A Tale of Two Species:
Clock Properties and Sleep Characteristics of
Drosophila melanogaster and *Drosophila ananassae*

Thesis

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Master of Science

By

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Dedicated to

Ma, Tuffy, Pi, and Limo, and to the loving memory of Minky and her daughters

Table of Contents

Declaration

I hereby declare that the contents of this thesis entitled '**A tale of two species: clock properties and sleep characteristics of *Drosophila melanogaster* and *Drosophila ananassae***', submitted to Jawaharlal Nehru Centre for Advanced Scientific Research for the degree of Master of Science, is my original work, done under the guidance of Dr. Sheeba Vasu. Wherever any part of the presented content has described findings of other investigators, due acknowledgements have been made within the text and in the references. To the best of my knowledge, the references and acknowledgements listed are exhaustive. However, any omissions made in this regard may have occurred due to oversight or misjudgment, and I sincerely apologize for them.

Date: 07. 08. 2017

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7th August, 2017

Certificate

This is to certify that the work described in the thesis entitled '**A tale of two species: clock properties and sleep characteristics of *Drosophila melanogaster* and *Drosophila ananassae***' is the result of investigations undertaken by **Ms Pritha Kundu** under my supervision at Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree, or fellowship.

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> *"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to Heaven, we were all going direct the other way – in short, the period was so far like the present period, that some of its noisiest authorities insisted on its being received, for good or for evil, in the superlative degree of comparison only."*
>
> (Dickens, 1859)

Synopsis

Circadian clocks are endogenous time-keeping mechanisms which enable organisms to schedule their biological processes at appropriate times of the day, giving rise to the observable rhythms in occurrence of various behavioral and physiological processes, called circadian rhythms. In absence of an external time cue, circadian clocks show self-sustained oscillations with intrinsic periods that are close to, but often significantly different from 24 hours. They are sensitive to temporal changes in the environment, and thus, when present in a rhythmically changing environment, circadian clocks can adjust the period and phase of their oscillation such that particular phases of the overt rhythm can occur at specific phases of the environmental cycle, giving rise to a stable phase relationship between the circadian rhythm and the environmental cycle. The phase relationship is determined by intrinsic properties of the clock such that, inter-species differences in these properties may result in species-specific phase relationships of different circadian rhythms. Thus, studying the differences in clock properties that can influence features of the overt circadian rhythm is useful for understanding the mechanism of circadian clock function. This is discussed in detail in the first chapter of my thesis where I provide a brief introduction to circadian rhythms, properties and organization of circadian clocks, and discuss how circadian clocks may be adaptive and how they can time biological processes at specific phases of the environmental cycle. In this chapter, I further discuss the studies of circadian rhythm in Drosophilid species and what they have revealed about the structure and function of circadian clocks, and state the rationale for my present study.

Previously in our laboratory, a comparative study of the circadian rhythm in activity/rest behaviour of two sympatric Drosophilid species, *Drosophila melanogaster* (DM) and *Drosophila ananassae* (DA), showed that the phasing and waveform of the activity/rest

rhythm is distinct in these species, indicating that the underlying circadian clocks are different. DM has a crepuscular timing of activity with high activity around dawn and dusk, while DA shows a diurnal activity pattern, restricting its activity to the light phase of a light-dark cycle, and this temporal pattern of activity in DA was shown to persist under a wide range of environmental conditions. The present study aimed to explore the differences in clock properties of these two species that may influence the differences observed in their activity/rest rhythm. In this regard, I examined clock properties like, the intrinsic period, amplitude, circadian photosensitivity, and strength of inter-oscillator coupling. The results show that the clocks of DM and DA do not have significantly different intrinsic period in constant darkness, even though DA shows delayed phase of morning peak as compared to DM. DA has a low amplitude overt rhythm as compared to DM. The differences in activity/rest rhythm were also found to be accompanied by differences in circadian photosensitivity, phase response of the clock, and possibly, in strength of inter-oscillator coupling. These results are discussed in detail in the second chapter of the thesis.

The differences in the activity/rest rhythm of DM and DA are also reflected in their pattern of sleep, which is a physiological process known to be regulated by the circadian clock as well as a homeostatic mechanism. While the timing of sleep-wake rhythm is under circadian control, homeostatic mechanisms regulate the quality of sleep. Sleep pattern of DM and DA are different, indicating that the underlying circadian as well as homeostatic mechanism may be different in the two species. Before examining the mechanistic differences underlying sleep regulation in the two species, it is necessary to have a systematic characterization of the behaviour in DM and DA. Previously in our laboratory, a preliminary characterization of sleep in DA has been done only for virgin males of this species. However, sleep in DM shows sexual dimorphism and is also affected by the mating status. Thus in the present study, I also characterized and compared the features of sleep in

DA and DM taking into account the sex and mating status of the individuals. Unlike DM, in DA, the sleep pattern was not found to be affected by mating status or sex. Overall, consistent with results of the previous study, DA showed lower day time sleep and higher night time sleep as compared to DM. Sleep in DA was also less consolidated during the day and more consolidated during the night as compared to sleep of DM. I also find that for the same strength of mechanical perturbation, a larger fraction of sleeping DA flies are aroused both during the day and during the night as compared to DM, suggesting that DA sleep is possibly deeper than DM sleep at these time points. The third and final chapter of my thesis discusses the results of this study.

Chapter I

Introduction

1.1 Circadian rhythms

For centuries, it has been observed that behavioral and physiological processes of most organisms occur in a rhythmic manner with different periodicities (see Daan, 2010). Trees flower and fruit at species-specific seasons every year, marine organisms with inter-tidal habitats show behavioral processes that occur periodically according to tidal rhythms, petals of flowers open and close rhythmically at particular times of the day, different animal species remain active at specific times of the day, and so on (Dunlap et al., 2004). These biological rhythms were thought to arise as a result of the organism's response to the cyclic temporal changes in its environment.

One of the first systematic documentations of the possible endogenous origins of such a biological rhythm was done by a French astronomer De Mairan in 1729 (De Mairan, 1729; Pittendrigh, 1965) who studied the daily, rhythmic movement of leaves in the *Mimosa* plant. Persistence of rhythmic leaf movements in an environment devoid of cyclic changes in light suggested that this rhythmic behaviour has an endogenous origin and is not a simple passive response to periodic changes in the environment. Subsequently, experiments over decades have provided support for the endogenous nature of such rhythms (Kleinhoonte, 1929; Bunning and Stern, 1930; see also, Daan, 2010). Under constant conditions, the persistent rhythm does not have a periodicity exactly equal to that of Earth's rotation, suggesting that

some endogenous mechanism is responsible for generating the rhythm (Pittendrigh, 1965). These near-24 hour period oscillations in biological processes were called *circadian* rhythms (Latin. *circa* = about, *dies* = day) for the first time by Franz Halberg (Pittendrigh, 1965). Circadian rhythms were found to be present in diverse physiological, metabolic, and behavioural process in all the taxa studied so far, ranging from cyanobacteria, fungi, plants, and animals, and were shown to be an innate response (Aschoff, 1954; Pittendrigh and Bruce, 1957; Bunning, 1958; Daan, 2010; Sheeba et al., 2001). These rhythms were shown to have a genetic basis as early as 1932 when Erwin Bunning found that crossing bean plants with short and long period of circadian rhythm produced progeny whose period values showed a normal distribution around the mean period value of both parents (Bunning, 1973).

1.2 Circadian clocks

The endogenous time-keeping mechanism that generates overt circadian rhythms, is referred to as the circadian clock. Studies in the past century have elucidated certain general features of circadian clocks (Pittendrigh and Bruce, 1957; Pittendrigh, 1960), which are: (1) circadian clocks possess an intrinsic period, called the free-running period (FRP), which is close to, but significantly different from 24 hours, and is manifested in the overt circadian rhythm when the organism is present in an environment devoid of time-cues. The intrinsic period shows inter-individual as well as inter-species variation. (2) They are sensitive to time-dependent changes in the external and/or internal environmental cycles, as a result of which, when present in a rhythmically changing environment, clocks can *entrain* to these environmental cycles i.e., they are able to adjust their period and phase so that particular phases of the overt rhythm occur at particular phases of the environmental cycle (Pittendrigh, 1960). Rhythmic changes in the environment (e.g. light-dark, temperature, and humidity cycles) can serve as time cues which the circadian clock can use to schedule

biological processes, and are thus called *zeitgebers* (German. *zeit* = time, *geber* = giver). When entrained to a zeitgeber, the period of the clock matches that of the zeitgeber. The *entrainment* of circadian clocks is discussed in greater detail later in the chapter. (3) Circadian clocks are temperature compensated, i.e., the FRP does not change drastically with changes in environmental temperature within physiological limits. (4) Exposure to light or temperature pulses of short duration can perturb the phase of the free-running circadian rhythm towards an advanced or delayed phase. The magnitude and direction of such phase-shifts depends on the phase of the rhythm at which the exposure occurred as well as on the intensity and duration (i.e. the strength) of the pulse. Thus, a Phase Response Curve (PRC) can be constructed for a circadian clock which depicts the magnitude and direction of these phase-shifts in the rhythm as a function of the phase of the rhythm at which the pulse occurred. (5) When phase-shift occurs due to exposure to brief light or temperature pulses, the phase of the rhythm may continue to shift for a few cycles before reaching a new steady-state. The intermediate cycles required to attain the steady state are known as transients. Such features of the circadian clock, which are quite similar to those of self-sustained physical oscillators, led to the proposition that the function of circadian clocks is also similar to those of physical oscillators, an idea which has been widely supported by further experiments (Pittendrigh and Bruce, 1957).

