Circadian Rhythms in Fruit Flies Drosophila Under Natural Conditions

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By

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Declaration

I declare that the matter presented in my thesis entitled "***Circadian Rhythms in Fruit Flies Drosophila Under Natural Conditions***" is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Dr. Sheeba Vasu and Prof. Vijay K Sharma and that this work has not been submitted elsewhere for any other degree.

In keeping with the general practice of reporting of scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of the other investigators. Any omission, which might have occurred by oversight or error of misjudgment, is regretted.

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This is to certify that the work described in the thesis entitled "*Circadian Rhythms in Fruit Flies Drosophila Under Natural Conditions*" is the result of investigations carried out by Mr. Joydeep De in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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Summary

The great majority of studies on circadian rhythms have been carried out under controlled laboratory conditions. Typically, they were either constant conditions or square-wave shaped cycles of light or temperature or food or in rare cases a combination of two time cues. Although these studies have given us tremendous insight into the oscillator properties of circadian clock entrainment to time-cues, we must acknowledge that we know very little about how organisms synchronize their clocks in the real world, where multiple environmental factors change simultaneously and gradually. Since life forms have evolved under such conditions, it is imperative to examine circadian entrainment under more natural conditions. There are only a handful of very recent investigations that have attempted to do so thus far. These studies have provided us with mostly descriptions of several unique features of activity-rest and eclosion rhythms in nature, rather than a systematic scrutiny of role of time-cues like light and temperature in bringing about these unique characteristics of the rhythms in nature.

In this study, I attempted to understand the role of light in the regulation of activity patterns in nature. I found that light modulates phase and amplitude of morning (M-peak) and evening (E-peak) activity peaks and determines the occurrence of the afternoon peak (A-peak). Morning and evening peaks are flexible enough to use humidity and temperature information when light is not present. But presence of natural light in the afternoon is crucial for A-peak to occur. When flies are provided with constant darkness in otherwise natural conditions or when afternoon light is specifically blocked or when natural light intensity is cut down by 50% or more in otherwise natural conditions, afternoon peak does not occur. Therefore, natural light induces A-peak whereas it modulates timing and amplitudes of M and E-peaks.

I also attempted to gain insights about role of canonical clock gene *period* in the regulation of activity peaks in nature. I found that *period* gene has subtle roles to play in regulation of activity peaks in nature which could be masked by natural light or other environmental factors. E-peak of activity was found to be clock-dependent but A-peak was not.

I carried out behavioral observations in parallel with activity recording using conventional DAM systems to closely inspect the nature of the very high activity in the warm and bright afternoons in nature, as it intuitively does not make much sense for a fly to be very active at that time of the day which incurs high risk of desiccation. Interestingly, my study revealed that A-peak is an artifact of experimental paradigm. High afternoon activity is not a natural behavior of flies. Observing flies housed in activity tubes inside the *Drosophila Activity Monitor* (DAM) system reveals that this high amount of activity basically arises due to the shade seeking behavior of flies in the IR beam area of the recording apparatus, in bright and warm afternoons. Even though flies are largely at rest in the afternoon clarified through visual observation, the DAM system detects high activity. In petridishes, flies placed solitarily or in groups do not exhibit high afternoon activity.

I asked what could be functional significance of these activity peaks observed in nature. Visual observation of fly behavior in petridishes where flies are housed in groups shows that M-peak of activity correlates to courtship related activities. A-peak of activity was not seen in flies housed in petridishes and is largely an artifact of the conventional activity recording protocol. E-peak of activity is associated with general locomotion, the significance of which remains unclear.

I attempted to mimic natural-like light and/or temperature cycles in the laboratory to see whether it is possible to reproduce features of activity rhythm in nature and also to tease apart

relative roles of light and temperature in bringing about these features. I found that M-peak phase is more temperature-dependent whereas, E-peak phase is light-dependent. A-peak mainly depends on temperature. The proportion of flies showing A-peak increases with increasing daily temperature.

I also examined another behavioral rhythm in fruit flies, namely adult emergence rhythm, to understand how seasonal variations in the environmental parameters affect the parameters of the rhythm. I find that gate width of emergence, fraction of flies emerging during night, day-to-day variance in the timing of the peak of emergence and peak amplitude are lower in cooler and wetter conditions.

Chapter 1: Introduction

Circadian rhythms

Life forms on the earth have to experience and cope with several diurnal and annual geophysical cycles such as light and temperature. It is believed that organisms have evolved mechanisms in order to accomplish the task of synchronizing their behavioral, physiological and biochemical processes to the periodic environmental cycles (Vaze & Sharma, 2013), through the evolution of biological timekeeping systems or clocks. Many of the rhythmic phenomena in a diverse range of organisms have been shown to be regulated endogenously by their clocks (Pittendrigh, 1960), rather than just a mere response to the periodic variations in the environmental conditions. Biological oscillators with a periodicity of close to 24 hours (circa = almost, dian = day) are referred to as circadian clocks. In addition, these oscillators need to need to satisfy a few criteria in order to be called circadian clocks, such as,

1) they must be endogenously generated

2) they must be self-sustained – i.e., the oscillation must persist (free-run) even in the absence of any periodic time-cue (or, zeitgeber; zeit = time, geber = giver)

3) they can be synchronized by periodic light/dark cycle by a process known as 'entrainment'

4) they must be temperature-compensated - the period of the oscillator should remain unaltered within a physiologically tolerable range of temperatures (Pittendrigh, 1960).

Rhythmic behaviors with close to 24 hours periodicity (circadian rhythms) have been extensively studied in many organisms including insects and mammals. Studies in fruit flies, *Drosophila melanogaster*, have attempted to examine several aspects of these rhythms such as

understanding the behavior, its genetic, molecular and neuronal bases and its evolution (Dunlap, 1999; Sheeba, 2008). In flies, myriad behavioral and physiological processes are found to be under the regulation of circadian clocks. The process of eclosion of adult flies from the pupal cases is gated in a circadian manner, such that it happens at maximum around dawn (Pittendrigh, 1954; Saunders, 2002). Activity-rest rhythm is shown to have two peaks close to dawn and dusk. There are circadian rhythms in physiological processes like oviposition (Howladar et al., 2006), gustation, olfaction (Krishnan et al., 1999, reviewed in Allada and Chung, 2010) and metabolism (Xu et al., 2008).

It is believed and now widely accepted that evolution of clocks has an adaptive advantage. This advantage could be of two types: extrinsic and intrinsic (Sharma, 2003). Having functional clocks helps organisms synchronize their rhythmic behavioral, physiological and biochemical processes to the external geophysical cycles (Vaze and Sharma, 2013), therefore providing an 'extrinsic' advantage. But then, this cannot justify several observations in different species of organisms that live in aperiodic environmental conditions for generations after generations, they still show persistence of rhythmicity. Millipedes, *Glyophiulus cavernicolus*, inhabitants of deep recesses of caves that are never exposed to light, are shown to exhibit entrainment to light-dark cycles (LD) and free-running rhythms under constant darkness (DD) (Koilraj et al., 2000). Fly populations maintained in aperiodic condition of constant light (LL) for more than 600 generations in the lab, can still entrain their eclosion (Sheeba et al., 1999), oviposition (Sheeba et al., 2001) and locomotor activity rhythm (Sheeba et al., 2002) to LD cycles. In all the above examples it is not clear why if they do not experience environmental cycles, why would they need to retain the daily clock? It is possible that having functional

clocks might help organisms in the coordination of metabolic processes; therefore providing some 'intrinsic' advantage (Vaze and Sharma, 2013).

Entrainment of circadian rhythms to light/dark cycles

Light has been regarded as the primary driving force for the evolution of circadian clocks (Hastings et al 1991). Phase resetting of *Drosophila* clock by light occurs via modulation of levels of TIM protein (Suri et al., 1998). Constant light induces behavioral arrhythmicity (Konopka et al., 1989) and disruption of the molecular clock (Myers et al., 1996). Light pulse given in early subjective night delays the phase of the rhythm and the same in early subjective day advances it. This phase shift depends on TIM protein levels and its location in the cell (Suri et al., 1998).

Drosophila uses five photoreceptors/pigments for reception of light information to the clock. These are intracellular photoreceptor CRYPTOCHROME (CRY) (Stanewsky et al., 1998; Emery et al., 2001), the compound eyes, the ocelli, HB eyelets and unknown photoreceptors in the dorsal neurons. Cryptochrome is a blue-light photoreceptor (420 nm) (Stanewsky et al., 1998) and it can function cell-autonomously (Emery et al., 1999). CRY overexpression in pacemaker neurons increases behavioral photo-receptivity (Emery et al, 1999). CRY is degraded by light (Lin et al., 2001), involving JET-LAG (peschel et al., 2009). CRY transmits information about light to the clock through interacting with TIMELESS (Ceriani et al., 1999). CRY is also shown to play the role of a core clock protein in the peripheral organs like in the antennae (Krishnan et al., 2001). CRY is not the only photoreceptor that sends light signal to the clock, since cry^{01} flies can also 'phase-shift' to light pulses (Kistenpfennig et al., 2012). Other than CRY, flies can entrain to light using compound eyes, ocelli and H-B eyelets. Compound eyes

possess rhodopsin (Hanai et al., 2008) and are responsible for entrainment to extreme photoperiods (Rieger et al., 2003). Specific role(s) of the ocelli and HB eyelets remain difficult to assess (Rieger et al., 2003). Nevertheless, H-B eyelets are shown to co-ordinate PER and TIM protein expressions in clock neurons (Veleri et al., 2007). On removal of all photoreceptors in the visual system plus cryptochrome makes the fly visual and citrcadian-blind (Helfrich-Forster et al., 2001).

Entrainment of circadian rhythms to temperature cycles

Another crucial time-cue for entrainment of *Drosophila* clock is temperature (Konopka et al., 1989). Temperature cycles of as low as 2^0C can rescue behavioral rhythmicity induced by constant light (Matsumoto et al., 1998). Temperature cycles can also drive molecular oscillations of PER and TIM (Yoshii et al., 2005). Dorsal (DNs) and lateral posterial neurons (LPNs) are crucial for temperature entrainment (Yoshii et al., 2005, Miyasako et al., 2007) and therefore, entrainment to light (mainly by lateral neurons) (Stoleru et al., 1994) and temperature probably happens through two different sets of clock neurons and the efficient coupling between them enables *Drosophila* to keep proper phase relationship to light and temperature cycles (Miyasako et al., 2007). A recent study has shown that different sets of clock neurons mediate synchronization to temperature cycles with different amplitudes: dorsally located neurons to higher and ventrally located neurons to lower temperature cycles (Gentile et al., 2013). Chordotonal organs in flies have been identified as the major temperature sensory centre and the gene *nocte* expressed in these organs plays a crucial role in temperature synchronization (Sehadova et al., 2009).

Recent studies probing entrainment of circadian rhythms in nature

The role of light and temperature in entraining behavioral rhythms such as locomotor activity has been extensively studied in *Drosophila* (Helfrich-Forster, 2002; Ashmore and Sehgal, 2003; Peschel and Forster, 2011; Konopka et al., 1989; Matsumoto et al., 1998), but most, if not all, of these studies have used rectangular profiles of such time-cues, which are far removed from the reality of nature where multiple time-cues are simultaneously present. Light and temperature, among other environmental parameters in nature, varies gradually quite unlike what many studies have been able to replicate in the laboratories, where it is a step up and step down situation, in most of the cases. The quality of these time-cues is also very different in nature as compared to lab. For example, light used in most studies in laboratory is monochromatic, whereas, organisms experience the entire spectra of light in nature. The intensity of light also is quite high outside than what is used in the lab (mostly around 100-lux in the lab, as opposed to around 1000-2000-lux in the nature). Fluctuations in the environmental conditions are quite high in nature than in relatively much controlled lab protocols. Given all these salient differences between lab and nature, one might wonder how much of the inferences drawn from the lab studies could be stretched and generalized to understand how organisms keep time in nature. Therefore, it remains very crucial to examine circadian rhythms under natural environment where the life forms have evolved. There has not been any study entirely dedicated to address this issue until Supriya Bhutani's PhD thesis which was published in 2009. I will briefly summarize her work and follow that up with few very recent studies looking for insights on how organisms synchronize with environmental time-cues in nature.

Supriya Bhutani's thesis (2009)

This work reported several unique features of the activity-rest rhythm in natural conditions, for the first time. The activity pattern of wild-type flies was found to be unimodal and bimodal in cooler and warmer weather conditions, respectively. One important feature of the rhythm in natural condition was the presence of an additional activity peak (henceforth, afternoon peak or A-peak) in the middle of relatively warmer days with average day temperature greater than 29^0C. The activity in the middle of the light period in lab is relatively very low, which is what is generally called a 'siesta', and it is thought to be a means for the fly to escape hot temperatures in the afternoon (Majercak et al., 1999). Thus, this high amount of activity in the afternoon seen under nature was thought to be a stress-response to hot temperatures. Nevertheless, the possibility that this high movement is due to shade-seeking was discarded in this work as the recording systems were not placed under direct sunlight. Some of the salient results of Supriya Bhutani's thesis are discussed below.

Role of light on morning and evening activity: This study attempted to examine the roles of light and temperature in the regulation of morning (M) and evening (E) activity of flies under natural conditions. Light plays crucial role in the regulation if M-activity. M-activity is a response to twilights (very low levels of light in the morning such as 0.0006 to 0.06-lux), and it is not clock-controlled and intracellular photoreceptor *cryptochrome* (*cry*)-dependent, as the clock and *cry* mutants show similar response to such low light. The northern Europe fly-strain HU had a M-onset later than the southern strain WTALA, implying that northern strain is responding to higher light intensities compared to the southern strain, which is expected based on the notion that flies experiencing extreme photoperiods (the northern one) would evolve decreased light sensitivity (Tauber et al., 2007). The dependence of M-onset on the ancestry of fly-strain supports that M-activity is responsive mainly to the environment, and is unlikely to be

endogenously regulated. No such difference was observed between these two strains in terms of the E-peak onset. Light also affected E-activity in that when flies were covered from light, E-peak occurred later. Unlike M-activity, the role of light on E-peak was probably mediated through CRY.

Role of temperature on morning and evening activity: The position of M-onset was negatively correlated with the average nighttime temperature such that the onset occurs later in warmer nights. Similar correlation existed between E-onset and average day temperature, such as E-onset occurred earlier on cooler days.

Role of canonical clock genes on the locomotor activity rhythm in nature: The canonical clock gene mutants and flies lacking fully functional clock neuron network exhibited similar activity patterns like their genetic controls. Additionally, the correlations between activity and environmental parameters only marginally got affected in the clock mutants. Nevertheless, the E-onset was advanced in per^s and delayed in per^L. Stopping the clock in the M and E cells (Grima et al., 2004; Stoleru et al., 2004) did not bring any dramatic changes in morning or evening behavior, nonetheless, there were subtle effects.

These might reflect a very limited role of circadian clocks under natural conditions. Then the question one might ask is how important is it to have a functional clock in the natural environment? And, how do these 'clockless' flies keep time under natural conditions? Supriya Bhutani attempted to provide a threefold explanation for this. She argued that firstly, there could be some underlying clock-independent physiological process which results in some residual behavior which the clock could enhance. Secondly, there could be some interval timer which measures intervals between periodic environmental events and using this information, the

organism performs an activity. Thirdly, she pointed out the possibility of existence of a residual clock in these apparently 'clockless' flies under natural conditions.

Vanin, Bhutani and others' work (2012)

Following up to what Supriya Bhutani attempted to examine, this study pointed out several unique features of the locomotor activity rhythm under natural conditions. Several lab-phenotypes with respect to activity-rest rhythm such as anticipation to light transitions, less activity in the afternoon ('siesta'), crepuscular activity pattern and the dominance of light over temperature as time-cue were not observed under natural conditions (Vanin et al., 2012). These authors question the lab-based assumptions about circadian rhythms to infer how organisms keep time in the real world. These authors support and further illustrate findings of Bhutani's thesis such as M-onset is dependent on temperature and twilight whereas E-onset is temperature and clock-modulated. They also conclude that A-peak is clock-modulated as onset of A-peak is advanced in per^s and per^0 compared to their genetic controls. Nevertheless, presence of A-peak in per^0 contradicts this claim (Vanin et al., 2012).

De and others' work (2012)

Previous studies by myself and others in the laboratory examined adult emergence rhythm of *Drosophila* under natural conditions. Like activity-rest rhythm (Bhutani, 2009; Vanin et al., 2012), several parameters of this rhythm showed differences between lab and natural conditions. Emergence was more robust in nature than in lab such as the gate width of emergence, fraction of flies emerging in the night and day-to-day variation in the timing of the peak of emergence was lower in natural conditions than in the lab. Absence of the canonical clock gene *period* did not affect the rhythmicity in emergence under natural conditions but

emergence was less tightly gated in these flies compared to genetic controls, which is suggestive of a very little role of *period* gene in regulation of this rhythm in nature, similar to activity-rest rhythm (Bhutani, 2009; Vanin et al., 2012). Rather, seasonal variations in the environmental factors appeared to affect the rhythm to a large extent.

Studies in the laboratory mimicking natural-like conditions

There have been a couple of studies in recent times in the lab which have attempted to replicate features of natural environment in contrast to traditional 12:12 light:dark or warm:cold rectangular cycles most frequently used to understand light and temperature entrainment, respectively. These modified lab protocols included simultaneous exposure to light and temperature cycles, seasonal variation in thermoperiods and photoperiods and diurnal variation in the wavelength of light.

Light and temperature act synergistically to affect behavioral and molecular rhythms: Although entrainment by light and temperature has been studied quiet extensively (Helfrich-Forster, 2002; Ashmore and Sehgal, 2003; Peschel and Forster, 2011; Konopka et al., 1989; Matsumoto et al., 1998) in the lab but how both light and temperature simultaneously entrain the behavioral and molecular rhythms, like in nature, has rarely been examined. When both light and temperature cycles are imposed, they seem to affect behavioral and molecular rhythm synergistically (Yoshii et al., 2009). When both cycles were present, behavioral rhythm entrained better and resulted in in-phase entrainment of the clock neurons with greater amplitude of molecular oscillation compared to light or temperature cycle alone (Yoshii et al., 2009).

Seasonal variations in photoperiod affect activity patterns: A recent study examined how seasonal variations in the photoperiod affect the activity-rest pattern in three wild type strains of

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Drosophila (Rieger et al., 2012). This study subjected flies to varying photoperiods and assessed their ability to entrain. The ability to entrain to longer days was found to be restricted to a certain phase angle difference between morning and evening peaks and this ability to entrain also depended on simulated twilight, ambient temperature and the fly-strain.

M and E- activity peaks depend on temperature, in different extents: Exposing flies to different thermoperiods revealed that morning peak synchronizes with temperature rise in the morning and evening peak with temperature fall in the afternoon (Bywalez et al., 2012). Phase of E-peak was more dependent on the absolute temperature levels than the M-peak. In the light of dual oscillator model (Pittendrigh and Daan, 1976), the authors proposed that M and E-oscillators have different sensitivities to temperature (Bywalez et al., 2012).

Color of light can cue circadian rhythm: In nature, organisms are not only exposed to simultaneous light and temperature cycles with seasonally varying photo and thermoperiods but they also experience diurnal variation in the colour of light. Lab studies conventionally use monochromatic light quite unlike natural skylight. Therefore, whether varying wavelength of light, as in natural skylight, can serve as a time-cue for rhythmic behaviors remains unknown. A recent study on two species of cichlid fishes showed that diurnal changes in colour, not intensity, times circadian behavior (Pauers et al., 2012).

Pamela Menegazzi and others' work (2012, 2013)

From Supriya Bhutani (2009) and Vanin and others' work (2012), the role of canonical circadian clock genes was not conclusive. Bhutani concluded that known circadian clock genes may have subtle roles in regulation activity behavior in nature, whereas, Vanin et al., 2012 claimed that A and E-peaks are clock modulated, albeit with certain internal inconsistencies such

as presence of A-peak in per^0. Therefore, the present evidence from these two studies, the role of clock genes in the regulation of behavior in nature is not yet conclusive. More in the line of the role of canonical clock gene *period,* Pamela Menegazzi and other workers (2012) closely inspected this issue and showed that having a functional clock probably helps the fly suppress 'unproductive' activity during midday, as a response to changes in the environment, especially increase in temperature in the afternoon. Higher fraction of per^0 flies showed afternoon peak compared to their genetic controls. Given this little role of clock gene *period* in the regulation of activity-rest rhythm in nature as opposed to what is known from lab studies, Menegazzi and co-workers (2013), in a subsequent study, attempted to examine the neuronal expression patterns of clock proteins PER and TIM in nature. These authors found that these protein expression patterns differ between lab and natural conditions, to a large extent, and were also affected by seasonal variations in the environment. This study reveals that (1) the co-ordinated nuclear entry of PER and TIM oscillations seen in most laboratory light/dark cycles was not seen under summer conditions. (2)There is a difference in phase of PER/TIM expression across subsets of clock neurons in that irrespective of seasons, expression of PER and TIM peaks in dorsal neurons occurs earlier than lateral neurons.

Major questions of this thesis

At present, the available literature is limited to descriptions of features of rhythms under natural conditions (Bhutani, 2009; Vanin et al., 2012; Menegazzi et al., 2013) rather than a systematic and rigorous scrutiny of the role of different zeitgebers in determining unique activity/rest patterns in nature. Several questions about the role of light, temperature, and humidity as zeitgebers in regulating circadian rhythms under natural conditions remain unanswered. Vanin and co-workers (4) showed that phase of the morning (M) and evening (E)

activity peaks maintain a consistent relationship with mean daily temperature. But most of the conclusions on how light and temperature affect M and E activity in this study are mostly based on correlations. Proportion of flies displaying afternoon (A)-peak increased with increase in mean daytime temperatures. Other studies have shown that in the LAB, under gradually varying temperature cycles of different thermoperiods, M-peak was synchronized to temperature rise in the morning and E-peak to temperature fall in the evening (Bywalez et al., 2012). However, thus far, only few studies have examined the role of light in the natural context. A previous study under natural conditions reported that both photoperiod and twilight durations influence precision of locomotor activity rhythm (Aschoff, 1972). While simulated twilight conditions did alter activity profile of flies (Yoshii et al., 2009), it is still not known how different parts of the natural light profile affect activity pattern. Moreover, there has never been a comprehensive study wherein natural light profile was mimicked, nor has there been any attempt made to determine which aspects of light information are crucial or dispensable for timing circadian rhythms in nature. In this thesis I attempted to understand the role of light in the regulation of activity peaks in nature. I aimed to examine how natural light modulates the M, A, and E-peaks of activity by modified light information in various ways under otherwise natural conditions. Only light information was altered in our study while allowing other time-cues to change naturally. I hypothesize that this approach would reveal which aspects of the rhythm in nature are dependent on distinct facets of light information, and to what extent.

I ask what is the role of the known clock gene period in regulating activity-rest behavior in nature. In the presence of multiple reliable cues to time rhythmic behaviors in nature, do organisms really need a clock to do the job of keeping time? Is there a possibility that there is some role of clock genes in this regard, and that it is masked by environmental factors in nature,

such as light. I examined activity-rest behavior of per mutants under natural conditions and in constant darkness under otherwise natural conditions to answer these questions.

I also examined more carefully the nature of the high activity in the afternoon, a salient feature of the activity rhythm in nature. Since afternoons are associated with high temperature and low humidity, it is not intuitive to imagine such high activity in such hours which incur a high risk of desiccation. To address this issue, I made visual observations of flies placed solitarily in activity tubes in the monitor or outside and solitarily or in groups in petridishes. By corroborating data from visual observations and activity recordings using conventional DAM systems, I attempted to closely inspect the nature of the activity in the afternoon.

I asked what is the functional significance of the three activity peaks seen in nature (Vanin et al., 2012). If a fly is active during a particular section of the day, what is for the significance of this activity? Are they active to enable them to finding mates or for courtship and mating or foraging? The DAM system is highly unlikely to provide any insight into this question since it just records movement. I attempted to know what this movement is for, by making round-the-clock visual observations. I scored behaviors of flies housed solitarily or in groups and plotted the time-course of its occurrence in what is called a 'chronoethogram'.

I also attempted to mimic natural-like light and/or temperature profiles in the laboratory incubator. This was done for two reasons: (1) firstly, to see whether these protocols could reproduce features of rhythm in nature and (2) secondly, to dissect out relative contributions of light and temperature in the regulation of these activity peaks in nature.

Rhythmicity in the event of eclosion of adult fly out of the pupal case (adult emergence) under laboratory conditions has been demonstrated many decades ago and studied to some extent

(Saunders, 2002) and recently under natural conditions (De et al., 2012). I have attempted to understand how seasonal variations in the environmental factors affect rhythmic behavior of flies in natural condition by studying adult emergence rhythm in summer and winter months of the year in three species of fruit flies.

Major findings of this thesis

Light differentially affects the three activity peaks in nature: Light modulates phase and amplitude of morning (M) and evening (E) activity peaks and determines the occurrence of the afternoon peak (A-peak). Morning and evening peaks could also use humidity and temperature information when light is not present. Therefore, light information is dispensable for M and E-peaks. But presence of natural light in the afternoon is crucial for A-peak to occur. When flies are provided with constant darkness in otherwise natural conditions or when afternoon light is specifically blocked or when natural light intensity is cut down by 50% or more in otherwise natural conditions, afternoon peak does not occur. Therefore, natural light induces A-peak whereas it modulates timing and amplitudes of M and E-peaks.

Canonical clock gene period has a very little role in the regulation of activity peaks in nature: period gene has subtle roles to play in regulation of activity peaks in nature, and some of which could be masked by natural light or other environmental factors. *period* influences the phase of the E-peak but not A-peak. Nevertheless, afternoon activity levels get modulated due to *period*. However, this is found to be masked by natural light.

Afternoon peak is an artifact of experimental paradigm: High afternoon activity is not a natural behavior of flies. Observing flies housed in activity tubes inside the *Drosophila Activity Monitor* (DAM) system reveals that this high amount of activity basically arises due to the shade seeking

behavior of flies in the IR beam area of the recording apparatus, in bright and warm afternoons. Even though flies are largely at rest in the afternoon clarified through visual observation, the DAM system detects high activity. In petridishes, flies placed solitarily or in groups do not exhibit high afternoon activity.

Functional significance of the activity peaks: Visual observation of fly behavior in petridishes where flies are housed in groups of three males and three females shows that M-peak of activity is associated with courtship related behaviors such as chasing, wing expansion and copulation. A-peak of activity was not seen in flies housed in petridishes and is largely an artifact of the conventional activity recording protocol. E-peak of activity is associated with general locomotion, the significance of which remains unclear.

Reproducing natural behavior in lab by mimicking light and temperature profiles: I attempted to examine whether some of the unique characteristics of activity-rest pattern could be reproduced in the lab by mimicking natural-like gradual light and/or temperature cycles in the laboratory incubator. This approach was also used to tease apart contributions of light and temperature in bringing about the features of the rhythm seen in nature. I show that M-peak phase is more temperature-dependent whereas, E-peak phase is light-dependent. A-peak mainly depends on temperature. The proportion of flies showing A-peak increases with increasing daily temperature.

Seasonal variations in the environment greatly influence emergence rhythm: I examined another rhythm in fruit flies, namely adult emergence rhythm, to understand how seasonal variations in the environmental parameters affect the parameters of rhythm. Gate width of emergence, fraction

of flies emerging during night, day-to-day variance in the timing of the peak of emergence and peak amplitude are lower in cooler and wetter conditions.

Chapter 2: Role of light in regulation of the activity peaks under SN

Introduction

The role of circadian clocks in the temporal organization of behaviors has been mainly studied

under standard laboratory conditions. These studies are limited due to simplified laboratory

(LAB) protocols, often far removed from the reality of natural conditions in the wild. For

instance, LAB studies have mostly used square-waves of a singular zeitgeber (in rare cases, two)

(Saunders, 2002) quite unlike multiple, simultaneous, stochastic, gradually changing time-cues

existing in nature (Bhutani, 2009; Vanin et al 2012; De et al., 2012). In theory, one would

expect to see a tighter and more robust rhythm in nature compared to standard LAB regimes

mainly because of the availability of information-rich cues that can time behaviours. Two recent

studies attempted to inspect circadian behaviors such as activity/rest and adult emergence of fruit

flies *Drosophila melanogaster* under semi-natural (SN) conditions and both reported behaviours

significantly different in several key aspects from those observed under LAB conditions (Vanin

et al., 2012, De et al., 2012). For instance, the unique afternoon (A) peak of activity under

natural conditions (Vanin et al., 2012) had never been seen in any standard LAB assay

conditions. Adult emergence under SN conditions was reported to be more robust compared to

LAB and rhythmicity was observed in the loss-of-function *period* mutant (*per*[0]), which is not the

case in the LAB (De et al., 2012). Similarly in a study on a subterranean rodent *Tuco tuco*, it

was observed that these animals are day-active in the wild owing to foraging and burrow

maintenance behaviors, and therefore calling this species nocturnal based on LAB data may be

misleading and inaccurate (Tomotani et al., 2012). Vanin et al. (2012) also pointed out that

several LAB based assumptions do not hold under natural conditions such as anticipation of

time-cue transitions, mid-day siesta, crepuscular behavior and dominance of light as a zeitgeber

over temperature. Nevertheless, this study did not tease apart the roles of the different zeitgebers and therefore the nature of influence of light, temperature or humidity, in regulating activity/rest rhythm under natural conditions has not been addressed systematically. The scanty literature on circadian rhythms under natural conditions offers only a descriptive narration on features of rhythms under natural environment (Vanin et al., 2012, De et al., 2012) rather than a systematic and rigorous scrutiny of the role of different zeitgebers in determining these unique features of the rhythms. It was suggested from previous observations that the mean daily temperature is a better estimator for phase of the morning and evening activity peaks than the photoperiod and therefore, light is unlikely to play a dominant role in regulating activity/rest rhythm under natural conditions (Vanin et al., 2012). Interestingly, studies showed that when assayed in the LAB under nature-like temperature cycles with varying day-lengths (Bywalez et al., 2012), the M and E activity peaks were synchronized, to temperature increase in the morning and decrease in the afternoon respectively. However, there are only few studies that examined the role of light in a natural context. A study by Aschoff and co-workers examined the precision of activity rhythm under natural condition and suggested that photoperiod and twilight duration affect precision of the rhythm (Aschoff et al., 1972). However, the role of light in the regulation of different aspects of activity/rest rhythm in the natural context has never been distinguished from the influence of other potential zeitgebers and examined rigorously. In the present study, we aim to examine the morning (M), afternoon (A) and evening (E) activity peaks in the light of several modifications to the SN conditions. These modifications are likely to address issues such as how intensity and duration of light, cycling *versus* non-cycling LD, direct or proximate effect of changes in light under otherwise SN condition affect activity/rest rhythm. Our light modification protocols include (i) constant darkness (DD) and constant light (LL) of different intensities with

other semi-naturally varying cues to check how absence of light information affects the activity peaks, (ii) blocking light in different portions of the day to examine whether and how exposure to natural light profile at different time of the day modulates activity/rest pattern differently, and (iii) cutting down amplitude of SN light variation to see whether there is a direct effect of light intensity on activity peaks. In all such modifications, only light information was altered in multiple ways, while other potential zeitgebers varied semi-naturally. This approach would help us understand how much and which aspects of the rhythm in nature is light and clock dependent.

