with the oscillation of the protein in the same cluster of neurons in WT-ALA brains.
The experiment was carried out in July 2010; NLD~16:8, Tmax=36,2°C, Tmin=25,8°C.
PER fluctuations are statistically significant (One way ANOVA, $p<0.05$).

5.3 Discussion

PDF signalling is not involved in advancing the phase of PER accumulation within the DN1s

To test whether the phase advance of PER oscillation within the DN1s under natural conditions is caused by PDF signalling we analyzed PER expression profile in flies in which PDF expressing cells were killed using a construct driving the expression of apoptotic genes *hid* and *rpr* under the control of the same *pdf*-promoter driver.

We found that ablating PDF and PDF producing cells do not affect at all the phase of PER cycling within the dorsal neurons. Moreover, we observed that also PER cycling within LN$_d$s and the 5th s-LNvs was not different if compared to wild-type.

These findings fit with previous results obtained by Bhutani *et al.*, submitted, analyzing the behaviour of these flies under natural conditions. In fact, it has been observed that either killing the PDF expressing cells or stopping their clock does not generate dramatic effect on locomotor phenotype but it only enhances the temperature sensitivity of the evening peak of activity. These results are at odd with previous observations: flies lacking the morning cells do not show a morning peak of activity under laboratory LD 12:12 at constant temperature and appear to be arrhythmic in constant darkness (Grima *et al.*, 2004; Stoleru *et al.*, 2007; Stoleru *et al.*, 2004). We can now say that, most probably, it was not possible to detect any evident disruption of behavioural phenotype because the molecular clock of these genetically manipulated flies is not affected by the absence of both cluster of clock neurons and PDF. Moreover, it is important to keep in mind that the natural environment is much more complex than the laboratory one and that environmental cues are probably stronger in nature: it seems likely that under natural conditions every mutations that is known to compromise the clock mechanism (from lab studies) can be easily by-passed or buffered in their effects.

Altered pattern of PER accumulation within the clock neurons of MO+ flies

Since dorsal neurons are located in the dorsal brain near to the ocelli, it may be that these cells sense, differently from other clusters of clock neurons, specific stimuli coming from the environment. If this is the case, dorsal neurons could then communicate the information to the other clock cells.

To verify this hypothesis we looked at PER oscillation within the s-LNvs and l-LNvs in flies carrying a functional oscillator only in these two groups of cells (MO+ flies). To restrict the presence of a functional clock only to these eight cells PER expression was driven under control of *pdf* promoter in *per^0* background.

We found that in these flies the oscillation profile of PER was really different from what observed in the wild-type control WT-ALA under the same environmental conditions: under

Summer conditions the peak of PER was found to be far after dawn in all cluster of clock neurons whereas in MO+ flies we found a high level of the protein before dawn.

It is conceivable that the observed phase shift could be due to the absence of clock neurons other than the PDF expressing ones. Upon careful analysis of PER oscillation profile it is possible to see that in this case PER shows a pattern that is really similar to that observed under short days with a peak around lights-on (See **Fig 3.3**).

It has been shown by Majercak and colleagues that at low temperature and short photoperiods mimicking Winter conditions, the splicing of the *per* 3' UTR is enhanced, leading to an early accumulation of PER protein and a subsequent advance in the evening peak of activity (Majercak *et al.*, 1999). At high temperatures and long photoperiods, representative of Summer, *per* splicing is inhibited and the peaks of activity are shifted towards dawn and dusk, leading to a "siesta", presumably during the hotter parts of the day and thereby ensuring that the majority of the activity occurs during the cooler parts of the day.

The fact that PER oscillation in MO+ flies under Summer conditions resembles the one of wild-type flies under short day conditions may be caused by compromised splicing of PER due to the intrinsic "architecture" of the construct use to express the transgenic protein. Indeed the UAS*per16* construct miss a part of the 3' UTR (Blanchardon *et al.*, 2001).

Chapter 6:

PER and TIM can oscillate in antiphase

Light:dark regimes mimicking seasonal changes cause behavioural adjustments while altering clock genes expression (Majercak *et al.*, 1999; Shafer *et al.*, 2004; Stoleru *et al.*, 2007). Moreover, in response to light pulses administered at the beginning or at the end of the night the molecular clock can be delayed or advanced respectively due to modifications in PER and TIM abundance and interactions. Therefore, it is probable that behavioural seasonal adjustments in the real world reflect adjustments in clock genes and proteins interactions, with PER and TIM playing a crucial role.

In this study the oscillation profiles of PER and TIM under natural conditions have been studied in detail over the year. We found that PER and TIM accumulation patterns are different under different environmental conditions as they adjust to the different thermal profiles and photoperiods that characterize seasonality. In **Chapter 4** we report that the peak of PER is delayed under long days and hot temperature whereas the peak of TIM moves in the opposite directions as it advances when days are long and delays when days get shorter (see **Fig. 4.9** and **4.10**). The main effect of this phase shift between PER and TIM oscillation is that the two proteins are found to be almost out of phase (PER peaks almost 12 hours later than TIM) under Summer conditions, at least in some groups of clock neurons (**Fig. 4.5** and **5.4**). This unexpected finding appear to be at odd with what is known about the interaction between PER and TIM (**Section 1.2**). In fact, PER and TIM heterodimerize in the cytoplasm of the clock cells in order to enter the nucleus and repress their own transcription (Curtin *et al.*, 1995; Gekakis *et al.*, 1995); in the absence of TIM, PER is phosphorylated by the kinase DBT and undergoes degradation (Price *et al.*, 1998). Therefore, if under Summer conditions PER is still accumulating for many hours after TIM disappearance within the clock neurons of our WT-ALA flies something else than TIM must stabilize PER and allows its translocation into the nucleus.

In order to better understand this phenomenon the nuclear accumulation of both PER and TIM was analyzed in detail under Summer conditions (**Section 6.1**). Moreover, with the aim of indentifying the main environmental parameter inducing this striking phase shift, we analyzed the phenomenon in laboratory controlled conditions where we mimicked natural long day photoperiods at warm or hot temperatures (**Section 6.2**). The latter experiments have been performed in collaboration with the laboratory of Prof. Charlotte Helfrich-Förster, Department of Neurobiology and Genetics, Julius Maximilians Universität Würzburg (DE).

The patterns of PER and TIM nuclear accumulation under natural conditions as well as PER and TIM oscillation profiles under laboratory conditions mimicking Summer days are reported and commented below.

6.1 PERIOD and TIMELESS nuclear accumulation under natural conditions

Shafer and colleagues have analyzed some years ago the effects of changing day length on PER and TIM accumulation in laboratory conditions (Shafer *et al.*, 2004). The authors found that under long days, at constant 25°C, the nuclear entry of TIM stops at lights-on whereas the nuclear accumulation of PER continues until two hours after lights-on. After that levels of PER in the nucleus are slowly decreasing. Notably, under these conditions it appears that the translocation of PER and TIM into the nucleus begins at the same time.

In this study we have shown that under long days (NLD~ 16:8) and high temperatures (25- 35°C) PER continues to accumulate within the clock neurons even after TIM disappearance (**Fig. 4.5**). Notably, in **Chapter 4** the total fluorescence signal for PER and TIM was quantified on the entire neuronal cells, without a distinction in cytoplasmic and nuclear cell district.

Upon careful analysis of the images acquired for both PER and TIM experiments under Summer conditions we found that, as previously show by Shafer *et al.* (2004),the two clock proteins enter the nucleus at the same time: when TIM level is already high whereas PER just starts to accumulate (**Fig. 6.1**). Thus, TIM seems still to be necessary for PER translocation into the nucleus while the observation that the level of PER is still increasing after TIM is degraded may be true only for the cytoplasm.

In order to better define the pattern of nuclear accumulation of both clock proteins, PER and TIM nuclear stainings were considered. The fluorescence signals were quantified from the images already acquired from the two ICC experiments performed under Summer conditions (See **Section 4.2**). In this case only the signal relative to the area of the cell corresponding to the nucleus was taken into account. In **Fig. 6.2** the nuclear accumulations of PER and TIM under Summer conditions are shown.

