THERMOSENSITIVE SPLICING OF A CLOCK GENE AND SEASONAL ADAPTATION

by

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Abstract of the Dissertation

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Prior work showed that the thermosensitive splicing of an intron found in the 3' untranslated region (UTR) of the *Drosophila melanogaster period* (*per*) gene, termed dmpi8 (*Drosophila melanogaster per* intron 8), is critical for temperature-induced adjustments in the distribution of daily activity. Using a simplified cell culture system, we showed that an intricate balance between multiple suboptimal splicing signals is the underlying molecular basis for the thermosensitive splicing of dmpi8. We confirmed the physiological significance of this model in transgenic *Drosophila* by altering the splice site strengths of dmpi8. Presumably, at higher temperatures, the interaction between the spliceosome and the sub-optimal splicing signals is weaker and hence results in less efficient splicing.

Further studies of *Drosophila* species from different geographical regions strongly suggest that the thermal regulation in the splicing efficiency of the *D. melanogaster per* 3'-terminal intron is an important mechanism for seasonal adaptation in this species. Temperature dependent splicing of dmpi8 contributed to the ability of cosmopolitan *D.*

melanogaster to adapt to temperate regions by providing a mechanism that can extent midday siesta during the long warm days typical of temperate climates. However, *Drosophila* species indigenous to Afro-equatorial regions, wherein temperature undergoes little seasonal variation, do not exhibit thermal adjustments in their daily activity patterns. Intriguingly, 3'-terminal introns were also found in their *per* genes, but these introns have strong splice sites and are not spliced in a thermosensitive manner. Thus, the strengths of key splicing signals underlies species-specific differences in the thermosensitivities of *per* 3'-terminal intron removal that correlate with the ability to adjust daily activity patterns in a temperature dependent manner.

In related work we identified natural polymorphisms in non-intronic regions of the *per* 3'-UTR that modulate dmpi8 splicing. Preliminary analysis suggests that the effects of some of these polymorphisms might be mediated by SR proteins. Finally, we also identified a novel role for the *per* 3'-terminal intron on sleep homeostasis. In summary, this thesis utilized a multi-faceted strategy, including simplified mechanistic studies and comparative analysis, which led to new ecological and evolutionary perspectives on the role of circadian clock function on thermal and seasonal adaptation.

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Chapter 1. Introduction

1.1 Overview of circadian rhythms

As the stream of solar energy – light and heat – reaches our planet, the Earth's rotation around its axis and its orbiting around the Sun leads to cyclical changes in many environmental signals on Earth. Instead of reacting to daily environmental changes, such as sunrise, organisms on Earth can anticipate predictable environmental changes by means of an internal time measuring device. This occurs because environmental stimuli synchronize these time measuring device to local time, allowing organisms to carry out their biochemical, physiological, and behavioral activities at biologically advantageous times during the day and undergo characteristic seasonal responses. Circadian clocks have been found to be present almost ubiquitously among organisms and in the absence of external time cues, have an endogenous period of approximately 24 hours, which earned the term circadian (in *latin*, *circa* means around and *dies* refers to a day). Such widespread existence implicates the importance of circadian clocks. For example, malfunction or disruption of the circadian clock causes many disorders in human; e.g. Seasonal Affective Disorder (SAD), a mental mood disorder that usually occurs in the winter when daytime become significantly shorter (Bhattacharjee, 2007); exacerbation of cardiovascular disease (Martino et al., 2008); and increased risk of developing cancer (Straif et al., 2007). Therefore, it is important to understand the underlying mechanisms of how a clock measures the passage of daily time as well as senses and adapts to the external environment. In order to achieve that, it is critical for us to be informed of some of the well-established key features of circadian clocks.

In the absence of external stimuli, circadian rhythms persist or free-run with periods of approximately 24 hours. A wide variety of physiological and behavioral phenomena manifest circadian rhythms, such as our daily wake-sleep cycles. One of the earliest realizations that internal circadian clocks exists was the observation that leave movements (opening and closing) of the plant *Mimosa pudica* repeats approximately every 24 hours in the absence of light (De Mairan, 1729). Circadian rhythms were also shown to persist, under constant dark condition, from generation to generation in flies and mice (Aschoff, 1960; Sheeba et al., 1999) even though individuals in each generation were never exposed to daily light-dark cycles. Such self-sustaining rhythmicity indicates that circadian rhythms are driven by genetically encoded autonomous clocks within the organism and are not merely driven by daily changes in environmental stimuli. The fact that the period of circadian rhythms under constant darkness and temperature usually slightly varies from 24 hours, further suggested that these rhythms are internally generated and not driven by some undetected geophysical force due to the rotation of the Earth (Vitaterna et al., 2001).

Since the free-running period (FRP) of endogeneous circadian clocks are not exactly 24 hours, they would be constantly in and out of phase with the 24 hour solar day throughout the year. However, the internal oscillator is reset daily by external time cues, maintaining synchrony to local time. External time cues that synchronize circadian rhythms are known as zeitgebers (German for "time giver" or synchronizer). The most prominent and reliable daily time cues or zeitgebers are visible light and to a lesser extent temperature. There are also other less influential zeitgebers, like food intake (reviewed in Stephan, 2002), social cues (reviewed in Mistlberger and Skene, 2004), and

electromagnetic fields (reviewed in Yoshii et al., 2009). By synchronizing circadian clocks to local time, light-dark changes ensure that circadian-gated processes will occur at a specific time relative to a particular phase of the environment (e.g. sunrise or sunset). The essence of such an entrainment mechanism lies at the differential responses of circadian oscillators to entraining time cues. The internal oscillator is delayed when the organism is exposed to light in the early night, whereas it is phase advanced when exposed to light in the late night (Decoursey, 1960; Pittendrigh, 1960). In addition to light, daily changes in ambient temperature are also a potent entraining cue in most organisms (Dubruille and Emery, 2008). This is not surprising because temperature fluctuation is closely associated with the daily solar cycle of light and darkness. It tends to be warmer during the day and cooler after sunset. Evidence has shown that even warm blooded organisms (not just non-thermoregulated organisms, like insects) are entrainable by daily temperature cycles (Lindberg and Hayden, 1974; reviewed in Rensing and Ruoff, 2002; Tokura and Aschoff, 1983; Yoshii et al., 2002). This implies that the oscillator itself has an intrinsic input mechanism that is responsive to temperature.

Despite being responsive to temperature, the speed of circadian oscillators does not change significantly over a wide range of physiologically relevant temperatures. Normally, increases in temperature lead to acceleration in the rates of biochemical reactions. The free running period of circadian clocks is strongly temperature compensated and remains approximately the same over a range of ambient temperatures (Hastings and Sweeney, 1957; Pittendrigh, 1954). It was proposed and later experimentally suggested that the circadian system is composed of two opposing biochemical reactions, whereby temperature equally increases both reactions, leading to

counter-balance of each other (Baker et al., 2009; Mehra et al., 2009). This temperature compensation, not thermo-insensitivity, is essential for accurate timekeeping under diverse environmental conditions. This makes biological sense because a solar day is 24 hours long no matter if it is a cold winter or a warm summer day. Thus, while changes in temperature can adjust the phase and amplitude of circadian clocks it does not alter its frequency.

The circadian system has been conceptualized as being composed of three interconnected parts: 1) the input pathways that connect the clock to the external environment; 2) the core oscillator that is able to generate and sustain rhythms in the absence of time cues; and, 3) the output pathways that manifest temporally regulated downstream biological functions. Single cells can operate as circadian clocks. In multicellular organisms, circadian clocks can be found operating in many different tissues (Schibler, 2009). The internal temporal order is achieved by a multi-oscillatory circadian organization. For instance, in higher organisms, such as mice, circadian clocks are not confined to the brain but also reside in non-innervated peripheral organs with different tissue-specific functions that may be coordinated by the master pacemaker in the brain (Liu et al., 2007). Clock cells of both the master pacemaker and peripheral oscillators contain similar molecular components, widely known as clock genes, essential for generating oscillation autonomously at the cellular level. Nevertheless, it is not just genes in the cell but also the intercellular communications between different clock cells of different tissues that play a role in the circadian physiology of organisms as a whole (Cuninkova and Brown, 2008). Even within the brain, the central pacemaker works as an integrated circuit, in which different clock neurons contribute separately, but

coordinately, to run physiological and behavioral rhythms of the organism (Nitabach and Taghert, 2008).

The roles of circadian clocks can be best appreciated when we examine what happens when circadian rhythms are disrupted. Many travelers who have flown across time zones have experienced the groggy realization that while your day is just beginning in NYC, the night you just left in San Francisco is hardly over. This is usually associated with gastrointestinal disturbances, decreased vigilance and attention span, and general feeling of malaise, which are all indications of desynchronized internal physiological rhythms with local time (Panda et al., 2002). Similarly, shift work personnel are normal, healthy subjects who experience abrupt changes in light-dark cycles, which leads to different rates of resynchronization amongst oscillators found in various peripheral tissues (Stokkan et al., 2001). In addition, there are also disease-related disorders due to altered zeitgeber sensing, core oscillator malfunction, or failure to synchronize among peripheral oscillators. For example, there is a chronic disorder of timing of sleep relative to societal norms that is known as familial advanced sleep-phase syndrome (FASPS). The patient is consistently feeling sleepy in the early evening (7:30pm) and spontaneously awaken very early in the morning (4:30am). This was shown to be an inheritable disorder characterized by intrinsically short circadian period (Jones et al., 1999). And interestingly, Toh et al. (2001) later revealed that such heritable disorder is due to a point mutation in one of the human clock genes, *Period2* (*hper2*), which affects the phosphorylation of hPER2 by casein kinase 1-epsilon (discussed in more detail below).

The studies of FASPS highlights a recent trend in the circadian field. Traditionally, circadian researchers were limited to examining the physiological and behavioural outputs of the clock and establishing a conceptual model to describe the clock. Genetics and molecular analysis of the circadian clock bloomed after Konopka and Benzer (1971) discovered the first clock gene, *period* (*dper*), in the model organism *Drosophila*. Not until almost two decades later, another 2 clock genes, *timeless* (*dtim*) and *cryptochrome* (*dcry*) were discovered as a result of induced mutations in the same model organism (Hsu et al., 1996; Myers et al., 1995; Sehgal et al., 1994; Stanewsky et al., 1998). The findings immediately accelerated the search of orthologs in mammals, based on homology search: *cry1, 2* (Hsu et al., 1996); *per1, 2*: (Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997); *tim*: (Zylka et al., 1998). In addition, forward genetics common to *Drosophila* researchers was adapted in mice, leading to the discovery of the *clock* (*mclk*) gene (Takahashi et al., 1994; Vitaterna et al., 1994). Surprisingly, an independent forward genetics studies in *Drosophila* later uncovered a circadian gene homologous to *mclk* (Allada et al., 1998; Bae et al., 1998; Darlington et al., 1998). It is now well established that the clock mechanism in *Drosophila* and mammals, including humans, are very similar. In addition, many clock related physiological functions in mammals are also found in the invertebrate, like sleep-wake cycles (Hendricks et al., 2000). Hence, the availability of tractable genetic systems, rapid generation of mutants, and conservation of genes and physiology across species underscores the importance of the use of *Drosophila* in understanding the fundamental mechanism underlying circadian regulation of complex behaviors.

1.2 Overview of the *Drosophila melanogaster* circadian clock

The important role of *Drosophila* as a model organism to study circadian rhythms is highly attributed to the ease of quantifying overt phenotypes driven by the internal circadian clock. Earlier characterization of endogenous circadian clock properties (temperature compensation, entrainment properties) relied on measuring the daily emergence of young adults from their pupal case at the end of metamorphosis (eclosion) (Pittendrigh, 1981). With the development of automated systems, it was possible to measure locomotor activity, the cycles of activity and rest, of individual flies, which provides a much efficient, versatile, robust and reliable readout of the circadian clock. The locomotor activity of *Drosophila melanogaster* exhibits a bimodal or crepuscular pattern under standard 12 hours light and 12 hours dark cycles (12:12 LD). There is a "morning" peak and an "evening" peak of activity at the dark-to-light and light-to-dark transitions, respectively, separated by a midday siesta and nighttime inactivity (Figure 1.3). The "evening" activity component is usually assayed as a *bona fide* readout of the circadian system because it is most visibly persistent in constant darkness whereas the "morning" peak usually coincides with a direct stimulatory effect of light (or "startle" response) following dawn (Wheeler et al., 1993). This behavioral rhythmicity persists for the life-time of the animal many days after switching to constant dark condition, revealing the intrinsic endogenous free-running period (Klarsfeld et al., 2003). By identifying mutant flies with altered behavior rhythms, this led to the identification and characterization of the major components in circadian timekeeping. At least a dozen clock relevant genes have been discovered so far: *period* (*dper*), *timeless* (*dtim*), *clock* (*dclk*), *cycle* (*cyc*), *cryptochrome* (*dcry*), *vrille* (*vri*), *Par domain protein 1 Epsilon*

(*Pdp1ε*), *clockwork orange* (*cwo*), *double time* (*dbt*), *shaggy* (*sgg*), *Casein kinases 2 alpha* and *beta* (*Ck2α* and *β*), and *Pigment dispersing factor* (*Pdf*). Indeed, many of them were isolated by genetic screens based on pupal emergence and/or locomotor activity (e.g., *dper*, *dtim*, *dclk, cyc, dcry, dbt, CkIIα* and *β*). Assisted by a formidable arsenal of genetic tools and biochemical analysis, a very complex working model describing the circadian system has emerged.

As in all model organisms analyzed to date, the *Drosophila* central clockwork is based on the rhythmic abundance or activity of one or more clock proteins. A component whose rhythmic change is central to progression of the clock is termed a "state variable" of the clock. Not all clock factors are state variables but help maintain the rhythmic properties of the "state variable". Cycles in clock factors are usually achieved by complex negative feedback loops. The feedback loop is mainly transcriptional, in which the rhythmicity of transcription activator function is dependent on the transactivation of negative elements that then will feed back (directly or indirectly) to negatively regulate its own expression by blocking the transcription activator. The negative element, dPER is a *bona fide* "state variable", in which the daily fluctuations in the abundance or activity of protein, as opposed to simply their presence, are inextricably linked to the phase of the clock. Based on the current model of the *Drosophila* molecular clock, there are 3 interlocked transcriptional-translational feedback loops (Figure 1.1): (1) the *dper/dtim* loop that is driving the rhythmic expression of dPER and dTIM (transcription repressors) to ensure a self-sustainable oscillator; (2) the *dClk* loop that is responsible for cycling of *dClk* transcript (transcription activator) serving to fine-tune the oscillator; (3) the *cwo* loop that helps maintain high amplitude of the molecular oscillation.

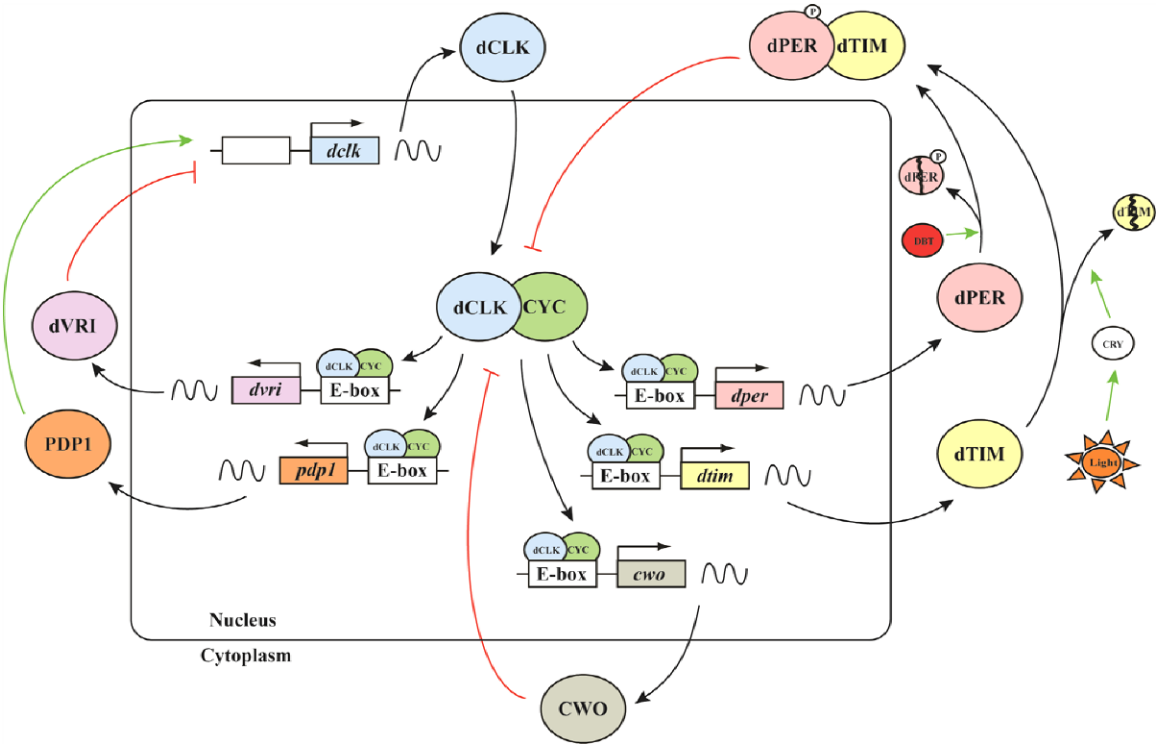


Figure 1.1. Overview of transcriptional/translational circuits underlying the *Drosophila* clock.

Shown are the components and control logic of the core clock in *Drosophila*. It is made up of three transcriptional/translational feedback loops that interlock via the transcription factors, dCLK and CYC (see text for details). Lines represent pathways of the clock circuitry; green arrows denote activating effect; red lines terminating in bar represent repressing effect; small p in circle indicates phosphorylation; squiggly lines show cycling in levels.

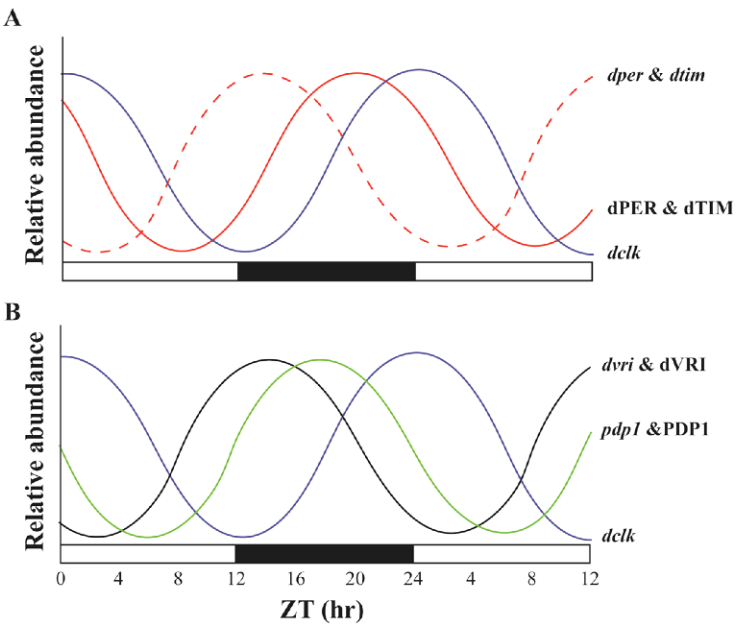


Figure 1.2. Daily abundane cycles for clock mRNAs and their protein products in *Drosophila*.

Daily cycling profiles for clock RNAs and proteins in fly heads are shown during a 12hr light and 12hr dark cycle. Note that temporal profile of the *dClk* protein product was not shown because it is relatively constant throughout a daily cycle (see text). White bar denotes light phase; black bar represents dark phase.

In the *dper/dtim* feedback loop, the bHLH/PAS (basic helix-loop-helix/PER-ARNT-SIM) containing transcription activators, dCLK and CYC heterodimerize and turn on the expression of *dper* and *dtim* by binding to E-box elements on their promoters (Darlington et al., 1998; Hao et al., 1997; Hao et al., 1999). The mRNA levels of *dper* and *dtim* rise at midday and reach maximum values during the early night (Figure 1.2A). Their protein products accumulate several hours later (Edery et al., 1994; Hardin et al., 1990; Marrus et al., 1996; Zeng et al., 1996; Zerr et al., 1990). Without dTIM, dPER is subjected to phosphorylation by DBT, homolog of the mammalian CK1 Ɛ/δ kinase followed by proteosome degradation (Figure 1.1) (Kloss et al., 1998; Kloss et al., 2001; Ko et al., 2002). Once threshold levels of monomeric dPER and dTIM are achieved in the late day/early night, dTIM binds dPER to protect it from degradation and helps stimulate transportation of dPER into the nucleus, which occurs during the midnight (Gekakis et al., 1995; Meyer et al., 2006; Shafer et al., 2002). In the nucleus dPER represses the transactivation activity of dCLK/CYC, forming a negative feedback loop (Figure 1.1) (Chang and Reppert, 2003; Darlington et al., 1998; Kim and Edery, 2006; Lee et al., 1998, 1999; Rothenfluh et al., 2000; Yu et al., 2006). As a result, the mRNA levels of *dper* and *dtim* start declining to trough values in the early day (Figure 1.2A). In the absence of the transcript and hence absence of *de novo* protein synthesis, the repression of dCLK/CYC starts to wear off in the early day as dPER and dTIM protein level decline (Figure 1.2A). As a result, another cycle of dCLK/CYC dependent transcription begins. In addition to driving cyclical expression of clock genes, such as *dper* and *dtim*, rhythmic activation and repression of dCLK/CYC can also drive cyclical transcription of downstream effector genes that ultimately lead to circadian rhythms in

physiology and behavior (Jin et al., 1999; Lopez-Molina et al., 1997; Ripperger et al., 2000). For instance, the expression of a transcription activator, *Pdp1ε*, which is part of the clock (discussed below) and also plays a role in circadian output locomotor activity rhythms of *Drosophila*, is rhythmically activated by dCLK/CYC (Benito et al., 2007; Zheng et al., 2009).

The current working model includes an additional interlocked transcriptional feedback loop that governs the rhythmic transcription of the positive element, *dClk*. Like *dper* and *dtim*, the transcript level of *dClk* is rhythmic but it cycles in antiphase, accumulating right after dusk and peaking at dawn (Figure 1.2A) (Bae et al., 1998). This rhythmic expression is driven by the alternative activity of a transcription repressor, VRI and a transcription activator, PDP1ε. Interestingly, the expression of *vri* and *Pdp1ε* are rhythmically regulated by dCLK/CYC. VRI protein accumulates first and represses *dClk* expression. PDP1ε protein then peaks and out-competes VRI on the *dClk* promoter to activate *dClk* transcription after VRI-mediated repression ends in the middle of the night (Cyran et al., 2003; Glossop et al., 2003). Intriguingly, the level of *dClk* protein abundance stay relatively constant throughout a daily cycle (Kim and Edery, 2006; Yu et al., 2006). Nevertheless, the role of *dClk* cyclic expression regulated in this second interconnected feedback loop is to add robustness to the molecular clock (Preitner et al., 2002).

The complexity of this interconnected transcriptional feedback system is further revealed by recent identifications of a new player, called *clockwork orange* (*cwo*) (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). Transcription of the *cwo* is activated by dCLK/CYC via E-box element (Figure 1.1) and

it oscillates in phase with *dper* and *dtim* (Figure 1.2B). The protein product of *cwo* feedbacks to repress its own transcription and represses other dCLK/CYC dependent transcription by binding to the E-box elements. As a result, this particular feedback mechanism helps sustain a high amplitude molecular oscillation (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). However, it is unlikely to be central to clock function.

1.2.1 Transcriptional and post-transcriptional regulation

Within the transcriptional feedback loops, cycling of *dper* mRNA plays a critical role in fine-tuning the molecular oscillator. Although dPER levels cycle in the absence of mRNA cycling (Cheng and Hardin, 1998a; Frisch et al., 1994; Vosshall and Young, 1995; Yang and Sehgal, 2001), rhythmic *dper* transcription allows additional precision in timing of the appearance and disappearance of the protein ensuring a robust and precisely phased clock (Chen et al., 1998; Kadener et al., 2008; So and Rosbash, 1997; Stanewsky et al., 2002). Kadener et al. (2008) have shown that although constitutive expression of *dper* can rescue behavior rhythmicity, the period distribution of individual flies is unusually broad. This less precise period determination is consistent with earlier studies in which constitutive expression of *dper* under different promoters (Cheng and Hardin, 1998b; Vosshall and Young, 1995) or without promoter (Frisch et al., 1994) showed weak rhythms in dPER abundance with low amplitudes and altered phases. In addition, improving the transcription rate of *dper* by enhancing the transactivation properties of the dCLK/CYC, alters the rhythmicity of the dPER oscillation, consistent with a critical role for RNA cycling (Hao et al., 1999; Kadener et al., 2008). Such manipulations drive

increased *dper* transcription that leads to more rapid accumulation of *dper* RNA and protein, which then phase advances subsequent dPER-mediated repression.

In addition to transcriptional regulation, post-transcriptional regulation also plays an important part in sustaining the oscillation of *dper* mRNA. The mRNA metabolism of clock components is temporally regulated through rhythmic post-transcriptional modifications, contributing to the cycling amplitude of the oscillation as well as fine-tuning the phase of the oscillation (Chen et al., 1998; So and Rosbash, 1997; Stanewsky et al., 2002). Comparison of *dper* transcription rates and its transcript accumulation profile indicates a significant delay in the daily rise of mRNA upon transcription activation, whereas the daily downswing in *dper* mRNA immediately follows the drop in transcription activity. These differences suggest a temporal regulation of *dper* RNA stability (So and Rosbash, 1997). Indeed, a transcript that is stable throughout the circadian cycle would tend to lower or even abolish the overall amplitude of the transcriptional oscillation (So and Rosbash, 1997; Wuarin et al., 1992). To have a relatively short transcript half-life, the dynamic range occurring at the transcriptional level can also be more accurately reflected (Edery, 1999). Contribution of RNA stability on phasing of circadian rhythms is also implicated in a study whereby the 3'UTR of *dper* was switched with that from tubulin. Such modification presumably rendered the transcript more stable causing earlier accumulation of *dper* RNA/protein and therefore shorter period of behavior rhythm (Chen et al., 1998).

Post-translational regulation plays a critical role in temporally separating the inhibition and activation events of the feedback loops in order to prevent dampening of the oscillation. Such modification at the protein level appear to be the primary means to

bring about biochemical time constraints in order to generate a molecular cycle that takes approximately 24 hour to complete. One of the most common and well-studied post-transcriptional regulations is the modulation of the phosphorylation state of one or more clock proteins. A key player in the feedback loops, namely dPER was shown to undergo daily changes in phosphorylation (Edery et al., 1994). The phosphorylation state of dPER serves as a tag to define its stability and subcellular localization thereby affecting the dynamic of the molecular oscillator and hence setting the pace of the oscillator (reviewed in Bae and Edery, 2006). Hyperphosphorylated dPER is preferentially bound by an E3 ligase, SUPERNUMERARY LIMBS (SLMB) and subjected to the ubiquitin-proteosome degradation pathway (Grima et al., 2002; Ko et al., 2002). Delayed accumulation of dPER protein compared to its mRNA (Figure 1.2A) is due to the coordinated activity of DBT and PHOSPHATASE PROTEIN 2A (PP2A) (Kloss et al., 1998; Ko et al., 2002; Price et al., 1998; Sathyanarayanan et al., 2004). The regulation of dPER accumulation is further dictated by the role of dTIM. dTIM binding to dPER prevents DBT dependent dPER degradation (Kloss et al., 1998; Kloss et al., 2001). Also dTIM levels are regulated through phosphorylation by an ortholog of human GLYCOGEN SYNTHASE KINASE 3β (GSK3β), called SHAGGY (SGG) (Martinek et al., 2001). This introduces another layer of time constraint that regulates the timing of nuclear translocation of dPER and dTIM proteins (Figure 1.1). The nuclear import of the dPER/dTIM complex was found to be affected by DBT, PP2A, SGG, and CASEIN KINASE 2 (CK2) (Akten et al., 2003; Lin et al., 2002; Nawathean and Rosbash, 2004). Once in the nucleus, dPER undergoes progressive phosphorylation.

Besides setting the pace of the clock, phosphorylation-dependent changes in clock protein stability also helps adjust the phase of the clock in response to light cues. In other words, it is part of the underlying mechanism on how the circadian clock entrains to daily light dark cycles. The initial accumulation of dPER in the cytoplasm at early evening and degradation/turnover of dPER in the nucleus at late night/early day are intimately gated by light via dTIM. The light signal is perceived by a blue-light photoreceptor, CRYPTOCHROME (CRY) (Stanewsky et al., 1998). Upon activation by light, dCRY promotes the degradation of dTIM via the ubiquitin-proteosome pathway (Figure 1.2A) (Koh et al., 2006; Naidoo et al., 1999; Peschel et al., 2009; Stanewsky et al., 1998). Since the stability of dPER is dependent of dTIM, this ensures that the dPER cycling is synchronized to the light-dark cycle. When sunset is later than usual, light exposure extends into the early night and delays the accumulation of dPER in the cytoplasm and hence the timing of its nuclear entry (phase delay); whereas, when sunrise is earlier than usual, premature exposure to light during the late night accelerates the degradation of dPER/dTIM complex in the nucleus (phase advance) allowing earlier start of another round of dCLK/CYC-mediated transcription (Myers et al., 1996; Lee et al., 1996).

1.2.2 *Drosophila* pacemaker circuit

The classic forward genetic approach (screening mutants) has been instrumental in advancing our understanding of the basic clockworks through identifying molecular components that constitute a clock. Despite system-level complexities, such analyses indicated that circadian systems largely operate in a cell-autonomous manner. Nevertheless, several recent observations have suggested that the circadian wake-sleep is

driven by extensive neural interactions among functionally distinct neuronal clock cells in the adult fly brain (reviewed in Nitabach and Taghert, 2008).