Materials and Methods

Fly strains used

All experiments were performed on virgin male flies (unless specified) starting at the age of 3-4 days. *Canton-S* (*CS*), *yellow-white* (*yw*) and *white-eye* (w^{1118}) strains were considered as wild-type. To examine the role of circadian clocks in the regulation of activity peaks under SN, I used the null mutant of circadian *period* gene (per^0) in *white* eye-color (w^{1118}) genetic background.

The assay conditions

Semi-natural condition (SN)

The locomotor activity assays were done in June 2012, within JNCASR, Bangalore campus (12°59'N 77°35'E), inside an enclosure constructed under a leafy canopy (De et al., 2012). The enclosure was an iron cage ($122 \times 122 \times 122$ cm^3) with grids (6×6 cm^2) allowing free flow of air, and covered only on top with a sloping translucent plastic sheet. Fly activity was recorded using Drosophila Activity Monitor (DAM) system (TriKinetics, USA). The daily profiles of light, temperature, and humidity were also monitored simultaneously using DEnM, Trikinetics, USA.

LL or DD under otherwise SN condition (LL+SN or DD+SN)

In order to create LL conditions of varying intensities, under otherwise SN condition, activity monitors were placed inside light-tight metal boxes ($44 \times 27 \times 20$ cm^3) fitted with light baffles and a small fan such that the temperature and humidity inside the box closely matched that of the outside environment. Temperature and humidity inside and outside the boxes were recorded continuously and were found to be stable throughout the study. The LL intensities used in this experiment were 10, 100 and 1000 lux. Light intensity was measured using LiCor luxmeter, USA. Similar boxes were used to create DD condition under SN (DD+SN).

Light blocking experiments

The activity monitors were kept in boxes mentioned above and covers were placed for different durations of the day, every day, for 7 days - morning-cover (MC - 4:00 to 10:00 h), afternoon-cover (AC - 10:00 to 16:00 h) and evening-cover (EC - 16:00 to 22:00 h) and morning and evening-cover (MC/EC - 4:00-10:00 h and 16:00-22:00 h). These durations were chosen based on the average light intensity profiles under SN recorded for several days, just before the assays. MC filters out the rising part of light intensity, whereas AC and EC the plateau and decreasing parts of the SN light profile, respectively.

Semi-natural (SN) light intensity filtering experiments

Neutral density filters (Lee Filters, Andover England) from *www.studiodepot.com* were used to cover the monitors to create three regimes where the semi-naturally varying light amplitude was reduced by 90% (SN90), 75% (SN75) and 50% (SN50). Other than causing reduction in the amplitude of light waveform, these filters did not cause any alteration in the qualitative profile of light.

Statistical analyses

The activity profiles in the figures are based on bin size of 15 min. Three intervals of the day were considered to be Morning (4:00 to 10:00-h), Afternoon (10:00 to 16:00 h) and Evening (16:00 to 22:00-h) for determine presence/absence, phase, amplitude of activity peaks and activity performed in a specific interval. To determine the presence of M, A, and E-peaks, average activity profiles (15-min bin) for each genotype or protocol were plotted. An interval (M, A, or E) was considered to have a peak based on qualitative assessment of the activity profile averaged across flies and days of recording. Phases of M, A, and E activity peaks were estimated by scanning 7-day average activity records of each fly, and identifying that time-point corresponding to the highest activity counts observed within that interval. In the afternoon, when there are multiple peaks, the peak closest to maximum light and temperature in the environment was considered and its phase and amplitude were calculated. Phase and amplitude for each peak were averaged across 32 flies from each genotype and each regime. One-way ANOVA was carried out to see whether there is any statistically significant effect of genotype or regime on the phase and amplitude of activity peaks. Post-hoc multiple comparisons of phase and amplitude data was performed using Tukey's HSD test. The p value of 0.05 was considered as level of statistical significance throughout all the analyses. Total activity was calculated by taking average across 32 flies for each regime and each genotype, while for each fly total activity was calculated by taking average across 7 days. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's test was performed to see whether there is any significant effect of regime/genotype on the total activity, and which of the regimes/genotypes is significantly different from others in pair-wise comparisons. Activity during morning, afternoon and evening hours was calculated as the sum of activity counts in the three durations for each fly and then

averaged across 32 flies. The durations were the same as mentioned above for phase and amplitude calculations. Two-way ANOVA followed by post-hoc multiple comparisons using Tukey's test was done on activity data in different durations for different regimes, having regimes and durations as fixed factors. Dawn anticipation index (AI) was calculated as the ratio of activity counts for 3-h duration prior to dawn (the time-point when the light intensity value first rose above 0-lux) over activity counts for 6-h duration prior to dawn (Harrisingh et al., 2007). The error bars on the waveforms in all the figures are SEM, whereas error bars on the bar plots for quantification of phase, amplitude, total activity and activity in different durations are 95% CI (95% Confidence Interval), which could be used for visual hypothesis testing for difference between means.

Results

All wild-type strains exhibit two peaks, while some exhibit three peaks of activity under SN
Studies were conducted during June 2012, when in the experimental enclosure, light intensities reached ~2000-lux and nighttime light intensity was undetectably low, temperatures ranged between ~23 and ~30°C while relative humidity levels rose up to ~85% and fell down to ~50%. Under such environmental conditions, out of the three wild-type strains examined, *CS* ($n = 27$) and *w^{1118}* ($n = 14$) showed three peaks of activity corresponding to morning, afternoon and evening hours. In contrast, *yw* ($n = 9$) showed only one prominent peak in the afternoon and a weak peak in the evening (Figure 1A). Both *CS* and *w^{1118}* flies showed similar phase and amplitude of the M-peak (one-way ANOVA, $p > 0.05$; Figure 1B), while this peak was not detectable in *yw* flies. In all three strains, flies did not appear to 'anticipate' dawn (Table 1) unlike standard LAB LD12:12. A-peak was displayed by all three wild-type strains, with no

significant difference in phase ($F_{2,59} = 0.11$, $p = 0.89$) coinciding with maximum mid-day light intensity (Figure 1A, B). A-peak was composed of several sub-peaks, which coincided with fluctuations in natural light intensity. One-way ANOVA ($F_{2,59} = 6.87$, $p < 0.002$) followed by post-hoc multiple comparisons using Tukey's test revealed that amplitude of A-peak in *yw* flies was significantly lower compared to *CS* ($p < 0.002$) and w^{1118} ($p < 0.01$; Figure 1B). The E-peak was significantly phase-delayed in w^{1118} compared to *CS* and *yw* ($F_{1,45} = 9.44$, $p < 0.003$; Figure 1B) and its amplitude was greater in w^{1118} flies than *CS* and *yw* ($F_{1,45} = 65.03$, $p < 0.001$; Figure 1B). The total activity of *yw* was significantly lower than w^{1118} and *CS* ($F_{2,56} = 14.49$, $p < 0.05$, Figure 1C). I also examined activity/rest behavior of *per^0* flies ($n = 16$) under SN (Fig 2A) and found that they show lower amplitude of A-peak than w^{1118} ($F_{1,30} = 16.27$, $p < 0.003$), while amplitude of other peaks and their timing did not differ (M-peak: $F_{1,30} = 4.05$, $p = 0.17$; A-peak: $F_{1,30} = 0.18$, $p = 0.66$, E-peak: $F_{1,30} = 2.86$, $p = 0.10$) (Fig 2B). Total activity of *per^0* flies was also not different from w^{1118} ($F_{1,30} = 3.39$, $p = 0.05$; Figure 2C). In summary, under SN, wild-type and *per^0* flies showed three peaks of activity (including one in the afternoon) except *yw*, which did not display the M-peak and had overall lower activity.

Under SN conditions, LL abolishes M-peak, advances E-peak and makes flies more active

Since it is known that LL under constant temperature abolishes the activity/rest rhythm of flies under LAB conditions, I asked how it might affect the rhythm in presence of several environmental conditions of nature. Under SN, LL of intensity 10 ($n = 28$, LL_{10}+SN), 100 ($n = 21$, LL_{100}+SN) or 1000-lux ($n = 29$, LL_{1000}+SN) (LL+SN) abolished the M-peak (Figure 3A, top row). In contrast, flies maintained under DD with access to other time-cues of SN (DD+SN) ($n = 31$) showed a prominent M-peak (Figure 3A, bottom row). Thus LL abolishes M-peak despite the presence of non-photic time-cues in nature. Flies did not anticipate dawn under any of the

LL+SN conditions as estimated by AI values which lay close to 0.5 (Table 1). However, when a low intensity (100-lux) LD cycle (LD+SN) ($n = 26$) was imposed in a similar apparatus as that employed for LL+SN (Figure 3A), slightly greater anticipation was detectable (Table 1; significantly greater than LL_{1000}+SN; $p = 0.01$, although not statistically different from LL_{10}+SN and LL_{100}+SN). Under DD+SN where light information was completely blocked, flies exhibited anticipatory activity probably to temperature trough and humidity peak (Table 1; Figure 3A, bottom right panel). ANOVA on arcsine transformed AI data revealed that flies show greater anticipation to dawn under DD+SN than under SN, LD+SN, or all LL+SN ($F_{5,175} = 17.62$, $p < 0.0001$). E-peak was phase-advanced under DD+SN and in all LL+SN compared to SN and LD+SN, with the sole exception of LL_{1000}+SN (ANOVA followed by post-hoc multiple comparisons using Tukey's test, phase: $F_{5,163} = 75.18$, $p < 0.0001$; amplitude: $F_{5,163} = 4.23$, $p < 0.001$; Figure 3B). This suggests that under protocols such as LL+SN and DD+SN, where flies do not have access to changes in natural light, they probably use humidity and temperature to phase their E-peak, whereas under SN flies use photic cues to time their E-peak such that it occurs only after it is dark, i.e., when light intensity has fallen below 0-lux. However, the LL_{10}+SN and LL_{100}+SN experiments were conducted separately from all the others (about two weeks later), when profiles of both temperature and humidity were slightly altered, such that high humidity levels persisted for shorter time and warm temperature conditions lasted for longer duration (Figure 3A). Under LL_{1000}+SN, where the E-peak was even more delayed compared to other LL+SN protocols and SN, probably because of the fact that during this experiment, slope of humidity rise and temperature fall was more gradual than during the other LL+SN protocols.

ANOVA on total activity revealed a statistically significant effect of protocol ($F_{5,537} = 23.1$, $p < 0.0001$), time-interval ($F_{2,537} = 28.73$, $p < 0.0001$) and protocol × time-interval

interaction ($F_{10,536}$ = 18.36, p < 0.0001; Figure 3C). The total activity of flies mostly comprised of activity during evening hours for LL+SN (or late evening in case of LL$_{1000}$ + SN), unlike DD+SN and LD+SN, where activity was distributed between morning and evening hours (Figure 3C). In contrast, under SN, total activity was mostly contributed by movements in the afternoon. ANOVA followed by post-hoc multiple comparisons revealed that overall activity of flies was highest in all three LL+SN protocols compared to DD+SN ($F_{4,180}$ = 9.07, p < 0.0001; Figure 3C) but not significantly different from SN. Based on these results, I conclude that constant light under otherwise SN conditions abolishes M-peak; nevertheless, in nature light information is dispensable for M-peak to occur just as it is for the E-peak; presumably because information regarding changing temperature and/or humidity is sufficient.

Different portions of natural light profile have differential modulatory effects on activity/rest pattern of flies

Since light was found to affect the phase and amplitude of activity peaks even in presence of other environmental cues in nature (Figure 3), we asked how depriving flies of different portions of natural light profile might affect the three activity peaks. I hypothesized that each of the three distinct activity peaks is regulated by natural light during morning, afternoon and evening hours. To test this hypothesis, I blocked natural light from reaching flies by covering activity monitors during the morning (MC; n = 24) or afternoon (AC; n = 26) or evening (EC; n = 32), and morning plus evening hours (MEC; n = 30), while allowing light to be perceived during mid-day (Figure 4A, shaded horizontal bars). I then estimated anticipation to dawn, phase and amplitudes of each of the activity peaks and total activity of flies as described previously. Compared to SN, flies showed greater anticipation to dawn under MC, AC, and DD+SN (Table 1; $F_{5,172}$ = 9.45, p < 0.0001). Furthermore, under MC, flies showed greater dawn anticipation than in both EC (p <

0.006) and MEC ($p < 0.006$; Figure 4A, Table 1). Thus, it appears that flies normally do not anticipate light in nature but when deprived of it, show enhanced anticipatory activity, probably in response to temperature and/or humidity changes as seen under MC and DD+SN.

Phase of M-peak was affected when flies were deprived of light during different times of the day (ANOVA, $F_{4, 133} = 2.75.55$, $p = .03$, Figure 4B). Blocking natural light during morning (MC) significantly advanced the phase of M-peak compared to SN ($p < 0.02$), such that it now occurred at a phase similar to that under DD+SN ($p = 0.99$; Figure 4B). Interestingly, blocking light in the evening (EC) affected the M-peak most profoundly, in that, it was completely suppressed even though these flies had access to the rising portion of the natural light profile (Figure 4B). Amplitude of M-peak was affected by the blocking of light ($F_{4,133} = 6.10$, $p < 0.01$, Figure 4B) at different intervals, in that, M-peak was diminished under AC compared to all other protocols (Figure 4A, B). When both morning and evening light was blocked (MEC) such that flies now experienced a near-rectangular light profile, phase and amplitude of the M-peak remained similar to SN (Figure 4B).

Both phase ($F_{5,153} = 147.89$, $p < 0.0001$) and amplitude ($F_{5,153} = 5.89$, $p < 0.0001$) of E-peak were affected by the light blocking protocol. Neither MC nor AC affected phase or amplitude of E-peak, and they remained identical to that observed under SN (Figure 4B). EC however, caused phase-advancement ($p < 0.0002$) of E-peak relative to SN ($p < 0.0002$, Figure 4B). Interestingly, the phase of E-peak under EC was even more phase-advanced compared to DD+SN ($p < 0.0002$, Figure 4B), suggesting that exposure to light during afternoon followed by an abrupt fall causes sudden increase in activity much before civil dusk.

A-peak was significantly affected by light covering protocol in terms of both phase ($F_{4,129}$ = 5.95, $p < 0.001$) and amplitude ($F_{4,129}$ = 53.58, $p < 0.0001$). MC and MEC altered neither the phase nor amplitude of A-peak compared to SN (Figure 4B). AC caused drastic reduction of A-peak amplitude, suggesting that this peak is highly dependent on bright light exposure during afternoon (Figure 4A, B). However, in EC, A-peak was phase-advanced compared to SN ($p < 0.001$) and its amplitude was significantly higher than SN ($p < 0.001$), probably as a result of lowered morning activity (Figure 4A, B). Total activity was drastically reduced in the AC protocol compared to SN and all other light blocking protocols (Figure 4C), suggesting that exposure to natural light in the afternoon makes flies more active. The amount of activity during afternoon under AC was also significantly lower than SN and any other light blocking protocols. When flies experienced natural light in the afternoon (SN and MC, EC and MEC), much of total activity was contributed by that during mid-day, whereas, in AC, total activity was distributed nearly equally throughout the day without any preference for mid-day (Figure 4C). ANOVA on activity data during the three intervals under different protocols (Figure 4C) revealed a statistically significant effect of protocol ($F_{5,536}$ = 38.79, $p < 0.0001$), time-interval ($F_{2,536}$ = 29.18, $p < 0.0001$) and protocol × time-interval interaction ($F_{10,536}$ = 11.07, $p < 0.0001$; Figure 4C). Except AC and DD+SN, in all other protocols where flies experienced light in the afternoon, greater proportion of activity was seen during mid-day compared to any other time. ANOVA on total daily activity (Figure 4C) revealed a statistically significant effect of protocol ($F_{5,171}$ = 147.89, $p < 0.0001$). Flies exposed to AC and MEC showed a significant reduction in overall activity compared to SN, whereas those under MC or EC did not differ from SN (Figure 4C). Given that the total amount of light reaching the flies under AC is even more reduced than

that under MC, or EC protocols, it is likely that daily activity level of flies is proportional to the amount of light they are exposed to.

Thus, for the occurrence of A-peak, bright light in the afternoon is critical unlike the M and E-peaks, where light exposure during morning or evening can only modulate phase and amplitude of the peaks. Thus blocking natural light at different times of the day affects the activity profiles of fruit flies to different extents, with more severe effect in the afternoon compared to morning or evening.

Level of natural light intensity determines presence of A-peak modulates phase of M-peak but not of E-peak and alters overall activity

To determine how activity is influenced by intensity of natural light, I subjected flies to SN conditions with altered levels of light without changing its waveform. I employed neutral density filters which reduced light intensity by 50% (SN_{50}; $n = 30$), 75% (SN_{75}; $n = 26$) or 90% (SN_{90}; $n = 25$) (Figure 5A). Anticipation to dawn was significantly greater when natural light intensity was cut down by 50% or more compared to SN ($F_{4,149} = 16.76$, $p < 0.0001$, Table 1). Reduction in light intensity by 50% or more caused significant advancement of M-peak relative to SN ($F_{4,147} = 5.88$, $p < 0.0002$, Figure 5B). This further confirms the earlier finding (Figure 5B) that flies phase their M-peak close to temperature minima and/or humidity maxima when light information is not available in the morning. Amplitude of M-peak was not affected by such reduction in light intensity ($F_{4,147} = 0.94$, $p = 0.44$; Figure 5B).

Unlike the M-peak there was no detectable change in the phase of E-peak in flies exposed to reduced light intensity compared to SN (SN_{50}: $p = 0.26$, SN_{75}: $p = 0.08$, SN_{90}: $p = 0.35$), which suggests that flies are able to track the fall of light intensity even when it falls from levels much

lower than that under SN. However, when evening light information was not available, such as in EC or MEC, E-peak was phase-advanced, probably in response to the abrupt fall in light intensity to 0-lux. Amplitude of E-peak was also affected by light intensity ($F_{4,147} = 10.89$, $p < 0.0002$). Flies exposed to reduced intensity had higher E-peak amplitude compared to SN and DD+SN. ANOVA on the activity levels during different times of the day across these protocols revealed a statistically significant effect of protocol ($F_{4,465} = 6.17$, $p < 0.0001$), time-interval ($F_{2,465} = 48.17$, $p < 0.0001$) and protocol × time-interval interaction ($F_{8,465} = 17.88$, $p < 0.0001$; Figure 5C). Under filtered light intensity protocol, activity of flies was found to be distributed mainly into M and E-peaks quite like DD+SN, and quite unlike SN where the major fraction of total activity was contributed by afternoon activity (Figure 5C). Among the three filtered light intensity protocols (SN_{50}, SN_{75}, and SN_{90}), total activity of flies was not different from each other or from DD+SN ($F_{4,149} = 6.21$, $p < 0.0001$; Figure 5C). However, there was a general trend of reduced activity in all three protocols compared to SN, though only the difference between SN and SN_{50} was statistically significant.

Discussion

The amount of light perceived determines activity levels of flies under SN

The total activity in all the three LL+SN protocols was higher than DD+SN, which was also lower than SN (Figure 3C). Similarly, when flies were subjected to truncated natural light profiles by blocking light at different time of the day, MEC and AC caused significant reduction in activity compared to that under SN, whereas total activity under MC or EC conditions did not differ from SN. The fact that blocking only afternoon light or light during both morning and evening hours cuts down substantial amount of light reaching the flies compared to blocking

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only in the morning or evening hours, suggests that amount of light perceived influences total activity levels. Similarly, when natural light intensity was below 50% of SN, the total activity became lower than SN, pointing towards the possibility that amount of light received is crucial in determining activity levels of flies.

M and E-peaks require natural light information mainly to determine phase

Under SN, M-peak occurred about an hour after sunrise (Figure 1A), coinciding with rise in light intensity and temperature. Flies under MC, MEC and DD+SN have advanced M-peak relative to SN. Similarly, flies experiencing reduced natural light intensity have M-peak phase-advanced compared to SN. Thus, when morning light was blocked or if only diminished light profile was available, M-peak became advanced and coincided with temperature minimum and humidity maximum. Therefore, as far as presence of M-peak is concerned, time-cue in the form of changing light quality is dispensable, whereas its phase is altered by light. The E-peak seems to respond directly to fall of light or temperature or to increase in humidity. Under EC, where the light profile dropped steeply compared to SN, E-peak became phase-advanced relative to SN, such that it now occurred immediately after light intensity dropped to 0-lux, which indicates a strong dependence of E-peak on the time of light fall. However, similar to M-peak, light information was dispensable for E-peak to occur, as seen under DD+SN. The phase of E-peak under DD+SN was advanced relative to that in SN, and coincided with temperature fall and humidity rise at the end of the day. Thus under SN, neither M, nor E-peak seems to depend on light for their occurrence, although their phases are affected by light.

Natural light induces A-peak

Vanin and co-workers proposed that like the M and E-peaks, A-peak is also circadian clock-controlled, because short period per^s mutants showed phase-advanced A-peak while long period per^l mutants exhibited phase-delayed A-peak compared to controls (Vanin et al., 2012). However, the fact that per^0 flies also showed an A-peak (Fig 2A), not different from wild-type flies (Vanin et al., 2012), weakens the case for A-peak to be clock-driven. Among all the different light protocols used in this study, A-peak was prominent only when natural light was available during afternoon, which had a direct effect on the level of activity, manifested in an A-peak. This peak consisted of several sub-peaks that coincided with light intensity spikes during mid-day, indicating a direct and instantaneous response to fluctuations in light intensity. There was very little variation in the phase of A-peak among different genotypes and between protocols whenever A-peak was manifested, which suggests that occurrence of A-peak at a particular phase is greatly influenced by afternoon light. When light was blocked only during afternoon, A-peak was diminished, whereas, blocking light at other time of the day (morning and/or evening) did not affect the A-peak amplitude. However, A-peak can be reproduced even in the laboratory when provided with gradually changing high amplitude light intensity and temperature cycles (Vanin et al., 2012).

Constant light inhibits M-peak probably through clock-independent mechanisms

I tried to address the question of how changing natural light intensity modulates the activity profile of flies, by providing flies with otherwise SN conditions in the backdrop of either LL or DD, intending to examine how crucial light information is for the activity of flies when they are anyway exposed to other naturally varying time-cues. I observed that LL+SN abolished M-peak of activity. This could mean that change in light intensity is a prerequisite for M-peak. However, under DD+SN, which is another way of achieving a situation of absence of light
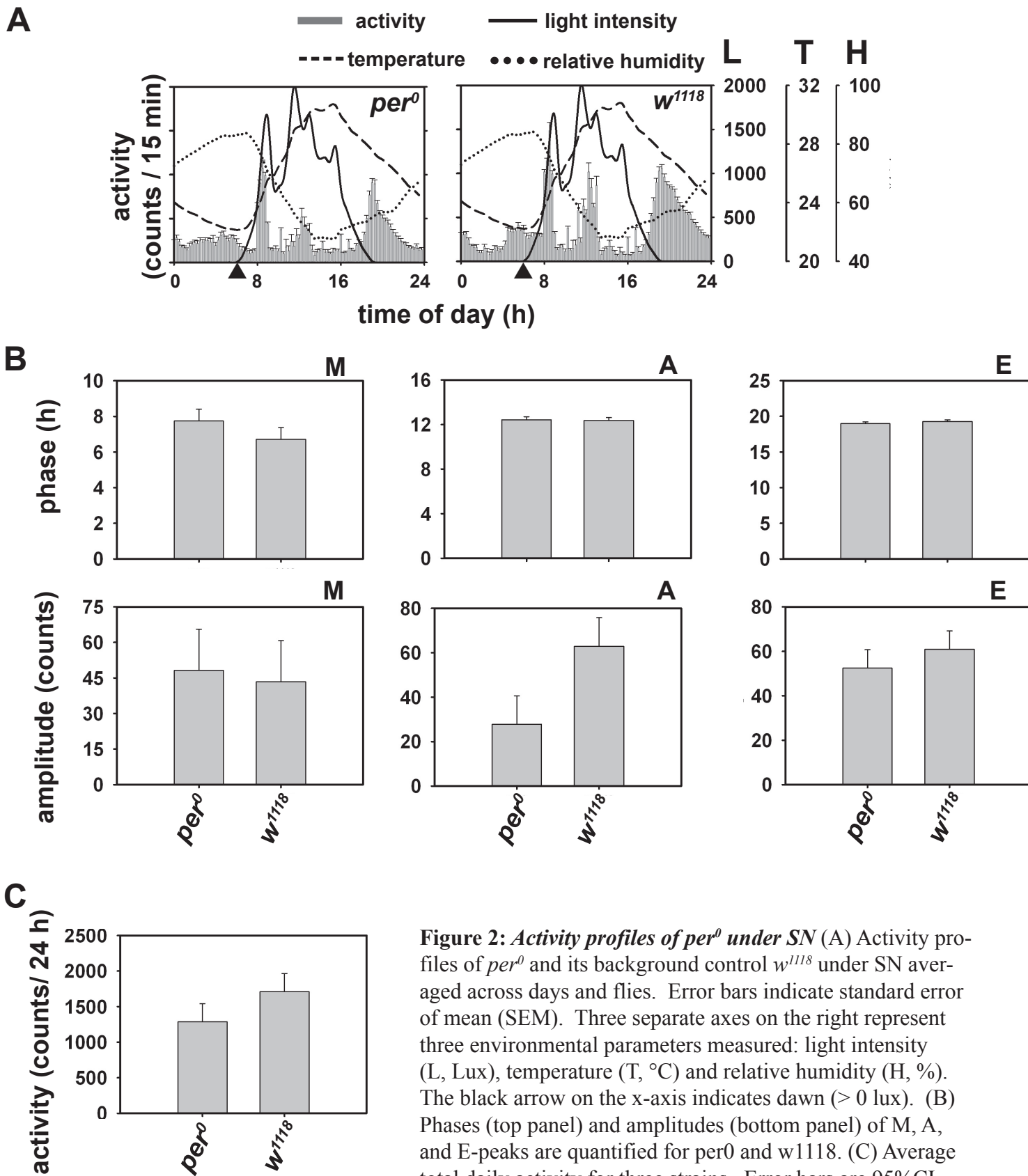
information, a clear M-peak is seen. Therefore, M-peak is not dependent on changing light intensity. Instead, I hypothesize that constantly available light inhibits M-peak, even when other potential time-cues are available to flies. This is consistent with previous findings (Matsumoto et al., 1998), where discrete temperature cycles resulted in strong anticipatory morning activity under DD but only a small startle response in LL. The startle is absent here probably due to the gradual change in temperature under SN. As previous laboratory studies have shown that LL disrupts molecular clocks, I compared the behavior of wild-type flies under LL with that of per^0 flies under SN, where the clock is genetically abolished. However, I find that per^0 flies do exhibit M-peak under SN, which confirms that constant light may have clock-independent effects on activity patterns.