In **Fig. 6.**2**a** it is evident that PER enters the nucleus as soon as it starts to accumulate. This can be easily explained since when PER starts to accumulate TIM levels are already high in the cytoplasm.
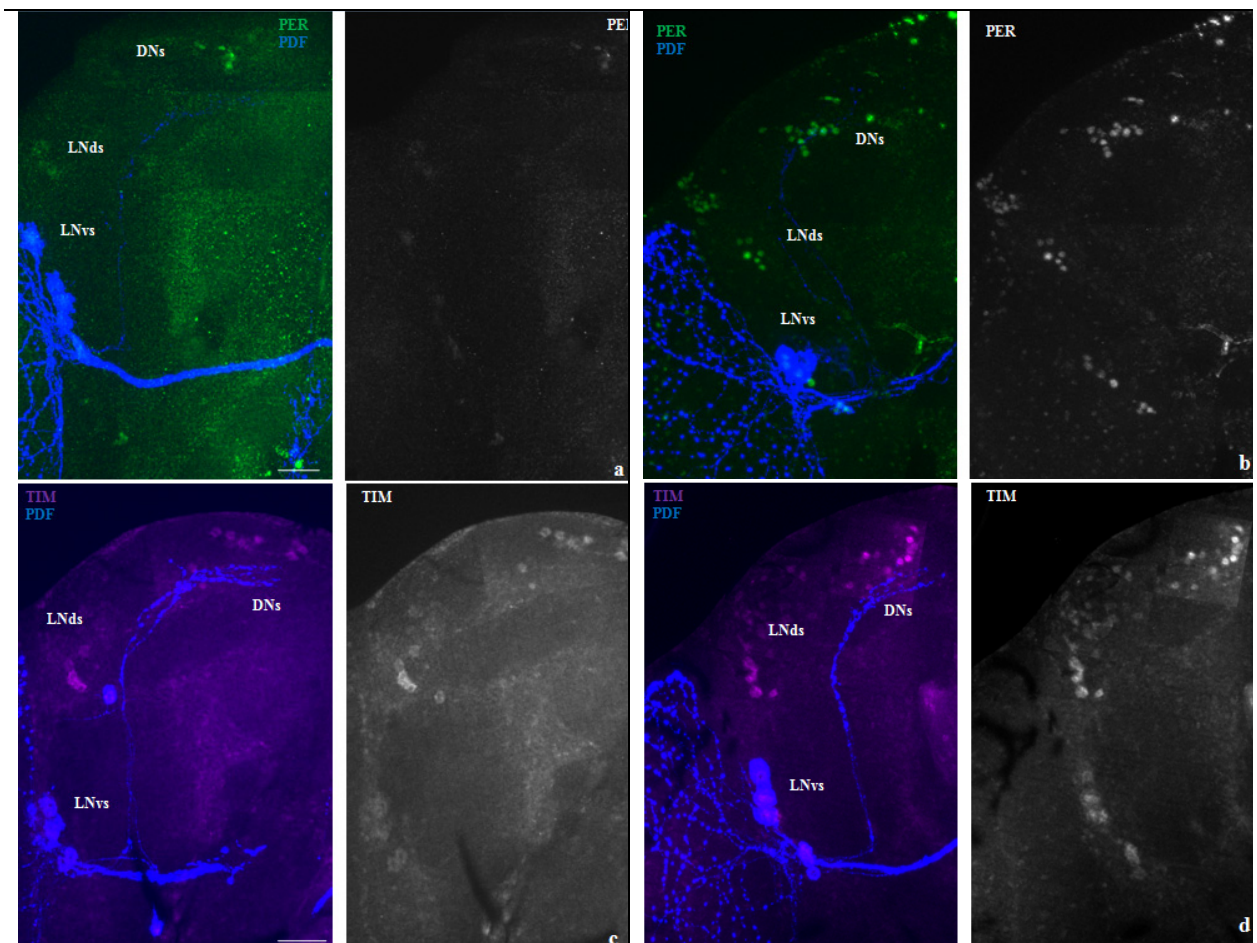
Fig. 6.1 PER, TIM and PDF immunoreactivity within the clock neurons of WT flies collected at 3 a.m. (**a** and **c**) or right before dawn (5 and 4:30 a.m. in **b** and **d**, respectively) under Summer conditions in 2008 (PER) and 2009 (TIM). See **Table 4.2** and **4.3**. Scale bar = 20μm either for PER/PDF or TIM/PDF images. In **a** and **c** the staining appear to be cytoplasmic for both PER and TIM, and PER fluorescence is low if compared to the TIM one. Notably, the time point reported in **a** (3 a.m.) is the first in which PER is detectable under Summer conditions. Two hours later (**b** and **d**) the staining appears to be high and nuclear for both proteins.

The nuclear accumulation pattern of PER and TIM within the dorsal neurons is similar to the one usually observed under laboratory conditions. The peak of TIM occurs about three hours earlier than that of PER as it does for example under standard laboratory conditions (e.g. **Fig. 3.3**)

On the contrary PER and TIM nuclear accumulation within the lateral cells appears to be completely unusual:

- TIM starts to accumulate slowly and its peak in the nucleus is reached right before dawn;

- PER level in the nucleus is already high three hours after it starts to accumulate and it is staying high until midday, when TIM is not present anymore.
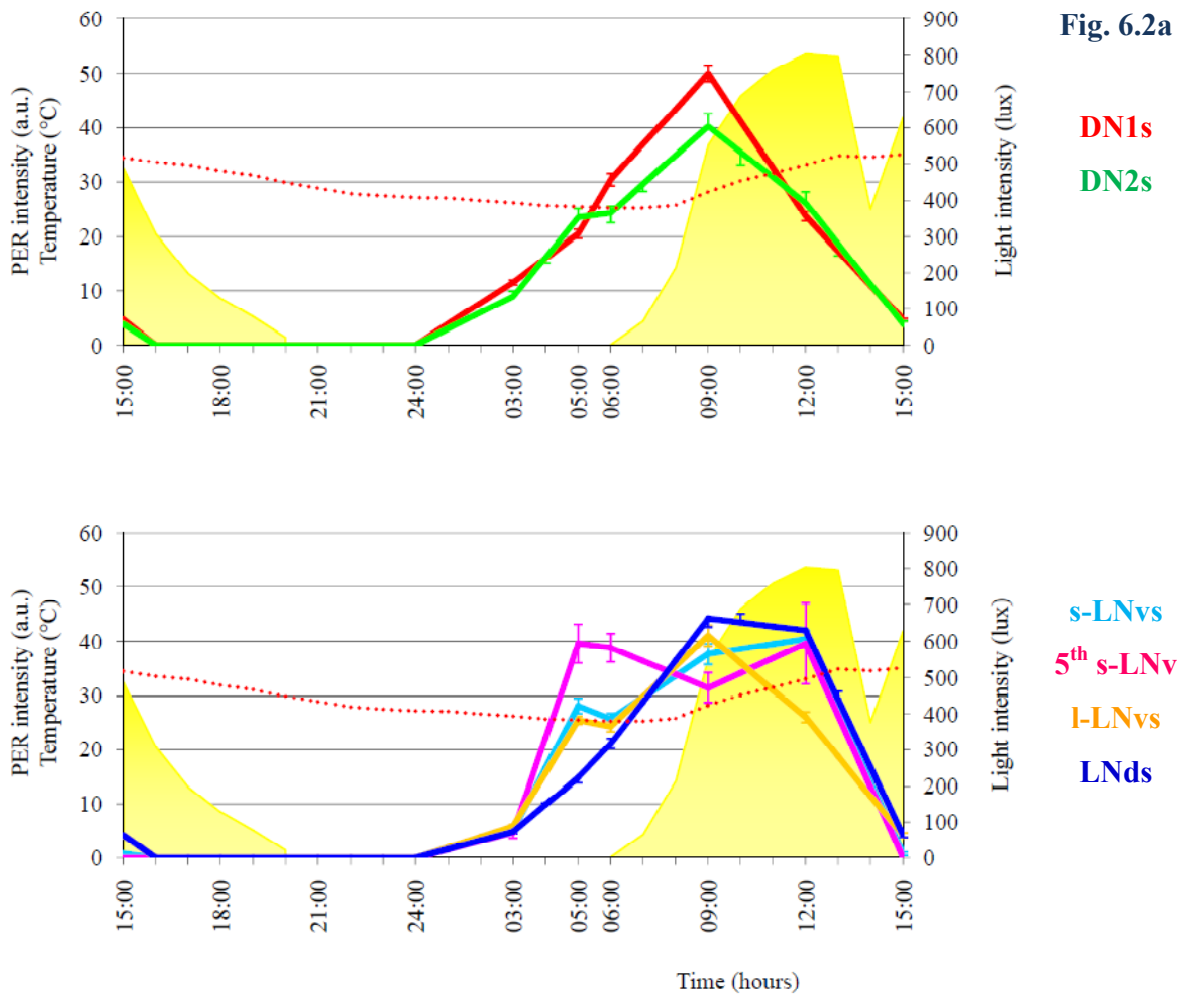

Fig. 6.2: PER (**a**) and TIM (**b**) oscillation profiles within the nucleus of clock neurons of WT-ALA flies entrained under Summer conditions (See **Tab. 4.2** and **4.3**). The yellow area represents the light intensity, the red dotted line is temperature. PER shows a peak within the nucleus of the DNs at 9 a.m. and then its level decreases. Within the LNs, PER reaches a high level at about dawn (5 a.m.) but then it stays high until midday. TIM oscillation appear to be more synchronized among the different clusters of clock neurons if the nuclear quantification is compared to the whole cell one (**Fig. 4.5b**).In any case, in both DNs and LNs the peak of TIM occurs at 4:30, right before dawn, and the level drastically decreases until the protein is undetectable at about 7:30, three hours later.

Fig. 6.2b

DN1s

DN2s

s-LNvs

5th s-LNv

l-LNvs

LNds

Time (hours)
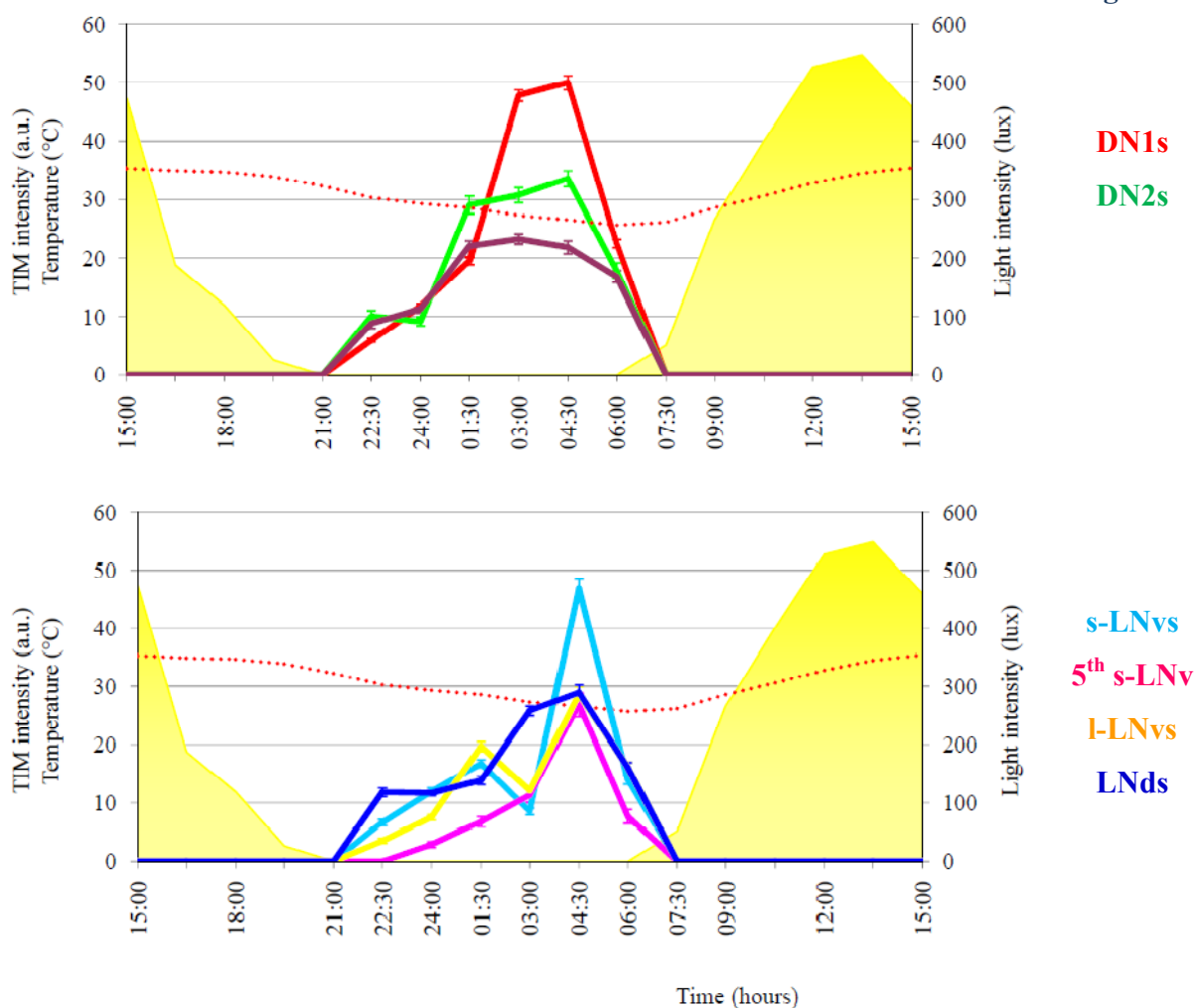
6.2 TIM is necessary to trigger PER translocation into the nucleus

