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Integration of Light and Temperature in the Regulation of Circadian Gene Expression in Drosophila

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INTEGRATION OF LIGHT AND TEMPERATURE IN THE REGULATION OF CIRCADIAN GENE EXPRESSION IN *DROSOPHILA*

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by

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Clocks are aligned to the environment via inputs from both daily light and temperature cycles. Previous molecular and behavioral studies in *Drosophila* have largely focused on light-dependent regulation of circadian clocks and their outputs. Although light is the strongest and best understood *Zeitgeber* for the circadian clock, temperature is also an important factor. This thesis aims to understand better the role of temperature on gene expression and behavior in the fly, as well as to examine how information from both light and temperature are integrated by the clock to regulate circadian gene expression.

Genome-wide expression profiles of transcripts from wild-type, light-entrained flies show a similar transcriptional response in entrainment and free-run. In contrast, expression profiles from wild-type, temperature-entrained flies show a dramatic difference in the presence or absence of a thermocycle. Whereas almost all transcription appears to be modified by changes in temperature, there is a limited number of transcripts that continue to oscillate in constant conditions following temperature entrainment. This

suggests two distinct responses to temperature: clock-independent temperature-driven oscillations and clock-dependent circadian oscillations.

The clock-dependent transcripts oscillating in constant conditions following temperature entrainment show a significant overlap with those transcripts oscillating in response to photocycles. Further, they maintain the same mutual phase relationships after entrainment by temperature or light. That is, the phase observed at the onset of the thermophase is systematically advanced by about six hours relative to the phase at the onset of light. A similar phase relationship is observed at the level of protein expression and locomotor activity behavior. These observations indicate that entrainment by light and temperature would occur cooperatively and be integrated by the fly under natural circumstances, given the size of the delay that is commonly found between environmental temperature profiles and light/dark cycles.

For Omi

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Abbreviations

AA	constant 25°C
AC (or AC 12:12)	Ambient:Cool cycle (12 hours of 25°C followed by 12 hours of 18°C, in constant darkness)
bp	base pair
CT	circadian time (reflective of prior entrainment scheme, where CT0 is subjective lights-on and CT12 is subjective lights-off)
DD	constant darkness
ECL	enhanced chemiluminesence
GSP	gene specific primer
LD (or LD 12:12)	Light:Dark cycle (12 hours of light followed by 12 hours of darkness at 25°C)
n	number tested
PCR	polymerase chain reaction
ph	phase
PRC	phase response curve
QQ	quantile-quantile
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RMA	robust multi-average single algorithm
RNAi	RNA interference
RPA	RNAse protection assay
rpm	rotations per minute
RT	reverse transcription
UAS	upstream activating sequence
UTR	untranslated region
wt	wild-type
ZT	*Zeitgeber* time (ZT0 is lights-on and ZT12 is lights-off)

Chapter 1: Overview and background

The Earth's rotation on its axis results in a daily cycle of light and darkness. In order to time their physiologies and behavior in a biologically advantageous manner, most organisms on earth, from microbes to men, have evolved an internal time-keeping system. This biological clock enables them not only to align their physiologies and behavior to the 24-hr environmental day, but also to predict when it will be light and dark. The resulting circadian (*circa* = about, *diem* = day) rhythms are incredibly precise and persist even in the absence of external time cues, or *Zeitgebers*.

Androsthenes, a scribe of Alexander the Great, made the first recorded observation of a circadian rhythm in the fourth century BC, noting that the leaves of the tamarind tree open during the day and close at night. The French astronomer Jean Jacques d'Ortous de Mairan observed a similar behavior in the leaves of the heliotrope in 1729. De Mairan, however, demonstrated that this was not simply a response to sunrise and sunset by showing that the leaves continued to open and close in the absence of a daily light/dark cycle. Although it wouldn't be understood for another 250 years, de Mairan illustrated a fundamental requirement of all circadian clocks: that they are endogenous.

A second prerequisite of a biological clock is that it must be able to be "set" or entrained to the 24-hr environmental day, as the endogenous, or free-running rhythms of many organisms are slightly shorter or longer than 24 hours. Although the daily light/dark cycle is the predominant *Zeitgeber* for the circadian clock, other entrainment signals such as daily changes in temperature, food availability and social cues also impart time-of-day information to the organism. In addition to being entrained the clock needs to be able to be reset in response, for example, to seasonal changes in the timing of light and darkness or to travel across time zones.

Most biochemical reactions are temperature sensitive. As a result, a third requirement for a biological clock is the ability to maintain a relatively constant period length over a range of physiological temperatures. How temperature compensation occurs is poorly understood.

An example of a behavior regulated by the internal clock is the emergence of adult *Drosophilae* from the pupal case, or eclosion. This circadian-phase dependent event occurs preferentially at dawn, allowing the fly's wings to expand and its cuticle to harden when it is cool and moist. Pittendrigh (1954) showed that by simulating the daily light/dark cycle with 12 hours of light followed by 12 hours of darkness (LD 12:12) over a range of temperatures, adult flies emerged from the pupal case just before lights-

on. If he then removed the LD cycle and allowed the pupae to free-run, eclosion continued to occur approximately every 24 hours at subjective dawn.

Dissecting the *Drosophila* clock

Identifying clock components. Despite some criticism, Konopka and Benzer (1971) decided to attempt to understand the molecular underpinnings of the *Drosophila* circadian clock by mutagenizing the components involved. By testing EMS-mutagenized flies for abnormal eclosion rhythms, they isolated three mutations that all mapped to the same locus on the X chromosome, which they called *period (per)*. One strain resulted in 19-hr rhythms (per^{Short}; per^{S}), another resulted in 29-hr rhythms (per^{Long}; per^{L}) and the third resulted in aperiodic eclosion rhythms (per^{0}). Importantly, these rhythm phenotypes were also exhibited in another circadian phase-dependent behavior in the fly, locomotor activity, indicating that a single oscillator can dictate diverse rhythms at various stages of the fly life cycle.

Framing a skeletal feedback loop. *per* was cloned independently by two labs (Bargiello and Young, 1984; Reddy et al., 1984). Although the sequence analysis did not initially reveal much information about its

biochemical function, it did uncover a domain that is shared with the basic helix-loop-helix (bHLH) transcription factors *single-minded (sim)* and *aryl hydrocarbon receptor nuclear translocator (arnt)*. This shared PAS domain (for PER-ARNT-SIM) is responsible for protein-protein binding and dimerization (Huang et al., 1993). Although PER does not contain a DNA binding domain, it was thought that perhaps PER could inhibit transcription by binding to other bHLH transcription factors through its PAS domain.

per RNA levels were shown to oscillate with a circadian rhythm in adult fly heads (Hardin et al., 1990). Further, the oscillatory period matches the locomotor rhythm phenotypes in per^S and per^L flies, and ceases altogether in per^0 flies. *per* levels in per^0 flies are intermediate to high throughout the day, which suggested PER protein may feedback to regulate its own transcription.

PER protein also cycles with a 24-hr rhythm, but it is delayed by several hours compared to RNA levels (Siwicki et al., 1998; Zerr et al., 1990). It was found that PER is expressed in 20-30 lateral neurons, or pacemaker cells, in the adult fly brain, and that it enters the nucleus with a circadian rhythm (Curtin et al., 1995; Vosshall et al., 1994). Accumulating PER is cytoplasmic. When it reaches a critical threshold it translocates to

the nucleus where it was proposed that it regulates its own transcription (Hardin et al., 1990).

It was an additional ten years before another clock gene, *timeless (tim)*, was isolated from a P-element screen (Sehgal et al., 1994). As with *per*, short, long and arrhythmic mutations all mapped to the same locus. And as in per^0 flies, *per* is at intermediate to high levels in tim^{01} flies (Price et al., 1995; Sehgal et al., 1994). *tim* was cloned and sequenced, and both the RNA and protein behave similarly to *per* (Myers et al., 1995; Sehgal et al., 1995). The exception is that TIM protein oscillates in per^0 flies, implicating TIM in the light entrainment pathway (Hunter-Ensor et al., 1996; Myers et al., 1996). Although TIM does not have a PAS domain, it was isolated with PER in a yeast-two-hybrid screen (Gekakis et al., 1995) and was shown to co-immunoprecipitate with PER from fly head extracts (Zeng et al., 1996). PER nuclear localization is blocked in tim^{01} flies, suggesting that TIM is a regulator of PER subcellular localization (Vosshall et al., 1994).

With *per* and *tim* in hand, a skeletal clockwork involving the negative transcriptional feedback loop originally proposed by Hardin et al. (1990) emerged (Figure 1.1). PER and TIM dimerize as they accumulate in the cytoplasm during the circadian day. In the evening, when they reach a

critical threshold, they translocate to the nucleus and down-regulate their own transcription, possibly through another bHLH-PAS domain protein.

Building on the core clock components: Closing the molecular loop. Another screen for circadian mutants using chemically mutagenized flies yielded two additional bHLH transcription factors, *dClock (dClk)* and *cycle (cyc)/dBmal*, both with mammalian orthologs (Allada et al., 1998; Bae et al., 1998; Rutila et al., 1998). *dClk* and *cyc* were shown to heterodimerize and bind to upstream, cis-acting elements called E-boxes in the promoters of *per* and *tim*, thereby activating their transcription (Darlington et al., 1998, 2000; Hao et al., 1997; McDonald et al., 2001). This activity was shown to be inhibited by PER and TIM, thus closing the molecular loop. *dClk* levels, but not *cyc*, oscillate with a circadian rhythm, which is antiphase to that of *per* and *tim* (Bae et al., 1998; Darlington et al., 1998; Glossop et al., 1999).

With the addition of *dClk* and *cyc*, the picture of the core circadian clock mechanism was more complete (Figure 1.1). In the late morning the dCLK/CYC heterodimer activates transcription of *per* and *tim* through E-box binding. PER and TIM levels rise throughout the day, peaking in the early evening. At a critical level PER and TIM heterodimerize and translocate to the nucleus, where they inhibit the transcriptional activity of

dCLK/CYC and thus repress their own transcription. PER and TIM are degraded before dawn, which relieves repression of their cognate genes and allows another cycle to commence.

A second feedback loop. The model became slightly more complicated when it was found that *dClk* levels are constitutively low in per^{0} and tim^{01} null mutants, suggesting that PER and TIM activate *dClk* transcription. At the same time, *dClk* levels are constitutively high in *dClk* and *cyc* mutants, suggesting that dCLK/CYC negatively regulate *dClk* transcription (Bae et al., 1998; Glossop et al., 1999). A secondary feedback loop was proposed in which dCLK/CYC repress *dClk* transcription, most likely through an additional factor, while PER/TIM somehow relieve this repression (Glossop et al., 1999).

A candidate for the dCLK repressor was the bZIP, PAR domain-containing transcription factor VRILLE (VRI) (Blau and Young, 1999; Cyran et al., 2003; George and Terracol, 1997; Glossop et al., 2003). *vri* was identified as a circadian gene in a differential display screen in adult *Drosophila* heads that selected for clock-controlled genes (Blau and Young, 1999). *vri* RNA cycles in the same phase as *per* and *tim*, and like these genes is regulated by dCLK/CYC binding to E-boxes in its promoter region.

Overexpression of *vri* results in long locomotor activity periods or arrhythmicity, and *dClk* levels are significantly reduced in these flies (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003). Importantly, VRI has been shown to bind directly to the *dClk* promoter *in vitro*, along with another bZIP, PAR domain-containing protein called PAR DOMAIN PROTEIN 1 (PDP1) (Cyran et al., 2003; Glossop et al., 2003). The expression of the *Pdp1ε* isoform is regulated by dCLK/CYC, and it oscillates with a similar (although slightly later) phase as *per*, *tim* and *vri*. Reduction of *Pdp1ε* expression lengthens the locomotor activity period, while removal of PDP1ε, as tested in third instar larvae, abolishes PER and TIM cycling in the pacemaker cells (Cyran et al., 2003).

Both VRI and PDP1ε were shown to compete for access to binding sites in the *Clk* promoter (Cyran et al., 2003). The resulting balance between VRI-mediated repression and PDP1ε-mediated activation regulates cycling levels of *dClk* expression. Newly produced dCLK is initially inactive due to the presence of PER. As PER is degraded, dCLK/CYC can simultaneously commence another round of transcription of both the *per*/*tim* and *vri*/*Pdp1ε* feedback loops (Figure 1.2).

Refining the core clock mechanism: Post-translational regulation.

The interlocking negative and positive feedback loops described so far provide a solid framework for the molecular clock. However, additional components are required to refine the core mechanism in order to prevent the oscillator from damping out, to confer robustness on the system and to stretch the period length of the oscillation to 24 hours (Figure 1.3).

Many clock proteins are phosphorylated in a circadian-dependant manner. It is therefore not surprising that several kinases, including *double-time (dbt)*, *shaggy (sgg)* and casein kinase 2 (*CK2*), and a phosphatase, protein phosphatase 2A (PP2A), have been implicated in the molecular clock.

dbt was identified in a screen for aberrant locomotor activity behavior (Price et al., 1998). Cloning and sequencing revealed that *dbt* encodes the *Drosophila* ortholog of mammalian Casein Kinase 1ε (CK1ε) (Kloss et al., 1998). The oscillatory period lengths of both *per* and *tim* RNA and protein were shown to be altered in a short (dbt^S) and long (dbt^L) allele of *dbt* (Price et al., 1998). A severely hypomorphic strain carrying a P-element insertion (dbt^P) was identified. Although it is homozygous lethal, most third instar larvae pupate and die later in pupal development. Oscillations of *tim* RNA and protein cease in dbt^P third instar larval brains. Further, PER is expressed

at constitutively high levels, suggesting a role for *dbt* in the regulation of PER accumulation and degradation (Price et al., 1998). Indeed, DBT was shown to physically interact with PER and PER/TIM complexes *in vitro* (Kloss et al., 1998).

sgg, the *Drosophila* ortholog of mammalian glycogen synthase kinase 3 (GSK-3), was identified in a behavioral screen of *tim*-(UAS)-GAL4-driven EP lines (Martinek et al., 2001). Like *dbt*, both *sgg* RNA and protein are constitutively expressed. Overexpression of *sgg* results in a shortened locomotor activity period, with concomitant effects on TIM protein cycling. In addition, PER/TIM nuclear entry is advanced in *sgg*-overexpressing flies. Reduction in expression of *sgg* results in a lengthened locomotor activity period, an increase in PER and TIM levels and a reduction in TIM phosphorylation. These data, along with the observation that TIM can be directly phosphorylated by GSK-3 *in vitro*, indicate that *sgg*-dependent TIM phosphorylation regulates nuclear entry of the PER/TIM complex (Martinek et al., 2001).

It appears that *dbt* and *sgg* exert opposing effects on the clock. While *dbt* delays nuclear entry by phosphorylating PER, thus targeting it for degradation (by *slmb*, see below) and delaying nuclear entry, *sgg* promotes nuclear entry by phosphorylating TIM. This finely tuned balance is akin to

that of VRI and PDP1 repression and activation, respectively, of *dClk* transcription. It is this fine-tuning and push/pull that is critical for a 24-hr circadian clock.

A third constitutively expressed protein kinase that also regulates PER/TIM nuclear entry is CK2. Two mutations of CK2 were identified in locomotor behavioral analyses: *Tik*, which encodes the catalytic α-subunit of CK2 (*CK2α*), and *Andante*, which encodes the regulatory β-subunit of CK2 (*CK2β*) (Akten et al., 2003; Lin et al., 2002a). Both mutations result in long behavioral phenotypes and both subunits are expressed predominantly in the pacemaker cells in the *Drosophila* brain. PER and TIM protein levels are elevated in these mutants, and in agreement with the long locomotor activity rhythms, nuclear entry is delayed (Akten et al., 2003; Lin et al., 2002a). Phosphorylation of PER and TIM is also altered in these mutants, and CK2 has been shown to directly phosphorylate PER *in vitro* (Lin et al., 2002a). It is thus likely that, like *sgg*, CK2 promotes nuclear entry of PER/TIM.

Rhythmic levels of phosphorylation of clock proteins in the presence of constitutively expressed kinases could be achieved by rhythmic dephosphorylation. Levels of the regulatory subunits of PP2A, *twins (tws)* and *widerborst (wdb)*, have been shown to cycle in adult fly heads (Sathyanarayanan et al., 2004). RNAi against *tws*, *wdb* or the catalytic

subunit of PP2A *mutagenic star (mts)* in cell culture results in increased PER levels, indicating a role for PP2A in PER stability. Increased levels of PP2A in flies, through overexpression of *tws* or *mts*, result in a short locomotor activity rhythm with eventual arrhythmicity, while decreased levels of PP2A result in long behavioral rhythms. Overexpression of wild-type *mts* also results in constant, high levels of PER that is localized to the nucleus of pacemaker cells, while overexpression of a dominant-negative form of *mts* results in extremely low levels of PER. This suggests that PP2A is required for the cycling expression, stability and regulation of nuclear entry of PER. Interestingly, whereas overexpression of *tws* results in a short behavioral phenotype that degenerates to arrhythmia, overexpression of *wdb* results in period lengthening. This observation, along with the more robust cycling of *tws*, could indicate that *tws* is more relevant to the clock. Indeed, while a severe hypomorphic form of *tws* (tws^{60}) results in the delayed nuclear accumulation of PER, hypomorphic *wdb* mutants show no circadian phenotype.

Although protein phosphorylation is an important component of the molecular clock, it is unclear how phosphorylated PER and TIM are targeted for degradation. A clue came from the identification of *slimb (slmb)*, a member of the F-box/WD40 protein family in the Skp1/Cullin/F-box (SCF)

ubiquitin ligase complex, as an essential clock gene (Grima et al., 2002; Ko et al., 2002). The ubiquitin ligase family of proteins is responsible for targeting phosphorylated proteins for degradation by the 26S proteasome (Kipreos and Pagano, 2000). Flies with reduced levels of *slmb* are behaviorally arrhythmic with highly phosphorylated forms of PER and TIM present at all times, suggesting that the degradation of both proteins is SLMB-dependent (Grima et al., 2002). Both hypo- and hyper-phosphorylated forms of PER co-immunoprecipitate with SLMB in wild-type fly head extracts, indicating that SLMB can physically interact with either form of PER. Overexpression of *slmb* results in long behavioral rhythms, which could be a result of high levels of SLMB causing increased cytoplasmic PER degradation and thus delayed nuclear entry of PER/TIM complexes (Grima et al., 2002; Ko et al., 2002). Similarly, low levels of SLMB could delay nuclear PER degradation, which would in turn delay the start of the next transcriptional cycle.

Clock coordination. PER and TIM are expressed in several cell types in the *Drosophila* nervous system, including photoreceptor cells, glial cells and dorsal (DN) and lateral (LN) neurons (Kaneko and Hall, 2000). There are three subsets of DNs, namely DN_1, DN_2 and DN_3, as well three clusters

of LNs. Five to eight LNs are found dorsally (LN_ds), while four to six large LNs (l-LN_vs) and five small LNs (s-LN_vs) are found ventrally. *disconnected (disco)* mutant flies, most of which lack the LN_ds and LN_vs, are behaviorally arrhythmic (Dushay et al., 1989). However, rhythmic locomotor activity behavior is seen in the few *disco* flies that still retain the s-LN_vs and their projections, indicating that these neurons are critical to the regulation of rhythmic behavior (Helfrich-Förster, 1998).

Four of the s-LN_vs, as well as the l-LN_vs, rhythmically express the neuropeptide pigment-dispersing factor (PDF) (Helfrich-Förster, 1995; Kaneko et al., 1997). pdf^{01} null mutants are behaviorally rhythmic in LD cycles and the first day or two of DD, but then gradually become arrhythmic (Renn et al., 1999). Based on the expression pattern of PDF and the morphology of the LN_vs and their projections, secreted PDF is thought to be responsible for synchronizing individual clock neurons (Lin et al., 2004; Park et al., 2000; Peng et al., 2003). The receptor for PDF has recently been identified, which should further help elucidate how this is achieved (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005).

Summary: A moment in time. Although much has been learned about the inner workings of the *Drosophila* clock over the past 35 years, it is

evident that all the relevant cogs and wheels have not yet been identified. What we do know is that the fly clock is a precise, 24-hr time-keeping mechanism composed of interlocking negative and positive feedback loops whose actions are modified by several critical components (Figure 1.3).

The negative and positive feedback loops are connected by the transcription factors dCLK and CYC. In the negative feedback loop, dCLK/CYC heterodimers activate *per*/*tim* transcription. As PER accumulates in the cytoplasm, it is phosphorylated by DBT and targeted for degradation by the proteasome. An important role for TIM is to stabilize PER and protect it from degradation. When sufficient PER/TIM levels are reached, nuclear entry occurs. This event is mediated by several players, including SGG, CK2 and PP2A. Once inside the nucleus, PER has been shown to independently repress its own transcription (Ashmore et al., 2003; Chang and Reppert, 2003; Nawathean and Rosbash, 2004).

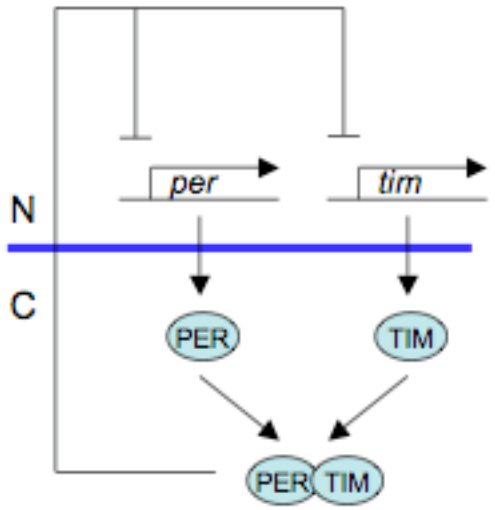
In the positive feedback loop, *dClk* transcription is mediated by VRI and PDP1. Competing access to the *dClk* promoter by VRI and PDP1 results in sequential repression and activation, respectively. Newly translated dCLK, which is responsible for the transcription of *vri* and *Pdp1* in addition to *per* and *tim*, is repressed by PER. As PER is degraded, dCLK can once again commence another round of transcription.

It should be noted that components of this model have recently come into question based on imaging of single cells expressing fluorescently tagged PER and TIM (Meyer et al., 2006). Using fluorescence resonance energy transfer (FRET) measurements in non-oscillating Schneider 2 (S2) cells, Meyer et al. (2006) showed that fluorescently-tagged, heat-shock-induced PER and TIM interact rapidly within discrete cytoplasmic foci upon their production. They further observed that FRET levels decline as PER and TIM move from the cytoplasm to the nucleus. By following PER and TIM separately, this group showed that PER and TIM independently translocate to the nucleus. Thus they propose that PER and TIM serve as part of an "intracellular interval timer," perhaps acting in conjunction with other proteins within the identified cytoplasmic foci. This opens up the possibility of a new, previously unobserved level of regulation that may be the determining factor in establishing the period length of the biological clock.

Figure 1.1. Early model of the negative feedback loop underlying the *Drosophila* clock.

(a). The original model of the molecular clock proposed a negative feedback loop in which PER and TIM dimers translocated to the nucleus to repress the transcription of their cognate genes. (b). With the identification of dCLK and CYC as transcriptional activators of *per* and *tim*, the molecular loop was closed. Proteins are represented in uppercase, RNA in lowercase. Events occurring above and below the blue line are nuclear (N) and cytoplasmic (C), respectively.

a.



b.

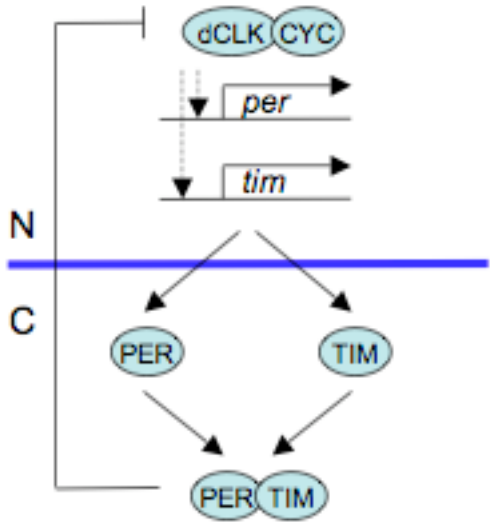


Figure 1.2. Negative and positive interlocking feedback loops of the *Drosophila* clock.

Further work showed that the *Drosophila* clock is composed of negative (-ve) and positive (+ve) interlocking feedback loops. The negative loop involves the dCLK/CYC-mediated transcription of *per* and *tim,* whose proteins feed back to inhibit their own transcription. The positive loop involves the regulation of *dClk* transcription by VRI and PDP1. Proteins are represented in uppercase, RNA in lowercase. Events occurring above and below the blue line are nuclear (N) and cytoplasmic (C), respectively.

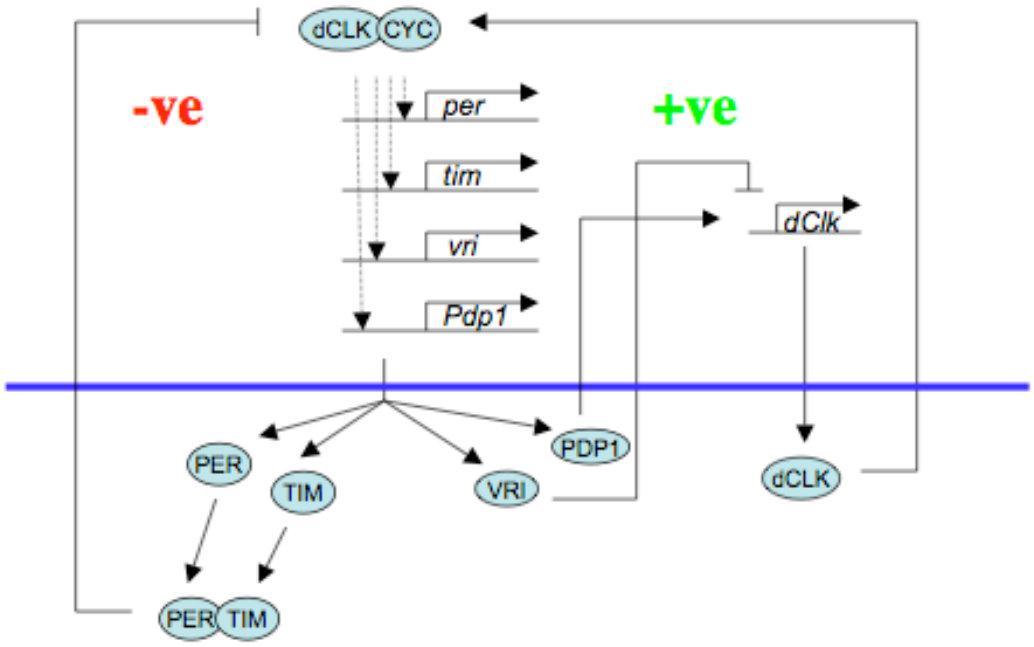
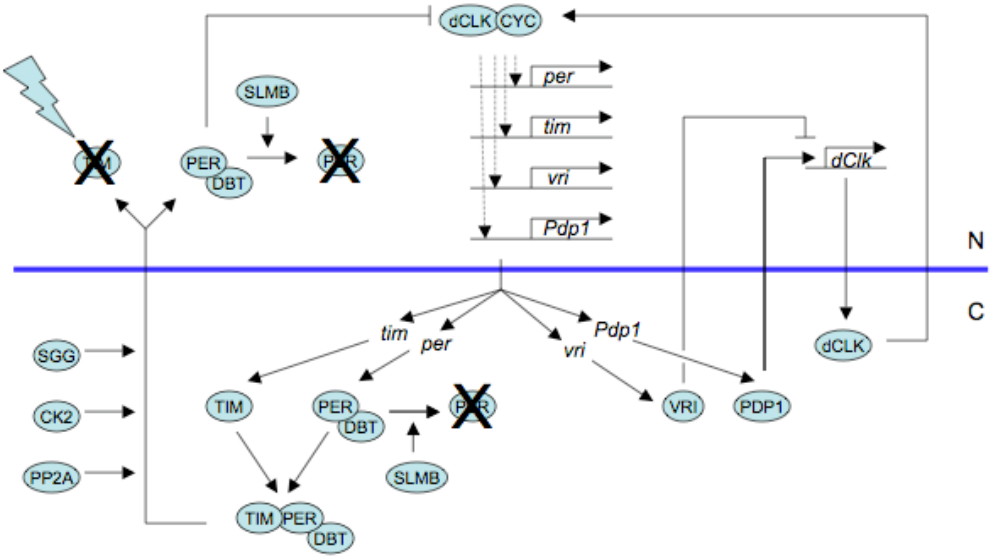

dCLK
CYC
-ve
+ve
per
tim
vri
Pdp1
dClk
PER
TIM
VRI
PDP1
dCLK
PER
TIM

Figure 1.3. Contemporary model of the *Drosophila* clock.