As mentioned earlier, clock cells are widespread throughout *Drosophila* (Plautz et al., 1997), but only a small number of neurons found in the *Drosophila* adult brain show molecular oscillations in clock gene expression and are responsible for circadian regulation of wake-sleep cycles (Nitabach and Taghert, 2008). These 150 out of 100,000 neurons are anatomically sub-divided into seven groups of neurons: the small and large ventral lateral neurons (s-LN_vs and l-LN_vs, respectively), the dorsolateral neurons (LN_ds), three groups of dorsal neurons (DN1-3), and the lateral-posterior neurons (LPN) (Kaneko and Hall, 2000; Nitabach and Taghert, 2008; Rieger et al., 2006; Shafer et al., 2006). These anatomically separate neuronal groups can be functionally separated into distinct rhythmic centers controlling different episodes of rhythmic behavior that define the fly's overall daily activity profile. For example, the morning bout of activity is driven by the s-LN_vs (defined as morning oscillator), which were found to send a resetting signal, a rhythmically secreted neuropeptide called PDF, to the evening oscillator that includes a combination of the LN_ds, non-PDF s-LN_vs, and a subset of DNs (Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2005). Rhythmic secretion of PDF in cells including l-LN_vs as well as s-LN_vs, are required for synchronizing different clock neurons and hence maintenance of self-sustained behavioral rhythms in constant darkness (Lin et al., 2004; Renn et al., 1999). Also, several recent studies reveal that various group of neurons appear to have differential roles in light and temperature entrainment of behavioral rhythms: evening oscillator plays a role in perceiving light cues (Murad et al., 2007; Picot

et al., 2007; Stoleru et al., 2007), whereas, LPNs appear to be preferentially entrained by daily temperature cycles (Busza et al., 2007; Miyasako et al., 2007; Shafer et al., 2006).

1.3 Role of circadian clock in seasonal adaptation: lessons from *Drosophila*

As the Earth makes its yearly orbit around the Sun, the planet's 23.5º axial tilt leads to cyclical environmental changes that define our seasons (e.g. summer and winter) through increases and decreases in the angle of incidence of sunlight hitting the Earth (e.g. equinox and solstice). Seasonal changes expose organisms to pronounced variations in external conditions such as photoperiod (day/night length), weather changes, food availability, and associated ecological niche. Such annual variations would be increasingly wide as one moves further away from the equator (temperate region, Arctic). Therefore, it is critical for organisms, especially from temperate regions, to anticipate transitions of seasons through developing appropriate behavioral and physiological adaptations to protect adults from severe seasonal conditions or to restrict their reproduction to the optimal time of year for survival of the young (Dunlap et al., 2003). Examples of seasonal adaptations include, entering a quiescence state to conserve energy during food scarcity; migratory flight away from harsh winter environment; molting into thicker fur or feathers for insulation against the cold; seasonally timed regression of reproductive systems.

In addition to keeping in-sync with daily local time, circadian clocks also play a critical role in adapting to cyclical annual environmental challenges. Many studies on seasonal adaptation have shown a strong interplay between seasonal responses and circadian controlled activities that suggest a role of circadian clock in decoding seasonal

cues. Small endotherms, such as bats and hamsters, which cannot afford thermoregulation throughout a cold winter day because they are extremely metabolic and with great surface loss of heat, go through circadianly regulated heterothermy during winter (Körtner and Geiser, 2000). They remain homeothermic when active but reduce their metabolisms drastically (poikilothermic) when at rest to conserve energy. Nocturnal migratory birds, like warblers, which are normally day active, develop additional locomotor activity at night in preparation for night time migratory flight during autumn and spring (return to warmer region) (Gwinner, 1996). The birds also exhibit changes in synchronization properties of their circadian systems in such a way that circadian rhythms adjust faster to new conditions after long transmeridian flight. Thus, circadian changes in physiology and behavior are closely associated with yearly rhythms in these species.

In order to understand how circadian controlled activities respond to seasonal changes, it is important to understand the role of seasonal variables, namely day length and temperature in the regulation of circadian rhythms. The duration of day length (photoperiod) can modify the temporal alignment between a circadian rhythm and local time. The daily distributions of physiological and behavioral rhythms are not rigidly locked to local time but can be adjusted for seasonal changes in day length. In the field, the circadianly regulated locomotor activity of sleep lizard (*T. rugosa*) becomes prevalently more bimodal, as opposed to unimodal, in long summer day, presumably to avoid hot midday weather (Ellis et al., 2008; Firth and Belan, 1998). Indeed, there is a neural network of two distinct cellular pacemakers (morning and evening) in the *Drosophila* adult brain, which in turn, dominate the circadian neural circuitry governing

circadian locomotor behavior in a day length dependent manner, presumably imposing activity patterns appropriate for particular seasons. For example, during long nights, the morning oscillator dominates, whereas during long days, effects of the evening oscillator are predominant (Stoleru et al., 2007).

Ambient temperature is also a key environmental modality regulating the timing of daily activity patterns in animals (Rensing and Ruoff, 2002). This makes intuitive sense because in temperate latitudes, seasonal changes in day length are also accompanied by predictable changes in average daily temperatures. Attending to multiple seasonal cues can ensure precise seasonal timing under seasonal environment with year-to-year variability. It was shown that the steady-state phases of daily behavioral rhythms can vary as a function of temperature even during entrainment by daily light/dark cycles (Ellis et al., 2007; Majercak et al., 1999; Sweeney and Hastings, 1960). For example, garter snakes, which display a bimodal distribution of activity at moderate temperatures, are mainly nocturnal at warm temperatures, and diurnal at cold temperatures (Heckrotte, 1962). In the water midge, long photoperiod will delay the timing of activity when kept at 20ºC while the same photoperiodic regiment will not delay its activity at colder temperatures (Kureck, 1979). In general, this directional response has a clear adaptive value, displaying a greater proportion of their activity during the cooler nighttime hours on hot days and conversely the warmer daytime hours during cold days (Sweeney and Hastings, 1960).

In search for the underpinnings governing how changes in average daily temperatures modulate activity rhythms entrained by daily light:dark cycles, our lab has presented the first description of molecular mechanisms that underlie the effects of

temperatures on the daily distribution of activity through regulation of circadian clock genes (Chen et al., 2006; Majercak et al., 2004; Majercak et al., 1999). Using *Drosophila melanogaster* as a model system, we showed that post-transcriptional regulation at the 3' UTR region of *dper* is responsible for the phasing of daily wake-sleep cycles in response to temperature cues (Majercak et al., 1999). In response to low temperature (cold winter day), removal of an intron found in the 3'UTR region of *dper*, called *Drosophila melanogaster period* intron 8 (dmpi8) was enhanced. Temperature has a major effect in setting the mean daily splicing efficiency independent of the clock (Collins et al., 2004; Majercak et al., 2004). More splicing is accompanied by increases in *dper* mRNA and a more rapid accumulation phase, presumably underlying the earlier timing of evening activity (Figure 1.3) (Majercak et al., 1999). With increasing temperature (summer), the splicing efficiency is reduced and evening activity becomes progressively more nocturnal and midday inactivity more pronounced, likely ensuring that flies avoid activity during the hot midday sun when they are at increased risk of desiccation. Mutations that inhibit splicing of dmpi8 led to lower levels of *dper* mRNA and protein, and display nocturnal evening activity even on cold days (Majercak et al., 1999). It was suggested that removal of the 3'UTR intron stimulates 3'-end formation leading to an increase in mature transcripts (Figure 1.3) (Majercak et al., 1999). Temperature also has a strong influence on clock regulation of dmpi8 splicing (Collins et al., 2004; Majercak et al., 2004). The clock helps maintain lower dmpi8 splicing levels during the day and stimulates it during the night. The amplitude of the clock-regulated daytime repression in splicing increases as temperature rises, suggesting another level of adaptive response to avoid hot midday condition.

In addition to temperature, light has small but significant effects on the splicing efficiency of this intron, with shorter day shown to stimulate splicing (Collins et al., 2004; Majercak et al., 2004). Hence this suggests that regulation of *dper* splicing acts as a seasonal sensor in the circadian clock, in which multiple external cue (temperature, and photoperiod) are integrated. And this sensing mechanism is mediated by the post-transcriptional differential splicing of the 3'-terminal intron of *dper*.

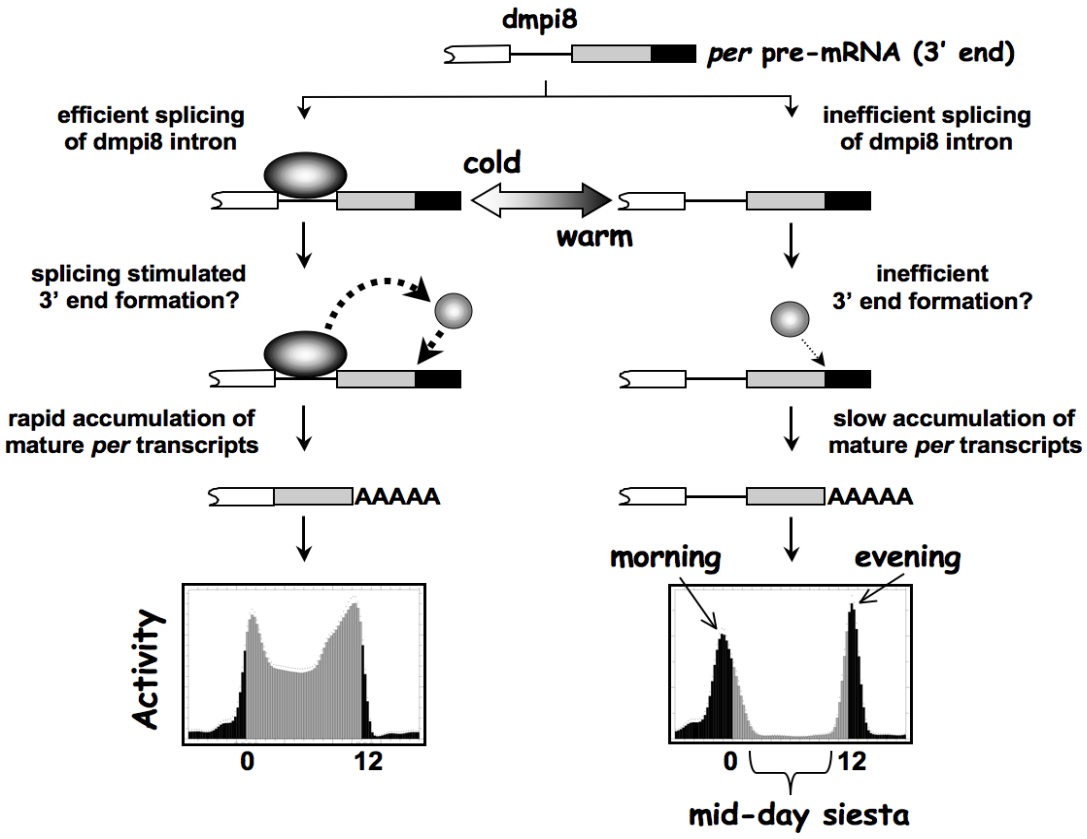


Figure 1.3. Model for how temperature regulates the phase of daily activity in *Drosophila melanogaster*.

Shown at the top is the 3' end of the *dper* precursor mRNA (pre-mRNA); (white box) sequences from translation stop codon to just upstream of 5' splice site of dmpi8; (horizontal line) dmpi8 intron; (gray box) sequences following 3' splice site of dmpi8 until 3' cleavage site; (black box) transcribed sequences downstream from 3' cleavage site. Cold temperatures enhance binding of spliceosome (large oval), which stimulates binding of 3'-end formation factors (small circle), hence more rapid accumulation of *dper* transcripts, leading to advanced evening activity and less prominent midday inactivity. Conversely, on warm days, the inefficient splicing of dmpi8 leads to an extended midday siesta and preferential nocturnal evening activity, events that enhance the ability of

Figure legend of Figure 1.3 continued.

Drosophila to avoid the deleterious effects associated with the hot midday sun. The histograms (bottom) represent daily locomotor activity patterns of *Drosophila* under 12 hr of light and 12 hr of darkness at either 18°C (left) or 29°C (right). The grey bars represent day time activity and the black bars represent night time activity. (Adapted from Majercak et al., 1999)

The thesis work presented here sought to understand the molecular basis for the thermosensitive splicing of the dmpi8 intron. This was done through analysis of daily activity patterns coupled with molecular studies of transgenic flies and natural populations of *Drosophila* originating from different localities. It was shown that multiple suboptimal splicing signals are the key to temperature dependent dmpi8 splicing, enabling *Drosophila melanogaster* to prolong its midday "siesta", a mechanism that likely diminishes the deleterious effects of heat during the longer summer days in temperate climates. In addition, the thesis work identified natural polymorphisms that affect dmpi8 splicing efficiency that explain strain-specific differences in daily activity patterns. Intriguingly, a comparative study of *Drosophila* species with worldwide distributions and those with more restricted ancestral distributions in Afro-equatorial climates, suggest that the thermosensitive splicing of the *dper* 3'-terminal intron contributed to the ability of some species to adapt to temperate regions. On a broader implication, results in this thesis suggest that RNA:RNA interactions with the ability to form base-pair interactions varying in strength appear to be ideal targets for constructing intracellular molecular mechanisms that can be targeted by natural selection to establish species-specific thermal adaptation.

1.4 Precursor mRNA splicing

Because much of my thesis work involved pre-mRNA splicing, a brief overview of this pathway is presented (summarized in Figure 1.4):

RNA splicing is a modification of newly transcribed nascent pre-mRNAs in which introns are removed via two sequential transesterification steps followed by fusion of the

flanking exon sequences (reviewed in Staley and Guthrie, 1998). Such modifications are part of the pre-mRNA maturation process before it can be used to produce correct protein through translation. This splicing reaction involves two major components: (1) the *trans*-factors that consist of highly dynamic RNA-containing protein machinery, called spliceosome and (2) the *cis*-elements that assist the splicing machinery in recognizing the proper exon-intron boundaries.

The spliceosome is a constantly changing conglomerate of protein complexes called small nuclear ribonucleoprotein (snRNP). There are five major snRNPs (U1, U2, U4, U5, U6 snRNPs), in which the identities are defined by the uridyl-rich RNA components enclosed by the specific subset of protein subunits. Base-pairing of these snRNAs to the consensus *cis*-splicing elements facilitates binding of target intron. These protein subunits and snRNAs assemble anew on each nascently transcribed mRNA. Such assembly is assisted by four main splicing determinants that generally control the efficiency of splicing: (i) 5' splice site (ss), (ii) 3' ss, (iii) branch point sequence, and (iv) uridine-rich (U-rich) polypyrimidine tract. In metazoans, additional exonic and intronic *cis*-acting regulatory sequences are also found to play critical roles in supplementing proper exon-intron recognition by the splicing machinery (Chasin, 2007). In addition, RNA splicing also involves a conserved family of serine-arginine rich splicing factors called SR proteins, which mainly play a regulatory role. These SR proteins consist of serine-arginine (SR) dipeptide repeat that facilitate binding with other SR containing proteins and at least one RNA recognition motif (RRM). It has been suggested that SR proteins functions as bridging factors between components in the spliceosome and regulatory factors (Graveley, 2000).

A series of highly ordered regulatory steps has to be completed in a stepwise manner to set the stage for the two catalytic phosphoryl transfer reactions. These involve a series of extensive rearrangements of intermolecular as well as intramolecular RNA:RNA and RNA:protein interactions facilitated by snRNPs, which require ATP hydrolysis, presumably to unwind the prevalent secondary or tertiary structures. This is because a lot of the interactions are mutually exclusive, in which the formation of one interaction required the disruption of another. The actual catalytic reactions, however, are mainly RNA based and was shown to occur independent of protein *in vitro* (Michel and Ferat, 1995). The splicing reaction begins when U1 snRNP recognizes the 5' SS, whereas U2snRNP binds the branch point region with the help of SR protein, U2AF (AF=auxiliary factor) that binds to the polypyrimidine tract usually located approximately 30nt upstream of 3' ss (Figure 1.4). This is followed by entry of a complex of three snRNPs, U4/U5/U6 that initiates a series of RNA-RNA rearrangements. As a result of such rearrangements between the snRNAs, U1snRNP is competed off by U5/U6 snRNP from the 5'ss and U4snRNP together with U1 snRNP are released from the spliceosome after escorting U5/U6 snRNP to the intron. The first transesterification reaction is initiated when 2'-OH group of the conserved branch point adenosine nucleophilic attacks 5' phosphate group of 5'ss guanosine forming a 5' to 2' phosphodiester bond. The subsequent phosphodiester bond, however, is formed between the 3'-OH group of upstream exon and 5' phosphate group of downstream exon resulting in formation of the spliced mRNA and release of the branched "lariat" intron for degradation (Figure 1.4).

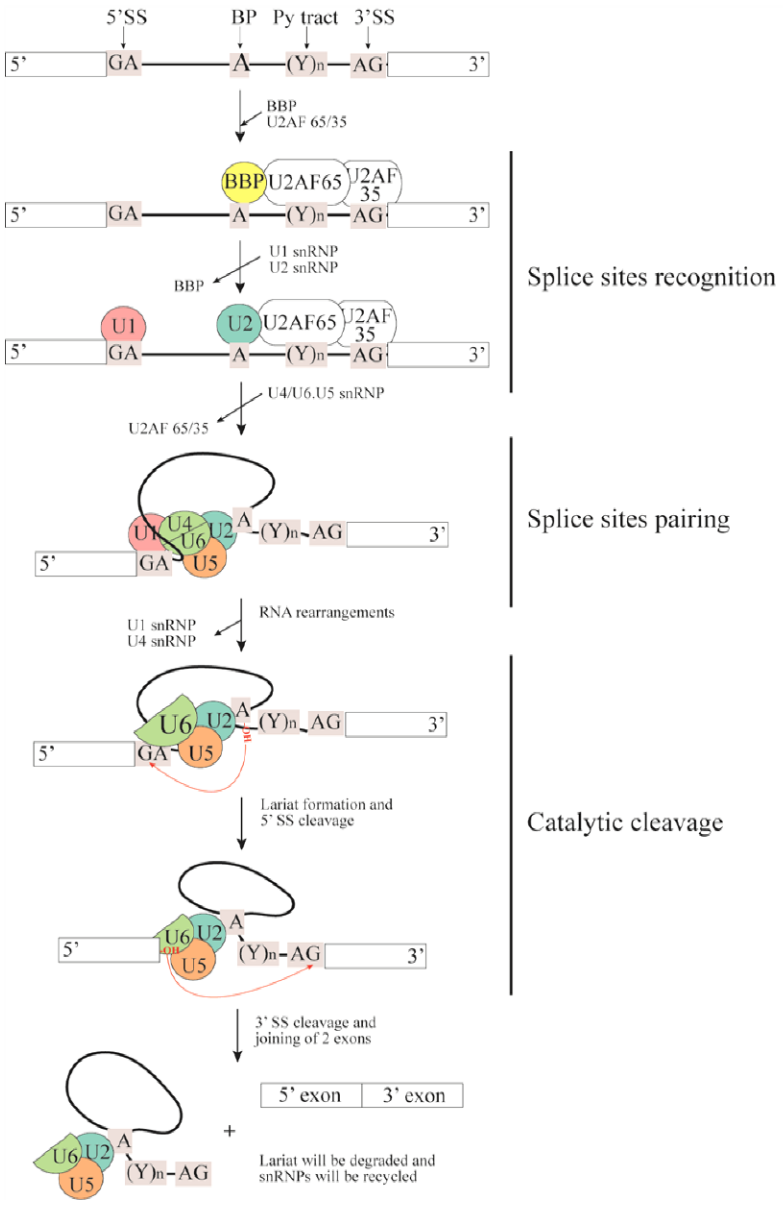


Figure 1.4. Overview of metazoan pre-mRNA intron splicing by a highly dynamic spliceosomal complex.

Shown are spliceosome assembly, rearrangement, and disassembly at critical steps of the splicing reaction (see text for details). Sequence elements required for splicing are shown in gray shade. The 5' end of the intron is mainly defined by the 5'ss 'GA' dinucleotide

Figure legend of Figure 1.4 continued.

whereas the 3' end is defined by the branch point adenosine, 'A'; polypyrimidine tract, '(Y)n'; and 3' ss 'AG' nucleotides. Exon sequences are denoted by open boxes and intron sequences are represented by line. The splicing reaction involves three major steps: (1) initial recognition of 5' and 3' end of the intron; (2) bringing together the correct pairs of 5' and 3' ss across the intron; and (3) two catalytic transesterification steps (Adapted from Alberts, 2008).

The description of the splicing reaction mentioned above is based mainly on vertebrate systems. It is important to keep in mind that there are some species-specific differences in splicing regulation (Mount et al., 1992). Fortunately, the consensus of splicing determinants (except polypyrimidine tract) of *Drosophila* introns and the participating *trans*-acting factors are conserved in flies compared to vertebrates. However, the average length of *Drosophila* introns are relatively short (~63nt) compared to vertebrate's (minimum required is 70nt) (Mount et al., 1992). In fact, there are two groups of introns that can be found in fruit flies, i.e. small (<81nt) and large (>80nt) introns. The large introns act similarly as those found in vertebrate. On the contrary, the small introns can only be spliced in *Drosophila* cell extracts but the mechanism is still not clear. Small introns in *Drosophila* usually lack the conventional U-rich polypyrimidine rich tract located between the branch point and 3' ss. Interestingly, the 3'-terminal dmpi8 intron of *dper* is 89 nt, a length which falls in the range of the upper and lower limits of the *Drosophila* small and large classes of introns, respectively. Understanding the molecular mechanism of dmpi8 splicing phenotype might shed some light on the underlying regulation of small intron splicing.

Chapter 2. Natural variation in the splice site strength of a clock gene and species-specific thermal adaptation

2.1 Introduction

Many animals exhibit a bimodal distribution of activity, with 'morning' and 'evening' bouts of activity that are separated by a midday dip in activity or 'siesta'. Cell-based circadian (≅24 hr) pacemakers drive these wake-sleep cycles, in addition to a multitude of other daily rhythms in physiological and behavioral phenomena. A physiologically relevant feature of circadian clocks is that they are synchronized (entrained) by environmental cues, most notably visible light and ambient temperature. Light is almost certainly the predominant entraining agent in nature that aligns circadian rhythms to local time, enabling life forms to anticipate environmental transitions and perform activities at biologically advantageous times during the day (Edery, 2000; Hastings et al., 1991). Ambient temperature is also a key environmental modality regulating the daily timing of circadian rhythms (Rensing and Ruoff, 2002; Sweeney and Hastings, 1960). For example, diurnal animals usually respond to colder temperatures by displaying a greater proportion of their activity during the warmer day-time hours, whereas night-time activity predominates at warmer temperatures. This directional response has a clear adaptive value, ensuring that the activity of an organism is maximal at a time of day when the temperature would be expected to be optimal for activity (Sweeney and Hastings, 1960).

Several years ago we used *Drosophila melanogaster* as a model system to understand how temperature evokes changes in the daily distribution of activity. Over a wide range of photoperiods and temperatures, the morning and evening bouts of activity in *D. melanogaster* are roughly aligned with the dark-to-light and light-to-dark

transitions, respectively (Majercak et al., 1999; Qiu and Hardin, 1996; Rieger et al., 2003). Nonetheless, temperature modulates both the morning and evening activity components by 'fine-tuning' their temporal distributions. As temperature increases, there is less midday activity and the morning and evening bouts are increasingly shifted into the cooler night-time hours (Majercak et al., 1999). The increase in nocturnal activity during warm days is almost certainly an adaptive response that ensures *D. melanogaster* minimizes the detrimental effects of the hot midday sun.

We showed that thermosensitive splicing of the 3'-terminal intron (termed dmpi8) from the key clock gene period (per) plays a major role in temperature-induced changes in the daily activity profile of *D. melanogaster* (Majercak et al., 1999). Expression of per is under circadian regulation, contributing to daily cycles in per RNA and protein levels, molecular oscillations that are inextricably linked to the state of the clock and its normal progression (Edery, 2000; Hastings et al., 1991). On seasonably cold days the proportion of dmpi8 spliced per mRNA compared to the unspliced variant is enhanced, leading to more rapid daily increases in total per transcript levels and earlier evening activity (Majercak et al., 1999). Active splicing of dmpi8 is required for increasing the abundance of per mRNA levels, leading to the hypothesis that assembly of spliceosomes at the 3'-terminal intron somehow produces more mature transcripts, possibly by facilitating 3'-end formation. Transgenic flies bearing variant per transgenes where splicing of dmpi8 was abrogated, manifested preferential nocturnal evening activity even on cold days (Majercak et al., 1999). Furthermore, the splicing efficiency of dmpi8 is regulated by the clock and photoperiod, with long days inhibiting intron removal (Collins et al., 2004; Majercak et al., 2004). The interplay between day length and temperature

makes intuitive sense because in temperate latitudes seasonal changes in day length are also accompanied by predictable changes in average daily temperatures. Together, the results suggest a model wherein dmpi8 splicing plays a central role in the seasonal adaptation of *D. melanogaster*, most notably by adjusting the timing of evening activity in response to changes in average daily temperatures.

A rather unique feature of *D. melanogaster* is that it has a wide distribution pattern from tropical to temperate regions, colonizing in a manner closely associated with human migration. In this report we sought to determine if a similar mechanism is operating in *D. yakuba*, a species closely related to the cosmopolitan *D. melanogaster* (Ko et al., 2003; Lachaise et al., 1988; Russo et al., 1995) but with a more ancestral distribution indigenous to Afro-equatorial regions wherein day length and temperature exhibit little fluctuation throughout the year. We show that although the per gene from *D. yakuba* also has a 3'-terminal intron, it is efficiently spliced over a wide range of temperatures, consistent with the little effect of temperature on the daily rhythms of per RNA levels and behavior in this species. The species-specific thermal splicing phenotypes are based on differences in the strengths of key splice sites, whereby multiple suboptimal splice signals on the 3'-terminal intron from *D. melanogaster* lead to gradual reductions in splicing efficiency as temperature increases, presumably because binding of the spliceosome to weak splicing signals is favoured at cold temperatures. A causal link between the strengths of canonical splice sites on per 3'-terminal introns and the thermal responsiveness in splicing efficiencies and daily activity patterns is also supported by studies in *D. santomea* and *D. simulans*, species closely related to *D. yakuba* and *D. melanogaster*, respectively. Our findings indicate that the weak splicing signals on

dmpi8 enables *D. melanogaster* to manifest a more robust and longer midday siesta, possibly facilitating adaptation to temperate climates where the longer days of summer are accompanied by prolonged periods of heat.

2.2 Materials and methods

2.2.1 Fly strains and general handling

The wildtype *D. melanogaster* data shown was obtained with the laboratory strain, Canton S. Similar results were observed with other standard strains of *D. melanogaster* (e.g., Oregon R, y w; data not shown). We show results from two different *D. yakuba* strains: In figure 2.1, we used descendents of a strain originally captured in 1955 in the Ivory Coast (Burla strain) and obtained from the Tucson Drosophila Stock Center (stock number; 14021-0261.00). In Figure 2.8, we used the Tai18E2 strain, a gift from Dr. Coyne, University of Chicago. This line is derived from an isofemale line termed Tai18 collected in 1981 in the Tai rainforest on the border between Liberia and Ivory Coast (described in, Coyne et al., 2004) and subsequently laboratory inbreeding led to the subline Tai18E2. Similar results were obtained using other *D. yakuba* isofemale lines that we received from Dr. Coyne (e.g., Tai30, SJ2, *D. yakuba* 2 and *D. yakuba* 45; data not shown). *D. santomea* (isofemale line ST0.4) was a gift from Dr. Coyne and originally collected in 1998 by Lachaise and co-workers (Cariou et al., 2001; Lachaise et al., 2000), whereas *D. simulans* (sim4 strain; originally captured in New Caledonia, Scotland) was obtained from the Tucson Drosophila Stock Center (stock number; 14021-0251.216). The generation of transgenic flies is described below. All flies were

routinely reared at room temperature (22-25°C) and maintained in vials or bottles containing standard agar-cornmeal-sugar-yeast-Tegosept-media.

2.2.2 Tissue culture constructs

We used the pUChsNeoAct5C vector (kindly provided by Dr. K. Irvine, Rutgers University, USA) as the backbone for generating constructs that express the luciferase (luc) open reading frame (ORF) fused to the dmper 3' UTR and flanking 3' genomic sequences. PCR was used in the presence of a previously described CaSpeR-4 based transformation vector containing a 13.2kb genomic dmper insert (termed CaSpeR13.2) (Cheng et al., 1998; Lee et al., 1998) to amplify dmper sequences from the stop codon to 90bp after the presumed poly(A) cleavage site (nucleotides 6869 to 7465, numbering according to (Citri et al., 1987). In addition, during the PCR we introduced a StuI restriction site just upstream of the dmper stop codon and a SalI site immediately after position 7465. This dmper-containing fragment was digested with StuI and SalI, then purified. In a second PCR we used the pGL3 plasmid (Promega, USA) as a template to amplify the luc ORF and introduce an EcoRI site just upstream of the start codon and a StuI site immediately before the luc stop codon. This luc-containing fragment was digested with EcoRI and StuI, then purified. Subsequently, a three-way ligation was performed with the two purified fragments and the backbone of pUChsNeoAct5C after digestion with EcoRI and SalI, resulting in a luc-dmper hybrid gene downstream of the pAct5C promoter (termed 8:8; Fig.2.3). Finally, we used standard PCR-based techniques to introduce an XhoI site 9bp upstream of the dmpi8 5'ss, and a KpnI site 10bp downstream of the 3'ss, yielding 8:8kx. We also generated a derivative of this plasmid by performing the same general procedure but further introducing BamHI sites

immediately 3' to the XhoI site and 5' to the KpnI site. Digestion with BamHI followed by ligation generated a construct that still retains the XhoI and KpnI sites but now linked via a BamHI site eliminating the dmpi8 intron to yield Δ8:8kx. To simplify the swapping of intronic sequences we digested the Δ8:8kx plasmid with EcoRI and SalI and subcloned the released luc-per fragment into the smaller pGEMT-Easy vector (Promega, USA), resulting in the intermediate vector termed Luc- Δ8-TA. Intron-containing sequences were first subcloned into Luc- Δ8-TA at the XhoI and KpnI sites. Subsequently, the plasmid was digested with StuI and SalI and the released fragment subcloned into either the 8:8kx or Δ8:8kx backbones digested with the same restriction enzymes.