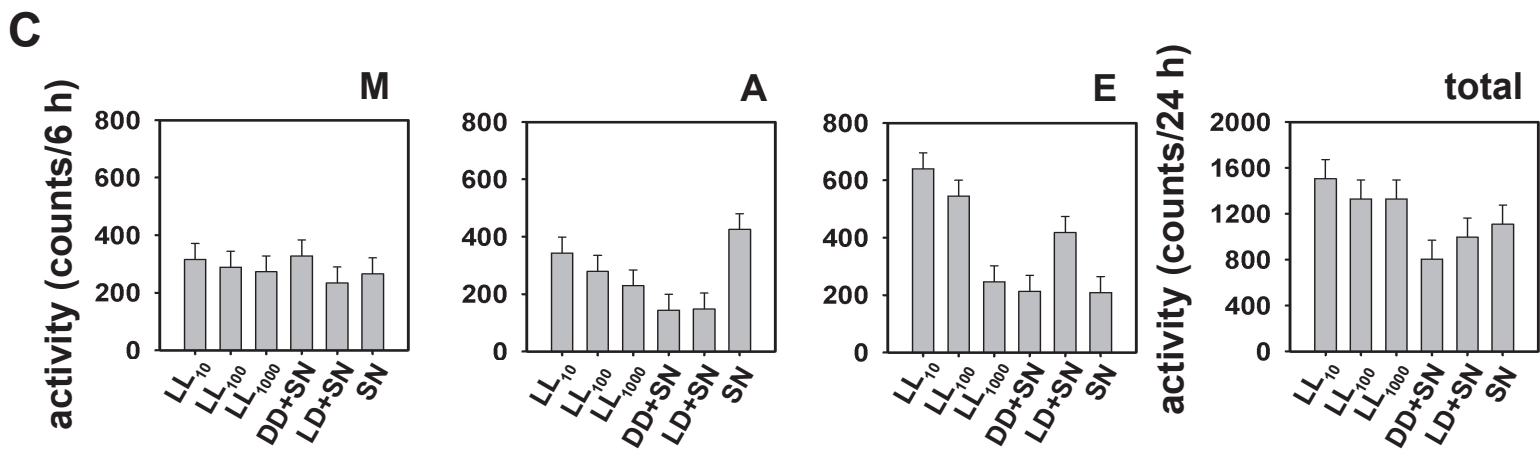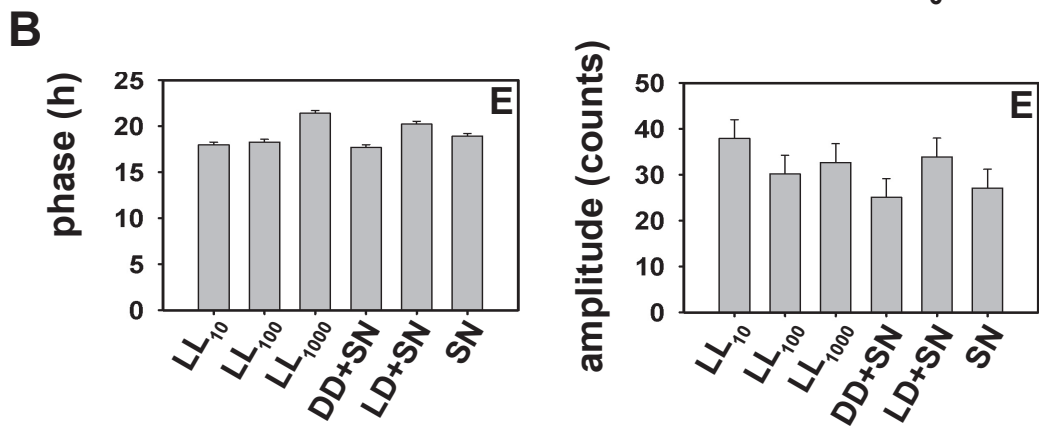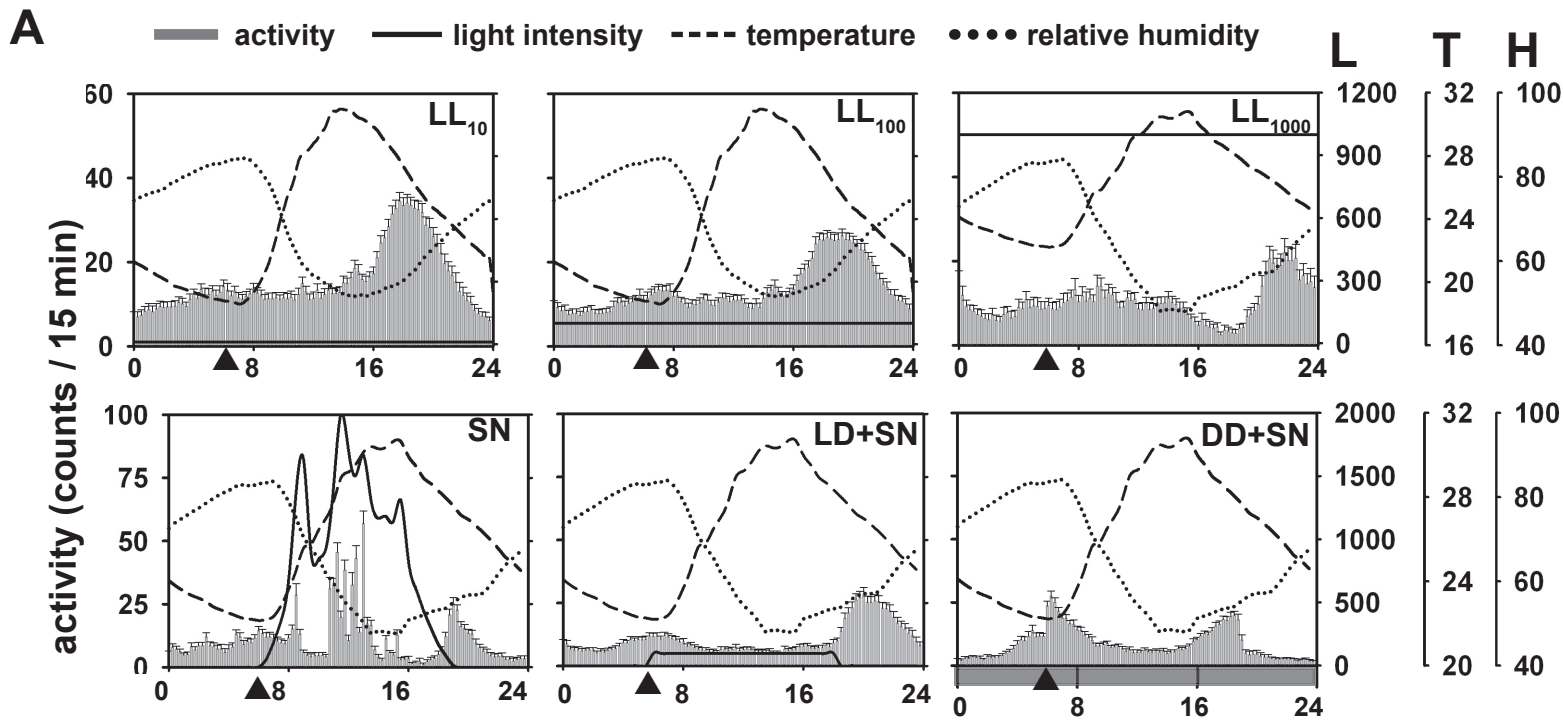### *Among the three activity peaks, E-peak is least dependent on light information*

In all the light-modification protocols, E-peak was the most persistent, which suggests that it is least dependent on light information compared to the M and A-peaks. None of these experimental protocols modified temperature information, and we found E-peak to be present albeit with minor alterations in phase based on the light protocol. Furthermore, cutting down light intensity to values 50%, 75%, and 90% of SN, did not alter the phase of E-peak, which again points toward the possibility of light-independent nature of the E-peak. Interestingly enough, when natural light was blocked in the evening, the M-peak disappeared, which indicates that natural light information during evening is necessary for the M-peak. A previous study has shown that dim light (moonlight) in the night advances M-peak by 1-h (Bachleitner et al., 2007). In a study on humans, additional evening light was reported to reduce circadian phase advances to morning bright light (Burgess HJ, 2012). This study also supports the notion that evening light plays a critical role in timing the M-peak.

In summary, I report that natural light mainly modulates the phase of M and E-peaks, whereas it directly influences the occurrence of A-peak. Phases of M and E-peaks are dependent on light information; although these peaks can occur even in absence of light, presumably timed by environmental temperature and/or humidity. In fact, there is not any evidence of a major role for the known circadian clock mechanisms in determining the activity peaks under SN. I feel that as pertinent as these processes are in understanding the basic properties of circadian behavior, they are relatively less relevant in shaping the behavior of flies under SN.

Figure 1: *Activity profiles of three wild-type strains of Drosophila under SN* (A) Activity profiles of *CS*, *w[1118]* and *yw* strains under SN averaged across days and flies. Error bars indicate standard error of mean (SEM). Three separate axes on the right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrow on the x-axis indicates dawn (> 0 lux). (B) Phases (top panel) and amplitudes (bottom panel) of M, A, and E-peaks are quantified for the three strains. (C) Average total daily activity for three strains. Error bars are 95%CI (95% Confidence Interval) for B and C.

Figure 2: *Activity profiles of per⁰ under SN* (A) Activity profiles of *per⁰* and its background control *w¹¹¹⁸* under SN averaged across days and flies. Error bars indicate standard error of mean (SEM). Three separate axes on the right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrow on the x-axis indicates dawn (> 0 lux). (B) Phases (top panel) and amplitudes (bottom panel) of M, A, and E-peaks are quantified for per0 and w1118. (C) Average total daily activity for three strains. Error bars are 95%CI (95% Confidence Interval) for B and C.

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Figure 3: *Activity profiles of CS flies under modified light protocols in SN.* (A - top panel) Left to right are activity profiles of *CS* flies under different constant light intensities 10 (LL_{10}+SN), 100 (LL_{100}+SN) and 1000-lux (LL_{1000}+SN) in otherwise SN conditions. (A - bottom panel) Left to right are profiles under SN, square LD cycle (LD+SN) and in constant darkness in SN (DD+SN). Error bars indicate standard error of mean (SEM). Three separate axes on the right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrow on the *x*-axis indicates dawn (> 0 lux). (B) Phases and amplitudes of E-peak for all six protocols. § denotes no detectable peak. (C) Activity in the morning (M) (4:00-10:00-h), afternoon (A) (10:00-16:00-h) and evening (E) (16:00-22:00-h) durations and total daily activity in each protocol. Error bars in B and C represent 95%CI.

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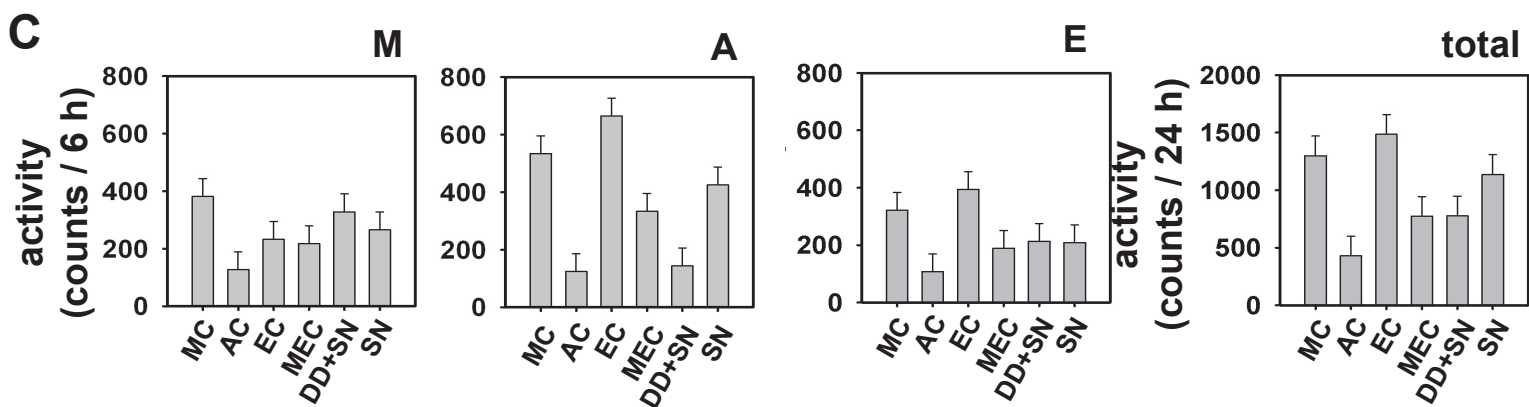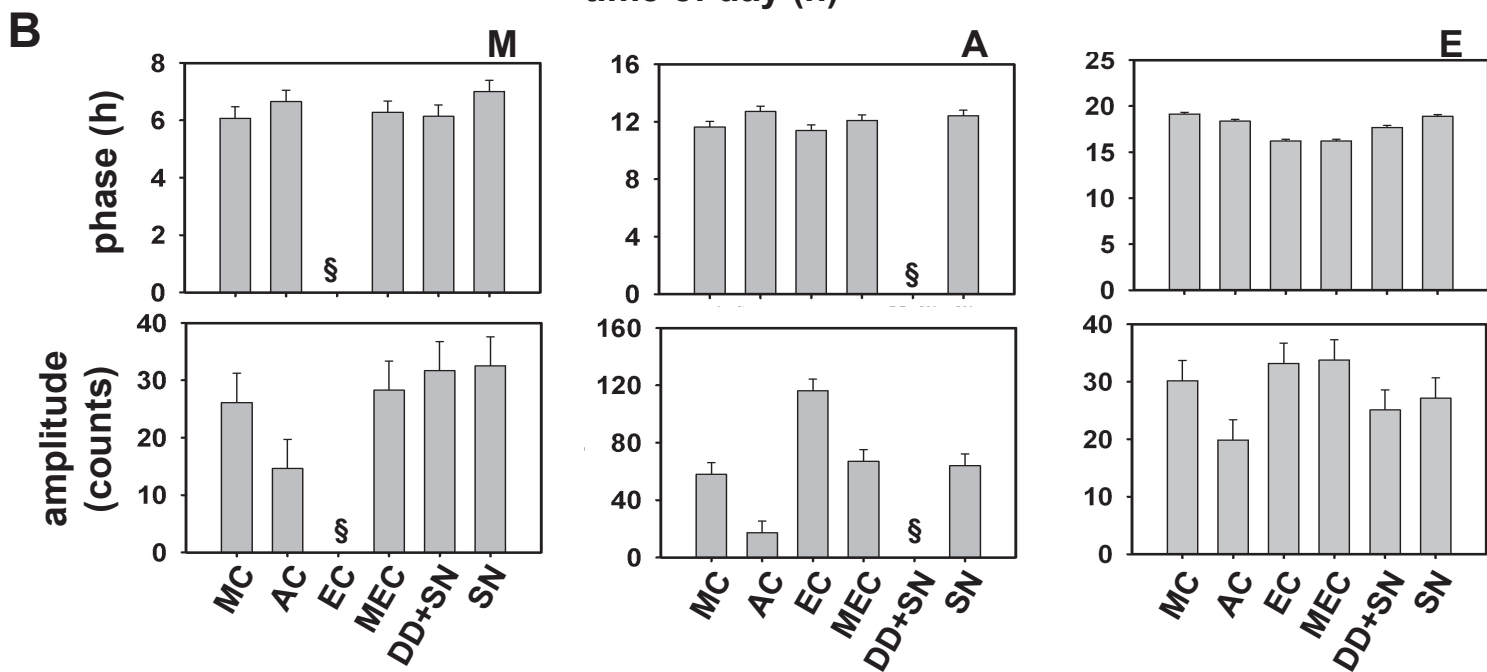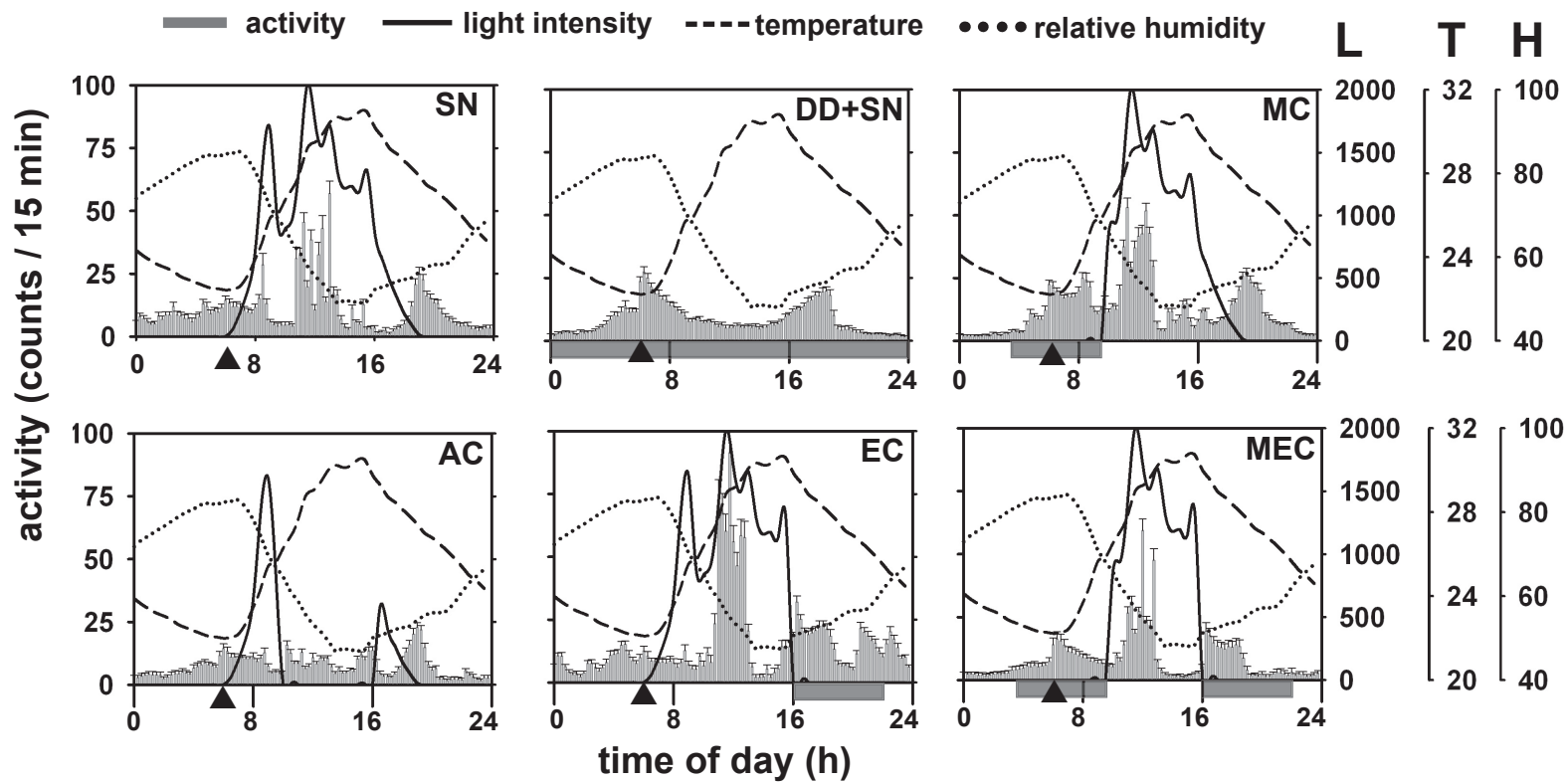
Figure 4: *Average activity profiles of CS under protocols where natural light was blocked for specific durations*. (A) Average activity profiles in different light blocking protocols; constant darkness (DD) in otherwise SN (DD+SN) and SN (bottom panel) for comparison. Error bars indicate standard error of mean (SEM). Three separate axes on the right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrow on the *x*-axis indicates dawn (> 0 lux). (B) Phases and amplitudes of M, A, and E-peaks for above protocols. § denotes no detectable peak. (C) Activity in the M, A and E intervals and total daily activity averaged across days and flies. Error bars in B and C represent 95%CI.
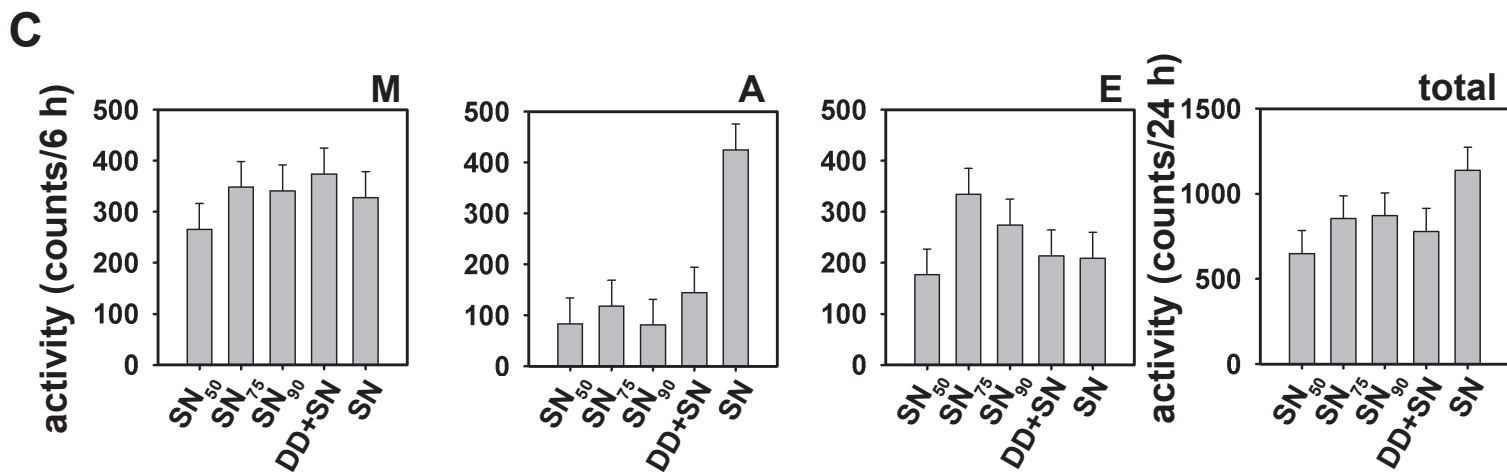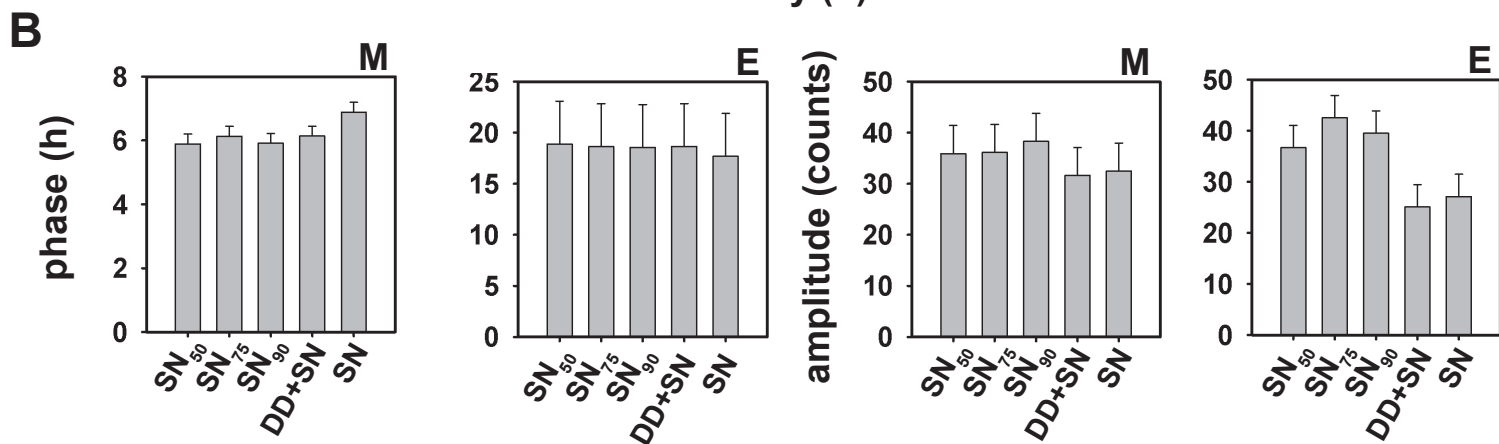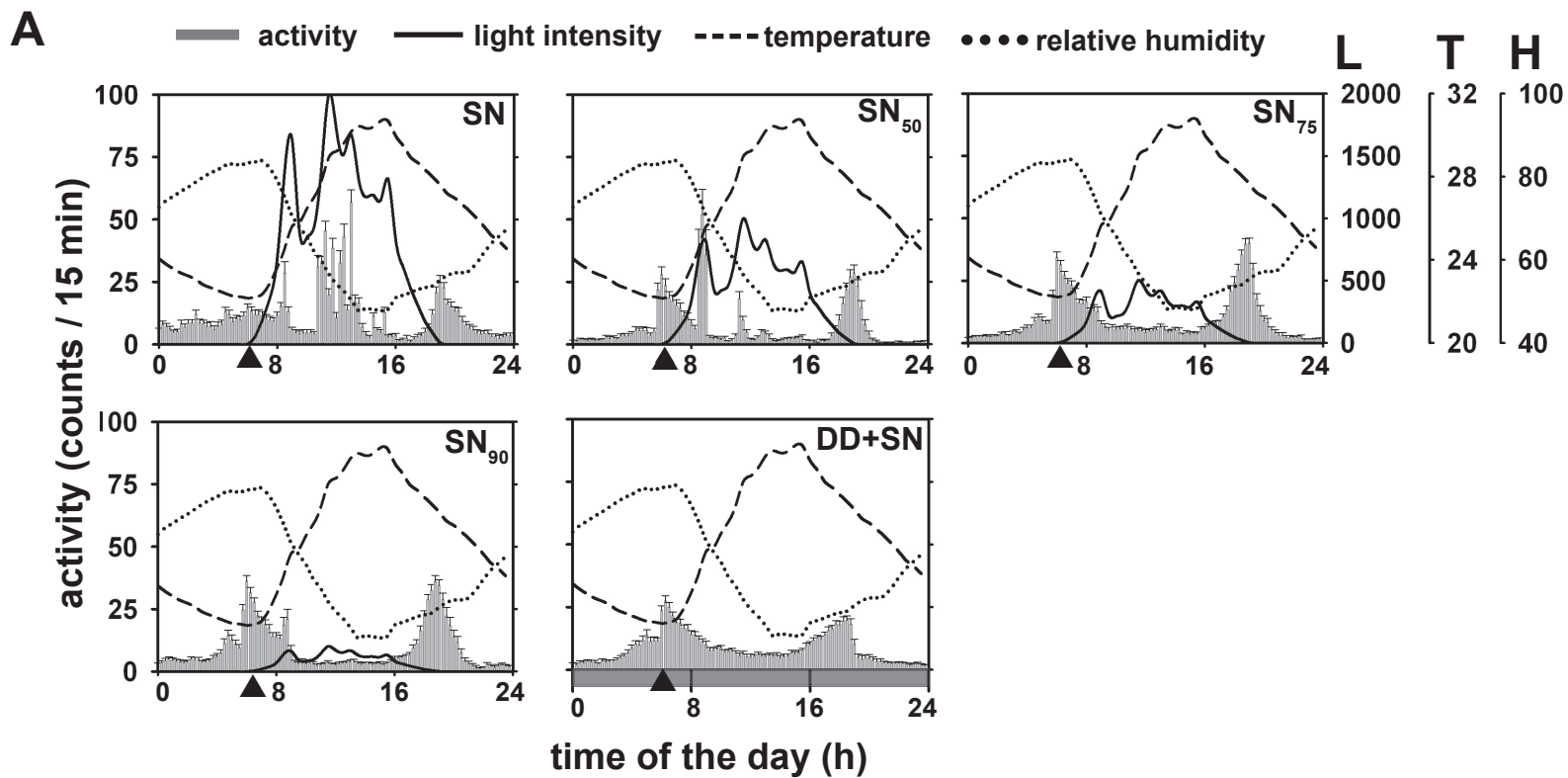
Figure 5: *Average activity profiles under filtered semi-natural (SN) light intensity.* (A) Average activity profiles of *CS* flies when naturally varying light was reduced by 50% (SN_{50}), 75% (SN_{75}) and 90% (SN_{90}). Other than causing reduction in the amplitude of light waveform, these filters did not cause alteration in the qualitative profile of light. DD+SN and SN are also plotted for comparison. Error bars indicate standard error of mean (SEM). Three separate axes on the right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrow on the *x*-axis indicates dawn (> 0 lux). (B) Phases and amplitudes of M and E-peaks. (C) Activity in the M, A and E durations and average total daily activity. Error bars in B and C represent 95%CI.

Table 1: Dawn anticipation indices of various strains under SN followed by CS flies under different light protocols and SN.

Genotype/Protocol	Anticipation Index
CS	0.57 ± 0.02
w^{1118}	0.54 ± 0.01
yw	0.59 ± 0.04
LL_{10} +SN	0.59 ± 0.01
LL_{100} + SN	0.53 ± 0.01
LL_{1000} + SN	0.51 ± 0.02
DD + SN	0.77 ± 0.02
LD + SN	0.62 ± 0.02
MC	0.79 ± 0.02
AC	0.74 ± 0.03
EC	0.66 ± 0.02
MEC	0.66 ± 0.02
SN_{50}	0.81 ± 0.02
SN_{75}	0.77 ± 0.03
SN_{90}	0.78 ± 0.02

Table 1: *Anticipation indices of wild-type flies in different protocols under different light modification protocols in SN.* Dawn anticipation index (AI) for *CS* flies under light-filtered, light-blocked and constant light protocols under otherwise SN and mutant flies under SN and DD+SN was calculated as the ratio of activity counts for 3-h duration prior to dawn (light intensity > 0-lux) over activity counts for 6-h duration prior to dawn. All error values are Standard Error of Mean (SEM).

Chapter 3: Role of canonical clock gene *period* on activity-rest rhythm under SN

Introduction

Over the past 4-5 decades, we have learnt a great deal about the many genetic and cellular

components that make up the underlying machinery of the circadian clock in *Drosophila*

melanogaster. Yet, the role of these circadian clock genes in regulating behavioural rhythms

under natural conditions where there are multiple simultaneously varying strong time-cues are

available remains largely unanswered. Therein the presence of multiple reliable cues to time

rhythmic behaviors, do organisms really need a clock to do that job? Until recently, there was

not any attempt to examine this question. Supriya Bhutani's research (Bhutani, 2009) and those

of Vanin, Bhutani and co-workers (Vanin et al., 2012) showed that several signatures of the

clock's role in regulating rhythmic behaviors were absent in SN, for example, the anticipation of

transitions in zeitgeber cycles. Nevertheless, one of the unique features of the activity-rest

behavior in SN, the afternoon peak (A-peak), seemed to be affected by the canonical clock gene

period. The onset of activity was significantly advanced in short-period mutant (per^s) and

period-null mutant (per^0) compared to other genotypes (Vanin et al 2012). However, the

presence of A-peak in per^0 flies similar to their controls does not make the case for circadian

clock to be involved to a great extent in regulation of this peak under SN. A subsequent study

proposed that *period* helps to modulate activity in the middle of the day, such that per^0 flies

showed A-peak more frequently than their controls (Menegazzi et al., 2012). Thus the authors

propose that the role of circadian clock gene *period* might be in regulating the amount of activity

in the middle of the day as flies with a functional clock are better able to avoid unproductive

activity in the middle of the day (Menegazzi et al., 2012).

In chapter 1, I presented results of activity-rest rhythm of per^0 under SN. There was no major

difference in terms of presence/absence, phase and amplitude of the activity peaks, except that

A-peak amplitude was reduced in per^0 compared to its control w^{1118}. This reflects very little role of canonical clock gene *period* on the activity-rest rhythm under SN. But there is a possibility that per^0 flies do as good as their controls under SN, probably because their behavior is masked by natural light.

In this chapter, I present data for few other period mutants along with per^0 – short period mutant per^s and long period mutant per^L, under both SN and in constant darkness in otherwise semi-natural conditions (DD+SN). Unlike what Vanin and co-workers found, A-peak phase was not altered among these different genotypes. Nevertheless, E-peak was delayed in per^L. Using SN and DD+SN protocols, I show that canonical clock gene *period* has subtle roles to play in the regulation of activity peaks under SN, which may be masked by light.

Materials and Methods

Fly strains used

The activity recordings were done in January-February 2013. Mutants of the circadian gene *period* (per^0, per^S, and per^L) and their controls (w^{1118} and *CS*) were assayed for activity-rest rhythm. Flies were of age 3-4 days at the beginning of the assay.