Briefly, *per* and *tim* transcription is activated in the early day by dCLK/CYC heterodimers. PER and TIM protein levels accumulate in the cytoplasm during the day. Until sufficient levels of TIM are reached, DBT phosphorylates PER and targets it for degradation by SLMB (although see work by Meyer et al. [2006], above). About 4-6 hours after peak transcription levels, PER/DBT and TIM heterotrimerize and translocate to the nucleus, a step that is dependent on SGG, CK2 and PP2A. Once inside the nucleus, PER binds to dCLK/CYC thereby inhibiting *per* and *tim* transcription, while TIM is degraded in a proteasome-dependent manner. At the same time dCLK/CYC activate *Pdp1ε* and *vri* expression, which in turn regulates *dClk* expression via VRI-mediated repression and subsequent PDP1ε-mediated activation. While dCLK levels are increasing PER is being degraded, thus allowing another cycle to ensue. Proteins are represented in uppercase, RNA in lowercase. Events occurring above and below the blue line are nuclear (N) and cytoplasmic (C), respectively.


dCLK
CYC
per
tim
vri
Pdp1
dClk
SLMB
PER
DBT
N
C
tim
per
vri
Pdp1
dCLK
SGG
CK2
PP2A
TIM
PER
DBT
SLMB
VRI
PDP1
TIM
PER
DBT

Marking time in mammals

From flies to men. Located in the suprachiasmatic nuclei of the hypothalamus (SCN), the mammalian central clock shares many components of the *Drosophila* clock (Figure 1.4). Indeed, many of the core mammalian clock genes were isolated by homology to their *Drosophila* counterparts.

Similar to *Drosophila*, the mammalian central clock is composed of interlocking positive and negative feedback loops. The positive regulators of the mammalian clock are the bHLH/PAS domain transcription factors CLK and BMAL1 (a homolog of CYC, also known as MOP3). In the early circadian day, CLK and BMAL1 heterodimerize and activate transcription of *mPer* through E-box binding (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999; Kume et al., 1999). mPER protein peaks towards the end of the circadian day and in turn exerts a negative action on CLK/BMAL1.

Despite the conservation of the clock framework between flies and mammals, there are several key differences. First, gene duplication has resulted in many of the clock genes being represented by multiple orthologs in mammals (e.g. *mPer1, 2* and *3*). Secondly, whereas in *Drosophila Clk* RNA and protein cycle and *cyc* RNA and protein are constitutively expressed, in mammals *Bmal1* cycles and *Clk* does not (Shearman et al.,

2000a). Thirdly, whereas there is both strong and TIM-dependent repression of dCLK/CYC in *Drosophila*, repression of CLK/BMAL1 is somewhat weaker and TIM-independent in mammals (Jin et al., 1999). Closer inspection of *mTim* reveals that it is more similar to another *Drosophila* gene, *timeout (to)*, which doesn't appear to be necessary for the maintenance of fly circadian rhythms (Gotter et al., 2000; Reppert and Weaver, 2000). In mammals, CRYPTORCHROMEs (mCRY1 and mCRY2) appear to take the place of TIM in the negative feedback loop. Whereas *Drosophila* CRY is a blue-light photoreceptor (see below), mCRY1 and mCRY2 play a light-independent role in the mammalian clock (Griffin et al., 1999). *In vitro*, the mCRYs associate with all three mPERs, as well as CLK and BMAL (Griffin et al., 1999; Kume et al., 1999). Further, mCRY1 and mCRY2 have been shown to promote nuclear entry of the mPERs in cell culture (Kume et al., 1999). *In vivo*, *mCry* levels are low in *Clk* mutant mice (Kume et al., 1999), supporting its role as a target of CLK/BMAL1 transcription. And consistent with the mCRYs serving as players in the negative feedback loop, *mPer1* and *mPer2* are expressed at constant high levels in *mCry1* and *mCry2* mutant mice (van der Horst et al., 1999; Vitaterna et al., 1999).

Despite the evident role of mCRY in the mammalian negative feedback loop, there is some data supporting mTIM as an essential

component of the mammalian clock (Barnes et al., 2003; Tischkau et al., 1999). There are two transcripts of *mTim* that encode a large (mTIM-fl) and a small (mTIM-s) protein (Li et al., 2000). Only mTIM-fl has been shown to oscillate in SCN slices, while antisense oligonucleotides against this isoform abolish rhythmic neuronal firing (Barnes et al., 2003). mTIM-fl has also been shown to interact with all three mPERs in cell culture, leading researchers to believe that mTIM plays a similar role as dTIM in the negative feedback loop (Barnes et al., 2003). Further work should clarify the role of mTIM in the mammalian clock.

There are three *mPer* alleles whose transcripts cycle with slightly different phases. mPER3 does not appear to play a role in the central clock as its disruption, either alone or in combinations with *mPer1* or *mPer2*, does not significantly affect wheel-running behavioral rhythms (Bae et al., 2001; Shearman et al., 2000b). In contrast, both mPER1 and mPER2 knockout mice exhibit severe locomotor activity defects. mPER2 has been shown to act as a positive regulator of CLK/BMAL1 transcription (Bae et al. 2001; Shearman et al., 2000a; Zheng et al., 1999). mPER1 does not act as an activator or repressor but instead functions at the level of input to the entrainment pathway (Akiyama et al., 1999; Albrecht et al., 1997).

The positive (CLK/BMAL1) and negative (PER/CRY) interlocking feedback loops are connected by the retinoic acid-related orphan receptor (ROR) *Rev-Erbα*, (Preitner et al., 2002). Like *mPer* and *mCry, Rev-Erbα* transcription is activated by binding of CLK/BMAL1 to E-boxes in its promoter region. REV-ERBα protein binds to ROR element (RORE) sites in the *Bmal1* promoter, thereby down-regulating *Bmal1* transcription. As REV-ERBα is degraded, *Bmal1* transcription resumes, resulting in antiphase cycling of *Bmal1* and *Rev-Erbα* RNA. A second gene, *Rora*, was identified as an activator of *Bmal1* transcription in a cell-based screen. *In vitro*, REV-ERBα and RORA compete for RORE binding sites in the *Bmal1* promoter (Sato et al., 2004). REV-ERBα levels peak slightly earlier than those of RORA, suggesting successive repression and activation, respectively, of *Bmal1*. This is reminiscent of VRI and PDP1 regulation of *dClk* in *Drosophila*.

Fine-tuning the mammalian clock. As in flies, post-translational modifications are necessary components of the core clock mechanism. The short running wheel activity period of a Syrian hamster mutant, dubbed *tau*, was discovered to be the result of a point mutation in CKIε (Lowrey et al., 2000). mPER, mCRY and BMAL1 are all phosphorylated by CKIε (Eide et

al., 2002, 2005; Lee et al., 2004). Familial Advanced Sleep Phase Syndrome (FASPS), an autosomal dominant human sleep disorder that is manifested by early bedtimes and awakenings, is a result of the inactivation of a CKIε site on hPER2 (Toh et al., 2001). A second kinase, CKIδ, also associates with mPER and mCRY proteins and is likely to play a similar role as CKIε (Lee et al., 2001).

As in flies, GSK-3β (the ortholog of *sgg*) is constitutively expressed. Its phosphorylation has been shown to cycle with a circadian rhythm both *in vivo* and *in vitro* (Iitaka et al., 2005). In cell culture, inhibition of GSK-3β results in an increase in phosphorylation of GSK-3β and period length, while overexpression of GSK-3β results in a short rhythm. GSK-3β has been shown to phosphorylate and promote the nuclear translocation of mPER2. It has also recently been shown that treating cultured mouse fibroblasts with lithium, an inhibitor of GSK-3β, results in degradation of REV-ERBα and induction of *Bmal1* (Yin et al., 2006). These data all suggest that GSK-3β plays a crucial role in modulating the mammalian clock.

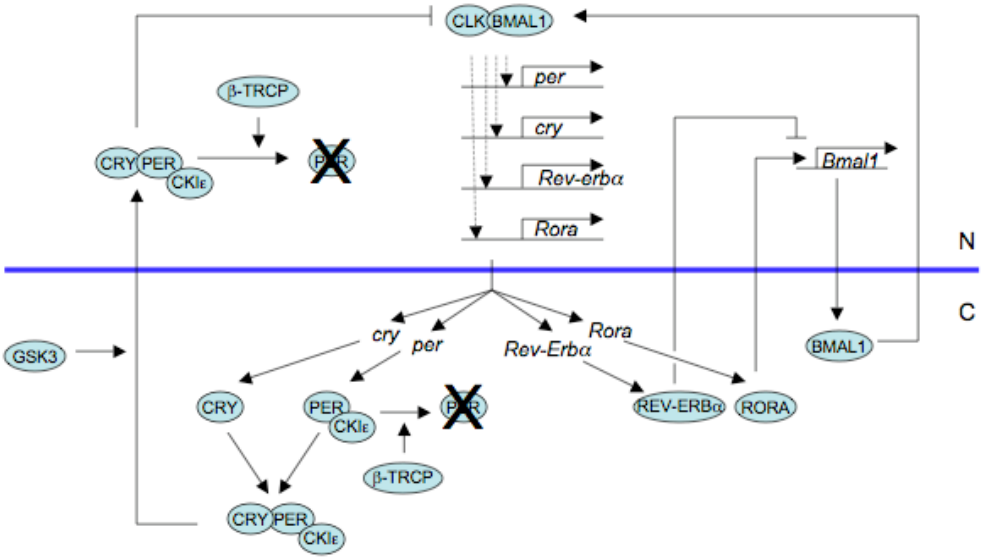
The identification of the role of *slmb* in the *Drosophila* clock led researchers to investigate a role for ubiquitin ligases in the mammalian clock. It has been shown that UchL1, a deubiquitinating enzyme (DUB), is highly expressed in the SCN, although without a circadian rhythm (Dong et

al., 2005; Zhang et al., 2004). β-TRCP1 and β-TRCP2, the mammalian orthologs of *slmb*, show a phosphorylation-dependent association with mPER1 *in vitro* (Eide et al., 2005; Shirogane et al., 2005). Inhibition of β-TRCP1 and β-TRCP2 increases the stability of mPER1, while overexpression of β-TRCP1 and β-TRCP2 results in increased transcription of CLK/BMAL1-dependent reporter constructs. These data strongly suggest a role for β-TRCP1 and β-TRCP2 in regulating the turnover of mPER1.

Another recently identified post-translational modification event in the mammalian clock is the SUMOylation (covalent linkage of small ubiquitin-related modifier protein to lysine residues) of BMAL1 (Cardone et al., 2005). CLK-dependent SUMOylation of BMAL1 was shown to occur with a circadian rhythm that parallels cycling BMAL1 expression *in vivo*. *In vitro*, inhibition of SUMOylation by mutating the target lysine residue abolishes BMAL1 cycling and increases its abundance, indicating a role for SUMOylation in both the rhythmic expression and turnover of BMAL1.

Figure 1.4. Model of the mammalian clock.

The mammalian central clock is composed of positive and negative interlocking feedback loops. Briefly, CLK/BMAL1 activate transcription of m*Per* and *mCry* RNA. mPER and mCRY proteins accumulate in the cytoplasm and when critical levels are reached, translocate to the nucleus where they inhibit their own transcription by binding to CLK/BMAL1. At the same time, BMAL1 levels cycle due to REV-ERBα-mediated repression and subsequent RORA-mediated activation of *Bmal1* transcription. A new cycle ensues when mPER is degraded and repression of CLK/BMAL1 is relieved. Post-translational modifications by CK1ε, GSK3-β and β-TRCP all contribute to the regulation of the core clock components. Proteins are represented in uppercase, RNA in lowercase. Events occurring above and below the blue line are nuclear (N) and cytoplasmic (C), respectively.


CLK
BMAL1
β-TRCP
per
cry
Rev-erbα
Rora
Bmal1
CRY
PER
CKIε
N
C
GSK3
cry
per
Rev-Erbα
Rora
BMAL1
CRY
PER
CKIε
REV-ERBα
RORA
β-TRCP
CRY
PER
CKIε

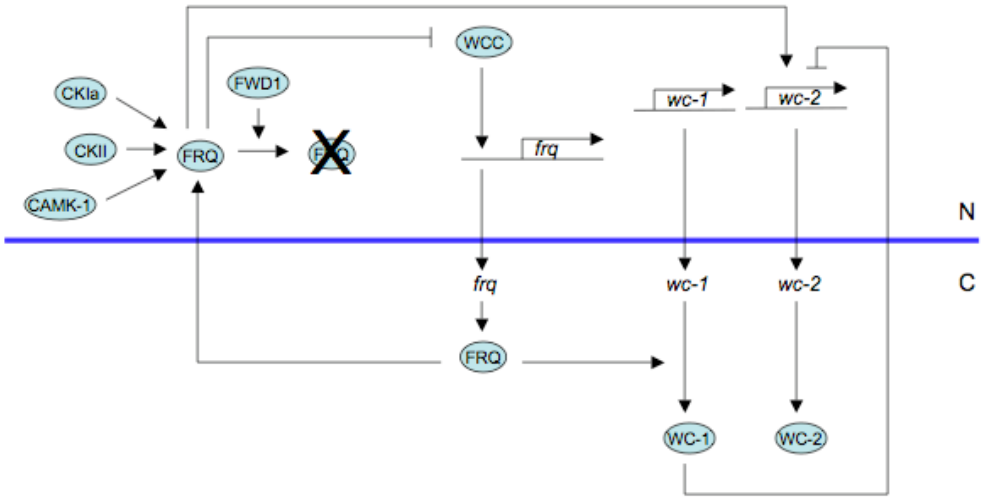
Timekeeping in *Neurospora*

Neurospora has been used as a genetic tool for over 50 years and its conidial banding pattern makes it a good system in which to study circadian rhythms (Loros and Dunlap, 2001). As in the *Drosophila* and mammalian clocks, the *Neurospora* clock is composed of coupled negative and positive feedback loops (Figure 1.5). The core negative feedback loop includes the proteins White Collar-1 (WC-1) and White Collar-2 (WC-2), as well as *frequency (frq)* RNA and protein (Aronson et al., 1994; Ballario et al., 1996; Crosthwaite et al., 1997; Linden and Macino, 1997). WC-1 and WC-2 are both zinc finger, PAS domain proteins that heterodimerize to form a White Collar Complex (WCC), which is reminiscent of *Drosophila* CLK and CYC (Lee et al., 2000). WCC binds to two sites in the *frq* promoter during the late night, thereby activating *frq* transcription (Froelich et al., 2002, 2003). *frq* RNA is alternatively spliced, determining the ratio of the two forms of FRQ protein produced (Garceau et al., 1997; Liu et al., 1997). Peak levels of FRQ protein are reached about 4 hours after maximal *frq* RNA levels. FRQ proteins dimerize and begin to enter the nucleus in the early morning (Cheng et al., 2001a). Inside the nucleus, FRQ interacts with WCC, thereby inhibiting *frq* transcription (Garceau et al., 1997; Cheng et al., 2001a; Froelich et al., 2003). This is reminiscent of *Drosophila* PER.

In a second positive feedback loop, FRQ promotes the translation of constitutively expressed *wc-1* message. The resulting WC-1 protein oscillates antiphase to FRQ (Cheng et al., 2001b; Lee et al., 2000). FRQ is also responsible for regulating the transcription of *wc-2*, whose constitutively expressed protein binds to and stabilizes WC-1 (Cheng et al., 2001b, 2003). The resulting WCC is in turn rendered inactive by FRQ, inhibiting *frq* transcription (Garceau et al., 1997; Liu et al., 2000; Froehlich et al., 2003). FRQ is progressively phosphorylated by CKIa, CKII and calcium/calmodulin-dependent kinase 1 (CAMK-1) (Gorl et al., 2001; Yang et al., 2001, 2002, 2003). Phosphorylated FRQ is ultimately degraded in a ubiquitin-mediated manner that is dependent on F-box and WD40 repeat-containing protein 1 (FWD1), the *Neurospora* homolog of *Drosophila* SLMB (He et al., 2003). This liberates WCC and allows it to resume transcription of *frq* (Froelich et al., 2003; Garceau et al., 1997; He et al., 2003; Liu et al., 2000).

Figure 1.5. Model of the *Neurospora* clock.

The *Neurospora* clock is composed of positive and negative interlocking feedback loops. WCC activates *frq* transcription in the late night. FRQ protein levels peak about 4 hours after maximal *frq* transcription, when they enter the nucleus and inhibit *frq* transcription by interacting with WCC. FRQ is also responsible for transcription of *wc-2* RNA, as well as promoting the translation of *wc-1*. Cycling WC-1 binds to and is stabilized by constitutively expressed WC-2, forming WCC. Phosphorylated FRQ is degraded in a ubiquitin-mediated manner, thus liberating WCC and allowing another cycle of *frq* transcription to ensue. Proteins are represented in uppercase, RNA in lowercase. Events occurring above and below the blue line are nuclear (N) and cytoplasmic (C), respectively.


WCC
CKIa
FWD1
wc-1
wc-2
CKII
FRQ
frq
CAMK-1
N
C
frq
wc-1
wc-2
FRQ
WC-1
WC-2

A conserved timekeeping mechanism. Diverse organisms have evolved a single solution—an endogenous, biological clock—in order to respond to and anticipate environmental changes resulting from the Earth's 24-hr rotation on its axis. This internal timekeeping mechanism is recapitulated in organisms as diverse as fungus and flies. Although the molecules involved may differ, the key features illustrated in the clocks of the organisms described here, namely autoregulatory transcriptional feedback loops and regulation of protein stability, are conserved features of all characterized Eukaryotic biological clocks.

Setting the clock

As mentioned above, one of the requirements for a biological clock is its ability to be entrained to the 24-hr environmental day. This is achieved through *Zeitgebers* ("time givers"), such as daily oscillations in light and dark, ambient temperature, food availability and social cues. The roles of light and temperature as *Zeitgebers* for the *Drosophila* clock will be discussed here.

Flicking the switch. The circadian clock is differentially sensitive to light exposure depending on the time of day. If, for example, light is given during the subjective day, there is little or no effect on phase since light is expected at that time anyway. If, however, a light pulse is given at subjective dawn or dusk, there is a resulting phase advance or delay, respectively. This allows the organism to align its behavior to the environmental day, as well as adapt to seasonal changes in day length. A phase response curve illustrates the relationship between a light pulse and both the direction and magnitude of the resulting phase shift.

In *Drosophila*, TIM is thought to be responsible for the light-induced resetting of the circadian clock as it is rapidly degraded in response to light. This effect is mediated by CRY, a blue light photoreceptor in the family of

flavoproteins (Ceriani et al., 1999). A strong hypomorphic mutation, *cry*^{baby} (*cry*^{b}), was isolated in a *per*-luciferase assay (Stanewsky et al., 1998). This mutation completely abolishes *per* and *tim* oscillations in whole adult fly heads, although it does not affect locomotor activity rhythms (Emery et al., 1998, 2000; Stanewsky et al., 1998). The mutation also results in abnormal responses to light in terms of both resetting and in that *cry*^{b} flies are behaviorally rhythmic in constant light. It is thought that CRY is responsible for relaying environmental light/dark information to the central oscillator. CRY has been shown to associate with TIM during the light phase of the circadian day, resulting in ubiquitination and degradation of TIM by a proteasome-mediated pathway (Ceriani et al., 1999; Naidoo et al., 1999). This in turn relieves PER/TIM inhibition of dCLK-mediated transcription.

The fact that *cry*^{b} flies can still be synchronized to a LD cycle indicates the presence of other pathways involved in the photoentrainment of the clock. The light-sensing, photopigment-expressing organs in the fly are the compound eye, the ocelli and the Hofbauer-Buchner (H-B) eyelet, a structure beneath the retina that makes neuronal contacts with the pacemaker cells (Helfrich-Förster et al., 2001). Locomotor activity behavior can still be entrained in phospholipase C (PLC) mutant no-receptor-potential-A *(norpA)*

flies, in which the compound eye and ocelli are disrupted. *norpA; cry*^{b} double mutants can also be entrained to an LD cycle (Stanewsky et al., 1998). It is only when CRY and all the light-sensing organs, including the H-B eyelet, are removed that the fly becomes completely blind to light (Helfrich-Förster et al., 2001).

Turning up the heat. In addition to daily variations in light exposure, temperate zones undergo daily changes in temperature. Much is known about how the fly uses light to align its behavior to the daily LD cycle but less is known about how the fly uses temperature as a *Zeitgeber*. It was established that *D. pseudoobscura* eclosion rhythms could be entrained to temperature cycles in the 1960s (Zimmerman et al., 1968). Further, it was shown in this population that temperature step-ups, step-downs and pulses result in accompanying phase shifts in behavior (Sweeney and Hastings, 1960).

Short, high temperature heat pulses result in rapid down-regulation of both PER and TIM (Sidote et al., 1998). This results in a phase delay if the heat pulse is administered in the early night. However, a heat pulse given in the late night does not result in a phase advance, as is the case with a light pulse given at this time. This is thought to be due to a rapid increase in PER

and TIM production after the initial down-regulation, ultimately resulting in a constant period.

Locomotor activity in *D. melanogaster* can be entrained to temperature cycles of as little as 3°C (Wheeler et al., 1993). The locomotor activity rhythms of arrhythmic clock mutants (per^0, tim^0, Clk^{Jrk}, cyc^0) can be driven by temperature (Yoshii et al., 2002). As with an LD cycle, they do not truly entrain as they don't anticipate the temperature transitions and rhythmicity does not persist when they are released into constant conditions. Interestingly, wild-type flies can be entrained to temperature in constant light (LL), a situation that would normally result in arrhythmicity. There is anticipation of temperature transitions but as with the clock mutants, the animals become arrhythmic when the temperature cycle is removed (Glaser and Stanewsky, 2005; Yoshii et al., 2002, 2005).

Molecularly it was shown that both PER and TIM oscillate during temperature entrainment in DD and that these oscillations are maintained during constant conditions following entrainment (Stanewsky et al., 1998). It thus follows that temperature acts on at least some of the same molecular components of the circadian clock as light. Temperature cycles can also drive PER and TIM oscillations, as well as locomotor activity behavior (see above), during constant light, a situation that normally results in behavioral

and molecular arrhythmicity (Glaser and Stanewsky, 2005; Yoshii et al., 2005). This result implies that the temperature cycle must somehow override the light-induced degradation of TIM by CRY. However, this result does not distinguish between direct behavioral or molecular masking and functional entrainment of the clock since the animals revert to arrhythmicity when the temperature cycle is removed.

Much of the recent molecular work on temperature and circadian rhythms has focused on alternative splicing of an 89-bp intron in the 3' untranslated region (UTR) of *per*, an event thought to be important in seasonal adaptation (Majercak et al., 1999). At low temperatures, more of the *per* spliced variant is produced than the unspliced variant. The result is an earlier increase in PER protein abundance and an advanced phase of locomotor activity, although the overall period length is maintained. The presumable adaptive advantage is that during short cold days, the fly would move its behavior towards the (brighter and warmer) middle part of the day. Indeed, long photoperiods counteract the behavioral advance seen at cold temperatures by delaying the accumulation of TIM. Without TIM, any prematurely produced PER would be rendered unstable (Majercak et al., 1999). It thus seems that information from both light and temperature are

integrated by the fly to enable it to align its behavior to the environmental day.

Further work implicated not only light and temperature in aligning behavior and the environment, but also the clock (Collins et al., 2004; Majercak et al., 2004). While short cold days lead to increased amounts of spliced *per*, warmer temperatures result in less of the spliced variant, especially during the day. This appears to be a clock-dependent effect that results in the fly moving its behavior to the later (cooler) part of the day. Thus *per* splicing allows the fly to adapt to changes in both temperature and photoperiod by regulating the amount of available PER protein, while TIM appears to be responsible for relaying environmental light/dark information to the clock by regulating the rate of PER accumulation.

PLC also plays a novel non-photic role in relaying information about temperature to the clock by down-regulating *per* splicing at warmer temperatures (Collins et al., 2004; Majercak et al., 2004). While wild-type flies show inhibition of splicing at warmer temperatures, *norpA* flies, which are deficient in PLC, do not. Indeed, these flies exhibit the cold temperature splicing phenotype regardless of the temperature and time of day. This appears to be a light-independent effect, however, as the ability of a light pulse to down-regulate *per* splicing is still maintained in these flies.

The product of another gene, *nocte*, has also recently been implicated in temperature entrainment of the circadian clock (Glaser and Stanewsky, 2005). *nocte* was identified in EMS-mutagenized BG-*luc* flies with aberrant luciferase oscillations during temperature entrainment in LL. Rhythmic locomotor activity of *nocte* flies can be driven by temperature cycles in LL. However, as with arrhythmic clock mutants, this is not true entrainment as they become arrhythmic once the temperature cycle is removed. Another indication that *nocte* flies are merely responding to temperature changes is their immediate behavioral shift in response to an advance in the temperature cycle. Despite their arrhythmicity in response to temperature entrainment, *nocte* flies are behaviorally rhythmic in LD and DD over a range of constant temperatures. Oscillations of PER and TIM are abolished in whole head extracts of *nocte* flies that are entrained to a temperature cycle in LL. This is analogous to what happens to PER and TIM in cry^b flies in DD.

Aims of this thesis

The intention of this thesis is to characterize the regulation of circadian gene expression by light and temperature in *Drosophila*. The global approach of generating genome-wide transcriptional profiles is used to define a discrete set of genes that oscillate in the wild-type adult fly head in response to daily light/dark and temperature cycles. The generation of gene expression profiles in an arrhythmic clock mutant also determines whether all 24-hr periodic expression requires the presence of a functional clock. The responses of the core clock genes to temperature cycles, both in the presence and absence of a clock, are further analyzed using Northern, Western and behavioral analyses. By monitoring oscillations at the transcript, protein and behavioral level, it will be shown that daily environmental information is integrated by, and relayed through, a single clock in the adult fly head.

Chapter 2: Materials and methods

Fly strains

The wild-type strains used were *yellow white (y w)*, *cinnabar brown (cn bw)* and *Canton-S*. The clock mutant strains used were $Clock^{Jrk}$ (Clk^{Jrk}) (Allada et al., 1998), $cryptochrome^{baby}$ (cry^{b}) (Stanewsky et al., 1998), $cycle^{0}$ (cyc^{0}) (Rutilla et al., 1998), *white; period*^{0} (*w; per*^{0}) (Konopka and Benzer, 1971) and *y w; timeless*^{01} (*y w; tim*^{01}) (Myers et al., 1995). The flies were raised on standard yeast cornmeal medium.

Time course collection

For light experiments, adult flies were entrained to 12 hours of light followed by 12 hours of darkness (LD 12:12, where ZT0 is lights-on and ZT12 is lights-off) at 25ºC for five days and subsequently released into constant darkness (DD, where CT0 is subjective lights-off). They were harvested onto dry ice every four hours (unless specified) during the last day of entrainment and for one to two days of DD (as specified: Day 1 = CT0-24, Day 2 = CT24-48).

Temperature experiments were conducted entirely in the dark except for the initial seeding of parental bottles. Adults (parental) were placed in

fresh media and allowed to lay at 25ºC for five days. The parents were cleared and the next generation was kept at 25ºC until pupae, when they were then transferred to 12 hours of 25ºC followed by 12 hours of 18ºC (AC, where AC0 is onset of 25ºC and AC12 is onset of 18ºC) until eclosion. The newly eclosed flies were transferred to fresh media and allowed to entrain for an additional four days and then released into a constant temperature of 25ºC (AA). They were harvested onto dry ice every four hours during the last day of entrainment and for one to two and a half days in AA (as specified: Day 1 = AA14-34, Day 2 = AA34-58, partial Day 3 = AA58-70). The frozen flies were vortexed and passed through a series of sieves in order to isolate the heads for RNA or protein extraction.

Behavioral analysis

Individual flies were monitored and their locomotor activity analyzed with the *Drosophila* Activity Monitoring System IV (TriKinetics). For LD/DD experiments, the flies were reared under standard lighting conditions and monitored at 25ºC in LD and/or DD as specified. Temperature experiments were conducted entirely in the dark (unless otherwise specified). The flies were raised at 25ºC until the pupal stage and were then entrained to AC until eclosion. Individual flies were monitored and their

locomotor activity analyzed as above both during entrainment (AC) and free-run (AA). Period length and time of activity onset were calculated using ClockLab Software (ActiMetrics).

Northern blot analysis

Total RNA was extracted from approximately 100µl of adult heads per time point using either RNA-STAT60 (Tel-Test, Inc.) or guanidinium thiocyanate followed by centrifugation in Cesium chloride solution. 15-30µg of total RNA were denatured for five minutes at 65ºC and resolved on a 1% Formaldehyde-Agarose gel (20mM MOPS, pH7; 5mM NaOAc; 1mM EDTA). The resolved RNA was transferred to Nytran membrane (Schleicher & Schuell) in 10x SSC overnight. Sequence-verified probe templates were obtained from the *Drosophila* Gene Collection (Berkelely *Drosophila* Genome Project) and were radio-labeled as described in the DECAprime II kit (Ambion). Hybridizations were carried out at 55ºC in UltraHyb solution (Ambion) and 10mg denatured fish sperm DNA (Roche). The blots were visualized and quantitated with either a Storm or Typhoon Phosphorimager (Molecular Dynamics) and the results plotted in Microsoft Excel. Fourier analyses were also performed on the Northern data to be able to directly compare them to the microarry data when appropriate.

RNAse protection assay (RPA)

Total RNA was extracted from approximately 100µl of adult heads per time point using RNA-STAT60 (Tel-Test, Inc.). The RPA was performed as described in the RPAIII kit (Ambion). 10µg of total RNA were used for each sample. The oligonucleotides used to generate riboprobes from *CG5798* cDNA were as follows:
exon 5/6 boundary (ex5-6) 5'-CAGCCGAGGAATGTGGAG-3' and 5'-TAATACGACTCACTATAGGNNNNNNNNNNNNGTTGCTCAGGCACTGCAG-3';
exon 6/7 boundary (ex6-7) 5'-GAGGTGGCTGCACTCATC-3' and 5'-TAATACGACTCACTATAGGNNNNNNNNNNNNGAGTGCAGCCAGTCCATC-3'. The riboprobes were α-^{32}P[UTP] labeled using T7 DNA polymerase according to instructions in the MAXIscript kit (Ambion).

RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

RNA was isolated from adult heads collected at ZT6 using RNA-STAT60 (Tel-Test, Inc.) and polyA$^+$-purified over a column (Qiagen). The RLM-RACE reaction was carried out as described in the GeneRacer^{TM} kit (Invitrogen). GeneRacer^{TM} Oligo dT was used for the reverse transcription

reaction. The reverse Gene-Specific Primer (GSP) used to amplify cDNA ends was 5'-GTAAAAACCGAAAGGAAACCGTAGCGAA-3'. The reverse nested GSP was 5'-GCACTGATCATCTCCCGCTGACGGG-3'. The PCR product was gel-purified and cloned into a pCR®4-TOPO vector (Invitrogen) according to the manufacturer's instructions. Sequencing was carried out using the built-in M13F primer.

Western blot analysis

Total protein was extracted from about 35µl of adult heads per time point in 75µl Head Extraction Buffer (100mM KCl; 20mM Hepes, pH 7.5; 10% glycerol; 10mM EDTA, pH 8; 0.1% Trition X-100; 50mM NaF; 1mM DTT) with 1x protease and phosphatase inhibitors (Roche) using a handheld homogenizer (Kontes). Samples were centrifuged at 14k rpm for 15 minutes at 4ºC. The supernatant was transferred to a new tube and centrifuged as above for an additional 10 minutes. 15-30µg of total protein were resolved on 6% SDS polyacrylamide gels and transferred to nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked for at least one hour at room temperature with 5% non-fat dry milk in 1x TBST. Primary antibodies were diluted in blocking solution (1:10000 for α-PER [rabbit], 1:2000 for α-TIM [rat], 1:10000 for α-Hsp70 [mouse], 1:5000 for α-SGG [mouse]) and

incubated with the membranes at 4ºC overnight. The membranes were washed three times for 10 minutes each in 1x TBST and incubated with secondary antibodies (1:10000) (Jackson ImmunoResearch) for one hour at room temperature. The membranes were washed as before and detection was carried out using ECL (Amersham Pharmacia Biotech).

RT-PCR analyses

RNA was extracted with RNA-STAT60 (TelTest, Inc.) in the same manner as for Northern blots. cDNA was generated using the ThermoScript RT-PCR System (Invitrogen) as described by the manufacturer with one exception: cDNA synthesis was carried out at 50ºC for 90 minutes with Oligo$(dT)_{20}$. Three μl of cDNA were used in subsequent PCR reactions with AccuPrime Pfx DNA Polymerase (Invitrogen).

Generation of transgenes and transgenic lines

Ctim3^{FL}: *tim* cDNA (Meyers et al., 1995) was digested with ApaI and NotI and cloned into a plasmid containing ~4 kb of genomic DNA upstream of the *tim* transcription initiation site as well as 3560 bp of the 5' genomic region of *tim* (pGEM-117BA) using the same restriction sites. The

resulting 117BA-tim3 construct was digested with NotI and XhoI and cloned into pCaSper4.

Ctim3^{FL+S}: PCR amplification from cDNA was performed (95°C for 15 s, 5 cycles [95°C for 15 s, 63°C for 30 s, 68°C for 1 m 45 s], 25 cycles [95°C for 15 s, 59°C for 30 s, 68°C for 1 m 45 s], 68°C for 5 m) with TBamHIoF (5'-CACCCTGTTGGAATACGATGACCTG-3')/Tin14R2 (5'-TTAGCCAGGTCGAAATCTTGGCTGG-3') and Tin14F2 (5'-GTCGGTTGGCACACAGCTTAGAAG-3')/TBamHIoR (5'-AAATGTACAATCCTTGTGCCGGGTG-3') primer pairs. 1μg product from each reaction was combined in a total volume of 25μl and heated to 95°C for 5 m and then slowly cooled to 37°C over 10 m. The resulting annealed product was amplified (68°C for 15 s, 95°C for 15 s, 5 cycles [95°C for 15 s, 63°C for 30 s, 68°C for 2 m 30 s], 25 cycles [95°C for 15 s, 59°C for 30 s, 68°C for 2 m 30 s], 68°C for 5 m) with TBamHIiF (5'-CGAGGATGCGGAGTATCAGAGAACT-3')/TBamHIiR (5'-GTACAATTCTTTCCAGCTGGGTGGG-3') primers. The resulting product was digested with BamHI, cloned back into *tim* cDNA and oriented to make tim3^{FL+S}. tim3^{FL+S} was then cloned into pCaSper4 as described for the Ctim3^{FL} construct.

Ctim3^{mS}: BamHI digested Ctim3^{FL+S} was cloned into pBluescript for mutagenesis, which was carried out using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The primers used were TmutGA1-F (5'-GGAGAAGGAATTAAAATGAGTGCGATTGAGC-3')/TmutGA1-R (5'-GCTCAATCGCACTCATTTTAATTCCTTCTCC-3') and TmutGA2-F (5'-CTCATGATCATTGCAACAGCGACAATGTCTC-3')/TmutGA2-R (5'-GAGACATTGTCGCTGTTGCAATGATCATGAG-3'). Sequence-verified clones were digested with BamHI, cloned back into *tim* cDNA and oriented to make tim3^{mS}. tim3^{mS} was then cloned into pCaSper4 as described for the Ctim3^{FL} construct.

The constructs were injected into *Drosophila* embryos by standard means (Spradling and Rubin, 1982). The resulting lines were crossed into a *tim^{01}* background.

Microarray experiments

RNA was extracted from adult heads with guanidinium thiocyanate followed by centrifugation in Cesium chloride solution. 50μg of the RNA was further purified over RNeasy columns (Qiagen) according to instructions from the manufacturer. 25μg of the purified RNA was used to generate biotin-labeled cRNA probe as described in the Affymetrix

GeneChip manual. T7-d24 primers (MWG Biotech), Superscript Choice (Life Technologies) and enzymes from New England Biolabs were used to synthesize cDNA. The ENZO Bioarray High Yield RNA transcript labeling kit was used for *in vitro* transcription reactions. Hybridization, washing, staining and scanning of the target cRNA to the Affymetrix *Drosophila* Genome 1 arrays were carried out according to the Affymetrix GeneChip manual.

Microarray data analyses

The robust multi-array average (RMA) single algorithm (Irizarry et al., 2003) was used to prepare the microarray data from each experiment. Fourier scores were calculated for appended time course experiments. The probability values were calculated against a permutation null hypothesis. In the null model, the real data were permuted 1000 times to give a background distribution of Fourier scores. These were divided into a number of quantiles equal to the original number of probe sets and compared to the Fourier values from the real data.

The primary ranking filter used to select the 172 transcripts that oscillate in LD/DD demanded that the $pF24_{LDDD}$ be ≤ 0.001. To eliminate noisy transcripts, the average daily range had to be ≥ 0.3. A Kruskal-Wallis

test (KW) demanded that the autocorrelation between like time points be $\leq$ 0.05. Finally, to avoid light-driven effects, the $pF24_{DD}$ had to be ≤ 0.05. Similar filters were applied to the temperature data to identify the 143 AC/AA cycles, except that pKW_{AA} had to be ≤ 0.01. To select the 164 temperature-driven transcripts, the $pF24_{wt+timAC}$ had to be ≤ 0.05, the average daily range had to be ≥ 0.3 for both wild-type alone and the combined wild-type and *tim*^{01} data and the $pKW_{wt+timAC}$ had to be ≤ 0.025.

Chapter 3: A comprehensive analysis of circadian gene expression in the adult *Drosophila* head

Introduction

The CLK/CYC controlled expression program of the core clock transcripts restricts the possible phases of gene expression to early (*Clk, cry*) or late (*per, tim, vri, Pdp1ε*) in the circadian day. It is possible that all clock-controlled gene expression is limited to phases overlapping with those of the core clock genes. However, given the diverse clock-controlled physiologies and behaviors such as vision, olfaction, metabolism, locomotor activity, eclosion and courtship, it is likely that there are other expression patterns.

In order to address the existence of multiple expression patterns, high density oligonucleotide microarrays were used to identify rhythmically expressed transcripts in the adult *Drosophila* head (Claridge-Chang et al., 2001). Samples were collected from three independent wild-type (*y w* and *cn bw*) populations every four hours for one day of entrainment (LD) and one day of free-run (DD). Fourier analyses were used to identify transcripts with 24-hr periodic oscillations and a subset of these were confirmed by Northern blot analyses. This strategy revealed a set of about 400 oscillatory

transcripts, with a subset of 158 showing particularly robust circadian behavior. Further, the phases of the oscillatory transcripts were distributed throughout the circadian day, indicating that the *Drosophila* clock regulates gene expression through both known and novel transcriptional mechanisms.

Several others labs subsequently published similar studies (Ceriani et al., 2002; Lin et al., 2002b; McDonald and Rosbash, 2001; Ueda et al., 2002), although with dramatically different results. Of the 548 combined oscillatory genes identified by all five groups, only seven (~1%) are in common (Figure 3.1). Even if the very small set found by Lin et al. (2002b) is excluded, no more than about 25% of the transcripts overlap between any two groups. In addition to the poor overlap, some of the core clock transcripts (*per, Clk, Pdp1* and *cry*) are absent in one or more of the published sets. There are many possible hypotheses for the extreme variability but the most likely cause is the different analytical methods used to identify cycling transcripts in combination with slightly different experimental designs. The poor overlap is also a strong indication that there is no clear "circadian" versus "non-circadian" delineation, especially in the set of complex tissues composing the adult head.

In this chapter, a single algorithm that emphasizes the uniformity in period length and peak phase between experiments is applied to all the

available published and unpublished microarray data. As a result, a more comprehensive picture of the circadian expression program in the adult *Drosophila* head is revealed. These data are further used to determine whether all rhythmic transcription is circadian or whether oscillations with other periods are present. These data are also compared to gene expression profiles obtained from an arrhythmic mutant to ascertain whether 24-hr periodic transcription depends on known clock circuits.

Results

A comprehensive analysis of all available clock microarray data reveals an extensive program of circadian gene expression. An integrative analysis of all the available microarray data (17 days), from both published and unpublished sources, was performed in order to define a more comprehensive system of circadian gene expression (for a thorough description of microarray data analyses see Materials and Methods). The quantile-quantil (QQ) plot in Figure 3.2 shows the distribution of 24-hr oscillatory statistics by plotting the distribution of squared Fourier scores in the wild-type data against a null permutation model. Each point of the y-axis corresponds to the real Fourier score of an individual transcript, while the same data are permuted 1000 times to give a background distribution of

Fourier scores represented as quantiles on the x-axis. The large number of transcripts that deviate from the diagonal suggest significant enrichment for circadian gene expression.

Separate analyses were also performed for time course data collected in LD only (8 days) and DD only (9 days) (Figure 3.2). As evidenced by the increased enrichment for 24-hr oscillatory transcripts in the LD data, rhythmicity is somewhat improved by the presence of a daily photocycle. This is most likely due to greater synchrony of the clock and higher amplitude of gene expression during entrainment.

Several filters were applied to the data in order to define a discrete set of robustly oscillating transcripts (see Materials and Methods). These selections yielded a set of 172 genes listed in Table 3.1 and represented by phase in Figure 3.3. All of the oscillating core clock transcripts are represented in this set. As can be seen from the diagonal nature of the phasegram, all circadian phases are represented. This indicates the presence of a program of circadian expression that is much more extensive than would be predicted by a CLK/CYC-controlled model.

Ten of the 172 transcripts are known to be circadian from the literature (Table 3.1, Column 3). An additional 17 transcripts from this set were tested on Northern blots representing one day of LD followed by one

day of DD (Table 3.1, Column 4 and Figure 3.4). The Northern data were Fourier fit in order to obtain a quantitative measure of any oscillation (Table 3.1, Column 5). Of the transcripts tested, 13 are confirmed circadian, and one (*Rab-RP1*) is a false positive. The remaining three (*puc, CG9400, 5-HT2*) do not statistically oscillate. However, as can be seen in Figure 3.4, there does appear to be an oscillation that is probably masked in the algorithm by a single time point (*puc*), a large difference in amplitude between the two days measured (*CG9400*) or an overall "slanting trend" of the data (*5-HT2*). Given that additional blots will most likely confirm these transcripts as oscillatory, one can estimate an overall false positive rate of less than 1% in this comprehensive set.

There is a clear bias toward transcription with a circadian period.

In order to test for the existence of transcription with ultradian or infradian periods, a subset of the wild-type data (four 2-day LD/DD experiments) was fit to sin waves of periods ranging from 12 to 48 hours. Figure 3.5 shows the data as QQ plots in the same format as previously described. It is evident that substantial enrichment, seen as an upward deviation from the diagonal, is present for the 24-hr period. Further, the distribution of real Fourier scores is much greater for the 24-hr period (up to ~0.8), while those

for the 12- and 16-hr periods are indistinguishable from the range of permuted values (up to ~0.4). While there is some enrichment for the 48-hr period, this is most likely due to the contribution of light-dependent transcripts (see Wijnen et al., 2006). Indeed, a transcript that is light-regulated would appear to have a 48-hr period due to the LD/DD experimental format which is essentially 12 hours of light followed by 36 hours of darkness. Therefore the data strongly suggest that the dominant period of rhythmic transcription is indeed circadian.

Circadian gene expression depends on the presence of a functional clock. The free-running oscillations of individual circadian transcripts have uniformly shown a dependence on a functional circadian pacemaker. It would therefore follow that the specific enrichment observed for transcripts with a period of 24 hours in wild-type flies would be absent in arrhythmic mutants. This prediction was tested by comparing a subset of the wild-type data (four 2-day LD/DD experiments) to expression profile data of the same size and format obtained from arrhythmic tim^{01} flies.

A direct comparison between the two data sets shows specific enrichment for 24-hr periodic oscillations in wild-type as compared to tim^{01} flies (Figure 3.6.a). Separate comparisons of data from both wild-type and

tim^{01} flies to permutated data further demonstrates that circadian profiles are enriched in the wild-type data, but not in the *tim*^{01} data (Figures 3.6.b and c). If enrichment for 24-hr periodic oscillations is present in the *tim*^{01} flies, it is much weaker than that seen in the wild-type data. Examining different periods (F12 and F16) does not enrich for oscillating *tim*^{01} transcripts (data not shown).

The apparent absence of enrichment of circadian gene expression in the *tim*^{01} data cannot, however, be taken as evidence that 24-hr periodic oscillations of all transcripts are abolished in this context. Figure 3.6.c shows that the squared Fourier scores for many of the *tim*^{01} transcripts are greater than the maximal value of 0.4 seen in permuted wild-type data. Furthermore, several wild-type genes with Fourier scores in the range seen for some of the *tim*^{01} transcripts have been confirmed as circadian. To address these issues, three selections were made to identify transcripts that carried the highest prediction of showing circadian oscillations in *tim*^{01} flies. Several members of each group were independently tested by Northern blot analyses. The first selection was directed solely at selecting candidate circadian oscillators in the context of *tim*^{01}. The candidates in this list have no obligation to be circadian genes in the context of wild-type circadian rhythms and could even be masked in such an instance. In a second

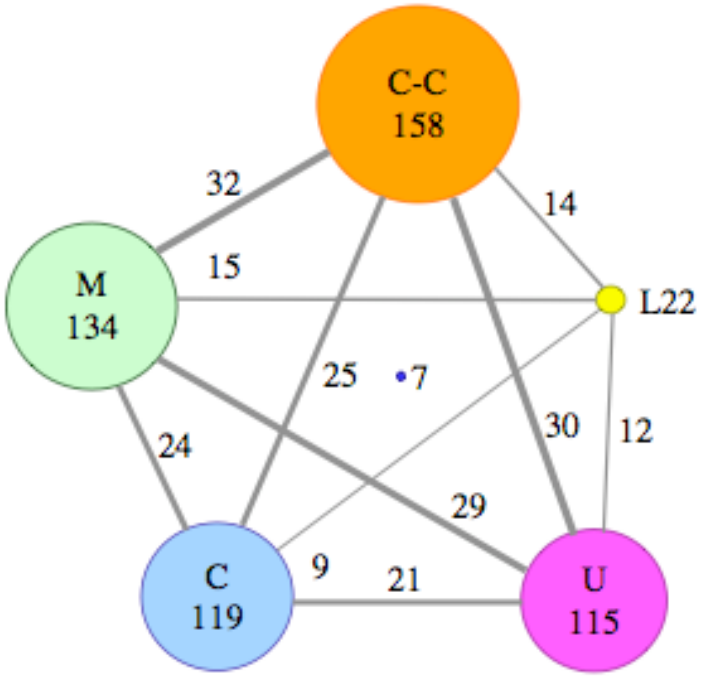
selection it was required that the transcripts also show a significant circadian oscillation in the wild-type data, although the phases need not match. This requirement was further expanded in a third selection in which it was demanded that the predicted oscillations for transcripts from both tim^{01} and wild-type flies should have the same phase. Transcripts in this last selection could be candidates for a clock independent of the central oscillator. Top-ranking candidates from each of the three ranked selections are shown in Table 3.2. A total of 14 transcripts were tested on Northern blots that contained samples from a 2-day circadian time-course experiment (LD/DD) conducted in tim^{01} flies (Figure 3.7). The Northern 24-hr Fourier component values for these transcripts are shown in Table 3.2. None of the transcripts show a significant circadian oscillation with the exception of *Slob* and the lower transcript of *Epac*. It was determined that these two transcripts are not circadian but instead are light dependent (Figure 3.7 and data not shown). Taken together, these observations strongly suggest that all circadian transcript oscillations depend on the known molecular clock circuits.

Summary. In recent years several groups have tried to define a set of circadian transcripts in the adult *Drosophila* head. There has been little consensus, resulting in a small overlap among publications and the exclusion of many confirmed oscillatory transcripts. In this chapter, an integrative analysis was carried out that emphasized uniformity in period and phase while tolerating inter-experimental differences in amplitude. As a result, a comprehensive set of circadian transcripts was defined. This set includes all of the known cycling clock genes, as well as genes involved in diverse processes such as vision, detoxification, metabolism, signal transduction and synaptic transmission. A number of newly identified oscillatory transcripts were further confirmed by Northern blot analyses. These data indicate there is an extensive and diverse program of circadian gene expression.

Using the results of the integrative analysis, it was further suggested that the dominant period of transcription is 24 hours. The enrichment for 24-hr periodic oscillations was lost in a tim^{01} arrhythmic mutant, indicating that a functional, TIM-dependent clock is required for circadian transcription.

Figure 3.1. Analyses of circadian gene expression in the fly: Consensus between published reports.

There is little consensus between the published analyses of circadian gene expression in the adult fly head. Of the combined 548 transcripts identified as circadian by all five groups, only seven are in common (blue center circle and listed to the right). The sizes of the circles are proportional to the number of transcripts identified by each group. Agreement between any two groups is indicated by lines connecting the circles, with the numbers corresponding to the number of transcripts in common. C = Ceriani et al., 2002, C-C = Claridge-Chang et al., 2001, L = Lin et al., 2002b, M = McDonald and Rosbash, 2001, U = Ueda et al., 2002.



Common:
CG10553
CG11891
CG5798
Slob
tim
Ugt35b
vri

Figure 3.2. An integrative analysis reveals an extensive program of circadian expression.

A QQ plot of spectral power (squared Fourier components) is shown. Points on the y-axis correspond to the real Fourier scores for individual transcripts, while the values on the x-axis are quantiles of 1000 permutations of the real data. Enrichment is indicated by an upward deviation from the diagonal (brown). The ticks on the right show the approximate number of transcripts above the corresponding Fourier value. Analyses were done separately for 17 days of combined LD and DD data (black), 8 days of LD data only (red) and 9 days of DD data only (green). Data are from Ceriani et al., 2002; Claridge-Chang et al., 2001; Lin et al., 2002b; McDonald and Rosbash, 2001; Wijnen et al., 2006.

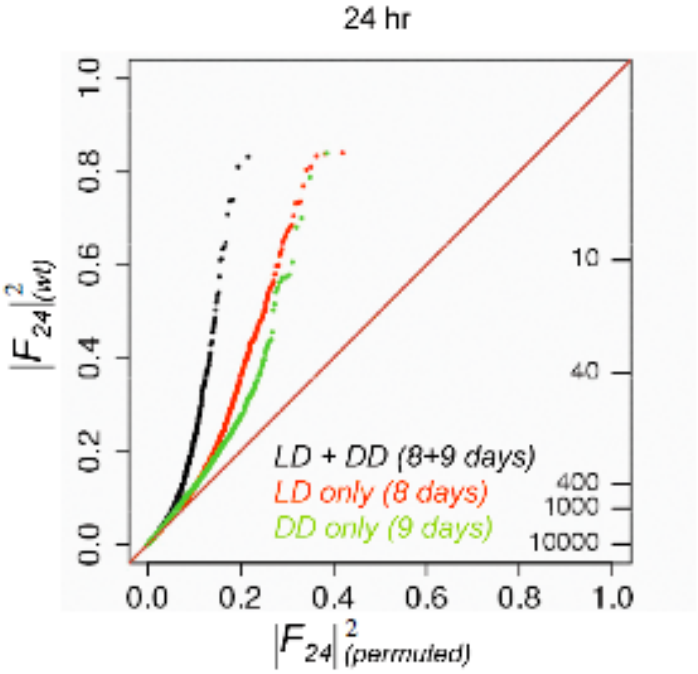

24 hr
$|F_{24}|^2_{(wt)}$
$|F_{24}|^2_{(permuted)}$
0.0
0.2
0.4
0.6
0.8
1.0
10
40
400
1000
10000
LD + DD (8+9 days)
LD only (8 days)
DD only (9 days)

Table 3.1. 172 transcripts show robust oscillations in LD and DD.

The data were filtered to define a set of oscillatory transcripts. It was demanded that pF24 $\leq$ 0.001, pKW $\leq$ 0.05, average range:noise ratio > 3.0, and $pKW_{DD} \leq 0.05$ (see Methods and Materials). Column 1 = Transcript name; Column 2 = Phase; Column 3 = Confirmed from literature; Column 4 = Confirmed by Northern; Column 5 = p-value of Northern data.

Figure 3.3. Organization of the expression patterns by phase of the 172 oscillating transcripts.

Columns correspond to time points and genes (listed to the right) are represented by row. Pink indicates up-regulation, turquoise indicates down-regulation. The open bars above and below the phasegram denote the entrainment scheme, with the white bars indicating light time points, the black bars indicating dark time points and the gray bars indicating free-run time points taken during subjective light. As can be seen from the diagonal nature of the phasegram, all circadian phases are represented.

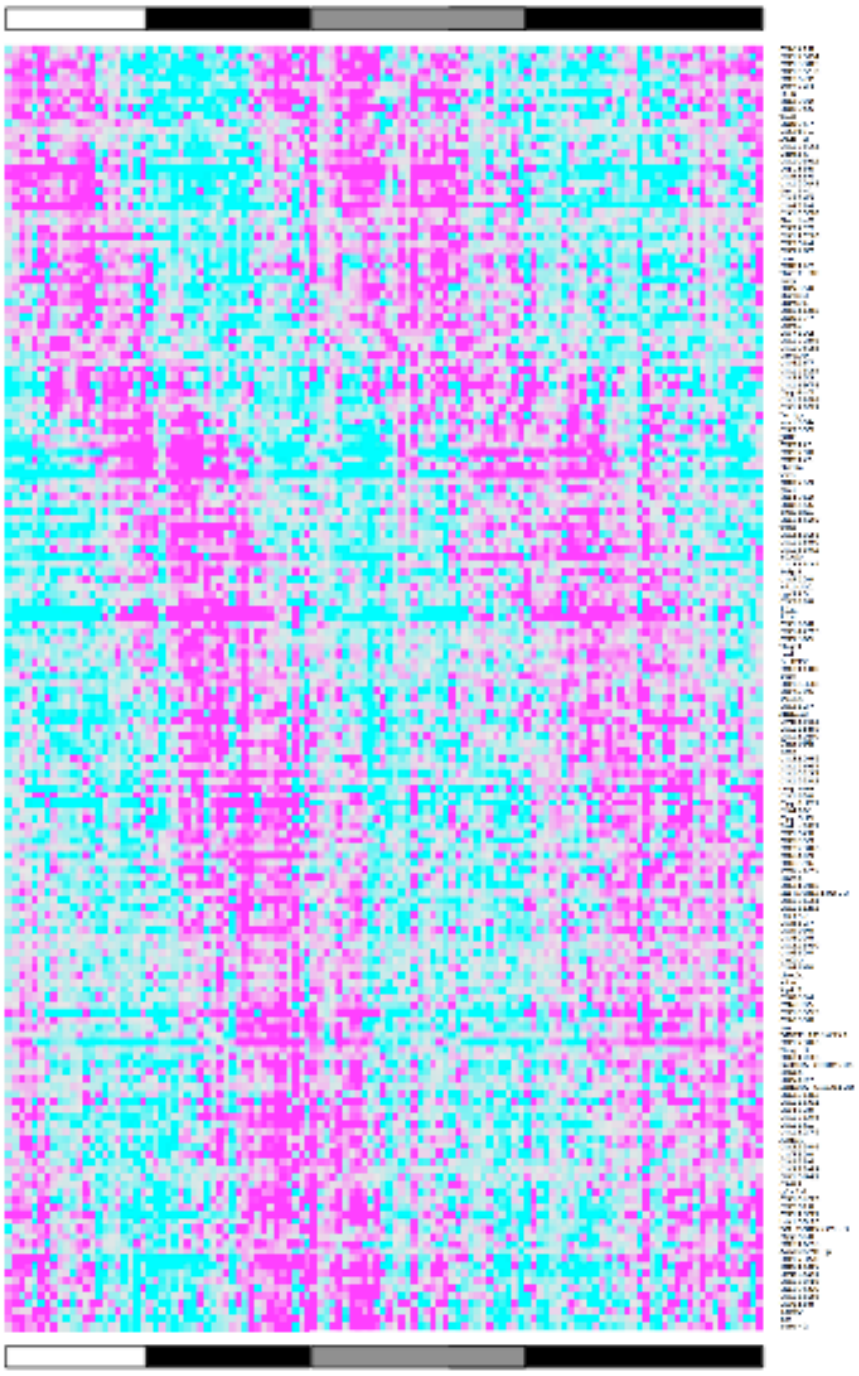
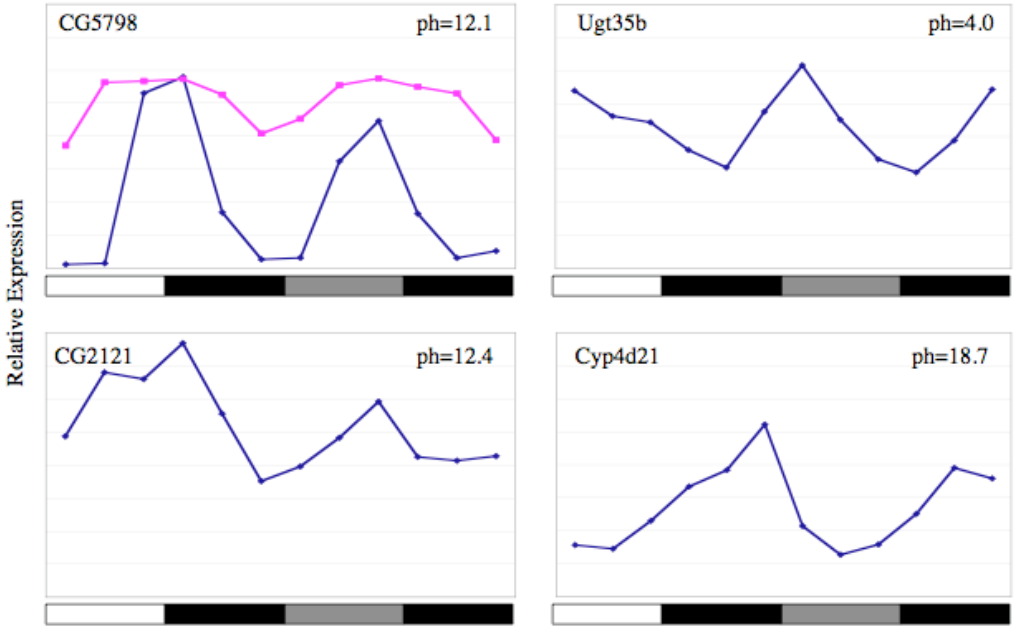
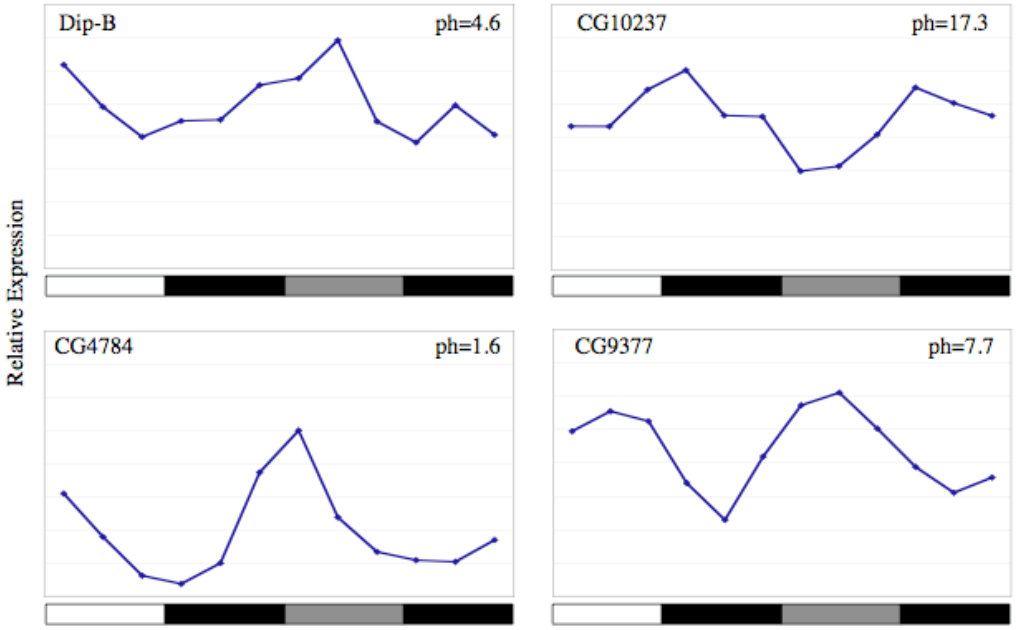
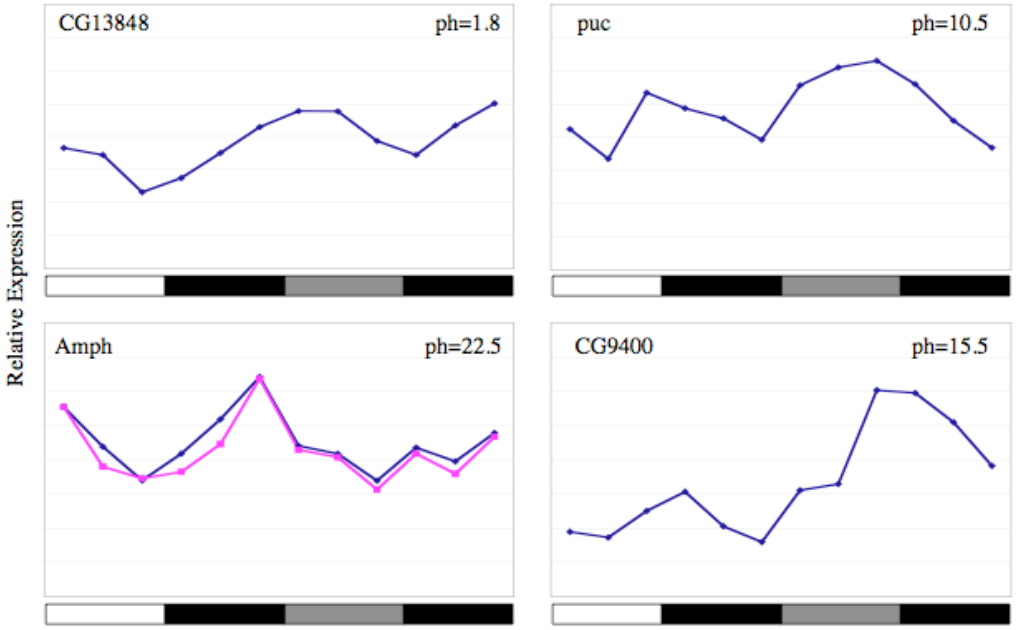


Figure 3.4. Northern blot analyses of the predicted oscillatory transcripts.

y w or *cn bw* flies were entrained to LD 12:12 for five days and subsequently released into DD. Time points were taken every four hours during the last day of entrainment and the first day of DD starting at either ZT2 or ZT4. RNA from whole heads was run on Northern blots and probed with cDNA encoding the indicated transcript (upper left-hand corners). An *rp49*-specific probe was used as a loading control in all cases. The data were Fourier fit (see Materials and Methods) in order to quantitatively measure any oscillation (see Table 3.1 for corresponding p-values) and calculate the phase (ph; upper right-hand corners). The bars below the plots denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points and gray bars indicating free-run time points taken during subjective light. Blue line = lower (or only) transcript, pink line = upper transcript, if present.