1.3 Number and location of circadian clock(s)

It has been observed that individual organisms show more than one circadian rhythm, each manifesting in different physiological or behavioral processes. In mammals, for instance, locomotor activity, body temperature variation, corticosteroid levels, etc. show circadian rhythms. Each of the rhythms within an individual has their own temporal relationship with the external environment as well as with other rhythms, thus leading to an internally

synchronized state. The hypothesis, that there are multiple oscillators within an individual, each of which governs a different rhythm (Pittendrigh, 1960), has been shown to hold in case of metazoan circadian systems which have been studied. When individuals were subjected to aperiodic environment, the different rhythms, which were otherwise in mutual synchrony, showed desynchronization from each other and free-ran with their inherent periodicities (Aschoff, 1965; Sulzman et al., 1979). In other cases, circadian rhythms have been shown to show a phenomenon called *splitting*, whereby a single rhythm splits into two or more component rhythms each of which free-runs with their own periodicity (e.g. locomotor activity rhythm in rodents upon changing light intensity of constant illumination; Pittendrigh, 1960; Hoffman, 1971). Such observations provided indirect evidence for existence of multiple oscillators within an individual that govern different circadian rhythms. So where are these clocks located within an organism?

In many species of multicellular organisms, discrete anatomical regions in the nervous system and/or other parts of the body have been implicated to be the possible location of circadian clocks (Pittendrigh, 1981a). The suprachiasmatic nuclei (SCN) in the hypothalamus of mammals (Ralph et al., 1990), optic lobes in cockroach and cricket (Page, 1982; Tomioka and Chiba, 1984; see also Helfrich-Förster et al., 1998), pineal gland in sparrows (Zimmerman and Menaker, 1979), eyes of *Aplysia* (Jacklet, 1969) are known to house groups of cells which show self-sustained circadian oscillations and are believed to be the site of circadian clocks in these species.

Furthermore, circadian oscillators have also been shown to exist in peripheral organs of insects (Giebultowicz, 1999; Plautz et al., 1997). For instance, cockroach epidermis shows a rhythm in cuticle secretion when observed *in vitro* (Weber, 1995). The first "clock" gene to be discovered (*period*, in *Drosophila melanogaster*; Konopka and Benzer, 1971) was subsequently shown to have rhythmic expression in various central and peripheral tissues

of *D. melanogaster*, suggesting the existence of multiple, self-sustained oscillators (Plautz et al., 1997). Thus, apart from oscillators in the central nervous system, peripheral oscillators also exist in non-nervous tissues within an individual. How are these multiple oscillators organized to form the circadian system?

1.4 Organization of the circadian system

A multi-oscillatory clock organization has been found in all the metazoan species studied although there is wide variation across species. Despite several exceptions to the rule, the circadian system can be thought to be composed of three distinct components, input pathways that are sensitive to environmental factors, a central pacemaker or oscillator that integrates the input and keeps time, and output pathways which convey the time information from the central pacemaker and bring about the overt rhythms.

How do multiple oscillators interact with each other and respond to the external environment to bring about the overt rhythms? In order to answer this, various models have been proposed (Pittendrigh, 1974) which consider the different ways in which the oscillators may be interacting in order to generate the overt rhythms.

The *hierarchical model* of circadian organization posits that one central oscillator is responsible for controlling the phase of the oscillations in the peripheral oscillators which, in turn, bring about overt rhythms. In this case, the "master oscillator" does not receive any feedback from the peripheral oscillators i.e., the interaction is "unilateral". The master oscillator is sensitive to inputs from environmental time cues. Thus, one oscillator entrains to environmental zeitgebers and the others entrain to this oscillator. This model has been shown to hold in case of the circadian system of various species including that of mammalian species. In mammals, SCN can entrain to light-dark cycles and can, in turn,

entrain the peripheral oscillators and the overt rhythms that they drive (Yamazaki et al., 2000; Yoo et al., 2004). In *Drosophila pseudoobscura*, the gating of eclosion was thought to be regulated by a hierarchically interacting pair of coupled oscillators (Pittendrigh, 1974). The 'A-oscillator' is entrained by the light cycle and it in turn, entrains the 'B-oscillator', which is involved in the actual gating of eclosion.

The *non-hierarchical* model, on the other hand, proposes that each oscillator is able to entrain to environmental time cues as well as to each other in order to achieve the required temporal organization. Studies suggest such an organization may be present in circadian systems of *Drosophila* (Plautz et al., 1997), cockroach (Nishiitsutsuji-Uwo and Pittendrigh, 1968), and zebrafish (Whitmore et al., 1998) amongst that of other species (Pittendrigh, 1974).

While the gating of eclosion rhythm in *Drosophila pseudoobscura* is believed to be under the control of hierarchically interacting pair of oscillators (Pittendrigh and Bruce, 1959), the locomotor activity rhythm in *Drosophila melanogaster* seems to be regulated by a pair of oscillators which interact non-hierarchically (Grima et al., 2004; Stoleru et al., 2004). Thus, the organization of the circadian system may involve elements of both hierarchical as well as non-hierarchical interactions, depending on the overt rhythm being considered. Also, the organization of oscillators governing the same overt rhythm may involve elements of both the kinds of organization discussed above (Pittendrigh and Bruce, 1959).

1.5 Why do organisms have circadian time-keeping systems?

Circadian clocks are seen to have a ubiquitous existence as a biological time-keeping mechanism and inherent periodicities of circadian clocks are very similar to that of environmental cycles produced due to rotation of the Earth. Moreover, the molecular

mechanisms (discussed later in the chapter) underlying the generation of circadian rhythms appear to follow certain general principles and remain largely conserved across taxa. These findings suggest that circadian systems probably have an adaptive significance for organisms that possess it, and are likely to have been shaped by natural selection. Charles Darwin had suggested that the daily leaf movement of plants may be an adaptation against the deleterious effect of constant illumination on chlorophyll bearing leaves (Darwin, 1881). Since then, numerous studies have hinted at the possible adaptive role of circadian clocks. Three, mutually non-exclusive major hypotheses have been proposed, and are being put to test, regarding the possible adaptive benefits of circadian clocks (reviewed in Vaze and Sharma, 2013).

Circadian resonance hypothesis (Pittendrigh and Bruce, 1959) was put forth to explain the benefit of possessing clocks with near-24 hour periodicities. It suggests that, when the endogenous period of the circadian clock is similar to, or matches the period of the environmental cycle that it is entraining to, the amplitude of the clock is enhanced, which results in better time-keeping abilities of the clock.

Extrinsic advantage hypothesis suggests that circadian clocks can reliably schedule different biological processes at specific times of the day such that maximum survival and reproductive benefit can be gained (Aschoff, 1964). Thus, circadian clocks enable organisms to attain an optimal temporal niche in the environment by entraining to external environmental cycles.

Intrinsic advantage hypothesis (see Pittendrigh, 1993) suggests that circadian clocks have an adaptive advantage as they bring about a temporal order *within* the organism by adjusting the timing of the different metabolic and behavioral processes such that they occur in concert

with one another in a coordinated manner, resulting in the physiological well-being of the organism.

1.6 How do circadian clocks keep time?

Adaptive advantage that can be associated with having an innate and robust time-keeping mechanism should arise from the ability of the mechanism to reliably convey time information. So, how do circadian clocks time biological processes at particular times of the day? As stated previously, the oscillations driven by circadian clocks are not mere passive responses to rhythmic environmental conditions, i.e. the environmental rhythms do not *force* circadian oscillations. Oscillators within organisms are able to *couple* to environmental oscillations (e.g. external light-dark cycles, daily variation in temperature, etc.) and the system can be conceptualized as a pair of coupled oscillations (Pittendrigh, 1974). In this case, the coupling is unilateral, whereby, the circadian clocks, which are sensitive to zeitgebers (environmental rhythms), are able to change their period and phase of oscillation such that the period of the clock matches that of the zeitgeber. This process is known as entrainment of the clock to the zeitgeber. As a consequence of entrainment, the rhythms driven by the clock attain a stable *phase relationship* with the zeitgeber i.e. particular phases of the circadian rhythm occur at specific phases of the zeitgeber, thus timing the biological process at specific times of the day. Entrainment is different from *synchronization* in the sense that synchronization is a phenomenon where the waveform of the circadian rhythm coincides with that of the zeitgeber (see Johnson et al., 2003), whereas, the circadian rhythm need not do so when the clock is entrained to the zeitgeber (which is evident from the existence of stable phase relationships).