Assay conditions

Semi-natural condition (SN)

The locomotor activity assays were done in February 2013 within JNCASR, Bangalore campus (12°59'N 77°35'E), inside an enclosure constructed under a leafy canopy (De et al., 2012). The enclosure was an iron cage ($122 \times 122 \times 122$ cm^3) with grids (6×6 cm^2) allowing free flow of air, and covered only on top with a sloping translucent plastic sheet. Fly activity was recorded

using Drosophila Activity Monitor (DAM) system (TriKinetics, USA). The daily profiles of light, temperature, and humidity were also monitored simultaneously using DEnM, Trikinetics, USA. During this run, maximum light intensity reaching the enclosure was around 2500-lux. The minimum and maximum temperatures were around 20^0C and 30^0C, respectively. Humidity varied from around 40% to 80% in a day. On comparison to the weather conditions (June 2012) during activity runs described in chapter 1, this was slightly cooler and brighter.

DD under otherwise SN condition (DD+SN)

In order to create DD conditions of varying intensities, under otherwise SN condition, activity monitors were placed inside light-tight metal boxes ($44 \times 27 \times 20$ cm^3) fitted with light baffles and a small fan such that the temperature and humidity inside the box closely matched that of the outside environment. Temperature and humidity inside and outside the boxes were recorded continuously and were found to be stable throughout the study.

Statistical analyses

The activity profiles in the figures are based on bin size of 15 min. Three intervals of the day were considered to be Morning (4:00 to 10:00-h), Afternoon (10:00 to 16:00 h) and Evening (16:00 to 22:00-h) for determine presence/absence, phase, amplitude of activity peaks and activity performed in a specific interval. To determine the presence of M, A, and E-peaks, average activity profiles (15-min bin) for each genotype or protocol were plotted. An interval (M, A, or E) was considered to have a peak based on qualitative assessment of the activity profile averaged across flies and days of recording. Phases of M, A, and E activity peaks were estimated by scanning 7-day average activity records of each fly, and identifying that time-point corresponding to the highest activity counts observed within that interval. In the afternoon, when

there are multiple peaks, the peak closest to maximum light and temperature in the environment was considered and its phase and amplitude were calculated. Phase and amplitude for each peak were averaged across 32 flies from each genotype and each regime. One-way ANOVA was carried out to see whether there is any statistically significant effect of genotype or regime on the phase and amplitude of activity peaks. Post-hoc multiple comparisons of phase and amplitude data was performed using Tukey's HSD test. The p value of 0.05 was considered as level of statistical significance throughout all the analyses. Total activity was calculated by taking average across 32 flies for each regime and each genotype, while for each fly total activity was calculated by taking average across 7 days. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's test was performed to see whether there is any significant effect of regime/genotype on the total activity, and which of the regimes/genotypes is significantly different from others in pair-wise comparisons. Activity during morning, afternoon and evening hours was calculated as the sum of activity counts in the three durations for each fly and then averaged across 32 flies. The durations were the same as mentioned above for phase and amplitude calculations. Two-way ANOVA followed by post-hoc multiple comparisons using Tukey's test was done on activity data in different durations for different regimes, having regimes and durations as fixed factors. Dawn anticipation index (AI) was calculated as the ratio of activity counts for 3-h duration prior to dawn (the time-point when the light intensity value first rose above 0-lux) over activity counts for 6-h duration prior to dawn (Harrisingh et al., 2007). The error bars on the waveforms in all the figures are SEM, whereas error bars on the bar plots for quantification of phase, amplitude, total activity and activity in different durations are 95% CI (95% Confidence Interval), which could be used for visual hypothesis testing for difference between means.

Results

Period gene improves anticipation to dawn transition and affects timing of E-peak.

Although unlike the summer (May-June 2012; chapter 1) a sharp M-peak was not detected, per^0 flies showed lesser anticipation to dawn compared to w^{1118} (Table 1) under SN. This improvement of anticipation is more under DD+SN (Table 1), which reflects that clock's role could be masked by natural light. Similarly, in per^L, dawn anticipation was poorer than CS in both SN and DD+SN (Table 1). While per^L flies also showed poor anticipation to dawn, flies from other strains anticipated dawn fairly well albeit to variable extents (Table 1). E-peak was phase-delayed and was of lower amplitude in per^L flies relative to CS ($F_{4,87} = 17.19$, $p < 0.001$) (Figs 1A, B, 2A), which suggests that E-peak is clock-modulated. However, the difference in amplitude can also be attributed to reduced overall activity in per^L flies.

Period gene modulates activity in the midday, the effect of which could be masked by natural light

These recordings were carried out in the month of February 2013. In these environmental conditions, a prominent M-peak was not seen in any of the genotypes studied except in w^{1118}, which is consistent with activity patterns observed in winter conditions (Priya Prabhakaran and Sheeba Vasu, unpublished manuscript). Nevertheless, A and E-peaks of activity were seen. The A-peak in all three *per* mutants [per^S ($n=8$), per^0 ($n>23$), and per^L ($n=20$)] as well as in their genetic backgrounds ($n>15$) occurred at a similar phase ($F_{4,87} = 1.32$, $p = 0.2$) and amplitude ($F_{4,81} = 1.71$, $p > 0.05$) under SN (Fig 1A, B), which is suggestive of clock-independent nature of this peak. In some flies, multiple peaks were seen in the afternoon corresponding to the spikes in light intensity profile which further confirms the role of light in determining the occurrence of

this peak, quite independent of the genotype. Under DD+SN, there is an increase in activity throughout the daytime without any detectable peak in per^0, unlike the w^{1118} controls, where there are clear M and E bouts of activity, and a small afternoon-peak, probably in response to high temperature (Fig 3D). Activity in the afternoon under DD+SN is higher in per^0 compared to w^{1118} ($p < 0.001$) (Figs 1D, 2E), which indicates a role of clock in suppressing activity during the warmest time of the day (Menegazzi et al., 2012), but this difference is not manifested in SN, probably due to light, which masks circadian clock's role by suppressing afternoon activity.

In summary, while A-peak appears to be clock-independent, presence of clock helps in the modulation of activity in the afternoon.


Discussion

Canonical clock gene period has subtle roles to play in regulating activity peaks under SN

The A-peak is clock-independent because even the per^0 flies showed A-peak quite similar to their wild-type controls (Figs 1A, B, 2A). Unlike the findings of previous studies (Vanin et al., 2012, Menegazzi et al., 2012), I find that the phase of A-peak in per^S, per^0, and per^L mutants did not differ among themselves or from their wild-type controls, suggesting that A-peak is clock-independent, or at least its occurrence does not require the presence of PER (Fig 1B). In the light of clear divergence in phase (up to 3-h) reported by Vanin et al, this result is surprising. We propose that such differences may be due to the differences in experimental protocols and/or in environmental conditions prevailing in tropical and temperate regions; however, this would require further investigation.

 Taken together, the results of this study clearly suggest that A-peak under SN is not likely to be a natural behavior of flies and it is not circadian clock-controlled. Nevertheless, weaker dawn

anticipation in per^0 and per^L and delayed E-peak in per^L indicate a role of clock in timing the M and E-peaks. Since the results described in our study are based on activity/rest behavior conducted under cyclic conditions of SN, it is likely that the three peaks of activity are directly driven by environmental factors, such as light. Therefore, we cannot rule out the subtle effects of circadian clock mechanism in the regulation of these peaks.
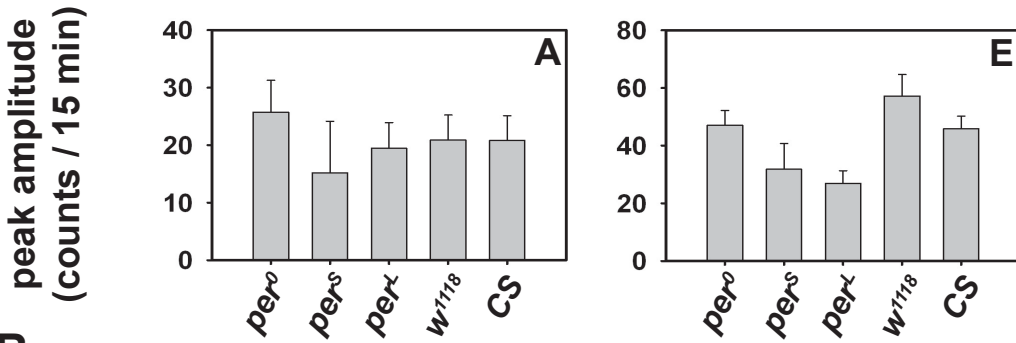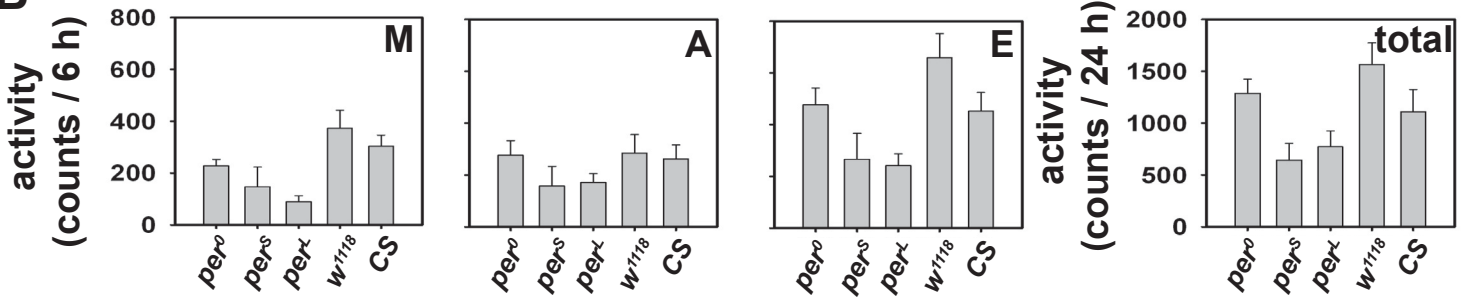
A *per* mutants under SN

C *per* mutants under DD+SN

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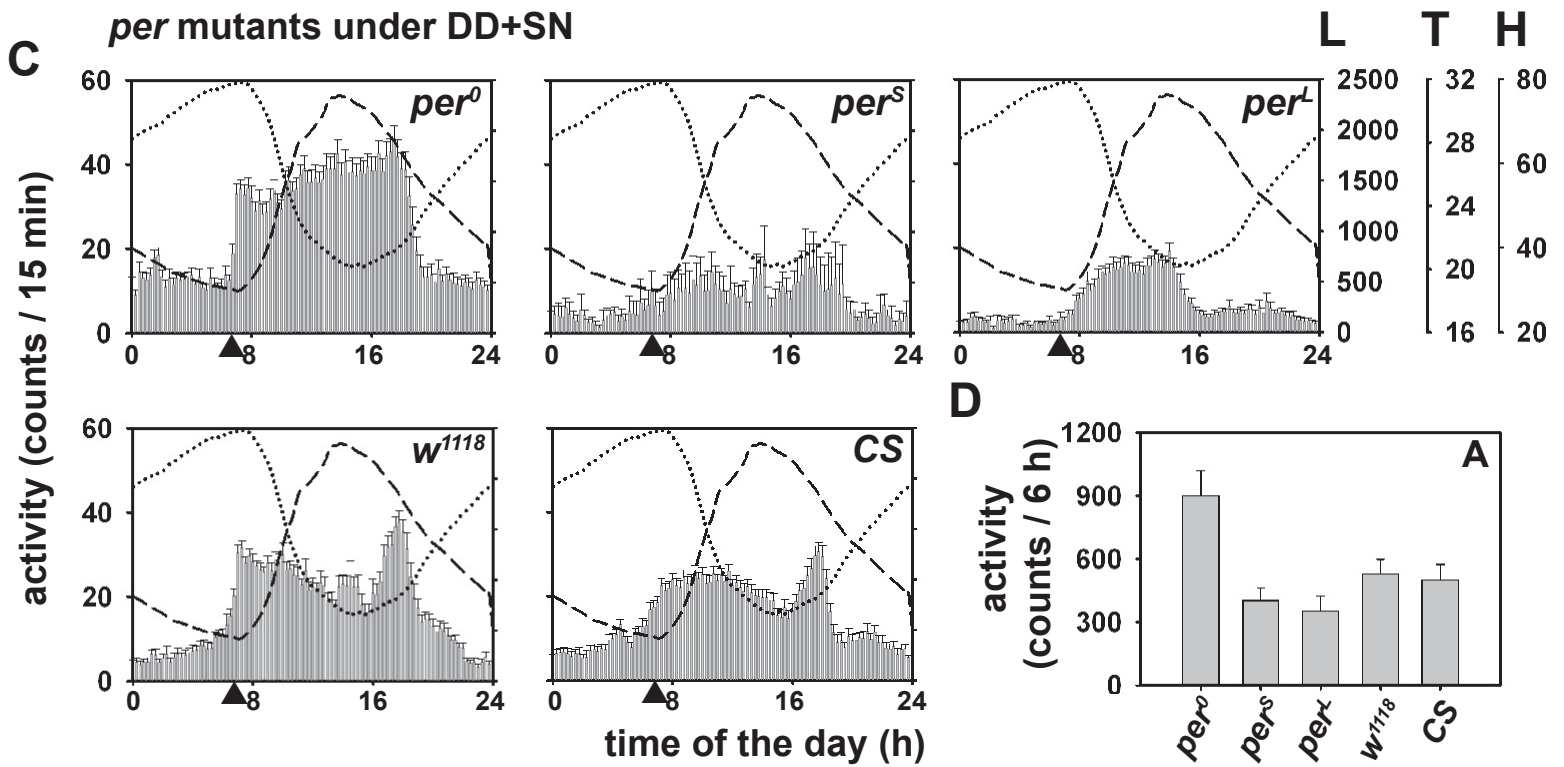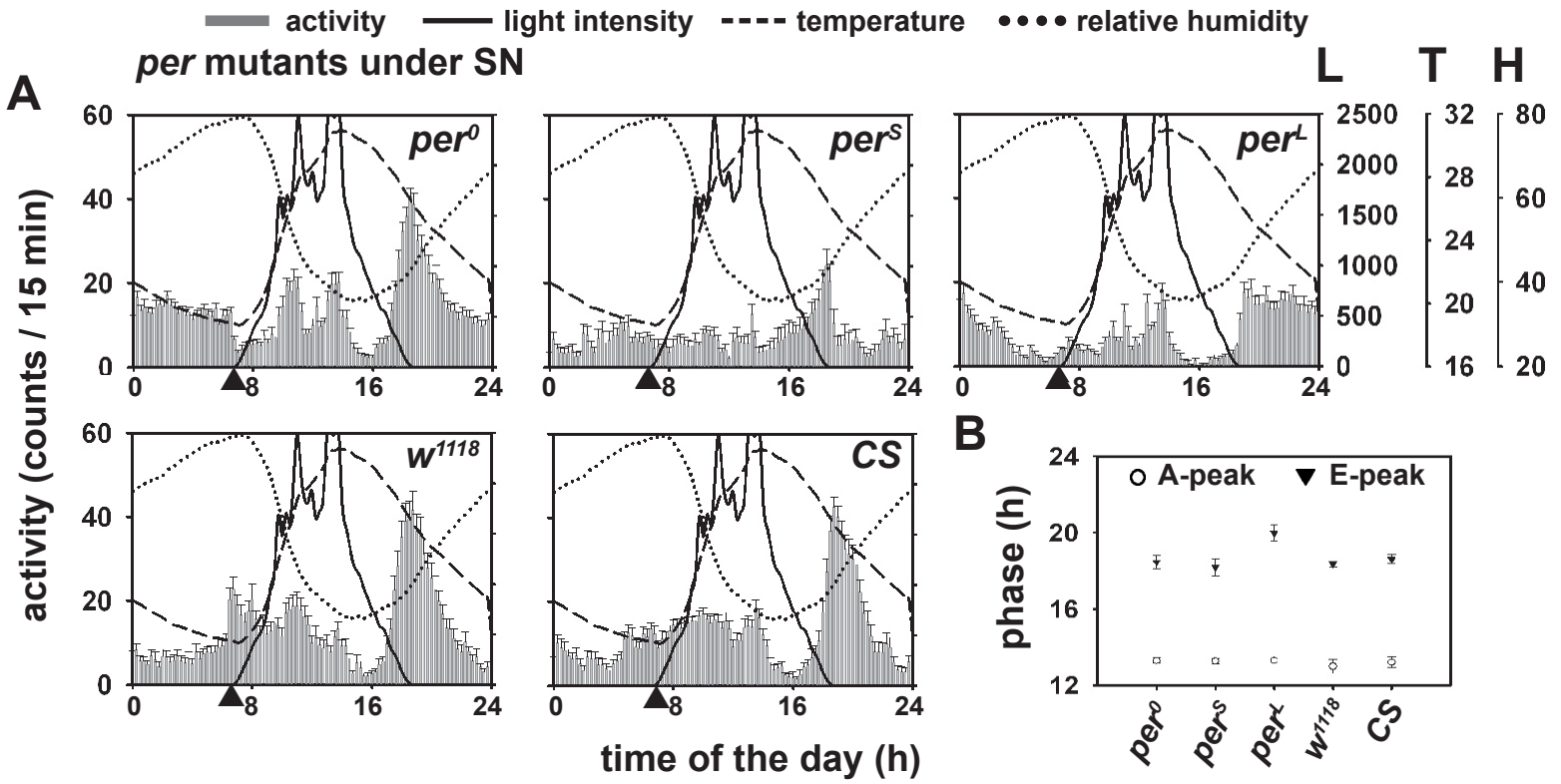
Figure 1: *per mutants under DD+SN* (A) Average activity profiles of *period* mutants (*per^0*, *per^S*, and *per^L*) and their genetic background controls (*w^{1118}* for *per^0*, and *CS* for *per^S* and *per^L*) under SN assayed in February 2013. Error bars are standard error of mean (SEM). Three separate axes on extreme right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). The black arrowhead on the *x*-axis indicates dawn (> 0-lux). (B) Phase of A- and E-peaks in these genotypes under SN. A-peak phase was not different across genotypes but E-peak was delayed in *per^L* compared to its control. (C) Average activity profiles of *period* mutants (*per^0*, *per^S* and *per^L*) and controls under DD+SN. (D) Afternoon (A) activity level is greater in *per^0* compared to *w^{1118}*, although *per^S* and *per^L* do not differ from *CS*. Error bars in B and D represent 95%CI.
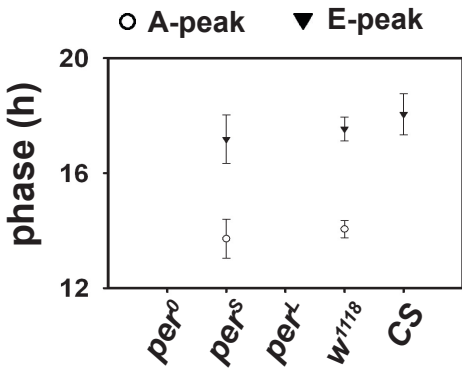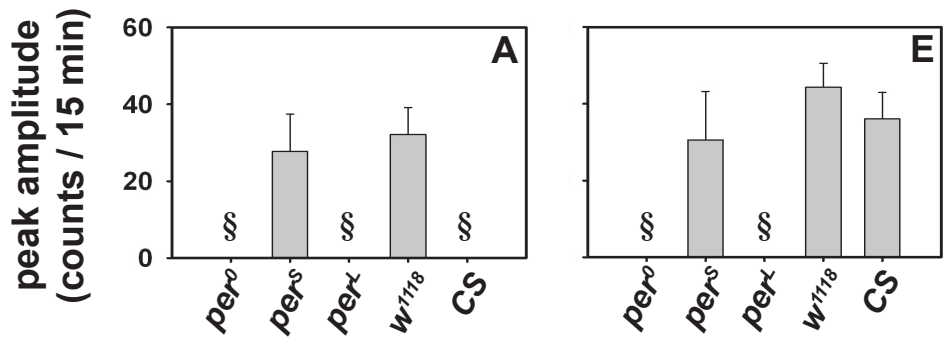
per mutants under SN

per mutants under DD+SN
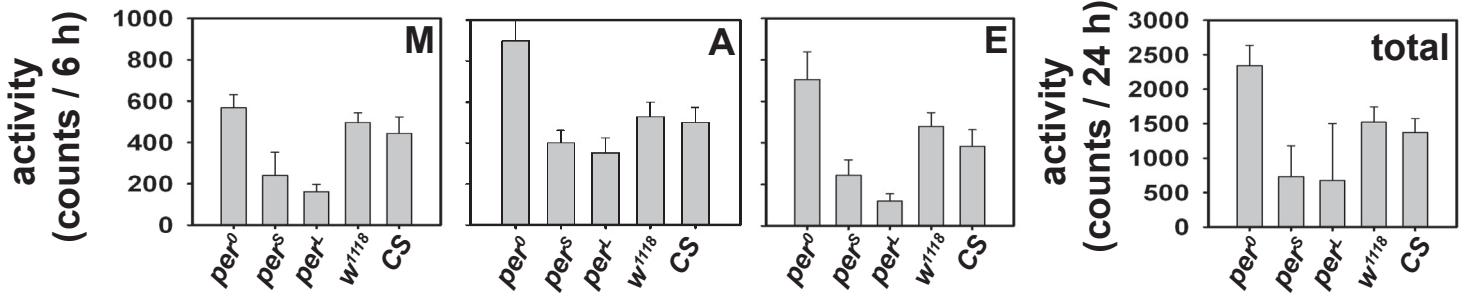
Figure 2: *Phase and amplitude of M, A and E-peaks, activity levels in M, A and E-intervals and total daily activity of per mutants and controls under SN and DD+SN conditions.* (A) Average activity recorded at the A-peak of *per* mutant flies with null (*per^0*), short period (*per^S*), and long period (*per^L*) alleles and their wild-type controls, w^{1118} and *CS* under SN conditions showed no significant effect of genotype whereas E-peak was affected. (B) Average activity in the M, A and E-intervals showed significant main effects of genotype ($F_{4,258} = 39.35$, $p < 0.001$), interval ($F_{2,258} = 52.23$, $p < 0.001$) and genotype × interval interaction ($F_{8,258} = 4.37$, $p < 0.001$). (Right) Total daily activity showed a significant effect of genotype ($F_{4,85} = 15.56$, $p < 0.001$). (C) Average phase of the A ($F_{1,23} = 1.14$, $p > 0.05$) and E-peaks ($F_{2,38} = 1.63$, $p > 0.05$) of *per* mutant flies and their controls did not show significant effect of genotype under DD+SN. (D) Average activity recorded at the A ($F_{1,23} = 1.18$, $p < 0.05$) and E-peaks ($F_{2,38} = 3.17$, $p < 0.05$) of locomotor activity of *per* mutant flies were not different from their controls under DD+SN. (E) Average activity in the M, A and E-intervals showed main effects of genotype ($F_{4,243} = 75.19$, $p < 0.001$), interval ($F_{2,243} = 15.61$, $p < 0.001$) and genotype × interval interaction ($F_{8,243} = 3.01$, $p < 0.005$). (Right) Total daily activity also showed significant effect of genotype ($F_{4,94} = 26.57$, $p < 0.001$). All error bars represent 95% Confidence Intervals.

Table 1. Dawn anticipation indices of various strains under different protocols.

		Anticipation Index (AI)	
		February 2013	
Strain/protocol	June 2012	SN	DD+SN
CS	0.57 ± 0.06	0.55 ± 0.08	0.60 ± 0.05
w^{1118}	0.59 ± 0.12	0.54 ± 0.06	0.60 ± 0.06
per^0	0.56 ± 0.05	0.47 ± 0.03	0.48 ± 0.03
per^S	–	0.66 ± 0.14	0.52 ± 0.21
per^L	–	0.21 ± 0.04	0.48 ± 0.06
SN_{50}	0.81 ± 0.07	–	–
SN_{75}	0.77 ± 0.07	–	–
SN_{90}	0.78 ± 0.07	–	–
MC	0.79 ± 0.08	–	–
AC	0.74 ± 0.12	–	–
EC	0.66 ± 0.09	–	–
MEC	0.66 ± 0.09	–	–
DD+SN	0.77 ± 0.07	–	–
$LL_{10}+SN$	0.59 ± 0.05	–	–
$LL_{100}+SN$	0.53 ± 0.04	–	–
$LL_{1000}+SN$	0.51 ± 0.07	–	–

Table 1. *Anticipation indices of wild-type flies in different protocols and mutants under SN and DD+SN.* Dawn anticipation index (AI) for *CS* flies under light-filtered, light-blocked and constant light (LL) protocols under otherwise SN and mutant flies under SN and DD+SN was calculated as the ratio of activity counts for 3-h duration prior to dawn (light intensity > 0-lux) over activity counts for 6-h duration prior to dawn. One set of experiments with the light-filtered, light-blocked and constant light protocols in otherwise SN were conducted in June 2012. *per* mutants and their controls were assayed in February 2013 under SN and DD+SN to determine the role of circadian clocks in timing of peaks and anticipation to dawn. All error values are 95% Confidence Intervals.

Chapter 4: Behavioral observations provide insights on plausible functional significance of activity peaks under SN

Introduction

Drosophila exhibited several unique features of activity-rest rhythm under SN which were not characteristics of any standard laboratory protocol studied so far. The activity peak in the afternoon (A-peak: Bhutani, 2009; Vanin et al., 2012; Menegazzi et al., 2012, Menegazzi et al., 2013, chapters 1 and 2 of this thesis), reduced anticipation to light-dark transition (Vanin et al., 2012, chapter 1 of this thesis), very limited role of canonical clock gene '*period*' in regulation of the rhythm (chapter 2 of this thesis) and absence of crepuscular activity pattern (Bhutani, 2009; Vanin et al., 2012; Menegazzi et al., 2012, Menegazzi et al., 2013, chapters 1 and 2 of this thesis) were the most important features observed in the SN, quite unlike what has been observed under lab-based protocols, in many extensive investigations. Under SN, three peaks of activity – morning, afternoon and evening (M, A and E), have been detected by the conventional Drosophila Activity Monitor (DAM) systems, where fly movement is detected through the breaking of an infra –red (IR) beam in the middle of the tube in the monitor. Using this method, the experimenter is able to quantify activity only in terms of how many times flies have crossed the region where the IR beam is located, but this conventional method is unable to give any information about the qualitative behavior of flies. If a fly is active during a particular section of the day, what is for the significance of this activity? Are they active to enable them to finding mates or for courtship and mating or foraging? The DAM system is highly unlikely to provide any insight into this question. Nevertheless, it would be of considerable interest if we can reliably assign some functional significance to this extremely robust rhythmic locomotor behaviour which fly circadian biologists have been using as a readout of the functioning of the core circadian clock.

In this chapter, I present results of experiments designed to gain information on the functional significance of the activity peaks seen under SN, mostly through behavioral observations of fly behavior, in addition to conventional DAM recordings. In addition to finding behavioral correlates of the activity peaks, behavioral observations were also made in order to better understand the nature of the increased activity in the afternoon, as observed across several studies carried out under SN (Bhutani, 2009; Vanin et al., 2012; Menegazzi et al., 2012, Menegazzi et al., 2013, chapters 1 and 2 of this thesis). Vanin and co-workers postulate that A activity is clock-modulated based on the phase advanced onset of A activity in per^s and per^0 flies compared to their wild type controls. A later study (Menegazzi et al., 2012) found that *period* gene modulates A-activity, in such a way that a null mutation of that gene results in very high 'unproductive' midday activity. In chapter-1 of this thesis, I describe results of experiments which showed that A-peak is a response to bright afternoon light and not quite likely to be natural behavior of fly, therefore even highly unlikely to be regulated by circadian clocks. In chapter 2, further studies which showed that *period* gene does not regulate timing of A-peak were described. Nevertheless, *period* gene may have some subtle role in modulating activity in the afternoon which could be masked by natural light. Thus, taken together, all these studies suggest very small role, if any, of circadian clocks on A-peak of activity and suggest that it is more likely to be a response to harsh environmental conditions in the midday. Intuitively, it does not make much sense for a fly to be highly active in the warm and dry afternoons due to high risk of desiccation. Studies described in this chapter based on visual observations of fly behaviour suggest that the M-peak is associated with courtship-related activities. On the other hand, we find that the afternoon peak in activity revealed does not appear to be a natural behavior of flies; rather it is an artifact of experimental protocol. In the afternoon, higher proportions of flies seek

shade in the shaded region of the monitor, where eventually IR beam detects activity, even though the fly is not moving much. When provided with a relatively larger arena than activity tubes, such as petridishes, flies do not show high afternoon activity, either under solitary or grouped conditions. I conclude that M-peak of activity accounts for courtship-related activity, A-peak of activity is an artifact of experimental protocol and E-peak, I speculate to be related to food-searching behavior.

Materials and Methods

Observing behaviors under semi-natural (SN) conditions

Visual observation of DAM2 monitors: Single male flies placed in glass activity tubes (*n*=32) in DAM2 monitors kept under SN were manually observed every 1 hr from 7:00 to 19:00-h for 11 consecutive days during January 2013 while automated recording occurred in parallel (Fig. 2B). Three consecutive visual scans were made at every time point and the location of flies (near food, middle, near cotton) and whether they were moving was noted. The proportion of flies in each zone at each time point was used as the basic unit of data.

Visual observation of flies in activity tubes: Single male flies in glass activity tubes were placed flat on a tray in the same SN enclosure. Tubes were either left completely unshaded, shaded near food, in the middle or near cotton (*n*=4 tubes each) using a black tape (~18 mm long). Tubes were manually observed every 2 hr throughout the day for five consecutive days during July 2012. Three consecutive visual scans were made at every time point and the location of flies (near food, middle, near cotton) was noted. The proportion of flies in each zone at each time point was used as the basic unit of data.

Visual observation of flies in petridishes: Flies were housed in petridishes with a thin layer of standard corn-meal fly-food at the bottom. Flies were either assayed in groups of 3 males and 3 females per dish, or as solitary males, with 6 replicates of each type. At every 2-h interval, the number of instances of behaviors (locomotion, wing-expansion, chasing and copulation) was recorded by visually scanning each dish. Three consecutive visual scans were made at every time point and the behaviour was noted. The proportion of flies performing a particular behavior at each time point, was used as the basic unit of data. Prior to these assays, I and all other experimenters involved in conducting behavioral observations made a series of observations in parallel and ensured that experimenter-bias was kept to a minimum by using similar criteria for all behaviors to be scored. These observations were done in February 2013, parallel to the recordings described in chapter 2.