Relative Expression
CG5798
ph=12.1
Ugt35b
ph=4.0
CG2121
ph=12.4
Cyp4d21
ph=18.7


Relative Expression
Dip-B
ph=4.6
CG10237
ph=17.3
CG4784
ph=1.6
CG9377
ph=7.7


Relative Expression
CG13848
ph=1.8
puc
ph=10.5
Amph
ph=22.5
CG9400
ph=15.5

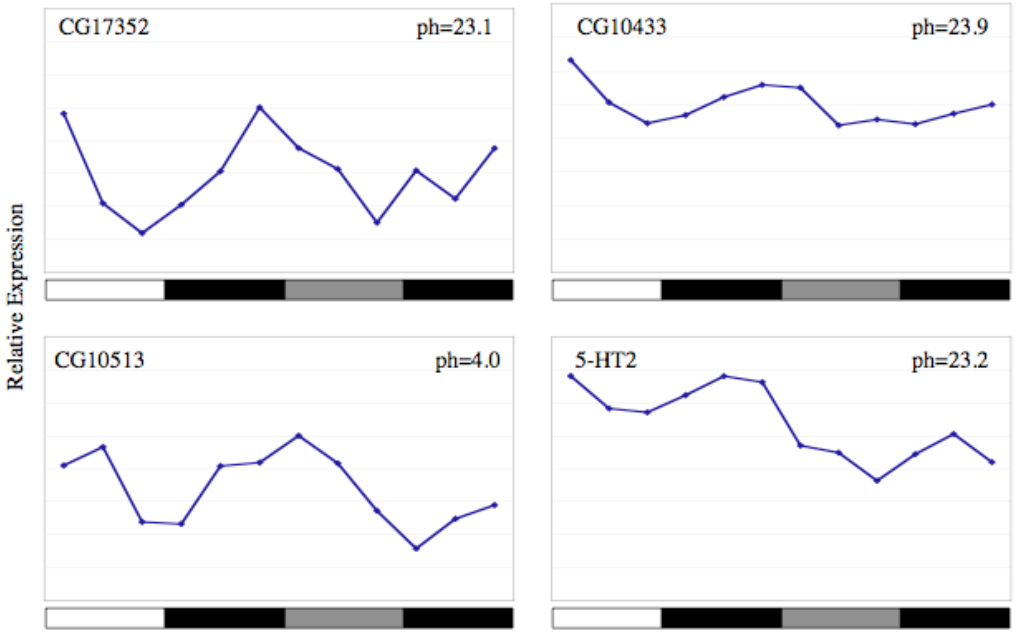

Relative Expression
CG17352
ph=23.1
CG10433
ph=23.9
CG10513
ph=4.0
5-HT2
ph=23.2

Figure 3.5. The dominant period of rhythmic transcription is 24 hours.

QQ plots of spectral power at periods of 12, 16, 24 and 48 hours, in the same format as Figure 3.2, are shown. Analyses were performed on data from four 2-day LD/DD collections. Substantial enrichment can be seen for the 24-hr period. While there is some enrichment at the 48-hr period, it is most likely due to the presence of light-regulated transcripts. There is no difference between the real and permuted data at the 12-hr and 16-hr periods.

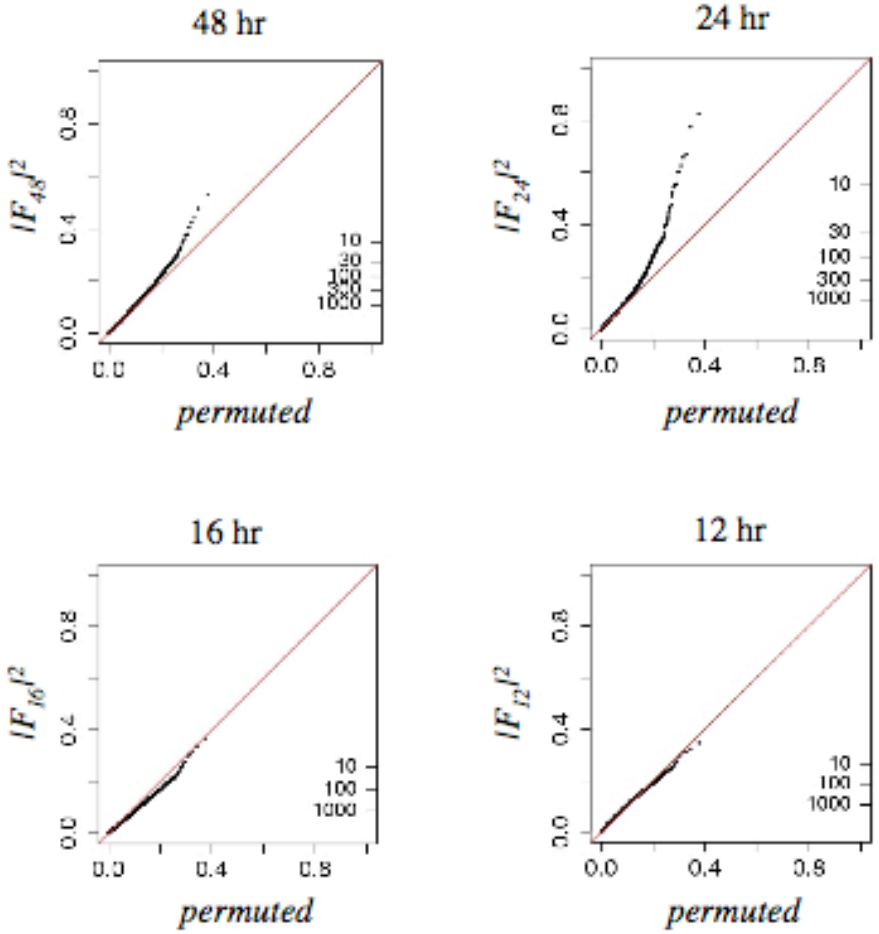
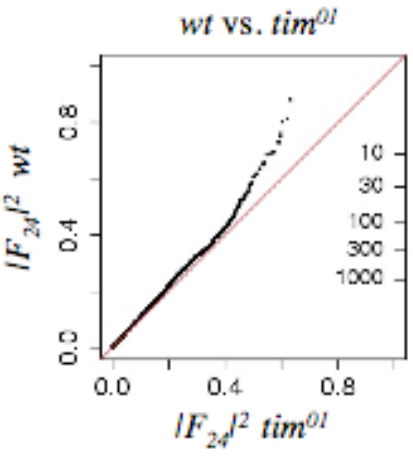

48 hr
24 hr
16 hr
12 hr
$IF_{48}I^2$
$IF_{24}I^2$
$IF_{16}I^2$
$IF_{12}I^2$
permuted
0.0
0.4
0.8
10
30
100
300
1000

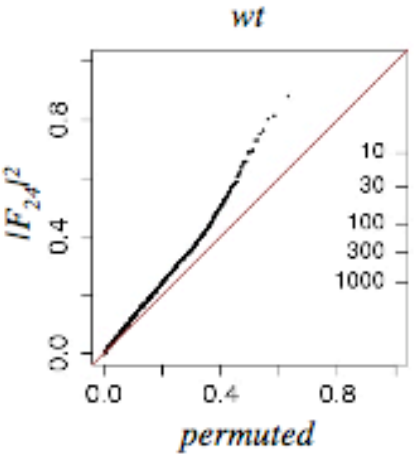
Figure 3.6. Wild-type transcript profiles are enriched for 24-hr periodic oscillations as compared to *tim*^{01} data.

(a). QQ plots (in the same format as Figure 3.2) comparing expression profile data from wild-type flies on the y-axis to data from *tim*^{01} flies on the x-axis demonstrate enrichment for circadian transcript profiles in the wild-type data. Each data set consists of two LD/DD time course collections. Enrichment is indicated by an upward deviation from the diagonal (brown). QQ plots comparing the wild-type data (b) and *tim*^{01} data (c) to a permutation null model further illustrate that 24-hr periodic oscillations are enriched in wild-type, but not *tim*^{01} flies.

a.


wt vs. tim01
|F24|2 wt
|F24|2 tim01
0.0
0.4
0.8
10
30
100
300
1000

b.


wt
|F24|2
permuted
0.0
0.4
0.8
10
30
100
300
1000

c.

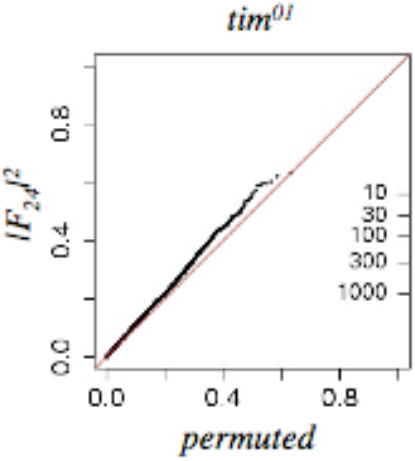

tim01
|F24|2
permuted
0.0
0.4
0.8
10
30
100
300
1000

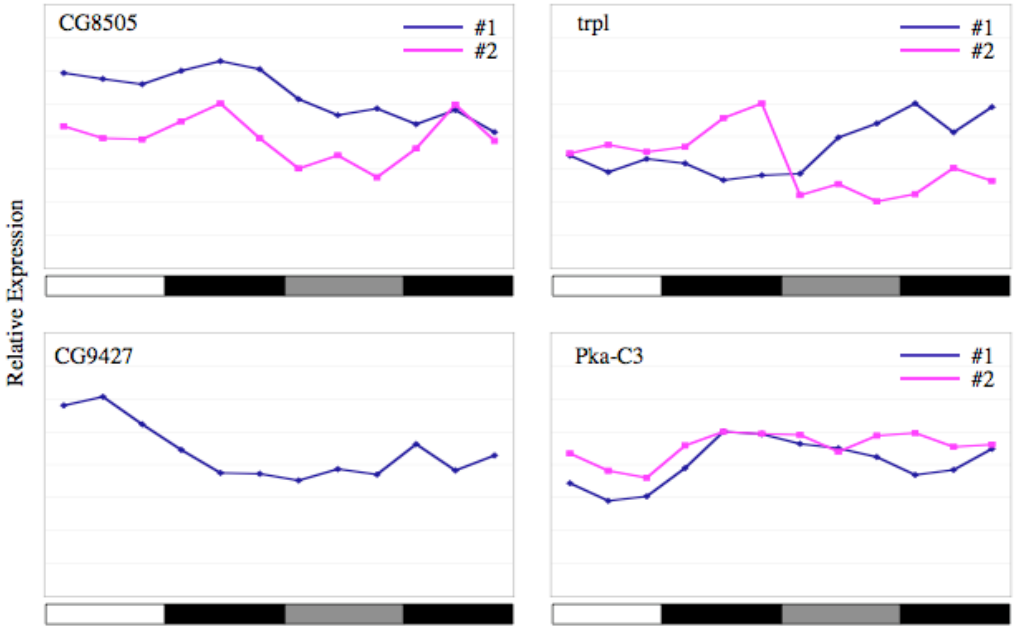
Table 3.2. *tim*^{01} transcripts with the highest probability of showing 24-hr periodic oscillations.

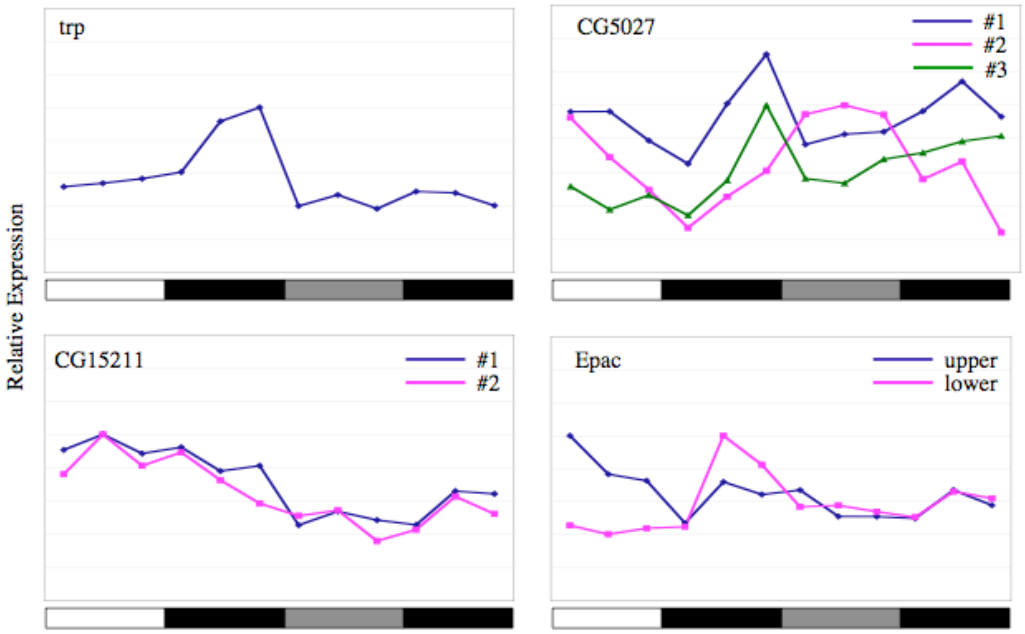
Three selections (see text) were carried out to identify those transcripts with the greatest probability of showing a circadian oscillation in *tim*^{01} fly heads. The 14 top-ranking transcripts chosen for Northern blot analyses are listed in Column 1, with the selection they came from indicated by an "X" in Columns 2-4. The 24-hr Fourier scores and probabilities (pF24) are shown in Columns 5 and 6. None of the transcripts, with the exception of *Slob* and the lower transcript of *Epac*, show a significant oscillation in *tim*^{01} flies. Closer examination of those two transcripts reveals that they are light-driven, not circadian (see Figure 3.7).

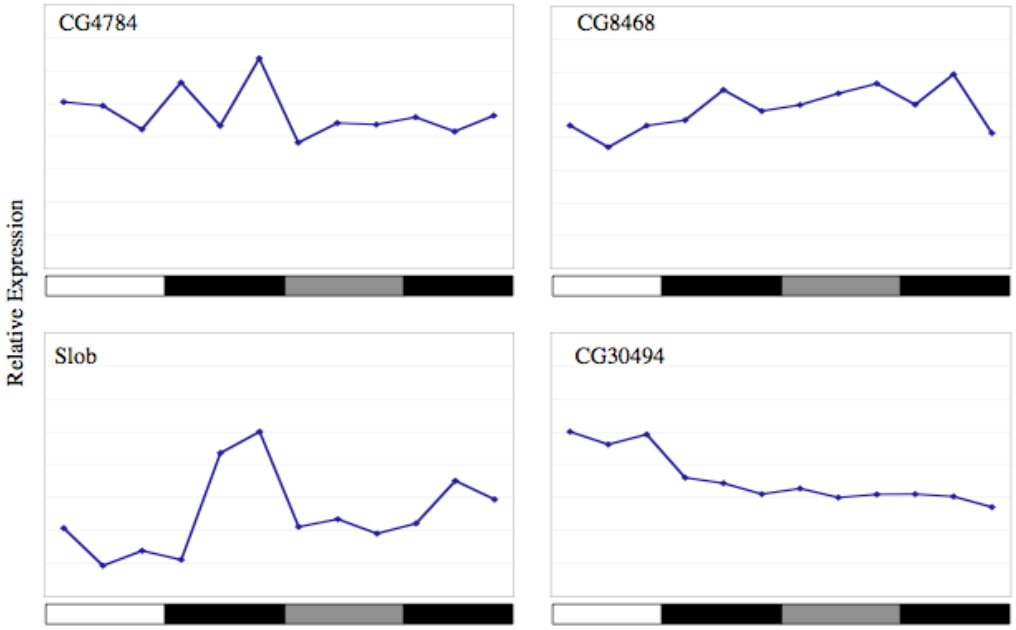
Transcript (experiment #)	**Selections**			**Northern data**	
	1	**2**	**3**	**F_{24}**	**pF_{24}**
CG8585 (#1)	X	X	X	0.38	0.12
CG8505 (#2)				<0.01	0.99
CG8505 (average)				0.18	0.39
trpl (#1)	X	X	X	0.08	0.68
trpl (#2)				0.13	0.51
trpl (average)				0.31	0.15
CG9427	X	X	X	0.16	0.43
Pka-C3 (#1)	X		X	0.32	0.18
Pka-C3 (#2)				0.33	0.17
Pka-C3 (average)				0.32	0.18
trp	X			0.19	0.41
CG5027 (#1)	X			0.35	0.15
CG5027 (#2)				0.37	0.13
CG5027 (#3)				0.34	0.15
CG5027 (average)				0.22	0.32
CG15211 (#1)		X	X	<0.01	0.98
CG15211 (#2)				<0.01	0.98
CG15211 (average)				<0.01	0.99
Epac (upper)		X	X	0.29	0.21
Epac (lower)				0.52	0.03
CG4784		X	X	0.05	0.81
CG8468		X		0.16	0.44
Slob		X		0.59	0.02
CG30494			X	0.29	0.22
CG2121			X	0.14	0.49
Inos (#1)			X	0.07	0.71
Inos (#2)				0.18	0.41
Inos (average)				0.10	0.62

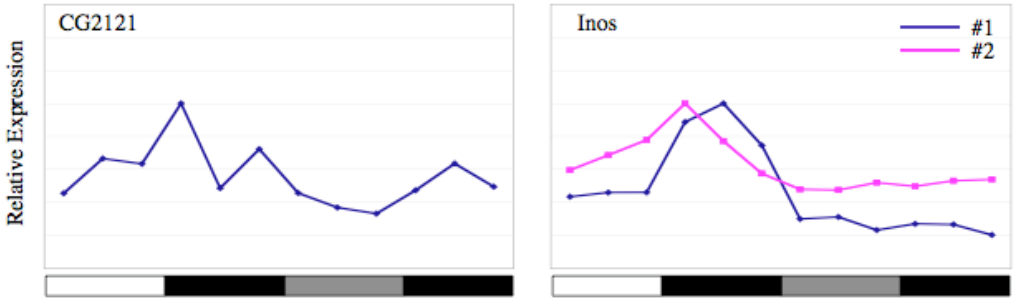
Figure 3.7. Northern blot analyses of *tim*^{01} transcripts with the highest probability of showing 24-hr periodic oscillations.

tim^{01} flies were entrained to LD 12:12 for five days and subsequently released into DD. Time points were taken every four hours during the last day of entrainment and the first day of DD starting at ZT2. RNA from whole heads was run on Northern blots and probed with cDNA encoding the indicated transcript (upper left-hand corners). An *rp49*-specific probe was used as a loading control in all cases. The data were Fourier fit (see Methods and Materials) in order to quantitatively measure any oscillation (see Table 3.2 for corresponding Fourier scores and p-values). The bars below the plots denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points and gray bars indicating free-run time points taken during subjective light.


Relative Expression
CG8505
#1
#2
trpl
#1
#2
CG9427
Pka-C3
#1
#2


Relative Expression
trp
CG5027
#1
#2
#3
CG15211
#1
#2
Epac
upper
lower


Relative Expression
CG4784
CG8468
Slob
CG30494


Relative Expression
CG2121
Inos
#1
#2

Chapter 4: The deubiquitinating enzyme *DUB*^{5798}

Introduction

One of the transcripts whose expression was recently identified as circadian by microarray analysis (see Chapter 3) is the deubiquitinating enzyme *CG5798 (DUB*^{5798}*)*. Ubiquitination and subsequent degradation by the proteasome is a widely employed regulatory mechanism in the cell. Ubiquitin, a 76-aa residue, is covalently attached to the ε-group of a lysine residue via a cascade of enzymatic reactions involving an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) (Wilkinson and Hochstrasser, 1998). Targeted proteins are then recognized and degraded by the 26S proteasome, and ubiquitin is recycled through the action of DUBs. There are two classes of DUBs: ubiquitin C-terminal hydrolases (Uch) and ubiquitin processing proteases (Ubp). Twenty-three DUBs have been identified in the *Drosophila* genome, four of which are Uchs and 19 of which are Ubps (Rubin et al., 2000). Catalysis by Ubps is dependent on two conserved catalytic domains, the His and Cys boxes. DUBs have been shown to be target-specific, recognizing either the substrate itself or the conjugated protein, which results in a tight regulatory process of ubiquitination and proteolysis. An example of a well-characterized

Drosophila Ubp is the *fat facets (faf)* gene, which is essential for proper patterning during eye development (Chen et al., 2002). *faf* has been shown to prevent the degradation of its substrate, *liquid facets (laf)*, by cleaving the ubiquitin tagging it for degradation.

The ubiquitin-proteasome system has emerged as an important and conserved post-translational regulatory mechanism in the circadian clock. SLMB, an F-box/WD40 protein in the SCF ubiquitin ligase complex, has been shown to regulate the degradation of phosphorylated PER in *Drosophila* (Grima et al., 2002; Ko et al., 2002). β-TRCP and FWD1, the mammalian and *Neurospora* orthologs of SLMB, respectively, show similar regulation of mPER and FRQ (Eide et al., 2005; He et al., 2003; Shirogane et al., 2005). *Drosophila* TIM is phosphorylated and targeted to the proteasome by a yet unknown kinase in response to its light-dependent interaction with CRY, which is itself degraded by the ubiquitin-proteasome pathway (Lin et al., 2001; Naidoo et al., 1999).

Results

Hierarchical clustering of gene expression profile data revealed that DUB^{5798} is grouped with the clock genes *per*, *tim* and *vri* based on phase of peak expression in wild-type flies and overall expression levels in clock

mutants (Claridge-Chang et al. and data not shown). *DUB*^{5798} encodes a 3206 nt non-oscillatory transcript and a smaller transcript (~1400 nt) (*s-DUB*^{5798}) that appears to be under circadian control by Northern blot (Figures 4.1 and 4.4).

s-DUB^{5798} is primarily expressed in adult heads, mainly in the eye (Figure 4.2 and data not shown). The expression of *s-DUB*^{5798} appears to be dependent on the clock as it is almost completely undetectable in *Clk*^{Jrk} flies (Figure 4.3.a). Although *s-DUB*^{5798} oscillates in both LD and DD in wild-type flies, it is up-regulated in *per*^{0} and *tim*^{01} null mutants during the light phase of entrainment. This suggests dual regulation by the central clock and light (Figure 4.3.b and data not shown). A set of transcripts primarily expressed in the *Drosophila* eye that are dually controlled by the clock and light, including *DUB*^{5798}, was recently identified by microarray analysis (Wijnen et al., 2006). These transcripts may be important for processes requiring both anticipation of, and a response to, changes in the daily LD cycle.

Using Northern blotting, RNAse protection assays (RPAs) and rapid amplification of cDNA ends (RACE), it was determined that *s-DUB*^{5798} is composed of the 3'-terminal three exons of the full-length *DUB*^{5798} transcript, as well as a unique piece from the fifth intron of about 184-187 bp

in length (see Materials and Methods; data not shown). If this unique piece is used to probe a Northern blot, only the oscillating transcript is seen (Figure 4.4). Both the residues critical for the activity of a DUB, the His and Cys catalytic domains, are encoded by *s-DUB*^{5798}, suggesting the oscillating transcript is functionally significant.

Discussion

The ubiquitin-proteasome pathway has been shown to be critical to circadian regulation of the clock, a feature that is highly conserved among organisms. This chapter describes the initial characterization of the oscillatory transcript of the deubiquitinating enzyme *DUB*^{5798}. It is likely *s-DUB*^{5798} plays an important role in the circadian clock for several reasons. First, *s-DUB*^{5798} cycles in phase with the dCLK/CYC-regulated core clock genes. Secondly, the expression of *s-DUB*^{5798} is dependent on the activity of dCLK, which is a central clock protein. Thirdly, *s-DUB*^{5798} encodes the DUB His and Cys functional domains. Fourthly, *s-DUB*^{5798} was identified with a group of transcripts showing dual regulation by the clock and light, possibly implicating it in the light input pathway to the clock. It is possible that the alternative splicing of *DUB*^{5798} is itself regulated by a complex response to signals from the clock and light. Future work will build on these

data and hopefully elucidate how *DUB*^{5798} is involved in the *Drosophila* clock.

Figure 4.1. Expression of *DUB*^{5798} in adult fly heads in an LD cycle.

(a). *DUB*^{5798} encodes two transcripts, only one of which is under circadian control. Wild-type flies were entrained to LD12:12 for three days and subsequently released into DD. Time points were taken every four hours during the first two days in DD starting at CT2. RNA from whole heads was run on a Northern blot and probed with cDNA encoding the entire *DUB*^{5798} transcript. An *rp49*-specific probe was used as a loading control. The asterisk indicates the oscillatory *s-DUB*^{5798} transcript. The bars denote the entrainment scheme, with gray bars indicating time points taken during subjective light and black bars indicating dark time points. (b). Quantitation of the Northern data from (a). Green line = non-oscillatory transcript, pink line = oscillatory transcript.