A circadian oscillator is considered as entrained to a zeitgeber when (a) there is *period match* i.e. the period of the circadian clock matches that of the zeitgeber, (b) a stable and

reproducible phase relationship exists between the two oscillations i.e. a phase in the oscillation of the clock occurs at a specific phase of the zeitgeber, and this phase relationship stays same over repeated cycles of oscillation, and across experiments, and (c) there is *phase control* i.e. upon removal of the entraining zeitgeber, the circadian clock starts to free-run from a prior phase that is determined by the zeitgeber (see Daan and Aschoff, 2001).

How does entrainment occur? In order to address this question, the following two major models were proposed. The *discrete model* of entrainment (Pittendrigh and Minis, 1964; Pittendrigh, 1965; Pittendrigh and Daan, 1976a; Pittendrigh, 1981b) posits that perturbation of the circadian oscillation by zeitgebers at particular phases can cause the phase of the circadian oscillation to shift (advance or delay) such that the phase-shift is equal to the difference in periodicities of the circadian clock and that of the zeitgeber (Phase-shift = FRP – *T*, where *T* is the period of the zeitgeber). The phase resetting thus results in period matching. The model requires that the phase of the circadian clock oscillation be sensitive to perturbations by a zeitgeber in a phase-dependent manner. The phase of the oscillation at which the zeitgeber perturbation can bring about the required phase-shift can be known from the Phase Response Curve (PRC) of the clock, as discussed previously. Stable-phase relationship can be attained by such a mechanism of entrainment as the phase-shift required for stable entrainment will be determined by the FRP of the clock. Clocks with different FRPs will require different phase-shifts and hence, the zeitgeber would need to fall on distinct phases of the oscillation (as determined by the PRC) depending on the FRP. Thus, clocks with different FRPs are expected to have distinct phase relationships with the zeitgeber. The discrete model of entrainment has been useful in explaining entrainment of some organisms like *Drosophila pseudoobscura* and nocturnal rodents (Pittendrigh, 1965; 1981b; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976a).

The discrete model assumes that the zeitgeber does not affect the FRP of the circadian clock, and entrainment occurs by changes in only phase of the clock which is why this model is also referred to as the *non-parametric* model. However, the FRP of the clock has been reported to undergo changes along with the phase when exposed to light pulses that produce single phase-shifts (reviewed in Daan and Aschoff, 2001), facilitating the construction of a Period Response Curve. Furthermore, this model implies that discrete time-cues are sufficient to entrain the clock. For example, entrainment to light-dark cycles can be brought about by dawn and dusk transitions. However, the role of continuous presence of light during the rest of the photophase needs to be considered as well. In many organisms, especially mammals, continuous presence of light is known to modulate the FRP of the clock (Aschoff, 1960) which is manifested as *aftereffects* of the entraining cycle on the intrinsic period (see Daan and Aschoff, 2001). Based on the observations that the FRP can be modulated by the tonic effects of light, the *continuous model* of entrainment was proposed (Aschoff, 1960; see also Daan and Aschoff, 2001). This model suggests that the circadian clocks can entrain to light-dark cycles by changing the FRP due to tonic effects of light. This is also known as the *parametric model* of entrainment as it suggests that entrainment is occurring by modifying a parameter (FRP) of the clock.

1.7 Unravelling the proximate and ultimate principles underlying circadian clock function with *Drosophila spp.*

A large body of work in circadian biology involving the mechanism of clock function (proximate questions) as well as the functional significance of clocks (ultimate questions) in poikilotherms has revolved around studies in different species of fruit fly *Drosophila.* This is partly because circadian rhythms observed in large number of diverse physiological and behavioral processes in *Drosophila* offers a robust and easily assayable system for

studying the underlying oscillators. Moreover, being a sufficiently simple system to study, it is amenable to molecular genetic manipulation using the host of tools available, which offers the opportunity to study the potential genes, proteins, molecular mechanisms as well as the neuronal groups involved in the circadian machinery (reviewed in Simpson, 2009). The following discussion elucidates the results of various studies on the circadian system in different *Drosophila* species, with emphasis on those in *Drosophila melanogaster*.

Circadian rhythms in *Drosophila*

The emergence of adult flies from pupae (eclosion) was shown to be under the control of circadian oscillators in *Drosophila pseudoobscura* (Pittendrigh, 1954) and extensive study of the circadian rhythm of eclosion in this species has provided seminal insights into the mechanism of circadian clock function. Other Drosophilid species were also shown to exhibit circadian rhythm in eclosion (Myers et al., 2003; Prabhakaran and Sheeba, 2013a). Circadian rhythm in the activity/rest pattern of *Drosophila sp.* is one of the most extensively studied rhythms and is discussed in detail in subsequent sections. Sleep-wake cycle in *Drosophila melanogaster* is another such widely studied circadian rhythm. Numerous other biological processes that are regulated by circadian clocks include, but are not limited to, feeding behaviour (Xu et al., 2008), courtship and mating behaviour (Sakai and Ishida, 2001; Fujii et al., 2007), and egg-laying rhythm (Sheeba et al., 2001; Howlader and Sharma, 2006).

Drosophila melanogaster has emerged to be one of the most popular organisms for studying the underlying molecular mechanisms and neuronal circuits of circadian clocks governing many of the above-mentioned rhythms and is discussed in the following sections.

Molecular mechanisms of circadian clock function in *Drosophila melanogaster*

The molecular mechanisms that regulate overt circadian rhythms are brought about by interactions between products of "clock" genes. Numerous clock genes have been identified till date which include *period* (*per*) (Konopka and Benzer, 1971), *timeless* (*tim*) (Sehgal et al., 1994), *clock* (*clk*) (Allada et al., 1998), *cycle* (*cyc*) (Rutila et al., 1998), *doubletime* (*dbt*) (Price et al., 1998), *cryptochrome* (*cry*) (Stanewsky et al., 1998), *shaggy* (*sgg*) (Martinek et al., 2001), *casein kinase* 2 (*CK2*) (Akten et al., 2003), and several others (reviewed in Hardin, 2011). The products of these genes serve diverse functions. Some are transcriptional activators or repressors, while there are others, which alter protein stability, act as degraders, etc. The function of circadian oscillators is believed to be maintained by rhythmic transcription-translation feedback loops. The transcription of genes is regulated by its protein products. There is a post-translational regulation of the levels and the sub-cellular localization of protein products such that a rhythmic transcription of genes occurs (Hardin, 2005). The two important intracellular feedback loops in gene expression that occur in this regard are the PER/TIM loop and the CLK/CYC loop (Hardin et al., 1990; Glossop et al., 1999).

The DNA-binding heterodimer CLK/CYC binds to the specific target promoters and drives the expression of *per* and *tim* from mid-day to early in the night (Darlington et al., 1998; Hao et al., 1997; McDonald et al., 2001; Wang et al., 2001). The levels of *per* and *tim* transcripts peak during early night but the corresponding protein levels do not do so until late at night (Hardin et al., 1990; Zerr et al., 1990; Edery et al., 1994; Sehgal et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). This delay is caused by a destabilization of PER by DBT mediated phosphorylation and stabilization of PER-DBT complex by TIM (Price et al., 1998; Kloss et al., 1998; Akten et al., 2003; Nawathean and Rosbash, 2004). In the PER-TIM-DBT complex, SGG phosphorylates TIM (Meissner et

al., 2008) while PER is phosphorylated by CK2 (Price et al., 1998). Following this, the complex is translocated to the nucleus where PER represses the CLK-CYC dependent transcription (Jui-Ming et al., 2002; Akten et al., 2003; Martinek et al., 2001; Kloss et al., 2001; Ashmore and Sehgal, 2003; Shafer et al., 2002). TIM is degraded in light dependent manner via CRY binding (Ceriani et al., 1999; Naidoo et al., 1999; Busza et al., 2004). PER-DBT complex is then degraded via ubiquitin-proteosome pathway in the early part of the day, thus relieving the repression of CLK-CYC dependent transcription (Lee et al., 1996; Zeng et al., 1996; Grima et al., 2002; Ko et al., 2002). Such transcription-translation feedback loops thus give rise to oscillations in the mRNA and protein levels of the core clock genes, driving the molecular clock within the cells.