For the dyp3' plasmid (Figs. 2.3 and 2.4), the dyp3' intron with 9bp of 5' and 10bp of 3' flanking sequences were amplified with XhoI and KpnI sites using PCR and D. yakuba genomic DNA (using the Ivory Coast strain used in this study) as template, and subcloned into the Luc- Δ8-TA backbone followed by the steps described above. A similar strategy was used to generate the 3:3 plasmid (Fig. 2.4) using PCR in the presence of CaSpeR13.2 to amplify intron 3 from dmper. Oligonucleotides with overhanging XhoI and KpnI sites were used to generate the 8:3 and 3:8 constructs (Fig. 2.4), which are hybrids between dmpi8 and intron 3 of dmper fused at the putative branchpoint (both have the same sequence, CTAAC). We used PCR to generate the hybrids between the dmpi8 and dyp3' introns (i.e., dyp3':8, 8:dyp3' and 8:dyp3'(3'ss)) (Fig. 2.4). Finally, mutants of dmpi8 with altered 5' and 3'ss (i.e., M1, M2, M3, M2M1 and M3M1; Fig. 2.3) were generated using the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA) and the 8:8kx vector as template. All final constructs used in this study (i.e., Figs. 2.3 and 2.4) were validated by DNA sequencing prior to further use.

2.2.3 Constructs for transgenic flies

We first generated a construct that contains a hybrid between dmper cDNA and genomic sequences with a StuI site just 5' upstream of the dmper stop codon (termed 8:8-CRS/hs/cper). This was generated by amplifying genomic dmper sequences from positions 5903 (137bp upstream of the SfiI site in exon 5 of dmper) to 7529 (225bp downstream of the Bsu36I site in the 3' UTR of 13.2 dmper genomic sequence) using CaSpeR13.2 (referred to as perG in Cheng et al (1998) as a template and introducing an AatII and SfiI sites at the 5' and 3' ends of the fragment, respectively. The amplified fragment was digested with AatII and EcoRI and subcloned in the shuttle vector, pSP72 (Promega, USA) to yield pSP72-per13.2-3'end. We then used the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA) to introduce a StuI site immediately upstream of the stop codon (pSP72-per13.2-3'endStuI). Subsequently, the StuI-to-Bsu36I fragment spanning from the stop codon to 234bp downstream of the dmpi8 3'ss was replaced with variants from the tissue culture constructs (8:8kx, dyp3', M2M1). Finally, the resulting constructs were digested with SfiI and Bsu36I and subcloned into the previously described CRS/hs/cper transformation vector (Hao et al., 1999) to yield 8:8-CRS/hs/cper, dyp3'-CRS/hs/cper and M2M1-CRS/hs/cper. Transgenic flies were generated by Genetic Services, Inc. (Sudbury, MA, USA) in a w1118 background and subsequently crossed into a w per01 background with a double balancer line (w per01;Sco/Cyo;MKRS/TM6B), resulting in the transgenic lines termed P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}. At least three independent lines for each construct were obtained. The results shown in this manuscript were derived by pooling

data from the following lines: P{dmper/8:8}, f9, f19, f46; P{dmper/dyp3'}, f6, f14, f22; P{dmper/M2M1}, f13, m17, m32.

2.2.4 Locomotor activity

Locomotor activity was continuously monitored and recorded in 15-min bins by placing individual adult male flies (three to seven day-old males) in glass tubes and using a Trikinetics (Waltham, MA, USA) system, as previously described (Rosato and Kyriacou, 2006). Briefly, throughout the testing period flies were maintained at the indicated temperature (18°, 25° or 29°C) and subjected to 5 days at the indicated photoperiod (LD; where zeitgeber time 0 (ZT0) is defined as lights-on), and in some cases followed by 5-7 days of constant dark conditions. Cool white fluorescent light (~2000 lux) was used during LD and the temperature did not vary by more than 0.5oC between the light and dark periods. Data analysis was done on a Macintosh computer with the FaasX software (kindly provided by M. Boudinot and F. Rouyer, CNRS, France), which is based on the Brandeis Rhythm Package (originally developed in the laboratories of J. Hall and M. Rosbash, Brandies University, MA, USA). The histograms (eductions) showing the distribution of locomotor activity through a 24 hr period (e.g., see Fig. 2.1) were obtained using the 'eduction' option of the FaasX software. The last 3 days worth of LD data were averaged for each fly, and data pooled to generate the group averages shown in 15-min or 30-min bins, as indicated in the figure legends. This included multiple independent experiments and for the transgenics, pooling results from at least two independent lines for eachs genotype. A correction applied to neutralize "startle response" (i.e., increased bout of fly activity following the light-to-dark and dark-to-light environmental transitions; essentially the activity counts in the bin right after the environmental

transition is replaced by an average of the activity counts in the bins just before and after) (Wheeler et al., 1993). In figure 2.5, daily locomotor activity profiles were normalized such that the peak of evening activity was set to 1, facilitating visual comparison of the different transgenic genotypes.

Free-running periods and power (amplitude or strength of the rhythm) were obtained using the Chi-square periodogram module available within the FaasX program using activity data collected in 30 min bins during at least 5 consecutive days in DD. Flies with power □10, width □2, and periods between 20-30 hr were designated rhythmic. Values for individual flies were pooled to obtain an average value for each genotype. The timing of morning and evening peaks, 50% morning offset and 50% evening onset were determined on a Unix command line version of the Brandeis Rhythm Package (BRP) Phase module. The values were based on pooling data from multiple individual flies over the last three days of LD using data collected in 30 min bins. ANOVA and appropriate post-hoc analysis were performed using SPSS 16.0 (SPSS Inc., Chicago, USA). Similar results were obtained when we varied the onset and offset phase reference points from 25 to 75% of peak values (data not shown), and results with 50% are shown as they were the most reproducible.

2.2.5 Tissue culture transfection and collection

The S2 cells and DES expression medium were purchased from Invitrogen and all procedures were performed according to manufacturer's instructions. To generate stable transformants, the Calcium Phosphate Transfection Kit (Invitrogen, USA) was used according to the manufacturer's instructions. Transient transfections were performed using Effectene (Qiagen, USA) according to manufacturer's instruction. Briefly, 0.5 mg

of plasmid were mixed with 4 ml of Enhancer and 5 ml of Effectene and incubated with 3.0 x 106 cells for 12 to 16 hr. Subsequently, cells were transferred to the indicated temperatures for overnight incubation before collection. During collection, cells were resuspended and washed twice with PBS on ice. Cell pellets were subjected to RNA extraction and further analysis as described below. The results shown in figures 2.3 and 2.4 were based on pooling data from at least two independent stable transformants for the stable cell lines and at least three independent experiments for the data obtained using transient transfections.

2.2.6 Splicing assay

For RNA analysis in flies, vials containing ~100 young (2- to 6-day-old) adult flies were placed in controlled environmental chambers (Percival, USA) at the indicated temperature and exposed to at least five 24-h photoperiods of alternating LD cycles as described above for recording locomotor activity. At selected times during LD, flies were collected by freezing and heads isolated.

Total RNA was extracted and the relative levels of dmpi8 spliced and unspliced per RNA variants in fly heads and S2 cells were measured using a semi-quantitative reverse transcriptase-PCR (RT-PCR) assay as previously described (Majercak et al., 2004; Majercak et al., 1999). Briefly, RNA was collected from isolated fly heads and S2 cells using Tri-reagent (Sigma). Approximately 2µg of total RNA was reversed transcribed using oligo(dT)20 and Thermoscript RT enzyme (Invitrogen) in a 20□l reaction. Gene specific primers flanking the 3' UTR intron of the different dmper variants were used to amplify both the spliced and unspliced forms in a 50µl reaction using 2µl of RT product as template. The following primers were used to amplify the

target regions: for *D. melanogaster* (Canton S) flies and S2 cells, sense primer P6869 (5' TAGTAGCCACACCCGCAGT 3') and antisense primer P7197 (5' TCTACATTATCCTCGGCTTGC 3'), as previously described (Majercak et al., 2004); for *D. simulans*, sense primer P6890 (5' CTGCTGACCGACGTACACAAC 3') and antisense primer P7184 (5' GGCTTGAGATCTACATTATCCTC 3'); for *D. yakuba* and *D. santomea*, sense primer yakF1 (5' AGCACGGCGATGGGTAGTAG 3') and antisense primer yakR1 (5' CCTTAGGGCTGAGCCACTCTAG 3'); for transgenic flies, we used sense primers P6851 (5' ACACAGCACGGGGATGGGTAGT 3') and P6851-StuI (5' ACACAGCACGGGGATGGGAGGC 3') to differentiate between the endogenous *per01* mRNA transcripts and the dmper transgene derived RNA, respectively. The latter primer will only amplify transgenic dmper RNA that contains the engineered StuI site upstream of the stop codon. All RT-PCRs included gene specific primers targeting the non-cycling Cap Binding Protein 20 (CBP20) gene as an internal control (Majercak et al., 2004). Species-specific primer sets were used to amplify CBP20 from *D. melanogaster, D. simulans, D. yakuba* (for both Tai18E2 and Ivory Coast, Burla strain) and *D. santomea*, as follows: for *D. melanogaster* (Canton S) flies and S2 cells, sense primer CBP540F (5' GTCTGATTCGTGTGGACTGG 3') and antisense primer CBP673R (5' CAACAGTTTGCCATAACCCC 3'); for *D. simulans* (sim4), sense primer CBP540F (5' GTCTGATTCGTGTGGACTGG 3') and antisense primer CBP500R (5' TGTGACAACAGTTTGCCATAACC 3'); for *D. yakuba* and *D. santomea*, yakCBP2066 (5' ACTGATTCGCGTGGACTGG 3') and yakCBP2207 (5' CTTCTGCGACAACAGTTTGC 3'). PCR products were separated and visualized by electrophoresis on 2% agarose gels containing Gelstar (Cambrex Co., USA), and the

bands were quantified using a Typhoon 9400 Imager. The values of per-containing amplified products were normalized relative to CBP20 and expressed as either total RNA or the proportion with the 3'-terminal intron removed. Total RNA was calculated by adding the values for the two RT-PCR products; i.e., with and without the dmpi8 intron. We routinely collected samples after different cycle lengths to ensure that the PCR products were in the linear range.

2.3 Results

2.3.1 Daylength but not temperature modulates the daily distribution of activity in *D. yakuba*

To investigate the effects of temperature on the daily activity pattern of *D. yakuba* we initially entrained flies to standard cycles of 12hr light/12 hr dark (12:12LD; where zeitgeber time (ZT) 0 is lights-on), and evaluated them at several different temperatures previously shown to modulate the timing of daily activity in *D. melanogaster* (i.e., 18, 25 and 29°C) (Majercak et al., 1999). We also included *D. melanogaster* flies that were treated contemporaneously as a benchmark for comparative analysis. To better quantify the effects of temperature on daily activity patterns we measured the onsets, peaks and offsets of the clock-controlled morning and evening bouts of activity. In addition, we also measured the less well-documented midday siesta, herein defined as the time interval between the offset and onset of the morning and evening components, respectively. We found that morning offset and evening onset were the most reliable phase markers for temperature-induced changes in the timing of the two major activity bouts, although calculating the morning component is sometimes less reliable due to an occasional light-driven burst in activity ('startle response') at the dark-to-light transition. Similar results were obtained when we varied the onset and offset phase reference points from 25 to 75% of peak values (data not shown), and results with 50% are shown as they were the most reproducible.

While not the focus of this current study we examined a wide variety of standard laboratory and natural strains of *D. melanogaster* and noted that they exhibit similar temperature induced changes in daily activity patterns, indicating that this thermal

response is a general feature of this species (data not shown; results obtained with the standard Canton S strain are shown). Most notably, increases in temperature are associated with slight advances in morning activity, a more robust and longer siesta time and significant delays in evening activity (e.g., Figure 2.1 A-C and Tables A1-3 for results from ANOVA analysis) (Majercak et al., 1999). For example, at 29°C the offset of morning activity is ~2.0 hr earlier, the siesta time 6 hr longer and the onset of evening activity 3.5 hr later compared to 18°C (Figure 2.1 and Table A1). In prior work we also observed a preferential effect of temperature on the timing of the evening activity component compared to the morning bout (Majercak et al., 1999). Indeed, although temperature has broad circadian-regulated and direct ('masking') effects on the diurnal distribution of activity in *D. melanogaster* (e.g., (Yoshii et al., 2002)), the role of the per (herein referred to as dmper; *D. melanogaster* per) 3'-terminal intron (dmpi8) has been most closely linked to the timing of evening activity (Majercak et al., 1999) (see Introduction). As previously reported, the mean splicing efficiency of dmpi8 throughout a daily cycle decreases as temperature rises and there is a clock-controlled daily fluctuation, especially at warm temperatures where it reaches a nadir between ZT6 to 12 (Figure 2.1G) (Collins et al., 2004; Majercak et al., 2004; Majercak et al., 1999). In summary, our results confirm prior findings using D. melanogaster and the more detailed behavioural analysis indicates that midday activity levels are particularly sensitive to temperature (Tables A1-3).

D. yakuba also displays a bimodal activity pattern (Figure 2.1D-F and Tables A1-3). However, over a broad range of temperatures (18° to 29°C) there is little effect on the timing of the morning and evening bouts of activity and especially the length of siesta

time, which remains at ~7 hr (Tables A1-3; ANOVA for comparison between temperatures, P=0.78). We saw similar responses in all the *D. yakuba* strains we analyzed, whether the progeny tested were derived from strains that had been reared under laboratory conditions for several decades or isofemale lines established from recently wild-caught flies (e.g., Fig. 2.8A and B; and data not shown). *D. yakuba* strains also display a pronounced midday dip in activity even during cold days (Figure 2.1D) in contrast to *D. melanogaster*. Thus, unlike *D. melanogaster, D. yakuba* exhibits preferential daytime activity over a broad range of temperatures with a pronounced decrease in activity levels during a relatively fixed time window in the middle of the day when hot temperatures are expected in its natural environment. The period lengths of *D. melanogaster* and *D. yakuba* show little variation at the different test temperatures (Tables A4 and A5), as expected based on a hallmark feature of circadian clocks termed 'temperature compensation', a not well understood mechanism that results in roughly constant free-running periods over a wide range of physiologically relevant temperatures (Hastings et al., 1991). Therefore, variations in period length cannot account for the temperature dependent changes in the daily activity profile of *D. melanogaster*. Together, the results indicate that *D. melanogaster* and *D. yakuba* have stably heritable differences in the responsiveness of their daily activity patterns to temperature.

Besides temperature, changes in day-length (photoperiod) modulate the timing of evening activity in *D. melanogaster* (Majercak et al., 1999), a response that is based on the light-induced degradation of TIMELESS (TIM), the critical partner of PER (Ashmore and Sehgal, 2003). To examine whether the daily distribution of *D. yakuba* changes as a function of day-length we exposed the flies to a shorter photoperiod (9:15LD). When

aligned with the dark-to-light transition it is clear that the timing of evening activity in *D. yakuba* changes as a function of day-length in a manner similar to that of *D. melanogaster*, peaking earlier under shorter photoperiods (Figure2.2 and Tables A1 and A2; ANOVA comparison of evening peak and onset at the two different photoperiods, $P<0.0001$). Thus, with regards to the daily distribution of activity, *D. yakuba* displays a preferential insensitivity to thermal but not photic adaptation.

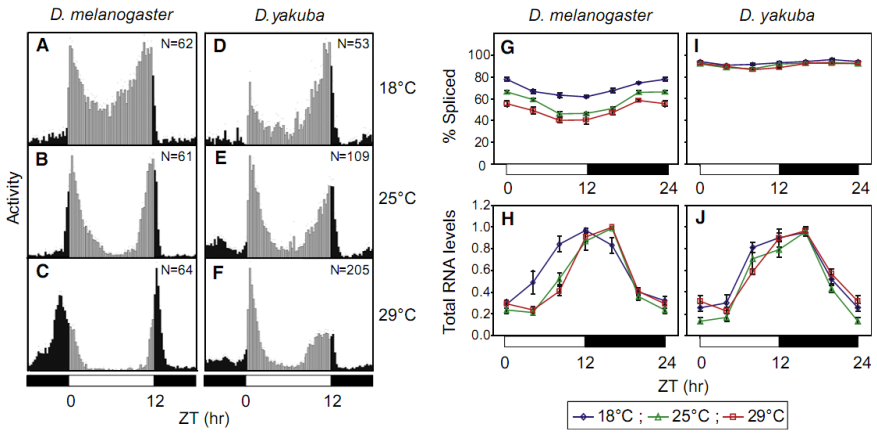


Figure 2.1. Little effect of temperature on the daily distribution of activity and *per* 3'-terminal intron splicing and RNA cycles in *D. yakuba*

(A-F) Histograms represent the distribution of locomotor activity for *D. melanogaster* (Canton S) and *D. yakuba* (Ivory Coast, Burla strain) flies during 12:12LD cycles at the indicated temperatures. The number of total flies used for each genotype x temperature is shown in the panels. Black and gray vertical bars (15-min bins) indicate relative activity levels during the light and dark periods, respectively. (G, I) Splicing efficiency of dmpi8 (G) and dyp3' (I) introns in *D. melanogaster* and *D. yakuba*, respectively. The splicing efficiency of dmpi8 shows significant temperature effects at all times in the day (ANOVA, $P<0.005$), whereas for dyp3' the effect of temperature is not significant except for ZT8 and 20 when comparing 18^{o} and 29^{o}C (ANOVA, $P<0.01$). (H, J) Total *per* RNA levels in *D. melanogaster* (H) and *D. yakuba* (J) flies (n=3). Peak values at each temperature were set to 1 and the rest of the values normalized. ANOVA analysis showed significant effect of temperature on the daytime values (ZT4, 8, 12) of *D. melanogaster per* RNA ($P<<0.001$) but no effect of temperature at any time throughout a daily cycle on *per* RNA values in *D. yakuba*. White and black horizontal bars; 12hr light, 12hr dark periods, respectively.

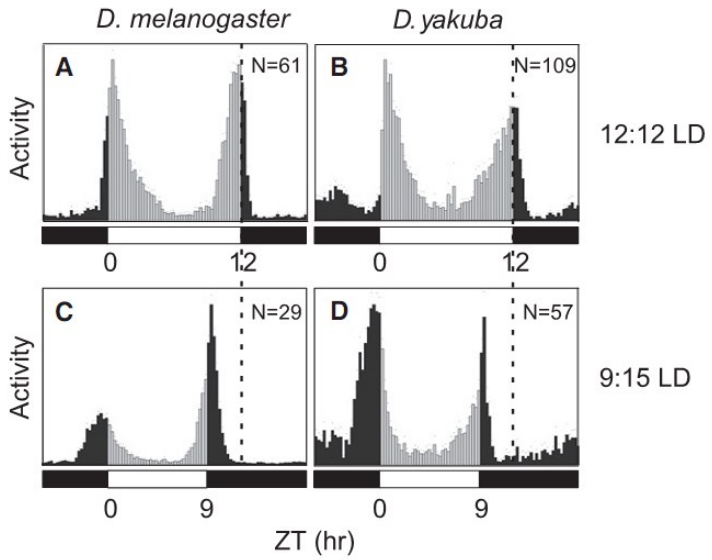


Figure 2.2. The timing of evening activity in *D. yakuba* responds to photoperiod. Histograms represent the distribution of activity for *D. melanogaster* (Canton S) and *D. yakuba* (Ivory Coast, Burla strain) flies at 25^{o}C during either 12:12LD (A, B) or 9:15LD (C, D) cycles (number of flies used for each genotype x photoperiod are shown in panel). Black and gray vertical bars (15-min bins) indicate relative activity levels during the light and dark periods, respectively. White and black horizontal bars; 12hr light, 12hr dark periods, respectively. Note that the timing of evening activity (vertical dashed line; aligned with evening peak under 12:12LD) occurs earlier in both *D. yakuba* and *D. melanogaster* under the shorter photoperiod of 9:15LD compared to 12:12LD.

2.3.2 Lack of thermosensitivity in the splicing efficiency of the *D. yakuba* 3'-terminal intron

We identified the presence of an 85-nt intron in the 3' UTR of *D. yakuba per* (herein termed dyp3') that is almost identical in size and relative position to dmpi8 in *D. melanogaster* (Thackeray and Kyriacou, 1990) (Fig. A1A). The 3' UTRs from at least 7 independent *D. yakuba* isolates were analyzed and all had identical sequences for the dyp3' intron and nearby 5' and 3' flanking regions (data not shown). Remarkably, over a wide range of temperatures (and photoperiods) the splicing efficiency of dyp3' is constitutively high (Figure 2.1I; dyp3' is excised in ~80-95% of dy*per* transcripts) and the daily profiles of dy*per* transcripts are largely insensitive to changes in temperature (Figure 2.1J; ANOVA results shown in legend to figure). These results are strikingly different from our earlier work using *D. melanogaster*, whereby cold temperatures (e.g., 18°C) stimulate the splicing efficiency of dmpi8, leading to an earlier upswing in dm*per* RNA levels and higher peak values (Figure 2.1G and H, and Fig. 2.7E and F) (Majercak et al., 1999). Thus, there is a very tight link between the thermal responsiveness in the splicing efficiencies of *per* 3'-terminal introns and temperature effects on the daily profiles of *per* mRNA levels and activity in two different species of *Drosophila*.

2.3.3 Recapitulating the species-specific thermal splicing phenotypes in a simplified tissue culture system

To better understand the molecular underpinnings governing the thermal sensitivities in the splicing efficiencies of the dmpi8 and dyp3' introns, we developed a simplified cell culture system whereby *per* genomic sequences encompassing the entire *D. melanogaster* 3' UTR followed by 90 bp of 3' flanking non-transcribed region were fused downstream

of a *luciferase* (*luc*) reporter gene (Figure 2.3A). Expression of the hybrid gene was placed under the control of the constitutive actin 5C promoter (pAct). To enable the simple introduction of different intron and nearby flanking exon sequences, we also engineered XhoI and KpnI restriction sites 9 or 10 bp upstream and downstream of the dmpi8 5' and 3' splice sites (ss), respectively (Figure 2.3A, Figure 2.4A and A1A). The commonly used *Drosophila* Schneider 2 (S2) cells were either stably or transiently transfected and at least two independent transformants analyzed for each construct. Cells were incubated at different temperatures, total RNA extracted and the relative levels of spliced and non-spliced products determined.

When we evaluated the control plasmid containing the dmpi8 intron (herein denoted as the 'luc/8:8' plasmid), there was ~2 to 3 fold increase in the proportion of spliced to unspliced RNA at 12° compared to 22°C (Figure 2.3B; ANOVA analysis is summarized in figure legend). More extensive analysis showed a linear relationship between the proportion of spliced products and temperature (data not shown). We could not use the same temperatures as those in our fly studies because the S2 cells did not grow well above 23°-24°C (data not shown). Nonetheless, we note that the temperature differential between our standard 'cold' and 'warm' treated S2 cells is 10^{o}C, similar to what we used when evaluating flies (i.e., 18^{o} and 29^{o}C). As is the case for dm*per* RNA in fly head extracts, comparable results were obtained if cDNA synthesis was primed with poly(dT), or if the requirement for polyadenylation was bypassed by using gene specific primers (data not shown). Importantly, there is little effect of temperature on the splicing efficiency of the dyp3' intron in our reporter-based S2 cell culture system (Figure 2.3B; if anything, luc/dyp3' splicing is slightly inhibited at cold temperatures).

Thus, the species-specific differences in the splicing thermosensitivies of dmpi8 and dyp3' can be faithfully recapitulated in transfected S2 cells, providing a powerful approach to investigate mechanistic issues. Moreover, these results obtained in S2 cells indicate that the thermal sensitivity in dmpi8 splicing does not require a functional clock.

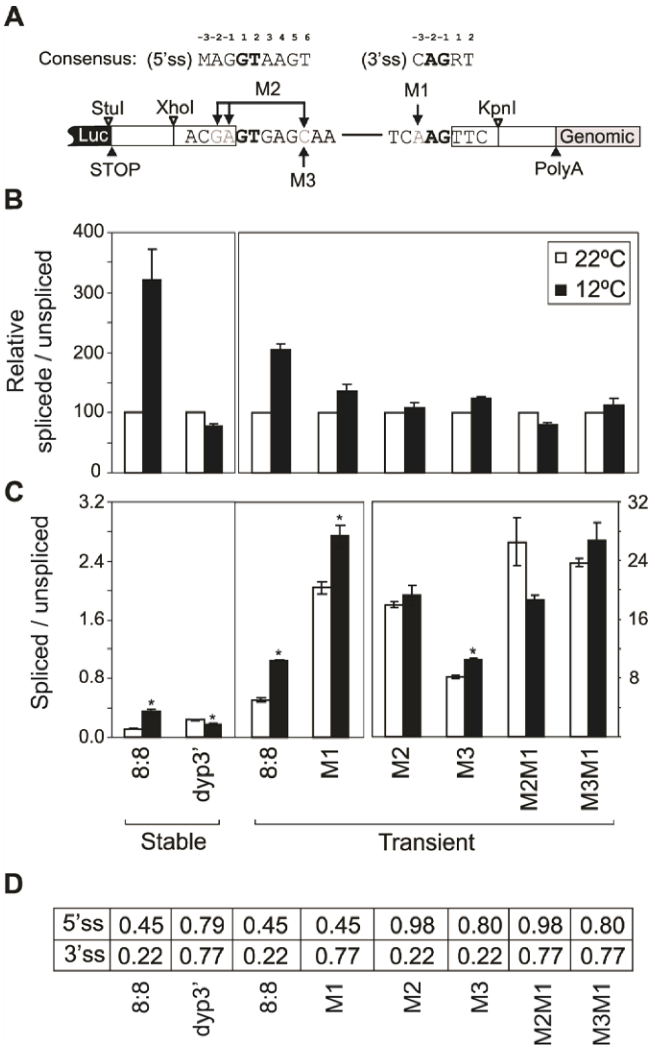


Figure 2.3. Recapitulation of species-specific thermal responses in *per* 3'-terminal intron splicing using a simplified *Drosophila* cell culture system.

(A) Shown at top are the *Drosophila* consensus sequences for the 5'ss (where the G at the 5' end of the intron is designated the +1 position) and 3'ss (where the G at the 3' end of the intron is designated the -1 position). The canonical GT and AG dinucleotides at the 5' and 3' ends of introns are in bold; M=A or C, R= A or G. Shown at bottom is a schematic of the hybrid construct containing the *luciferase* open reading frame (Luc) followed by dm*per* 3' sequences (entire 3' UTR and downstream genomic sequences). The dm*per* 3' UTR begins at the translation stop codon (STOP) and ends at the 3' cleavage

Figure legend of Figure 2.3 continued

/polyadenylation site (PolyA). Also indicated; (1) engineered XhoI and KpnI sites upstream and downstream of the 5' and 3'ss, respectively; (2) M1, M2 and M3 mutations that change the indicated bases (vertical arrow) to those of the consensus (top); (3) horizontal bar, intronic sequences. (B, C) The levels of spliced and non-spliced RNA were determined and expressed as a ratio (i.e., RNA levels for spliced, divided by RNA levels for unspliced). Results are an average of at least two independent experiments and derived from either stably or transiently transfected cells, as indicated (bottom). (B) For each construct, the spliced to unspliced ratio at 22^{o}C was set to 100 (white bars) and the corresponding value at 12^{o}C normalized, which facilitates visualizing the relative splicing thermosensitivities of the different constructs. (C) ANOVA analysis revealed that there are significant effects of changing the predicted splice site strengths on the splicing efficiency of the dmpi8 intron ($P<<0.0001$; rank-order beginning with most highly spliced variant; M3M1, M2M1, M2 > M3 > M1, 8:8), and that thermosensitivity in splicing efficiency varies as a function of dmpi8 variant. ANOVA analysis was further performed to compare values obtained at the two test temperatures for each construct; *, denotes $P < 0.01$. Note that there is a separate scale for the four values shown in the extreme right box as the spliced/unspliced ratio was much higher for these constructs. (D) Predicted strengths of the 5'ss and 3'ss for the different constructs (range is 0 to 1, with higher values predicting stronger splice sites).

2.3.4 Weak 5' and 3' splice sites underlie thermosensitivity of dmpi8 splicing

Using a splice site prediction program that is trained to predict 5' and 3' splice sites in *D. melanogaster* (www.fruitfly.org/seq_tools/splice.html; the output of the network is a score between 0 and 1 for a potential splice site, with 1 being highly likely) (Reese et al., 1997), we noted that of all the *D. melanogaster per* introns, dmpi8 has the lowest predicted scores for both the 5'ss (score=0.45) and 3'ss (score=0.22) (Figure 2.3D and data not shown). In *Drosophila* the consensus 5'ss is (-1)G**G**TAAGT(+6) (where the bold G is the 5' start of the intron; defined as position +1), the branch point signal (BPS) is CTA**A**T (the bold A is where lariat formation occurs) and the 3'ss is a polypyrimidine tract (PPT) followed by (-3)CA**G**(-1) (where the bold G is the 3' end of the intron and defined as position -1) (Figure 2.3A and Figure 2.4A, top). The predicted 5'ss and 3'ss scores for the dyp3' intron were significantly higher compared to dmpi8 (Figure 2.3D). We were intrigued by the putative weak 5' and 3'ss for dmpi8 because earlier pioneering work by Murphy and co-workers showed that multiple weak splicing signals can result in thermosensitive splicing, whereby cold temperatures enhance splicing efficiency (Ainsworth et al., 1996; Touchman et al., 1995). It is thought that low temperatures stabilize suboptimal RNA-RNA or RNA-protein interactions between the splicing machinery and the pre-mRNA (see Discussion).

To investigate the possible role(s) of suboptimal 5'ss and 3'ss in the thermal regulation of dmpi8 splicing, we mutated predicted weak sites to the consensus at that position and assayed the splicing phenotypes of the resultant substrates containing either individual changes or in several combinations (Figure 2.3A). Of note, the main differences in the 5' and 3'ss between dmpi8 and dyp3' are position +6 at the 5'ss and

position -3 at the 3'ss, which are consensus in *D. yakuba* but suboptimal in D. melanogaster (Fig. A1A). Indeed, increasing the predicted strength of the dmpi8 5'ss (e.g., luc/M2 and luc/M3), 3'ss (e.g., luc/M1) or both (luc/M2M1 and luc/M3M1) not only enhanced overall intron removal as expected (Figure 2.3C), but diminished the thermal regulation in splicing efficiency (Figure 2.3B). We observed a graded response whereby the ability of temperature to modulate splicing efficiency was attenuated by single mutations that targeted either the 5'ss (M3) or 3'ss (M1), and eliminated when individual mutations were combined (e.g., M2M1 and M3M1).