It is well known that the formation of PER/TIM heterodimers is necessary both for PER accumulation and its translocation into the nucleus of clock neurons (Curtin *et al.*, 1995; Gekakis *et al.*, 1995; Meyer *et al.*, 2006; Price *et al.*, 1998). In this study, the oscillation profile of PER and TIM under different natural environmental conditions was analyzed and something at odd with the observations reported above has been found.

\In **Section 4.**2 we showed that PER and TIM appear to oscillate in antiphase under particular environmental conditions (**Fig. 4.**5), namely, long days and hot temperatures.

Moreover, in **Section 6.1** we reported that PER not only persists to accumulate in the absence of TIM but most probably also continues to enter the nucleus. Therefore, it is likely that there is something else other than TIM acting on PER accumulation and translocation into the nucleus, since these two processes can go on even when TIM is not detectable anymore within the clock neurons due to its degradation mediated by light signalling (Ceriani *et al.*, 1999).

In order to verify this hypothesis, *tim^{01}* flies (a TIM null mutant; see **Section 2.2**) were entrained under natural conditions (Summer 2010: NLD~ 16:8; Tmax= 35,8°C; Tmin= 26,2°°C) and then collected every three hours throughout a day for dissection. Dissected brains were then stained with α-PER and α-PDF antibodies to reveal the PER accumulation profile (if any) within the clock neurons. WT-ALA flies were used as a wild-type control.

In order to avoid problems due to the sensitivity characteristics of the semiconfocal microscope usually utilized for the acquisition of the images (Nikon Eclipse80i), a confocal microscope was used in this case (Nikon Eclipse E600).

Upon analysis of all images relative both to *tim^{01}* mutant and WT-ALA control flies it appeared clear that PER staining was not detectable at any of the time points analyzed in the mutants whereas the oscillation pattern of the protein was normal in the wild-type flies.

Fig. 6.3a and **b** show an example our findings; images are relative to a time point (6 a.m.) at which a high level of PER signal within the nucleus of the clock neurons was expected, according to the results reported in **Section 6.1**.
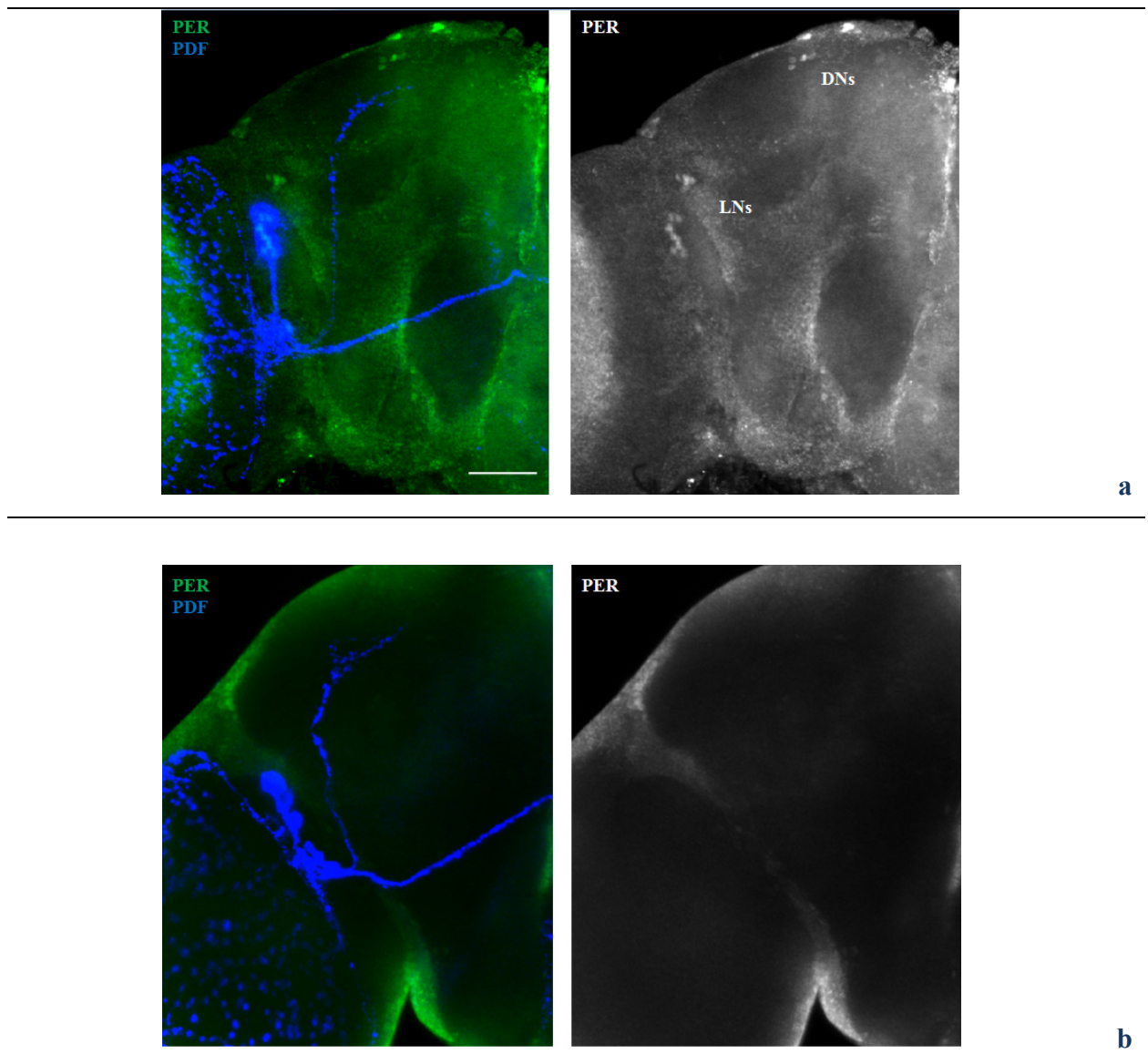
Fig. 6.3: PER and PDF immunoreactivity within the clock neurons of WT-ALA (**a**) and *tim⁰¹* (**b**) entrained under natural conditions in Summer 2010. The brains shown were collected at 6 a.m.. Scale bar = 20μm. In WT-ALA all clock neurons appear to express PER (according to the oscillation profile reported in **Fig. 6.2a** whereas no PER staining was detectable within the *tim01* brain at all time points analyzed.

6.3 Identifying the cause of PER phase delay

Albeit TIM is necessary to initiate PER accumulation even under natural conditions (**Section 6.2**) as it is under standard laboratory conditions (Sehgal *et al.*, 1995), we found that under long days and hot temperatures the peak of PER is delayed of almost twelve hours at least in s-LN$_v$s and

LNds (**Section 4.2**, **Fig.4.5**). Moreover, we found that when days get longer and temperature higher, the peak of PER is delayed whereas the peak of TIM is advanced (**Fig. 4.9** and **Fig. 4.10**). Because of these phase shifts of PER and TIM oscillations towards opposite directions, PER continues to accumulate in the clock neurons even after TIM has been completely degraded.

This phase shift between PER and TIM oscillation appear is more evident in Summer than in Spring, when photoperiods were very similar (**Section 4.2**, **Tab. 4.2** and **Tab. 4.3**). Comparing the two seasons it is evident that the main environmental difference is temperature: in Spring the average temperatures during the experiments were about 20°C while average temperatures in Summer were about 30°C. We therefore hypothesize that the bigger phase shift between PER and TIM peaks recorded in Summer was mainly due to the high temperature.

In order to test this hypothesis it was necessary to asses PER and TIM accumulation patterns under controlled conditions. We carried out an experiment in collaboration with Prof. Charlotte's Helfrich-Förster's lab, Department of Neurobiology and Genetics, University of Würzburg (DE). WT-ALA flies were entrained to LDR conditions (light/dark cycles with ramping of light to simulate dawn and dusk) for three days: flies were experiencing a long photoperiod (LDR 16:8) either at constant 20°C or 30°C. More precisely light intensity was either increases from 0 to 500 lux to simulate dawn or decreased from 500 to 0 lux to simulate dusk in 1 hour and a half time interval. To get a finest resolution of the oscillation pattern, flies were collected every two hours instead than every three hours as usually. The confocal microscope available at the Department of Neurobiology and Genetics of the University of Wurzburg was used for the acquisition of the images; the possibility to use the Leica DM 5500 Q confocal microscope allowed us to obtain two important improvements in our experimental approach:

- higher sensitivity, as already reported in **Section 4.2**;
- the possibility to perform a triple staining (α-PDF, α-TIM; α-PER antibodies used at the same time to incubate the dissected brains) that otherwise would have been impossible with the equipments available at the Department of Biology of the University of Padova (IT).