Neuronal circuit regulating activity/rest rhythm in *Drosophila melanogaster*

Anatomical identities of central pacemaker neurons: The potential neuronal components of the circadian machinery have been identified using cytological staining for presence of clock gene products (Zerr et al., 1990; Ewer et al., 1992; Kaneko et al., 1997; reviewed in Taghert and Shafer, 2006). Among the 100,000 neurons estimated to be present in the brain of *D. melanogaster*, the central pacemaker is thought to comprise about 150 neurons (Nitabach and Taghert, 2008; Kaneko and Hall, 2000; Shafer et al., 2006; Rieger et al., 2006). These circadian neurons are divided into subsets based on their anatomical location and include the ventrolateral neurons (LN) and the six dorsal lateral neurons (LNd), three lateral posterior neurons (LPN), dorsal neurons (DN1), DN2, and DN3. Based on their size and gene expression, the LNv are classified into small (sLNv) and large (lLNv). Four out of the five sLNvs express the neuropeptide PDF (Pigment Dispersing Factor) and hence the sLNvs are PDF positive or PDF negative. A subset of the LNds express CRY, while the anterior

DN1 (DN1a) express IPNamide and CRY and posterior DN1 (DN1p) express only CRY. All of these neurons express PER (see Dubruille and Emery, 2008).

Light input pathways: Clock neurons in *Drosophila* can entrain to light inputs which may reach these neurons via three independent pathways (Ashmore and Sehgal, 2003): through compound eyes and ocelli (Stanewsky et al., 1998), Hofbauer-Buchner eyelets that are situated behind each compound eye (Veleri et al., 2003; Helfrich-Förster et al., 2001), and through blue light pigment CRY mediated photo transduction (Stanewsky et al., 1998; Emery et al., 1998). There may also be a CRY and compound eye-independent pathway (Stanewsky et al., 1998; Helfrich-Forster et al., 2001).

Neuronal circuits: Under 12 hour light and 12 hour dark cycles (LD 12:12), the activity/rest rhythm of *D. melanogaster* shows a bimodal profile with a peak in the morning and in the evening. There is an anticipatory behaviour, where the activity increases gradually prior to lights on and off. Based on the dual oscillator model of Pittendrigh and Daan (Pittendrigh and Daan, 1976b), distinct subsets of the clock neurons have been postulated to be the M and E oscillators which are coupled to dawn and dusk respectively, and regulate the corresponding morning and evening components of activity (Grima et al., 2004; Stoleru et al., 2004). The PDF expressing sLNvs are believed to constitute the so called "morning or M cells", whereas the PDF^{-ve} CRY^{+ve} cells, 3 LNds and the 5^{th} sLNv, have been implicated to be the "evening or E cells".

The sLNvs are required for maintaining activity/rest rhythms under constant darkness (DD) while the lLNvs are not believed to have significant role in DD (Grima et al., 2004). Molecular oscillations in the lLNvs appear to dampen under constant conditions (Yang and Sehgal, 2001; Lear et al., 2005; Lin et al., 2004; Veleri et al., 2003). The sLNvs strongly determine the amplitude and phase of morning activity under LD cycles (Grima et al., 2004;

Stoleru, 2004). It is speculated (Collins et al, 2005; Helfrich-Förster, 2007) that the lLNvs help in gating of light input. The LNds and 5th sLNvs are believed to be part of oscillators that play a role in regulating the evening activity peak (Grima et al., 2004; Stoleru et al., 2004). However, the PDF-expressing sLNv neurons were shown to also contribute to the evening activity (Rieger et al., 2006).

The clock neurons express various neuropeptides, of which PDF has been shown to be important for regulating various aspects of the activity/rest rhythm both under entrained as well as free-running conditions. The lLNvs and sLNvs except the 5th sLNv express PDF. When PDF-expressing clock neurons were selectively ablated, flies showed arrhythmic behaviour in DD and a loss of morning anticipation in LD conditions (Stoleru et al., 2004; Renn et al., 1999). *Pdf* null flies show a phenotype similar to that of the flies who lack PDF-expressing neurons. (Renn et al., 1999; Blanchardon et al., 2001). PDF over expression rendered flies arrhythmic in DD and some showed complex rhythms (Helfrich-Förster et al., 2000, Wülbeck et al., 2008). PDF shows a daily, gated release from the dorsal projections of the sLNvs, near the dorsal neurons (Park et al., 2000). However, some *Drosophila* strains showed rhythmic behaviour even though PDF-staining rhythms were not observed (Kula et al., 2006). A subset of the dorsal neurons express a receptor for PDF (Mertens et al., 2005; Lear et al., 2005). Shafer et al. (2008) showed that majority of dorsal neurons and the sLNvs seem to respond to PDF. In absence of PDF, the molecular oscillations show reduced amplitude and are not properly in phase, which indicates that PDF is required for coordinated functioning of different subsets of circadian neurons, bringing about persistent rhythms under constant darkness (Peng et al., 2003; Lin et al., 2004). Overall, the neuronal network regulating activity/rest rhythm in *D. melanogaster* is composed of distinct subsets of neurons, which express characteristic neuropeptides, and

seem to regulate specific features of the overt rhythm under different environmental conditions.

1.8 Studies of circadian system in other Drosophilid species

A large body of work in circadian biology, which focussed on the circadian system of *Drosophila melanogaster*, has provided major advances in the field, as discussed in the previous sections. However, in order to understand the general principles that underlie the structure and function of circadian systems, comparative studies of circadian systems across species are more useful. This is especially because comparative studies are capable of providing an understanding of the inter-species variation in the circadian systems and the factors that are likely to bring about such variation.

Circadian rhythms of a few Drosophilid species apart from that of *Drosophila melanogaster* have been studied previously. Different Drosophilid species, and even different strains of the same species have been found to show variations in the properties of circadian rhythms as well as in the underlying molecular and neural mechanisms, which appear to be correlated with differences in their geographic localization. Lankinen and colleagues (1993) found that strains of *D. pseudoobscura* show variation in the period and phase of their eclosion rhythm, and that this variation exhibits a latitudinal cline. Such intra-species latitudinal clines were also observed in eclosion rhythm of *D. auraria* strains (Pittendrigh and Takamura, 1989) and oviposition rhythm of *D. ananassae* strains (Satralkar et al., 2007). Inter-species studies have also shown variation in properties of activity/rest rhythm depending on the location which the species inhabited. Simunovic and Jaenike (2006) studied the activity rhythm of 11 Drosophilid species spread over latitudes ranging from 19°N to 60°S in North America and reported that the activity profile of the species differed

widely depending on the latitude as well as the microhabitats that they inhabited. Species from the higher latitudes showed greater midday activity as compared to the others. When species occupying similar latitudinal ranges were compared, the ones inhabiting wet microhabitats showed greater midday activity than those who were localized in woodlands. More recently, Menegazzi et al. (2017) have shown that four Drosophilids belonging to sub-group *virilis*, found in the higher latitudes, show distinctly different activity/rest profile as compared to *D. melanogaster*, which inhabits lower latitudes (Menegazzi et al., 2017; Kauranen et al., 2012; Bahn et al., 2009). The variation in activity/rest rhythm is along similar lines to that reported by Simunovic and Jaenike. The species from higher latitudes show deviations from the bimodal activity/rest profile seen in *D. melanogaster* in the sense that they have reduced morning activity, lack a midday siesta, and are able to prolong the evening activity under long photoperiods.

Much like latitude-dependent variation, altitudinal clines have also been reported to exist in circadian rhythms of Drosophilid species who inhabit similar latitudinal ranges. Khare et al. (2002) reported that the ability to entrain eclosion rhythm to LD cycles in high altitude Himalayan strain of *D. ananassae* (HA) was largely affected by the ambient temperature as compared to low altitude strains (LA) where such an effect was not observed. When subjected to conflicting conditions of LD and temperature cycles, with photophase and cryophase coinciding, the HA strain limited its eclosion to the thermophase in the dark while the LA strain showed eclosion in the cryophase when light was present. The HA strain continued to show preference for thermophase when subjected to temperature cycles, both in DD and LL, which was not the case for the LA strain. The authors suggest that due to the conflicting nature of environmental condition in the location which the HA strain inhabits (e.g. low temperature with high intensity light and little variation in photoperiod), the clock has adapted such that it predominantly responds to more reliable temperature cues.

The activity/rest rhythm of a high altitude strain of *D. helvetica* (haH) showed unimodal profile of activity rhythm with delayed onset of morning activity whereas, in a low altitude strain (laH), bimodal activity was seen (Vanlalhriatpuia et al., 2007). The authors have proposed that the difference in timing of activity may be explained in context of the different light and temperature profile that the two strains face in their respective habitats.

Comparative studies have also been useful in understanding how the underlying molecular and neuronal network of circadian systems vary with environmental conditions. For, instance, two studies by Majercak et al. (1999), and Low et al. (2008) showed how the core components of the circadian clock could interact with environmental factors in order to bring about significant differences in the overt rhythm. Majercak et al. (1999) showed that thermosensitive splicing of a 3`-terminal intron of *per* mRNA is important for various aspects of *D. melanogaster* activity rhythm, like prolonged midday siesta under high temperatures and an advanced evening peak under low temperature. Moreover, long photoperiod counteracted the advancement of cold-induced evening peak to some extent. Subsequently, Low et al. (2008) found that this is not the case for *D. yakuba* where, the splicing of 3`-terminal intron of *per* mRNA was robust under a wide range of temperatures and no thermal calibration was observed in the daily *per* mRNA profiles or in the activity profile of the species. The authors reasoned that *D. melanogaster*, which shows a wide distribution at most latitudes and altitudes including the temperate latitudes, faces high variation in temperature and day-length throughout the year, whereas *D. yakuba*, whose distribution is around the Afro-equatorial region, faces low variation in day-length and temperature. The authors suggested that the existence of temperature-dependent splicing in a core clock gene mRNA, which affects the timing of activity, helped in the adaptation of *D. melanogaster* to a wide range of temperate habitats, whereas in *D. yakuba*, the absence of such a mechanism is consistent with environmental conditions of its native habitat.