Statistical analyses

For manual observation data from tubes in DAM2 monitor, one-way ANOVA was done on proportion of flies found in the middle zone of activity tubes to test for time-dependent preference for middle zone of the tube. Similar analysis was done on proportion of flies exhibiting locomotion. For data from visual observation experiment where tubes were not placed in monitors one-way ANOVA was done on proportion of flies found in the shaded region of activity tubes to test for time-dependent preference for shaded part of the tube. For chronoethogram data from grouped flies, separate one-way ANOVA were carried out on courtship related movement and on general locomotion to test for time of day effects. For solitary flies, similar test was done for general locomotion.

Results

Courtship related activities peak in the morning

I made manual visual observations for several easily scorable behaviors in flies experiencing SN, such as movement and courtship to assess the significance of what is recorded as general activity in the activity tubes of DAM monitors. These observations were made simultaneously with the activity recordings using DAM monitors. In a series of experiments both in solitary and grouped flies, two peaks of locomotion were observed (grouped: Fig 1, upper panel, solitary: Fig 2). Courtship-related behaviors such as wing-expansion, chasing and copulation exhibited by the male flies, peak in the morning under grouped conditions (Fig 1, bottom panel) which probably corresponds to enhanced activity in the morning seen in solitary flies (Fig 2), while a second activity peak was also seen in the evening under both grouped (in petridishes) and solitarily housed conditions (Figs 1 and 2), the significance of which remains unclear.

Visual observation of flies does not detect increased activity in the afternoon

Flies were placed in petridishes in solitary and grouped conditions and locomotion and resting behaviors were noted down. These observations did not detect increased activity in the afternoon in either solitary or grouped flies (Figs 1 and 2), whereas activity recording in parallel using DAM2 monitors detected A-peak of activity (Fig 1, chapter 2). In petri-dishes, both single and grouped flies showed only two prominent peaks of activity in the morning (few hours around sunrise) and evening (few hours around sunset) hours (Figs 1 and 2). In solitary flies, the effect of time-point on activity was statistically significant ($p < 0.01$; number of flies performing locomotor activity at 6-h was significantly greater than that at 14-h, although the evening peak was not statistically distinguishable, Fig 2). The grouped flies also showed two prominent peaks of general activity although unlike solitary flies the effect of time point was not statistically significant ($p > 0.05$). Under no circumstance, did flies kept solitarily or in groups in petri-plates

show enhanced activity during afternoon (Figs 1, 2). This might indicate that there might be a space constraint in the locomotor activity tubes of 5-mm diameter, due to which flies probably exhibit movement attempting to escape the tubes in response to harsh afternoon conditions giving rise to high A-activity, which does not occur when flies are housed in a bigger arena such as in petridishes.

To further investigate what the flies do in the activity tubes of DAM2 monitors especially in the middle of the day, we made behavioral observations of flies housed in activity tubes in the DAM2 monitor, during the day, from 7 in the morning till 6 in the evening. Activity of these flies was also being recorded in parallel through conventional DAM2 system. Observations revealed higher preference for the middle zone of tubes in the afternoon (Fig 3, top panel, left). Locomotion as determined by visual observations showed only two peaks – M and E, with a trough in the afternoon (Fig 3, top panel, right). Nevertheless, simultaneous recording in DAM2 showed a distinct A-peak (Fig 3, top panel). I propose that the A-peak is predominantly a result of the flies occupying the middle zone of the tubes where the IR emitter-receiver combination is located, even though flies do not exhibit locomotion. Flies tend to occupy the shaded area in the DAM2 monitors in the middle of the day. Therefore, A-activity is probably an artifact of flies seeking shade.

Afternoon activity is an artifact of shade-seeking behavior

To further clarify whether flies seek shade during midday, two sets of experiments were performed. In the first, we used a flatter version of the recording apparatus – DAM5 (Trikinetics, Waltham MA) and provided additional shade in the mid-region where the IR beam is located (see schematic Fig 4A, left). Under SN, we find that flies with shade in the middle

zone show significantly higher afternoon activity compared to the unshaded controls (Fig 4A, right).

Secondly, we made observations on solitary flies placed in glass activity tubes normally used in DAM5 monitors, but three different zones of the tube were shaded - near the food (zone-1), middle (zone-2), or near the cotton plug (zone-3), while controls tubes were completely unshaded (Fig 4B). These tubes were not placed in the monitor, but laid flat on a tray in the SN enclosure. Flies did not show any time dependent preference towards any particular zone in the unshaded tubes (preference for zone-1: $F_{11,24} = 0.83$, $p = 0.60$; zone-2: $F_{11,24} = 1.15$, $p = 0.30$; zone-3: $F_{11,24} = 1.56$, $p = 0.17$; Fig 4B, top panel). There was no time specific preference for any of the three zones for 'shade near food' protocol, or 'shade near cotton' protocol, as flies always stayed close to food in the 'shade near food' protocol, and flies in 'shade near cotton' protocol did not show any preference to any particular zone much like flies in the unshaded tubes (Fig 4, top and bottom panels). However, under 'shade in the middle' protocol, flies preferred shaded middle zone (zone 2) particularly in the afternoon (preference for zone-1: $F_{11,24} = 2.76$, $p = 0.01$; zone-2: $F_{11,36} = 3.83$, $p = 0.002$; zone-3: $F_{11,36} = 1.64$, $p = 0.14$), which resulted in a significant effect of time-point for preferring middle zone of the tube ($p < 0.002$; average number of flies in shade in the middle protocol at 14-h was significantly higher than at 6-h, and the same was higher at 12-h and 14-h compared to 18-h; Fig 4, top panel, right). This indicates that flies prefer shaded portion of the tube in the afternoon when shade is in the middle of the tube, or prefer shade, independent of time of the day if it is close to food. They in general prefer shade unless the shade is far away from the food. Thus, shaded portion of tube in combination with availability of food determines the position of a fly in an activity tube.

This finding that flies prefer the shaded middle zone of the tube in the afternoon is consistent with the fact that flies in the DAM2 monitor show high amount of activity as they seek shade in the middle of the tube which sits inside the grove where IR beam passes. This confirms our speculation in the previous section that A-activity is an artifact of shade seeking behavior.

Afternoon activity is an artifact of experimental paradigm

Based on the above experiments we concluded that flies, in general, prefer shade in the afternoon unless the shade is far away from food and that the extent of shade-seeking depends on the location of the shade. If this were correct, one could expect to detect different levels of activity counts when recorded from different portions of the tube. This would suggest that not only the A-activity is a result of shade-seeking behavior, but also highly sensitive to experimental protocol, and therefore, highly unlikely to be an endogenous or clock-modulated behavior of flies. I carried out activity recordings from different parts of the tube (see schematic in Fig 5, top panel). A-peak is seen in all these three protocols (Fig 5, bottom panel). I found that A-activity is higher when recorded close to food compared to recording from middle followed by recording close to the cotton plug ($F_{2,61} = 21.43$, $p < 0.001$; Fig 5, bottom panel) which is in agreement with the results of the experiment where tubes were shaded at different locations. I also observed that the activity levels in the afternoon depends on the location of the fly in the monitor, such as the A-activity is higher in the flies placed in the uppermost row in the monitor than the other three rows ($F_{3,28} = 4.45$, $p < 0.01$) (data not shown). This could probably be explained by the possibility that greater illumination results in greater shade-seeking, an idea that we did not test further.

Discussion

M-peak of activity is probably associated with courtship-related behavior

Here I describe experiments in which we used a novel approach of obtaining 'chronoethograms'
in which we temporally monitor behaviours such as locomotor activity and rest, courtship-related
activities such as chasing, wing-expansion, and copulation, which enabled us to assign
behavioral correlates to the three activity peaks. Chronoethogram studies under SN on solitary
flies in petridishes revealed two distinct peaks in locomotion, which corresponded with dawn and
dusk (Fig 2). Activity peaks thus obtained were similar to those detected by automated activity
recording (Fig 1, chapter 1). Previous studies in the LAB have shown that mating frequency in
flies is highest around lights-on (ZT3-4; 12) (Sakai and Ishida, 2001). Courtship-related
activities decline around dusk and remain high during rest of the day (13). Mating (Sakai and
Ishida, 2001) and courtship rhythms (Fujii et al., 2007, Hamasaka et al., 2010) in *Drosophila*
have been shown to be under clock-control. Based on the chronoethograms of flies we report
rhythm in courtship-related behaviors under SN. These behaviors mostly consist of chasing,
wing-expansion, and copulation, which peak around dawn (Fig 4A), closely resembling previous
studies in the LAB (Fujii et al., 2007, Hamasaka et al., 2010). Therefore, I propose that M-peak
is due to locomotor activity associated with courtship behavior. During mid-day when
automated recordings of flies in tubes showed enhanced activity, majority of flies maintained
solitarily or in groups in petridishes were immobile. The E-peak corresponded to general
locomotion by flies to which no specific behavior could be assigned, hence the significance of
the E-peak remains unknown. While the inferences on the functional significance of the activity
are based on flies living in groups, I propose that activity related to key behaviors represent
innate tendencies that are expressed even in flies living solitarily.

A-peak of activity is basically an artifact, and not a natural behavior of flies

From chapter 1, we saw that A-peak was prominent only when sufficiently high intensity of natural light was available during the warm afternoon and consisted of several sub-peaks that coincided with light intensity spikes during mid-day, indicating a direct and instantaneous response to fluctuations in light intensity (Fig 1, chapter 1). We speculated that high afternoon activity is a result of harsh environmental conditions, inducing flies to seek shade in the IR-zone of DAM2 monitors, yielding abnormally high activity counts. Providing additional shade in an alternate version of the DAM monitor (DAM5) resulted in even higher activity counts in the afternoon (Fig 2A). Similar preference for the less-illuminated portion of the activity tube was apparent when we made observations on flies in tubes with shade provided in different regions, and upon automated recording of activity from different zones of the tubes (Figs 2C, S2A). Visual observations of flies whose activity was being recorded in the DAM2 monitors revealed that majority of them preferred the relatively shaded middle zone of the activity tube where the IR beam is located (Fig 2B). The fact that the A-peak is an artifact of activity recording protocol was further confirmed when solitary and grouped flies kept in petridishes, did not show A-peak (Figs 2D, 4).

In summary, I conclude that the M-peak of activity might be due to courtship-related activities and A-peak is an artifact of experimental protocol whereas, the behavioral significance of E-peak remains unknown.
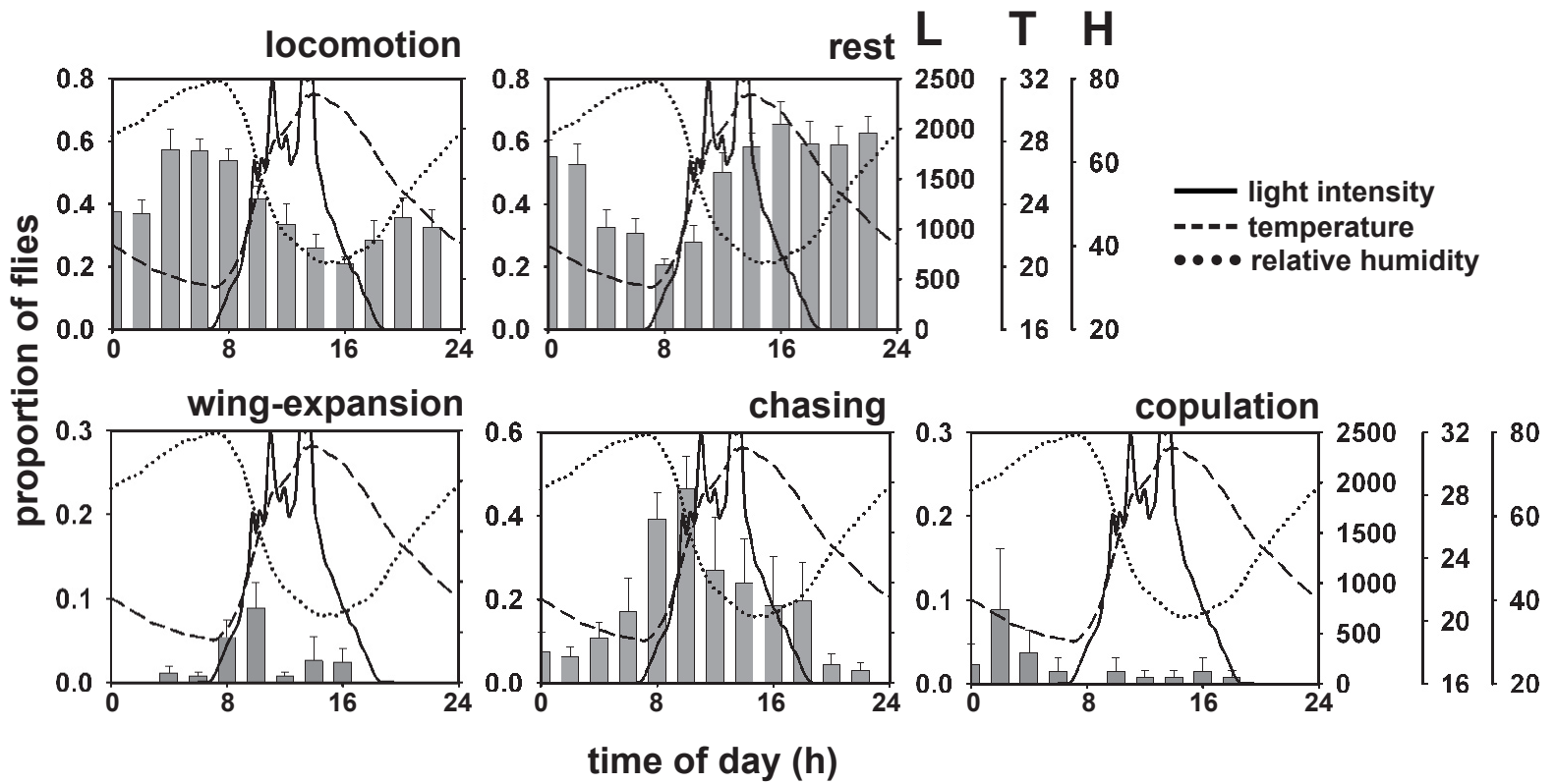
Figure 1: Chronoethograms of flies under SN. (Top) Profile of proportion of flies performing locomotion (left) or resting (right) in petridishes under group condition of 3 males and 3 females. (Bottom) Profile of proportion of flies performing courtship-related activities such as wing-expansion, chasing, and copulation. Three separate axes on extreme right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). Courtship-related behaviors peak during the morning hours. Significant main effect of time was seen for all behaviors except copulation.
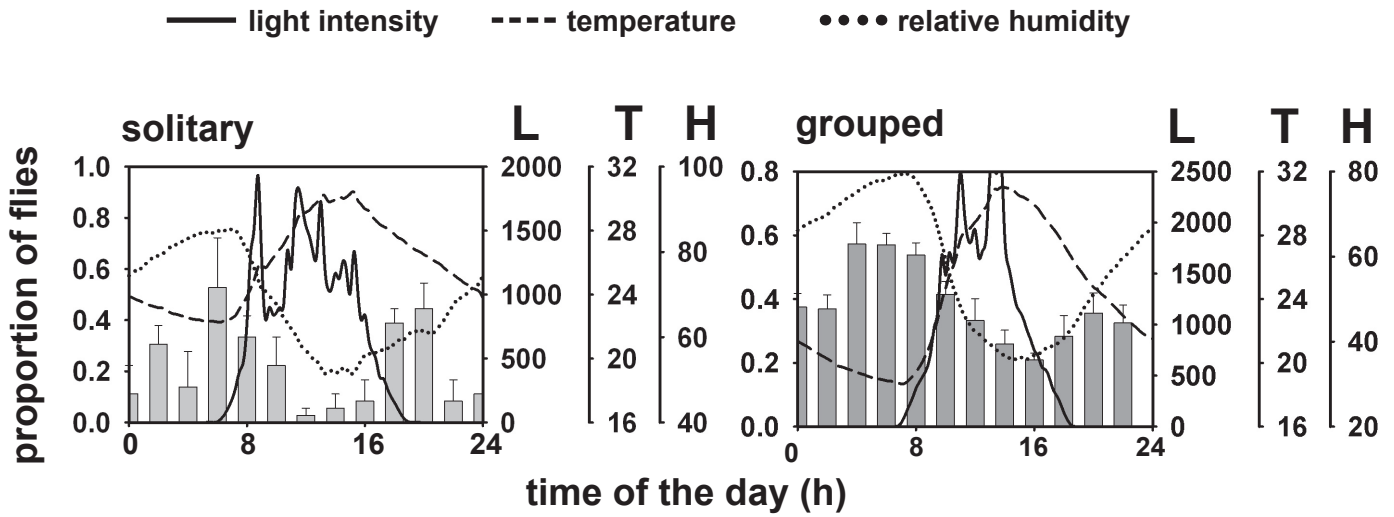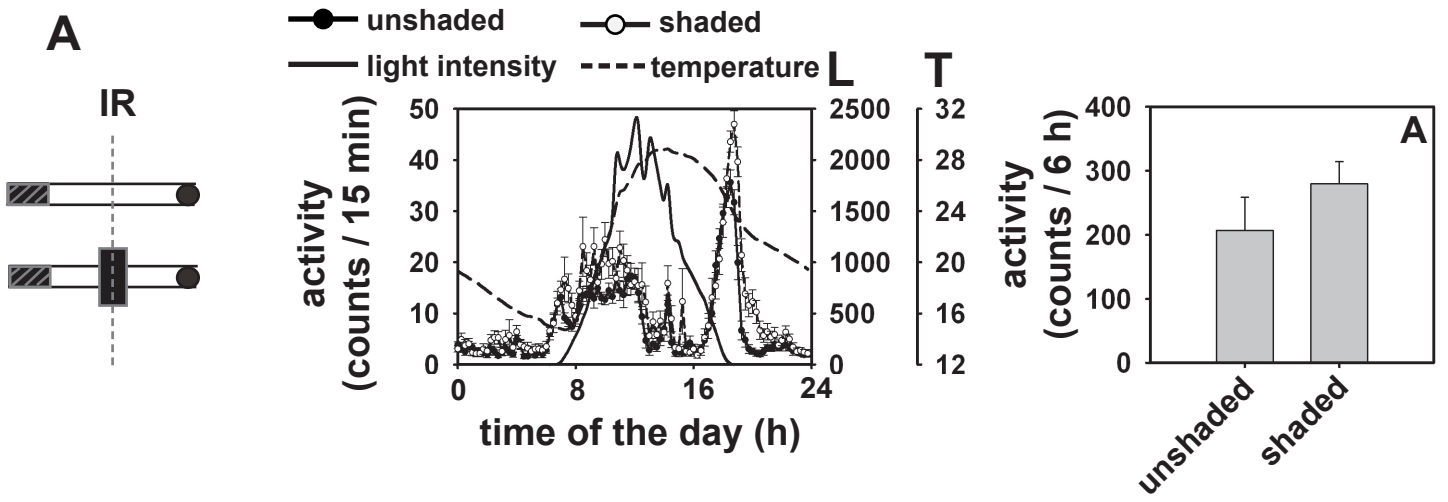
Figure 2: *Profile of proportion of solitary male flies performing locomotion in petridishes.* Three separate axes on extreme right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %).
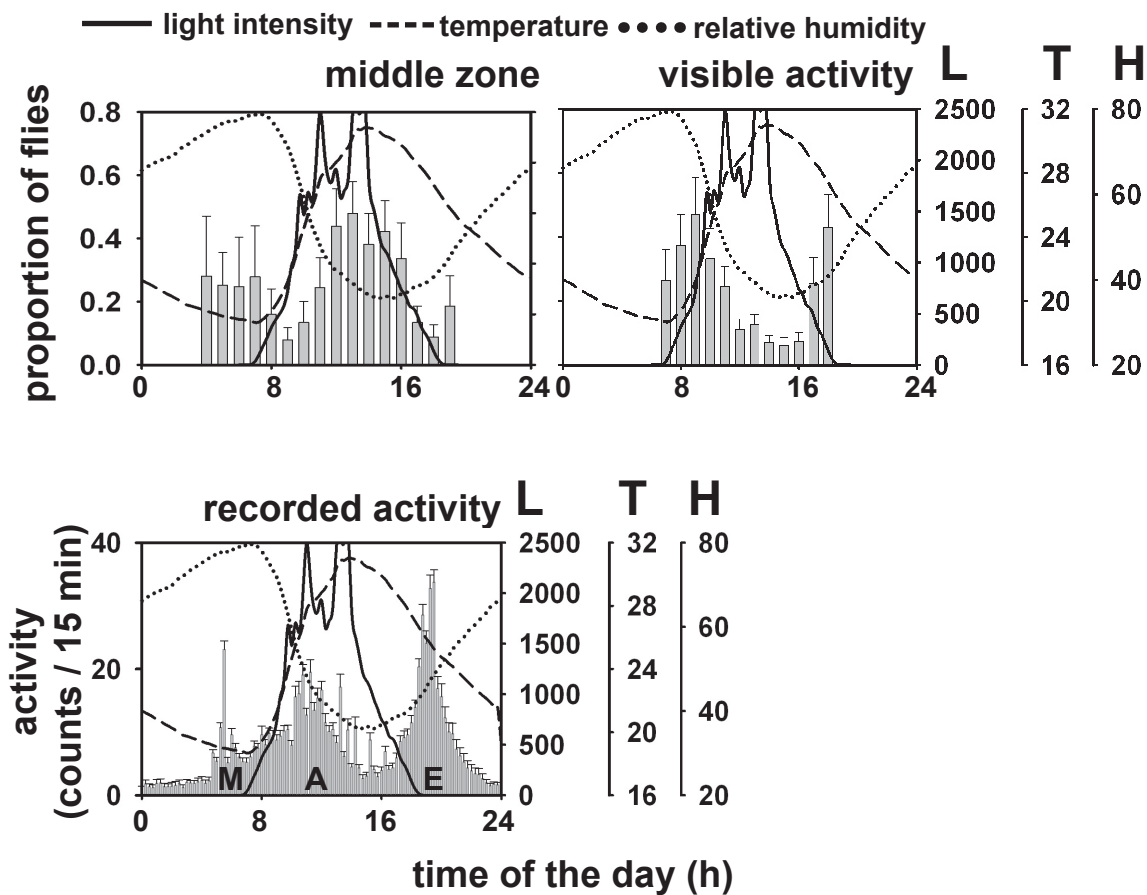
Figure 3: *Visual observation and parallel recording of activity of flies in a DAM2 monitor.* Proportion of flies preferring the middle zone of the activity tube (top, left), the part of the tube which is relatively shaded. Flies prefer the middle zone in the afternoon more than other time of the day. Visual observation of locomotion in the tubes placed in DAM2 monitor shows two peaks of locomotion (top, right). Error bars are SEM. (B, right) Average activity recorded in the same DAM2 monitor shows A-peak. Three separate axes on extreme right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %).
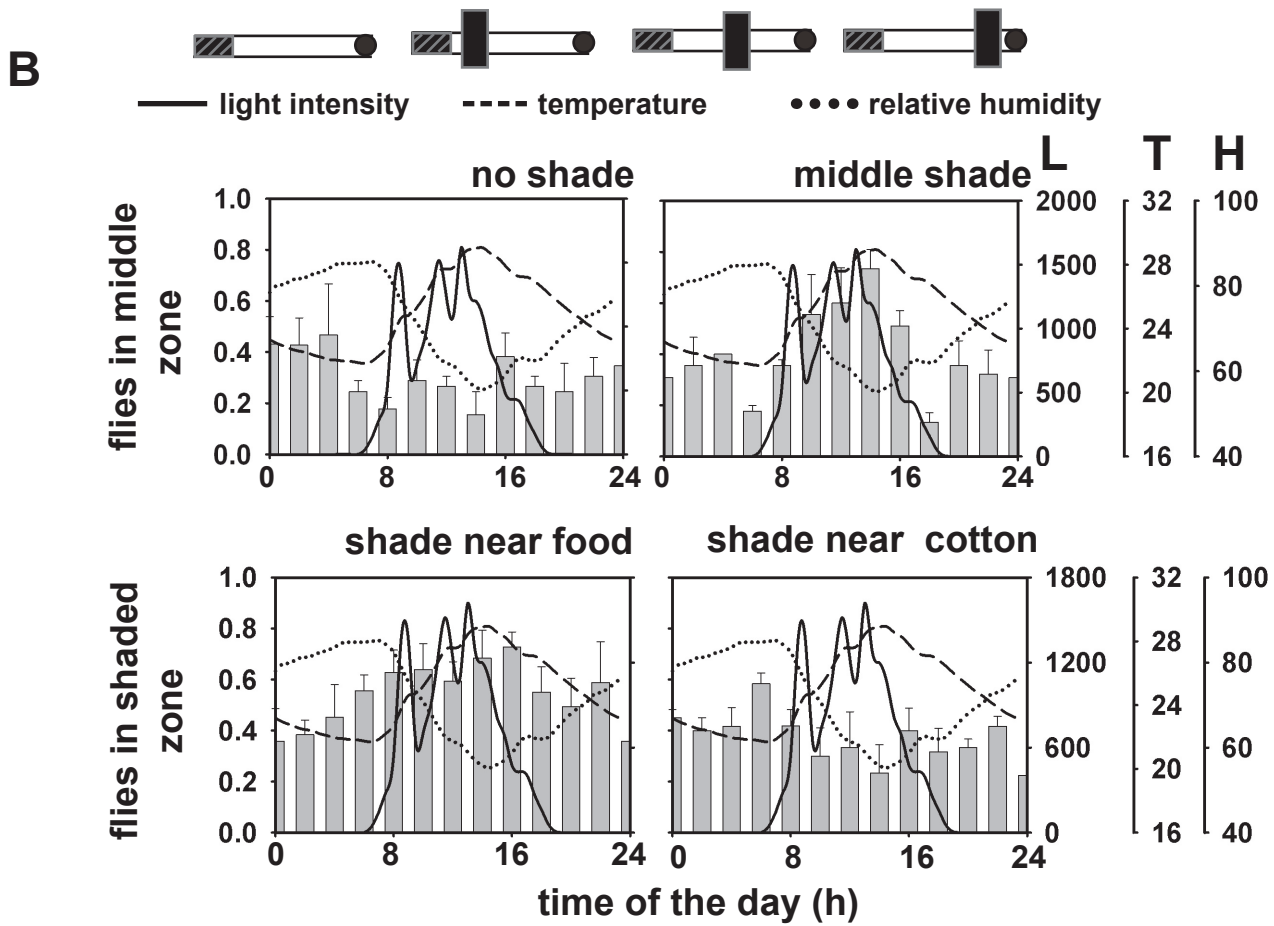
Figure 4: *Flies prefer shade in the afternoon.* (A, left panel) Schematic of experimental set-up. (A, middle panel) Average activity profiles of flies recorded in flatter version of DAM (DAM5) monitor, with (filled circles) or without shade (unfilled circles) in the middle. Error bars are SEM. Three separate axes on extreme right represent three environmental parameters measured: light intensity (L, Lux), temperature (T, °C) and relative humidity (H, %). (A, right panel) Activity in the afternoon interval is greater in shaded than unshaded tubes. (B, top panel) Schematic of experimental set-up shown above graphs indicates the position of shade in various regions of the tubes placed flat under SN. Proportion of flies located in the middle-zones of the unshaded and middle-zone shaded tubes as estimated by visual observation (first and third tubes of the schematic). When shaded in the middle zone, flies prefer the middle zone in the afternoon whereas no such time-specific preference for the middle zone was observed in tubes without shade. (B, bottom panel) Average number of flies in shaded region when shade was provided near food (left) or near cotton (right). Individual flies were placed in locomotor tubes with food at one end and cotton plug at the other and black tape was used to shade different zones of the tube ($n = 4$ tubes for each type of shaded protocol).
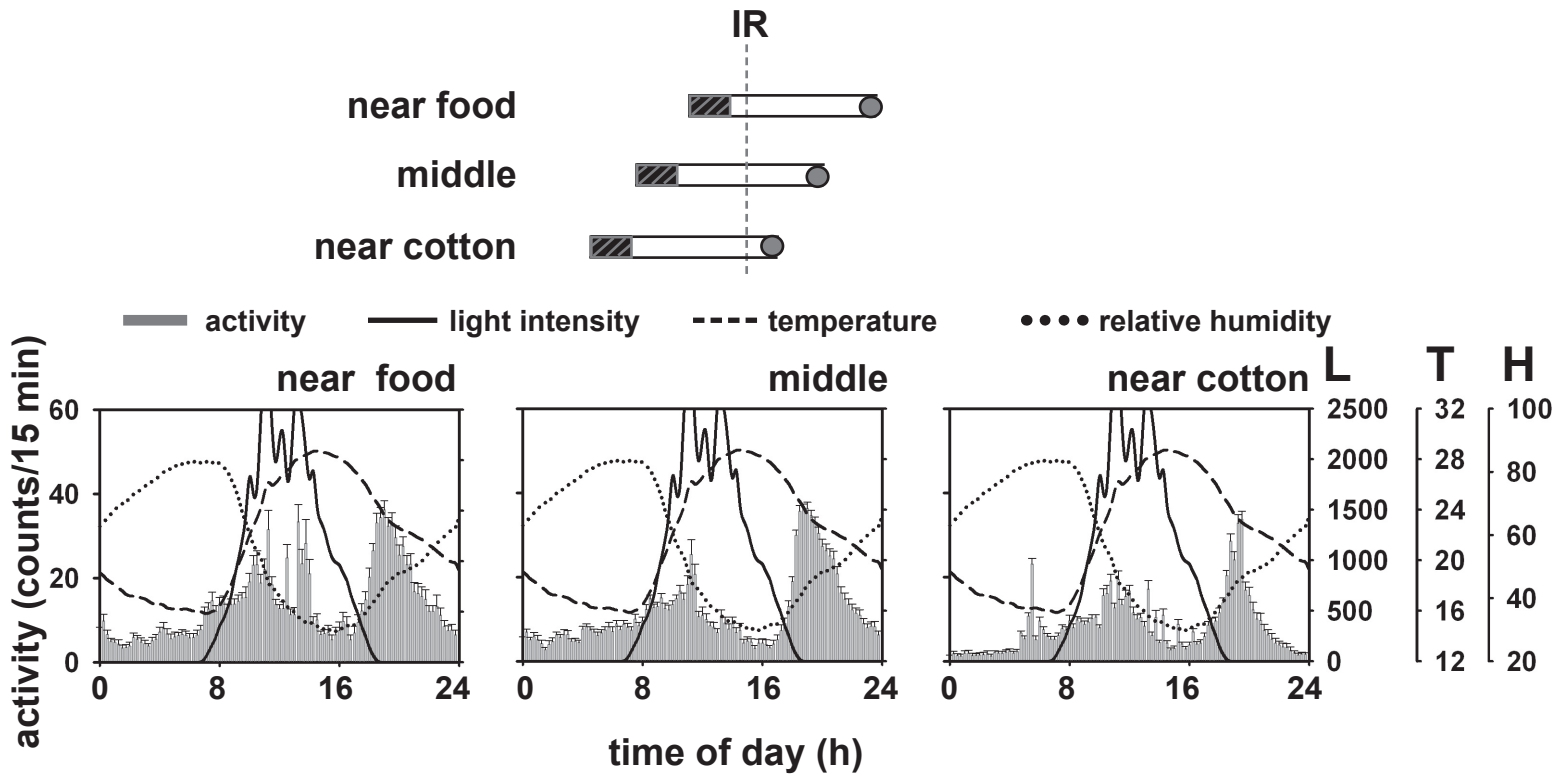
Figure 5: *Recording from different parts of the activity tube.* Schematic representation (top) of protocol for recording activity from different zones of the tubes by placing tubes in DAM2 monitors in such a manner that the IR beam detects activity either near food, through the middle as usual or near cotton. Average activity profiles (bottom) recorded near food, middle and near cotton of the activity tube.