We also generated a series of hybrid introns by fusing parts of dmpi8 with sequences from either dyp3' or intron 3 from *D. melanogaster* (Figure 2.4A and A1A). Intron 3 from *D. melanogaster per* was chosen for hybrid studies because it is a small intron (64 nt) that has the same predicted branch point signal (CTAAC) as dmpi8, yet contains a consensus 5'ss and a strong 3'ss (Fig. A1A). As observed for the results obtained with point mutants, hybrid introns with predicted stronger 5'ss or 3'ss significantly attenuated the influence of temperature on splicing. This appeared to be especially true for increasing the strength of the 5'ss, whereas increases in the 3'ss did not always lead to a strong reduction in the thermosensitivity of splicing efficiency (Figure 2.4B; e.g., hybrids luc/8:dyp3' and luc/8:3), suggesting a predominant role for 5'ss recognition in establishing the thermal range of dmpi8 splicing (see Discussion). Nonetheless, although the luc/8:dyp3'(3'ss) has more *D. melanogaster* sequence compared to luc/8:dyp3', the former does not exhibit temperature dependent splicing (Figure 2.4C). Intriguingly, the 8:dyp3'(3'ss) intron has a slightly stronger predicted 3'ss compared to 8:dyp3' (Figure 2.4D), likely underlying the attenuated thermal sensitivity

of the 8:dyp3'(3'ss) intron. Presumably, the combination of *D. melanogaster* and *D. yakuba* sequences used to generate the 8:dyp3'(3'ss) intron yields a novel 3' recognition signal with increased strength compared to its two parental constructs. Collectively, the data further support the notion that it is the overall strengths of key splicing signals as opposed to particular sequences that underlies the thermal phenotypes in the splicing efficiencies of dmpi8 and dyp3'.

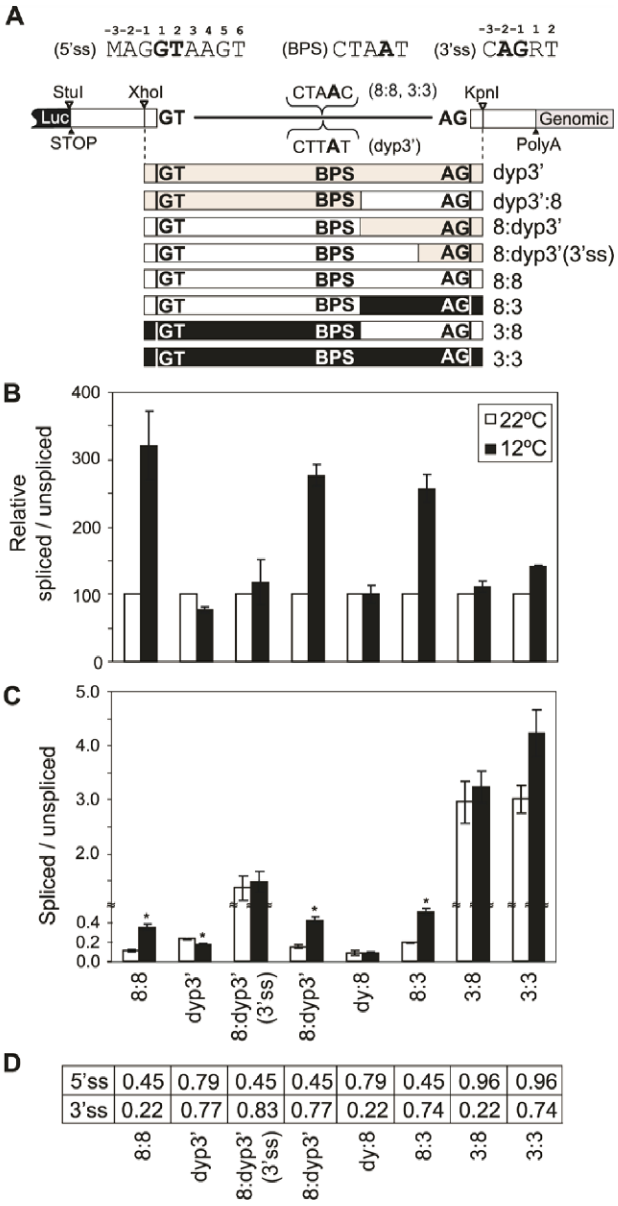


Figure 2.4. Hybrid introns reveal that the thermosensitive splicing phenotype of dmpi8 is based on suboptimal splice sites and not other intron specific information.

(A) Shown at top are the *Drosophila* consensus sequences for the 5'ss and 3'ss, and a schematic of the parental Luc-dmper construct, as explained in figure 3. At bottom are shown schematic representations of the different hybrid introns; gray, dyp3'; white, dmpi8; black, dm*per* intron 3 (further details are shown in Fig. A1A). (B, C) The levels of spliced and non-spliced RNA were determined and expressed as a ratio (spliced/

Figure legend of Figure 2.4 continued

unspliced). Results from at least three independent experiments were averaged. (B) For each construct, the spliced to unspliced ratio at 22°C was set to 100 (white bars) and the corresponding value at 12°C normalized, which facilitates visualizing the relative splicing thermosensitivities of the different constructs. (C) ANOVA analysis showed that both temperature and genotype have significant effects on splicing efficiency of the 3' intron; further ANOVA analysis was performed comparing values obtained at the two temperatures for each genotype; *, denotes $P < 0.01$. (D) Predicted strengths of the 5'ss and 3'ss for the different constructs (range is 0 to 1, with higher values predicting stronger splice sites).

2.3.5 Multiple weak splicing signals on dmpi8 confer the ability to manifest more robust and longer siesta times that extent beyond midday

To evaluate the physiological significance of the results obtained in cultured cells, we generated transgenic flies bearing the 8:8, dyp3' and M2M1 versions of dm*per* which were constructed using the corresponding 3' UTRs analyzed in the cell-culture assays (Figure 2.5). The '8:8' transformation vector is virtually identical to the wildtype dm*per* gene except that it has the introduced XhoI and KpnI sites flanking the native dmpi8 intron (Figure 2.5A). The host genetic background for the transgenic flies was w^{1118}, which generally exhibits a more prominent midday siesta compared to the Canton S flies shown in Figure 2.1 (data not shown). For each construct, at least three independent transgenic lines were analyzed for behavioral rescue in a *w per*^{01} genetic background, and all manifested robust rhythms with wildtype periods of ~23 to 24 hr (Table A9). We compared the activity profiles of the different genotypes (i.e., P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}) under a variety of temperatures (18°, 25° and 29°C) and photoperiods (11:13, 12:12, 13:11 or 14:10) (Figure 2.5). To more readily observe differences in daily activity patterns, the wave-forms for each genotype were superimposed.

Although not observed for all temperatures and photoperiods examined, we noted a general trend in that the P{dmper/dyp3'} and P{dmper/M2M1} flies showed earlier onsets of evening activity and shorter, less robust, midday siestas compared to the P{dmper/8:8} flies (Figure 2.5 and Tables A6-8). Most notably, P{dmper/8:8} flies exhibit an enhanced ability to prolong midday inactivity for several more hours into the afternoon. This was most readily observed at shorter photoperiods (11:13 and 12:12) and/or cooler temperatures (18° and 25°C). Differences in morning activity were

generally of lesser magnitude. There is a remarkably strong link between the intrinsic splice site strengths on the *per* 3'-terminal intron and the midday siesta (Figure 2.5K). For example, whereas the length of midday siesta is significantly different between P{dmper/8:8} and P{dmper/M2M1} at each entraining condition tested, results with P{dmper/dyp3'} were more intermediate, sometimes resembling P{dmper/8:8} and other times P{dmper/M2M1} (Figure 2.5K and Table A8). The free-running periods were almost identical in the different transgenic flies (Table A9), indicating that dm*per* 3'-terminal intron splicing does not influence the distribution of daily activity by changing the overall pace of the clock.

Differences in activity profiles between the different genotypes were less apparent at 29°C (Figure2.5). Prior work in *D. melanogaster* showed that increases in temperature directly inhibit daytime activity ('masking') (Tomioka et al., 1998) and longer photoperiods delay the timing of evening activity (Majercak et al., 1999; Shafer et al., 2004). Thus, there is likely to be a balance of opposing effects whereby higher temperatures and longer photoperiods partially override the degree to which highly efficient splicing of a *per* 3'-terminal intron can enable the manifestation of elevated midday activity (at least, in our experimental paradigm). This might explain why P{dmper/M2M1} flies do not exhibit a robust siesta at 29°C and 11:13LD, whereas this is not the case for P{dmper/dyp3'} (Figure 2.5H); i.e., the more efficient splicing of the 3'-terminal intron of P{dmper/M2M1} compared to that of P{dmper/dyp3'} (see below, Figure 2.6) is above a critical threshold sufficient to sustain increased midday activity despite the warm temperature which normally acts to diminish daytime activity. The increasingly stronger inhibitory effects of light as temperatures rise in *D. melanogaster*

likely also contribute to why the P{dmper/dyp3'} and P{dmper/M2M1} flies still exhibit temperature dependent changes in activity profiles, in contrast to wildtype *D. yakuba* (Figure 2.1). Thus, simply replacing the natural dmpi8 intron with its counterpart from *D. yakuba* does not abolish temperature effects on activity rhythms in *D. melanogaster*.

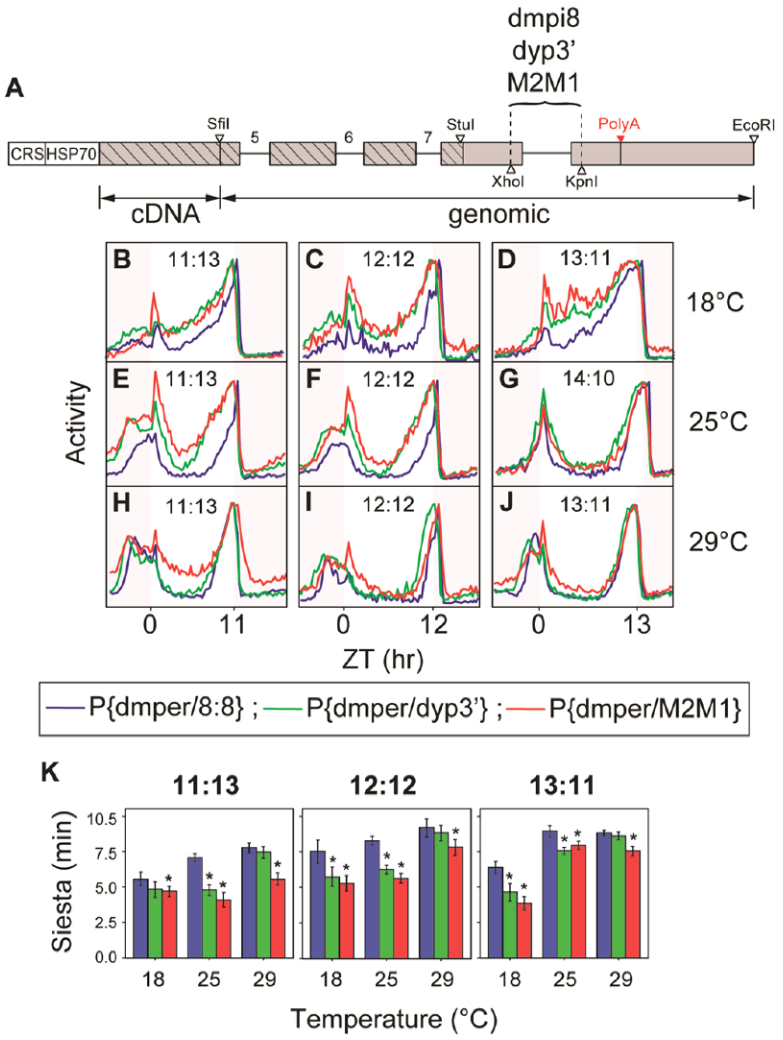


Figure 2.5. Suboptimal splice sites in dmpi8 enable *D. melanogaster* flies to exhibit robust and prolonged midday siestas.

(A) Schematic representation of *per*-containing plasmid used as a basis to generate the different transgenic flies used in this study; CRS, *per* circadian regulatory sequence; HSP70, *D. melanogaster* HSP70 basal promoter; hatched boxes, dm*per* coding sequence; engineered XhoI and KpnI sites in the dm*per* 3' UTR were used to insert the various introns, as indicated. (B-J) Shown are group averages of the daily activity rhythms for

Figure legend of Figure 2.5 continued

the different transgenic flies (i.e., *w* per^{01} flies bearing the P{dmper/8:8}, P{dmper/dyp3'} or P{dmper/M2M1} transgenes) maintained at the indicated temperatures (right of panels) and photoperiods (within panels). To facilitate comparisons, the peak value in daily activity for each genotype was set to 1.0 and the normalized profiles superimposed. For each genotype and entrainment condition, data from at least 20 flies was used to generate the activity profiles shown. (K) Length of siesta time for activity profiles shown in panels B to J. For simplicity we grouped results from LD14:10 with those of LD13:11. *, siesta time different from P{dmper/8:8}, $P < 0.01$ (see Tables A6-8 for further details).

2.3.6 Higher splicing efficiency leads to increased dm*per* RNA levels

To measure *per* 3'-terminal intron splicing efficiency we probed adult fly head extracts using our RT-PCR based assay (Majercak et al., 2004; Majercak et al., 1999) in the presence of primers that distinguish between transgene and per^{01} derived *per* transcripts (Figure 2.6). Splicing of the dyp3' and M2M1 introns were very efficient at both high and low temperatures (Figure 2.6A and B), similar to the situation in native *D. yakuba* flies (Figure 2.1I; ANOVA results shown in figure legend). Moreover, the better splicing efficiency of the M2M1 intron compared to that of the dyp3' intron in the transgenic flies (Figure 2.6B; ~90-100% for M2M1 versus ~80% for dyp3'), is consistent with the less robust siesta time observed for the former, which as pointed out above, is more obvious under certain environmental conditions (e.g., Figure 2.5E, F and H). In contrast, P{dmper/8:8} flies displayed a splicing phenotype similar to that of the endogenously expressed per^{01} RNA (which has the dmpi8 intron, similar to '8:8'), with lower overall splicing efficiency at warmer temperatures (Figure 2.6A and B), in agreement with our earlier findings (Majercak et al., 2004; Majercak et al., 1999) (and see Figure 2.1G). Finally, the overall levels of dm*per* transcripts were significantly higher in the P{dmper/dyp3'} and P{dmper/M2M1} flies (Figure 2.6C and D), again consistent with our prior work showing that inability to splice dmpi8 leads to decreased levels of dm*per* mRNA (Majercak et al., 1999). Parenthetically, the presence of functional dPER in the different transgenic flies rescues normal cycling of the endogenous per^{01} RNA, explaining why it behaves similar to the wildtype control 8:8 version (Figure 2.6C and D). The findings suggest that abnormally high levels of dm*per* mRNA during its

accumulation phase compromises the ability of *D. melanogaster* flies to mount a robust and prolonged midday siesta (Figure 2.5F and Figure 2.6).

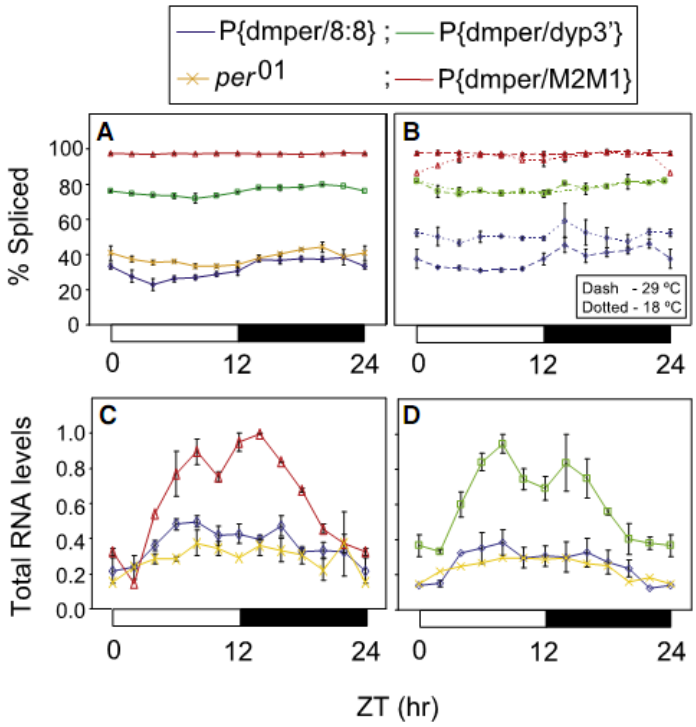


Figure 2.6. Highly efficient splicing at all temperatures and abnormally elevated dm*per* RNA levels in transgenic flies with 3' terminal introns that have strong splicing signals.

Transgenic flies (i.e., *w* per^{01} flies bearing the P{dmper/8:8}, P{dmper/dyp3'} or P{dmper/M2M1} transgenes) were entrained to 12:12LD cycles at 25°C (A, C and D) or 18°C and 29°C (B). RNA was extracted and used to measure either splicing efficiency of the *per* 3'-terminal intron (A, B) or total *per* RNA levels (C, D). Results from at least two independent experiments were averaged. per^{01}, indicates results for the endogenously expressed per^{01} RNA in the transgenic flies, whereby values from the different transformants were pooled. For assaying *per* transcripts expressed from the transgenes, primers were used that do not detect endogenously derived per^{01} RNAs. To determine relative total *per* RNA levels, values were normalized to CBP20 RNA. (A, B) ANOVA analysis showed that splicing efficiency varies as a function of genotype; also,

Figure legend of Figure 2.5 continued.

only the splicing efficiency of the *per* 3'-terminal intron in P{dmper/8:8} flies showed significant temperature effects (P<<0.0001). (C, D) Total *per* RNA levels in P{dmper/dyp3'} and P{dmper/M2M1} flies are significantly different from that in P{dmper/8:8} flies (ANOVA, P<<0.0001).

2.3.7 *D. santomea* and *D. simulans* exhibit similar thermal responses as their close relatives, *D. yakuba* and *D. melanogaster*, respectively

Although not the focus of this study we sought to determine if other *Drosophila* species also exhibit a correlation between splice site strength, thermal sensitivity in the splicing efficiency of the *per* 3'-terminal intron and the ability of temperature to modulate the daily distribution of activity. As an initial attempt we analyzed *D. santomea* and *D. simulans*. *D. santomea* is a very recently described species that is found on São Tomé, one of the Gulf of Guinea islands in west-equatorial Africa, where it co-exits with its closest relative *D. yakuba* (Cariou et al., 2001; Lachaise et al., 2000). It is estimated that *D. santomea* and *D. yakuba* diverged about 400,000 years ago. *D. simulans* is very similar to *D. melanogaster* from which it split about 2-3 million years ago (Lachaise et al., 1988). *D. santomea* and *D. simulans* along with *D. melanogaster* are part of the nine sister species that form the *D. melanogaster* subgroup. Although all these closely related species are endemic to Afro-tropical regions from where they likely originated, only *D. melanogaster* and *D. simulans* are cosmopoliton with a wide geographical range (Keller, 2007).

For both *D. santomea* and *D. simulans*, we confirmed the presence of a 3'-terminal intron that is located at a similar distance downstream of the translation stop codon (~110 bp) as that found in *D. melanogaster* (Fig. A1B). In addition to published sequences for *D. simulans* we also sequenced the 3' UTRs from several independent strains and all had the same 3'-terminal intronic and flanking sequences (data not shown). For *D. santomea* we sequenced the 3'UTRs from five independent isolates and all had the same intronic and flanking sequences (data not shown). In the case of *D. simulans* its 3-terminal intron (dsimp3') is 86 nt long and has predicted weak 5' and 3'ss (Fig. A1B). Interestingly, the

D. santomea 3'-terminal intron (dsanp3') is virtually identical to the *D. yakuba* intron with its stronger 5's and 3'ss, except that there is a 13 nt internal deletion in the 5'-half of the intron (Fig. A1B). Besides *D. simulans* and *D. santomea* we analyzed an independent *D. yakuba* strain (Tai18E2).

Similar to *D. melanogaster*, there is a clear delay in the timing of evening activity and more pronounced siesta time in *D. simulans* with increasing temperature (Figure2.7C and D), consistent with prior work (Rogers et al., 2004). Moreover, in *D. simulans* colder temperatures evoke increases in the splicing efficiency of dsimp3' and total *per* RNA levels, highly reminiscent of *D. melanogaster* (Figure2.7E-G). In striking contrast, *D. santomea* and *D. yakuba* Tai18E2 exhibit little change in daily activity as a function of temperature, especially during the daytime hours where *per* 3'-terminal intron splicing has its biggest effect in *D. melanogaster* (Figure 2.8A-D). Likewise, splicing of the *per* 3'-terminal introns in *D. santomea* and the Tai18E2 strain was very efficient at all temperatures (Figure 2.8E and F). This further supports the notion that multiple suboptimal splice sites on a *per* 3'-terminal intron forms the basis of a seasonal adaptation mechanism that enables some *Drosophila* species the ability to undergo temperature dependent changes in daily activity profiles.

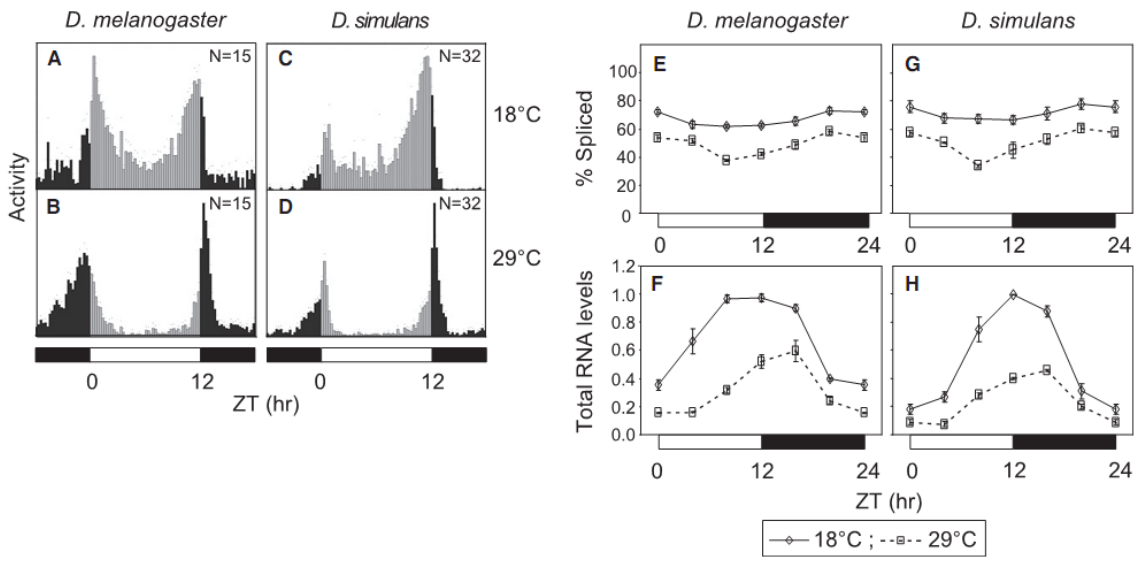


Figure 2.7. Prominent temperature effects on the daily distribution of activity, dsimp3' intron splicing and *per* transcript levels in *D. simulans*

(A-D) Histograms represent the distribution of locomotor activity for *D. melanogaster* (Canton-S) and *D. simulans* (sim4) flies that were contemporaneously subjected to 12:12LD cycles at the indicated temperatures. The number of flies used to generate the daily activity profiles is shown in the panels. Black and gray vertical bars (15-min bins) indicate relative activity levels during the light and dark periods, respectively. (E, G) Splicing efficiency of dmpi8 (E) and dsimp3' (G) introns in *D. melanogaster* and *D. simulans*, respectively. (F, H) Relative *per* RNA levels in *D. melanogaster* (F) and *D. simulans* (H). Peak values at 18°C were set to 1 and the rest of the values normalized. White and black horizontal bars; 12hr light, 12hr dark periods, respectively. Results from at least two independent experiments were averaged. For both D. melanogaster and D. simulans the splicing efficiencies of their respective per 3'-terminal introns and total RNA levels showed significant changes as a function of temperature (ANOVA, $P<<0.0001$).

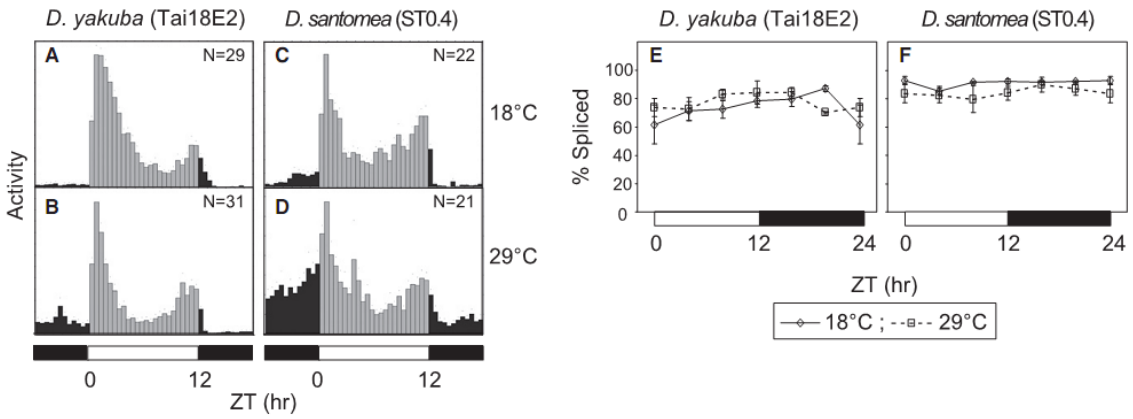


Figure 2.8. Little to no thermal response in the daily activity profile and splicing efficiency of the dsanp3' intron in *D. Santomea.*

(A-D) Histograms represent the distribution of locomotor activity for *D. yakuba* (Tai18E2) and *D. simulans* (ST0.4) flies during 12:12LD cycles at the indicated temperatures. The number of flies used to generate the daily activity profiles is shown in the panels. Black and gray vertical bars (30-min bins) indicate relative activity levels during the light and dark periods, respectively. (E, F) Splicing efficiency of dyp3' (E) and dsanp3' (G) introns in *D. yakuba* and *D. santomea*, respectively. ANOVA analysis revealed no significant effect of temperature on the splicing efficiencies of either dyp3' or dsanp3' introns ($P>0.01$). White and black horizontal bars; 12hr light, 12hr dark periods, respectively. Results from at least two independent experiments were averaged.

2.4 Discussion

Based on the rationale that the temperature regulated splicing of the dmpi8 intron plays a role in the seasonal adaptation of *D. melanogaster* we sought to determine if a similar mechanism occurs in *D. yakuba*, a related species but with a more restricted and ancestral location in equatorial Africa. We show that although there is a 3'-terminal intron in *per* from *D. yakuba* similar in length and relative position as that of dmpi8 in *D. melanogaster*, splicing of the dyp3' intron is highly efficient over a wide range of physiological temperatures, consistent with a lack of thermal regulation in the daily profiles of *per* RNA and activity in this species (Figure 2.1 and Figure 2.2). We investigated the molecular basis for the species-specific splicing phenotypes and found that multiple suboptimal splicing signals on dmpi8 underlie the thermosensitivity in its splicing efficiency (Figure 2.3, Figure 2.4 and Figure 2.6). The main effect of changing the splicing efficiency of dmpi8 was on the robustness and length of the midday siesta, whereby weak 5' and 3'ss enable *D. melanogaster* to prolong reduced inactivity beyond midday for several more hours into the afternoon (Figure 2.5).

Although requiring the analysis of a wider sample of different *Drosophila* species, results obtained using *D. santomea* and *D. simulans* also support a causal relationship between multiple suboptimal splicing signals, thermosensitive splicing of a *per* 3'-terminal intron and temperature dependent changes in daily activity profiles (Figure 2.7 and Figure 2.8). While our results cannot establish that the lack of thermal sensitivity in the splicing efficiencies of *per* 3'-terminal introns underlies the inability of temperature to evoke significant adjustments in the daily distribution of activity in *D. yakuba* and *D. santomea*, they raise the intriguing possibility that for at least some *Drosophila* species,

the presence of weak 5' and 3' splice sites on their respective *per* 3'-terminal introns underlies a thermal calibration mechanism that contributed to their successful colonization of temperate climates.

2.4.1 Species-specific differences in the ability to adjust midday siesta as a function of temperature

Our prior findings using transgenic flies where splicing of dmpi8 was blocked suggested that the main effect of splicing this intron is on the timing of evening activity in *D. melanogaster*, with little to no effect on the morning component. In this study we generated flies whereby the splicing efficiency of dmpi8 was increased and undertook a more systematic analysis of daily activity profiles under a wide range of temperatures and photoperiods. Although changes in the intrinsic splicing efficiency of the *per* 3'-terminal intron preferentially regulates the evening component consistent with earlier findings, it is clear that the timing of morning activity is also modulated. For example, the P{dmper/M2M1} and P{dmper/dyp3'} flies generally exhibit a later offset in morning activity as well as earlier onset in the evening peak compared to the wildtype control P{dmper/dmpi8} flies (Figure 2.5 and Tables A6-A8). In addition, while P{dmper/M2M1} and P{dmper/dyp3'} flies exhibit similar activity profiles, there are interesting differences. The most notable is that under certain environmental conditions, especially warmer and shorter days, P{dmper/M2M1} flies have higher midday activity levels compared to P{dmper/dyp3'} flies (e.g., Figure 2.5, panels E, F and H). Thus, there is a remarkably tight link between the intrinsic splicing efficiency of dmpi8 and the robustness and length of the midday siesta. In general, inefficient splicing enables *D. melanogaster* to manifest a more pronounced and longer siesta time, especially extending

for several hours beyond midday and delaying the onset of the evening bout of activity (Figure 2.5).