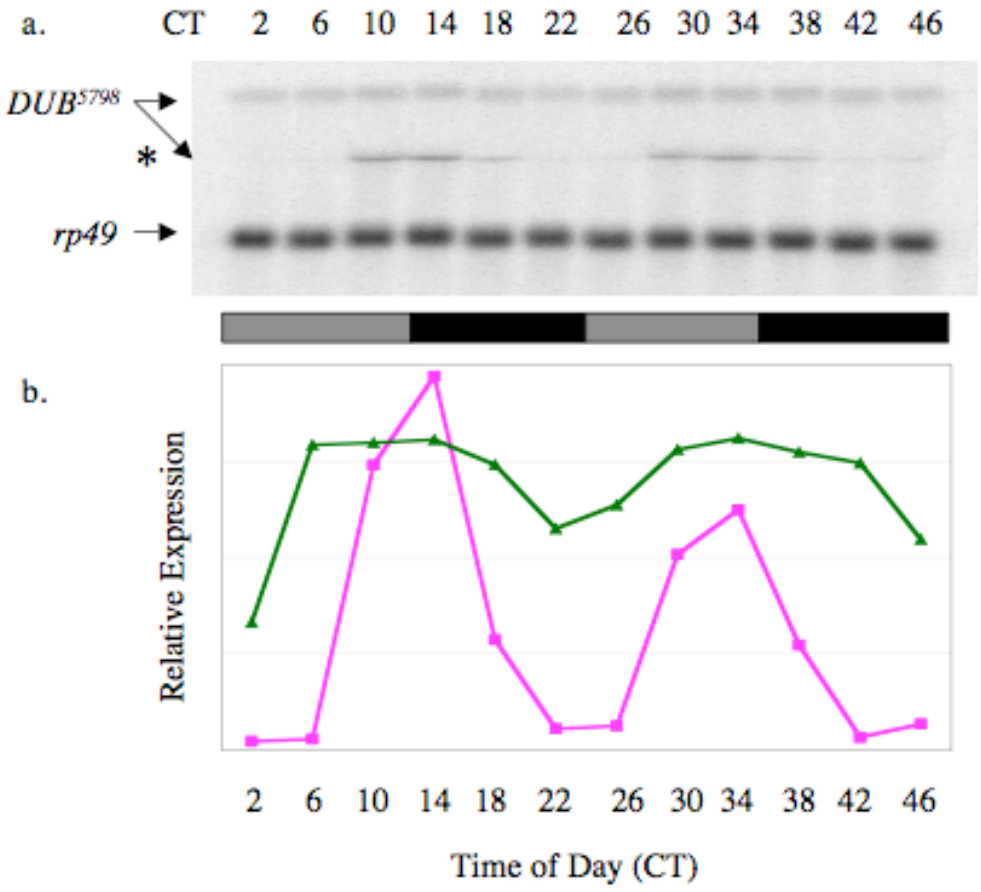

a.
CT 2 6 10 14 18 22 26 30 34 38 42 46
DUB5798
*
rp49
b.
Relative Expression
2 6 10 14 18 22 26 30 34 38 42 46
Time of Day (CT)

Figure 4.2. Pattern of expression of *s-DUB*^{5798}.

(a). *s-DUB*^{5798} is primarily expressed in adult fly heads, while the non-oscillating transcript is expressed ubiquitously. RNA was extracted from wild-type flies collected at the indicated developmental stages, run on a Northern blot and probed with cDNA encoding the entire *DUB*^{5798} transcript. An *rp49*-specific probe was used as a loading control. The asterisk indicates the *s-DUB*^{5798} transcript. (b). Quantitation of the Northern data from (a). Blue bars = non-oscillatory transcript, purple bars = oscillatory transcript.

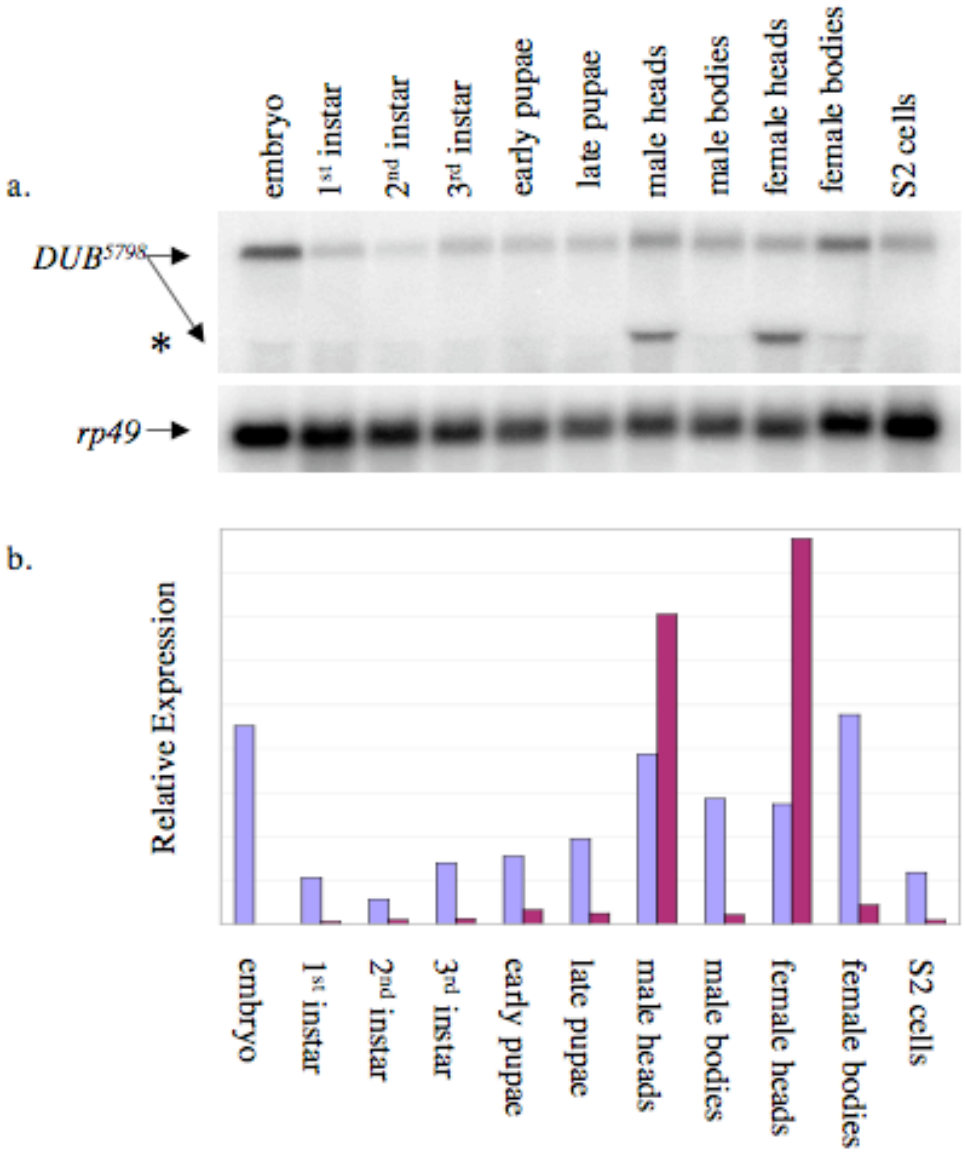

a.
embryo
1st instar
2nd instar
3rd instar
early pupae
late pupae
male heads
male bodies
female heads
female bodies
S2 cells
DUB5798
*
rp49
b.
Relative Expression
embryo
1st instar
2nd instar
3rd instar
early pupae
late pupae
male heads
male bodies
female heads
female bodies
S2 cells

Figure 4.3. *s-DUB*^{5798} is dually regulated by the clock and light.

Wild-type flies were entrained to LD 12:12 for five days and time points were taken for one day every four hours starting at ZT2. *Clk*^{Jrk} (a) and *per*^{0} (b) flies were entrained to LD 12:12 for five days and subsequently released into DD. Time points were taken every four hours during the last day of entrainment and the first day of DD starting at ZT2. RNA from whole heads was run on a Northern blot and probed with cDNA encoding the entire *DUB*^{5798} transcript. An *rp49*-specific probe was used as a loading control. The asterisk indicates the *s-DUB*^{5798} transcript. The bars denote the entrainment scheme, with the white bars indicating light time points, the black bars indicating dark time points and the gray bars indicating free-run time points taken during subjective light. (a). Severe down-regulation of *s-DUB*^{5798} can be seen in *Clk*^{Jrk} as compared to *y w*. (b). Up-regulation of *s-DUB*^{5798} can be seen during lights-on in *per*^{0} flies.

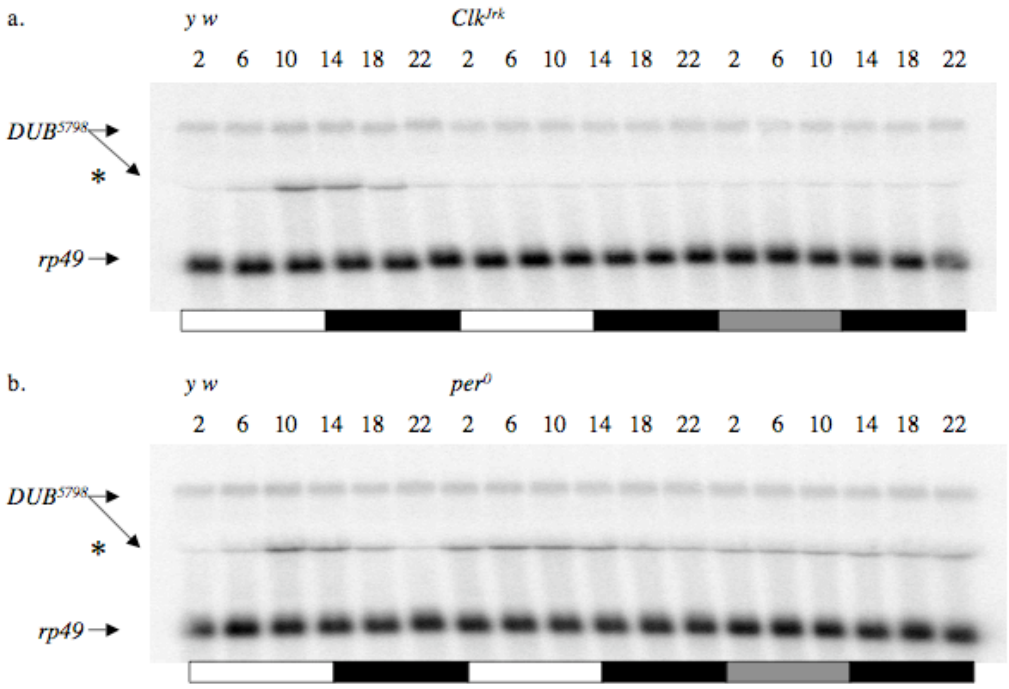
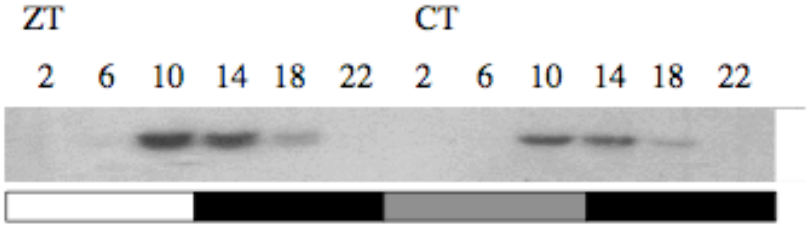

a.
y w
Clk^Jrk
2 6 10 14 18 22 2 6 10 14 18 22 2 6 10 14 18 22
DUB^5798
*
rp49
b.
y w
per^0
2 6 10 14 18 22 2 6 10 14 18 22 2 6 10 14 18 22
DUB^5798
*
rp49

Figure 4.4. Alternative splicing of *DUB*^{5798} .

s-DUB^{5798} is composed of a portion of the fifth intron and the last three exons of *DUB*^{5798} (a). Genomic organization of *DUB*^{5798}. Boxes denote exons, with translated regions being filled. The catalytic Cys and His are represented by a red line in exon 6 and a blue line in exon 8, respectively. The arrow indicates the putative start site of the oscillating transcript. (b). Flies were entrained to LD 12:12 and for five days subsequently released into DD. Time points were taken every four hours during the last day of entrainment and the first day of free-run starting at ZT2. RNA from whole heads was run on a Northern blot and probed with cDNA encoding a portion of the fifth intron of *DUB*^{5798} (green bar in [a]). An *rp49*-specific probe was used as a loading control. Only one transcript which corresponds to *s-DUB*^{5798} can be seen. The bars denote the entrainment scheme, with the white bar indicating light time points, the black bars indicating dark time points and the gray bar indicating free-run time points taken during subjective light.

a.

b.



Chapter 5: Temperature as a *Zeitgeber* for the *Drosophila* circadian clock

Introduction

Clocks are aligned to the environment via inputs from both daily light and temperature cycles. Previous molecular and behavioral studies in *Drosophila* have largely focused on light-dependent regulation of circadian clocks and their outputs. This chapter aims to better understand the role of temperature on gene expression and behavior in the fly.

Although light is the strongest and best understood *Zeitgeber* for the circadian clock, temperature also plays an important role in entrainment. Both LD cycles and temperature cycles can entrain locomotor activity independently (Figure 5.1). When light and temperature are superimposed (warm during light and cold during dark), locomotor activity always occurs during the warm/light period. If, however, light and temperature are counter-imposed (cold during light and warm during dark), the animals will be active during the warm/dark period. This is a direct effect on behavior independent from the internal clock (masking effect) because, when the flies are released into constant conditions (warm/dark), they "remember" when it used to be light and move their activity to the subjective light phase (Figure

5.1). Further, expression of the core clock proteins follows the light phase, not temperature (Figure 5.2).

Here the direct effects of temperature on gene expression and behavior are differentiated from clock-dependent effects. This is accomplished by generating genome-wide expression profiles of transcripts during temperature entrainment and subsequent constant conditions in both wild-type and arrhythmic tim^{01} flies. Analyses of individual genes at the transcript and protein level are used to confirm and extend these data. In examining the response to temperature, the ability of the clock to integrate multiple environmental inputs is better understood.

Results

Genome-wide expression profiles of transcripts during temperature entrainment. Two independent sets of temperature-entrained, *y w* flies were collected every four hours for one day in 12 hours Ambient:12 hours Cool (AC; Ambient = 25°C, Cool = 18°C) followed by one day in Ambient:Ambient (AA) conditions, all during constant darkness. A single set of tim^{01} flies was collected under the same conditions. An additional set of *cn bw* flies was collected in AA every four hours for one day starting 12 hours after the last period of 18°C. RNA was isolated from the heads,

labeled and hybridized to Affymetrix Genechips (see Materials and Methods).

In order to test for the presence of both ultradian and circadian periods in response to temperature entrainment, the gene expression profile data from wild-type flies were fit to sine waves of periods of 8, 12 and 24 hours. Figure 5.3 shows the distribution of squared Fourier scores of the indicated phases plotted against a null permutation model. As under light-entrained conditions (see Chapter 3), there is selective enrichment for the 24-hr period, with the real data for the 8- and 12-hr periods being indistinguishable from the range of permuted values.

In wild-type light-entrained animals, the same limited set of transcripts that oscillate during entrainment continue to cycle during free-run. In wild-type temperature-entrained animals, however, the entire transcriptional program appears to be modified by a thermocycle, with only a small subset of transcripts continuing to oscillate in constant conditions following temperature entrainment (Figure 5.4). This suggests two distinctive responses to temperature. The first, a clock-independent temperature-driven response, involves transcripts that are simply driven by temperature cycles and whose fluctuations are not mediated by the clock. The second, a clock-dependent circadian response, involves those transcripts

that continue to oscillate in constant conditions following temperature entrainment. These transcripts are dependent on the clock and their presence indicates that the clock can work against simple up- or down-regulation to generate meaningful phases of transcription.

Defining a set of temperature-driven transcripts. The behavior of clock-independent transcripts that merely respond to temperature cycles should be the same in the presence or absence of a functional clock. In order to define a set of temperature-driven transcripts, wild-type AC expression profile data were analyzed in combination with the tim^{01} AC data. The expression profile data were fit to a 24-hr Fourier wave and subjected to several filters (see Materials and Methods). The phasegrams for the resulting set of 164 temperature-driven transcripts in both wild-type and tim^{01} flies are shown in Figure 5.5. These transcripts respond with a simple pattern of either activation during the warm phase and repression during the cold, or vice versa. The phasegrams for the same 164 transcripts in light-entrained wild-type and tim^{01} flies are also shown in Figure 5.5. As can be seen from the complete disorganization of these LD/DD phasegrams, there is no apparent overlap between the set of temperature-driven transcripts and genes oscillating in, or driven by, light.

Figure 5.6 shows the phases of the temperature-driven transcripts in wild-type and *tim*^{01} flies during temperature entrainment. If the term AC0 is assigned to the onset of the warm phase and AC12 to the onset of the cold phase, the majority of the transcripts have a phase of either AC6 (in the middle of the warm period) or AC18 (in the middle of the cold period). A closer examination of the behavior of these transcripts reveals that the phases of peak expression are correlated with the rates of response to temperature changes (Figure 5.7). That is, the few transcripts that peak at the cold-to-warm or warm-to-cold transitions respond gradually to temperature changes. The majority of the transcripts, however, are rapidly up- or down-regulated in response to temperature changes, reaching their maximal or minimal levels about six hours later and then returning to baseline.

Defining a set of clock-dependent transcripts. The expression profile data from wild-type flies in AC/AA were analyzed in order to define a set of transcripts that oscillate during temperature entrainment and subsequent constant conditions in a clock-dependent manner. In order to avoid transcripts that are merely temperature-driven, it was required that they also show a significant oscillation in AA alone as well as AC/AA (see Materials

and Methods). The phasegrams for the resulting set of 143 clock-dependent transcripts are shown by phase in Figure 5.8, along with the phases of the same transcripts in LD/DD. Although the transcripts are aligned according their temperature phase, the tight banding pattern in the LD/DD phasegram indicates there is at least some overlap between these two sets of transcripts. By comparing this set of temperature-entrained circadian transcripts to the set of light-entrained circadian transcripts (from Chapter 3), it was revealed that 49 of the 143 AC/AA transcripts (34.3%) are also found in the set of 172 LD/DD transcripts (Figure 5.9). This significant overlap suggests an overall shared transcriptional response to light and temperature cycles. The fact that the overlap is incomplete, however, could indicate that there are both light- and temperature-specific transcriptional responses. To test this, four transcripts that were predicted to oscillate only in temperature cycles and not light were tested on Northern blots with samples from an LD/DD time course. Three of the transcripts show significant oscillations in LD/DD (Figure 5.10). The fourth transcript appears to be a false positive as it neither oscillates on an LD/DD or AC/AA Northern (data not shown). Thus the Northerns indicate there is no evident difference between the set of transcripts that oscillate in response to light vs. temperature entrainment. The statistical discrepancy is most likely due to limitations in methodology.

The relationship between the phases of transcripts oscillating in light and temperature cycles indicates an integrated response to these Zeitgebers. In contrast to the AC6/AC18 maxima found for most transcripts (Figure 5.6), the rhythm of each clock-regulated gene during and after entrainment to temperature cycles assumes a phase that is related to that produced during LD. Given AC0 represents the onset of the warm phase in AC and ZT0 represents the onset of the light phase in LD, each clock-controlled gene assumes an AC phase that is about six hours advanced relative to that measured in LD (Figure 5.11). This "advance" is an effect of nomenclature, since light and temperature cycles are not aligned under natural circumstances. Thus the "advance" in AC molecular rhythms is an indication that the animal is using the circadian clock to interpret temperature cycles in order to predict when it would have seen light or dark, had photic input also been provided. This is further emphasized by locomotor activity data (Figure 5.12). During LD cycles, flies are preferentially active around the dark-to-light and light-to-dark transitions, with a siesta in the middle of the day. In other words, behavior is triggered around lights-on and lights-off. During temperature entrainment, activity onset occurs during the cold period, with a drop-off in activity right around

the cold-to-warm transition and then a second burst of activity in the early to mid part of the warm period. This corresponds to when the animal would have seen lights on and off as temperature maxima and minima are late in the day and night, respectively. Thus the clock enables the animal to generate behavior that isn't simply informed by high or low temperature but instead reflects a phase appropriate to the presence of light, and vice versa.

Core clock components oscillate in temperature entrainment. The well-characterized core clock transcripts that are known to oscillate under light entrainment (*per, tim, Clk, cry, vri, Pdp1ε*) continue to cycle under temperature entrainment (Figures 5.13 and 5.14). Despite the fact that temperature cycles modify the overall transcriptional program (Figure 5.4), the non-cycling core clock transcripts (*cyc, dbt*) do not oscillate during temperature entrainment (Figure 5.14 and data not shown). This further illustrates that the clock can work against simple up- or down-regulation. As in light cycles, PER and TIM proteins also oscillate in both temperature entrainment and constant conditions following temperature entrainment (Figures 5.15 and 5.16). The RNA and protein phases are, as mentioned, "advanced" by about six hours (relative to the onset of the respective *Zeitgeber*) as compared to light entrainment.

The phase relationships between transcripts (i.e. *per*, *tim, vri* and *Pdp1ε* oscillate antiphase to *Clk* and *cry*) are largely maintained. Temperature cycles therefore appear to promote the same overall clock organization and function as light entrainment, although with an important distinction that involves *per* and *tim* RNA expression. In light entrainment and subsequent free-run, *per* and *tim* transcription is tightly coupled at all times (Figure 5.17.a). In temperature entrainment, peak *per* RNA expression occurs several hours earlier than peak *tim* RNA levels (Figure 5.17.b). This divergence is absent in constant conditions following temperature entrainment. The differential expression is also absent in protein levels, indicating the phenomena is transcriptional and not translational. The differential expression could be due to the effects of a thermosensitive splicing event in the 3'UTR of *per*, which is thought to enable flies to seasonally adapt to cold, short days. At low temperatures in an LD cycle, an 89-bp intron is preferentially spliced out, resulting in earlier accumulation of *per* transcript and a concomitant advance of the evening locomotor activity peak (Majercak et al., 1999).

tim is alternatively spliced at cold temperatures. Alternative splicing of *tim* at cold temperatures also could play a role in the differential

expression of *per* and *tim* mentioned above. While *per* peaks earlier in temperature entrainment than it does when released into constant conditions, *tim* peaks about two hours later in temperature entrainment than in free-run (Figure 5.14). Further, a second *tim* transcript (referred to as *tim*-upper) is clearly present during temperature entrainment, especially during the cold phase (Figure 5.18.a). This novel transcript is expressed at low levels in light-entrainment at 25°C (Figure 5.18.b), but is quite distinct in light entrainment performed at 18°C (Figure 5.18.c). While the predominant transcript of *tim* (referred to as *tim*-lower) is differentially regulated from *per* during temperature entrainment, *tim*-upper peaks in phase with *per* (Figure 5.17.c). RT-PCR and Northern analyses reveal that, in the novel transcript, the last *tim* intron (~850 bp) is retained (Figure 5.19). This unspliced form of *tim* has a premature stop codon that would result in a protein about 30 kDa smaller than in wild-type. The missing fragment corresponds to a small piece of the cytoplasmic localization domain (CLD) (Saez and Young, 1996). Western blots reveal the presence of two TIM isoforms at 18°C in light entrainment, the lower of which is absent at 25°C (Figure 5.19).

Three transgene constructs under the control of the *tim* promoter were created and injected into flies in order to test whether the alternatively spliced form of *tim* has any affect on the period or phase of locomotor

activity behavior (see Materials and Methods). The first construct, $Ctim3^{FL}$, expresses only the full-length TIM protein. The second construct, $Ctim3^{FL+S}$, can express both the full-length and truncated proteins due to the inclusion of the last intron. The third construct, $Ctim3^{mS}$, can only express the truncated TIM protein; although the last intron is present, both splice sites are mutated. Two lines were obtained for each construct. Protein expression was confirmed by Western blotting in both S2 cells and *in vivo* (data not shown).

The locomotor activity behavior of the six transgenic lines was tested at 25°C in LD and DD (Table 5.1). The average period length (tau) during DD was calculated for each line. There are no significant differences among the free-running period lengths of the $Ctim3^{FL}$ and $Ctim3^{FL+S}$ lines (avg = 23.1 ± 0.05). That is, flies that can make either the full-length or a combination of full-length and truncated proteins have the same locomotor activity period length. However, the period lengths of the $Ctim3^{mS}$ lines (avg = 22.5 ± 0.3) are significantly shorter when compared to the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines (Student's t test, $p < 0.01$). It therefore appears that production of only the short TIM isoform shortens the free-running period length of locomotor activity behavior at 25°C.

The average time of activity onset in LD was calculated to determine if there are phase differences between the lines. As for period length, there are no significant differences among the times of activity onset in the $Ctim3^{FL}$ and $Ctim3^{FL+S}$ lines (avg = ZT 10.4 $\pm$ 0.14). However, the average onsets of activity are significantly delayed in the $Ctim3^{mS}$ lines (avg = ZT 11.3 $\pm$ 0.18) as compared to the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines ($p < 0.05$). Thus, in addition to shortening the free-running period length, production of only the short TIM isoform appears to also result in a delayed phase of locomotor activity in LD cycles at 25°C.

Circadian transcript levels change in the presence of a thermocycle in arrhythmic mutants. Null mutations in any of the clock genes result in molecular arrhythmicity in light entrainment and subsequent free-run. Although per^0 and tim^{01} flies can be forced to display rhythmic locomotor behavior in an LD cycle, this masking effect has been shown to be due to a startle response at the light-to-dark and dark-to-light transitions (Wheeler et al., 1993). Clk^{Jrk} flies do not show the dark-to-light startle response and as a consequence, appear largely arrhythmic even in an LD cycle (Allada et al., 1998). Core clock transcripts do not cycle in any of these mutants in LD or DD, although there is a set of light-driven transcripts that cycles in tim^{01} LD-

entrained flies (Wijnen et al., 2006). It has previously been reported that *per*^{0}, *tim*^{01}, *Clk*^{Jrk} and *cyc*^{0} flies display rhythmic locomotor activity in temperature cycles of 25ºC/30ºC (Yoshii et al., 2002). None of the mutants, however, anticipate the temperature transitions or continue to display rhythmic behavior when the temperature cycle is removed, indicating that this behavior represents a masking effect. These mutants also display rhythmic locomotor activity behavior under 25ºC/18ºC temperature cycles (Figure 5.20).

In addition to forcing rhythmic behavior, temperature cycles also appear to promote cycling levels of clock gene expression in the arrhythmic clock mutants. *Clk*, *cry*, *per* and *tim* all cycle during temperature entrainment in *tim*^{01} and *per*^{0} flies, both on Northerns and, in the case of *tim*^{01}, by microarrays (Figures 5.21, 5.22 and data not shown). As with locomotor activity behavior, however, this cycling gene expression is directly driven by temperature as oscillations cease when the flies are released into constant conditions. Further, the phases of some of the core clock genes in *tim*^{01} and *per*^{0} flies are different than in wild-type. For example, in wild-type flies in AC, *per* and *tim*-upper peak at the beginning of the warm period, while in *tim*^{01} and *per*^{0} flies both are up-regulated during the cold period. This result likely reflects the two different responses to

temperature (i.e. thermal regulation without mediation by the clock and clock dependent control). Genes that are simply thermally regulated would have the same phase in a wild-type or mutant background. Core clock and clock-controlled genes do not simply follow the temperature cycle in wild-type flies but assume clock-programmed phases. However, since this regulation does not exist in an arrhythmic clock mutant, these transcripts become temperature dependent and their phases are realigned to simply follow temperature maxima and minima.

Despite the cycling of core clock transcripts in tim^{01} and per^{0} flies, only *tim*-upper and not *tim*-lower, *per, cry* or *Clk* cycle in cyc^{0} flies (Figure 5.23). This could be an indication that *tim* transcription, or at least that of the novel transcript of *tim*, can occur independently of *cyc* while expression of other transcripts cannot.

While some core clock genes, like *per* and *tim*-upper, cycle with a different phase in tim^{01} and per^{0} flies than in wild-type, others, like *tim*-lower, do not move their phases. As a result, *per* and at least one of the *tim* transcripts are out of phase with each other, further supporting the notion of differential regulation of these clock genes. A hint as to how this differential regulation is possible may come from the phase of *Clk* in these two mutants. In the case of tim^{01} flies, *Clk* cycles in phase with *tim*-lower, while in per^{0}

flies *Clk* cycles in phase with *per*. Thus *Clk* RNA (and presumably CLK protein) appears to be maintaining the appropriate anti-phase relationship with the remaining wild-type transcript. This may be a reflection of the close regulatory interactions between *per* and *tim*.

Summary. Although light is the strongest and best characterized *Zeitgeber* for the circadian clock, temperature is also an important factor. Temperature cycles promote rhythmic locomotor activity behavior and cycling gene expression. There is a temperature-driven, clock-independent transcriptional response to thermocycles, as evidenced by the large number of genes that directly respond to temperature cycles and the cycling of clock transcripts in arrhythmic mutants in AC. There is also a clock-dependent response to temperature, indicated by the limited number of transcripts that continue to oscillate in constant conditions following temperature entrainment. These transcripts show a significant overlap with those genes oscillating in light entrainment. The transcripts oscillating in both photo- and thermocylces exhibit a phase relationship reflective of the connection between light and temperature under natural conditions, indicating that information from these *Zeitgebers* would be integrated by the fly.

Figure 5.1. Light and temperature can independently entrain locomotor activity behavior.

The locomotor activity of an individual fly over a number of days is represented in each panel. The data are double plotted for visual continuity. (a). Flies were recorded during entrainment to LD 12:12 as indicated by the open and closed bars above the panel, respectively. They were then released into DD (arrow) and allowed to free-run for the remainder of the assay. (b). Flies were recorded during entrainment to AC 12:12 as indicated by the red and blue bars above the panel, respectively. They were then released into AA (arrow) and allowed to free-run for the remainder of the assay. (c) and (d). Flies were recorded during entrainment to LD 12:12, as indicated by the open and closed bars above the panel, respectively. A temperature cycle was then superimposed (c; warm during light, cold during dark) or counter-imposed (d; cold during light, warm during dark), as indicated by the red/blue shading. The flies were then released into AA (red shading) for the remainder of the assay.