Chapter 5: Mimicking natural light and temperature in the lab reproduces several features of activity-rest rhythm under SN

Introduction

Most organisms possess circadian clocks that possibly enable them to synchronize their behaviors or physiological processes to diurnally varying time-cues in the environment (Saunders, 2002). The mechanism of entrainment of these behavioral rhythms to light-dark cycle with 24-h periodicity has been studied for organisms across diverse taxa (Saunders, 2002; Rieger et al., 2003 and many other studies). Relatively recently, the cellular and molecular basis of temperature as an entraining agent has also been examined. High:low temperature cycles could entrain activity-rest rhythm in fruit flies even in constant light when the molecular clock is disrupted (Glaser and Stanewsky, 2005). However, the role of light and temperature in entraining circadian rhythms has been mostly studied using rectangular cycles of such time cues which are far removed from what happens in the real world where the organism experiences gradual, simultaneous and high amplitude variations in many potential time-cues (Bhutani, 2009). Few recent studies have shed some light on this aspect of circadian entrainment by performing behavioral (Bhutani, 2009, Vanin et al., 2012, De et al., 2012, Menegazzi et al., 2012) and cellular (Bhutani, 2009, Menegazzi et al., 2013) analysis under semi-natural conditions (SN). In chapters 1-3 of this thesis, I have presented results of my studies which attempted to answer some questions regarding how circadian entrainment occurs under SN. Several protocols were designed to assess the role of light in particular, in governing some salient features of the activity-rest behavior under SN. These protocols were designed in such a way that temperature is largely unaltered across protocols, only light was modified in different ways. Therefore, role of temperature and light and temperature together on the features of activity-rest rhythm remains yet to be explored. Even though we could gather information about the role of light from the studies discussed in chapters 1-3, some of those salient findings need to be verified in the lab under more controlled conditions where everything else except the factor

under study remains constant with a greater probability than what happens outside, where controlling noise arising from different other factors becomes a major obstacle.

In this chapter, I will discuss data from experiments done in the lab attempting to mimic light and/or temperature profiles outside in a systematic fashion, to be better able to tease apart roles of light and temperature in bringing about the activity-rest behavior we observe in SN. I ask if and how natural-like light and/or temperature cycles with different ranges and combinations affect the activity peaks. I use gradual step-wise increase and decrease in monochromatic light and temperature in order to mimic SN conditions as closely as possible. Although this approach is able to mimic naturally varying temperature profiles quite successfully, one need to be a bit skeptical about the same for light. Although these protocols in lab incubator reflect the intensity component of light as in SN, but it completely ignores various other aspects, of which perhaps the most crucial is the wavelength or spectral composition of light.

I show that light and temperature affect phases of E and M-peaks, respectively. A-peak depends on maximum daily temperature for it to occur, although light and temperature both are crucial determinants for A-peak. Light causes reduction in the amplitude of M-peak, which might be mediated through the compound eyes.

Material and methods

Fly strains used:

I assayed activity/rest rhythm of *Canton S* (*CS*) flies under all the protocols of gradient light and/or temperature. To specifically examine how under constant light in otherwise SN conditions, M-peak is abolished (chapter 1); I studied mutants of intracellular photoreceptor, phototransduction and compound eye under LL of 100 and 1000-lux intensities with gradient

temperature cycles. These photoreceptor and vision mutants are null mutants of intracellular photoreceptor *cryptochrome* (cry^{02}) (Dolezelova et al., 2007), phototransduction pathway phospholipase C mutant *norpA* (Hu et al., 1978, Pearn et al., 1996), double mutant *norpa;;cry^{02}*, eye morphology mutant *gl^{60j}* (Moses et al., 1989) and compound eye mutants *cli^{eya}* and *so^{1}* (Rieger et al 2003)). Canonical clock gene *period* null mutant (*per^{0}*) was also assayed.

Protocols:

1) *Gradient temperature cycles (T_r)*: Temperature was ramped from lowest 17 ^{o}C to maximum 32 ^{o}C in DD (T_r 17-32, DD) or from lowest 21 ^{o}C to maximum 28 ^{o}C in DD (T_r 21-28, DD) and LD (T_r 21-28, LD). The temperature reaches its highest value during midday and the lowest during late night and remains so until early morning to mimic natural conditions.

2) *Gradient light cycle (L_r)*: Light intensity was ramped from lowest 0-lux to highest 1800-lux under constant temperature 25 ^{o}C (L_r, 25).

3) *Gradient light and temperature cycles (L_r, T_r)*: Both light and temperature were ramped. Light ramping was same as L_r, 25. Temperature was ramped from lowest 17 ^{o}C to maximum 28 ^{o}C (L_r, T_r 17-28) or 32 ^{o}C (L_r, T_r 17-32).

4) *Gradient temperature cycles in constant light (T_r, LL)*: Temperature was ramped from lowest 17 ^{o}C to maximum 28 ^{o}C under 100-lux (T_r 17-28, 100) or 1000-lux (T_r 17-28, 100) constant light. The same was repeated with maximum temperature 32 ^{o}C for both 100 (T_r 17-32, 100) and 1000-lux (T_r 17-32, 1000).

Statistical analyses:

The activity profiles in the figures are based on bin size of 15 min. Phases of M, A, and E activity peaks were estimated by scanning 7-day average activity records of each fly, and identifying that time-point corresponding to the highest activity counts observed within that interval. In the afternoon, when there are multiple peaks, the peak closest to maximum light and temperature in the environment was considered and its phase and amplitude were calculated. Mean phase and amplitude for each peak was obtained for total number of flies from each genotype and each protocol. Two-way ANOVA was carried out to see whether there is any statistically significant effect of genotype or regime on the phase and amplitude of activity peaks. Post-hoc multiple comparisons of phase and amplitude data was performed using Tukey's HSD test. The p value of 0.05 was considered as level of statistical significance throughout all the analyses.

Results

Phase of M and E-peaks are modulated mainly by temperature and light, respectively

In L_r, 25 and T_r 21-28, LD, the M-peak occurs earlier than in all other protocols (Figs 1, 2 $F_{5,148}$ = 20.48, $p < 0.001$). M-peak tracks light when light is the only time-cue (L_r, 25) or when light is not gradual and is imposed in a step-up manner, such as in a standard rectangular cycle (T_r 21-28, LD). But when temperature is gradually varying with gradually varying light (L_r, T_r 17-28 and L_r, T_r 17-32) or without light (T_r 17-32, DD and T_r 21-28, DD), M-peak seems to track temperature changes giving rise to a phase delay (Fig 2). The effect of protocol on the phase of M-peak is statistically significant ($F_{5,148}$ = 20.48, $p < 0.001$). M-peak usually tracks changes in temperature more than light, except under certain conditions, for example when light is the only time cue (L_r T25 °C) or when light has a masking effect, such as in a rectangular LD cycle. The

phase of E-peak was significantly affected by protocol ($F_{5,147} = 39.37$, $p < 0.001$). E-peak, under light and temperature cycles, almost coincided with the phase at which light drops to zero. Protocols where light is not available, E-peak advances. But if light cycle is present, E-peak occurs coinciding with light intensity drop to 0-lux, irrespective of cyclic or constant temperature used. Therefore, fall in light intensity appears to have a greater influence on E-peak than temperature (Figs 1, 2). Nevertheless, when only temperature is available as time-cue, E-peak was advanced compared to when light is present, probably due to the behaviour now tracking fall in temperature (Figs 1, 2).

M-peak is suppressed by light

The amplitude of M-peak was lowered in protocols where light is ramped compared to others ($F_{5,148} = 9.59$, $p < 0.001$) (Figs 1, 2). However, when light profile is rectangular, this trend is not seen; probably light coming on abruptly elicits a startle response that surpasses its effect on the reduction of M-peak amplitude.

Occurrence of A-peak depends on maximum temperature

A-peak occurs in protocols with ramped temperature which reaches 32^0C in the middle of the day, and this is irrespective of whether light is present or absent, and whether LD cycle is rectangular or ramped (Fig 1). 80% of flies showed A-peak in T_r 17-32, DD. In T_r 17-28, DD, activity in the afternoon was high and dispersed through the mid-day duration, not giving rise to a prominent A-peak. A-peak was not detected in any fly under T_r 17-28, DD. The occurrence of A-peak was enhanced when T_r 17-28 was coupled with a rectangular LD, in that 39.13% flies showed A-peak, albeit of lower amplitude compared to T_r 17-32, DD. This frequency was increased when T_r 17-28 was coupled with L_r. In this case, 66% flies showed A-peak. However,

ramped light profile at constant temperature (L_r, 25) failed to induce A-peak. In L_r, T_r 17-32, 92% flies showed A-peak, which is highest among all the protocols. Thus, temperature greatly influences the occurrence of A-peak.

Presence of light consolidates activity peaks with clear peaks and troughs

When an LD cycle was imposed along with T_r 17-28, the activity pattern was consolidated into two distinct peaks of activity – morning and evening with relatively little afternoon activity bout, although clearly distinguishable unlike T_r 17-28, DD (Fig 1). Similarly, when a ramped light cycle was coupled with T_r 17-32, better consolidation of activity into three clearly distinguishable peaks of activity occurred. Light-dark cycle, either rectangular or gradual, consolidates activity around clearly distinguishable activity peaks (Fig 1).

Compound eyes probably mediate the suppression of M-peak in response to light

The results of studies described in chapter 1, showed that constant light in otherwise semi-natural conditions (LL+SN) abolishes M-peak of activity. To better understand this phenomenon, I examined activity-rest rhythm of phototransduction, photoreceptor and compound eye mutants where it is expected that the light input to the circadian clock is disrupted either partially or completely under constant light while providing temperature cycles as time cues. M-peak was diminished in most of the genotypes studied including wild type strains except mutants for compound eye (*cli^{eya}* and *so^1*) (Figs 3-7). Two-way ANOVA on M-peak amplitude with protocols (T_r 17-28, 100; T_r 17-28, 1000; T_r 17-32, 100 and T_r 17-32, 1000) and genotypes (*CS*, *cli^{eya}* and *so^1*) as the two factors, showed significant main effect of protocol ($F_{3,228} = 3.22$, $p = 0.02$), strain ($F_{2,228} = 17.46$, $p < 0.001$) and their interaction ($F_{6,228} = 3.92$, $p < 0.001$). Nevertheless, in T_r 17-28, 1000, M-peak amplitude was similar in the three genotypes (Fig 7).

The reduction of M-peak was also seen in L_r (Figs 1, 2). I speculate that compound eye mediates the effect of light in reduction of M-peak amplitude. In phototransduction mutant *norpA,* intracellular photoreceptor *cryptochrome* mutant *cry[02],* and their double mutant *norpA;; cry[02],* the M-peak was completely abolished in all four T_r, LL protocols (Figs 3-6).

Discussion

Features of the locomotor activity rhythms exhibited under natural light and temperature could be elicited by simulated conditions in the lab

It is possible to successfully reproduce some of the features of activity-rest rhythm under semi-natural conditions (Vanin et al., 2012) by attempting to mimic at least changes in light intensity and temperature. The protocols used in this chapter were successfully able to mimic some of the features seen under SN (discussed in previous chapters) such as presence of A-peak (Fig 1), dependence of phase of E-peak on light (Fig 1, 2) and effect of constant light on the M-peak (Figs 3-7). Using these protocols, one could better separate effects of light and temperature on activity peaks such as temperature profile affects M-peak phase more than light does, whereas fall in light intensity affects E-peak phase (Figs 1, 2). The level of maximum temperature is a crucial determinant of A-peak. E-peak depends on light information for its timing (Fig 1).

M and E-peaks depend on light and temperature information for phase

The aim of designing these lab protocols used in this chapter to mimic some of the features of SN was, to be better able to tease apart roles of light and temperature on regulating the activity peaks. In chapter 1, we saw that M- and E-peaks depend on light information mostly to determine phase. We were unable to comment on the role of temperature as it was unaltered in all the protocols used under SN, where the main idea was to modify light information in different

ways and to inspect the role of light. These ramped light and/or temperature protocols were able to give us some insight into the relative roles of light and temperature in regulating activity peaks. M-peak phase is temperature-dependent whereas, E-peak phase is light-dependent (Figs 1, 2).

A-peak depends mainly on temperature

In chapter 1, we saw that A-peak is dependent on presence of natural light in the afternoon. Using ramped light and temperature protocols, it became clear that temperature also has an important role in determining the occurrence of A-peak. Although A-peak did not occur in our ramped light protocol (Fig 1) role of light cannot be ruled out, since our studies managed to provide a light maximum of 1800-lux only, whereas we come across more intense afternoon light in SN. When rectangular or ramped light cycles were imposed along with ramped temperature cycles non-conducive for A-peak (Fig 1), higher fraction of flies showed A-peak. Light also consolidated the afternoon activity around the peak from a dispersed overall high afternoon activity under ramped temperature cycles in constant darkness (Fig 1). Furthermore, mimicking natural light is slightly trickier than mimicking temperature as the ramped light protocol was only able to mimic the intensity aspect of it and not other features of the spectrum that changes dramatically throughout the day, especially during twilight hours. It appears that harsh light and/or temperature could produce A-peak, and that their effect could be additive.

A-peak is a response to harsh afternoon conditions and not likely to be a natural behavior of flies

A-peak occurred more frequently when the maximum temperature crossed 30^0C (Fig 1). This supports the proposition we raised based on studies described in the previous chapters that A-

peak is a response to harsher weather conditions which is largely circadian clock-independent and not likely to be a natural behavior of flies.
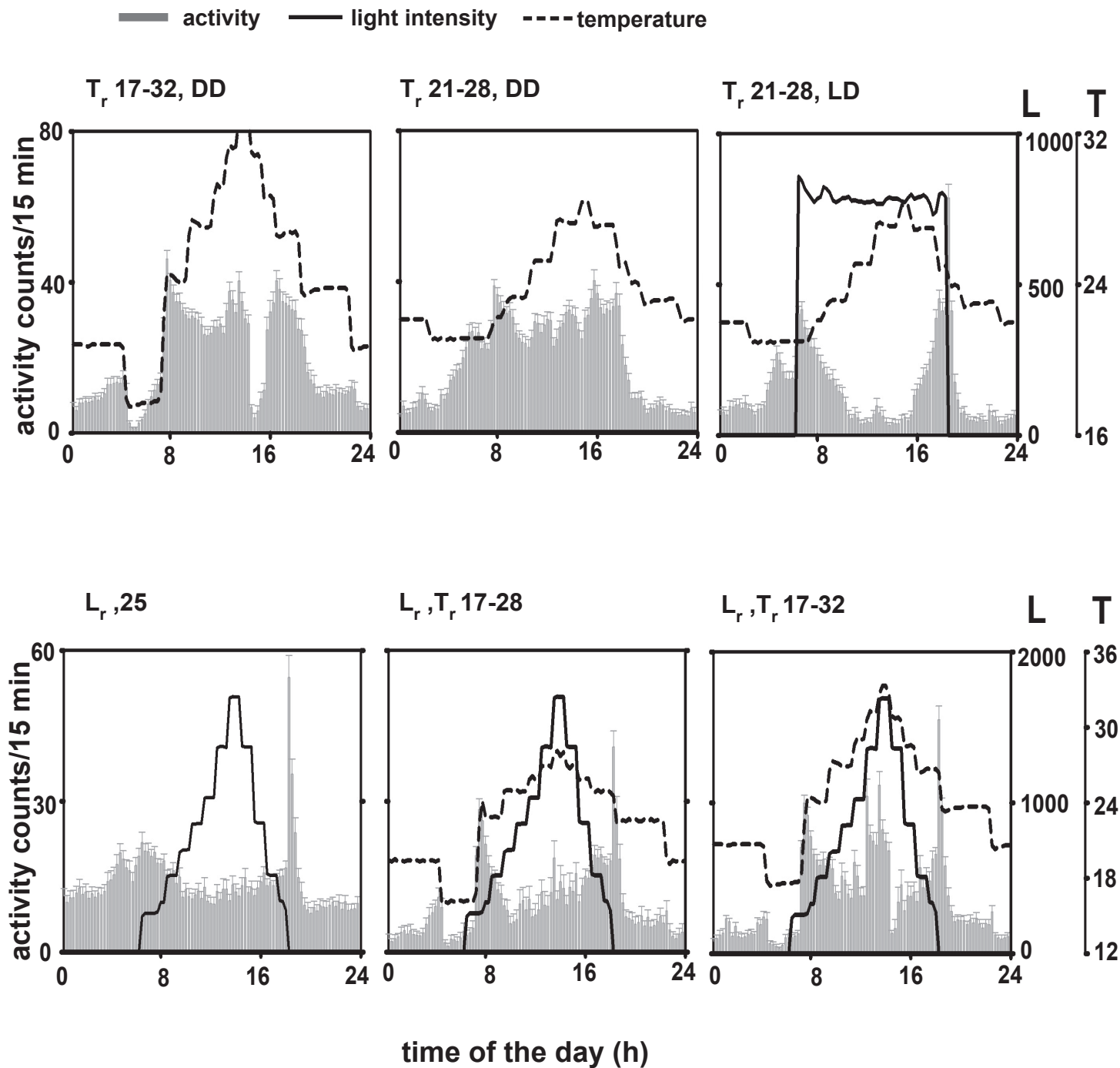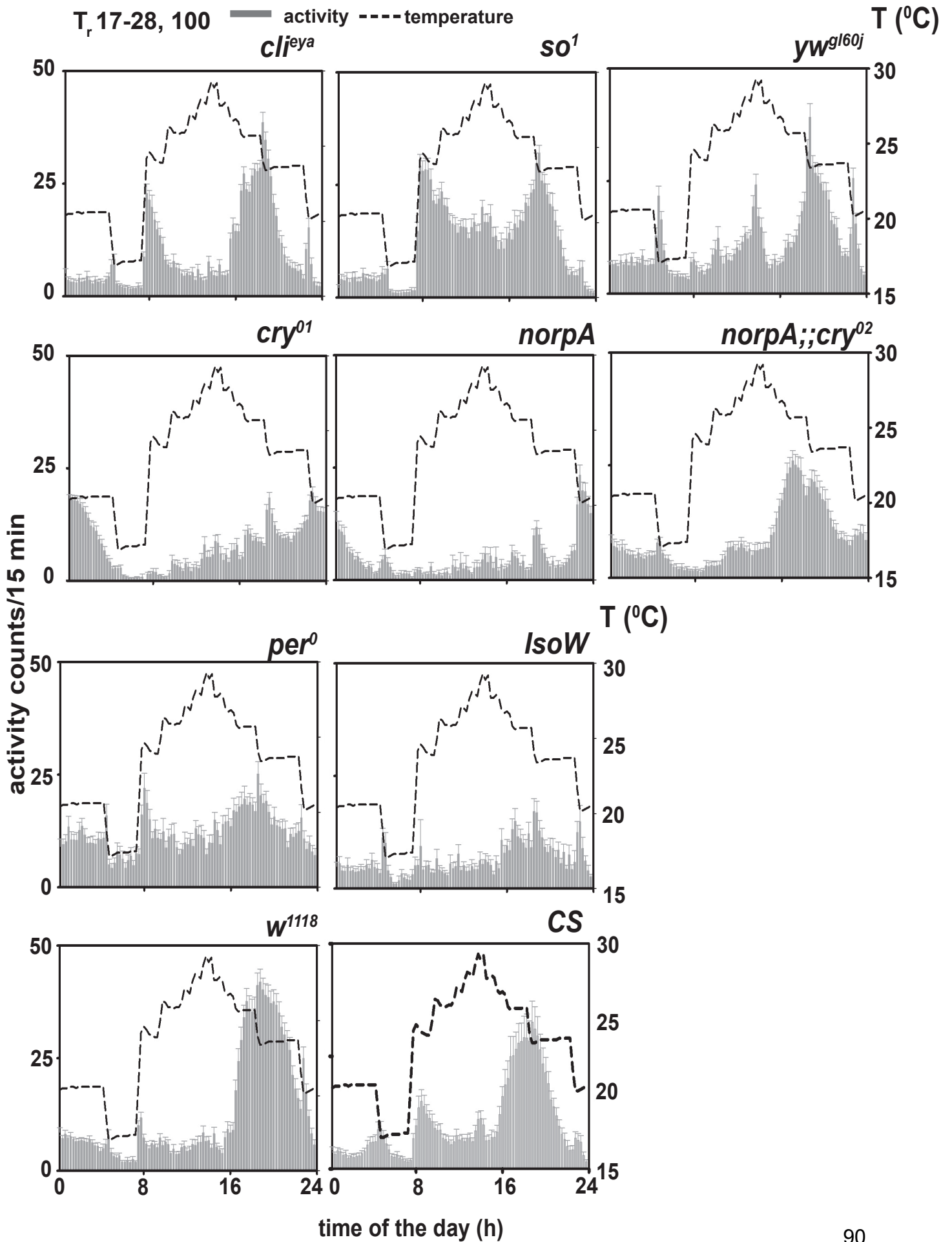
Figure 1: *Average activity profiles of Canton-S (CS) flies under different light and/or temperature protocols.* Temperature ramped from 17^0C to 32^0C under DD (T_r 17-32, DD) or from 21^0C to 28^0C under DD (T_r 21-28, DD) or LD (T_r 21-28, DD) (top panel). Light ramped from 0 to 1800-lux under constant temperature 25^0C (bottom, left). Both light and temperature were ramped (bottom, middle and right). Light was ramped from 0-1800-lux (L_r), and temperature was ramped from 17^0C to 28^0C (L_r, T_r 17-28) or 17^0C to 32^0C (L_r, T_r 17-32). Error bars are standard error of mean (SEM). Two separate axes on extreme right represent two environmental parameters measured: light intensity (L, Lux) and temperature (T, °C).
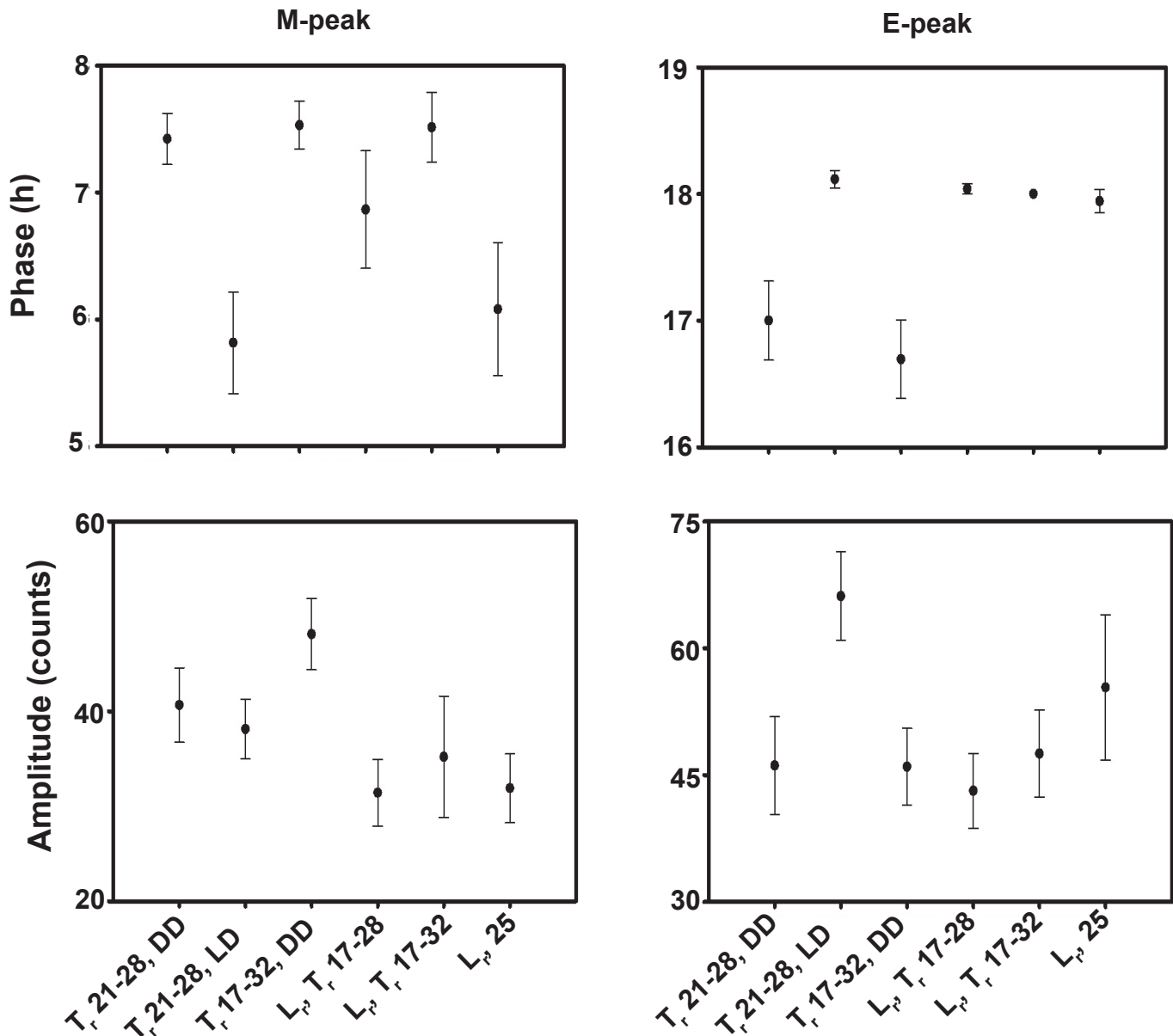
Figure 2: Phase (h) and amplitude (activity counts) of M- (top and bottom, left) and E-peaks (top and bottom, right) under different ramped temperature and/or light protocols. Error bars are 95% Confidence Intervals (95% CI).
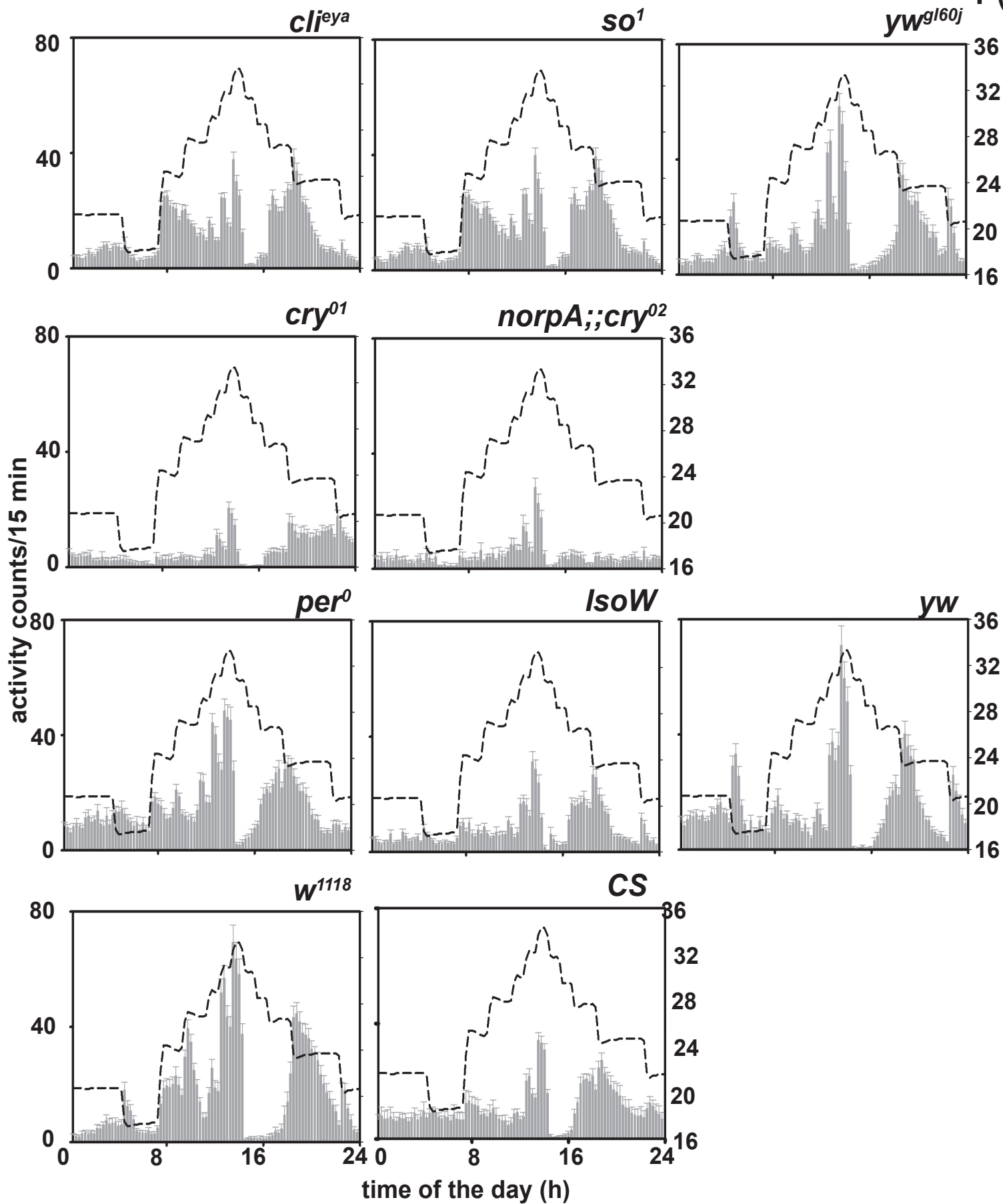
Figure 3: *Average activity profiles under ramped temperature cycle (ramped from 17^0C to 28^0C) in constant light of intensity 100-lux.* Compound eye mutants cli^{eya} and so^1 and eye morphology mutant gl^{60j} (topmost panel), *cryptochrome* (cry^{02}), phototransduction pathway phospholipase C mutant *norpA* and double mutant *norpa;;cry^{02}* (second panel), canonical clock gene *period* null mutant per^0 , and wild type backgrounds *IsoW* (for cry^{02}), w^{1118} (for per^0) and *CS* (for the rest). Error bars are standard error of mean (SEM). The axis on extreme right represents temperature (T, °C).
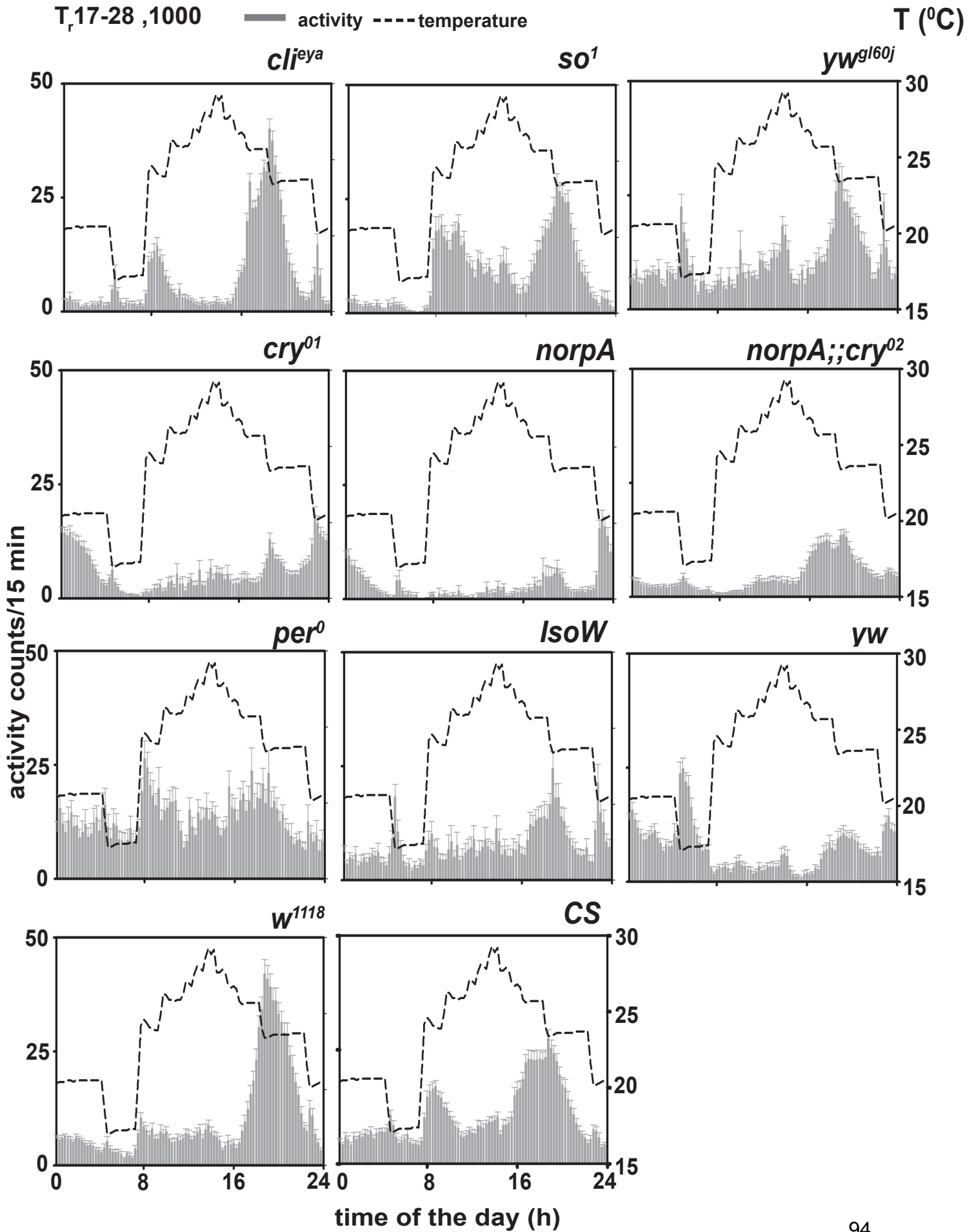
Figure 4: *Average activity profiles under ramped temperature cycle (ramped from 17^{0}C to 32^{0}C) in constant light of intensity 100-lux.* Compound eye mutants *cli^{eya}* and *so^1* and eye morphology mutant *gl^{60j}* (topmost panel), *cryptochrome* (*cry^{02}*), phototransduction pathway phospholipase C mutant *norpA* (second panel), canonical clock gene *period* null mutant *per^0* , and wild type backgrounds *IsoW* (for *cry^{02}*), *yw* (for *gl^{60j}*)*w^{1118}* (for *per^0*) and *CS* (for the rest). Error bars are standard error of mean (SEM). The axis on extreme right represents temperature (T, °C).
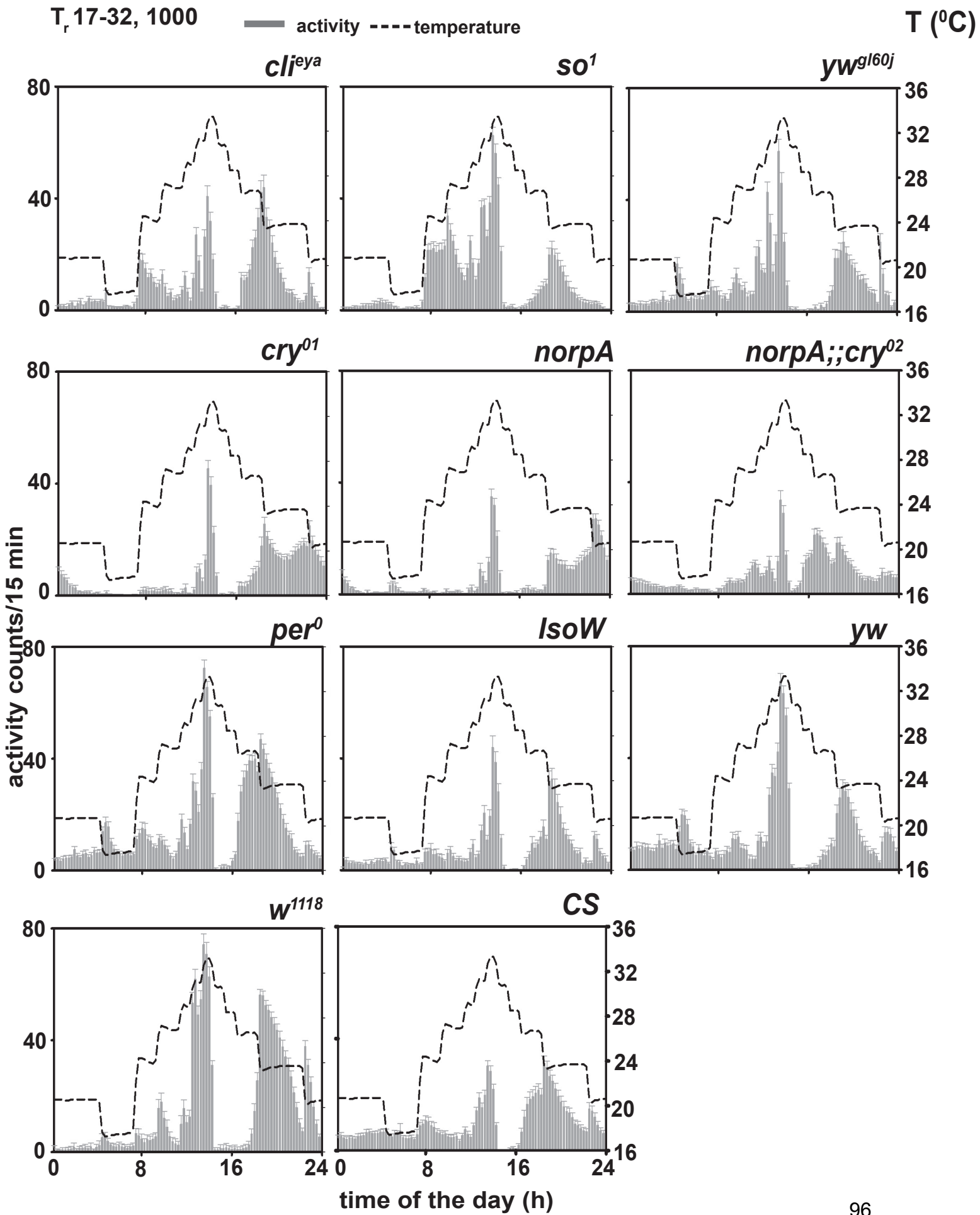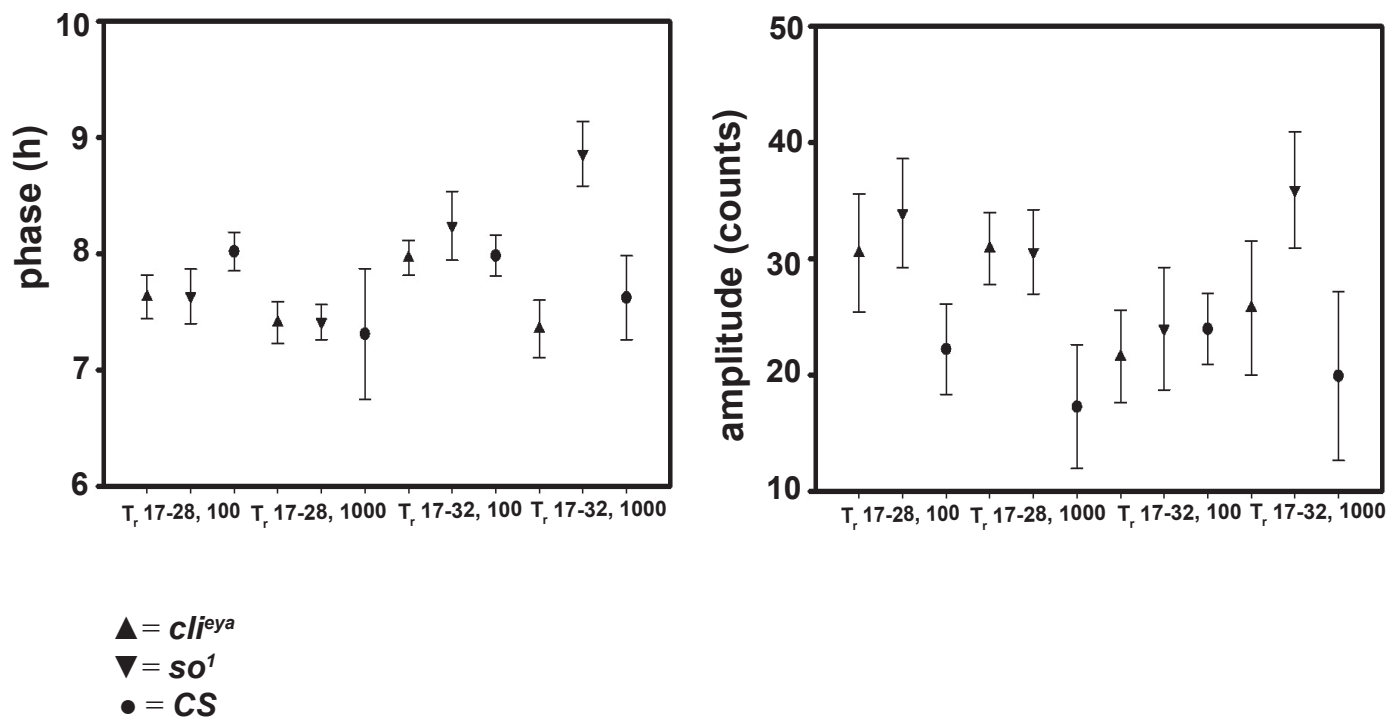
T$_r$17-28 ,1000 — activity --- temperature   T (^{0}C)