In this regard it is noteworthy that in natural conditions increases in ambient temperature lag those of light intensity, reaching peak values in the late afternoon. Avoiding exposure to heat is critical for small insects such as *Drosophila* that run the risk of desiccation. We suggest that the weak splicing of dmpi8 at elevated temperatures triggers a protective behavioural response culminating in a more robust and prolonged midday siesta in anticipation of extended periods of heat that accompany the longer daytime hours characteristic of warm days in temperate climates. Nonetheless, on colder days the enhanced splicing of dmpi8 enables *D. melanogaster* to exhibit relatively more activity during the day, presumably maximizing the warmer daytime hours normally associated with this part of the day in natural conditions. Thus, the regulation of dmpi8 splicing efficiency by temperature endows *D. melanogaster* with a dynamic mechanism that ensures its activity is maximal at a time of day when the temperature would be expected to be optimal for activity.

However, in the case of the equatorial *D. yakuba* (and *D. santomea*) species where day length is approximately 12 hr throughout the year, the timing and duration of the midday heat is relatively fixed and would not require a more dynamic clock-based mechanism that can adjust for seasonal changes in temperature and day length. Indeed, although *D. yakuba* manifests preferential daytime activity over a wide range of temperatures, it nonetheless exhibits a robust midday siesta even at cold temperatures (Figure 2.1). It therefore appears that *D. yakuba* evolved a largely temperature independent 'default' mechanism that mainly restricts activity during the middle of a

daily cycle when hot temperatures are expected in its natural environment. As such daytime activity *per se* does not appear to be detrimental as long as the hot hours of the day are avoided. Moreover, other adaptive strategies, such as the ability to resist desiccation, likely contribute to species or strain specific differences in shaping daily activity profiles.

That the effects of temperature on daily activity are fundamentally different in *D. melanogaster* and *D. yakuba* is further evidenced by the fact that although the dyp3' intron in P{dmper/dyp3'} transgenic flies is efficiently spliced at all temperatures, similar to the natural intron in wildtype *D. yakuba*, both the P{dmper/dyp3'} and P{dmper/M2M1} flies still manifest temperature dependent changes in the daily distribution of activity that are characteristic of *D. melanogaster*; e.g., longer siesta as temperature increases (Figure 2.5 and Table A6-8). Thus, while the splicing efficiency of the 3'-terminal inton of *per* has a prominent effect on the strength and length of the midday siesta in *D. melanogaster*, other environmental or genetic factors also contribute to how temperature influences the daily distribution of activity in this species.

A well-characterized example is the ability of light to 'directly' reduce activity levels at warm temperatures ('masking' effect) (Wheeler et al., 1993; Yoshii et al., 2002). This masking effect of light at warm temperatures is likely to be exaggerated under our experimental paradigm as we subjected flies to sharp light/dark transitions at a constant temperature and they cannot avoid exposure to light. A better evaluation of how the efficiency of dmpi8 splicing contributes to daily activity profiles would likely require analysis under more natural conditions. For example, the weak splicing of dmpi8 that triggers reduced activity beyond midday (Figure 2.5) could be accompanied by other

associated behavioural adaptations, such as seeking darker and cooler hiding places in anticipation of extended periods of heat. This would appear a clear advantage over hypothetical *D. melanogaster* flies with high dmpi8 splicing efficiency (e.g., P{dmper/M2M1}) and consequently intrinsically elevated 'base-line' activity levels during the afternoon. The difference being that although light can suppress activity on warm days, in the hypothetical flies this would occur in direct reaction to encountering warm temperatures. This reasoning is in line with the main adaptive feature of clocks, the ability to anticipate and hence prepare for changes in environmental conditions.

How do changes in the splicing efficiency of dmpi8 contribute to temperature-induced changes in the daily distribution of activity in *D. melanogaster*? Clearly, variations in the splicing efficiency of dmpi8 that are either evoked by temperature or changes in splice site strength lead to alterations in dm*per* RNA levels (Figure 2.6) (Majercak et al., 1999). Changes in the levels and/or timing of PER could modulate the dynamics of the clock leading to alterations in the distribution of daily activity. Therefore, although RNA cycles in dm*per* or *tim* are not required to manifest rhythmic behavior (Yang and Sehgal, 2001), it is likely that environmentally controlled modulations in their daily abundance rhythms, especially the rising phases, have biologically relevant roles in adjusting clock dynamics, presumably by contributing to determining the accumulation rates of PER and TIM proteins. In addition, the interaction of PER with TIM itself appears to be regulated by temperature (Kaushik et al., 2007).

2.4.2 Multiple suboptimal splicing signals as a basis for calibrating thermal responses

Although not extensively studied there are several examples of suboptimal splicing signals underlying thermosensitive splicing of pre-mRNAs. A classic example is the pioneering work by Murphy and co-workers where they identified a temperature dependent splicing event in the Maloney murine sarcoma *ts*110 (MuSVts110) RNA (Ainsworth et al., 1996; Touchman et al., 1995). It is thought that binding of the spliceosome via snRNA (or protein) contacts with suboptimal splicing signals on the pre-mRNA are favoured at cold temperatures and that increases in the strength of even one key *cis*-acting splicing signal can surpass a minimum threshold where interaction of the splicesosome with pre-mRNA is no longer rate-limiting over a broad range of physiologically relevant temperatures (Figure 2.9). Thus, although weak splice sites appear to underlie at least one class of thermosensitive splicing this does not demand that all inefficiently spliced introns are thermally regulated.

The 5'ss in metazoans provides 9 potential positions (positions -3 to +6) for U1 snRNA:5'ss base pairing, although 5 to 7 appears ideal as too much base-pairing inhibits further progress of the splicing machinery (Carmel et al., 2004). Like dmpi8 and dyp3', most introns in *Drosophila* are small (<100bp) and as a result are thought to contain all the necessary information for recognition by the splicing machinery (i.e., intron definition) (Lim and Burge, 2001; Talerico and Berget, 1994). The consensus sequence of the main 5'ss motif is G**G**TAAGT (where the bold G is the +1 position at the 5' end of the intron) (Lim and Burge, 2001; Sheth et al., 2006) (Figure 2.3A). Although the 5'ss for *per* 3'-terminal introns from both *D. melanogaster* and *D. yakuba* have a suboptimal A at position -1, they only differ at position +6, with a suboptimal C in *D. melanogaster*

and a consensus T for *D. yakuba*. Approximately 70% of introns in *Drosophila* have a T at position 6, whereas a C is present in less than 10% of cases, consistent with this position playing a non-essential but important modulatory role in regulating splicing efficiency (Sheth et al., 2006). Importantly, a suboptimal base at position +6 can be offset by an optimal base at position -1, and vice-versa (Carmel et al., 2004). Indeed, analysis of human splicing mutations in position -1, support the idea that a mismatch at this position can be compensated for by matches at positions +3 to +6, especially at position +6 (Ohno et al., 2005). Thus, although both dmpi8 and dyp3' introns are flanked by an A at position -1, the T at position +6 of dyp3' likely compensates, contributing to the more efficient and temperature independent splicing of dyp3' in *D. yakuba*.

We propose that the thermal range in the splicing efficiency of dmpi8 is mainly determined by the mismatches at positions -1 and +6 that yield gradually weakening interaction between the 5'ss and U1 snRNA as temperature increases (Figure 2.9A and B). However, this thermal responsiveness that ultimately manifests itself as changes in splicing efficiency of dmpi8 is only exhibited because the 3'ss is below a certain threshold whereby changes in the strength of the association between U1 snRNA and the 5'ss are rate-limiting for overall spliceosome binding to dmpi8 (Figure 2.9C). With short introns such as dmpi8, splicing factors that bind the 5' and 3' ends of the intron interact across the intron, stabilizing spliceosome assembly. Thus, stronger 3' splicing elements (BPS, PPT and 3'ss) would enhance the binding of key factors such as U2AF and U2 snRNA, which in turn could stabilize the interaction of U1 with a weak 5'ss, attenuating thermal sensitivity in splicing efficiency (Figure 2.9D). This could explain why even though both the 8:dyp3' and 8:dy3'(3'ss) hybrid introns have the same weak 5'ss from

dmpi8, 8:dyp3'(3'ss) does not exhibit thermosensitive splicing, presumably due to its higher C/T content in the polypyrimidine tract yielding a slightly stronger 3' splice signal (Figure 2.4 and A1A). In the case of dyp3', despite a non-consensus -1 position at the 5'ss, the strong +6 position of the 5'ss in combination with a moderate 3'ss likely provide enough stable contacts such that spliceosome binding to dyp3' is not rate-limiting over a wide range of physiologically relevant temperatures (Figure 2.9B). That the 5'ss has a greater effect on the thermosensitivity of dmpi8 splicing is also consistent with results obtained using hybrids between dmpi8 and either intron 3 of *D. melanogaster* or dyp3' (Figure 2.4).

Intriguingly, temperature dependent splicing based on suboptimal splicing signals was also shown to be the basis for at least one pathway underlying clock responses to temperature in *Neurospora*. In this system, the FREQUENCY (FRQ) protein undergoes daily oscillations in levels and phosphorylation that are central to clock progression (Dunlap and Loros, 2006). Earlier work from Dunlap and co-workers showed that temperature regulates the relative levels of two iso-forms of FRQ protein, a short (s-FRQ) and long (l-FRQ) version that arise from alternative use of translation initiation sites (Liu et al., 1997). More recent work from the Brunner and Dunlap labs demonstrated that the ratio of l-FRQ versus s-FRQ is regulated by thermosensitive splicing of an intron (*frq-l6*) that when excised removes the translation initiation site of l-FRQ (Brunner and Diernfellner, 2006; Colot et al., 2005; Diernfellner et al., 2005). This thermosensitivity is likely based on the presence of multiple weak splice signals, including a C at position -1 of the 5'ss in combination with a non-consensus BPS and 3'ss. A variant with more optimized 5' and 3'ss, showed increased splicing efficiency with little temperature

responsiveness (Diernfellner et al., 2005), similar to our results. Unlike the situation with dm*per* RNA, temperature does not affect *frq* transcript levels (Liu et al., 1998). Nonetheless, the 5' UTR of *frq* RNA contains several upstream non-consensus translation initiation signals, leading to the trapping of scanning ribosomes at lower temperatures (Diernfellner et al., 2005; Liu et al., 1997). As a result not only does the ratio of l-FRQ to s-FRQ increase as temperature rises but so does the overall abundance of l-FRQ (Colot et al., 2005; Diernfellner et al., 2005; Liu et al., 1997; Liu et al., 1998).

Thus, in two widely different species the clockworks adapts to changes in temperature by thermal adjustments in the levels of key state-variables (i.e., PER in *Drosophila* and FRQ in *Neurospora*) via a mechanism involving an initial thermosensitive splicing event that has ramifications for other more downstream aspects of mRNA metabolism or utilization, such as the abundance of *dper* or the translational efficiency of *frq* transcripts. Further similarities between the two systems include the observations that the splicing efficiencies of dmpi8 and *frq-l6* are not only regulated by temperature but also light and the clock, with the relative abundance of spliced transcripts peaking during the nadir in total RNA levels (Collins et al., 2004; Diernfellner et al., 2007; Majercak et al., 2004).

Yet it is important to emphasize that temperature has diverse effects on circadian systems that are likely to be governed by distinct mechanisms, most notably; (1) temperature dependent changes in the distribution of a daily rhythm—the focus of this study; (2) ability of clocks to be entrained by daily temperature cycles and be phase-shifted by temperature pulses or steps; (3) "stopping" the clock at temperatures outside those permissive for rhythm generation and; (4) temperature compensation of period

length (Rensing and Ruoff, 2002; Sweeney and Hastings, 1960). For example, whereas dmpi8 splicing is involved in adjusting the timing of daily activity in *Drosophila*, it is not required for synchronization to daily temperature cycles (Glaser and Stanewsky, 2005). Moreover, the aforementioned mechanism operating in *Neurospora* plays a role in 'temperature compensation' (Diernfellner et al., 2007; Liu et al., 1997). Nonetheless, the results in *Drosophila* and *Neurospora* suggest that thermosensitive splicing of a clock gene is a common mechanism in how circadian systems respond to a variety of temperature cues.

In summary, our findings based on comparative analysis of several evolutionary related species of *Drosophila* with widely different modern distributions suggest that temperature regulated splicing of a *per* 3'-terminal intron facilitated the adaptation of *D. melanogaster* and *D. simulans* to temperate climates. Natural polymorphisms in the coding region of dm*per* been shown to influence another temperature relevant effect on the clock, namely, temperature compensation (Kyriacou et al., 2008; Sawyer et al., 1997). Thus, it is possible that the dm*per* gene in *D. melanogaster* is a 'thermal responsive hot-spot' for optimizing clock function to a range of climates. Although it is not clear at present whether the thermal phenotype in the splicing of dmpi8 is a result of natural selection, the requirement for multiple suboptimal splicing signals suggests intricate co-evolution. It appears that the overall efficiency of dmpi8 splicing is optimized for not only thermal responsiveness, which is based on suboptimal splicing signals, but also balanced against sufficient splicing efficiency to influence global levels of *per* RNA. A similar mechanism is absent in *D. yakuba* and *D. samtomea*, two highly related species that do not face the challenge of large seasonal variations in temperature. On a broader

perspective, our data suggest that natural selection operating at the level of splice site strength is likely to be a significant mechanism underlying thermal adaptation of life forms.

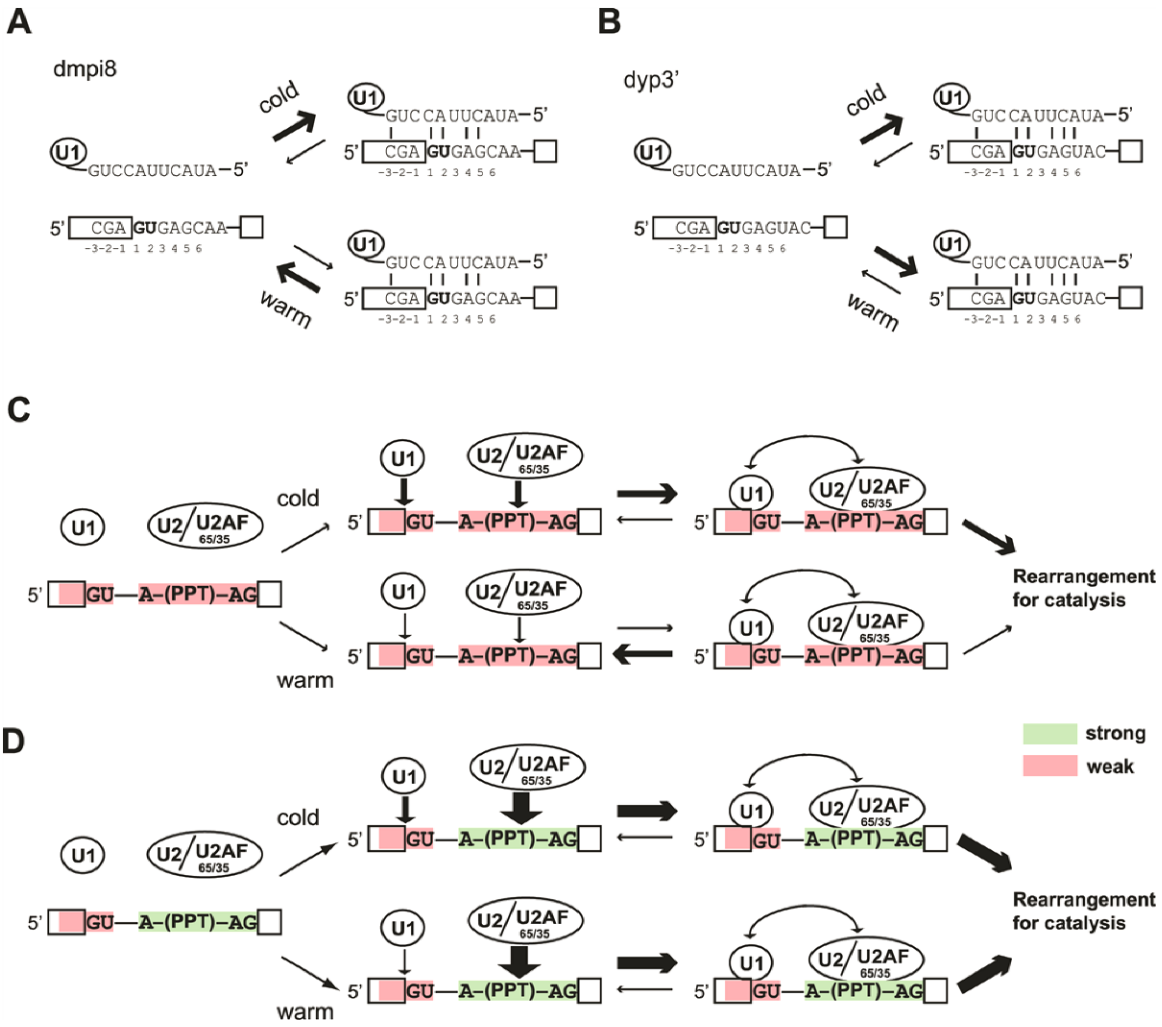


Figure 2.9. Model for how dmpi8 splicing is regulated by temperature.

(A, B) Shown are sequences around the 5'ss of dmpi8 (A) and dyp3' (B) and the predicted base-pairing contacts with U1 snRNA. Binding of U1 snRNA to the suboptimal 5'ss of dmpi8 is enhanced at cold temperatures, whereas temperature has little effect on this interaction with the stronger 5'ss of dyp3'. (C, D) During early steps in the splicing reaction, interactions between core splicing factors that recognize 5' and 3' canonical splicing signals (GU, 5'ss; A,j branch point; PPT, polypyrimidine tract; AG, 3'ss) stabilize splicesome assembly that after structural rearrangements leads to catalysis. (C) One or more suboptimal 3' signals (red) leads to transient binding of 3' factors,

Figure legend of Figure 2.9 continued.

minimizing their ability to stabilize the interaction of U1 snRNP to a weak 5'ss at warm temperatures, as is the case for dmpi8. (D) Stable binding of 3' factors to strong splicing signals (green) can promote the otherwise weak interaction of U1 with a suboptimal 5'ss at warm temperatures, attenuating the thermosenitivity of intron excision (e.g., as we noted for dmpi8:dyp3'(3'ss); Fig. 2.4). A similar lack of thermosensitivity in splicing efficiency can also be attained by having a strong 5'ss and a weak 3'ss (e.g., M2; Fig. 2.3).

Chapter 3. Natural polymorphisms in the 3' untranslated region of the *Drosophila melanogaster period* gene affect splicing of the dmpi8 intron and daily activity patterns

3.1 Introduction

Similar to many diurnal animals, the daily distribution of activity in *Drosophila melanogaster* exhibits a bimodal pattern with clock-controlled morning and evening peaks separated by a midday siesta (Rosato and Kyriacou, 2006). In prior work, we identified a putative adaptive mechanism that enables *D. melanogaster* the ability to optimize its daily distribution of activity in accordance with ambient temperature (Collins et al., 2004; Low et al., 2008; Majercak et al., 2004; Majercak et al., 1999). Most notably, the midday siesta is prolonged at higher temperatures, almost certainly an adaptive response to avoid the deleterious effects of extended periods of heat in the middle of a long hot summer day (Collins et al., 2004; Low et al., 2008; Majercak et al., 2004; Majercak et al., 1999). As opposed to behaviorally reacting to changes in temperature, we showed that such temperature-dependent behavioral responses are causally linked to a thermosensitive splicing event on the RNA from *period* gene (*dper*) (Low et al., 2008; Majercak et al., 1999), which is a key clock gene known for encoding species-specific circadian behavioral programs in *Drosophila* (Alt et al., 1998; Low et al., 2008; Petersen et al., 1988; Wheeler et al., 1991). The removal of a short intron in the 3' untranslated region (UTR) of *dper* RNA, named dmpi8, is inefficient at warmer temperature enabling a longer siesta and delaying the onset of the evening bout of activity (Low et al., 2008; Majercak et al., 1999). In contrast, splicing of dmpi8 is enhanced as temperature decreases, advancing the onset of the evening bout of activity and shifting

the daily distribution of activity to warmer daytime hours (Collins et al., 2004; Majercak et al., 2004; Majercak et al., 1999).

Earlier studies in *D. melanogaster* identified polymorphisms in the *dper* coding region that show a latitudinal cline and correlate with the ability of the clock to maintain temperature compensation (Costa et al., 1992; Dahlgaard et al., 2001; Sawyer et al., 1997; Sawyer et al., 2006; Simunovic and Jaenike, 2006). This raises the possibility that *dper* is a prime target for natural selection with regards to circadian thermal reponses. We sought to investigate the possibility of genetic variability harbored by natural populations of *D. melanogaster* from varying geographical locations that might affect the splicing efficiency of the dmpi8 intron, and hence the distribution of daily activity. Correlation of the fruit flies geographical location (variation in selective strength) with genetic variations affecting dmpi8 splicing efficiency could reveal mechanistic insights underlying the adaptation of circadian systems.

As an initial test case, Chen in our lab examined 10 isofemale lines – progeny originating from a single gravid female fly collected from the wild that gives an unbiased sampling of the wild living flies – from a collection of *D. melanogaster* caught along the east coast of the United States (Schmidt et al., 2000). Our rationale was based on the observation that the average annual coldest temperature from Vermont to Florida exhibits a robust latitudinal cline, suggesting we might identify one or more polymorphisms regulating dmpi8 splicing efficiency that vary as a function of latitude. Indeed, we did identify several polymorphisms in the *dper* 3' UTR. However, a latitudinal cline in either polymorphism or daily distribution of activity was not observed. All isofemale lines from this North American collection contain the identical suboptimal 5' and 3' splice

sites as the original lab strain of *D. melanogaster*, Canton-S (Figure 2.2). After sequencing *dper* 3' UTRs from many independent isofemale lines, Chen (2007) identified 2 allelic variants, namely VT1.1 and VT1.2 (Figure 3.1), named after an isofemale line captured from the wild in Whiting, Vermont (VT97.1) that carried both alleles. The VT1.1 and VT1.2 sub-lines, that are homogenous for a certain *dper* 3' UTR haplotype, were generated by multiple single-mated crosses and were confirmed by PCR and sequencing (Chen, 2007). The allelic variants consist of two combinations of 6 different polymorphisms, (4 Single Nucleotide Polymorphisms (SNPs) and 2 deletions/insertions; Figure 3.1).

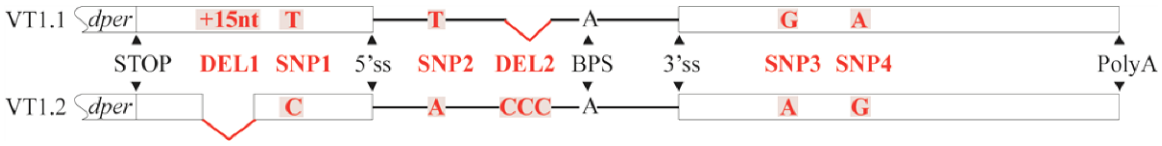


Figure 3.1. Two major haplotypes of the 3' UTR from *dper* are present in *D. melanogaster* from the eastern coast of the United States.

Shown are schematic representations of the *dper* 3' UTR from VT1.1 and VT1.2 inbred flies. The different natural variations are indicated (red). DEL: deletion; SNP: single nucleotide polymorphism; BPS: branch point sequence; ss: splice site; polyA: poly-adenylation site.

Interestingly, these 2 haplotypes differentially affect dmpi8 splicing efficiency and the profile in daily activity (Chen, 2007) in a manner consistent with previous findings (Low et al., 2008; Majercak et al., 1999). The inbred line (generated from isofemale line, VT97.1) carrying the VT1.1 allele exhibits higher splicing efficiency of dmpi8 and is associated with earlier accumulation of *dper* mRNA and shorter midday siesta (Figure A2, Page 139; Table 3.1) (Chen, 2007). Transgenic flies wherein the only functional copy of *dper* was a transgene carrying either the VT1.1 or VT1.2 haplotype further confirmed that variations in the circadian behavioral profiles observed in the inbred lines are due to differences in dmpi8 splicing efficiency (Figures A3, Page 140 and A4, Page 141) (Chen, 2007).

Table 3.1. VT1.1 inbred flies exhibit shorter siesta compared to VT1.2 inbred flies

Temperature (°C)	Genotype[1]	Morning Offset[2] (ZT hr ± sem)	Evening Onset[3] (ZT hr ± sem)	Siesta[4] (hr ± sem)	N[5]
18	VT1.1	2.9±0.1	7.9±0.2	5.0±0.2	81
	VT1.2	2.3±0.2	8.5±0.1	6.2±0.2**	90
29	VT1.1	2.7±0.2	9.6±0.2	6.9±0.4	52
	VT1.2	2.3±0.2	10.2±0.2	7.9±0.4*	54

[1]Young male flies were maintained under 12:12 LD cycle for four days. The last two days worth of activity data was pooled for each individual fly and then a group average was determined.

[2]Morning offset is defined as the time when 75% of peak morning activity was attained following the morning peak of activity.

[3]Evening onset is defined as the time when 75% of peak evening activity was attained prior to the evening peak of activity.

[4]Siesta time is defined as the length of time between 75% of morning offset and 75% of evening onset.

[5]N, number of flies that gave significant values for both morning offset, evening onset and that survived throughout the entire testing period.

*Student's t-test, $P<0.05$; **Student's t-test, $P<0.001$ comparing to VT1.1 inbred flies.

Following on the sequencing and behavioral studies, Chen used our simplified cell culture system (Chapter 2, Section 2.3.3) (Low et al., 2008) to evaluate the effects of the different polymorphisms on dmpi8 splicing efficiency. This analysis indicated that the splicing efficiency of VT1.1 is greater than that of VT1.2 (Figure 3.2, Page 95), in agreement with results obtained in flies (Figures A1, Page 128 and A3, Page 140). Also, comprehensive mutational studies in which each polymorphism of the VT1.1 haplotype was systematically replaced with the cognate VT1.2 version, revealed 2 non-intronic SNPs, namely SNP3 and SNP4, as key elements underlying the differential VT1.1. and VT1.2 splicing phenotypes (Figure 3.2, Page 95) (Chen, 2007). The objective of study presented in this chapter was to determine the possible physiological significance of SNP3/SNP4 using transgenic flies.

3.2 Materials and methods

3.2.1 Fly strains and general handling

All flies were routinely reared at room temperature (22-25°C) and maintained in vials or bottles containing standard agar-cornmeal-sugar-yeast-Tegosept-media. The generation of transgenic flies is described below.

3.2.2 Transgenic constructs and flies

To examine the physiological significance of SNP3 and SNP4 we utilized the VT1.1 transgenic construct generated by Chen (2007), which contains a 13.2 kb genomic *dper* insert with the 3' UTR from VT1.1 inbred flies, as a basis of our transgene, in which SNP3 and/or SNP4 were substituted with those found in VT1.2. Genomic DNA from

VT1.1 inbred flies was used as a template to amplify the *dper* 3' UTR from the STOP codon to nucleotide 7373 (numbering according to Citri et al., 1987), which is 68bp downstream of a unique Bsu36I site in the 3' UTR of d*per*. The fragment was amplified with primers KpnI_P6869 (5'-TAA***GGTACC***TAGTA GCCACACCCGCAGT-3'; KpnI site is bold italicized) and P7373 (5'- GTGGGCGTTGGCTTTTCG-3'). This introduces a KpnI site upstream of the STOP codon. The PCR products were then ligated into the pGEM-T easy vector (Promega). This parental construct was then used as a basis to introduce the VT1.2 SNP3, SNP4 or both using the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA). The KpnI and Bsu36I fragments were then reconstructed into a CaSpeR-4 based transformation vector, termed CaSpeR13.2-KA that contains a 13.2 kb genomic *dper* insert (Citri et al., 1987), yielding KpnI and ApaI sites upstream of the *dper* translation stop signal. Relevant regions of the transformation vectors were confirmed by sequencing before sending out to Genetic Services Inc (Sudbury, MA, USA) for injection into a w^{1118} background. The transgenes were subsequently crossed into a *w* per^{01} background with a double balancer line (*wper*^{0};*Sco*/*Cyo*;*MKRS*/*TM6B*), resulting in the transgenic lines termed P{VT1.1SNP3}, P{VT1.1SNP4}, P{VT1.1SNP3/4}. At least three independent lines for each construct were obtained. The results shown in this chapter were derived by pooling data from the following lines: P{VT1.1SNP3}: f36m1b, f5f1, f28m3b; P{VT1.1SNP4}: m23m1b, f7m1b, m43m3b, m44m1b; P{VT1.1SNP3/4}: m10m4, m17f1, m21f1.

3.2.3 Locomotor activity

Locomotor activity was recorded and analyzed as described in Chapter 2. Briefly, activity was continuously monitored and recorded in 15-min bins using the Trikinetics

(Waltham, MA, USA) system. Flies were entrained for at least 5 days of LD, followed by at least 5 days of DD under 2 different temperatures (18° and 25°C). For eductions and measuring behavioural values during LD cycles, at least two days worth of data were averaged and data from different lines pooled to generate the group averages. In Figure 3.5, daily locomotor activity profiles were normalized such that the peak of evening activity was set to 1, facilitating visual comparison of the different transgenic genotypes, as previously described (Low et al., 2008).

3.2.4 Splicing assay

The relative levels of the dmpi8 spliced type B' and unspliced type A *dper* RNA variants in wild type fly heads were measured using a reverse transcriptase-PCR (RT-PCR) assay as described in Chapter 2 (Section 2.2.6) (Low et al., 2008). In order to differentiate the transgenic *dper* mRNA transcripts from the endogenous per^{01} transcripts we used the forward primer P6851m2F (5'-ACAGCACGGGGATGGG***GGTACC***-3'; KpnI site bold italicized) with its 3' end base-pairing on the KpnI site which is not present in the endogenous transcript. As described in Chapter 2, P7197 was used as the reverse primer (5'-TCTACATTATCCT CGGCTTGC-3') and sense primer CBP540F (5' GTCTGATTCGTGTGGACTGG 3') and antisense primer CBP673R (5' CAACAGTTTGCCATAACCCC 3') were included in the same PCR reaction to target the non-cylcing Cap Binding Protein 20 (CBP20) gene as an internal control.