Bahn et al. (2009) found that differential expression of PDF in the central pacemaker neurons is partly responsible for the differences in activity profiles seen between *D. melanogaster* and a distantly related species *D. virilis.* Certain features of the activity/rest rhythm in *D. virilis* are similar to those observed for *pdf* null mutants in *D. melanogaster.* Activity profile of *D. virilis* lacks a morning peak of activity, and its activity is restricted to the light phase of the LD cycle. The species also exhibits arrhythmic activity/rest behaviour under DD. *D. virilis* PDF (DvPDF) expression was found to be absent in the sLNv neurons, suggesting that the role of sLNv neurons as "morning cells" and that of PDF in regulating activity/rest rhythm is not limited to only *D. melanogaster*. Moreover, *DvPdf* gene, when expressed in *D. melanogaster*, was able to drive expression of PDF in two PDF^{-ve} neuronal subsets (LNds and 5^{th} sLNv) as well as in all the endogenous PDF-expressing neurons of *D. melanogaster*, suggesting that some mechanism of inhibiting PDF expression in the LNds and 5^{th} sLNv must be in place in *D. melanogaster.* The authors also suggest that the expression of PDF in the sLNv of *D. virilis* is inhibited.

Kauranen et al. (2012) found that *D. montana,* which, like *D. virilis*, is also distributed in the higher latitudes, showed activity/rest behaviour similar to that found by Bahn et al. in *D. virilis.* Additionally, *D. montana* showed rhythmic behaviour even under high intensity light in LL, a condition that renders *D. melanogaster* activity/rest arrhythmic. *D. montana* showed similar expression pattern of PDF as that of *D. virilis.* Moreover, unlike *D. melanogaster,* CRY expression was not detected in lLNvs in *D. montana*, which may be the reason for their persistent rhythmicity in LL.

Menegazzi et al. (2017) also showed that the similarity in activity/rest behaviour of *D. ezoana* and *D. littoralis* with that of *D. virilis* and *D. montana* is accompanied by similarities in the expression pattern of CRY and PDF as well. Moreover, when the CRY and PDF expression pattern of these four species was mimicked in *D. melanogaster*, the activity/rest

rhythm of *D. melanogaster* under various environmental conditions resembled those of the species belonging to *virilis* group. It seems that the differential expression of neuropeptides and photopigments in the various neuronal subtypes underlies the differences observed in the circadian behaviour. This notion is further supported by the results from a study by Hermann et al. (2013). Comparison of the neuronal architecture of ten Drosophilid species by examining the expression of CRY, VRI, PDP1, and PDF molecules revealed that the organization of the neuronal network was similar in all the species studied.

Comparative studies have thus revealed that Drosophilid species exhibit variation in their activity/rest rhythm, which are correlated with the environmental conditions in their respective habitats. Some of these studies have also examined the underlying differences in neuronal network that are associated with the variations in the overt rhythm.

1.9 Rationale for the present study

Previously, the activity/rest rhythm of four Drosophilid species was studied under varying conditions of light and temperature, both in the laboratory and in semi-natural conditions (Prabhakaran and Sheeba, 2012, 2013b, 2014). These species were wild caught from the same geographical locations and maintained in the laboratory in large, outbred populations. Under LD 12:12, *D. melanogaster*, *D. malerkotliana*, and *Zaprionus indianus* were seen to have a bimodal activity profile with a morning and an evening peak around dawn and dusk respectively. In contrast, *D. ananassae* showed a unimodal activity profile with a morning peak, little or no activity in the evening, and restricted most of its activity to the light phase of the LD cycle. *D. ananassae* continued to restrict its activity to the light-phase under natural conditions of the environment, as well as when the natural conditions were simulated in the laboratory (Prabhakaran and Sheeba, 2013b, 2014).

Comparison of the circadian pacemaker neurons in these species revealed that the overall architecture is similar with regard to the broad neuronal subtypes that are present. Interestingly, *D. ananassae* and *D. melanogaster* showed differences in the number of cells present in some of the neuronal subsets, notably, the LNd, and the DN1, the neurons that are believed to be involved in regulating evening activity. Moreover, even though *D. ananassae* was reported to have a shorter FRP as compared to *D. melanogaster*, the former showed a delayed phase of morning peak than the latter. This is intriguing because, as mentioned in section 1.6, if entrainment is occurring by phase resetting of the clock, then clocks with different FRPs are expected to have different phase relationships under entrained condition, and clocks with shorter FRPs have earlier phases of entrainment as compared to those with longer FRPs (see Moore-Ede et al., 1982). Taken together, these results indicate that the clocks controlling activity/rest rhythm in *D. melanogaster* and in *D. ananassae* are different.

So what are the differences in the circadian clocks of the two species? The present study aimed to address this question.

As mentioned previously, comparative studies of circadian rhythms in Drosophilid species have identified components of the core pacemaker (neurons or neuropeptides) that may be responsible for the differences in the overt rhythm that are observed across species. At present, our knowledge about the neuronal network underlying the circadian clocks in the Drosophilid species that have been studied is not sufficient to understand, from a mechanistic view, how differences in the circadian clock function can bring about differences in the overt rhythm. In other words, even though there is some knowledge of the components that form the machinery of the clock, and what features of the overt rhythm they may be influencing, it is not enough to gain a holistic understanding of how the clock functions in its entirety.

In this regard, a classical chronobiological approach is useful, where the circadian clock is modelled after a physical oscillator. In doing so, experimentally testable predictions from theoretical studies can be used to explore estimable properties of this oscillator (e.g. FRP, sensitivity to zeitgeber, etc.) and how those properties affect the overt rhythm. Hence, a classical approach would be helpful for understanding how differences in circadian clock function can translate into inter-species differences in the timing and other features of overt rhythms.

Thus, in order to address the posed question, the present study aimed to examine whether there are differences in the properties of the circadian clocks in *D. melanogaster* and *D. ananassae*, as a first step to understanding if differences in clock properties can influence the differences in their activity/rest rhythm.

Chapter II

Clock properties of *D. melanogaster* and *D. ananassae*

2.1 Introduction

2.1.1 Introduction to *D. melanogaster* and *D. ananassae*

Drosophila melanogaster Meigen 1830 and *Drosophila ananassae* Doleschall 1858 belong to the sub-genus Sopophora and species group *melanogaster*. *D. melanogaster* (DM) is known to be a cosmopolitan species having an Afro-tropical origin. It has successfully colonized a wide range of geographic locations barring the extreme altitudes or latitudes (David and Tsacas, 1981; David and Capy, 1988). *D. ananassae* (DA) belongs to the species sub-group *ananassae*. DA was shown to have originated in Southeast Asia and ranges in distribution from tropical, subtropical, to mildly temperate regions (Das et al., 2004; Tobari, 1993; Dobzhansky and Dreyfus, 1943).

2.1.2 Previous studies

The study populations of DM and DA were obtained by capturing flies from the wild in Bangalore, India (described in Prabhakaran and Sheeba, 2012). The wild caught flies have been maintained in the laboratory as large, outbred populations (~1600 individuals) under LD 12:12 and constant temperature and humidity conditions for about 200 generations. Previous studies in these populations have found the activity/rest rhythm in these two species to be very different (Prabhakaran and Sheeba, 2012, 2013b, 2014). Under laboratory conditions of LD 12:12, DM shows a crepuscular timing of activity with the activity profile

showing two peaks, one in the morning around the time of lights-on and one in the evening around lights-off. DA, however, shows a diurnal timing of activity with the activity profile having one prominent peak in the morning after lights-on, and little or no activity in the evening. Moreover, the tendency of DA to restrict its activity to hours of the light phase persisted when observed under natural conditions as well as under simulations of natural conditions in the laboratory. The intrinsic period of the clock as assayed under constant darkness (DD), was reported to be slightly shorter in DA than in DM (Prabhakaran and Sheeba, 2012). Clocks with shorter FRP are expected to show earlier phase of entrainment as compared to clocks with longer FRP, but DA, with a shorter FRP, showed a morning peak whose phase was delayed as compared to that of DM. Furthermore, under long as well as short photoperiods, the evening bout of activity in DM became more pronounced as compared to the morning bout, whereas, in DA, the major fraction of activity was consistently seen towards the morning. If indeed different component oscillators within the circadian clock regulate the morning and evening activity in *Drosophila* (Pittendrigh and Daan, 1976b), this observation suggests that the organization of the clocks in DM and DA is likely to be different.