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Figure 5: ***Average activity profiles under ramped temperature cycle (ramped from 17^0C to 28^0C) in constant light of intensity 1000-lux.*** Compound eye mutants cli[eya] and so[1] and eye morphology mutant gl[60j] (topmost panel), cryptochrome (cry[02]), phototransduction pathway phospholipase C mutant norpA and double mutant norpa;;cry[02] (second panel), canonical clock gene period null mutant per[0], and wild type backgrounds IsoW (for cry[02]), yw (for gl[60j])w[1118] (for per[0]) and CS (for the rest). Error bars are standard error of mean (SEM). The axis on extreme right represents temperature (T, °C).

T$_r$ 17-32, 1000 — activity ---- temperature   T (^{0}C)

cli^{eya} so^1 yw^{gl60j}

cry^{01} norpA   norpA;;cry^{02}

per^0 IsoW   yw

w^{1118} CS

time of the day (h)

Figure 6: *Average activity profiles under ramped temperature cycle (ramped from 17^0C to 32^0C) in constant light of intensity 1000-lux.* Compound eye mutants cli^{eya} and so^1 and eye morphology mutant gl^{60j} (topmost panel), *cryptochrome* (cry^{02}), phototransduction pathway phospholipase C mutant *norpA* and double mutant *norpa;;cry^{02}* (second panel), canonical clock gene *period* null mutant per^0 , and wild type backgrounds *IsoW* (for cry^{02}), *yw* (for gl^{60j})w^{1118} (for per^0) and *CS* (for the rest). Error bars are standard error of mean (SEM). The axis on extreme right represents temperature (T, °C).

Figure 7: M-peak phase (h) and amplitude (activity counts) of compound eye mutants *cli^eya* and *so^1* and their control *CS*, under all T_r, LL protocols (T_r 17-28, 100; T_r 17-32, 100; T_r 17-28, 1000 and T_r 17-32, 1000).

Chapter 6. Seasonal Variations in the Environment Greatly Influence Adult Emergence Rhythm in Three Drosophilid Species

Introduction

Temporal regulation of rhythmic behaviors by environmental cycles is a fundamental property of most organisms, which enables them to maximally exploit resources in their environment, and to minimize the effects of adverse conditions (Pittendrigh, 1993; Saunders, 2002). In fruit flies *Drosophila melanogaster*, the act of adult emergence is clock-controlled, and entrainable to daily cycles of light and temperature (Saunders, 2002). Under standard laboratory (LAB) protocols, emergence is largely restricted to daytime with a sharp peak around dawn (Kumar et al., 2007). One popular hypothesis regarding the circadian regulation of emergence at dawn stresses upon the importance of temperature and humidity as key factors (Pittendrigh, 1993). Recently we have studied adult emergence rhythm under semi-natural conditions (SN) (De et al., 2012). This study has demonstrated that it is indeed true that much of the emergence occurs during early morning when the temperature is low and humidity is high in the environment (De et al., 2012). Similar to activity-rest rhythm (Bhutani, 2009; Vanin et al., 2012), several features of emergence rhythm also differs between SN and LAB (De et al., 2012). The gate width of emergence is lower in SN compared to LAB. Under semi-natural condition, gate-width of adult emergence rhythm and fraction of flies emerging in the night decreased significantly compared to the laboratory condition, which confirms that the strength of the natural environmental cycles is greater than the cycles imposed in the laboratory. This may due to the presence of multitude of zeitgebers in relatively higher contrast in nature unlike the laboratory where the only time cue present was in the form of presence and absence of relatively low intensity light (~100 lux). The richness of zeitgebers (e.g. light is polychromatic in nature as opposed to monochromatic in the lab) in the natural conditions might also play a crucial role in making the rhythm tight and consolidated. Also in nature, environmental factors vary in a gradual manner, which is likely to

provide multiple options for the circadian clocks of flies to phase-lock to a particular environmental time cue at an appropriate time of the day (Sharma et al., 1998). This was further confirmed in another recent study where flies with lab-evolved precise clocks and their controls showed enhanced peak and narrower gate width in SN compared to LAB (Kannan et al., 2012). The lack of the ability of per^0 flies to gate emergence rhythm to a particular time of the day confirms the notion that clocks are involved in the circadian gating of emergence rhythm under cyclic environmental conditions (De et al., 2012).

The temporal profile of emergence is shown to be dependent on seasonal variations in environmental parameters to a great extent such that in months with harsh conditions, much of the emergence occurs starting during late night to early morning whereas in months with milder weather conditions, emergence occurs till the afternoon (De et al., 2012). Therefore, the temporal correlations between emergence and weather conditions also vary from one weather condition to the other. In milder conditions, emergence correlates to light intensity but not with temperature and humidity but in harsh conditions, emergence correlates to humidity and temperature but not to light. One could argue that this lack of significant correlation with light may be due to poor resolution of the assay (2h). Emergence may correlate to light for shorter duration during dawn transition. We may have missed the 'meaningful' duration during which emergence correlates to light, because the binning was done in 2hrs while twilight occurs in a span of 30 minutes at this location (12°59'N 77°35'E) and light intensity saturates ~ 6 hrs after sunrise. Nevertheless, in the same study, a thorough inspection of emergence during dawn transition with a better resolution of 15-min revealed that emergence and light intensity was positively correlated during early morning. Although emergence is significantly correlated to some environmental parameters, it is not possible to infer causal relationships from these results.

One better, although indirect, way to understand how environmental parameters influence emergence profile could be by studying effect of seasonal variations in the environment on different parameters of emergence rhythm. In this study, I have assayed emergence rhythm in three wild-caught *Drosophila* species under SN, in five months of the year. These months include summer and winter seasons in Bangalore, India (12°59'N 77°35'E). I also compare the emergence profiles of these three species under LAB LD 12:12. This study aims to revisit some of the findings of De et al., 2012, particularly regarding how emergence depends on seasonal variations in the environmental parameters.

This study also aims to perform a comparative analysis on adult emergence rhythm on three species of *Drosophila* under semi-natural conditions. Sympatric species *Drosophila melanogaster* (*DM*) and *Drosophila ananassae* (*DA*) differ in several features of activity-rest rhythm (Prabhakaran and Sheeba, 2012). DM exhibited a bimodal activity pattern whereas DA had a predominantly morning-centered activity pattern which persisted under a range of photoperiods. *DA* was active in the light phase without any relative inactivity (siesta) during midday unlike *DM*. Prabhakaran and Sheeba, 2012 hypothesized that due to differences in their underlying clocks these two recently diverged sympatric species occupy different temporal niches. In this study, I attempted to understand how Drosophilids time their emergence under natural environment across seasons using three closely related wild-caught *Drosophila* species, *Drosophila melanogaster* (*DM*), *Drosophila malerkotliana* (*DK*) and *Drosophila ananassae* (*DA*). Even though these three species are closely positioned in the phylogenetic tree (Fig 1), there are prominent differences in their clock properties, which might be attributed to their ecological ancestry. This study will examine whether, with respect to adult emergence, these three species differentially respond to environmental conditions and the variations in them.

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Materials and Methods

Fly strains used:

Three wild-caught *Drosophila* species were used: *Drosophila melanogaster* (*DM*), *Drosophila malerkotliana* (*DK*) and *Drosophila ananassae* (*DA*).

Fly stock maintenance:

Flies were maintained in an enclosure under LD cycles at constant temperature (~25 ^{o}C) and relative humidity (~70%) on corn-meal medium. Food in the fly-vials was changed every alternate day. Freshly emerging adults were collected in plexiglass cages ($25 \times 20 \times 15$ cm^3), and to start a new generation, yeasted food plates were placed in these cages ~6-hr prior to egg collection. Dim red light was used to handle flies during night. For the assays, glass-vials containing eggs were placed in aluminum vial-racks and transferred immediately from laboratory to shelves in the outdoor enclosure (SN) / lab incubator (LAB).

The assay conditions

Semi-natural condition (SN)

The assays were done within JNCASR, Bangalore campus (12°59'N 77°35'E), inside an enclosure constructed under canopy. The enclosure was an iron cage ($122 \times 122 \times 122$ cm^3) with grids (6×6 cm^2) allowing free flow of air, and covered only on the top with a sloping translucent plastic sheet. The emergence rhythm assays on the three species were carried out under SN in the months of March, April, November and December in 2012 and February in 2013.

Laboratory condition (LAB)

The laboratory assay condition was 12:12 h LD cycles (lights-on at 10:00 hr and lights-off at 22:00 hr) at constant temperature ($25 \pm 0.5\,^{\circ}$C; mean $\pm$ SD), and relative humidity ($70 \pm 5\%$), inside an incubator.

Emergence rhythm assay

For the assays, eggs laid over a period of ~6-hr were collected and dispensed at high density (~300 eggs per vial) into glass-vials (18-cm height $\times$ 2.4-cm diameter) containing ~6-ml of corn-meal food. Twenty vials of each strain were used for the assay (10 each for SN and LAB). Eggs were collected in vials and transferred immediately into assay regimes, monitored for darkened pupae, and after the first flies began to emerge, emerging adults where cleared from the original vials at every 2-hr and counted. The daily profiles of light, temperature, and humidity were also monitored simultaneously using DEnM, Trikinetics, USA. The profiles of emergence and environmental variables shown in the figures are averages across four days.

Analyses of emergence data

The emergence profiles of each strain were plotted by averaging daily profiles of 10 replicate vials over four successive days. To compare emergence rhythm across species, regimes, and months, we quantified several parameters of the rhythm - gate-width, % nighttime emergence, peak timing and variance in peak timing. Gate-width was estimated at the time-interval between start and end of emergence in one complete cycle (using 5% of total emergence in that cycle as cut-off). First a spline was drawn on emergence data, and then the two time-points in every cycle at which emergence levels reached 5% of total were noted. The gate-width of every cycle was then estimated as the time-difference between these two 5% cut-off phases. Peak(s) of

emergence were determined using analysis of variance (ANOVA) with time-point as fixed

factor, followed by post-hoc multiple comparisons using Tukey's test. Variance in peak-timing

was estimated as day-to-day variation in timing of emergence-peak in each vial, averaged over

replicate vials. 'Nighttime' was considered from 22h to 4h, as in this interval the light intensity

remains at 0-lux across months. % nighttime emergence was averaged across vials and cycles.

The gate-width, peak timing, day-to-day variance in peak timing, peak amplitude and nighttime

emergence data were subjected to two-way ANOVAs to examine the main effect and interaction

of strain and assay month. Error bars shown in the emergence profiles are standard errors of

mean (SEM). Error bars in all other places are 95% Confidence Interval (95%CI). All statistical

analyses were implemented using STATISTICA^{TM} for windows.

Results

Adult Emergence in the LAB

To begin with I first examined the emergence profile of the three species under standard LAB

LD 12:12. Emergence peaked after lights ON, at ZT 2, in *DM* and *DK* (Fig 2). In both of these

species, emergence occurs mostly close to lights ON and then gradually tapers down as the day

progresses, whereas, in *DA*, emergence is distributed over the entire day with a peak during the

later part of the day (Fig 2). Gate width of emergence ($F_{2,26} = 1.17$, $p = 0.32$) fraction of flies

emerging in the night ($F_{2,26} = 2.75$, $p = 0.08$) and day-to-day variance in peak timing ($F_{2,22} =$

3.09, $p = 0.06$) did not differ among the three species in the LAB (Fig 2). The peak of emergence

was delayed in *DA* compared to *DM* and *DK* ($F_{2,22} = 14.06$, $p < 0.001$). Amplitude of the

emergence peak was higher in *DM* compared to *DK* and *DA* ($F_{2,24} = 5.58$, $p = 0.01$).

The peak of emergence coincides with humidity maxima and temperature minima in summer months

The temperature and humidity conditions in the five months in which the emergence assay was performed are summarized in figure 3. In March and April, maximum temperatures went above 30 °C, and average daytime temperature was between 25 and 30°C, whereas, in November, December and February, the maximum temperatures were between 25 and 30°C and average daytime temperature was below 25°C. With respect to humidity, March, April and February were harsher than November and December. In November and December, humidity was at overall higher levels with average day and nighttime humidity above 80%. On the contrary, the average day and nighttime humidity in the other three months were around 60%. Separation between phase of temperature trough and peak increased in winter compared to summer months, whereas phase of light onset and humidity trough remained mostly unaffected by season (Fig 3). Light intensity values were highly dependent on leaf movements and the extent of overhead canopy in SN, rather than reflection of features of a particular season. Therefore, light intensity information has not been used to assess how harsh or mild the weather was in a particular month. Nevertheless, the light reaching the experimental enclosure was highest in the month of February (Close to 2500-lux in the middle of the day as opposed to below 600-lux in other months). Based on temperature and humidity values, March and April conditions were considered as 'harsh', November and December as 'mild' and February as 'moderately harsh'. The peak of emergence is associated with temperature minima and humidity maxima in the months of March and April (Fig 4) (De et al., 2012). This trend breaks down as the weather conditions become milder in the other three months and peak of emergence occurred after humidity had peaked and temperature had reached its trough (Fig 4).

Gate width of emergence is narrower under harsher environmental conditions

Gate width of emergence differs across species ($F_{2,125} = 4.54$, $p = 0.012$) and months ($F_{4,125} = 15.74$, $p < 0.001$) but the interaction between species and months was not statistically significant ($F_{8,125} = 1.94$, $p = 0.05$). In March, gate width of emergence in two of the three species, is greater than November, December and February (Fig 5). In April, however, this trend persists for all the three species but only in case of *DK*, gate width is significantly narrower than November, December and February (Fig 5).

Day-to-day variation in peak timing is greater in relatively milder conditions

Day-to-day variation in peak timing does not differ among species ($F_{2,116} = 2.85$, $p = 0.06$) in any of the months of assay but it does differ across months ($F_{4,116} = 16.16$, $p < 0.001$) with significant interaction between species and months ($F_{8,125} = 3.64$, $p < 0.001$). The variation in timing of peak of emergence reduced in harsh environmental conditions of March and April compared to the other three months for two out of the three species, whereas in case of *DA*, such a trend was not statistically supported (Fig 5).

The average timing of the peak of emergence in all three species is delayed in milder conditions

The average timing of the peak of emergence in all the three species remained similar to each other ($F_{2,124} = 0.1$, $p = 0.9$) but was delayed in November and December and especially in February (Fig 5) (significant effect of months: $F_{4,124} = 73.85$, $p < 0.001$). The interaction between species and months was also statistically significant ($F_{8,124} = 7.95$, $p < 0.001$). In the milder weather conditions of November and December the peak shifted towards the day probably because the favorable conditions persist even quite past dawn which is in agreement with the findings of De et al., 2012, where the authors demonstrate that gate width and variance in peak timing increases in relatively wetter and cooler conditions. Along with the increase in

gate width in such conditions, the peak also shifts towards midday, unlike March and April, where the peak occurred around 8 AM (Fig 5). Onset of emergence is largely unaffected by season (Fig 3) except that in February, all the three species had a delayed onset. There is no consistent trend in terms of onset of emergence among different species across months (Fig 3).

The peak amplitude reduces in relatively milder conditions

As the weather conditions get milder in November to February, the amplitude of the peak of emergence reduced ($F_{4,129} = 74.97$, $p < 0.001$) (Fig 5). The peak amplitude also differed across species ($F_{2,129} = 4.38$, $p = 0.01$) with significant interaction between species and months ($F_{8,129} = 6.92$, $p < 0.001$). The reduction in peak amplitude can be considered as a by-product of broadening of the gate-width of emergence in milder conditions.

Fraction of flies emerging during night is greater when the day is warmer and drier

The percentage of nighttime emergence differed among species ($F_{2,128} = 6.03$, $p = 0.003$) and months ($F_{4,128} = 41.65$, $p < 0.001$) and the interaction between species and months was also statistically significant ($F_{8,128} = 15.85$, $p < 0.001$) (Fig 5). Fraction of flies emerging in the night was significantly higher in April compared to all the other months (including March) in case of *DM* and *DK*. *DA* had similar fraction of flies emerging during night across months (Fig 5).

Discussion

All the three species share mostly similar features of emergence rhythm under LAB

The three species studied namely *Drosophila melanogaster*, *Drosophila malerkotliana* and *Drosophila ananassae* share mostly similar characteristics of emergence rhythm in LAB (Fig 2). Gate width of emergence, fraction of flies emerging in the night and day-to-day variation in the timing of peak of emergence are similar in the three species in LAB. Nevertheless, *Drosophila ananassae* had a delayed peak compared to the other two species. *D. ananassae* emerges in

relatively higher numbers even in the later part of the day unlike the other two species, in which most of the emergence occurs close to lights ON. When activity-rest behavior of these three species was studied in LAB (Prabhakaran and Sheeba, 2012), *D. ananassae* was found to be active during midday while the other species showed siesta. Unlike activity-rest behavior, in emergence, there was no morning preference, *per se*, in *D. ananassae*. Rather, in *D. ananassae*, emergence was relatively shifted towards the day. However, it is unreasonable to assume that species-level differences seen for one behavioral rhythm would persist across behaviors.

The three species respond largely similarly to the environmental conditions

The weather conditions in this study consists of summer conditions with low average humidity and high average temperature and winter conditions with moderate to high average humidity and low average temperature. The three wild-caught species responded to these moderate to drastic seasonal changes, in terms of adult emergence behavior mostly in a similar manner. In harsher conditions, the rhythm is more tightened with narrower gate-width of emergence (consistent with De et al., 2012) and more robust with relatively higher peak amplitude. When weather conditions become milder, in that the average daily temperature comes down and humidity rises such as in the months of November and December, the gate-width broadens probably as the weather conditions favorable for emergence persists till the later part of the day. In these two months, the timing of the emergence peak is more variable across days than when the conditions are harsher, which indicates that emergence is temporally more flexible in mild conditions compared to conditions with high temperature and low humidity like in summer (again consistent with De et al., 2012). All these features are mostly shared by all the three species with little to no difference among each other, unlike activity-rest rhythm in LAB (Prabhakaran and Sheeba, 2012) and SN (Prabhakaran and Sheeba, unpublished).