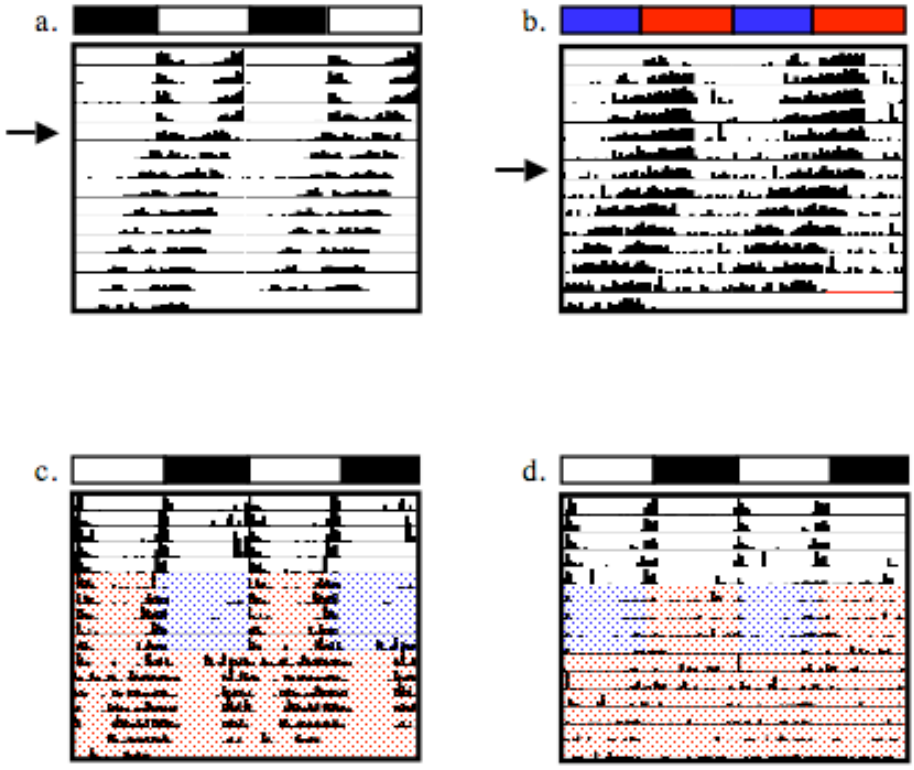
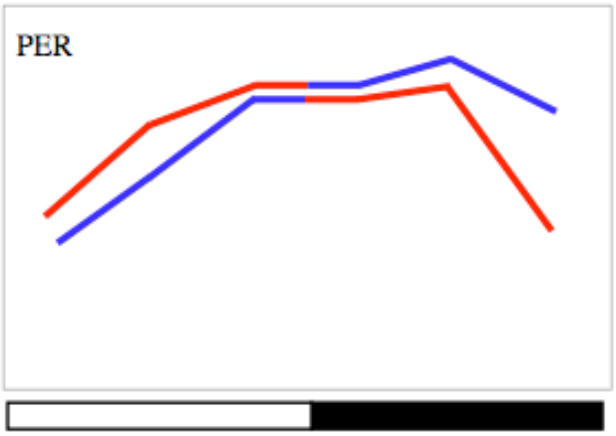

a.
b.
c.
d.

Figure 5.2. Light is the stronger *Zeitgeber.*

y w flies were entrained to LD 12:12 for five days. They were subsequently transferred to DD and a temperature cycle was either superimposed (warm during the prior light phase, cold during the prior dark phase) or counterimposed (cold during the prior light phase, warm during the prior dark phase). Flies were collected every four hours during the first day of DD/temperature cycles. Protein from whole heads was run on a Western blot and incubated with antibodies against PER (a) and TIM (b). Despite the fact that locomotor activity follows the temperature phase (see Figure 5.1), protein expression is aligned with the previous light cycle. The bars below the plots denote the light (white)/dark (black) entrainment scheme. The red and blue lines indicate time points collected at 25°C and 18°C, respectively.

a.



b.

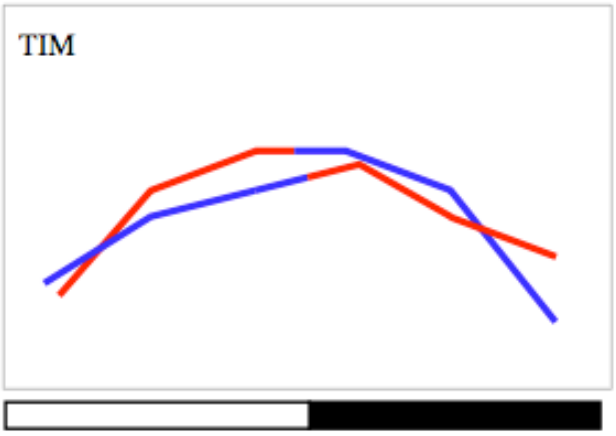
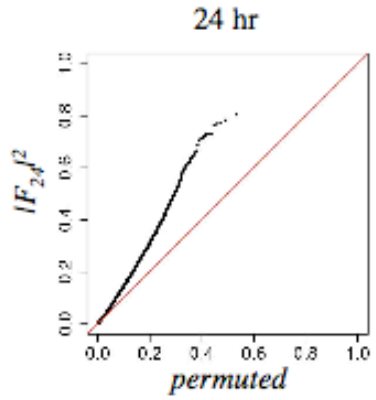
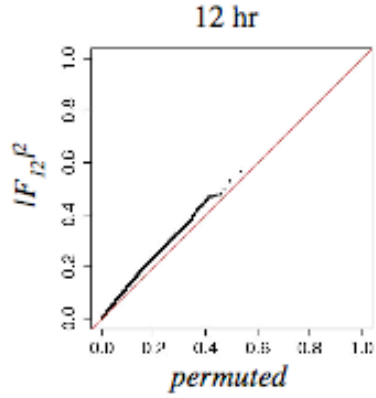


Figure 5.3. The dominant period of rhythmic transcription in temperature-entrained flies is 24 hours.

QQ plots of spectral power at periods of 8, 12 and 24 hours are shown. Points on the y-axis correspond to the real Fourier scores for individual transcripts, while the values on the x-axis are quantiles of 1000 permutations of the real data. Enrichment is indicated by an upward deviation from the diagonal (brown). Analyses were performed on data from two 2-day AC/AA collections and one 2-day AA collection. Substantial enrichment can be seen for the 24-hr period, while there is little difference between the real and permuted data at the 8- and 12-hr periods.


24 hr
$IF_{24}I^2$
permuted


12 hr
$IF_{12}I^2$
permuted

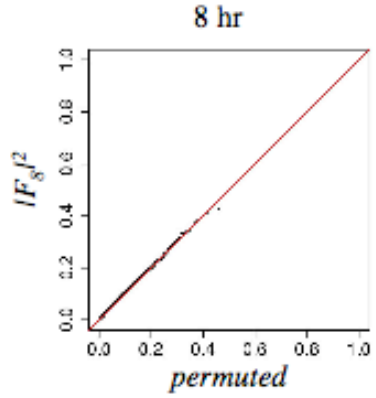

8 hr
IF_8I^2
permuted

Figure 5.4. Global responses to light vs. temperature entrainment.

QQ plots of 24-hrs, in the same format as Figure 5.3, are shown. Analyses were performed on data from (a) two 2-day LD/DD collections, (b) four days of DD, (c) two 2-day AC/AA collections and (d) four days of AA. Approximately the same number of transcripts oscillating in LD continue to do so in DD. However, almost all transcription is modified by thermocycles, with only a small subset of genes cycling in constant conditions following temperature entrainment.

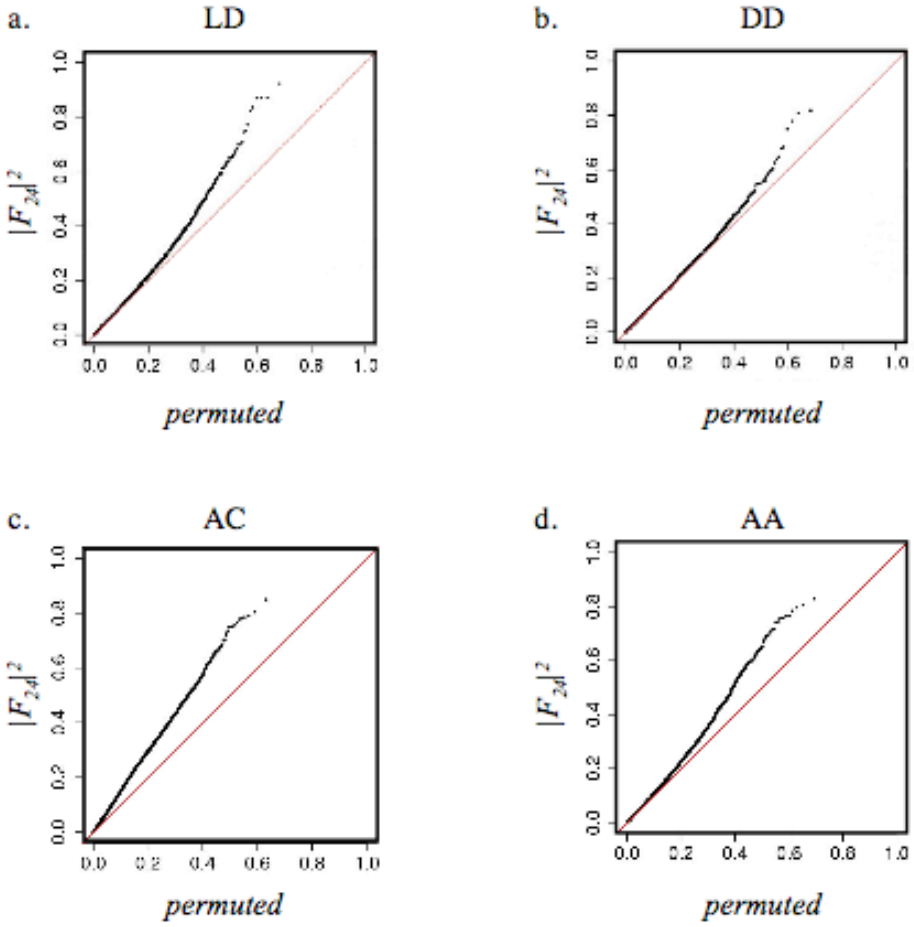

a.
LD
b.
DD
c.
AC
d.
AA
$|F_{24}|^2$
permuted
0.0
0.2
0.4
0.6
0.8
1.0

Figure 5.5. Organization of the expression patterns by phase of the 164 temperature-driven transcripts.

Columns correspond to time points and genes (listed to the right) are represented by row. Pink indicates up-regulation, turquoise indicates down-regulation. The phasegrams for transcripts from wild-type (wt) flies in AC/AA, tim^{01} flies in AC/AA, wt flies in LD/DD and tim^{01} flies in LD/DD are shown from left to right. The bars above and below the AC/AA phasegrams denote the temperature entrainment scheme, with the red bars indicating 25°C time points, the blue bars indicating 18°C time points and the violet bars indicating free-run time points taken during subjective 18°C. The bars above and below the LD/DD phasegrams denote the light entrainment scheme, with the white bars indicating light time points, the black bars indicating dark time points and the gray bars indicating free-run time points taken during subjective light.

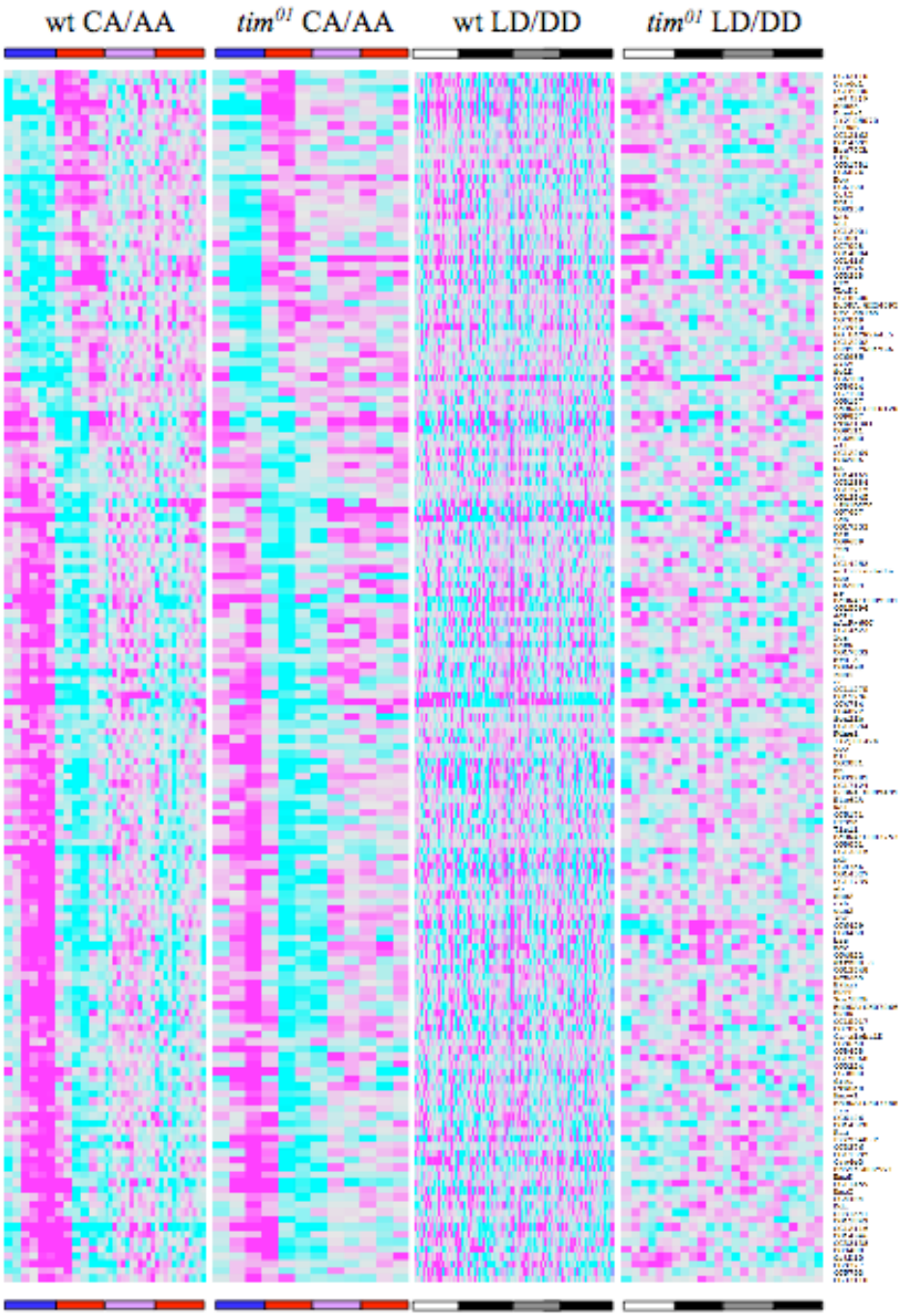

wt CA/AA
tim01 CA/AA
wt LD/DD
tim01 LD/DD

Figure 5.6. The majority of the temperature-driven transcripts in wild-type and *tim*^{01} flies have a phase of either the middle of the warm or cold period.

The histogram shows the number of temperature-driven transcripts (on the y-axis) with the indicated phase (on the x-axis). The red and blue bars above the plot indicate the warm and cold phases, respectively.

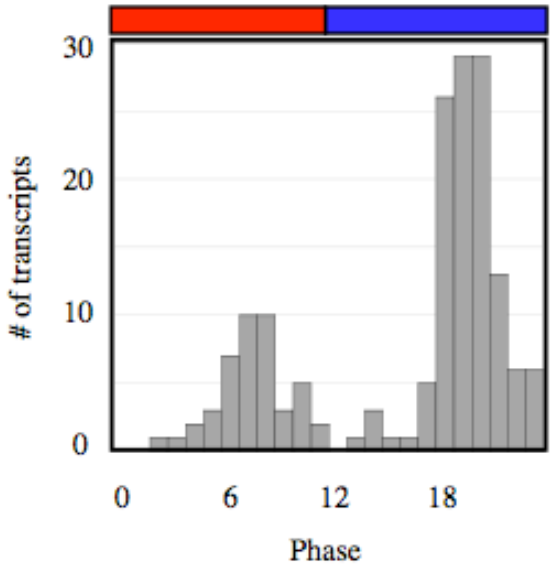
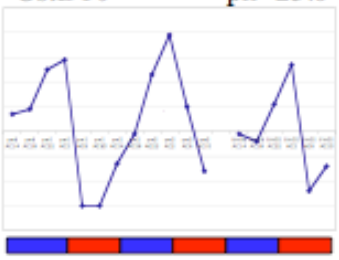

30
20
10
0
# of transcripts
0
6
12
18
Phase

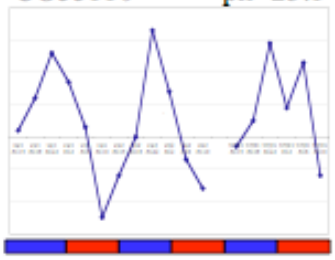
Figure 5.7. Kinetics of temperature changes.

The peak phases of expression (upper right-hand corners) of the temperature-driven transcripts are correlated with the rates of response to temperature changes. Gene expression profile data for two days of wild-type flies and one day of tim^{01} flies in AC are shown. The temperature-driven transcripts peaking around the cold-to-warm transition (a and b) and warm-to-cold transitions (c and d) show a gradual rate of response to temperature changes. The temperature-driven transcripts peaking in the middle of the warm phase (e and f) and the middle of the cold phase (g and h) show rapid up- or down-regulation in response to temperature changes, reaching their maximal or minimal values and then returning to baseline. The bars below the plots denote the temperature entrainment scheme, with red bars indicating 25°C time points and blue bars indicating 18°C time points.

a. *GstD10* ph=23.0



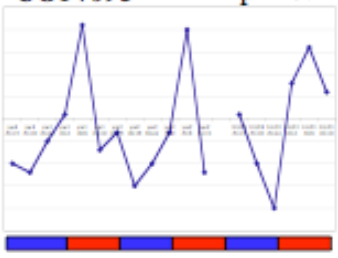
b. *CG33110* ph=23.1



c. *CG8187* ph=13.5

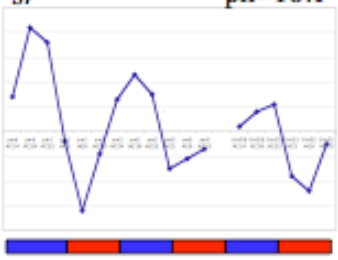
d. *CG9837* ph=14.1

e. *CG14693* ph=6.1



f. *CG6190* ph=6.5

g. *sr* ph=18.1



h. *GH02901* ph=18.1

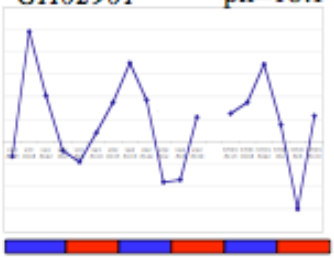


Figure 5.8. Organization of the expression patterns by phase of the 143 clock-dependent transcripts.

Format the same as in Figure 5.5. The behavior of the transcripts in AC/AA and LD/DD is shown on the left and right, respectively. Although the transcripts are aligned according to their temperature phase, the tight banding pattern in the LD/DD phasegrams indicates there is at least some overlap between these two sets of transcripts.


CA/AA
LD/DD

Figure 5.9. Organization of the expression patterns by phase of the 49 transcripts that oscillate in both AC/AA and LD/DD.

Format the same as in Figure 5.5. This significant overlap suggests an overall shared transcriptional response to light and temperature cycles.

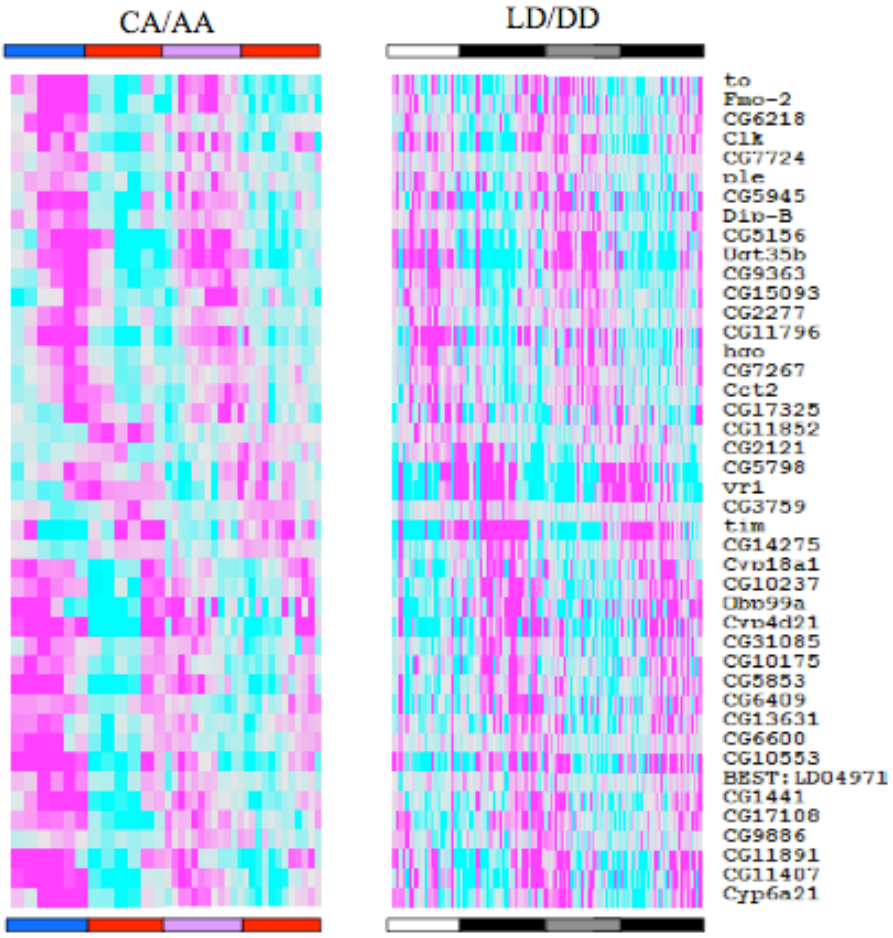

CA/AA
LD/DD
to
Fmo-2
CG6218
Clk
CG7724
ple
CG5945
Dip-B
CG5156
Ugt35b
CG9363
CG15093
CG2277
CG11796
hgo
CG7267
Cct2
CG17325
CG11852
CG2121
CG5798
Vri
CG3759
tim
CG14275
Cyp18a1
CG10237
Ubp99a
Cyp4d21
CG31085
CG10175
CG5853
CG6409
CG13631
CG6600
CG10553
BEST:LD04971
CG1441
CG17108
CG9886
CG11891
CG11407
Cyp6a21

Figure 5.10. Northern blot analyses suggest there are not light- and temperature-specific circadian transcripts.

The incomplete overlap between transcripts oscillating in response to photo- and thermocycles could indicate that there are both light- and temperature-specific transcriptional responses. Several transcripts predicted to oscillate only in temperature cycles and not light were tested on Northern blots (a). *cn bw* flies were entrained to LD 12:12 and subsequently released into DD. Time points were taken every four hours during the last day of entrainment and the first two days of free-run. RNA from whole heads was run on a Northern blot and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. The data were quantitated (b) and Fourier fit (see Materials and Methods) in order to determine the phase (ph; upper right-hand corners). The bars below the Northern images and plots denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points and gray bars indicating free-run time points taken during subjective light. The Northerns show that these transcripts oscillate in LD/DD, suggesting there is no difference between the set of transcripts oscillating in light vs. temperature.

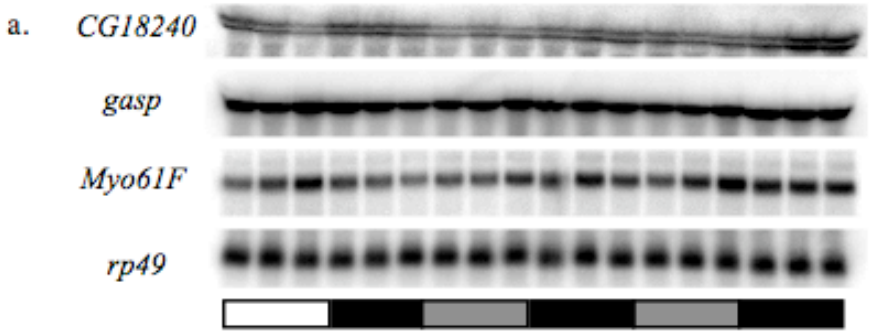

a.
CG18240
gasp
Myo61F
rp49
b.
CG18240
ph=18.4
gasp
ph=12.1
Myo61F
ph=12.0

Figure 5.11. Phases of transcripts oscillating in AC/AA and LD/DD.

The phases of transcripts oscillating in AC/AA are "advanced" (relative to the onset of the respective *Zeitgeber*) by about six hours as compared to LD/DD. ZT0 corresponds to lights-on in LD/DD and AC0 corresponds to the onset of the warm phase in AC/AA. The bars above and to the right of the plot denote the entrainment scheme, with the white bars indicating light time points, the black bar indicating dark time points, the red bar indicating 25°C time points and the blue bar indicating 18°C time points. Each red square on the plot corresponds to one of the 49 transcripts oscillating in both LD/DD and AC/AA, with their LD/DD phases indicated on the x-axis and their AC/AA phases indicated on the y-axis. The data were fit to a regression line (green), the fit of which is indicated in the lower right corner.

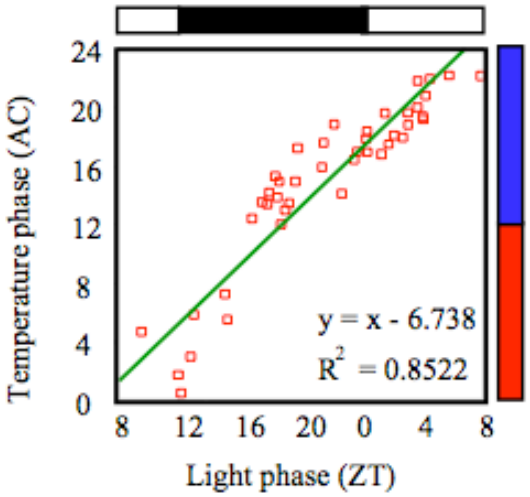

Temperature phase (AC)
24
20
16
12
8
4
0
8
12
16
20
0
4
8
Light phase (ZT)
y = x - 6.738
R2 = 0.8522

Figure 5.12. Locomotor activity behavior in AC vs. LD.

Locomotor activity behavior is "advanced" (relative to the onset of the respective *Zeitgeber*) in temperature entrainment as compared to light entrainment. The average locomotor activity counts from a group of flies in LD 12:12 (a; n=16) and AC 12:12 (b; n=16) are plotted. Entrainment conditions are denoted by the bars below each plot, with the white bar indicating light time points, the black bar indicating dark time points, the red bar indicating 25°C time points and the blue bar indicating 18°C time points.