The results obtained upon the quantification of the fluorescence intensity for PER and TIM under the experimental conditions adopted (two different constant temperature regimes) are reported in **Fig. 6.4** and **6.5**.

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In particular, we found that at constant 20°C (LDR 16:8, where ZT0 corresponds to lights-on and ZT16 to lights-off) PER starts to accumulate at about ZT 20 and its oscillation profile is different between dorsal and lateral clock cells.

- Lateral neurons: PER shows a slow accumulation, reaches a peak at ZT2 and starts to decrease right after that.
- Dorsal neurons: PER peaks at about ZT22 and then stays at high levels until ZT4, when the decrease starts.

Remarkably, in all clock neurons the levels of PER start to consistently decrease after ZT4 (**Fig. 6.4a**).

TIM starts to accumulate earlier that PER, right after lights-off, and reaches the maximum level at ZT20 in all clock neurons except of DN2s in which the peak is delayed of about 2 hours. The level of TIM drops in all clock neurons at the same time: at lights-on (**Fig. 6.4b**).

At constant 30°C, PER level starts to increase earlier than at 20°C; almost immediately after lights-off (ZT16) the signal raises in intensity. Notably, at 30°C PER is expressed also within the LPNs.

As previously found at 20°C, the accumulation profile of PER differs among the different clusters of clock cells (**Fig. 6.5a**).

- DN1s and LPNs: the maximum level is reached already at ZT 20; after this time point the fluorescence signal slightly decreases at ZT22 but a high level of fluorescence (at least 50% of the maximum intensity) is maintained until ZT4 for the LPNs and ZT6 for the DN1s.
- DN2s: the maximum intensity is reached already at ZT20 and it remains at about the same level until ZT6.
- 5th s-LNv: there is a clear peak in intensity reached at ZT2.
- s-LNvs and l-LNvs: the maximum level of intensity is maintained until ZT0 and ZT6.
- LNds: the level of PER intensity is already high at ZT20 but the peak is reached at ZT6.

Fig 6.4

a: PER oscillation profile within the Dorsal and Lateral neurons of brains dissected from flies entrained under LDR 16:8, at constant 20°C. PER fluctuations are statistically significant (One way ANOVA, p< 0,05).

DN1s   DN2s

s-LNvs   5th s-LNv

l-LNvs   LNds

b: TIM oscillation profile within the Dorsal and Lateral neurons of brains dissected from flies entrained under LDR 16:8, at constant 20°C. TIM fluctuations are statistically significant 8One way ANOVA, p<0,05).

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Fig 6.5

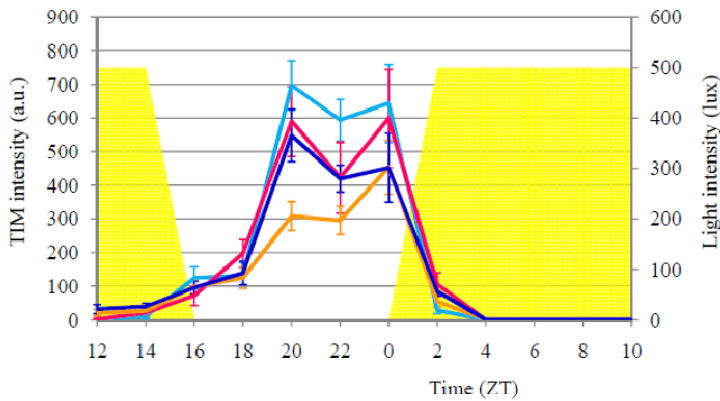**a**: PER oscillation profile within the Dorsal and Lateral neurons of brains dissected from flies entrained under LDR 16:8, at constant 30°C. PER fluctuations are statistically significant (One way ANOVA, $p < 0.05$).

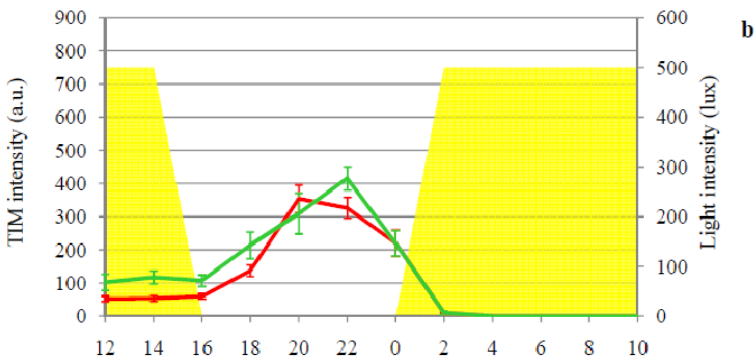**DN1s   DN2s   LPNs**

s-LNvs   5th s-LNv

l-LNvs   LNds

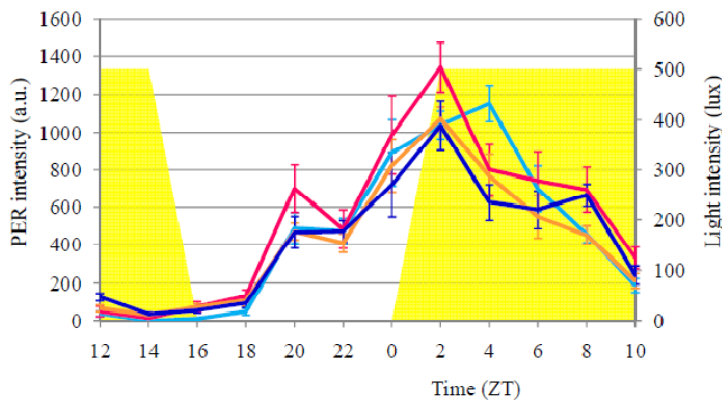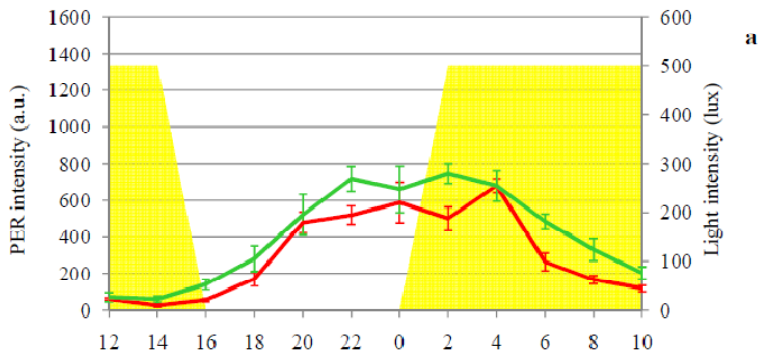**b**: TIM oscillation profile within the Dorsal and Lateral neurons of brains dissected from flies entrained under LDR 16:8, at constant 30°C. TIM fluctuations are statistically significant (One way ANOVA, $p < 0.05$).
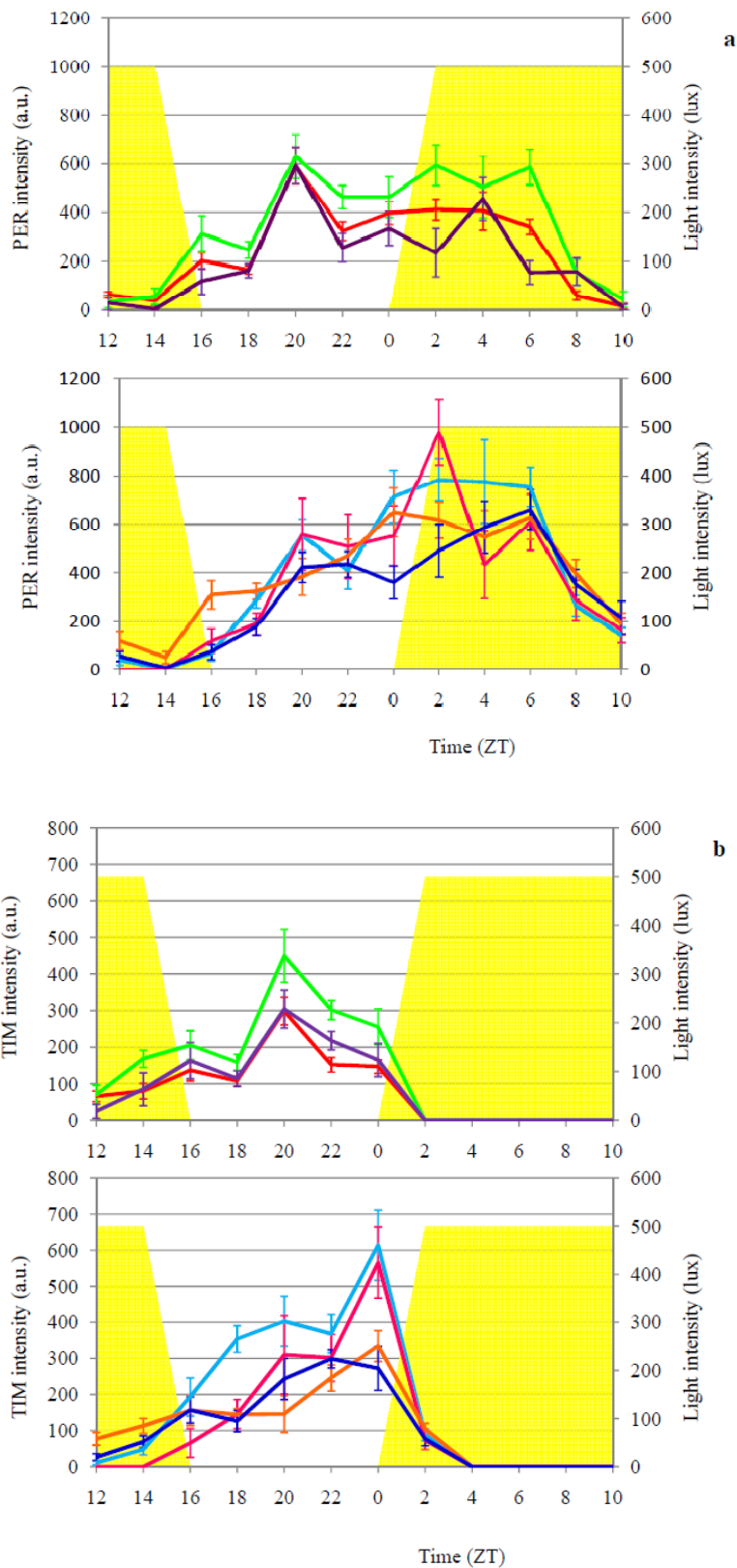
TIM oscillation profile also differs among the different clusters of clock neurons even if in this case there is a higher level of synchronization:

- DN1s, DN2s and LPNs: the peak of intensity is found at ZT20.
- Lateral neurons: the peak is at ZT0, exactly right before lights-on transition.