Taken together, the differences observed in the waveform and phasing of the activity/rest rhythm, and in the FRP indicate that the underlying circadian clocks in DM and DA are different. Thus, in the present study, I aimed to look at whether the clocks of DM and DA are indeed different, and if so, then in what way(s). In order to do so, I examined the circadian clocks of DM and DA with respect to the properties that define an oscillator and influence its function.

2.1.3 Properties that affect oscillator function

Free-running period: Among other features of the entrained activity/rest rhythm, the phase relationship of the rhythm is affected by the FRP of the circadian clock (see Moore-Ede, 1982). In order to entrain to a zeitgeber, the amount of phase resetting required by the clock is equal to the difference between the FRP of the clock and the period of the zeitgeber. Thus, clocks with different FRPs are expected to require different extents of phase-shifts in order to entrain to the same zeitgeber. For attaining the required phase-shift, the zeitgeber must perturb the clock at specific phases as dictated by the PRC of the clock. Therefore, clocks with different FRPs are expected to align with the zeitgeber differentially so as to enable distinct stable phase relationships.

Sensitivity to the zeitgeber: Under entrained conditions, the period of the clock changes to match that of the zeitgeber, and this change in period can be brought about by phase resetting or by modulation of the FRP of the clock, both of which require phase-dependent sensitivity of the clock to the zeitgeber. One way to assess the sensitivity of the clock to a zeitgeber is by examining the characteristics of the PRC, which is useful for analysis of entrainment of circadian clocks by light/dark cycles (see Moore-Ede et al., 1982). Differential sensitivity of circadian clocks are likely to be reflected in the shapes of their PRC. The PRC shape has been used previously to understand how the time-dependent modulation of the FRP and the phase of the clock can help organisms attain different phase relationships with the zeitgeber (Daan and Pittendrigh, 1976a).

Moreover, the *range of entrainment* of circadian clocks (i.e. the range of zeitgeber periods to which a clock can entrain) can be affected by the sensitivity of the clock to the zeitgeber and by the FRP, as is reflected in the PRC. For example, consider a clock with an FRP of 26 hours which can undergo a maximum delay phase-shift of 4 hours and a maximum advance phase-shift of 2 hours. Such a clock would be able to entrain to zeitgebers with

periods ranging from 24 hours (when it can undergo a phase advance of 2 hours) to 30 hours (when it can undergo a phase delay of 4 hours) assuming it is entraining by phase resetting alone.

The light sensitivity of the clock in *Drosophila* can also be assessed by recording the activity/rest rhythm in presence of constant light (LL), a condition which renders flies arrhythmic if the light intensity is above ~1 lux. However, under low intensity LL (~0.1 lux), a considerable proportion of wild type flies show free-running rhythms, while a smaller proportion may show arrhythmicity or presence of complex rhythms (rhythms with multiple periodicities) (Konopka et al., 1989; Rieger et al., 2006). Clocks which are less sensitive to light are expected to show persistent rhythmicity under these conditions, as opposed to more sensitive clocks which would be disrupted, resulting in arrhythmic behaviour.

Amplitude of the oscillator: The intrinsic amplitude of the circadian oscillator (A_0) has been shown to affect the phase resetting ability of the oscillator (i.e. sensitivity to the zeitgeber) as well as the entrainment range (Pittendrigh et al., 1991; Vitaterna et al., 2006; Brown et al., 2008; Abraham et al., 2010). The same strength of zeitgeber stimulus is expected to cause a larger phase response in an oscillator with smaller A_0 as compared to that with a larger A_0. The ratio of the zeitgeber amplitude (A_z) to A_0 affects the range of entrainment such that higher A_z / A_0 results in a larger range of entrainment (Abraham et al., 2010).

Strength of coupling between oscillators: Circadian clocks comprise networks of neurons that form constituent oscillators. These constituent oscillators show "coupling" amongst themselves through interactions by which they affect each other's oscillations. Depending on the nature of interaction, a number of coupling schemes may be present, each of which affects the overall properties of the circadian clock as a whole (see Welsh et al., 2010). When oscillators show mean-field coupling (Gonze et al., 2005; Locke et al., 2008;

Bordyugov et al., 2011), there is increased resonance among the coupled oscillators. Abraham et al. (2010) showed that, in such a case, the amplitude of the clock increases with increasing strength of coupling, and the range of entrainment is lower for a strongly coupled oscillator system. However, with diffusive coupling, amplitude reduction is seen (Bordyugov et al., 2011). Overall, irrespective of the nature of coupling, coupling strength of constituent oscillators is an important factor that affects various properties of the circadian clock.

2.1.4 The present study

In the present study, I examined some of the above-mentioned clock properties in *D. melanogaster* (DM) and *D. ananassae* (DA). The FRP of the clocks of the two species was studied in constant darkness. The phase of entrainment of the two species under LD cycles with varying periodicities (*T* cycles) was examined. It is expected that the clock would phase lag the zeitgeber (i.e. delayed phase of entrainment) when $T < \text{FRP}$, while it would phase lead the zeitgeber (i.e. advanced phase of entrainment) when $T > \text{FRP}$. Also, the differences in phase of entrainment between the two species is expected to be governed by their respective FRPs, provided they are entraining solely by phase-shifts. The sensitivity of the circadian clock to light was assayed by observing the activity/rest rhythm of the two species under dim LL. The amplitude of the clock in the two species was also examined by taking the amplitude of the overt rhythm (activity/rest rhythm) as a measure of the amplitude of the underlying clock. The intrinsic amplitude of the clock as well as the amplitude under entrained condition was examined. In order to see if the differences in the amplitude of the rhythm were reflected in the phase response of the clock, photic PRCs were constructed for DM and DA. A photic Dose Response Curve (DRC) was also constructed for the two species in order to see how the phase response varied with changing intensity and duration

of light pulse. The range of entrainment of DM and DA was also examined, given that the amplitude of the clock, sensitivity of the clock to the zeitgeber, and the FRP are known to affect the range of entrainment of the clock.

In order to see whether any of the prevailing models of entrainment could explain the entrainment of the clocks of DM and DA, two more experiments were done. The flies were entrained to a single brief light pulse occurring every 24 hours, and the phase relationship under entrainment was calculated. According to the discrete model of entrainment, the circadian clock is entrained by discrete light pulses which perturb the clock at a specific phase (as can be predicted from the PRC) such that the phase-shift incurred by the clock is equal to the difference between the period of the clock and that of the zeitgeber. Therefore, if the discrete entrainment model holds, then, when entrained to a single light pulse occurring every 24 hours, the phase relationship of the clock to the zeitgeber should be such that the pulse falls at a specific phase of oscillation which can be predicted from the PRC. On the other hand, continuous model of entrainment suggests that entrainment occurs via modulation of the FRP to match that of the zeitgeber. In fact, the effect of the entraining conditions on the FRP of the clock has been found to persist for a while after the organism is released in constant darkness, called *aftereffects* (Pittendrigh and Daan, 1976a). In order to entrain to different *T* cycles by parametric changes, the FRP of the clock needs to change to match the period of the *T* cycle. Therefore, the FRP of the clocks of DM and DA was examined before and after they were entrained to different *T* cycles in order to see if aftereffects were present. Thus, in the present study, these clock properties were studied in DM and DA.

2.2 Materials and methods

2.2.1 Fly strains and stock maintenance: Experiments were conducted on flies from populations of *Drosophila melanogaster* (DM) and *Drosophila ananassae* (DA) that were maintained in the laboratory. The populations were founded using flies collected from the wild in 2004-2005 within Bangalore, India (12°58'N, 77°38'E) using traps with fruit-bait or using net sweeps. The flies have been maintained as large, out-breeding populations (~1600 individuals) with a discrete generation cycle of 21 days. Standard cornmeal medium food is provided ad libitum and the population cages are kept in LD 12:12 (light intensity: 500 lux) at 25°C temperature and 70% humidity.

2.2.2 Activity recording: Activity of the flies were recorded using the Trikinetics *Drosophila* Activity Monitors (DAM) system (Trikinetics, Waltham, MA, USA). Individual flies were loaded into glass tubes of 5 mm diameter with food at one end and a cotton plug at the other. The tubes are placed in the channels of *Drosophila* Activity Monitors such that an infra-red beam passes through the middle of the tube. When the monitors are connected to a computer, movement of the flies in the middle region of the tubes, break the beam and each beam break is recorded as one activity count. 4-5 day old virgin male flies were used for the locomotor assays, unless specified otherwise.

2.2.3 Analysis of free-running period in DD: The flies were recorded in DD for 7 days and the activity counts were binned into 15 minute intervals. The data was used to calculate the free-running period using Chi-square periodogram in the Clocklab software (Actimetrics, Wilmette, IL, USA). The free-running period and amplitude of the Chi-square periodogram was calculated for 64 virgin male flies of each species in each of the six replicate experiments. These values were used to do a two-way ANOVA with species as a fixed factor and experiment as a random factor.