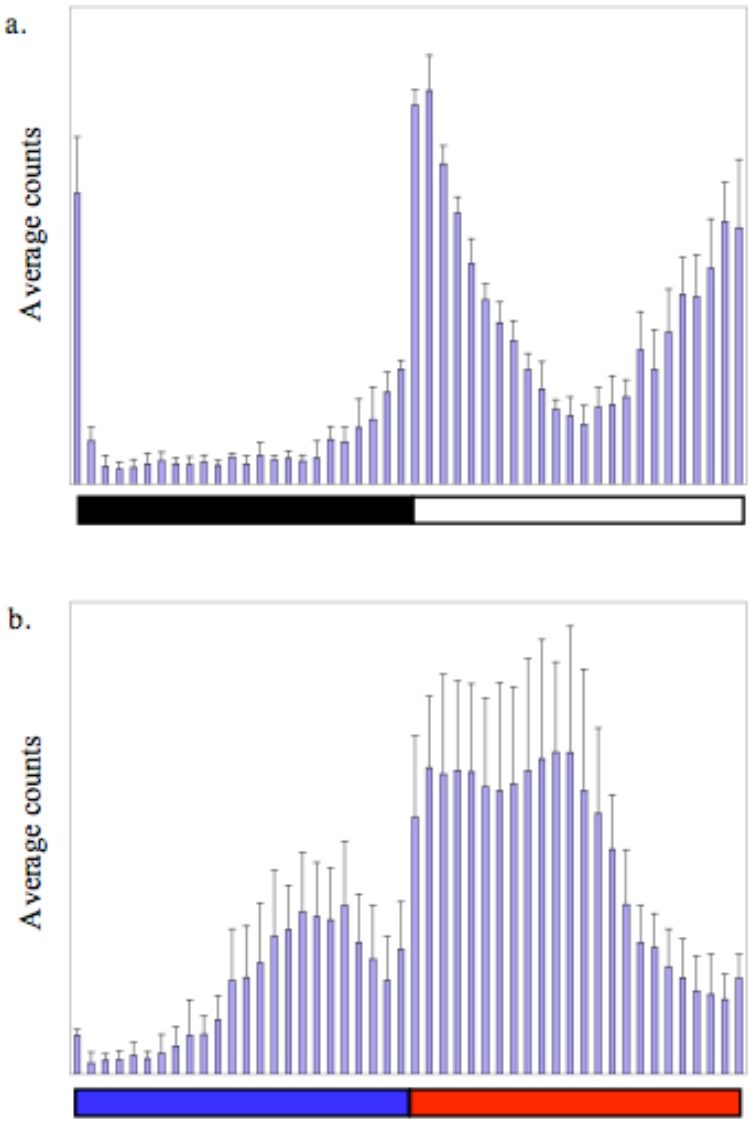
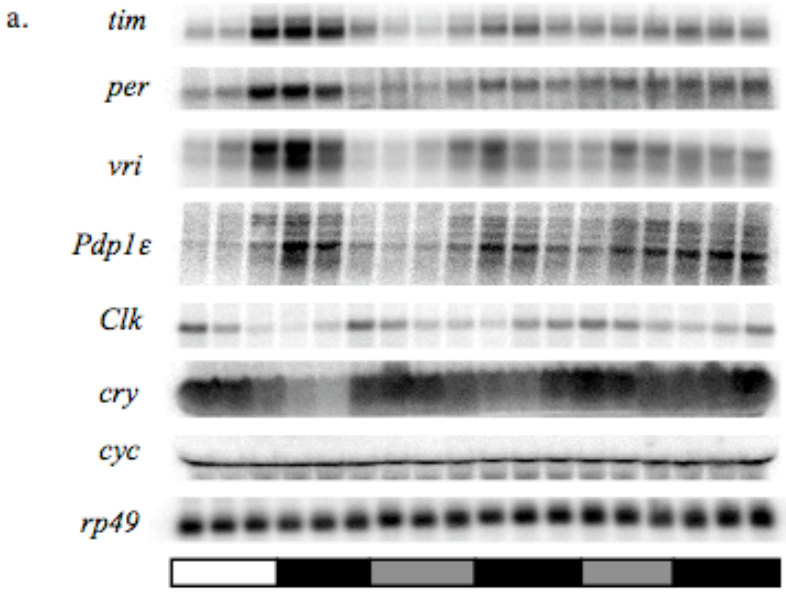

a.
Average counts
b.
Average counts

Figure 5.13. Expression of the core clock genes in LD/DD.

cn bw flies were entrained to LD 12:12 for five days and subsequently released into constant darkness. Time points were taken every four hours during the last day of entrainment and the first two days of free-run starting at ZT2. RNA from whole heads was run on a Northern blot (a) and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. The data were quantitated (b) and Fourier fit (see Materials and Methods) in order to determine the phase (upper right-hand corners) in LD alone (ph_{LD}) and DD alone (ph_{DD}). The bars below the Northern images and plots denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points and gray bars indicating free-run time points taken during subjective light. The different colored lines in the *vri* and *Pdp1* plots represent the different transcripts. At least two independent profiles were obtained for each transcript.


a.
tim
per
vri
Pdp1ε
Clk
cry
cyc
rp49

b.

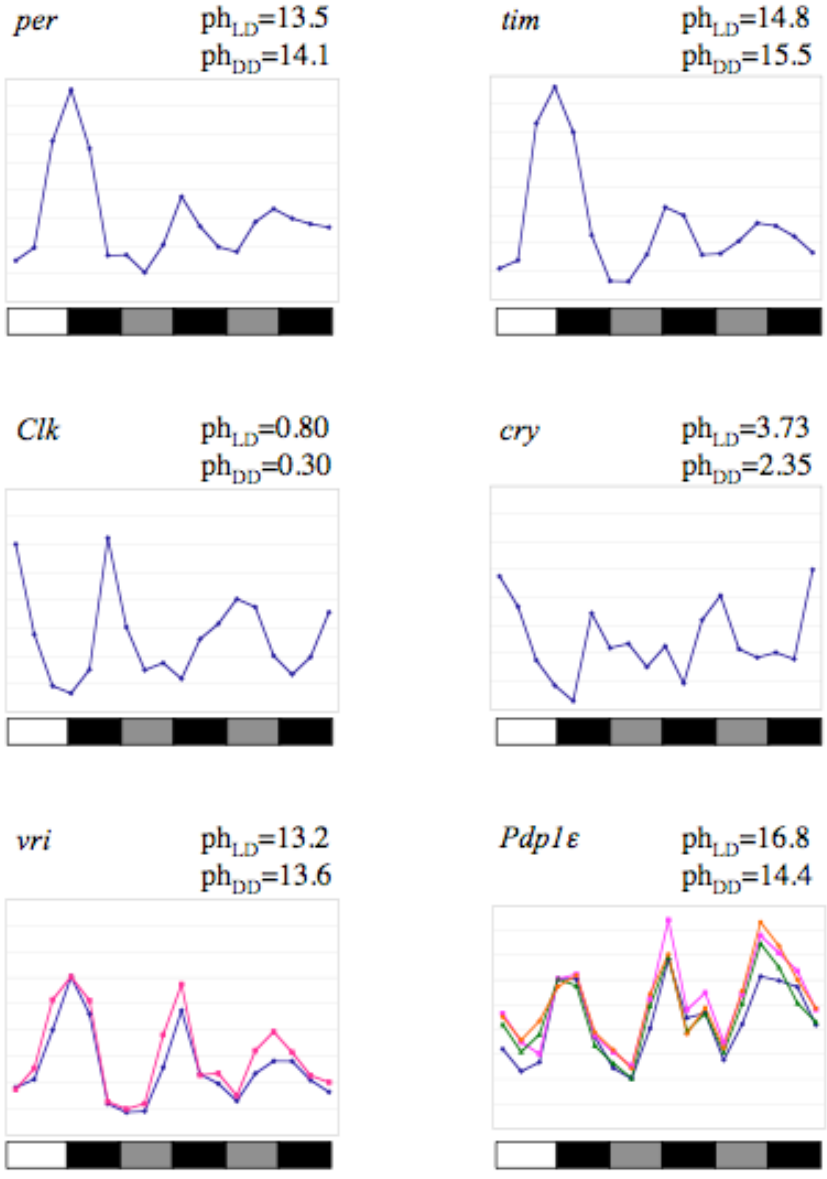
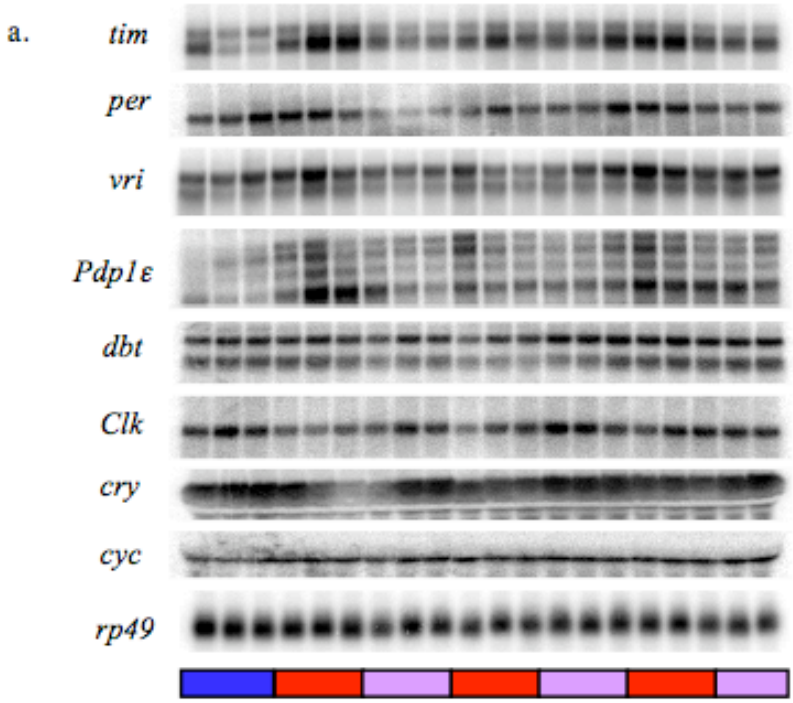

per
ph$_{LD}$=13.5
ph$_{DD}$=14.1
tim
ph$_{LD}$=14.8
ph$_{DD}$=15.5
Clk
ph$_{LD}$=0.80
ph$_{DD}$=0.30
cry
ph$_{LD}$=3.73
ph$_{DD}$=2.35
vri
ph$_{LD}$=13.2
ph$_{DD}$=13.6
Pdp1ε
ph$_{LD}$=16.8
ph$_{DD}$=14.4

Figure 5.14. Expression of the core clock genes in AC/AA.

cn bw flies were entrained to AC 12:12 (see Materials and Methods) and subsequently released into constant 25°C. Time points were taken every four hours during the last day of entrainment and the first two days of free-run. RNA from whole heads was run on a Northern blot (a) and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. The data were quantitated (b) and Fourier fit (see Materials and Methods) in order to determine the phase (upper right-hand corners) in AC alone (ph_{AC}) and AA alone (ph_{AA}). The bars below the Northern images and plots denote the entrainment scheme, with red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C. The different colored lines in the *vri* and *Pdp1* plots represent the different transcripts. At least two independent profiles were obtained for each transcript.


a.
tim
per
vri
Pdp1ε
dbt
Clk
cry
cyc
rp49

b.

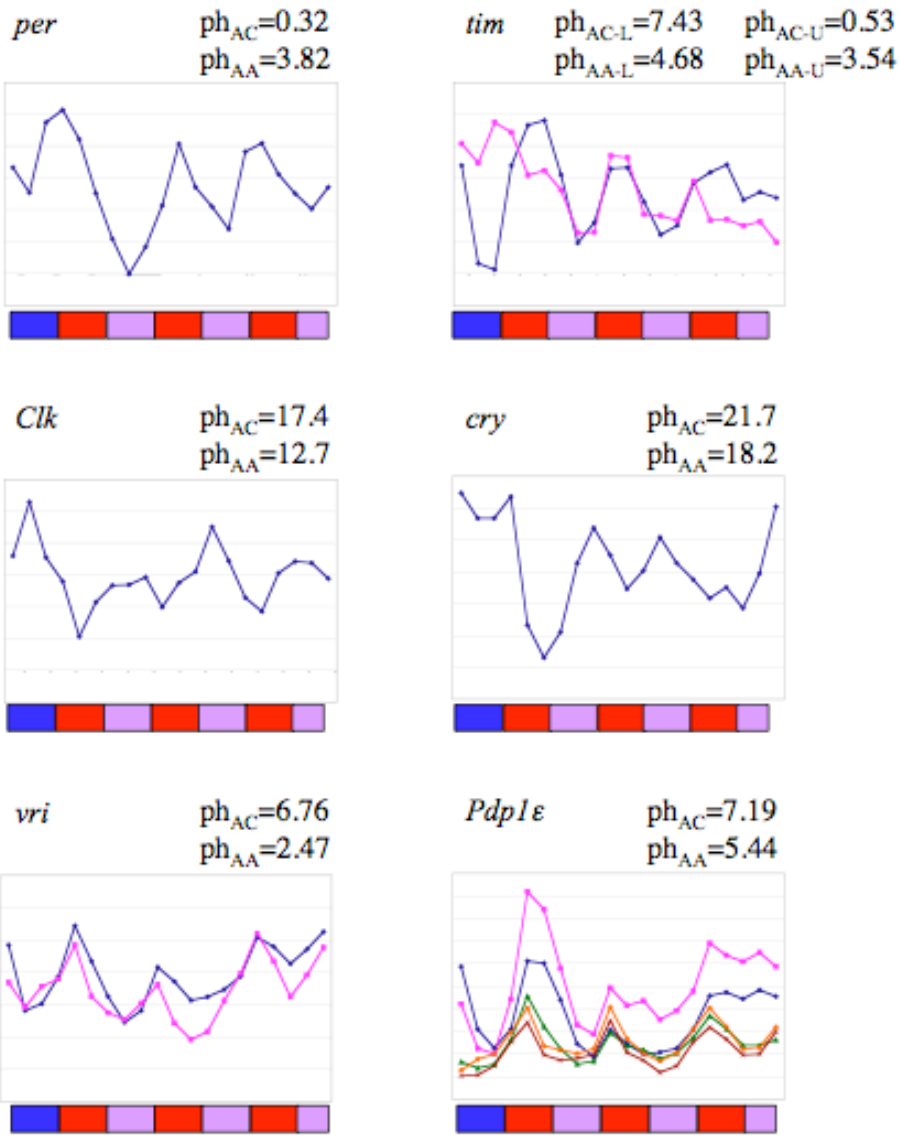
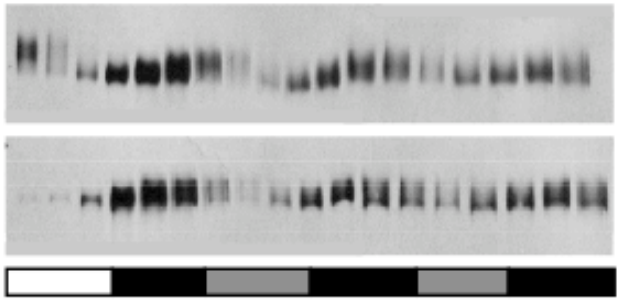

per
ph$_{AC}$=0.32
ph$_{AA}$=3.82
tim
ph$_{AC-L}$=7.43
ph$_{AA-L}$=4.68
ph$_{AC-U}$=0.53
ph$_{AA-U}$=3.54
Clk
ph$_{AC}$=17.4
ph$_{AA}$=12.7
cry
ph$_{AC}$=21.7
ph$_{AA}$=18.2
vri
ph$_{AC}$=6.76
ph$_{AA}$=2.47
Pdp1ε
ph$_{AC}$=7.19
ph$_{AA}$=5.44

Figure 5.15. PER and TIM expression in LD/DD.

cn bw flies were collected as in Figure 5.13. (a) Protein from whole heads was run on a Western blot and incubated with antibodies against PER and TIM, as indicated. (b) The data were quantitated and Fourier fit (see Materials and Methods) in order to determine the phase (upper right-hand corners) in LD alone (ph_{LD}) and DD alone (ph_{DD}). The bars below the Western images and plots denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points and gray bars indicating free-run time points taken during subjective light. At least two independent profiles were obtained.

a. PER

TIM



b. PER ph_{LD}=20.1 ph_{DD}=20.8 TIM ph_{LD}=17.6 ph_{DD}=17.9

Figure 5.16. PER and TIM expression in AC/AA.

cn bw flies were collected as in Figure 5.14. (a) Protein from whole heads was run on a Western blot and incubated with antibodies against PER and TIM, as indicated. (b) The data were quantitated and Fourier fit (see Materials and Methods) in order to determine the phase (upper right-hand corners) in AC alone (ph_{AC}) and AA alone (ph_{AA}). The bars below the Western images and plots denote the entrainment scheme, with red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C. At least two independent profiles were obtained.

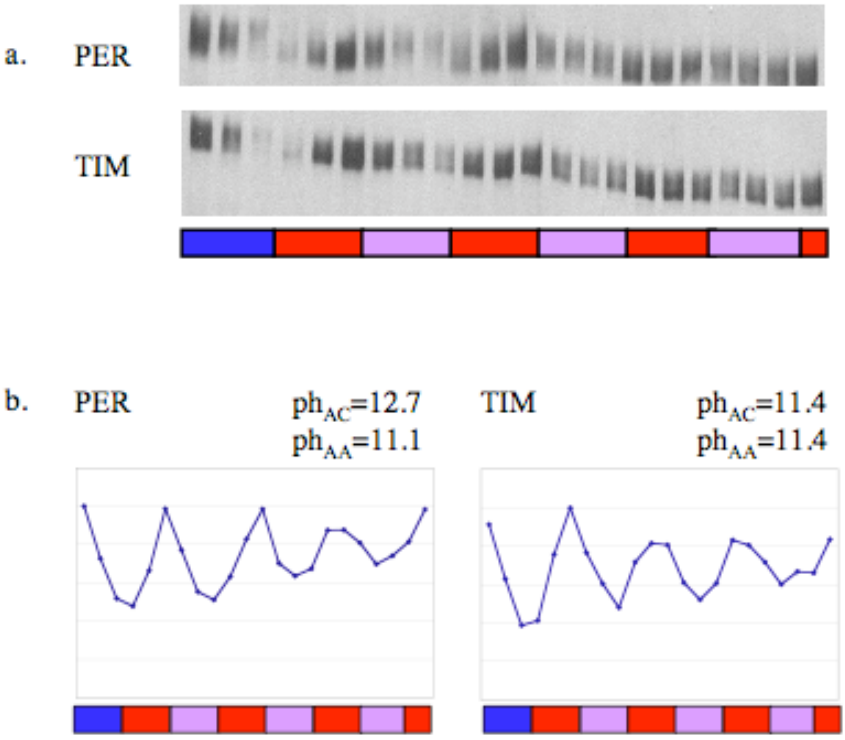
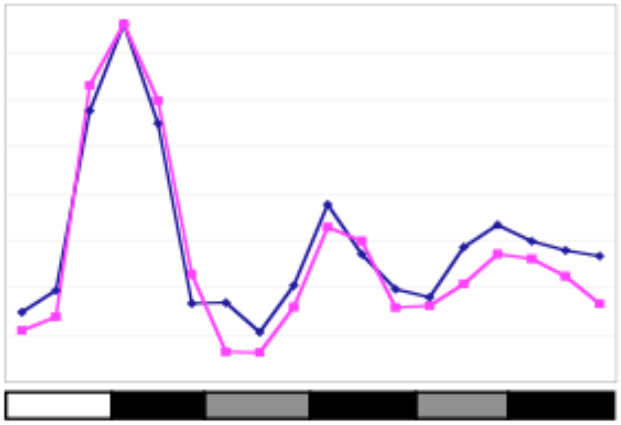

a.
PER
TIM
b.
PER
ph_{AC}=12.7
ph_{AA}=11.1
TIM
ph_{AC}=11.4
ph_{AA}=11.4

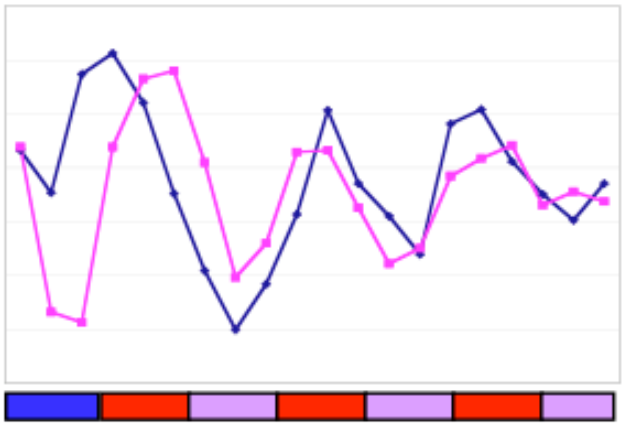
Figure 5.17. *per* and *tim* transcription during temperature entrainment.

per and *tim* transcription is uncoupled during temperature entrainment, but not free-run. The quantitations for *per* (blue) and *tim* (pink) in LD/DD (a) and AC/AA (b and c) from Figures 5.13 and 5.14, respectively, are shown. *per* and *tim* are tightly coupled at all times in LD/DD (a). In AC (b), peak *per* expression occurs several hours before peak expression of the predominant *tim* transcript. This differential expression is absent in AA. *per* cycles in phase with a novel transcript of *tim* during temperature entrainment (c). The bars below the plots denote the entrainment scheme, with the white bar indicating light time points, black bars indicating dark time points, gray bars indicating free-run time points taken during subjective light, red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C.

a.



b.



c.

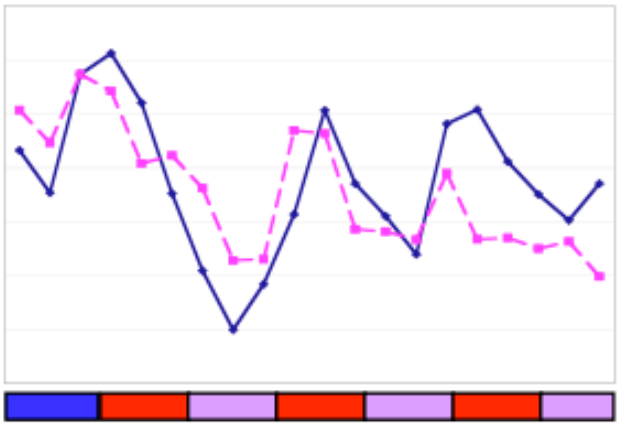


Figure 5.18. A novel *tim* transcript is present at cold temperatures.

An alternatively spliced form of *tim* (*tim*-upper; arrows) is present in *cn* bw flies during thermocycles (a) and in LD/DD cycles at 18°C (c). This splice form is less abundant in flies entrained to LD/DD at 25°C (b). The bars above the Northern images denote the entrainment scheme, with white bars indicating light time points, black bars indicating dark time points, gray bars indicating free-run time points taken during subjective light, red bars indicating 25°C time points, the blue bar indicating 18°C time points and the violet bar indicating free-run time points taken during subjective 18°C.

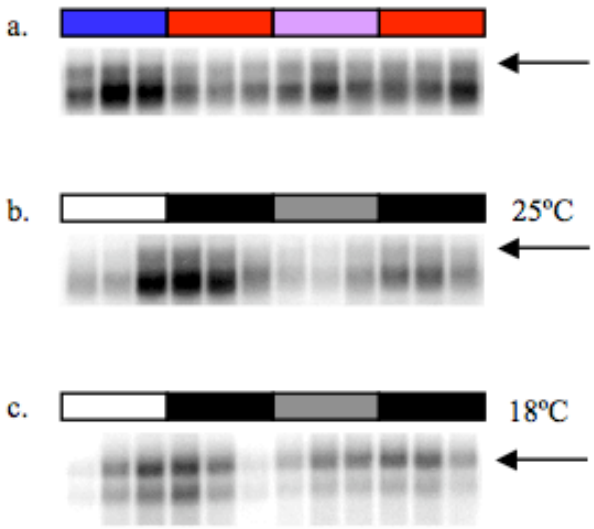
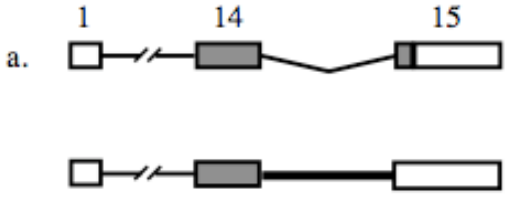

a.
b.
25ºC
c.
18ºC

Figure 5.19. Alternative splicing of the last *tim* intron.

(a) Genomic organization of the 3' region of *tim*. Boxes denote exons, with translated regions being filled. The last intron is retained in the novel transcript (bottom), which is predominantly expressed at cold temperatures. This results in a premature stop codon and hence a shorter TIM isoform (b; arrow), which can be seen in fly heads collected in LD 12:12 at 18°C but not 25°C.


a.
1
14
15

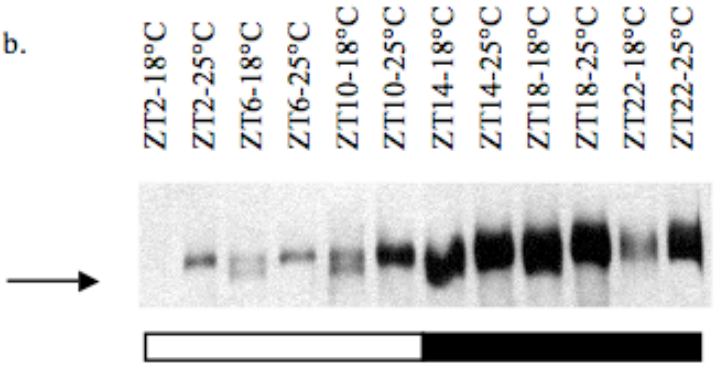

b.
ZT2-18°C
ZT2-25°C
ZT6-18°C
ZT6-25°C
ZT10-18°C
ZT10-25°C
ZT14-18°C
ZT14-25°C
ZT18-18°C
ZT18-25°C
ZT22-18°C
ZT22-25°C

Table 5.1. Locomotor activity behavior of *tim* transgenics.

Transgenic line	Tau ± SD	Onset ± SD	#AR/n
$Ctim3^{FL}$ 1	23.1 ± 0.19	10.3 ± 0.70	2/9
$Ctim3^{FL}$ 2	23.2 ± 0.35	10.2 ± 1.25	2/9
$Ctim3^{FL+S}$ 1	23.1 ± 0.17	10.4 ± 0.80	0/9
$Ctim3^{FL+S}$ 2	23.1 ± 0.17	10.6 ± 0.64	0/9
$Ctim3^{mS}$ 1	22.7 ± 0.26*	$11.4 \pm 0.39^{\#}$	0/9
$Ctim3^{mS}$ 2	22.3 ± 0.37*	$11.2 \pm 0.34^{\#}$	0/9

Locomotor activity data were obtained for three days in LD and eight days in DD for flies from each line. Average period length (tau) and time of activity onset, with associated standard deviations (SD), are shown in hours. Number of arrhythmic flies (#AR) and total flies (n) are indicated. There are no significant differences among the average taus of the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines. However, tau is significantly shorter in the $Ctim3^{mS}$ lines as compared to the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines (Student's t test, $p < 0.01$[*]). Similarly, there are no significant differences among the average times of onset of the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines. However, activity onset is significantly delayed in the $Ctim3^{-S}$ lines as compared to the $Ctim3^{FL}$ or $Ctim3^{FL+S}$ lines ($p < 0.05$ [$^{\#}$]).

Figure 5.20. Locomotor activity behavior of arrhythmic clock mutants is driven by temperature cycles.

The locomotor activity of individual flies of the indicated genotype is represented in each panel. The data are double plotted for visual continuity. Flies were recorded during entrainment to AC 12:12, as indicated by the red and blue bars above the panels. They were then released into constant 25°C (arrow) and allowed to free-run for the remainder of the assay. The flies show no anticipation of temperature transitions during entrainment and immediately become arrhythmic when the temperature cycle is removed, indicating their rhythmic behavior during entrainment is a direct response to the temperature cycle.

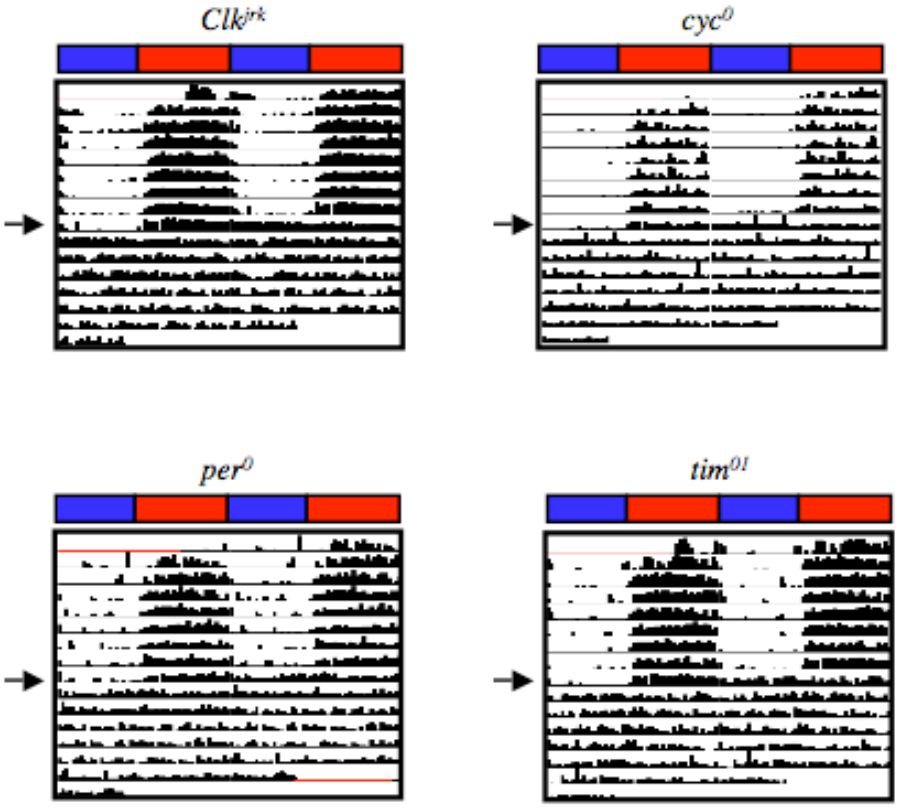

Clk^jrk
cyc^0
per^0
tim^01

Figure 5.21. Core clock transcripts are driven by temperature cycles in *tim^{01}* flies.

tim^{01} flies were entrained to AC 12:12 (see Materials and Methods) and subsequently released into constant 25°C. Time points were taken every four hours during the last two days of entrainment and the first day of free-run. (a) RNA from whole heads was run on a Northern blot and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. (b) The data were quantitated (light blue) and are shown along with the wild-type profiles from Figure 5.14 (dark blue). Phases were calculated for the two days of entrainment (unshaded) and are shown in the upper right-hand corners of each plot (ph_{wt} and ph_{tim}). The bars below the Northern images and plots denote the entrainment scheme, with red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C.