The main differences between TIM oscillation at 20°C or 30°C are as follow:

- with the exception of the Dorsal neurons, no TIM staining is found during the light period at 20°C whereas at 30°C TIM start to be detectable within the clock neurons already at ZT12;
- the peak of the protein is delayed within the Lateral cells at 30°C compared to 20°C;
- TIM is expressed within the LPNs at 30 but not at 20°C.

In order to give a better idea of what occurs under both environmental conditions within each group of clock neurons a comparison of PER and TIM accumulation profile in the different clusters of cells is shown in **Fig. 6.6**.

Interestingly, the intensity of the signal detected for both PER and TIM in all clusters of clock cells appear was lower at the higher temperature. The same microscope parameters have been used to acquire the images relative to both experiments and in this case data were not normalized (see **Section 2.4**). More details are in **Fig. 6.6**.

Fig. 6.6: PER and TIM immunoreactivity within the clock neurons of WT-ALA flies entrained under controlled lab conditions. LDR 16:8 at constant 20°C or 30°C. Light intensity is reported in yellow. PER oscillation is in red whereas TIM isn in blue. Oscillation profiles at 30°C are shown by full lines whereas those at 20°C by dotted lines.
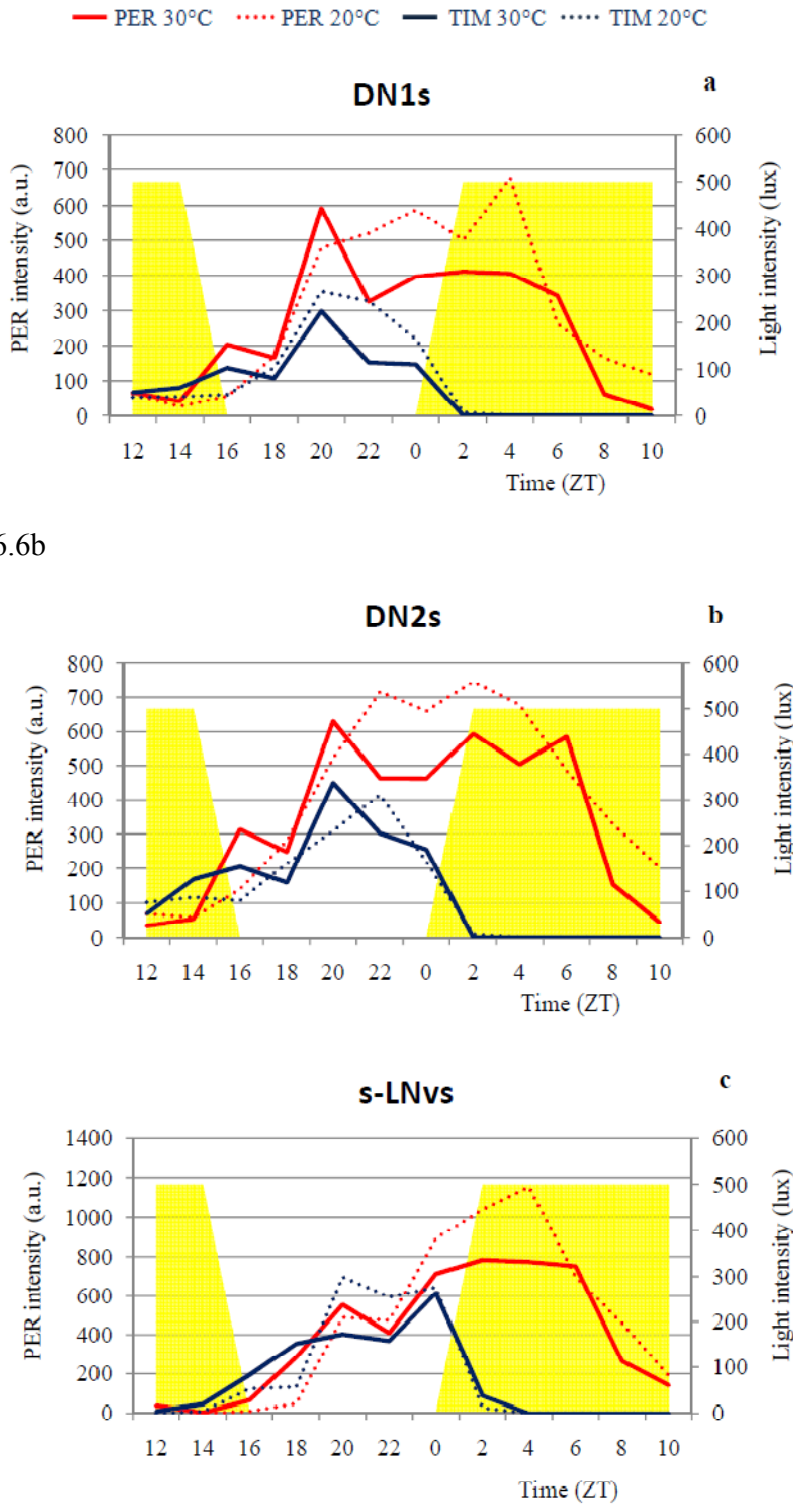
6.6b

a:
PER_ At 30°C PER peaks o at ZT20, then its level stays high until ZT6. At 20°C peak is delayed since it is found at ZT4. In any case, PER is showing high levels which last longer at 30°C.
TIM_ The protein peaks under both conditions at ZT 20. It seems likely that TIM oscillation mainly affected by photoperiod while not (or very little) affected by temperature.

b:
PER_ At 30°C PER peaks at ZT20 and its level then stays high until ZT6. At 20°C the peak appears narrower, since the level is high from ZT22 until ZT4.
TIM_ In this cluster of neurons TIM oscillation appears to be affected also by temperature; however the peak occurs earlier (ZT20) at higher temperature.

c:
PER_ Oscillations at 20°C and 30°C are not so different but again, as already found for DN1s and DN2s, PER level stays higher for a longer period: at 30°C the maximum level is maintained until ZT6 whereas at 20°C until ZT4.
TIM_ Within these cells TIM shows a sharper peak at 30°C (ZT0) whereas at 20°C the level is high from ZT20 until ZT0.
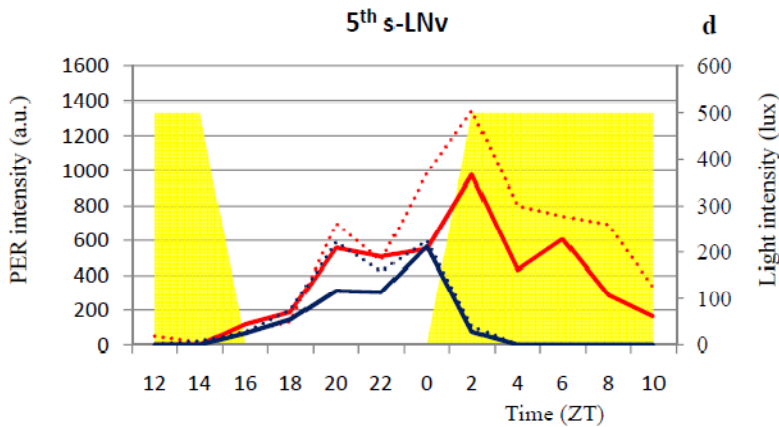
5th s-LNv

d:
PER_ PER accumulation within the 5th s-LNv appears less affected by temperature than in all other clock neurons. The peak occurs at ZT2 at both temperatures considered.

TIM_ The oscillation of TIM within the 5th s-LNv resembles very much the one within the s-LNvs at both temperature considered with the peak at ZT0 at 30°C and an high level between ZT20 and ZT0 at 20°C.

l-LNvs
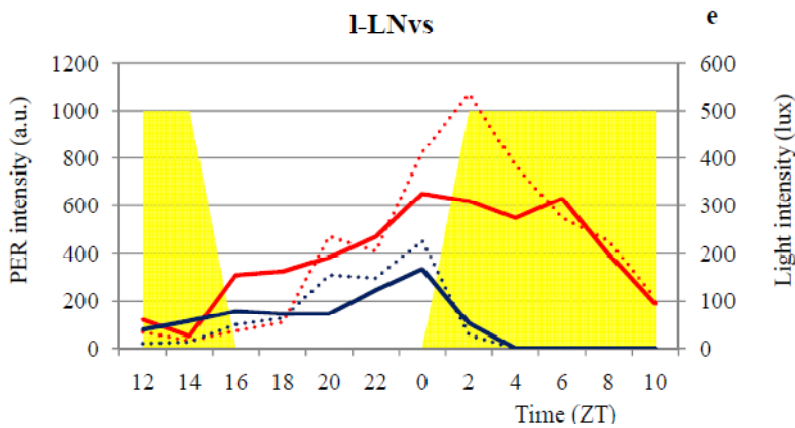
e:
PER_ At 20°C PER peak is clearly at ZT0 and at ZT2 the level of the protein is consistently decreased. At 30°C the maximum level is reached at ZT0 and maintained until ZT6.