In summary, adult emergence is dependent on seasonal variations in the weather conditions in the three *Drosophila* species and the nature of this dependence is very similar across species.
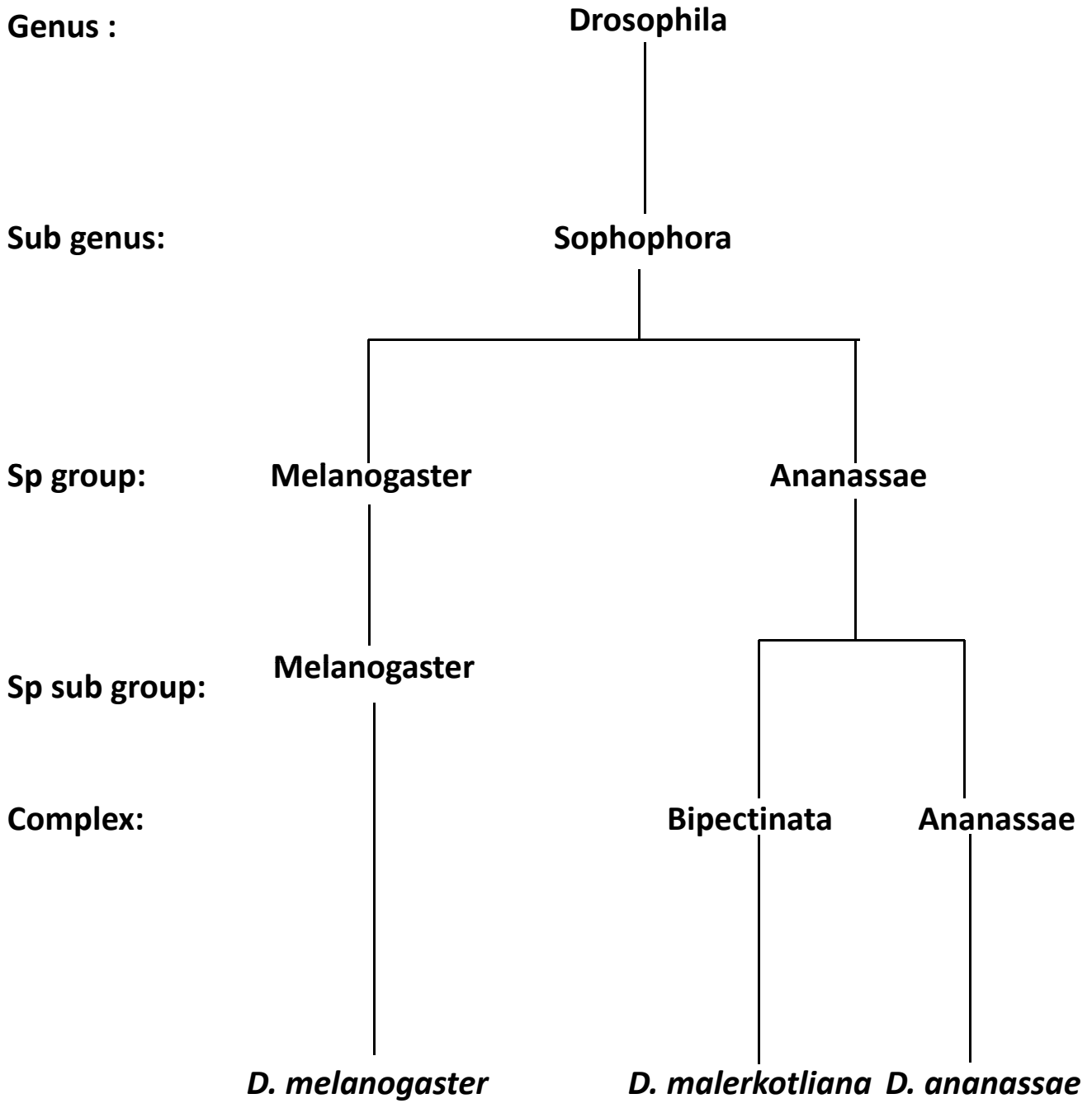
**Figure 1:** Phylogenetic tree of *Drosophilids* (*modified from Priya MP*).
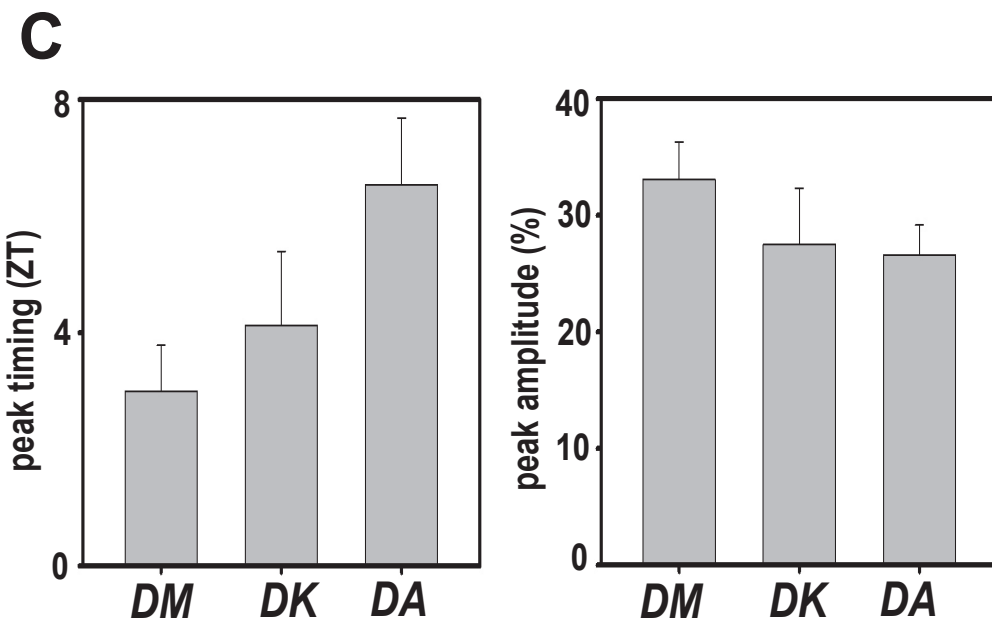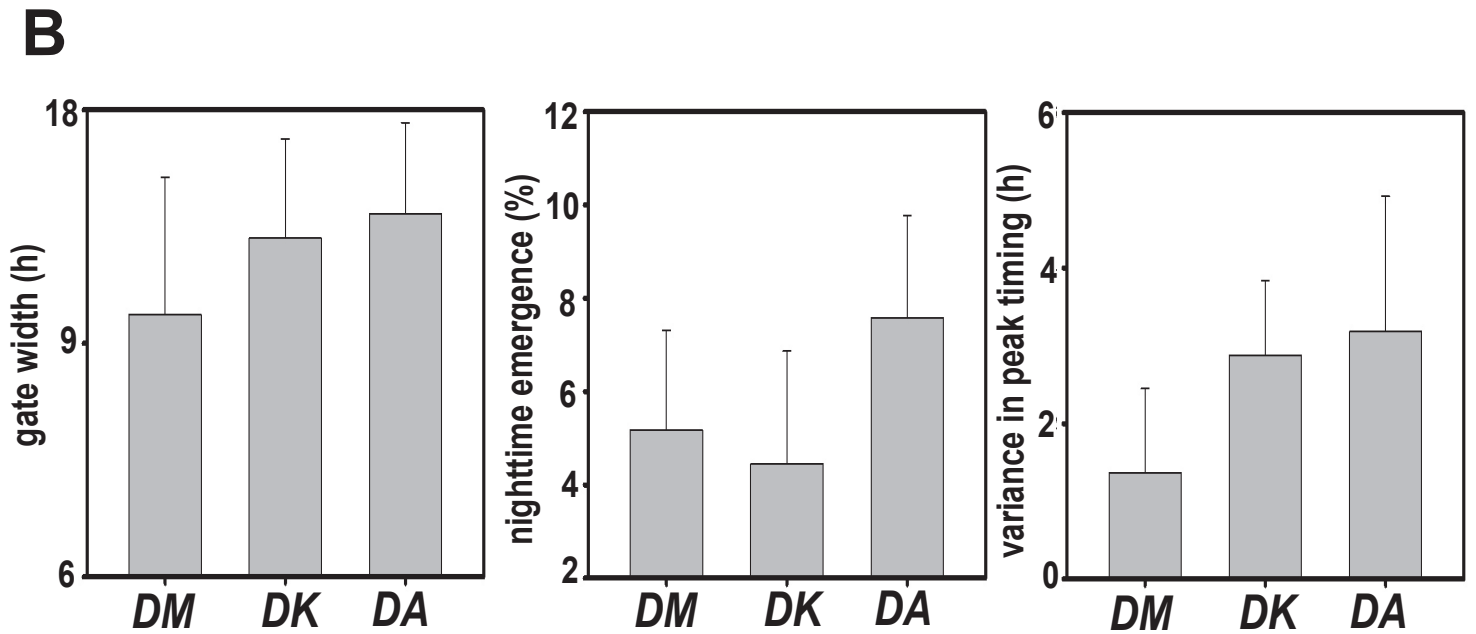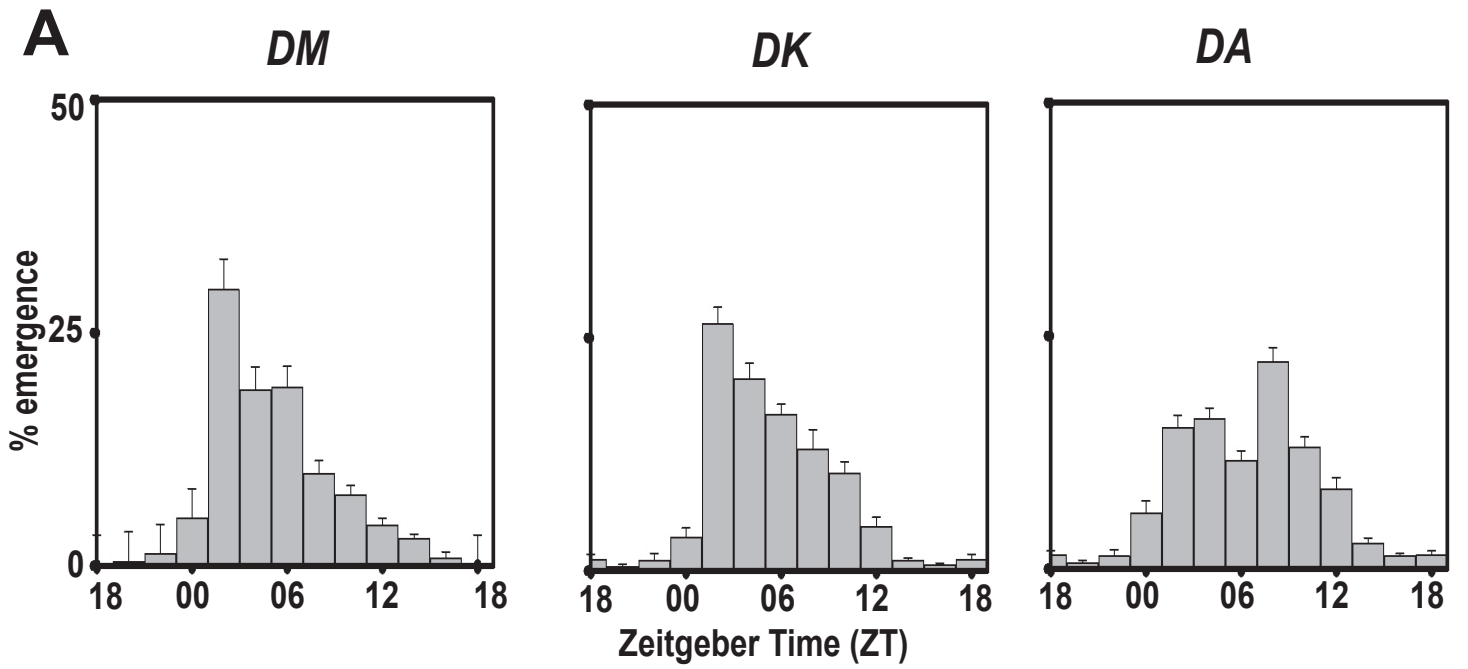
Figure 2: *LAB profiles of eclosion rhthm in the three species.* (A) Adult emergence rhythm profiles (for each strain averaged across 4-5 days and 10 vials) of *Drosophila melanogaster* (*DM*), *Drosophila malerkotliana* (*DK*) and *Drosophila ananassae* (*DA*) under laboratory 12:12 hr LD cycles. Zeitgeber Time 00 is lights ON (light phase: ZT00 - ZT12) and Zeitgeber Time 12 is lights OFF (dark phase: ZT12 – ZT00). The error bars indicate SEM. (B) Gate width of emergence (h), fraction of flies emerging during night (%), day-to-day variance in peak timing (h), peak timing (h) and peak amplitude (%) have been quantified for the three species under laboratory 12:12 hr LD cycles. Error bars are 95% Confidence Intervals (95% CI).
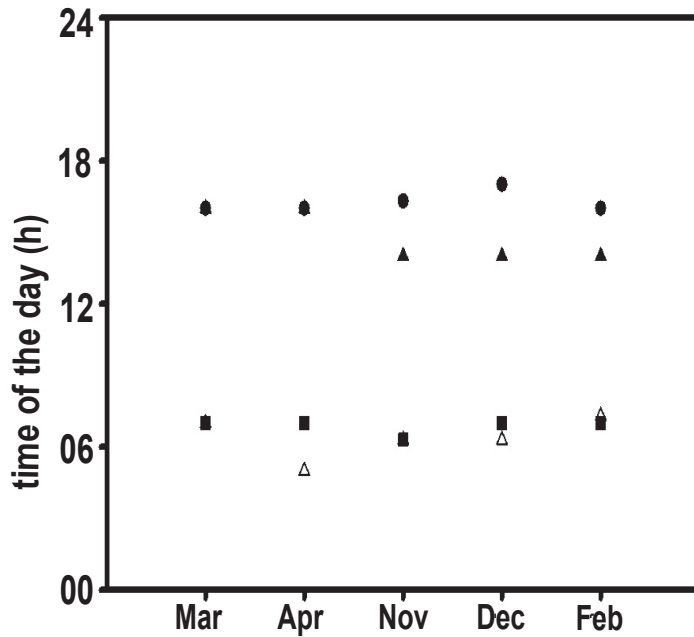
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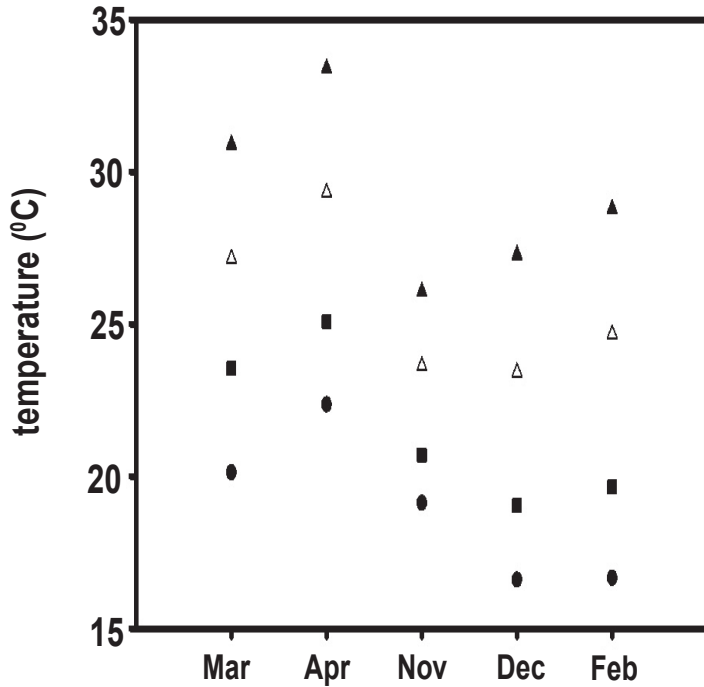
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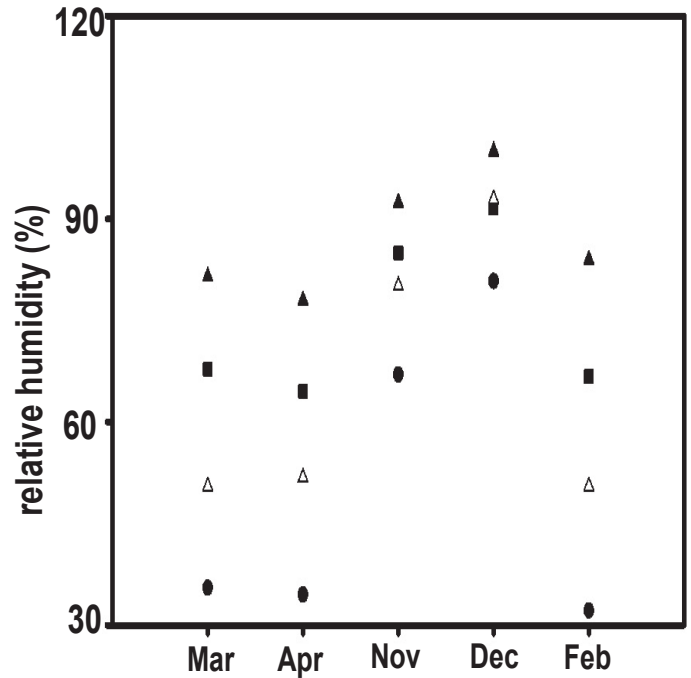
● = *temperature minimum*

■ = *average nighttime temperature*

△ = *average daytime temperature*

▲ = *temperature maximum*

● = *humidity minimum*

■ = *average nighttime humidity*

△ = *average daytime humidity*

▲ = *humidity maximum*

phase of environmental markers

■ *light onset*

△ *temperature trough*

▲ *temperature peak*

● *humidity trough*

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Figure 3: *Seasonal variations in the environmental parameters* (A) The temperature and humidity conditions in terms of minimum, daytime average, nighttime average and maximum temperature and humidity, are shown across five months. (B) Phases of four environmental markers; light onset, temperature trough, temperature peak and humidity trough are shown across months.
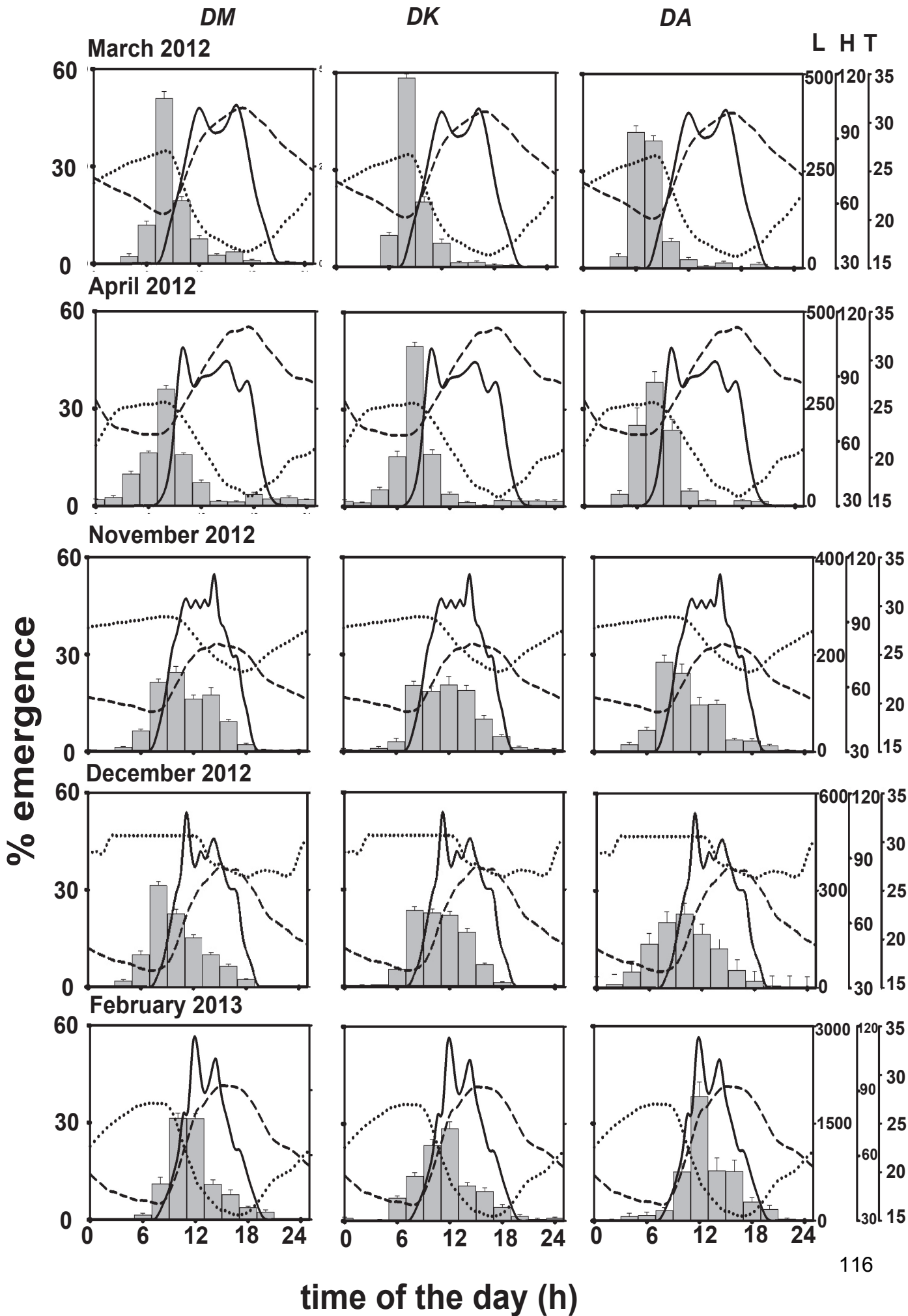
Figure 4: *Eclosion rhythm profiles under SN across months.* Adult emergence rhythm profiles (for each strain averaged across 4-5 days and 10 vials) of *Drosophila melanogaster* (*DM*), *Drosophila malerkotliana* (*DK*) and *Drosophila ananassae* (*DA*) in five different months of the years 2012 (March, April, November and December) and 2013 (February). % emergence as a function of the time of the day is plotted. The error bars indicate standard error of the mean (SEM). Three environmental factors were recorded simultaneously: light intensity (lux), relative humidity (%) and temperature (^{o}C). Three separate axes on the right of the panels are for these environmental factors - L = light intensity (lux), H = relative humidity (%) and T = temperature (^{o}C).
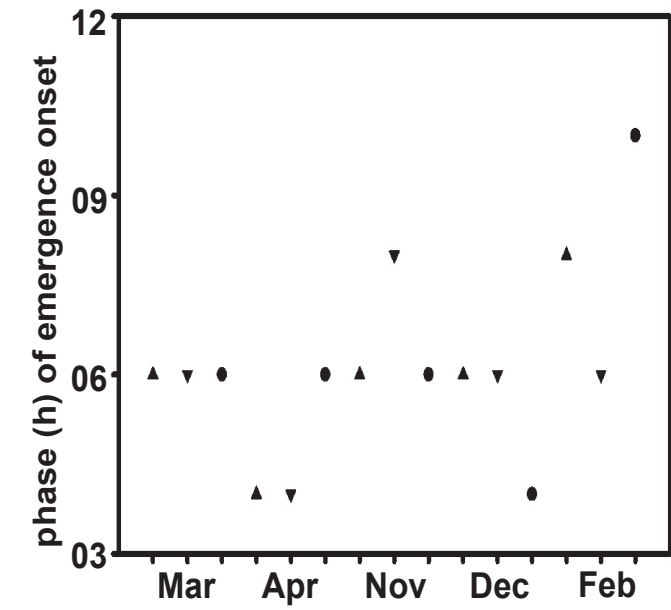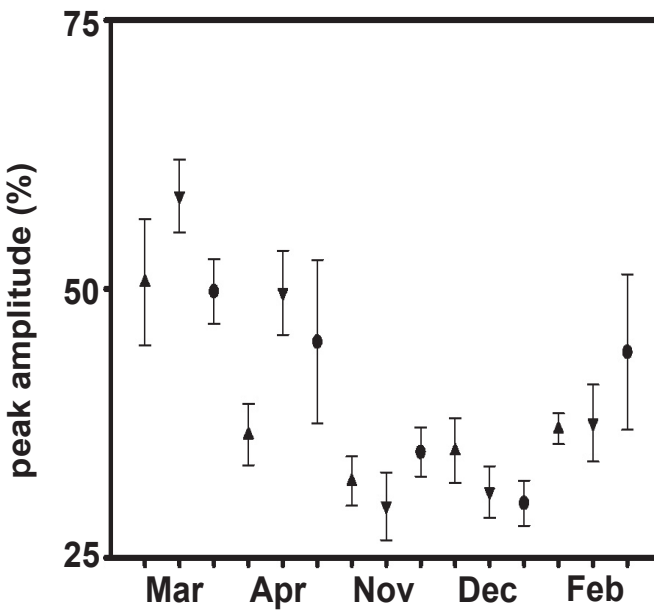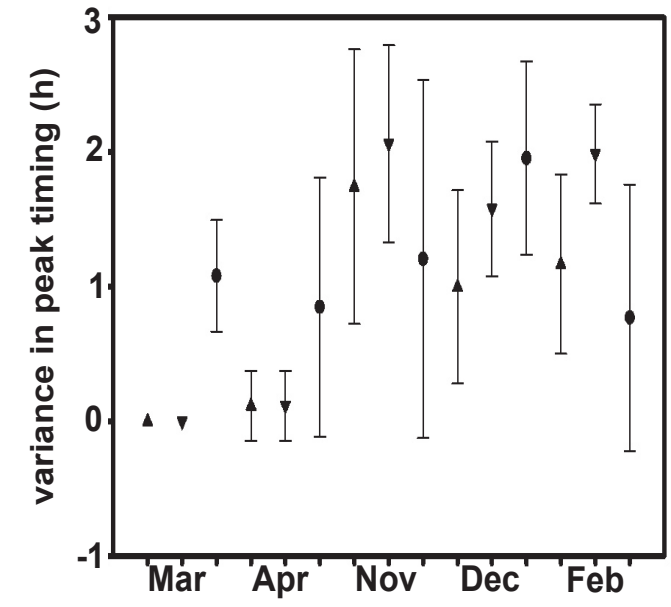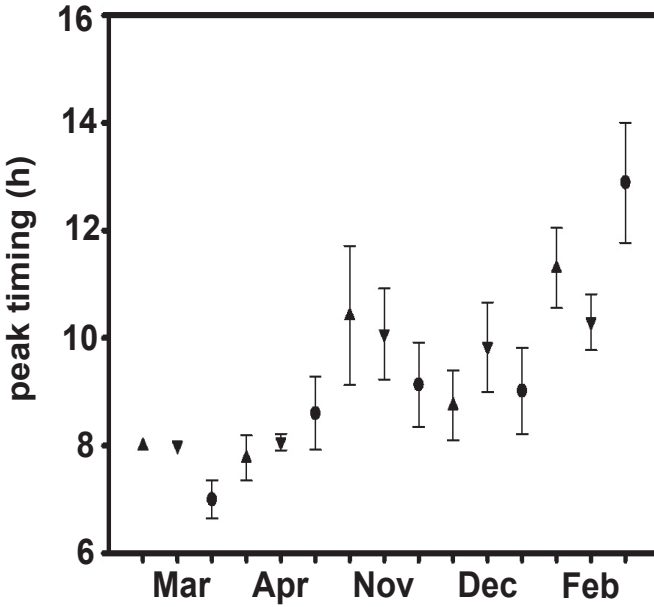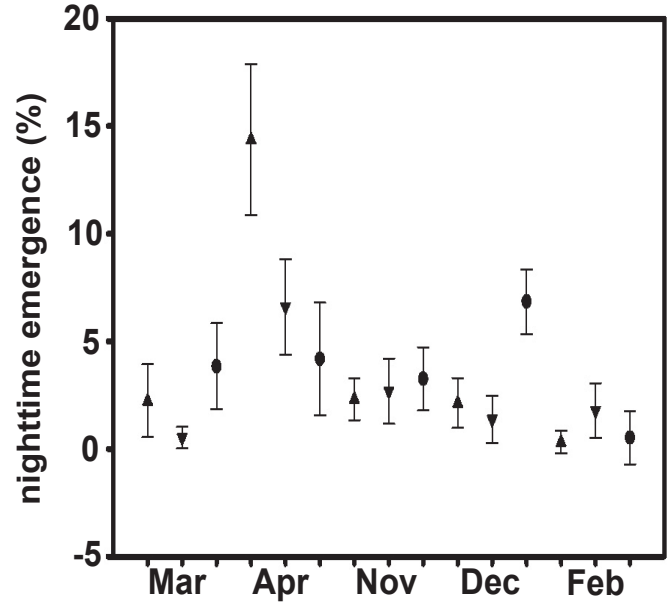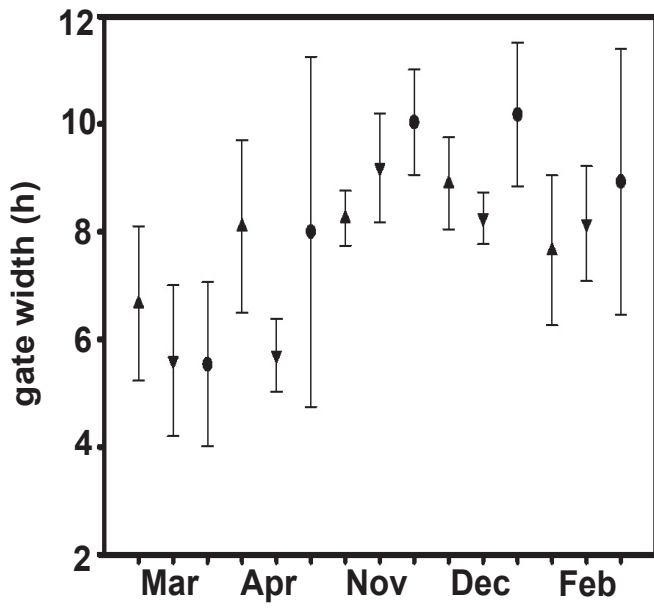
▲*Drosophila melanogaster* ▼*Drosophila malerkotliana* • *Drosophila ananassae*

Figure 5: *Seasonal variations in the parameters of adult emergence rhythm.* Gate width of emergence (h), fraction of flies emerging during night (%), day-to-day variance in peak timing (h), peak timing (h), peak amplitude (%) and phase of emergence onset (h) have been quantified for the three species across five different months under SN. Error bars are 95% Confidence Interval (95% CI). Phase of onset of emergence was estimated by qualitative assessment of the average profiles and therefore there are no error bars.

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