3.3 Results

3.3.1 *In vivo* effects of SNP3 and SNP4 on the efficiency of dmpi8 splicing are consistent with results obtained in cultured S2 cells

Previously, our lab established a simplified S2 cell culture system that recapitulates the thermal sensitive splicing efficiency of the dmpi8 intron observed in fly heads (Chapter 2, Section 2.3.3) (Low et al., 2008). In order to understand the underlying mechanism responsible for the differences in the splicing efficiency of dmpi8 from VT1.1. and VT1.2 flies, Chen in the lab replaced VT1.1 3' UTR polymorphisms with those from VT1.2 (Figure 3.2) (Chen, 2007). The idea was to identify SNPs from VT1.2 that when placed in a VT1.1 background reduced the splicing efficiency of dmpi8 to that observed with the control VT1.2 version. No significant effects on dmpi8 splicing efficiency were observed when single SNPs were replaced. However, several combinations of polymorphisms did reduce the splicing efficiency of the hybrid 3' UTR to that resembling the wildtype VT1.2 version. For example, SNP4 with either SNP 1, 2, or 3; also, SNP2 in combination with SNP3 (Figure 3.2). Among all combinations tested, introduction of the VT1.2 versions of SNPs 3 and 4 into the VT1.1 3' UTR background resulted in reducing dmpi8 splicing efficiency to a level very similar to that of the VT1.2 control (Figure 3.2), suggesting SNPs3/4 play a major role in the differential splicing efficiencies of dmpi8 in VT1.1 and VT1.2.

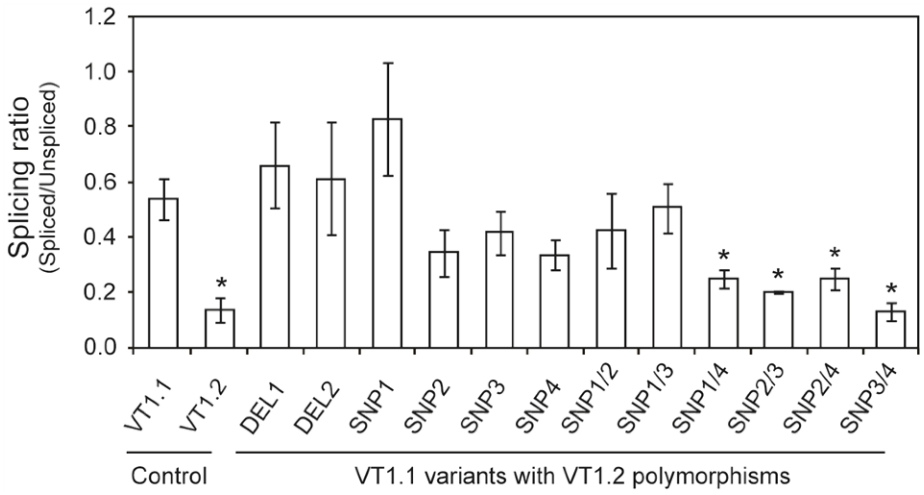


Figure 3.2. Analysis of natural polymorphisms that might underlie differences in dmpi8 splicing efficiency for the VT1.1 and VT1.2 versions of the *dper* 3' UTR.

Shown are splicing efficiencies of different variants of VT1.1, in which indicated polymorphisms (see Figure 3.1) are replaced by the corresponding one found in the VT1.2 haplotype. S2 cells were transiently transfected with reporter constructs with different 3' UTRs, grown at 22°C, collected two days later and assayed for dmpi8 splicing. Shown are the averages of five replicates. Student's t-test, * $P<0.05$, compared to values obtained for VT1.1 control (Courtesy of (Chen, 2007)).

Since combination of SNPs 3 and 4 gave the most striking effect and they are in close proximity (Figure 3.1), we decided to examine the physiological contributions of these two polymorphisms in flies. We generated transgenic flies and followed the same strategy as that used in the S2 cell studies, whereby the VT1.1 version of *dper* was modified by replacing SNP3, SNP4 or both with the corresponding VT1.2 version. The different *dper*-containing constructs were evaluated in a per^{01} background; thus, the only functional copy of *dper* in these flies is supplied by the recombinant transgene. At least 3 independent lines of each genotype (P{VT1.1SNP3}, P{VT1.1SNP4}, P{VT1.1SNP3/4}) were obtained and assayed for behavioral rhythms and dmpi8 splicing efficiency.

We first sought to measure the splicing efficiency of dmpi8. Mature poly(A)-tailed mRNA was extracted from adult fly heads and analyzed for transgene-derived dmpi8 splicing efficiency with a previously established RT-PCR based assay (see Section 3.2.4). Replacing SNP3 or SNP4 alone (P{VT1.1SNP3}, P{VT1.1SNP4}, respectively) did not cause any noticeable difference in dmpi8 splicing efficiency compared to the control VT1.1 under standard 12:12 light and dark conditions at 25°C (data not shown). Only when both SNPs were replaced (P{VT1.1SNP3/4}) did the splicing efficiency of dmpi8 exhibit significant reductions (Figure 3.3B; ANOVA, $P<0.001$). The effect of the SNP3/4 replacement is mainly apparent in the daytime, particularly ZT 4 and 12 (one way ANOVA, $P<0.01$). These *in vivo* splicing results are remarkably consistent with the results observed in the S2 cell-based splicing assay (Figure 3.2).

Interestingly, differences in dmpi8 splicing efficiency between the single replacements and the double were observed at all temperatures tested (Figure 3.3, A and

B). Indeed, all three transgenic flies (P{VT1.1SNP3}, P{VT1.1SNP4}, and P{VT1.1SNP3/4}) exhibit temperature-dependent splicing phenotypes, regardless of their intrinsic splicing efficiency (Figure 3.4 A, B, C). This is strikingly different from our recent work showing that increasing the strengths of the 5' and 3'SS on dmpi8 abolish the thermosensitivity in its splicing (Low et al., 2008). Thus, it appears that SNPs3/4 influence the intrinsic splicing efficiency of dmpi8 without altering its thermal range.

Higher splicing efficiency of dmpi8 is associated with an earlier daytime accumulation in *dper* RNA levels (Low et al., 2008; Majercak et al., 1999). In agreement with this relationship, *dper* transcript accumulation is delayed in the weaker splicing P{VT1.1SNP3/4} flies, an effect more clearly observed at 25°C (Figure 3.3). Intriguingly, the daily profiles in *dper* RNA levels even reflect subtle differences in dmpi8 splicing efficiency; for example, the daily splicing efficiency of dmpi8 in P{VT1.1SNP4} flies is intermediate between P{VT1.1SNP3} and P{VT1.1SNP3/4} (Figure 3.3, A and B), as is the timing in *dper* RNA accumulation (Figure 3.3, C and D).

Together, these data indicate that SNP3 and SNP4 function as a unit *in vivo* to regulate the splicing efficiency of dmpi8 without affecting its thermosensitivity. Moreover, enhancing the splicing efficiency of dmpi8 is associated with an earlier rise in the daily upswing in *dper* mRNA, further supporting the association between the splicing efficiency of dmpi8 and *dper* RNA levels (Figure 1.3).

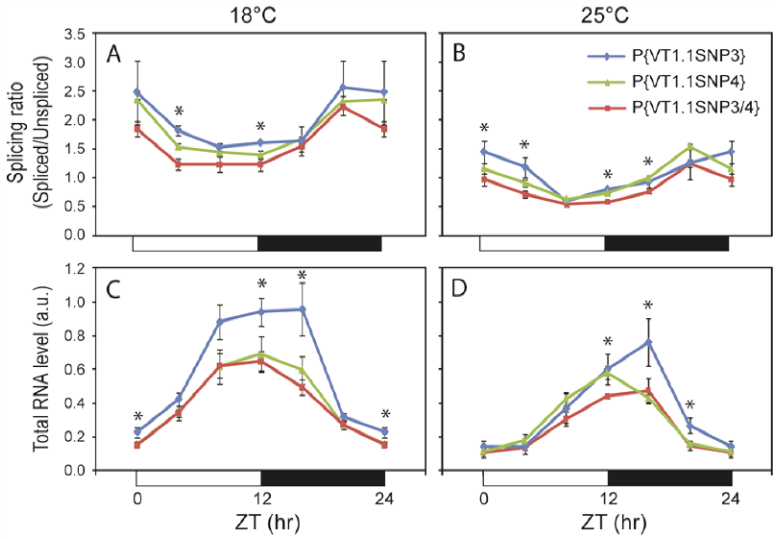


Figure 3.3. Substituting both SNP3 and SNP4 of VT1.1 with those from VT1.2 reduces the daily splicing efficiency of dmpi8 in fly heads and is associated with delayed accumulation of d*per* mRNA levels.

Transgenic flies were entrained for 5 12:12 LD cycles at 18°C (A, C) or 25°C (B, D), and collected once every 4 hours on the last LD for RNA extraction. The splicing ratio (A, B) and relative *dper* mRNA levels (C, D) of the transgene in *w per*^{01} genetic background were measured using semi-quantitative RT-PCR assay (see Section 3.2.4). Shown are data pooled from at least three independent lines for each genotype. (A and B) Splicing ratio (± SEM) of P{VT1.1SNP3/4} is significantly lower than the singly mutated genotypes (ANOVA, P<0.001; and post hoc multiple comparison with Tukey's HSD, P<0.0001). Astericks (*) indicate significant differences in splicing ratio as a function of genotype especially at daytime ZT hours (one way ANOVA, *, P<0.05). (C and D) ANOVA analysis showed that total RNA level (arbitrary unit (a.u.) ± SEM) of P{VT1.1SNP3} is significantly higher than all other genotypes (ANOVA, P<0.01, and post hoc multiple comparison with Tukey's HSD, P<0.0001). Astericks (*) indicate significant genotype effect on RNA level (one way ANOVA, *, P<0.05).

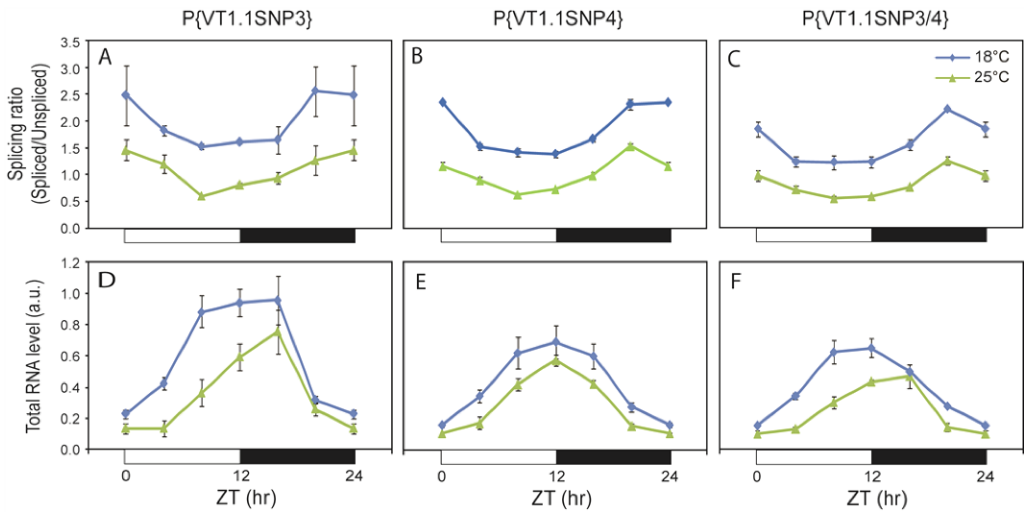


Figure 3.4. SNP3 and SNP4 regulate splicing efficiency of dmpi8 without affecting its thermosensitivity.

Splicing ratio (± SEM) (A-C) and total RNA level (arbitrary unit (a.u.) ± SEM) (D-F) are significantly higher at 18°C compare to 25°C (ANOVA, $P<0.00001$).

3.3.2 Substituting SNP3 and SNP4 of VT1.1 with those from VT1.2 lengthens siesta time

To further examine the physiological relevance of the SNP3/4-mediated dmpi8 splicing phenotype, we subjected the different transgenic flies to standard 12 hr light and 12 hr dark entraining scheme (LD) under different temperatures (18°C, 25°C, and 29°C) and compared daily locomotor behavioral profiles (Figure 3.5). For each genotype, we analyzed at least 3 independent lines and obtained an average profile.

All 3 transgenes rescued behavioral rhythmicity in a *w per*^{01} genetic background with identical free running periods (Table 3.3). This result is consistent with our prior work showing that the splicing efficiency of dmpi8 regulates the daily distribution of activity without altering the endogenous pace of the clock (Low et al., 2008; Majercak et al., 1999). The daily distributions in activity were similar for P{VT1.1SNP3} and P{VT1.1SNP4}. However, the midday siesta time was significantly more robust for P{VT1.1SNP3/4} flies compared to either P{VT1.1SNP3} or P{VT1.1SNP4}. This was the case at all temperatures tested (Figure 3.5D; Table 3.2), indicated by an earlier decline in morning activity (Figure 3.5E; Table 3.2) and/or delayed onset of evening activity (Figure 3.5F; Table 3.1). All three transgenic flies showed an increase in midday siesta with rising temperature, indicating that SNPs 3 and 4 do not abolish behavioral thermosensitivity, consistent with the effects of temperature on dmpi8 splicing efficiency (Figure 3.4)

Interestingly, the length of siesta or timing of morning/evening activity peak in P{VT1.1SNP4} flies are roughly intermediate between those of P{VT1.1SNP3} and P{VT1.1SNP3/4} flies (Figure 3.5 A, B, C). This is reminiscent of the splicing assay

data, in which the splicing efficiency of dmpi8 in P{VT1.1SNP4} appears intermediate to that observed in P{VT1.1SNP3} and P{VT1.1SNP3/4} flies (Figure 3.3 A, B).

As reported in the previous chapter, we have also noticed that as temperature increases, differences in the onset of evening activity between different genotypes are diminished (Figure 3.5A, B, C, F). This is likely due to the inhibitory effect of visible light (also known as "masking"), which suppresses activity during the day especially at higher temperatures (Matsumoto et al., 1998), overriding the effects of dmpi8 splicing (Low et al., 2008). Conversely, we noted differences in the timing of the offset in morning activity for the different genotypes at all temperatures tested, whereby genotypic differences were more prominent as temperature increases (Figure 3.5E). Indeed, at 29°C the offset in morning activity for P{VT1.1SNP4} was intermediate compared to P{VT1.1SNP3} and P{VT1.1SNP3/4} (Figure 3.5C). Thus, our findings identify a prominent role for dmpi8 splicing efficiency and SNPs3/4 in modulating the start of the decline in morning activity.

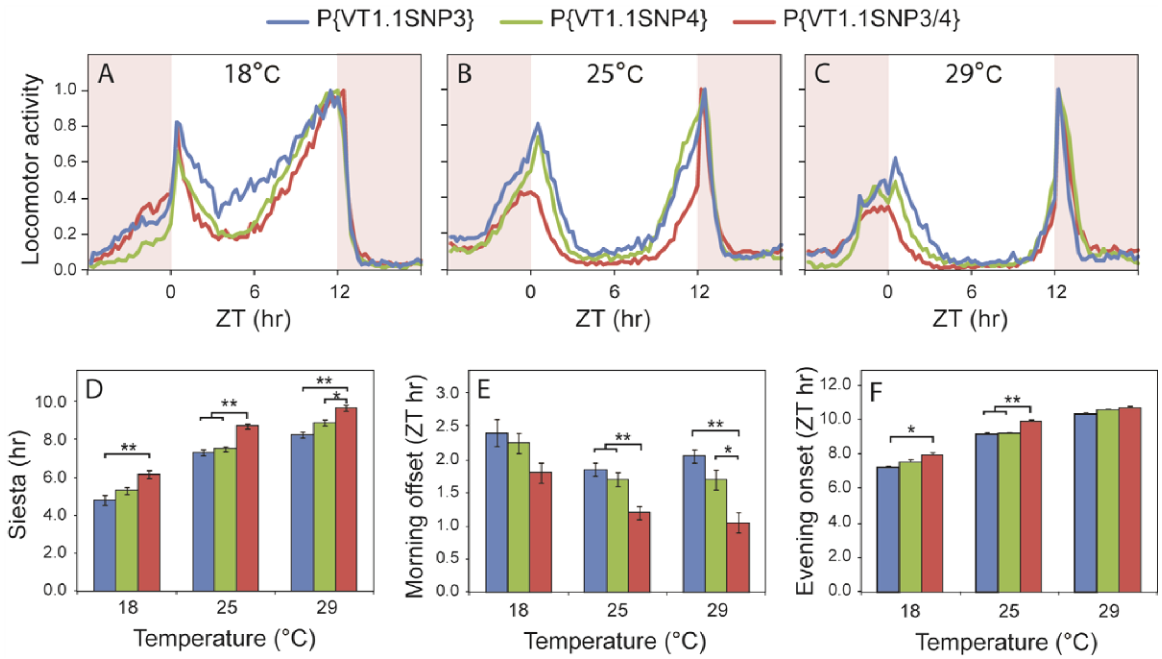


Figure 3.5. Replacing both SNP3 and SNP4 from VT1.2 prolongs the midday siesta. (A-C) Shown are the daily distributions of locomotor activity averaged over the last 2 LD cycles for different transgenic flies (i.e. *w per*^{01} flies bearing the P{VT1.1SNP3}, P{VT1.1SNP4}, or P{VT1.1SNP3/4} transgenes). Flies were entrained under 12:12 LD cycles (white and grey shade indicate light and dark periods, respectively) at three indicated temperatures indicated. For each genotype, at least 3 independent transformant lines were pooled and averaged. The peak of activity for each genotype was set to 1 to allow ease of comparison by overlaying the profiles. (D-E) Quantitative parameters of activity profiles shown in panel (A-C); i.e. length of siesta time (D), timing of morning activity offset (E), timing of evening activity onset (F). One way ANOVA followed by multiple comparison poct hoc Tukey's HSD **, P<0.0005; *, P<0.002.

Table 3.2. Timing of daily activity in P{VT1.1SNP3}, P{VT1.1SNP4} and P{VT1.1SNP3/4} flies.

Temperature (°C)	Genotype[1]	Morning Offset[2] (ZT hr ± sem)	Evening Onset[3] (ZT hr ± sem)	Siesta[4] (hr ± sem)	N[5]
18	**P{VT1.1SNP3}**	2.4 ± 0.2	7.2 ± 0.2	4.8 ± 0.3	89
	P{VT1.1SNP4}	2.3 ± 0.2	7.6 ± 0.2	5.3 ± 0.2	105
	P{VT1.1SNP3/4}	1.8 ± 0.2	8.0 ± 0.2	6.2 ± 0.2	86
25	**P{VT1.1SNP3}**	1.9 ± 0.1	9.2 ± 0.1	7.3 ± 0.2	110
	P{VT1.1SNP4}	1.7 ± 0.1	9.2 ± 0.1	7.5 ± 0.2	125
	P{VT1.1SNP3/4}	1.2 ± 0.1	9.9 ± 0.1	8.7 ± 0.2	94
29	**P{VT1.1SNP3}**	2.1 ± 0.1	10.3 ± 0.1	8.3 ± 0.2	80
	P{VT1.1SNP4}	1.7 ± 0.2	10.6 ± 0.1	8.9 ± 0.2	95
	P{VT1.1SNP3/4}	1.1 ± 0.2	10.7 ± 0.1	9.7 ± 0.2	63

[1]Young male flies were maintained at the indicated temperatures and photoperiod for four days. The last two days worth of activity data was pooled for each individual fly and then a group average was determined.

[2]Morning offset is defined as the time when 50% of peak morning activity was attained following the morning peak of activity.

[3]Evening onset is defined as the time when 50% of peak evening activity was attained prior to the evening peak of activity.

[4]Siesta time is defined as the length of time between 50% of morning offset and 50% of evening onset.

[5]N, number of flies that gave significant values for both morning offset, evening onset and that survived throughout the entire testing period.

Table 3.3. Periodicity parameters of P{VT1.1SNP3}, P{VT1.1SNP4} and P{VT1.1SNP3/4} flies.

Temperature (°C)	Genotype[1]	Period (hr ± sem)	Power[2] (± sem)	Rhythmicity[3] (%)	N
18	**P{VT1.1SNP3}**	23.5 ± 0.1	66.2 ± 4.0	84.1	63
	P{VT1.1SNP4}	23.5 ± 0.1	88.4 ± 4.4	85.5	62
	P{VT1.1SNP3/4}	23.5 ± 0.1	89.1 ± 4.1	92.1	63
25	**P{VT1.1SNP3}**	23.9 ± 0.1	80.2 ± 4.8	92.2	64
	P{VT1.1SNP4}	23.7 ± 0.1	96.6 ± 4.2	95.2	63
	P{VT1.1SNP3/4}	23.8 ± 0.1	93.5 ± 4.7	96.6	59
29	**P{VT1.1SNP3}**	23.6 ± 0.1	87.5 ± 5.6	96.9	32
	P{VT1.1SNP4}	23.6 ± 0.0	101.6 ± 4.0	100.0	32
	P{VT1.1SNP3/4}	23.5 ± 0.1	102.3 ± 5.8	100.0	29

[1] Young male flies were entrained for four 12:12LD cycles followed by five days in constant dark conditions at the indicated temperatures. The results are a subset of the same experiments used to calculate the data shown in Figure 3.5 A, B, C.
[2] Power is a measure of the strength or amplitude of the rhythm.
[3] Flies with a power value of greater than 10 and period ≥20 and ≤30, were defined as rhythmic.

3.4 Discussion

Based on the rationale that variations in geographical conditions could lead to intra-species variation in genetic components that enhance adaptation, our lab sought to identify naturally occurring *cis*-elements that regulate dmpi8 splicing. This was done by examining natural populations of *D. melanogaster* collected from a latitudinal cline that has a very robust gradient in average annual temperature. Although we did not observe genetic variation in the *dper* 3' UTR that correlates with latitude, we identified 6 naturally occurring polymorphisms that might play a role in the efficiency of dmpi8 removal. In this chapter, we described the role of 2 non-intronic polymorphisms (SNP3 and SNP4), working in combination, in mediating dmpi8 splicing efficiency without affecting thermosensitivity of this post-transcriptional process. These polymorphisms were also shown to influence daily cycles in *dper* RNA levels and daily activity patterns in a manner consistent with previous findings wherein better splicing is associated with earlier mRNA accumulation followed by less prominent siesta times and earlier evening activity (Low et al., 2008; Majercak et al., 1999). This data further supports the strong causal relationship between dmpi8 splicing efficiency, *dper* mRNA levels and daily activity profiles.

3.4.1 Regulation of dmpi8 splicing by non-intronic natural polymorphisms

In chapter 2 (Low et al., 2008), it was shown that increasing the splice site strength of dmpi8 improved its splicing efficiency, presumably by enhancing the recognition of intron/exon junctions by the spliceosome complex. Data in this chapter indicates that natural polymorphisms located outside of the dmpi8 intronic region can also influence dmpi8 splicing efficiency without affecting the strength of the intronic splicing signals.

As most splice sites sequences are relatively degenerate and the key splicing signals alone are not capable of efficiently directing spliceosome assembly, especially alternatively spliced introns, SNP3 and SNP4, which are located >70nt downstream of the 3'ss, are probably playing a modulatory role in dmpi8 splicing. For example, these SNPs might act as exonic splicing enhancers or silencers that can positively or negatively affects splice sites selection or recognition (Hertel, 2008). Indeed, preliminary analysis based on human consensus sequences for SR protein recognition sites (using web-based prediction algorithm , ESE finder) suggests that SNP3 found in VT1.1 is a better binding site for the SR protein termed SF2 (data not shown). This is not surprising because SR proteins are thought to play important roles in controlling spliceosome assembly (Stojdl and Bell, 1999; Yeakley et al., 1999) via direct interaction with alternative spliced pre-mRNAs and core splicing factors.

Even though the splicing efficiency of dmpi8 in P{VT1.1SNP3} flies is significantly higher than that observed in P{VT1.1SNP3/4} flies, splicing is still responsive to temperature in contrast to previous findings whereby strengthening the 5' and 3'ss of dmpi8 enhances dmpi8 splicing but reduces or eliminates its thermosensitivity (Low et al., 2008). Note that the overall splicing efficiency of dmpi8 in different SNP3 and SNP4 variants are within a range of 30% to 55% (percent spliced) at 25°C but transgenic flies with dmpi8 variants that have strong splice sites, i.e. P{dmper/M2M1} and P{dmper/dyp3'}, showed at least 80% of the mature mRNA with the dmpi8 intron spliced out. These differences suggest that below a certain threshold of overall splicing efficiency, thermal responsiveness is retained.

Alternatively, SNP3/4 can alter the overall splicing efficiency without affecting the binding of the splicesome to dmpi8. Prior work suggested that the initial recognition of the 5'ss suboptimal splicing signal underlies the temperature responsive splicing phenotype of dmpi8 (Low et al., 2008). On the contrary, SNP3/4 could influence the intrinsic splicing efficiency via their effect on subsequent splicing reaction steps, e.g. such as rearrangements necessary to carry out the catalytic step.

Some of the other polymorphisms that showed moderate effects in conjunction with SNP3 or SNP4 might be of interest to examine in terms of regulating dmpi8 splicing. Alternative splicing is known to vary in different tissues (Matlin et al., 2005) and S2 cells might not possess the right repertoire of cellular factors to allow dramatic effects of these other polymorphisms (Sakabe and de Souza, 2007). Splicing regulators such as SR proteins are normally associated with introns that have weak splicing signals and minimal sequence information for recognition by the spliceosome (Reed, 1996). Therefore, polymorphisms other than SNP3 and SNP4 might function cooperatively to regulate dmpi8 splicing. Since the functions of SR protein function are sometimes redundant (Tacke and Manley, 1999), delineating the role of individual SNPs might not be a straight-forward task. This might explain why mutating SNPs one at a time does not significantly influence the splicing efficiency of dmpi8 (Fig. 3.2).

Another reason we cannot discount the potential role of other polymorphisms is because all the SNPs identified in the inbred lines collected from Vermont are also found widespread in different lines of *D. melanogaster* collected from Africa and Europe (unpublished data from Cecilia Lim and Douglas Pike, personal communication). In fact, additional polymorphisms are also present in the *dper* 3' UTR region of more ancestral

flies from Africa. It is possible that this 3' UTR region is a "hot spot" for natural selection to select for regulator sequences that play a role in dmpi8 splicing. The 3' UTR region might be advantageous in terms of this molecular adaptation because it is not as constrained as coding regions.

3.4.2 Non-intronic natural polymorphisms regulate midday siesta length via differential effects on morning and evening activity bouts

The higher splicing efficiency of dmpi8 in P{VT1.1SNP3} and P{VT1.1SNP4} compared to P{VT1.1SNP3/4} transgenic flies (Figure 3.3 A and B) is not as dramatically different as the comparison between P{dmper/8:8} and P{dmper/M2M1} (Figure 2.6 A and B). However, differences in splicing efficiency of dmpi8 as a result of variations in SNP3/4 are still being translated into significant differences in midday siesta length (Table 3.1). This suggests there is a functional range of splicing dependent behavioural plasticity, in which if the splicing efficiency exceeds a certain upper threshold, no additional effect on the length of midday siesta time occurs.

In this study, we realized an interesting relationship between dmpi8 splicing and morning activity profile that we were not aware of previously. Differences in light induced locomotor activity right after dark-to-light transition (i.e., 'startle' response) are more pronounced as temperature increases (Figure 3.5 A, B, C). Interestingly, a recent study conducted by Zhang et al. (2010) showed that a subset of DN1 cells, $DN1_p$s, play a role in promoting morning activity and such a phenotype is inhibited at colder temperature. The $DN1_p$s might be a site-of-action for dmpi8 dependent behavioural program. The differential splicing of dmpi8 variants in $DN1_p$s can only be apparent at warmer temperatures as the light induced morning activity is not suppressed. In addition

to modulating morning activity, $DN1_p$s were also shown to modulate evening peak of activity that is tightly regulated by the environment, i.e. light and temperature. High light intensities and elevated temperature suppressed the evening locomotor activity in $DN1_p$s rescued *per*^{01} flies. This probably accounts for the masking effect of light and warmer temperature on differences in evening bout activity onset of different transgenic flies exhibiting variable dmpi8 splicing efficiency (Figure 2.5 C, F, I; Table A8; Figure 3.5 A, B, C, F; Table 3.1).

Reanalyzing the behavior of transgenic flies from the previous chapter, whereby dmpi8 splicing of the transgenic flies are altered by mutating splice site strength (Low et al., 2008), also showed more obvious differences in morning bout activity offset at warmer temperature (compare P{dmper/8:8} and P{dmper/M2M1} in Figure 2.5B, E, H; C, F, I; Tables A6 and A8). This suggests that splicing of dmpi8 in general, regardless of being affected by splice site strength or modulatory factors, regulates the onset of midday sleep in a temperature dependent fashion.

At 29°C, differences between morning activity bout offset (Figure 3.5 C) could be related to differential acute startle responses to light in these transgenic flies tested. Preliminary analyses indicated a surprisingly tight link between the intrinsic splicing efficiency of dmpi8 and daily sleep, especially daytime sleep (see Chapter 4). Prior work based on mosaic electrical excitation manipulation showed that the l-LN_vs function in light-dependent arousal (Shang et al., 2008; Sheeba et al., 2008). The results suggested that the hyperactive l-LN_vs increase arousal by antagonizing sleep homeostasis. Thus, it is possible that dmpi8 splicing preferentially regulates sleep propensity during the day by direct or secondary effects on arousal threshold via l-LN_vs, e.g. more efficient dmpi8

splicing leads to an increase in electrical excitation of the l-LN_vs through some unknown mechanism. Interestingly, l-LN_vs are also shown to function in the circadian photoreception circuit governing the morning activity bout (Shang et al., 2008). The PDF expressing l-LN_vs could act upstream of $DN1_p$s since $DN1_p$s express the PDF receptor (PDFR) and are highly sensitive to PDF resetting (Zhang et al., 2010). Future studies will be required to ascertain the site-of-action of dmpi8 splicing and its differential contributions to morning and evening activity bouts.