TIM_ The protein shows the same oscillation profile under the two thermal conditions with a peak at ZT0, right before lights-on.

LNds
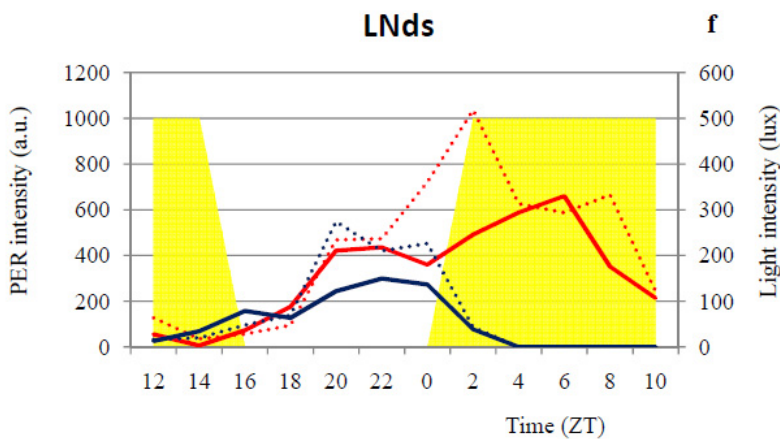
f:
PER_ Whereas at 20°C PER shows a clear peak at ZT2, its level is than maintained at more than 50% of the maximum until ZT8.

At 30°C there is already a high level of the protein at ZT20 but then the peak is reached at ZT6.

TIM_ The oscillation profiles of under are very similar, albeit at 30°C the level of the protein is lower.

LPNs
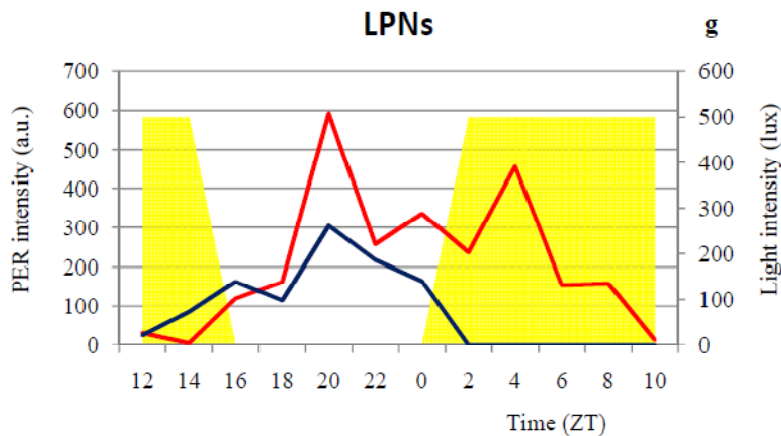
g:
Both PER and TIM were not detected at all within this cluster of cells at 20°C but they are expressed at 30°C.
PER_ The level of PER increases right after lights-off. The first peak is reached at ZT20 but then another peak, even if lower than the first one, occurs at ZT4.
TIM_ The protein increases before lights-off (at ZT14) and the peak is reached at ZT20.
TIM is again undetectable at ZT2.

In all cluster of clock neurons, both at 20 and 30°C, we have observed a phase delay between PER and TIM oscillations. Nevertheless, under our laboratory conditions, we didn't find a phase shift comparable to the one found under natural conditions (about 10-11 hours).

Worthy of note, at both temperature considered in our experiments, PER reaches a high level within the DN1s earlier than in the lateral cells (**Fig. 6.7 a** and **b**). This phase advance within DN1s occurs also for TIM oscillation (See **Fig. 6.7 c** and **d**) even if the difference between the DN1s and the lateral cells is much more pronounced at higher temperature in this case.

The Lateral Posterior Neurons are expressing both PER and TIM only at 30°; the phase of both PER and TIM oscillation within this cluster of neuron appears to be similar to the phase of the Dorsal cells (DN1s and DN2s, **Fig. 6.7 b** and **d**).

Fig. 6.7: Mean time (± s.e.m.) for PER (**a** and **b**) or TIM (**c** and d) antigenicity peak. In order to perform the immunoassays, flies were entrained under LDR 16:8 either at constant 20°C or 30°C (**a** and **c** or **b** and **d**, respectively).

a: PER, 20°C_ One way ANOVA shows a significant effect for neurons ($F_{5,1045}=7,8447$; p~0). DN1s show an earlier peak for PER relative to the Lateral Cells but no difference exists between DN1s and DN2s.

b: PER, 30°C_ One way ANOVA shows a significant effect for neurons ($F_{6,1008}=14,887$; p~0). DN1s appear to be significantly different from all the Lateral anterior cells (s-LNvs, 5th s-LNv, l-LNvs and LNds). No differences exist when comparing DN1s, DN2s and LPNs.

c: TIM, 20°C_ One way ANOVA shows a significant effect for neurons ($F_{5,576}=7,1601$; p~0). DN1s appear to be statistically different only from l-LNvs, whereas the l-LNvs show a significant phase delay if compared to all cluster of neurons except of the 5th s-LNV.

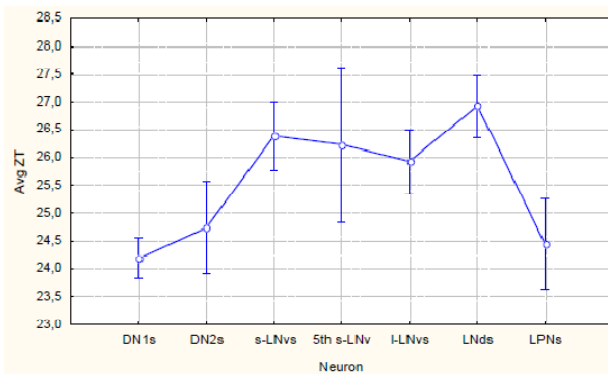**d**: TIM, 30°C_ One way ANOVA shows a significant effect for neurons ($F_{6,653}=8,3666$; p~0). DN1s appear to be significantly different from all Lateral anterior cells but not from DN2s and LPNs.

a
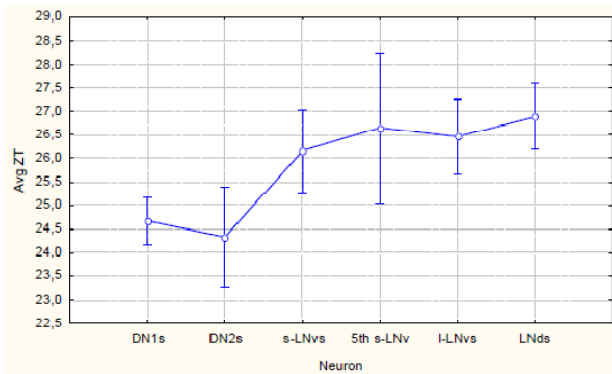
b

c
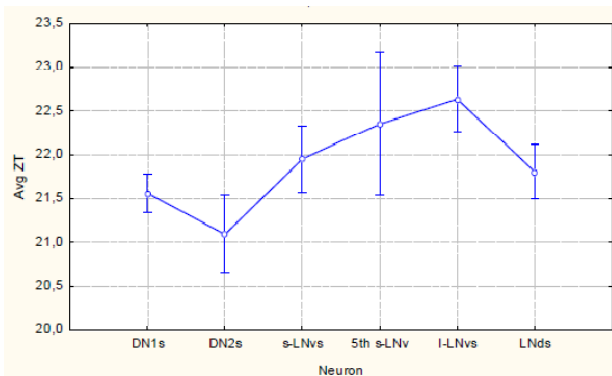
d
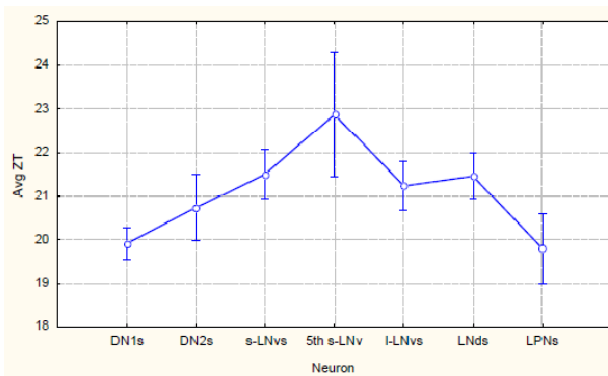
6.4 Discussion

Something else than TIM can stabilize PER and permit its translocation into the nucleus.

While it is well known that PER needs TIM to accumulate and translocate into the nucleus (Curtin *et al.*, 1995; Gekakis *et al.*, 1995; Price *et al.*, 1998) we have shown that, under specific natural environmental conditions, PER and TIM can oscillate in anti phase within the clock neurons of WT-ALA (**Chapter 4**, **Fig. 4.5**). Although this finding is at odd with all previous studies, we confirmed that the formation of PER/TIM heterodimer is necessary to trigger PER accumulation in the cytoplasm and most probably also to initiate its translocation into the nucleus. Indeed, in *tim^{01}* flies exposed to natural conditions PER cannot even accumulate because of TIM absence (**Fig. 6.3b**).

However we found that, in wild-type flies entrained under these particular environmental conditions, PER is still able to accumulate and enter the nucleus (**Fig. 6.2**.) even when TIM is not present anymore within the clock cells because it has been degraded in response to light exposure.

Landskron and co-workers (2009) have recently suggested that the formation of a PER:PER homodimers can fulfil some of the functions performed by the PER:TIM heterodimers (Landskron *et al.*, 2009). By disrupting the capability of the protein to form homodimers, the authors demonstrated that PER:PER homodimers are involved in PER repressive function by accomplishing its translocation into the nucleus: in flies unable to form the homodimer the level of PER within the nucleus of clock neurons is lower if compared to wild-type flies.

Thus, if under Summer natural conditions PER can still accumulate and enter the nucleus even when TIM has already been degraded, it may be PER itself, via the formation of the PER/PER homodimer, that is sustaining the process. This hypothesis is currently under investigation in our lab.