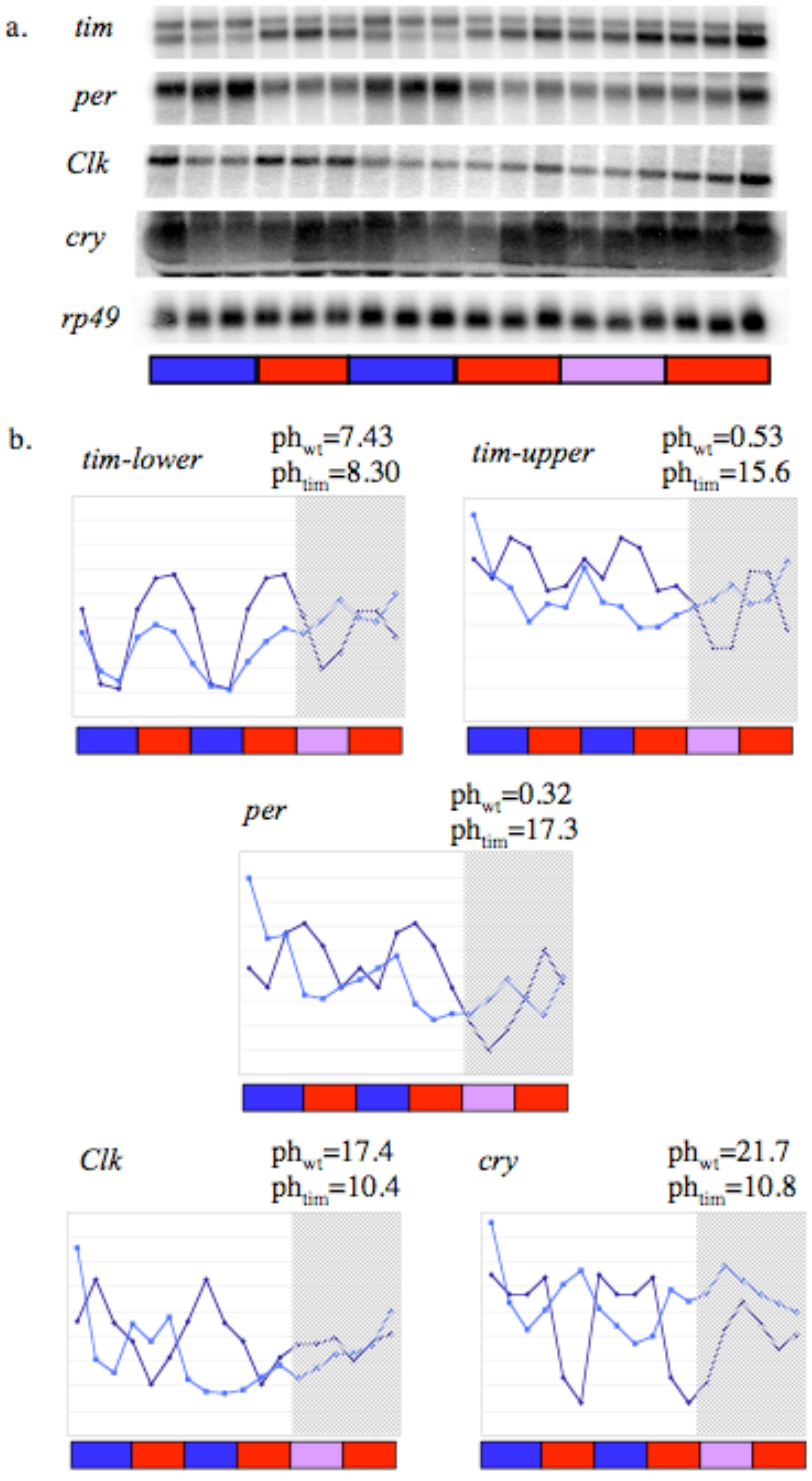

a.
tim
per
Clk
cry
rp49
b.
tim-lower
ph$_{wt}$=7.43
ph$_{tim}$=8.30
tim-upper
ph$_{wt}$=0.53
ph$_{tim}$=15.6
per
ph$_{wt}$=0.32
ph$_{tim}$=17.3
Clk
ph$_{wt}$=17.4
ph$_{tim}$=10.4
cry
ph$_{wt}$=21.7
ph$_{tim}$=10.8

Figure 5.22. Core clock transcripts are driven by temperature cycles in *per^0* flies.

per^0 flies were entrained to AC 12:12 (see Materials and Methods) and subsequently released into constant 25°C. Time points were taken every four hours during the last two days of entrainment and the first day of free-run. (a) RNA from whole heads was run on a Northern blot and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. (b) The data were quantitated (light blue) and are shown along with the wild-type profiles from Figure 5.14 (dark blue). Phases were calculated for the two days of entrainment (unshaded) and are shown in the upper right-hand corners of each plot (ph_{wt} and ph_{per}). The bars below the Northern images and plots denote the entrainment scheme, with red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C.

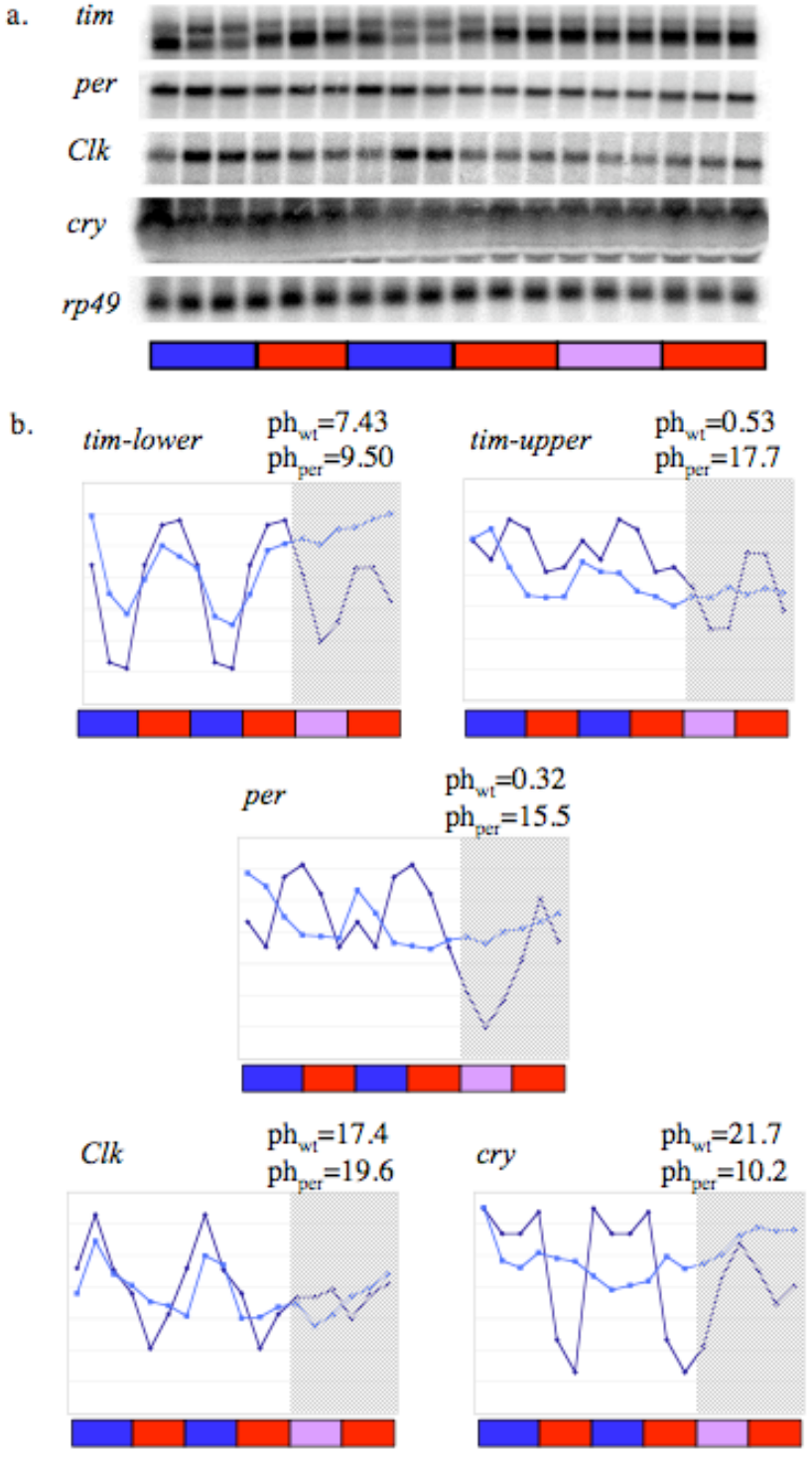
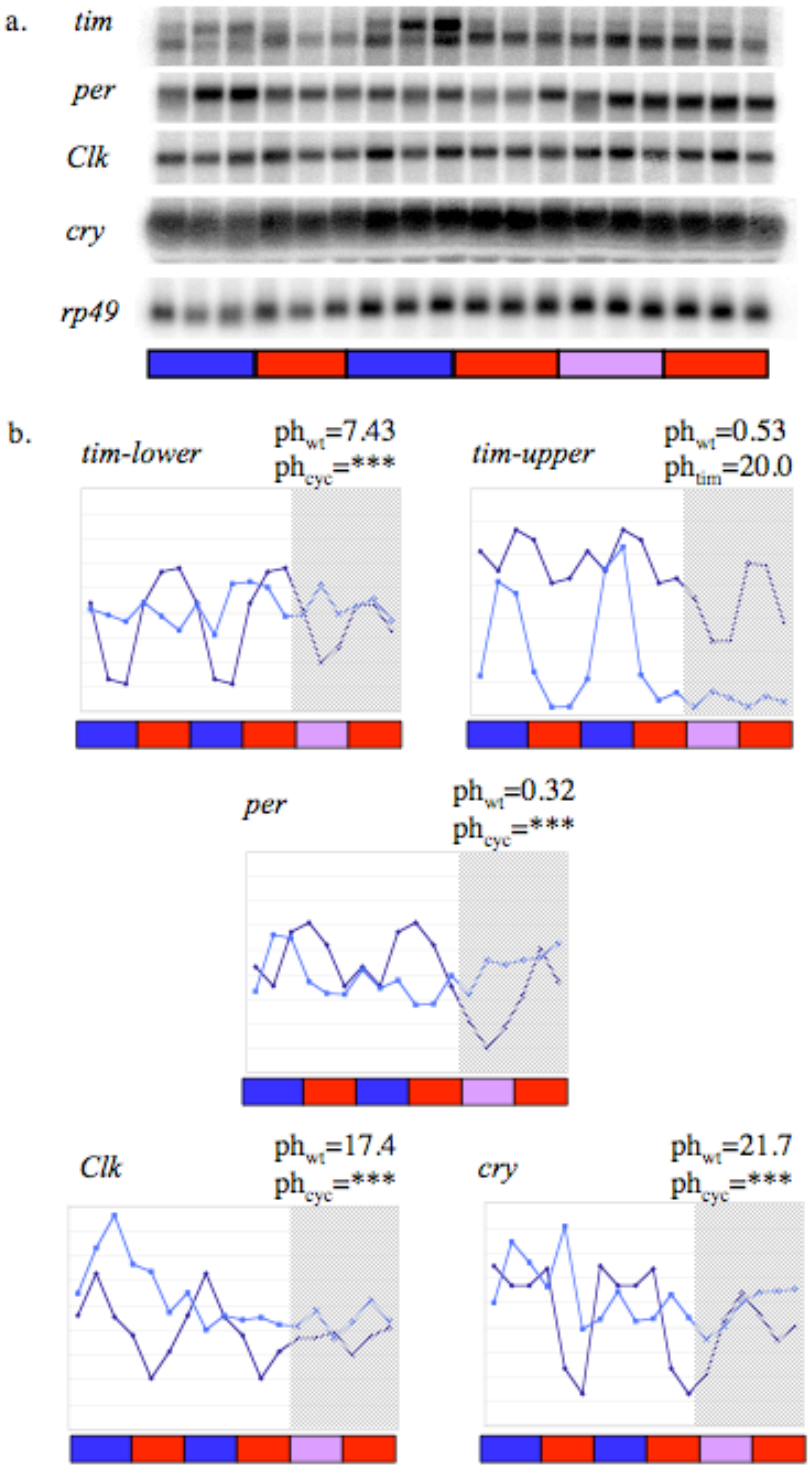

a.
tim
per
Clk
cry
rp49
b.
tim-lower
ph_wt=7.43
ph_per=9.50
tim-upper
ph_wt=0.53
ph_per=17.7
per
ph_wt=0.32
ph_per=15.5
Clk
ph_wt=17.4
ph_per=19.6
cry
ph_wt=21.7
ph_per=10.2

Figure 5.23. Only the novel transcript of *tim* is driven by temperature cycles in *cyc^0* flies.

cyc^0 flies were entrained to AC 12:12 (see Materials and Methods) and subsequently released into constant 25°C. Time points were taken every four hours during the last two days of entrainment and the first day of free-run. (a) RNA from whole heads was run on a Northern blot and probed with cDNA encoding the indicated transcripts (left). An *rp49*-specific probe was used as a loading control. (b) The data were quantitated (light blue) and are shown along with the wild-type profiles from Figure 5.14 (dark blue). Phases were calculated for the two days of entrainment (unshaded) and are shown in the upper right-hand corners of each plot (ph_{wt} and ph_{cyc}). Asterisks indicate no significant phase could be determined. The bars below the Northern images and plots denote the entrainment scheme, with red bars indicating 25°C time points, blue bars indicating 18°C time points and violet bars indicating free-run time points taken during subjective 18°C.


a.
tim
per
Clk
cry
rp49
b.
tim-lower
ph_wt=7.43
ph_cyc=***
tim-upper
ph_wt=0.53
ph_tim=20.0
per
ph_wt=0.32
ph_cyc=***
Clk
ph_wt=17.4
ph_cyc=***
cry
ph_wt=21.7
ph_cyc=***

Chapter 6: Discussion and perspectives

The intent of this project was to characterize the interplay of light and temperature in regulating gene expression in the fly. This was achieved by generating genome-wide transcription profiles in the presence of either daily light or temperature cycles in both wild-type and arrhythmic mutant flies. Northern, Western and behavioral analyses were used to confirm and extend the expression profile data.

Global analysis of circadian gene expression in light and temperature cycles. The emergence of microarray technology has allowed the field of circadian biology to move beyond the investigation of individual clock genes and their molecular circuits to the analysis of clock-controlled gene expression on a more global level. A number of labs have recently performed genome-wide surveys in an attempt to define a set of transcripts in the *Drosophila* head that oscillate with a circadian rhythm in response to light entrainment. While many genes have been identified, the overlap among the various surveys is very poor. In addition, several sets exclude several of the known oscillating clock components, such as *per, Clk, Pdp1* and *cry*.

Disparities among the studies are likely due to differences in experimental design and statistical analyses. In order to overcome these discrepancies, all of the available microarray data were subjected to an analysis that emphasized uniformity in period length and peak phase while tolerating inter-experimental variability in amplitude. As a result, a set of 172 transcripts that robustly oscillate in LD/DD in the adult fly head was defined. This set includes all of the known cycling clock transcripts, and Northern blot analyses confirmed a number of newly identified oscillatory genes. These data indicate there is a broad program of light-responsive circadian gene expression.

To determine whether periods other than 24 hours exist, the wild-type gene expression profile data were fit to periods ranging from 12 to 48 hours. Significant enrichment was seen only for the 24-hr period, strongly suggesting that the dominant period of rhythmic transcription is circadian. The enrichment was lost in a tim^{01} mutant, suggesting that a functional, TIM-dependent clock is required for 24-hr periodic transcription.

While light is the strongest and best understood *Zeitgeber* for the circadian clock, temperature is also an important input. Like light, temperature can entrain the locomotor activity behavior of *Drosophila*. Genome-wide expression profiles of transcripts during temperature

entrainment and subsequent constant conditions in both wild-type and arrhythmic *tim*^{01} backgrounds were generated in order to better understand the role of temperature on gene expression in the fly. Unlike as in light entrainment, where the magnitude of the overall transcriptional response in the presence or absence of a photocycle is largely maintained, there is a dramatic difference in transcriptional responses in the presence or absence of a thermocycle. Whereas almost all transcription appears to be modified by temperature cycles, there is a limited number of transcripts that continue to oscillate in constant conditions following temperature entrainment. This suggests two distinct responses to temperature: clock-independent temperature-driven oscillations and clock-dependent circadian oscillations (Figure 6.1).

A set of temperature-driven transcripts was defined by analyzing both the wild-type and *tim*^{01} data during AC. Since these transcripts are directly driven by a thermocycle, their behavior should be the same in the presence or absence of a functional clock. However, due to the inclusion of wild-type data, this set could, in theory, include transcripts that are both temperature-driven as well as clock-dependent. A set of dually light- and clock-regulated transcripts was recently identified in the context of an LD cycle (Wijnen et al., 2006) (Figure 6.1). In arrhythmic clock mutants, these transcripts are

simply induced or repressed in response to light. In wild-type flies, however, input from both light and the clock results in one phase and amplitude for these transcripts in LD and a slightly different one in DD. Transcripts such as these may be important for processes requiring both anticipation of and direct responses to light, such as in seasonal adaptation. If there are similarly dually regulated transcripts in the context of a temperature cycle, one question is whether their phases align in the presence or absence of a clock. It appears that, at least for the core clock transcripts, this is not the case. Core clock transcripts do not simply follow the temperature cycle in wild-type flies but assume clock-programmed phases. However, the core clock genes are driven by a thermocycle in arrhythmic tim^{01} and per^{0} mutants, and in this context they assume phases that are reflective of the temperature cycle. This suggests that the clock can act as a buffer to work against simple up- or down-regulation by temperature and generate meaningful phases of transcription.

Differential regulation of per and tim. Transcriptional regulation of *per* and *tim* appears to be different in light and temperature entrainment. Whereas in light entrainment *per* and *tim* RNA expression is tightly coupled at all times, in temperature entrainment *per* RNA levels peak before *tim*

RNA levels. This is a result of a temperature-induced advance in *per* expression and delay in *tim* expression. The uncoupling does not persist in constant conditions following temperature entrainment. It is also not present at the protein level, indicating the phenomenon is most likely transcriptional and not post-translational. It also follows that post-transcriptional controls must be added into the system in order to generate the appropriate phase of PER and TIM expression.

Transcriptional differences in *per* and *tim* regulation have been suggested earlier based on the observation that these transcripts show different rates of degradation in response to a light pulse (Rothenfluh et al., 2000). In addition, while at lower temperatures *per* expression is up-regulated in LD and DD, *tim* is down-regulated in LD and barely oscillatory in DD (Majercak et al., 1999). Further, while the phases of both *per* and *tim* are advanced at lower temperatures, the advance in *per* appears to be a result of faster accumulation, while the advance in *tim* is a result of slower degradation.

One of the factors involved in the observed differential expression of *per* and *tim* may be the alternative splicing of both transcripts. Alternative splicing of *per* has been extensively described and is an example of the cooperative effects of light and temperature (Collins et al., 2004; Majercak

et al., 1999, 2004). An 89-bp intron in the 3' UTR of *per* is preferentially spliced out during cold, short days. This is accompanied by an advance in both PER accumulation and locomotor activity rhythms. The alternative splicing is thought to be important in seasonal adaptation, as long photoperiods counteract the cold-induced behavioral advances by delaying the accumulation of TIM, in turn rendering prematurely produced PER unstable. Thus the fly is able to integrate information from both light and temperature to generate behavior that is aligned to the environmental day. The levels of the spliced form of *per* in temperature entrainment as compared to free-run need to be investigated.

Temperature-dependent alternative splicing of *tim* is described in this thesis. At low temperatures, the last intron of *tim* is preferentially retained, resulting in a premature stop codon and a truncated protein. Fly transgenic lines that could make only the full-length TIM isoform ($Ctim3^{FL}$), only the truncated protein ($Ctim3^{mS}$) or a combination of the two ($Ctim3^{FL+S}$) were generated. Locomotor activity analyses in LD and DD at 25°C suggest that $Ctim3^{mS}$ flies have both a significantly shorter behavioral period length in DD and a delayed phase of activity in LD than $Ctim3^{FL+S}$ or $Ctim3^{FL}$ flies. It is therefore possible that the observed delay in *tim* production during temperature entrainment as compared to free-run is a result of increased

levels of the (cold-induced) truncated protein. Taken together, the results may indicate that the alternative splicing and differential regulation of *per* and *tim* is responsible for finely tuning the clock in response to changing environmental conditions.

Two Zeitgebers, one clock. Different groups of clock-bearing cells in the fly have been shown to regulate different rhythmic processes. For example, locomotor activity and eclosion rhythms, arguably the best-characterized rhythmic behaviors in *Drosophila*, require the LN_vs and the neuropeptide PDF. Cyclic olfactory responses do not depend on the LNs or PDF, but instead depend on the antennal neurons (Tanoue et al., 2004; Zhou et al., 2005). Egg-laying rhythms also appear to be regulated independently of the LN_vs and PDF (Howlader et al., 2006). Thus the image of the circadian clock as a single entity is transforming into a more systematic model.

A system of two coupled oscillators was proposed for the *Drosophila* clock almost 50 years ago (Pittendrigh et al., 1958). In this model, the master or A oscillator is autonomous, light-sensitive and temperature-compensated. The slave or B oscillator, which is coupled to and driven by A, is responsive to temperature but not light. The evidence for this two-

oscillator model came from the different responses in eclosion rhythms to light and temperature. Whereas light pulses administered at different times of day resulted in steady-state phase advances or delays, the phase changes resulting from temperature pulses were transient. The researchers concluded that the steady-state phase changes in response to light were a result of the eventual realignment of the A oscillator to the light signal. The transient responses to temperature pulses were proposed to be a result of temporary temperature-induced disturbances in B, with the return to the previous phase reflecting the A oscillator's resumption of control over B.

Such a system of coupled oscillators has been demonstrated in several unicellular organisms. For example, different genes are expressed with different period lengths in some *Synechoccus* mutant backgrounds (Nair et al., 2002), and bioluminescence rhythms in *Gonyaulax* have been shown to be regulated by two oscillators that respond to different wavelengths of light (Morse et al., 1994). In *Neurospora*, strains carrying null alleles of *frq, wc-1* or *wc-2* still show a conidial banding rhythm. Although the rhythm of this "FRQ-less oscillator" (FLO) (Iwasaki and Dunlap, 2000) has lost most characteristics of a circadian clock, it can be entrained by *Zeitgebers* such as temperature cycles (Lakin-Thomas, 2006; Merrow et al., 1999), rhythms in nitrate reductase activity (Christensen et al., 2004) and transfer from light to

dark (Correa et al., 2003). This suggests that the FLO is a slave oscillator, requiring the master, FRQ-dependent clock for circadian rhythmicity yet with the ability to function independently of the master clock in a non-circadian manner (Bell-Pedersen et al., 2005).

A system of coupled oscillators has recently been demonstrated in the regulation of the morning and evening peaks of locomotor activity in the fly (Grima et al., 2004; Stoleru et al., 2004, 2005). The morning (M) oscillator requires the presence of the LN_vs, while the evening (E) oscillator requires the LN_ds. It was further shown that the E oscillator is set by the M oscillator by generating flies in which the M and E oscillators have different free-running periods (Stoleru et al., 2005). However, despite the parallels to Pittendrigh's original model, there is no published evidence that these or other oscillators would differentially respond to temperature, as opposed to light, as a *Zeitgeber*. So while it appears that there is a multicellular clock network in *Drosophila* that is reflected by coordinated yet independently regulated outputs, the data presented in this thesis suggest that the response to multiple inputs, such as light and temperature, would still be integrated by a single oscillator (Figure 6.1).

The following observations support the hypothesis of a single, integrative oscillator. First, there is most likely no difference between the

set of transcripts that cycle in response to light or temperature cycles given independently of one another.

Secondly, the phases of the transcripts that oscillate in both photo- and thermocycles maintain the same mutual phase relationship after entrainment by light or temperature. That is, the phase observed at the onset of the thermophase is systematically advanced by about six hours relative to the phase at the onset of light. Given the size of the delay that is commonly found between environmental temperature profiles and LD cycles, this is most likely a reflection of the cooperative entrainment by light and temperature that occurs under natural circumstances. It also indicates that a response to temperature would be well integrated with the expected light cycle were it also supplied, and vice versa.

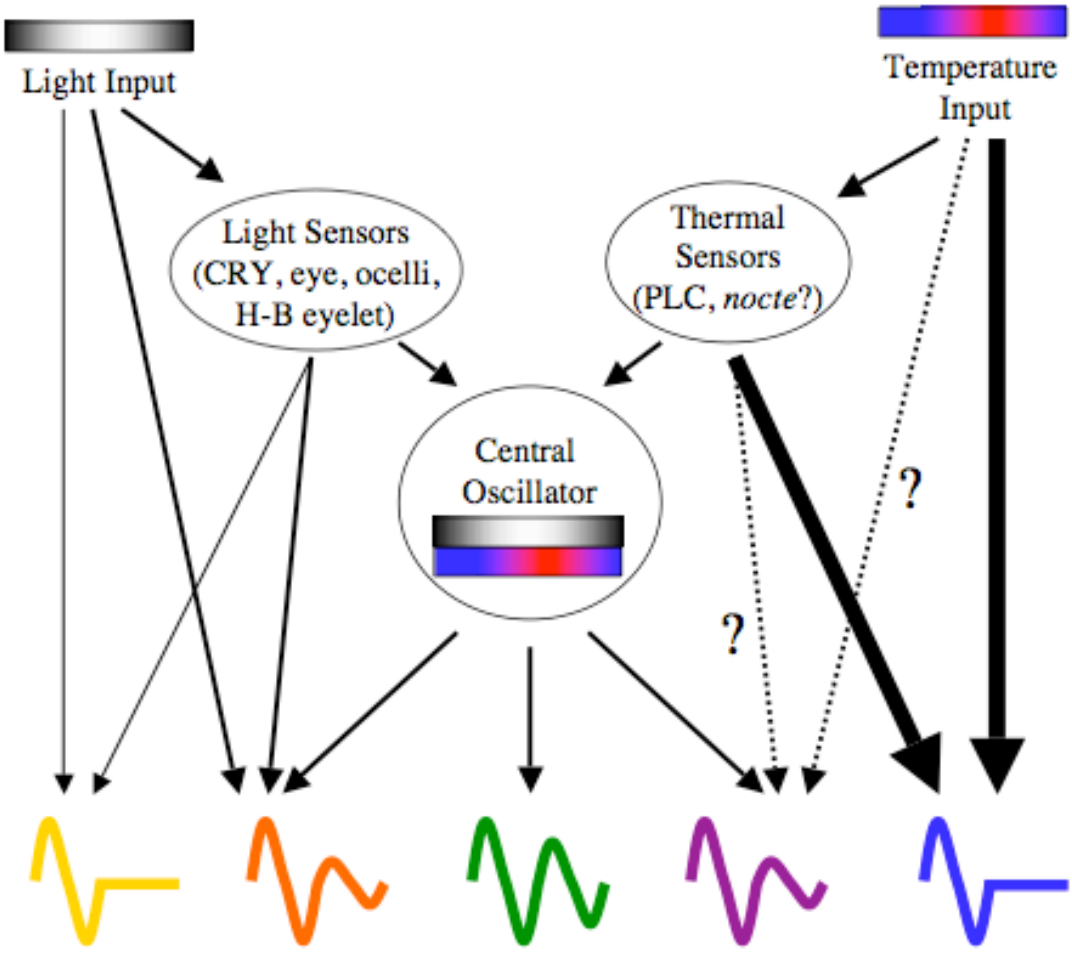
Thirdly, similar to the transcriptional relationship, the phases of PER and TIM proteins in temperature entrainment are correlated with the expected light cycle. A recent study reports that temperature entrainment in the presence of constant light, a condition that would normally result in arrhythmia, induces rhythmic bioluminescence levels in a *per-luc* construct (Glaser and Stanewsky, 2005). This entrainment is dependent on post-translational regulation as transgenics encoding only the *per* promoter fused to luciferase show no rhythmicity. These researchers further demonstrate

that both PER and TIM oscillate in wild-type flies maintained in a temperature cycle under constant light. While the expression of the proteins is "advanced" (relative to the onset of the respective *Zeitgeber*) as compared to LD/DD, the magnitude of the phase difference is less than is seen here for temperature entrainment during constant darkness. This indicates that, when uncoupled, light and temperature can independently modulate PER and TIM expression. However, under natural conditions, light and temperature act cooperatively to reinforce each other's effects on entrainment.

Summary. The data presented in this thesis support the existence of a single clock in the adult fly head that is able to integrate information from light and temperature. The ability to assimilate information from multiple sources may confer robustness and precision to the circadian timing system. It may also be critical to processes influenced by multiple factors, such as seasonal adaptation. Future studies examining gene expression and behavior in the presence of light and temperature cycles that are similar to those found under natural conditions should clarify the importance such an integrative clock.

Figure 6.1. Model of how information from light and temperature are processed by the fly circadian clock.

Light and temperature cycles are naturally staggered such that when the sun is at its highest point at around noon the temperature is still rising, usually reaching a maximum in the late afternoon. Information from light and temperature is relayed through the appropriate sensors to the clock. In the absence of photic or thermal input, the clock can predict when the fly would have seen light and dark or warm and cold, respectively. When both *Zeitgebers* are present, they are integrated by the clock to generate meaningful phases of transcription (green). Independently of the clock, light can directly affect the transcription of a small number of genes (yellow), whereas temperature can drive the expression of a larger number of transcripts (blue). There are also transcripts that are dually regulated by the clock and light (orange) or possibly temperature (purple), which would also be important for processes such as seasonal adaptation.


Light Input
Temperature Input
Light Sensors (CRY, eye, ocelli, H-B eyelet)
Thermal Sensors (PLC, *nocte*?)
Central Oscillator
?
?

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