2.2.4 Activity profile and phase of morning peak under *T* cycles: Flies of the two species were subjected to light-dark cycles of five different periodicities (*T* cycles) and the activity was recorded in the different *T* cycles for 10 cycles at 25°C and 1 lux intensity. Following completion of the 10 LD cycles, the flies were recorded under DD for 10 days in order to examine whether they had entrained to the *T* cycles. The *T* cycles were: *T* 18 (LD 9:9), *T* 20 (LD 10:10), *T* 24 (LD 12:12), *T* 28 (LD 14:14), *T* 30 (LD 15:15). 64 individuals of each species were recorded in each *T* cycle. The activity counts from the recordings were binned in 15 minute intervals and the activity profile was obtained for each cycle for each individual. Average profile for each species in each *T* cycle was then plotted by averaging the activity counts of each individual across cycles and then averaging across individuals for each species and each *T* cycle. The activity/rest data was visualized as actograms in Clocklab and these actograms were used to calculate phases of morning peak in DM and DA and evening peak in DM. The phase of highest activity count around ZT 0 (lights ON) in each cycle was taken as the phase of the morning peak of activity. The peak of activity exactly at ZT 0 is likely due to a startle response to lights-on and hence, was not considered as the true peak. Similarly, the phases of evening peak were marked as the phase of highest activity count around lights OFF (which was different according to the *T* cycle in question). The phases of peak for each individual was averaged across cycles. The mean values were used to perform pairwise comparisons using Mann-Whitney test. The *p*-value below which the differences are considered significant was determined by the Bonferroni correction according to the number of pairwise comparisons that were made. Only those individuals which had entrained to the LD cycles were used for the analysis (criteria used for entrainment has been discussed later).

2.2.5 Analysis of entrainment to *T* cycles: The activity count data from the DAM recording under different *T* cycles was binned in 15 minute intervals and visualized in actograms. The

phases of the onset and offset of activity were marked for each cycle and for the first day in DD following the 10 LD cycles. An individual was considered entrained if the onset and the offset of activity was found to be phase-locked to the LD cycle. In order to check for phase control, the phase of the marker (onset or offset) on the first day of DD was calculated and the standard deviation across the LD cycles in the phase of the marker was also calculated. If the phase of the marker on the first day of DD lay within 3 standard deviations of the phase of the marker under the *T* cycles, the rhythm was considered to be phase-locked to the *T* cycle. Individuals were classified as not-entrained (when neither the onset nor the offset was phase-locked to the *T* cycle or when either the onset or the offset was phase-locked to the *T* cycle) and entrained (when both the onset and the offset was phase-locked to the *T* cycle). Proportion of flies in each species which entrained to the *T* cycles was calculated. Since only one such experiment was carried out, a Chi-square test for goodness of fit was done in order to test for significant differences in proportion of flies that entrained to a *T* cycle, between the two species.

2.2.6 Analysis of activity/rest rhythm under constant light (LL): Activity/rest was recorded from flies of the two species under LL of 0.1 lux intensity and constant temperature of 25°C for 10 days. There were three such replicate experiments with recordings from 64 individuals from each species in each experiment. The data obtained from the DAM system was analyzed using Clocklab. The individuals were classified into the following categories based on their activity/rest rhythms: rhythmic and free-running (individuals with a single period in the activity/rest rhythm), complex rhythmic (individuals showing an activity/rest rhythm with more than one period), and arrhythmic (individuals showing activity/rest without any discernible rhythm). Chi-square periodogram method was used to determine the presence of statistically significant periods. Proportion of individuals showing free-

running rhythms, complex rhythms or arrhythmicity was statistically analyzed using Chi-square test for goodness of fit.

2.2.7 Amplitude of the activity/rest rhythm: Amplitude of the activity/rest rhythm when entrained to LD 12:12 (light intensity: 1 lux) was calculated from 7 days of DAM recording data for 64 flies of each species. The activity counts were binned into 15 minute intervals and used to plot the activity profile in Microsoft Excel (Microsoft, USA). When entrained to LD12:12 at 25°C constant temperature, DM shows a bimodal activity profile with a morning and an evening peak of activity while DA shows only one bout of activity in the morning. Therefore, only the amplitude of the morning activity was compared between the species. From the activity profile, the difference in activity counts between the trough and the peak of activity profile in the morning (ZT 20 - ZT 04) was taken as amplitude of the rhythm.

The amplitude of the activity/rest rhythm in DD at 25°C was calculated from 7 days of DAM recording data for 64 flies of each species. The activity counts were binned into 15 minute intervals and the activity profile was plotted using an Image J plugin, Actogram J (Image J, National Institute of Health, USA). From the activity profile, the difference in activity counts between the trough and the peak of the activity profile was calculated and taken as the amplitude of the rhythm in DD.

The amplitude values for each individual was averaged across days and a two-way ANOVA was done on these values with species and regime (DD and LD 12:12) as fixed factors.

2.2.8 Constructing a photic Phase Response Curve (PRC) and a Dose Response Curve (DRC): The flies were reared in LD 12:12 (Light intensity: 100 lux) for 4 days after eclosion. 4-5 day old flies were loaded into locomotor tubes and recorded in LD 12:12 for 7 days at 100 lux and 25°C temperature. On the 8^{th} day, four different sets of flies were

subjected to a light pulse of 100 lux for 5 minutes at 4 different circadian times (CT): CT 04, CT 10, CT 16 or CT 22. Assuming zeitgeber time (ZT) 0 hours to be CT 0 on the first day of DD, the circadian hour was calculated as: 1 circadian hour = FRP / 24. A particular CT (say CT n) was calculated as: CT n = CT 0 + n circadian hours. Following the pulse, the flies were recorded in DD for another 10 days. For subjecting the flies to the pulse, the monitors were placed into a light-box with appropriate light intensity for 5 minutes. Therefore, in order to account for any phase-shift caused due to disturbance alone, there were disturbance controls for each species at each of the 4 time points. Disturbance control flies were handled exactly the same way as the experimental flies, but they did not receive any light pulse as they were placed in a box without lights.

For constructing a DRC, the phase response of DA and DM was examined under three intensities of light (1 lux, 10 lux, and 50 lux) each with three durations (1 min, 10 min, and 50 min). The flies were reared in LD 12:12 for 4 days post eclosion. 4-5 day old flies were loaded into locomotor tubes and recorded in LD 12:12 for 7 days. On the 8^{th} day, 9 different sets of flies were subjected to a light pulse of 1 lux, 10 lux or 50 lux for a duration of 1 min, 10 min, and 50 min. As with the phase response curve, there were disturbance controls for each species and each duration of light pulse in order to account for any phase-shifts that may have been caused due to handling alone.

The activity/rest rhythm was recorded in 15 minute bins. The data was used to analyze the phase-shifts in the rhythm caused due to the light pulses at different circadian times. The activity data was visualized as actograms in Clocklab. The phase of offset of activity was marked for each day and a regression was drawn through the offsets from day 1 to day 7 and extrapolated to get the phase of offset on day 8. The offsets were also marked from day 11 to day 20 and the regression line drawn through them was extrapolated to get the phase of offset on day 8. The difference in phases between the two regression lines was taken as

the phase-shift. The difference in phase-shift between the experimental set and the disturbance controls was taken as the phase-shift caused due to light alone. These phase-shift values were calculated for all individuals and then a factorial ANOVA was done for statistical analysis. In case of the PRC, a two-way ANOVA was performed using the phase-shift values with phase and species as fixed factors. In case of the DRC, the phase response for each intensity of light for each phase was analyzed in two-way ANOVAs with species and duration as fixed factors.

2.2.9 Analysis of phase of onset when entrained to recurring single light pulses: Flies were reared in LD 12:12 for four days post eclosion. 5 day old flies were loaded in locomotor tubes and their activity was recorded in LD 12:12 with 100 lux intensity for 3 days. From the 4th day, they were subjected to a single light pulse of 100 lux intensity for five minutes every day. The light pulse occurred with a 9 hour delay as compared to the time of lights-on when the flies were in LD 12:12, so that the flies can re-entrain to the single light pulse occurring with a 24 hour period. After ten cycles of single light pulses, the flies were recorded in DD for two days to see if they had entrained. The individual actograms were analyzed for phase control in order to classify an individual as entrained or not. The phase of onset of activity was marked for the days in which the light pulse was given and also for the first day of DD. If the phase of the marker on the first day of DD lay within 3 standard deviations of the phase of onset on the previous 10 days, the rhythm was considered to be phase-locked to the light cue. The phase of onset of activity was marked for each cycle and then averaged across cycles for each individual. A one-way ANOVA was done on the onset phase values with species as a fixed factor.