High temperature is responsible for the phase delay in PER oscillation

In order to evaluate the effect of natural environmental parameters on the circadian clock of *D. melanogaster*, wild type flies were exposed to natural conditions and then sampled at precise time intervals in order to analyze PER and TIM expression in the brain. Experiments were carried out throughout the year to consider the main environmental changes characterizing seasonality. In particular, in our Spring and Summer experiments the photoperiods recorded were almost comparable whereas temperature was much higher (10°C on average) during Summer. Moreover during the Autumn experiment we recorded temperatures that were comparable to those recorded in Spring even though the photoperiod was much shorter.

It has been already highlighted that PER and TIM are adapting to the natural environment in different manners: the peak of PER is delayed when days are longer and temperature higher whereas the peak of TIM appears to advance (**Section 4.3**, **Fig. 4.9** and **4.10**).

The most striking result obtained in this study is the antiphase in expression between PER and TIM we observed under Summer conditions. As mentioned above, Summer conditions are very similar to Spring conditions in terms of photoperiods. Notably the average day temperature in Spring is about 20°C whereas the average day temperature in Summer is about 30°C. We therefore attempted to recreate in the lab a Spring-Summer like photoperiod to entrain wild-type flies: the two different constant temperatures of 20°C or 30°C were used in order to check whether the phase shift between PER and TIM is more prominent at higher temperatures.

We found that the phase shift between PER and TIM, albeit detectable, is not as evident in the lab as it is under natural conditions, not even at constant 30°C. Nevertheless, it is worth mentioning PER expression at relative high levels was lasting more at 30°C than at 20°C (**Fig. 6.4**, **6.5**, **6.6**).

These results can be explained if we consider that natural environmental cues are much more effective in driving the endogenous clock due to the complexity of their daily changes and interactions. This is further suggested by the immediate entrainment produced by the natural environment when flies are directly exposed to its effects while laboratory LD and TC cycles require normally a few days to synchronize the circadian rhythmicity (S. Bhutani, personal communication).

Consequently, the laboratory environmental stimuli we have used were not strong and complex enough to reproduce the extremely pronounced phase shift obtained in the wild. The effect of

much more articulated laboratory conditions, as for instance LDT 16:8 and TC cycles ranging from 25°C to 35°C (more representative of a typical Summer day) will be further investigated. An attempt will also be performed in order to mimic in the lab the natural daily variation in light spectrum and composition and intensity that may also play a role in modulating the levels of phase shift between the two proteins.

The advanced peak of PER oscillation within the DN1s can be reproduced in the lab

PER and TIM oscillations within the clock neurons of *D. melanogaster* is the basis for circadian clock functioning and it has been widely studied in the last decades. It is known that PER presents a peak of expression at the end of the night and a trough at the end of the day within all clock neurons (Zerr *et al.*, 1990). In this work, the synchronous PER oscillation among the different clusters of neurons under standard lab conditions has been proven in brains of WT-ALA flies (**Chapter 3**, **Fig. 3.3**). Nevertheless, we found that under natural conditions this synchronization is lost: irrespectively of the natural conditions in terms of temperature or photoperiod, the DN1s always show an earlier peak for PER oscillation than the lateral anterior cells (s-LNvs, 5th s-LNv, l-LNvs and LNds).

In this study, this desynchronization among different cluster of neurons has been observed for the first time also under laboratory conditions. In **Section 6.3** we reported that PER is peaking earlier within the DN1s under LDR 16:8, both at 20°C and 30°C, thus irrespectively of temperature. The main difference among the environmental conditions adopted to perform these experiments and the typical conditions usually adopted in the laboratory for these kinds of studies is the simulation of dawn and dusk. In fact, in the traditional in lab experiments flies experience rectangular light:dark cycles. It may be that the environmental cue that is advancing the phase of PER oscillation both under natural conditions and LDR is the ramping of light intensity.

Moreover the analysis of TIM oscillation under natural conditions revealed that the peak of the protein is advanced in DN1s only if compared to the 5th s-LNv and the LNds. Under laboratory conditions there are not significant differences between DN1s and the other clusters of cells (except of l-LNvs) at 20°C whereas at 30°C the peak of TIM is advanced in DN1s compared to all the Lateral anterior cells, thus the situation resembles in this case the result obtained for PER

oscillation. The fact that the advanced peaking of TIM within the DN1s appears to be more evident in the lab than under natural conditions may be due to the fact that TIM is sensing differently from PER information relative to the extension of the photoperiod or changes in temperature.

Chapter 7:

Discussion

The circadian clock of *Drosophila melanogaster* has been widely studied in the last decades, also because of its shared features with the mammalian clock (Glossop and Hardin, 2002; Helfrich-Forster, 2004). It is well known that the master clock of the fruit fly resides in the brain and relies on about 150 neurons bilaterally clustered in Lateral and Dorsal neurons (LNs and DNs). The LNs groups is composed of the 5 small and 4 large ventral Lateral Neurons (s-LNvs and l-LNvs), 6 dorsal Lateral Neurons (LNds) and 3 Lateral Posterior Neurons (LPNs). Among the Lateral neurons, 4 small and all the large LNvs express the neuropeptide PDF involved in the output (Helfrich-Forster and Homberg, 1993). The Dorsal Neurons are further divided in three groups: ~15 DN1s, 2 DN2s and ~40 DN3s.

Within the clock neurons of *D. melanogaster* clock proteins such as PERIOD and TIMELESS operate in interlocked feedback loops (Benito *et al.*, 2007; Collins and Blau, 2007). In the first loop, CLK and CYC directly activate the transcription of *per* and *tim*. CRY mediates light-dependent degradation of TIM and this is partly responsible for preventing PER and TIM accumulation during the day. At night, TIM can accumulate in the cytoplasm stabilizing PER, the two protein then heterodimerize and translocate into the nucleus, where PER inhibits CLK/CYC activity. In the second loop, PDP1 acts as an activator and VRI as a repressor of *Clk* transcription. As *Pdp1* and *vri* are direct targets of CLK/CYC, this creates a second feedback loop (See **Chapter 1: Introduction**, for further details).

Clock proteins oscillation within the clock neurons in the brain of the fruit fly results in many rhythmic behaviors among which the most studied in the fruit fly is for sure locomotor activity: *Drosophila*'s locomotor activity has a bimodal distribution during the 24 hours light dark cycle, characterized by morning and evening activity bouts that precede lights-on and lights-off, respectively.

To date, almost all the studies on fly circadian rhythms have been carried out in the lab under classical rectangular light:dark or thermophase:cryophase regimes, even if some attempts to mimic in the lab more articulated conditions have been sporadically made (Bachleitner *et al.*, 2007; Majercak *et al.*, 1999; Shafer *et al.*, 2004; Yoshii *et al.*, 2009). However, there are features of the natural environment that is almost impossible to recreate in the lab: outside light is continuously changing both in spectrum composition and intensity and also temperature is never the same.

The work reported in this thesis is part of a project jointly undertaken by our lab and Prof. Kyriacou's lab, granted by the European Commission (6th Framework Programme; Project

EUCLOCK N° 018741) and aimed to the understanding of the circadian clock functioning under real natural conditions. More precisely, in this thesis the oscillation profiles of PERIOD and TIMELESS at the level of the clock neurons of the fruit fly were determined and analyzed in detail.

In order to place the analysis of the fly circadian clock in a more ecological and realistic context, a natural strain of flies was used to carry out the experiment. The WT-ALA strain has been established in the lab starting from 37 isofemale lines collected in the North of Italy (Val Venosta, BZ; see **Section 2.2**, **Tab. 2.1**). In order to maintain the original high genetic variability, the adopted strain was integrated monthly with individuals from the 37 original independent isofemale lines which are also maintained in the lab. We have first shown (**Chapter 3**) that both PER and TIM oscillate normally in the brains of WT-ALA flies entrained under standard laboratory conditions of LD 12:12 and at constant temperature. Moreover, other PhD students involved in the project, S. Bhutani (Department of Genetics, University of Leicester, UK) and S. Montelli (Department of Biology, University of Padova, IT), have shown that also the locomotor activity as well as *per* and *tim* mRNA oscillation profiles in WT-ALA flies were comparable to those of every other wild-type lab strains studied so far (e.g. the American wild-type classical strain Canton-S or Oregon-R). Since WT-ALA flies displayed wild-type circadian clock and rhythmicity under standard lab conditions, the strain was considered suitable to be a reference wild type strain for studies on the functioning of the circadian clock in our European natural environment. Moreover, the higher genetic variability characterizing our "young" (as recently collected) strain of wild-type flies compared to every other wild-type strain maintained in the lab for decades was representing in our work a better guarantee that we were approaching the real natural conditions.

7.1 Are PERIOD and TIMELESS components of both daily and seasonal timers?

As already mentioned above, *D. melanogaster* shows a bimodal locomotor activity profile, which represents a common feature to most living organisms, both diurnal and nocturnal.
In 1976, Pittendrigh and Daan have suggested the presence of two coupled circadian oscillators to explain this phenomenon. A first argument for that comes from the analysis of the daily activity

pattern of the nocturnal rodent *Mus musculus*, which includes two different components: a morning and an evening one. In these animals, it has been shown that the position of the activity peaks changes with the day length, proving that the circadian clock, that controls this rhythm, is able to adjust itself according to the day-night cycles (Aschoff and Meyer-Lohmann, 1954).

Considering these data, Pittendrigh and Daan proposed the presence of two different oscillators, controlling the morning and evening activity peaks.

Evidence that several oscillators are involved in circadian activity rhythms comes from the "splitting" phenomenon that occurs in different species of mammals and birds, (Pittendrigh and Daan, 1976). The "splitting" phenomenon that they described accurately in the golden hamster *Mesocricetus auratus*, is the dissociation of a single activity bout into two components which become stably coupled in antiphase. The fact that these two components are coupled to each other means that these two oscillators are distinct but not independent.

The splitting phenomenon was observed also in *Drosophila* during the first few days in LL regimes in cry^b mutants (flies lacking a functional CRY). These flies show a strong activity burst that soon dissociates into two components, one running with a period of 22.5h and the other with a period of 25h (Yoshii *et al.*, 2004). This suggested that also in *Drosophila* there are two clock oscillators running with different periods that drive the locomotor activity rhythms.