Chapter 4. Summary

Using *Drosophila melanogaster* as a model system, our lab has been trying to understand the role of circadian clocks in regulating the pattern of daily activity in response to changes in temperature and day length. We showed that temperature regulates the daily distribution of activity in a manner that likely reflects a role for clocks in seasonal adaptation (Majercak et al., 1999). Cold temperatures induce an earlier rise and peak in evening-associated locomotor activity, resulting in a preference for diurnal activity. Conversely, at warm temperatures the evening bout of activity is mainly nocturnal and the midday siesta is enhanced, likely adaptive responses that minimize the risk of desiccation associated with the hot midday sun. The temperature-dependent behavioral plasticity is regulated via a post-transcriptional mechanism involving splicing of the 3' terminal intron in the RNA product from the *Drosophila per* gene (Majercak et al., 1999). Cold temperatures enhance the splicing efficiency of this intron (termed *Drosophila melanogaster period* intron, dmpi8), an event that is associated with advanced daily accumulation of *dper* RNA and protein (Majercak et al., 1999). Conversely, warm temperatures attenuate dmpi8 splicing efficiency contributing to delayed upswing in *dper* RNA levels and evening activity.

To further understand the adaptive role of this temperature responsive molecular mechanism, we employed a comparative approach with an evolutionary perspective. Sequence analyses of phylogenetically related *Drosophila* species (Figure 4.1) suggest that the presence of a 3' UTR terminal intron is a conserved feature of the *dper* gene across phyla (Low, 2008; Table 4.1). However, the splicing phenotype of the *dper* 3' terminal intron is variable between species examined (Low et al., 2008). The more Afro-

equatorial species of *Drosophila yakuba* and *Drosophila santomea,* exhibit a largely temperature independent splicing phenotype – highly efficient over a wide range of temperatures. Interestingly, the associated daily activity profile does not respond to changes in temperature, and the midday inactivity is mainly restricted at the middle of a daily cycle (when hot temperatures are expected in its natural environment) even at laboratory imposed colder temperatures (Low et al., 2008). This appears to make evolutionary sense as *D. yakuba* and *D. santomea* are species indigenous to Afro-tropical regions that experience little fluctuation in day length and temperature throughout the year (David et al., 2007). In contrast, the cosmopolitan *D. melanogaster* as well as a closely related species, *D. simulans*, exhibit widespread colonization in temperate parts of the world and are therefore challenged by seasonal variations in temperature. Thus, we view the temperature dependent splicing of *per* 3'-terminal introns as part of mechanisms that enabled *D. melanogaster* (and *D. simulans*) to adapt to temperature climates, where warm days are usually associated with long days (Low et al., 2008).

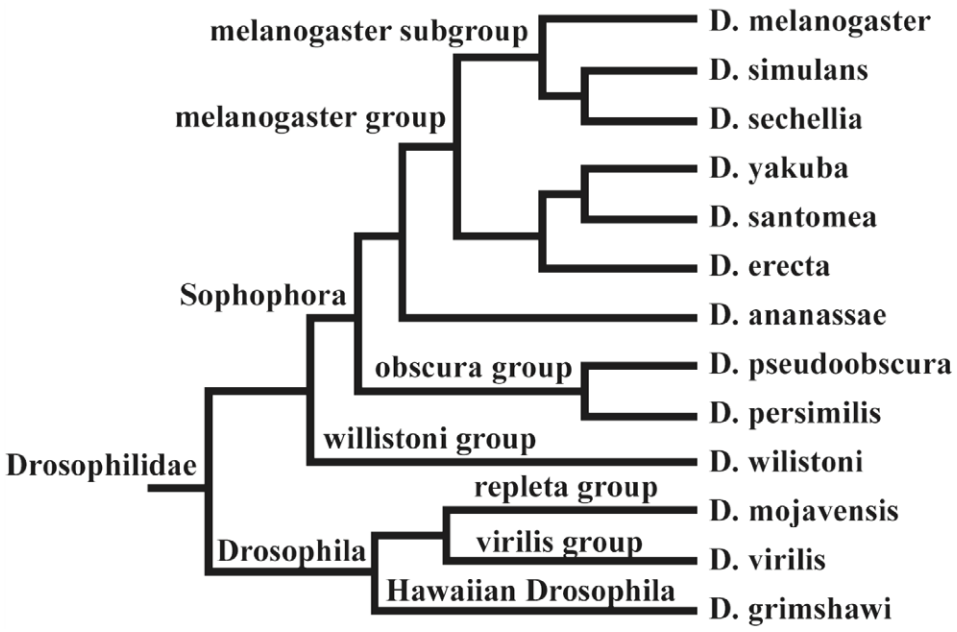


Figure 4.1. Phylogenetic tree relating *Drosophila* species.

Shown are 12 *Drosophila* species whose genomes were recently sequenced and analyzed in Stark et al. (2007). A newly discovered close relative of *D. yakuba*, *D. santomea* is adapted into the phylogenetic tree based on studies of Lachaise et al. (2000) (Adapted from Flybase).

Table 4.1. Known/predicted *dper* 3' terminal introns and their attributes

Species	Size (nt)	Predictive splice site score[1]		BPS[2,3]	polyA[3]	Distance between (nt)		
		5'ss	3'ss			STOP to 5'ss[4]	BPS to 3'ss[5]	3'ss to polyA[4]
D. melanogaster[6]	86	0.45	0.21	ctaAc	aataaa	111	24	277
D. simulans[6]	86	0.39	0.44	ctaAc	aataaa	111	24	250
D. sechelia[6]	86	0.21	0.41	ctaAc	aataaa	110	24	272
D. yakuba[6]	85	0.79	0.78	cttAt	aataaa	112	23	271
D. santomea[6]	72	0.80	0.78	cttAt	NA	112	23	NA
D. erecta[6]	83	0.77	0.26	ctaAt	attaaa	111	25	199
D. ananassae[6]	63	0.69	0.71	ttaAt	aataaa	127	20	162
D. pseudoobscura[6]	54	0.37	0.81	ctAtt	aataaa	121	13	159
D. wilistoni[6]	65	0.52	0.91	ttaAt	aataaa/aataaa/attaaa	122	12	133/183/406
D. mojavensis	59	0.87	0.89	cttAt	attaaa	126	16	864
D. virilis	78	0.83	0.85	ctaAc	aataaa	128	36	151
D. grimshawi	72	0.94	0.93	ctaAc/cttAt	attaaa/aataaa	111	44/29	111/116

[1] Prediction is based on a trained algorithm, NNSPLICE 0.9 (Reese et al., 1997).

[2] BPS: Branch point sequence.

[3] Putative BPSs and polyA signals are determined by visual inspection based on known consensus sequences (Mount et al., 1992; Retelska et al., 2006).

[4] Excluding STOP, 5'ss, 3'ss and polyA sequences.

[5] Including putative branchpoint A and 3'ss.

[6] Intron sequences were confirmed experimentally.

As an initial attempt to understand the molecular basis underlying the differential thermal responses of *dper* 3'-terminal introns from different *Drosophila* species, we sought to develop a simplified cell-culture-based assay that recapitulates the species-specific thermal splicing phenotypes (Low et al., 2008). Transfected *luciferase* (*luc*) reporter construct with *D. melanogaster* (Oregon-R strain) *dper* 3'UTR fused downstream showed increases in relative splicing efficiency at colder temperatures similar to that observed in flies (Figures 2.3 and 2.4). Importantly, the splicing efficiency of the dyp3' intron from *D. yakuba* does not change as a function of temperature. Furthermore, it was shown in the simplified tissue culture system that 3' terminal introns with higher splicing efficiency exhibit faster accumulation rates of the reporter mRNA (Figure 4.2), consistent with *in vivo* studies showing that increases in splicing efficiency are associated with higher *dper* RNA levels (Low et al., 2008; Majercak et al., 1999). Thus, when evaluated in cultured S2 cells, the *dper* 3'UTR is sufficient to manifest physiologically relevant thermal responses of *dper* 3'-terminal intron splicing, providing a powerful approach to investigate mechanistic issues.

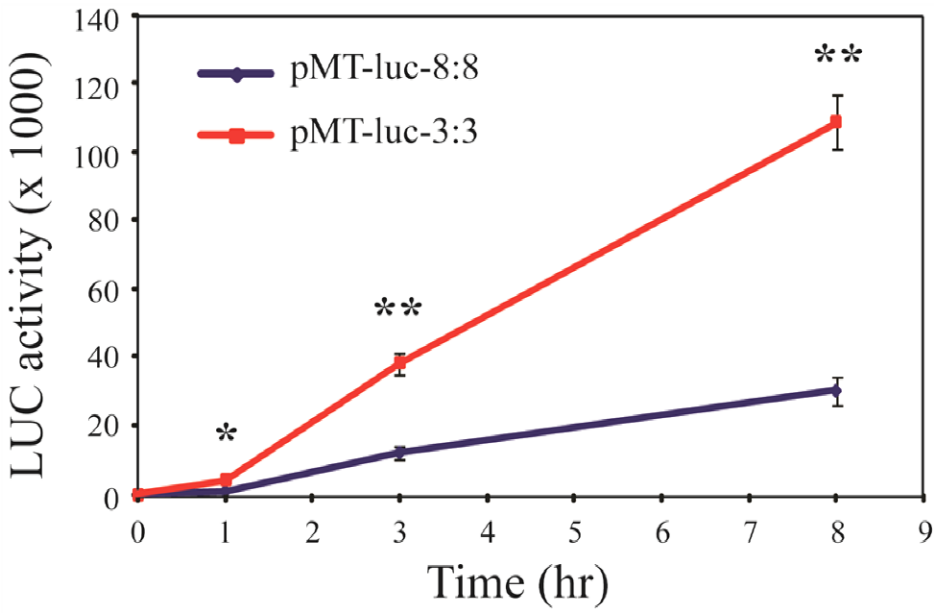


Figure 4.2. 3' terminal intron with better splicing efficiency are associated with greater amplitude increases in *dper* RNA abundance.

S2 cells were transfected with pMT (inducible metallothionein promoter) versions of either luc-8:8 or luc-3:3 (see Figure 2.4A), grown at 22°C and expression induced by the addition of Cu^{2+} (final concentration, 125µM) at time 0. Cells were collected at the indicated times (min), extracts prepared and levels of LUC activity measured. Values at time 0 were set to 0 and all other values normalized. For LUC activity values were also normalized to total protein content. **, $P<0.0005$; *, $P<0.001$, determined by two-tailed Student's t-test.

When the dmpi8 intron was inspected for notable features that might modulate its thermosensitive splicing efficiency, we noted that the 5' and 3'ss are predicted to be very weak (Mount et al., 1992) (Figure A1). To test this hypothesis we modified sequences defining 5'ss and 3'ss with sequences closer to the consensus, such as the one found in *D. yakuba* (Low et al., 2008) (Figures 2.3 and 2.4). Our findings indicate that temperature regulation of dmpi8 splicing involves an intricate balance of multiple suboptimal splice sites (Figure 2.3). The thermal range in the splicing efficiency of dmpi8 is mainly determined by the strength of the association between U1 snRNA and the non-consensus 5'ss (Figure 2.9A), which is gradually weakened as temperature increases or is stabilized as temperature decreases. This temperature responsive interaction between 5'ss and U1 snRNA would only be rate-limiting to the overall spliceosome binding of dmpi8 if the 3'ss recognition (determined by 3'ss sequence, branch point sequence, poly-pyrimidine tract) is below a certain threshold (Figure 2.9C and D).

Surprisingly, a similar thermosensitive splicing mechanism was also found in the central clock components of another well-studied model organism for circadian clocks, *Neurospora*. Earlier findings showed that temperature regulates the relative levels of two isoforms of FRQ protein, the negative elements in the circadian feedback loop of *Neurospora*, due to alternative use of translation initiation sites (Liu et al., 1997). The ratio of the long isoform (l-FRQ) versus the short ones (s-FRQ) is regulated by thermosensitive splicing of an intron (*frq-I6*) that when excised removes the translation initiation site of l-FRQ (Brunner and Diernfellner, 2006; Colot et al., 2005; Diernfellner et al., 2005). Mutational studies demonstrated that non-consensus splicing signals are the underlying molecular mechanism for such temperature responsiveness (Diernfellner et

al., 2005), similar to our findings. Additional steps including temperature-dependent effects on the rate of ribosome scanning at the different translation initiation recognition sequences allow for adjustments in FRQ levels that are appropriate for the local ambient temperature (Brunner and Diernfellner, 2006; Diernfellner et al., 2005). Thus, in two widely different species, the clockworks adapt to changes in temperature by thermal adjustments in the levels of key state variables via a mechanism involving an initial thermosensitive splicing event that has ramifications for other downstream aspects of mRNA metabolism or utilization, such as the efficiency of 3'-end formation in the case of *dper* or translation of *frq*.

The suboptimal splicing signals as the basis of thermosensitive splicing phenotype of dmpi8 and *frq-I6* was reminiscent of several prior studies from temperature sensitive mutants of murine retrovirus (MuSVts110) (Touchman et al., 1995), of Aspergillus (*bimG11*) (Hughes et al., 1996), and of *Arabidopsis* (*APETALA3-1*) (Sablowski and Meyerowitz, 1998). In all these cases, the mutations are located near the 5'ss that are likely to destabilize interaction with snRNAs known to form base pairs with sequences around the 5'ss or defective interaction between the transcript and protein factors that participate in 5'ss recognition.

In order to evaluate the physiological significance of the results obtained in cultured cells, we generated transgenic flies with sole functional copies of *dper* that either had the wildtype dmpi8 intron, a dmpi8 intron modified with strong 5' and 3'ss (M2M1), or dyp3' intron. As predicted by results obtained using our cell-culture splicing assay, transgenic flies either M2M1 or dyp3' showed significantly higher splicing efficiencies, leading to abnormally elevated *dper* RNA levels (Figure 2.6). Excitingly, the sequence

dependent enhancement in *dper* 3'-terminal intron splicing efficiency is sufficient to cause an earlier rise in the evening activity bout without altering the circadian period of the rescued per^{01} null background flies (Figure 2.5). This is direct evidence showing that the splicing efficiency of the *dper* 3'-terminal intron has a causal relationship to the phasing of the behavioral profile in *D. melanogaster*, presumably through influencing the daily cycles in *dper* RNA and protein levels.

Careful examination revealed that differential splicing efficiency associated changes in behavioral profile are not limited to timing of evening associated activity. Transgenic flies with increased splicing efficiency of dmpi8 (P{dmper/M2M1}) exhibit later offset in morning activity (Figure 2.5, Tables A6 and A7). In combination with the advanced onset in evening activity, flies manifest a shorter and less robust midday siesta (Figure 2.5, Tables A6 and A7). In other words, transgenic flies with the most efficiently spliced *dper* 3'-terminal intron show the least robust suppression in daytime activity. This raised the possibility that splicing of dmpi8 has a more direct role in sleep homeostasis.

Indeed, further sleep analysis of the behavioral data revealed an unanticipated role for dmpi8 splicing in regulating sleep architecture and reveal novel effects of temperature on sleep (Figure 4.2). It has been well-established in the *Drosophila* field that flies exhibit behavioral and physiological correlates of mammalian sleep (Cirelli, 2003; Hendricks et al., 2000; Hendricks and Sehgal, 2004; Shaw et al., 2000). After many years of research it is now widely accepted in the field that sleep in *Drosophila* is operationally defined as no observable locomotor activity for at least 5 consecutive minutes. Based on this criteria, we found that transgenic flies with high intrinsic splicing

efficiency of *dper* 3'-terminal intron (such as P{dmper/M2M1}) exhibit much reduced daily sleep, especially daytime sleep (Figure 4.3, A and C). In addition, the sleep bout length, a measure of sleep consolidation or fragmentation, is also shorter in P{dmper/M2M1}. These results indicate that not only do P{dmper/M2M1} flies sleep less during a daily cycle, but that the quality of sleep is less because sleep is more fragmented into shorter bouts. It is important to note that the average activity counts during waking periods were not significantly different among genotypes compared (data not shown). Therefore, the differences in sleep parameters can be comfortably accounted for by sleep regulation and not because of hyperactivity or health issues.

Intriguingly, the differences in these sleep parameters were more prominent in the light part of day/night cycles. Moreover, the differences in sleep parameters (most average notably sleep bout length) between P{dmper/M2M1} and P{dmper/8:8} flies were not observed after flies were transferred to constant darkness (Figure 4.3D), suggesting a light dependent role for dmpi8 splicing in sleep homeostasis. Intriguingly, two previous studies showed a light-dependent role of the l-LN_vs, in light-induced arousal (Shang et al., 2008; Sheeba et al., 2008). Thus, it is possible that splicing of dmpi8 affects daytime sleep directly or indirectly via the l-LN_vs. Indeed, the light promoting wakefulness is more pronounced in transgenic flies with more efficiently spliced *dper* 3'terminal introns (Figure 4.2 G), indicated by elevated sleep latency only in the day, which measures how soon the flies fall asleep after the dark-to-light transition. This phenotype appears similar to prior findings showing that increasing or decreasing the neuronal activity of l-LN_vs could cause flies to fall asleep later or faster, respectively (Parisky et al., 2008).

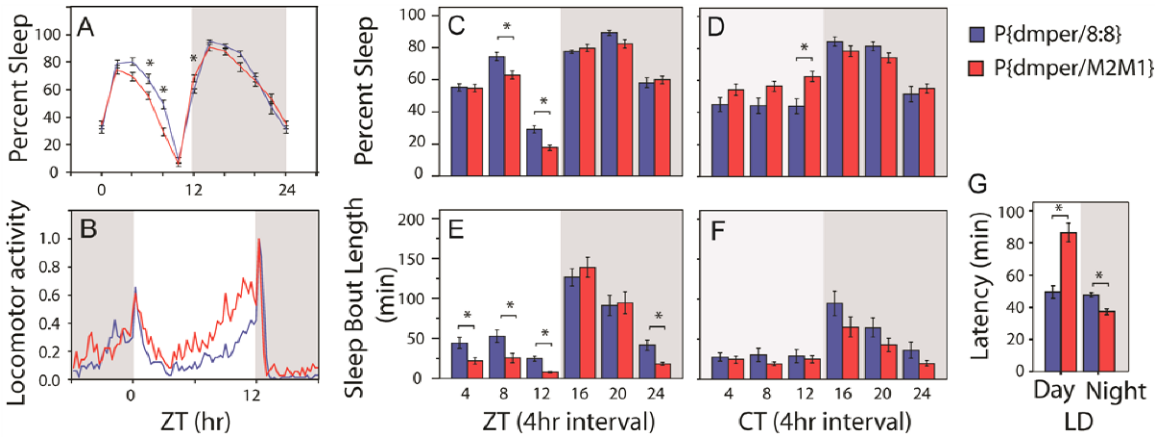


Figure 4.3. Altering the splice sites strength of d*per* 3'UTR intron affects sleep.

Male flies were exposed to several days of 12:12 LD at 25°C. For each genotype, data from at least 3 independent transgenic lines were pooled to obtain the group averages shown. Baseline sleep (A) and normalized locomotor activity (B) of representative LD cycle are shown. (C-G) Based on the data shown in A, other sleep parameters were analyzed for the time interval indicated. Light grey shade, subjective daytime; dark grey shade, night; ZT, zeitgeber time (time in LD); CT, circadian time (time in DD). Student's t-test, *, P<0.005.

In addition to interspecies comparisons, we investigated naturally occurring intraspecies variations surrounding the terminal intron in order to identify additional factors that might regulate the splicing dependent behavioural program. During our investigation, we discovered two polymorphisms (SNPs3 and 4) located 3' downstream of dmpi8 that appear to work as single unit in affecting dmpi8 splicing both in the simplified cell culture system (Figure 3.2) and transgenic flies (Figure 3.3, A and B). Since the more efficient splicing of dmpi8 in a VT1.1 3' UTR background does not involve changes in the 5' or 3'ss, unlike dyp3' or M2M1 introns, it is possible that these non-intronic polymorphisms act as binding sites for regulatory factors.

It is important to note that although SNPs3/4 do not alter the splice site strengths of dmpi8, they nevertheless regulate the phasing of daily behavioural profile (Figure 3.5) also in a manner similar to what we found by changing the 5' and 3'ss of dmpi8 (Figure 2.5). That is, the more efficient splicing of M2M1 or dmpi8 in a VT1.1 context are both correlated with earlier evening activity and less robust midday siesta compared to 8:8 and dmpi8 in a VT1.2 context, respectively. Therefore, whether it is the splicing signal strength or the surrounding regulatory *cis*-elements, they function within the same downstream molecular program linking splicing efficiency of dmpi8 to *D. melanogaster* daily distribution of locomotor activity. Furthermore, that the intrinsic splicing efficiency of dmpi8 modulates light-dependent arousal/sleep is further strengthened with data obtained from transgenic flies with different SNP3 and SNP4 variants. For example, flies with SNP3/4 from VT1.1 flies wherein the splicing efficiency of dmpi8 is reduced, also exhibit increased daytime sleep and longer sleep bout lengths (Figure 4.4).

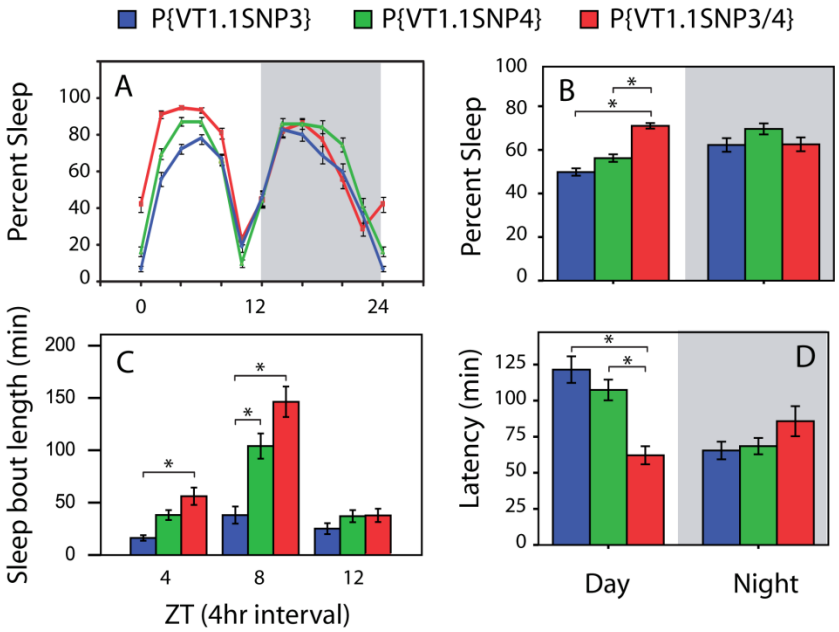


Figure 4.4. The splicing efficiency of d*per* 3'UTR intron affects sleep.

Male flies were exposed to several days of 12:12 LD at 25°C. For each genotype, data from at least 3 independent transgenic lines were pooled to obtain the group averages shown. (A) Baseline sleep of representative LD cycle are shown. (C-D) Based on the data shown in A, other sleep parameters were analyzed for the time interval indicated. White, daytime; dark grey shade, night-time. Student's t-test, *, P<0.005.

Thus, the splicing efficiency of dmpi8 can directly affect the timing and robustness of the midday siesta by direct effects on sleep regulation. In addition, it might also affect the daily distribution of activity by functioning in clock cells that secondarily regulate the activity profile. It will be of interest to dissect in which cells dmpi8 is exerting its effects on the daily distribution of activity.

In parallel to sequence analysis of *dper* 3' UTRs from different *Drosophila* species, we utilized our established cell culture system to perform RNA interference (RNAi) experiments to identify putative *trans*-acting factors in dmpi8 splicing regulation. As an initial attempt, we targeted members of the Ser-Arg-rich (SR) proteins. SR proteins comprise a large family of nuclear phosphoproteins that play roles in both constitutive and alternative pre-mRNA splicing, most notably by associating with other core splicing factors and the pre-mRNA via exonic or intronic binding elements (Bourgeois et al., 2004; Graveley, 2000; Hertel and Graveley, 2005). Double-stranded RNAs (dsRNAs) corresponding to each of the 7 SR proteins identified in *D. melanogaster* (SC35, ASF/SF2, B52/SRp55, 9G8, RBP1, RBP1-like and SRp54)(Mount and Salz, 2000) were generated and incubated with S2 cells that were transfected with reporter constructs containing either VT1.1 UTR or VT1.2 UTRs.

Preliminary data showed that down-regulation of B52/SRp55 significantly reduced splicing efficiency of VT1.1 haplotype at both cold and warm temperature, whereas VT1.2 haplotype was minimally reduced or not affected at cold or warm temperatures, respectively (Figure 4.5, A and B). This data suggests a sequence dependent stimulatory role of B52/SRp55. Silencing of B52/SRp55 almost completely diminished the differences between the two haplotypes but the thermosensitivity splicing of dmpi8 was

retained in both haplotypes (Figure 4.5, C and D, significant difference between temperatures $P<0.005$). Hence, B52/SRp55 is sufficient for the differences observed between VT1.1 and VT1.2, presumably via a modulatory role independent from the temperature responsive suboptimal splice sites recognition step (Chapter 2).

Interestingly, based on human consensus sequence (*Drosophila* SR protein recognition sites are poorly characterized at present), we identified a predicted B52/SRp55 binding site at SNP2 of VT1.1 UTR (nucleotide T) but it is abolished with an A variant found in VT1.2 haplotype (using the web-based ESE finder (Cartegni et al., 2003)). This might explain why silencing of B52/SRp55 had relatively minor effects on the splicing of dmpi8 within the VT1.2 haplotype (Figure 4.5, A and B).

Among all the other SR proteins tested, RBP1 was also shown to influence dmpi8 splicing in cultured cells. Down regulation of endogenous RBP1 expression seemed to attenuate the thermosensitivity of VT1.2 haplotype splicing efficiency (Figure 4.4 D) by enhancing the removal of dmpi8 at 22°C. These tissue culture results are highly preliminary but provide further testable predictions to dissect regulatory pathways that modulate overall splicing efficiency of dmpi8 as well as the thermosensitive aspect of it.

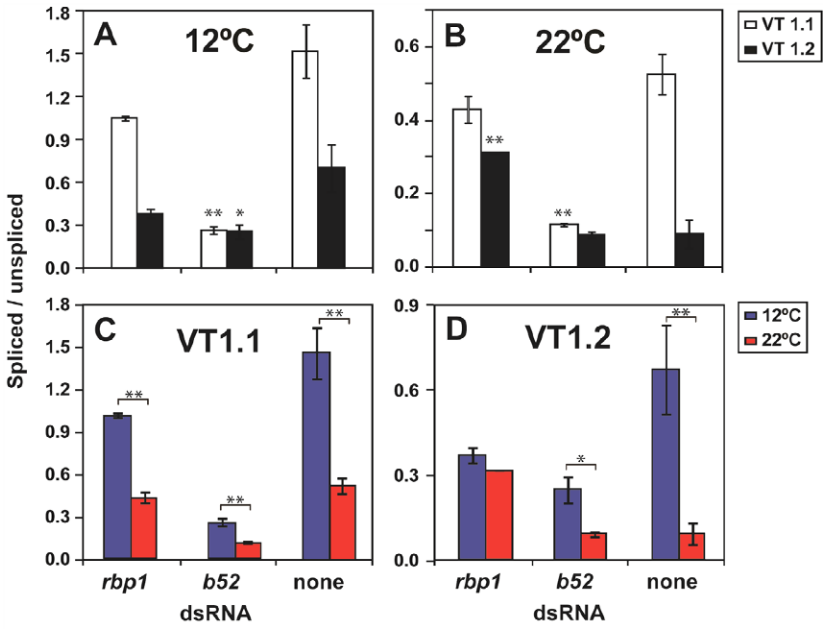


Figure 4.5. Differential effects of SR-proteins on the splicing efficiency of dmpi8 in S2 cells.

S2 cells were incubated with corresponding dsRNA for 2 days before transiently transfected with reporter luciferase constructs containing VT1.1 and VT1.2 3'UTR (see Section 2.2.2). Groups of S2 cells were placed at 12°C and 22°C one day after transfection and collected a day later for RNA extraction. Subsequent semi-quantitative RT-PCR was performed to assay dmpi8 splicing efficiency (as described in Section 2.2.6) and to verify the knockdown of the SR proteins. Shown are the averages of 3 replicates. (A and B) For each temperature tested, the effect of lowering SR protein level on splicing efficiency (expressed as a ratio of spliced/unspliced) of different haplotypes, i.e. VT1.1 and VT1.2 are compare side-by-side. Student's t-test, *, $P<0.05$; **, $P<0.005$, compared to no RNAi control ('none'). (C and D) The results shown in A and B are re-plotted to show the effect of SR protein knock-down on thermosensitivity of different dmpi8 splicing. Student's t-test, *$P<0.05$; **, $P<0.005$.

In conclusion, this thesis used a combinatorial approach to study the underlying molecular mechanisms of a temperature dependent behavioural program in *D. melanogaster*. These studies involved simplified tissue culture systems for mechanistic studies in conjunction with physiologically relevant comparative analysis based on phylogeny and ecological consideration. As a result, our work shed light in the molecular basis of clock evolution in shaping adaptation of life-forms to daily and seasonal changes in temperatures. It raises the intriguing possibility that RNA:RNA interactions are a key basis for thermal adaptation. The work in this thesis has also revealed an unanticipated role for dmpi8 splicing and ultimately *dper* function in regulating sleep. This will presumably lead to a better understanding of the interplay between circadian clock and the homeostatics regulation of sleep.

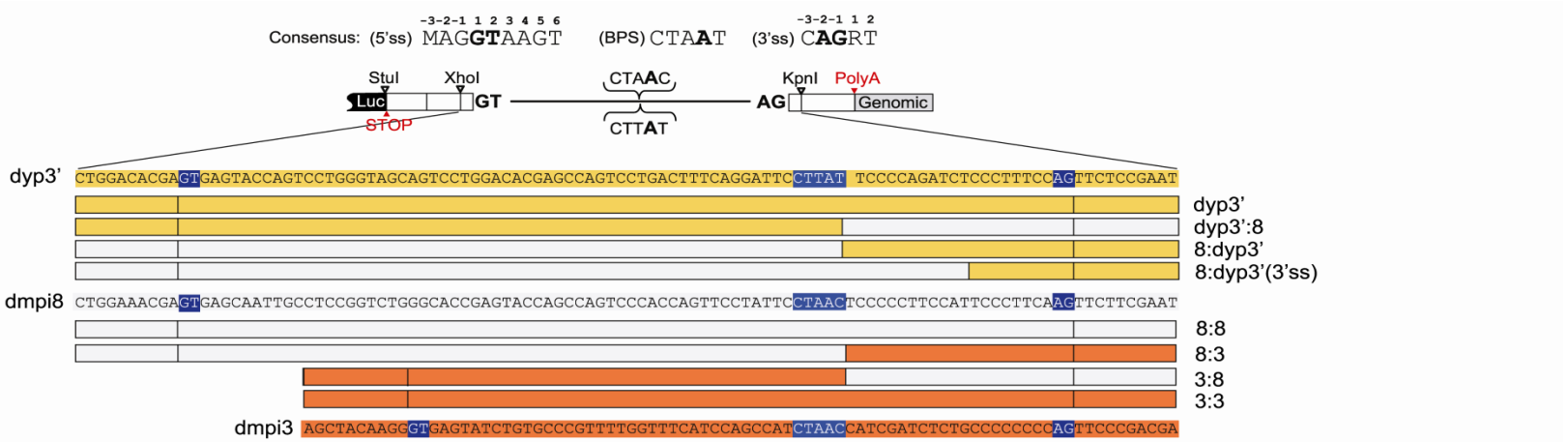
Appendix

Figures and tables relevant to chapter 2

Figure A1. Sequences and splice site strengths of *per* 3'-terminal introns and hybrids used in this study.

(A) Shown at top are the *Drosophila* consensus sequences for the 5'ss and 3'ss, as explained in figure 3. The schematic of the luc-dmper construct is identical to that shown in figure 3, except that here we also indicate the two branch point sites found in the different introns (CTAAC or CTTAT; where the presumptive branch point A is indicated in bold) used to generate the hybrid introns used in this study and indicated below as follows: Shown are the sequences of the different introns including 9bp and 10bp of flanking 5' and 3', respectively; yellow, dyp3'; gray, dmpi8; red, dmper intron 3. High-lighted in blue are the 5' and 3'ss and the presumptive branch point region for each construct. (B) Intronic and flanking sequences of the per 3'-terminal introns from *D. simulans* (sim4), *D. santomea* (ST0.4) and *D. yakuba* (Tai18E2) used in this study. (C) Predicted 5' and 3'ss strengths.

A



B

dmpi8	CTGGAAACGAGTGAGCAATTGCCTCCGGTCTGGGCACCGAGCACCAGCCAGTCCCACCAGTTCCCCCTATTCCTAACTCCCCCTTCCATTCCCTTCAAGTTCTTCGAATCAACGCGAAC
D. simulans (sim4)	CTGGAAACGAGTGAGCAGTTGCCTCCGGTCTGG---TCGAGCACCAGCCAGTCCCACCAGTTCCCCCCATTCCTAACTCCCCCTTCCATTCCCTTCAAGTTCTCCGAATCAACGCAAAC
D. yakuba (Tai18E2)	CTGGACACGAGTGAGTACCAGTCCTGGGTAGCAGTCCTGGACACGAGCCAGTCCTGACTTTCAGGATTCCTTATTCCCCAGATCTCCCTTTCCAGTTCTCCGAATCAGCACGAAC
D. santomea (ST0.4)	CTGGACACGAGTGAGTATCAGTCCTGG----------ACACGAGCCAGTCCTGACTTCCAGGATTCCTTATTCCCCAGATCTCCCTTTCCAGTTCTCCGAATCAGCACGAAC

C

Genotype	Splice site prediction score	
	5'ss	3'ss
dmpi8	0.45	0.21
D. simulans (sim4)	0.39	0.44
D. yakuba (Tai18E2)	0.79	0.78
D. santomea (ST0.4)	0.80	0.78

Table A1. Timing of daily activity in *D. melanogaster* and *D. yakuba* at different temperatures and photoperiods

Genotype[a]	Photoperiod[b] (L:D)	Temperature (°C)	n[c]	Morning peak (hr ± sem)[d]	Morning offset[e] (hr ± sem)[d]	Evening peak (hr ± sem)[d]	Evening onset[f] (hr ± sem)[d]	Siesta[g] (hr ± sem)
D. melanogaster	12:12	18	36	0.6 ± 0.1	3.1 ± 0.2	10.0 ± 0.1	7.1 ± 0.2	4.1 ± 0.3
		25	61	0.6 ± 0.1	2.6 ± 0.1	11.0 ± 0.1	9.2 ± 0.3	6.5 ± 0.3
		29	64	22.6 ± 0.1	0.7 ± 0.1	12.1 ± 0.0	10.6 ± 0.1	10.0 ± 0.1
D. yakuba		18	62	1.0 ± 0.3	2.7 ± 0.2	11.1 ± 0.2	9.3 ± 0.2	6.7 ± 0.3
		25	139	0.3 ± 0.1	2.0 ± 0.1	10.9 ± 0.1	9.0 ± 0.1	7.1 ± 0.2
		29	222	0.2 ± 0.1	2.1 ± 0.1	10.5 ± 0.1	8.7 ± 0.1	6.6 ± 0.1
D. melanogaster	9:15	18	35	23.7 ± 0.2	2.5 ± 0.3	7.3 ± 0.1	4.4 ± 0.2	2.3 ± 0.2
		25	59	23.2 ± 0.1	1.0 ± 0.2	8.9 ± 0.1	7.4 ± 0.1	6.4 ± 0.2
		29	62	21.4 ± 0.1	23.3 ± 0.1	10.1 ± 0.0	8.6 ± 0.0	9.2 ± 0.1
D. yakuba		18	25	23.5 ± 0.3	1.3 ± 0.4	8.4 ± 0.1	6.8 ± 0.2	5.7 ± 0.4
		25	36	23.5 ± 0.2	1.2 ± 0.3	8.6 ± 0.2	6.8 ± 0.3	5.7 ± 0.4
		29	43	23.0 ± 0.1	0.9 ± 0.2	8.6 ± 0.3	6.8 ± 0.2	6.0 ± 0.3

[a]Young male flies were maintained at the indicated temperature and photoperiod for five days. The last three days worth of activity data was pooled for each individual fly and then a group average was determined. *D. melanogaster* is Canton S and *D. yakuba* is Ivory Coast, Burla strain.
[b]The light/dark cycles were either 12hr light followed by 12 hr dark (12:12) or 9 hr light followed by 15 hr dark (9:15).
[c]n, number of flies that gave significant values for both morning peak, morning offset, evening peak, and evening onset and that survived throughout the entire testing period.
[d]Values denote zeitgeber time, with ZT0 defined as the start of lights-on.
[e]Morning offset is defined as the time when 50% of peak morning activity was attained following the morning peak of activity.
[f]Evening onset is defined as the time when 50% of peak evening activity was attained prior to the evening peak of activity.
[g]Siesta time is defined as the length of time between 50% of morning offset and 50% of evening onset.

Table A2. P-values for ANOVA analysis of results shown in Table A1

Comparison	Effect	Siesta[a]			P-value[c]			
		Statistic[b]		P-value[c]	Morning peak	Morning offset[a]	Evening peak	Eveningonset[a]
D. melanogaster and D. yakuba; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,568}$	= 169.9	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod	$F_{1,568}$	= 38.1	0.0000	0.0000	0.0000	0.0000	0.0000
	Genotype x Temperature	$F_{2,568}$	= 170.0	0.0000	0.0000	0.0000	0.0000	0.0000
	Genotype x Photoperiod	$F_{1,568}$	= 2.1	0.1451	0.0060	0.0060	0.3110	0.3184
D. melanogaster; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,234}$	= 741.20	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod	$F_{1,234}$	= 24.70	0.0000	0.0000	0.0000	0.0000	0.0000
D. yakuba; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,234}$	= 0.25	0.7811	0.0260	0.1160	0.2470	0.1327
	Photoperiod	$F_{1,234}$	= 14.98	0.0001	0.0000	0.0010	0.0000	0.0000

[a]Morning offset, evening onset and siesta are as defined in Table A1.
[b]F ratio from ANOVA analysis with degree of freedom shown in subscript.
[c]P-values of significance test from ANOVA analysis.

Table A3. Post-hoc Tukey HSD multiple comparisons test for data shown in Table A1[a]

Genotype	Comparison	P-value[c]				
		Siesta[b]	Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
D. melanogaster	18 °C VS. 25 °C	0.0000	0.0000	0.0000	0.0000	0.0000
	18 °C VS. 29 °C	0.0000	0.0000	0.0000	0.0000	0.0000
	25 °C VS. 29 °C	0.0000	0.0000	0.0000	0.0000	0.0000
D. yakuba	18 °C VS. 25 °C	0.8351	0.0770	0.1670	0.9970	0.5946
	18 °C VS. 29 °C	0.9972	0.0120	0.0740	0.2330	0.0556
	25 °C VS. 29 °C	0.7475	0.7840	0.9420	0.2060	0.3331

[a]Multiple comparisons test was done separately within each genotype with data from different temperatures and photoperiods.
[b]Morning offset, evening onset and siesta are as defined in Table A1.
[c]P-values of significance test from Tukey HSD test.

Table A4. Little effect of temperature on period length in *D. melanogaster* and *D. yakuba* flies

Genotype[a]	Temperature (°C)	n	Rhythmicity[b] (%)	Period (hr ± sem)	Power[c] (± sem)
D. melanogaster	18	32	84	23.7 ± 0.1	92.6 ± 6.9
	25	32	100	23.9 ± 0.1	91.1 ± 5.5
	29	32	91	23.7 ± 0.1	93.1 ± 6.6
D. yakuba	18	23	87	24.2 ± 0.2	46.0 ± 5.0
	25	31	81	24.0 ± 0.2	52.1 ± 6.0
	29	20	45	24.2 ± 0.5	17.1 ± 3.5

[a]Young male flies were entrained for five 12:12LD cycles followed by five days in constant dark conditions at the indicated temperature. The results are a subset of the same experiments used to calculate the data shown in Table A1.
[b]Flies with a power value of greater than 10 and period ≥ 20 and ≤ 30, were defined as rhythmic.
[c]Power is a measure of the strength or amplitude of the rhythm.

Table A5. P-values for ANOVA analysis of results shown in Table A4

Comparison	Effect	Statistic[a]	P-value[b]
D. melanogaster and D. yakuba; 18, 25 and 29ºC	Temperature	$F_{2,141} = 0.007$	0.9930
	Genotype	$F_{1,141} = 6.7$	0.0106
	Genotype x Temperature	$F_{2,141} = 1.1$	0.3477

[a]F ratio from ANOVA analysis with degree of freedom shown in subscript.
[b]P-values of significance test from ANOVA analysis.

Table A6. Timing of daily activity in P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1} transgenic flies at different temperatures and photoperiods

Genotype[a]	Photoperiod (L:D)	Temperature (°C)	n[b]	Morning peak (hr ± sem)[c]	Morning offset[d] (hr ± sem)[c]	Evening peak (hr ± sem)[c]	Evening onset[e] (hr ± sem)[c]	Siesta[f] (hr ± sem)
P{dmper/8:8}	11:13	18	51	0.2 ± 0.1	1.3 ± 0.1	9.9 ± 0.1	6.9 ± 0.2	5.6 ± 0.2
P{dmper/dyp3'}			39	23.9 ± 0.1	1.0 ± 0.3	9.3 ± 0.1	5.8 ± 0.2	4.8 ± 0.3
P{dmper/M2M1}			65	0.2 ± 0.1	1.6 ± 0.1	9.4 ± 0.1	6.3 ± 0.1	4.7 ± 0.2
P{dmper/8:8}	11:13	25	76	22.9 ± 0.1	1.1 ± 0.1	10.1 ± 0.1	8.2 ± 0.1	7.1 ± 0.1
P{dmper/dyp3'}			62	22.6 ± 0.2	1.6 ± 0.1	9.2 ± 0.1	6.4 ± 0.1	4.8 ± 0.2
P{dmper/M2M1}			91	23.5 ± 0.1	2.3 ± 0.2	9.4 ± 0.1	6.4 ± 0.2	4.1 ± 0.3
P{dmper/8:8}	11:13	29	79	22.3 ± 0.1	0.5 ± 0.1	10.1 ± 0.1	8.3 ± 0.2	7.8 ± 0.2
P{dmper/dyp3'}			91	21.6 ± 0.1	0.3 ± 0.1	9.9 ± 0.1	7.8 ± 0.1	7.5 ± 0.2
P{dmper/M2M1}			127	22.6 ± 0.2	2.1 ± 0.2	10.4 ± 0.1	7.7 ± 0.1	5.6 ± 0.2
P{dmper/8:8}	12:12	18	15	23.1 ± 0.4	1.7 ± 0.4	11.3 ± 0.1	9.3 ± 0.2	7.5 ± 0.4
P{dmper/dyp3'}			31	23.1 ± 0.3	1.7 ± 0.2	10.7 ± 0.2	7.5 ± 0.2	5.7 ± 0.3
P{dmper/M2M1}			45	0.6 ± 0.2	2.8 ± 0.3	10.8 ± 0.1	8.0 ± 0.1	5.3 ± 0.3
P{dmper/8:8}	12:12	25	77	22.7 ± 0.1	1.0 ± 0.1	11.2 ± 0.1	9.3 ± 0.1	8.3 ± 0.2
P{dmper/dyp3'}			62	22.8 ± 0.1	1.5 ± 0.1	10.4 ± 0.0	7.7 ± 0.1	6.2 ± 0.2
P{dmper/M2M1}			94	23..9 ± 0.1	2.4 ± 0.1	10.5 ± 0.1	8.0 ± 0.1	5.6 ± 0.2
P{dmper/8:8}	12:12	29	16	22.1 ± 0.2	0.7 ± 0.3	11.4 ± 0.1	9.9 ± 0.1	9.2 ± 0.3
P{dmper/dyp3'}			32	21.9 ± 0.2	0.3 ± 0.2	10.8 ± 0.1	9.1 ± 0.1	8.8 ± 0.3
P{dmper/M2M1}			46	23.0 ± 0.2	2.0 ± 0.2	11.6 ± 0.2	9.8 ± 0.2	7.8 ± 0.3
P{dmper/8:8}	13:11	18	44	0.6 ± 0.2	2.0 ± 0.2	11.4 ± 0.1	8.4 ± 0.1	6.4 ± 0.2
P{dmper/dyp3'}			25	0.8 ± 0.3	2.9 ± 0.3	11.3 ± 0.1	7.6 ± 0.3	4.6 ± 0.3
P{dmper/M2M1}			44	2.1 ± 0.2	4.2 ± 0.3	10.7 ± 0.1	8.0 ± 0.2	3.9 ± 0.3
P{dmper/8:8}	14:10	25	63	23.6 ± 0.2	2.0 ± 0.2	12.8 ± 0.0	10.9 ± 0.1	8.9 ± 0.2
P{dmper/dyp3'}			126	0.2 ± 0.1	2.4 ± 0.1	12.4 ± 0.0	10.0 ± 0.1	7.6 ± 0.1
P{dmper/M2M1}			91	0.1 ± 0.1	2.5 ± 0.1	12.7 ± 0.1	10.4 ± 0.1	8.0 ± 0.2

P{dmper/8:8}	13:11	29	186	23.2 ± 0.1	1.1 ± 0.1	11.9 ± 0.0	10.0 ± 0.1	8.8 ± 0.1
P{dmper/dyp3'}			152	22.7 ± 0.1	0.9 ± 0.1	11.6 ± 0.1	9.6 ± 0.1	8.6 ± 0.1
P{dmper/M2M1}			148	23.1 ± 0.1	2.0 ± 0.1	11.8 ± 0.1	9.6 ± 0.1	7.5 ± 0.2

[a]Young male flies were maintained at the indicated temperature and photoperiod for five days. The last three days worth of activity data was averaged for each individual fly and then a group average was determined. For each genotype, data from at least three independent lines was pooled.
[b]n, number of flies that gave significant values for both morning peak, morning offset, evening peak, and evening onset and that survived throughout the entire testing period.
[c]Values denote zeitgeber time, with ZT0 defined as the start of lights-on.
[d]Morning offset is defined as the time when 50% of peak morning activity was attained following the morning peak of activity.
[e]Evening onset is defined as the time when 50% of peak evening activity was attained prior to the evening peak of activity.
[f]Siesta time is defined as the length of time between 50% of morning offset and 50% of evening onset.

Table A7. P-values for ANOVA analysis of results shown in Table A6

Comparison[a]		Effect	Siesta[b]			P-value[d]			
			Statistic[c]		P-value[d]	Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}; 18, 25 and 29ºC; LD of 11:13, 12:12, 13:11		Genotype	$F_{2,2522}$	= 136.3	0.0000	0.0000	0.0000	0.0000	0.0000
		Photoperiod	$F_{2,2522}$	= 99.2	0.0000	0.0000	0.0000	0.0000	0.0000
		Temperature	$F_{2,2522}$	= 200.4	0.0000	0.0000	0.0000	0.0000	0.0000
		Photoperiod x Genotype	$F_{4,2522}$	= 1.8	0.1200	0.0250	0.1350	0.0440	0.0020
		Temperature x Genotype	$F_{4,2522}$	= 12.0	0.0000	0.0000	0.0000	0.0000	0.0000
		Photoperiod x Temperature	$F_{4,2522}$	= 35.7	0.0000	0.0000	0.0000	0.0000	0.0000
		Temperature x Genotype x	$F_{8,2522}$	= 6.4	0.0000	0.0000	0.0000	0.0000	0.0000
P{dmper/8:8}, P{dmper/dyp3'} and									
Photoperiod	Temperature								
11:13	18	Genotype	$F_{2,154}$	= 4.7	0.0110	0.1530	0.0170	0.0000	0.0000
11:13	25	Genotype	$F_{2,228}$	= 56.9	0.0000	0.0000	0.0000	0.0000	0.0000
11:13	29	Genotype	$F_{2,296}$	= 36	0.0000	0.0000	0.0000	0.0010	0.0100
12:12	18	Genotype	$F_{2,90}$	= 8.4	0.0000	0.0000	0.0080	0.0320	0.0000
12:12	25	Genotype	$F_{2,545}$	= 26.6	0.0000	0.0010	0.0000	0.0000	0.0000
12:12	29	Genotype	$F_{2,325}$	= 5.9	0.0030	0.0050	0.0000	0.0000	0.0000
13:11	18	Genotype	$F_{2,112}$	= 30.7	0.0000	0.0000	0.0000	0.0000	0.0300
14:10[e]	25	Genotype	$F_{2,279}$	= 19.6	0.0000	0.0020	0.0120	0.0000	0.0000
13:11	29	Genotype	$F_{2,485}$	= 27.5	0.0000	0.0010	0.0000	0.0000	0.0000

[a]ANOVA analysis was done with dataset specified.
[b]Morning offset, evening onset and siesta are as defined in Table A6.
[c]F ratio from ANOVA analysis with degree of freedom shown in subscript.
[d]P-values of significance test from ANOVA analysis.
[e]For simplicity, L:D of 14:10 was treated as 13:11 in the statistical analysis.

Table A8. Post-hoc Tukey HSD multiple comparisons test for data shown in Table A6[a]

Photoperiod (L:D)	Temperature (°C)	Comparison		P-value[c]				
		Genotype 1	Genotype 2	Siesta[b]	Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
11:13	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0810	0.1490	0.2720	0.0000	0.0000
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0110	0.8850	0.3570	0.0000	0.0300
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.8960	0.2770	0.0120	0.9970	0.1350
	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.3420	0.0150	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0020	0.0000	0.0000	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.0620	0.0000	0.0010	0.1950	0.9990
	29	P{dmper/8:8}	P{dmper/dyp3'}	0.5840	0.0120	0.7910	0.3010	0.0620
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.2730	0.0000	0.0860	0.0090
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.0000	0.0000	0.0000	0.0000	0.8320
12:12	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0080	0.9990	0.9990	0.0280	0.0000
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0020	0.0760	0.0710	0.0000
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.5340	0.0000	0.0130	0.7770	0.0160
	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.5100	0.7540	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0740	0.0000	0.0000	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.1140	0.0010	0.0000	0.2480	0.7010
	29	P{dmper/8:8}	P{dmper/dyp3'}	0.4140	0.1290	0.9040	0.0150	0.0080
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0020	0.5130	0.0000	0.0000	0.1630
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.1130	0.0030	0.0000	0.0000	0.0000
13:11	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.7870	0.0440	0.4190	0.0230
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0000	0.0000	0.0000	0.3980
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.1130	0.0020	0.0060	0.0020	0.2610
14:10	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.0020	0.0260	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0050	0.0160	0.5220	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.1100	0.9980	0.9240	0.0000	0.0000

13:11	29	P{dmper/8:8}	P{dmper/dyp3'}	0.5080	0.0010	0.3670	0.0000	0.0000
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.8580	0.0000	0.8290	0.0000
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.0000	0.0080	0.0000	0.0010	0.9890

[a]Multiple comparisons tests were done comparing two genotypes at the indicated temperatures and photoperiods.
[b]Morning offset, evening onset and siesta are as defined in Table A6.
[c]P-values of significance test from Tukey HSD test.

Table A9. Similar period lengths in P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1} flies

Genotype[a]	n	Rhythmicity[b] (%)	Period (hr ± sem)	Power[c] (± sem)
P{dmper/8:8}	140	89	23.4 ± 0.1	82.6 ± 3.1
P{dmper/dyp3'}	186	97	23.2 ± 0.0	96.5 ± 2.5
P{dmper/M2M1}	226	75	23.5 ± 0.1	62.6 ± 2.8

[a]Young male flies were kept at 25°C for five 12:12LD cycles followed by five days in constant dark conditions. The results are a subset of the same experiments used to calculate the data shown in Table A6.
[b]Flies with a power value of greater than 10 and period ≥ 20 and ≤ 30, were defined as rhythmic.
[c]Power is a measure of the strength or amplitude of the rhythm.

Figures relevant to chapter 3.

Figure A2. VT1.1 inbred flies manifest higher splicing efficiency of dmip8 and advanced *per* mRNA accumulation compared to VT1.2 inbred flies.

VT1.1 and VT1.2 flies were exposed to 4 days of 12:12LD at 29°C and collected at the indicated time during the last LD. The splicing efficiency (A) and relative *per* mRNA levels (B) were measured using semi-quantitative RT-PCR (from Chen, 2007).

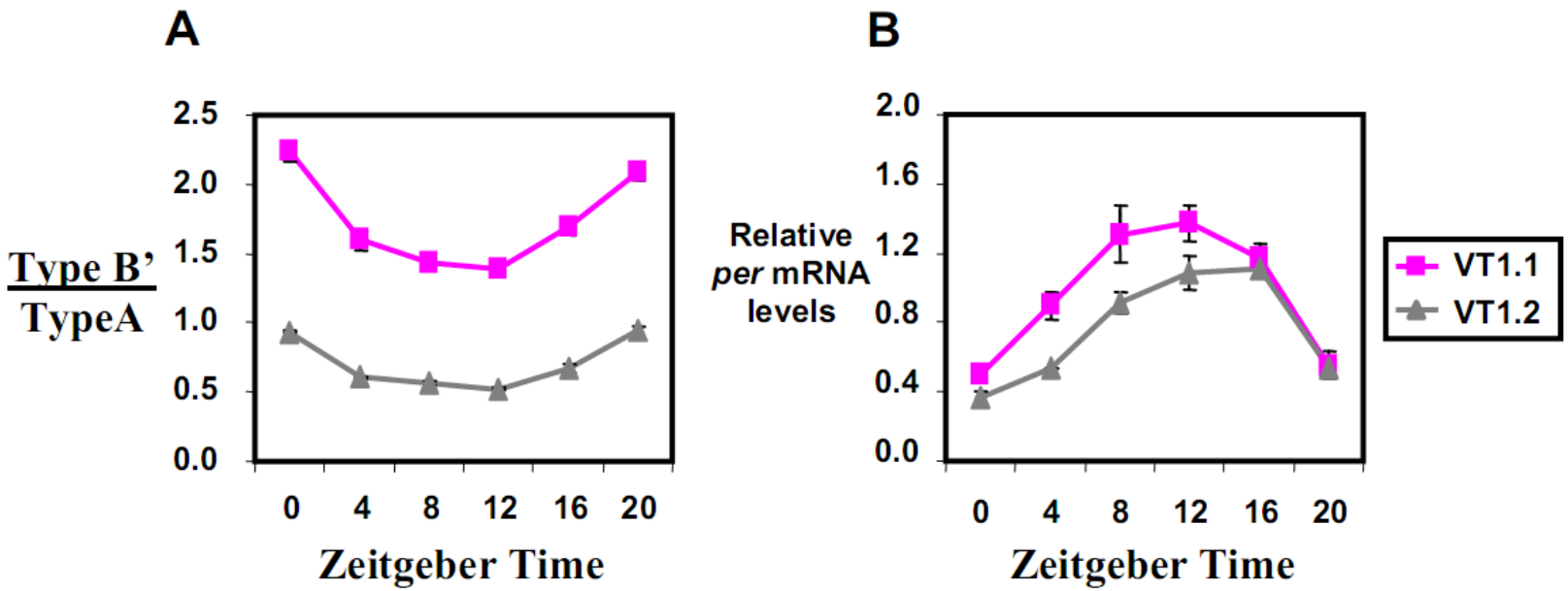


Figure A3. Splicing efficiency of dmpi8 is higher in VT1.1 transgenic flies compared to the VT1.2 version.

VT1.1 and VT1.2 transgenic flies (P{VT1.1} and P{VT1.2}) were generated with CaSpeR13.2-KA as backbone by swapping the KpnI and Bsu36I fragments of VT1.1 and VT1.2 3' UTRs into the transgenic construct. Flies were exposed to 4 days of 12:12LD at 29°C. Shown are results for the averages of two transformants for each genotype. Significant differences were found between P{VT1.1} compared to P{VT1.2} (Student's t-test, $P<0.01$) (from Chen, 2007).

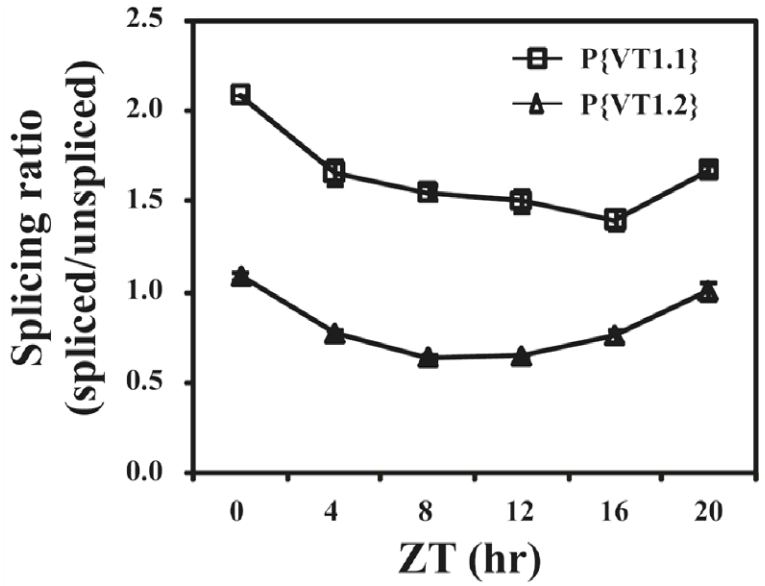
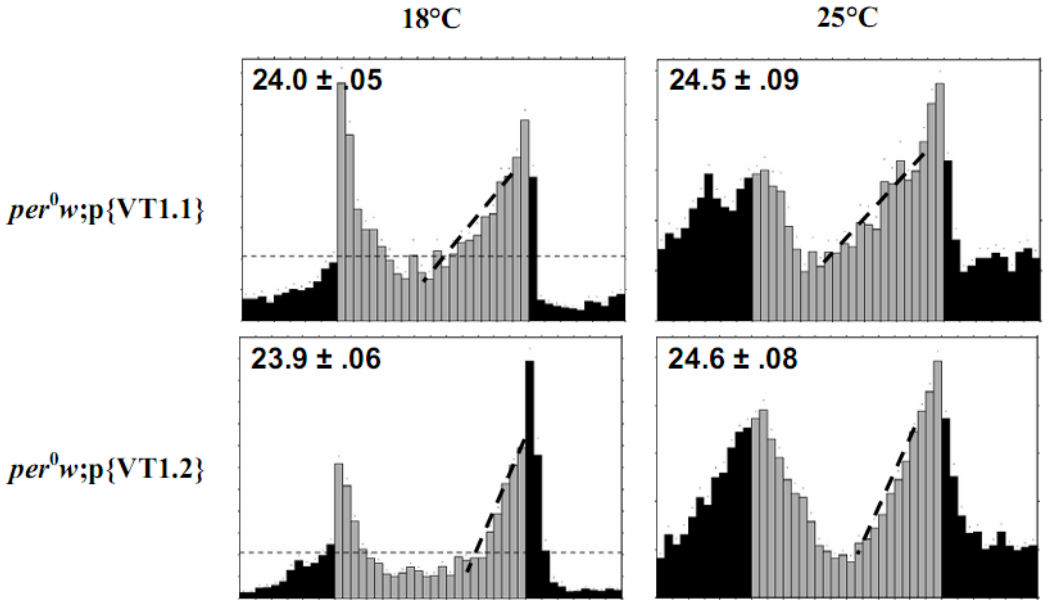


Figure A4. Transgenic flies with the VT1.1 transgene exhibit shorter midday siesta compared to the VT1.2 version.

Shown are the daily distributions of locomotor activity averaged over the third and fourth days of LD for male flies in a per^{01} genetic background. The flies were entrained to 12:12LD at either 18°C (left column) or 25°C (right column). Shown at the upper left corner are the periods (± SEM) for the behaviour of the same groups of flies kept in complete darkness after the LD cycles. No significant difference between the free-running periods was found for the transgenic flies at each temperature. The dashed lines indicate the approximate rising phase of evening activity of the flies (from (Chen, 2007)).



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Publications

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