It has been shown that the morning and evening peaks in *Drosophila melanogaster* are led by two clusters of different clock neurons: the ventral and dorsal lateral neurons respectively (Grima *et al.*, 2004; Picot *et al.*, 2007; Stoleru *et al.*, 2007; Stoleru *et al.*, 2004). Using two independent strategies of genetic manipulations, based on transgenic crosses with flies carrying the GAL4/UAS system these authors have found that the LNvs are able to drive the morning anticipation in LD condition and generate the rhythmicity when flies are released in DD whereas the LNds are responsible for driving the evening peak of activity in LD and maintaining behavioral rhythmicity in LL.

Moreover, it has been proposed by Stoleru and coworkers (2007) that the photoperiod to which flies are exposed determines the master clock identity: the long nights of winter-like photoperiods cause the morning cells to become the dominant clock, controlling the timing of both morning and evening cells output; the long summer days should prevent the morning cells from developing robust clock protein expression while the clock protein cycling in the evening cells becomes the master clock.

Upon analysis of PER and TIM oscillation within the different clusters of clock neurons throughout the seasons (**Chapter 4**) we didn't observe such a phenomenon as both PER and TIM do not show a more robust oscillation in a certain cluster of cells- or in other according to the outer photoperiod. Conversely, a maybe more striking phenomenon was occurring. In fact, we found that the two proteins are adjusting in opposite manner to the changes of photoperiods and temperature that occur over the year (**Fig. 4.9** and **4.10**). Thus, when days are longer and hotter the peak of PER is delayed whereas the peak of TIM is advanced. Interestingly, we have associated these shifts in the phase of the peak of the two clock proteins with the seasonality found in the behaviour. We have shown in Bhutani *et al.*, submitted, that under long day conditions the morning peak of activity is advanced while the evening peak is delayed, whereas under short days conditions the morning peak of activity is delayed while the evening one is advanced. Otherwise stated, it is evident that the phase shift in PER protein abundance tracks the displacements of the evening peak of activity whereas the phase shift of TIM oscillation appear to be more related to the morning peak of activity.

If the seasonality observed in the behaviour of the fruit fly can be explained by the phase shifts in PER and TIM oscillation profiles within the clock neurons then we might hypothesize that the reciprocal phase of the two proteins could be involved in some mechanistic aspects of the photoperiodic clock. The photoperiodic clock is generally defined as the clock that, either alone or in association with the circadian clock, allows organisms to anticipate the seasonal changes. While it seems unlikely that the photoperiodic clock coincides with the circadian clock, the possibility that the two clocks are sharing at least some molecular components has not been verified yet.

Over decades, many models to explain animal's photoperiodism have been proposed.

In the hourglass timer hypothesis, based the studies performed by Anthony D. Lees (Lees, 1950, 1960), the involvement of the circadian clock is excluded. According to this theory the photoperiodic clock consists of a series of biochemical processes that should be completed during the dark period. If these processes are completed after dawn, then the organism sense that it is exposed to a short night.

The first who tried to explain photoperiodism making use of the circadian clock was Edward Bünning in 1936. According to his theory, if the circadian cycle is composed of a photophilic phase (subjective day) and a scotophilic phase (subjective night), short days effects are produced when light is perceived only during the photophilic phase whereas long days effects when light is

perceived also during the scotophil phase. After Bünning, Pittendrigh and Minis proposed the external coincidence model; according to this model light is not only necessary for the daily circadian entrainment but also to illuminate the most light sensitive phase of the cycle (photoinducible phase that coincides with the second half of the night). When light is perceived during the photo-inducible phase long day effects are induced (Pittendrigh and Minis, 1964; Pittendrigh, 1966). Again in 1966, the internal coincidence model was proposed by Tyschenko. According to his model, short days exert their effects when critical phases in two circadian oscillators coincide in time; one oscillator should be linked to dawn and the other to dusk. This last model was developed in the dual oscillator model (Pittendrigh and Daan, 1976) in which at least two oscillators are entrained by the light cycle and either short or long days effects are produced depending on their phase relationship.

The internal coincidence model as well as the dual oscillator model nicely fit with the results we have obtained for PER and TIM oscillation under natural conditions. It was already mentioned that under long days PER peak is delayed (as the evening peak of activity) whereas TIM peak is advanced (as the morning peak of activity). Thus, the two peaks are far from each other during the good season, and they appear to be in phase in autumn. The phase relationship between PER and TIM oscillation could then be interpreted as an n important signal to anticipate the forthcoming cold season.

Temperature sensing also appears to be important in order to adjust the reciprocal phase of PER and TIM oscillation. In **Chapter 6** (**Section 6.3**) we have shown that at high temperatures the phase shift between PER and TIM increases whereas at very low temperatures (**Chapter 4**, **Section 4.2**) PER and TIM appear to be expressed at low levels and it seems they are not entering the nucleus anymore, suggesting that in these conditions the two protein still oscillate in phase but their action is compromised. Therefore, the reciprocal phase of PER and TIM could be used as a photoperiodic sensor, to anticipate the forthcoming new season, and the capability to perceive changes in photoperiod appears to be modulated by temperature, so that both environmental parameters are finely monitored.

This hypothesis does not exclude the double oscillator model based on different cluster of cells proposed in the past years and mentioned above. We have shown that a particular cluster of cells, the LPNs, express either PER or TIM only under specific temperature conditions. It is already know that light inhibits the output of the morning cells whereas activates the output of the evening cells and *vice versa* under constant darkness conditions (Grima *et al.*, 2004; Picot *et al.*,

2007; Stoleru *et al.*, 2007; Stoleru *et al.*, 2004). Thus, different clusters of neurons are differently activated under different environmental conditions and this could be functionally useful to regulate in a specific manner, according to PER and TIM reciprocal phases, circadian behaviours or physiological processes. Conversely, the continuous changes in PER and TIM phases at the level of the whole neuronal network could be used to interpret the drawing of the seasons.

7.2 Does each cluster of neurons preferentially sense for a specific environmental stimulus?

The contribution of each neuronal group to the circadian behaviour has been studied in detail in the last decade.

It is well known that s-LNvs are required for maintaining rhythmicity in DD and driving the morning peak of activity (Grima *et al.*, 2004; Stoleru *et al.*, 2004) whereas LNds and the 5th s-LNv are driving rhythmicity in LL, when CRY is inactive, and controlling the evening burst of activity (Picot *et al.*, 2007; Stoleru *et al.*, 2007).

It has been suggested that each cluster of clock neurons differently sense the environment and the clock protein oscillation within each cluster of cells appears to be mainly entrained either by light or temperature. In fact, CRY positive neurons are mainly light entrainable cells while CRY negative neurons are mainly temperature entrainable cells (Yoshii *et al.*, 2010).

We also have shown that this tendency of each cluster of neurons to be more sensitive to either one or the other environmental stimulus holds true under real natural conditions at least for the LPNs and the DN1s.

First of all, the hypothesis that the LPNs appear to be temperature entrainable cell (Miyasako *et al.*, 2007; Yoshii *et al.*, 2005) was confirmed. These cells do not express PER and TIM under laboratory conditions of LD 12:12 and constant temperature (i.e. 20°C and 23°C, **Chapter 3**). Under natural conditions we found that TIM is cycling within the LPNs all over the year whereas PER was expressed only at very high temperatures (**Chapter 4**, **Section 4.2**). The fact that TIM is always cycling in this cells under natural conditions may suggest that also PER is actually expressed and cycling within the LPNs but may be at too low levels to be detected.

Albeit temperature cycles appeared initially to be necessary for clock protein expression within the LPNs, we found that PER and TIM expression can actually be driven in this cells also in the

laboratory environment at constant high temperature. Comparing PER and TIM oscillation under LDR 16:8 at either 20 or 30°C we observed that LPNs express both proteins at 30°C while not at 20°C. In conclusion, TIM expression within the LPNs requires temperature cycles or very high temperatures whereas PER is expressed in these cells apparently only at very high temperatures. The fact that the clock within these cells can be turned off or on according to the environmental temperature could provide a specific signalling to the other components of the neuronal network.

In a recent work it has been proposed that DN1s are able to drive particular aspects of the circadian locomotor activity depending on the environmental conditions that flies are experiencing: if flies are exposed to bright-light:dark cycles these cells can promote morning activity whereas under constant darkness and thermo cycles they can generate a robust evening peak of activity (Zhang *et al.*, 2010). Obviously, under natural conditions temperature cycles and light:dark cycles (with changing light intensities and composition either during the day or night) are tightly linked together so that DN1s do not respond to a single stimulus but have to integrate a complex set of environmental signals.

We reported that under natural conditions the DN1s show an earlier peak in PER accumulation irrespective of the season (**Fig. 4.8a**). The earlier upswing in TIM accumulation within these neurons is less evident (even if statistically significant when compared at least to the 5th s-LNvs and LNds) but this could depend on TIM rapid dropping after sunrise and so maybe simply there's not enough time for the protein to continue its accumulation within the other cells (**Fig. 4.8b**).

Initially three possible explanations for the earlier PER and TIM accumulations within DN1s were considered:

1. since they express the receptor for the neuropetide PDF which in turn is expressed within the s-LNvs projecting towards the dorsal brain, DN1s could respond to specific stimuli coming from the lateral neurons;
2. they could run a faster clock;
3. considering their position in the brain they could sense directly stimuli coming from the environment and eventually transmit this information to the other cells.

With our results it has been possible to exclude that DN1s are showing this earlier upswing of PER accumulation via PDF signalling as their advanced phase persists even in the absence of both PDF producing cells and PDF itself (**Fig. 5.2**). While we cannot exclude that this cluster of

cells runs a faster clock. Nevertheless we were able to reproduce this phase shift for the first time in laboratory conditions mimicking sunset and sunrise (**Chapter 6**, **Fig 6.7**). Thus, it seems plausible that this earlier accumulation of PER could represent a direct response to the outer environment than a peculiarity of the internal clock of the DN1s.

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