2.2.10 Analysis of the aftereffects of entrainment to *T* cycles: The flies were reared in LD 12:12 for 4 days post eclosion. 5 day old flies were loaded into locomotor tubes and the activity was recorded in DD for 7 days. On the 8^{th} day, the flies were subjected to *T* cycles

with periodicities of 18 hr (LD 9:9), 20 hr (LD 10:10), 24 hr (LD 12:12), 28 hr (LD 14:14) or 30 hr (LD 15:15) and recorded for 10 cycles. The intensity of light was 1 lux and temperature was constant at 25°C. After 10 cycles were complete, the flies were subjected to another 10 days of DD in order to check for phase control and aftereffects of the respective *T* cycles. A set of flies was recorded in DD throughout the experiment which served as the age-controls. The free-running period in DD was calculated using Chi-square periodogram method for the first 7 days in DD (DD1) and the 7 days in DD following the *T* cycle (DD2). The difference in period of the clock between DD1 and DD2 was compared for the experimental and control flies of each species for each *T* cycle using two way ANOVA with species and treatment (experimental or control) as fixed factors.

2.2.11 Statistical analysis: The ANOVA and Mann-Whitney tests were done in Statistica 7 (Statsoft.inc). The Chi-square tests for goodness of fit were done using Microsoft Excel (Microsoft, USA).

2.3 Results

2.3.1 *D. melanogaster* (DM) and *D. ananassae* (DA) do not have significantly different free-running periods but they differ in the amplitude of the periodogram

I estimated the free-running period of the circadian clock of male flies in constant darkness (DD) and found it was not significantly different between DM and DA (DM = 23.72 ± 0.04 and DA = 23.78 ± 0.04, mean ± 95% CI; $F_{1, 492} = 4$, $p > 0.05$; Two-way ANOVA, Tukey's HSD) (Figure 2.1A, Table 2.1). The percentage of rhythmic flies in DD was also not significantly different in the two species (DM = 93.29 ± 1.59 and DA = 94.65 ± 1.03, mean ± SEM; Chi-square test, $p > 0.05$) (Figure 2.1B). The amplitude of the Chi-square periodogram, (which is a measure of the relative contribution of a given period to a periodogram) was significantly larger for DA as compared to that of DM (DM = 205.50 ± 11.29 and DA = 262.60 ± 11.29, mean ± 95%CI; $F_{1, 492} = 87.13$, $p < 0.05$; Two-way ANOVA, Tukey's HSD) (Figure 2.1C, Table 2.2). My results are in contrast to that reported in a previous study (Prabhakaran and Sheeba, 2012), and upon re-examining the older data set, I found the following differences which may explain this discrepancy. Firstly although the older data set consisted of 4 separate experiments, each used a small number of flies (n ~ 16). Moreover, the time series considered had 6-7 days and the periodogram analysis used was based on the Lomb-Scargle method which is not recommended for short duration data that may have irregularities in their daily profiles (Refinetti et al., 2007). When a Chi-square periodogram was used to analyze the previous time series, the periods were not found to be significantly different, which is consistent with the present results. Thus, I submit my finding that the two species do not differ in the period of their underlying circadian clock, based on time series as long as 7 days, consisting of 6 independent experiments, each consisting of at least 30 individuals.

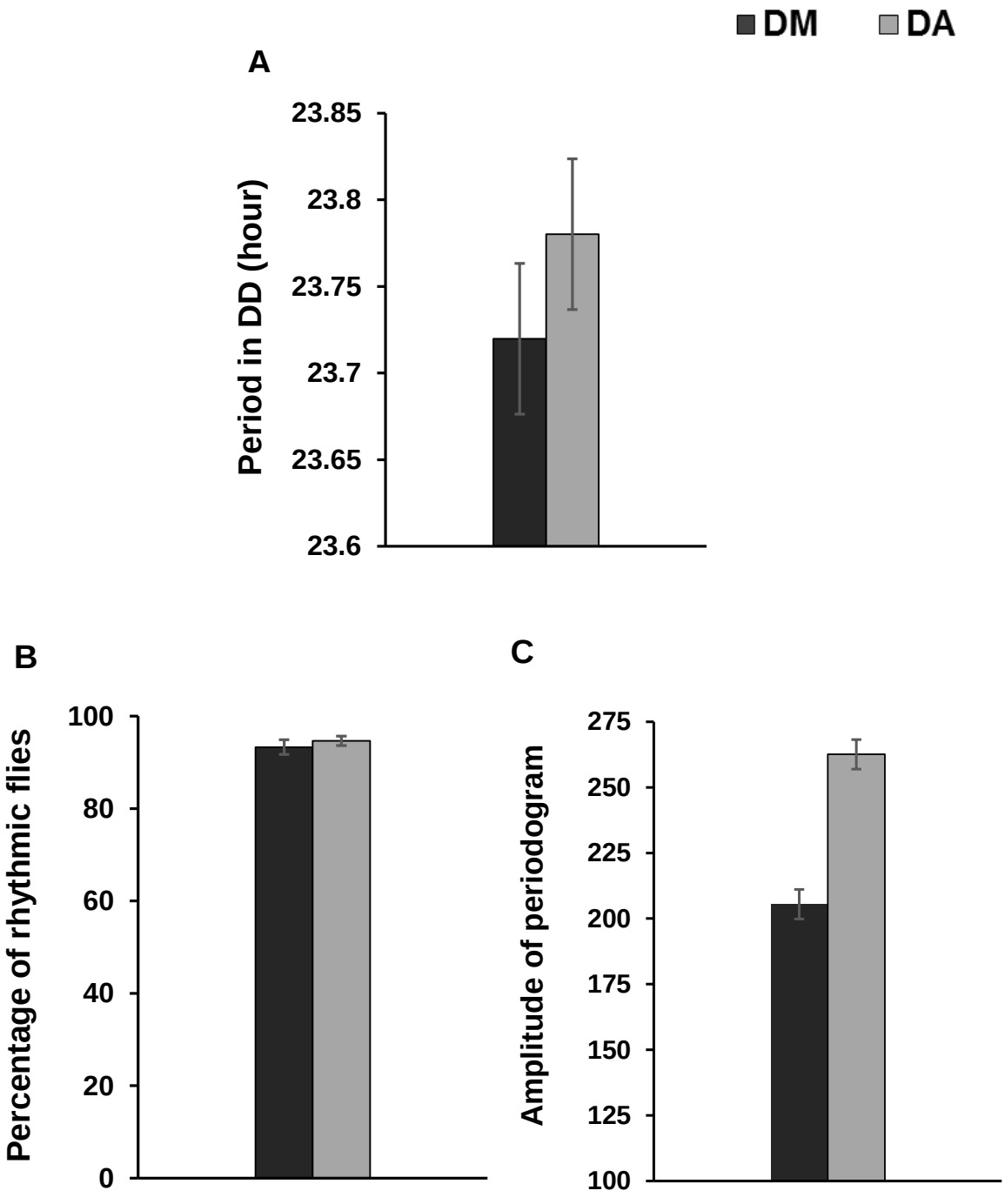

Figure 2.1. (A) Mean free-running period of the clock in DM and DA as assayed under constant darkness (DD) (Two-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing. **(B)** Percentage of flies that were rhythmic in DD; Chi-square test for goodness of fit, $p > 0.1$. Error bars indicate SEM. **(C)** Mean amplitude of the Chi-square periodogram (Two-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing.

2.3.2 Phase of entrainment under light-dark cycles of different periodicities in *D. melanogaster* and *D. ananassae*

The intrinsic period of the clock is known to reflect the phase of entrainment. Individuals with shorter period show an advanced phase of entrainment and individuals with longer period show a delayed phase of entrainment (see Moore-Ede et al., 1982). However, even though the period of DA and DM are not different, under LD 12:12, DA shows a delayed morning peak as compared to DM (Prabhakaran and Sheeba, 2012) (Figure 2.2 [*T* 24]). The two species were subjected to five *T* cycles, namely, *T* 18 (LD 9:9), *T* 20 (LD 10:10), *T* 24 (LD 12:12), *T* 28 (LD 14:14), and *T* 30 (LD 15:15) which had period lengths of 18 hr, 20 hr, 24 hr, 28 hr, and 30 hr respectively. Under each of the *T* cycles, phase of the morning peak in DA was significantly delayed as compared to that of DM (Figure 2.3 A, Table 2.3). DM individuals continued to show a bimodal activity profile with an increasing phase advance of morning (Figure 2.2, 2.3A) and evening peak (Figure 2.3B) of activity as the length of the *T* cycle increased. In DA, however, predominantly the activity bout remained consolidated to the light-phase of the *T* cycle with a consistent decrease in evening activity as the *T* cycle length increased (Figure 2.2). The morning peak in DA was phase delayed with respect to lights-on in *T* 18, *T* 20, and *T* 24 and did not differ significantly among the three *T* cycles. In *T* 28 and *T* 30, the phase of the morning peak in DA was not significantly different, but it was significantly advanced as compared to the morning peak in *T* 20 and *T* 24. In the extreme short *T* cycle (*T* 18), DA showed some activity around dusk and in the extreme long *T* cycle (*T* 30), DA shifted a part of its morning activity to dawn. It appears that the phasing of activity of DA is more tightly phase-locked to the light-phase as compared to that of DM.

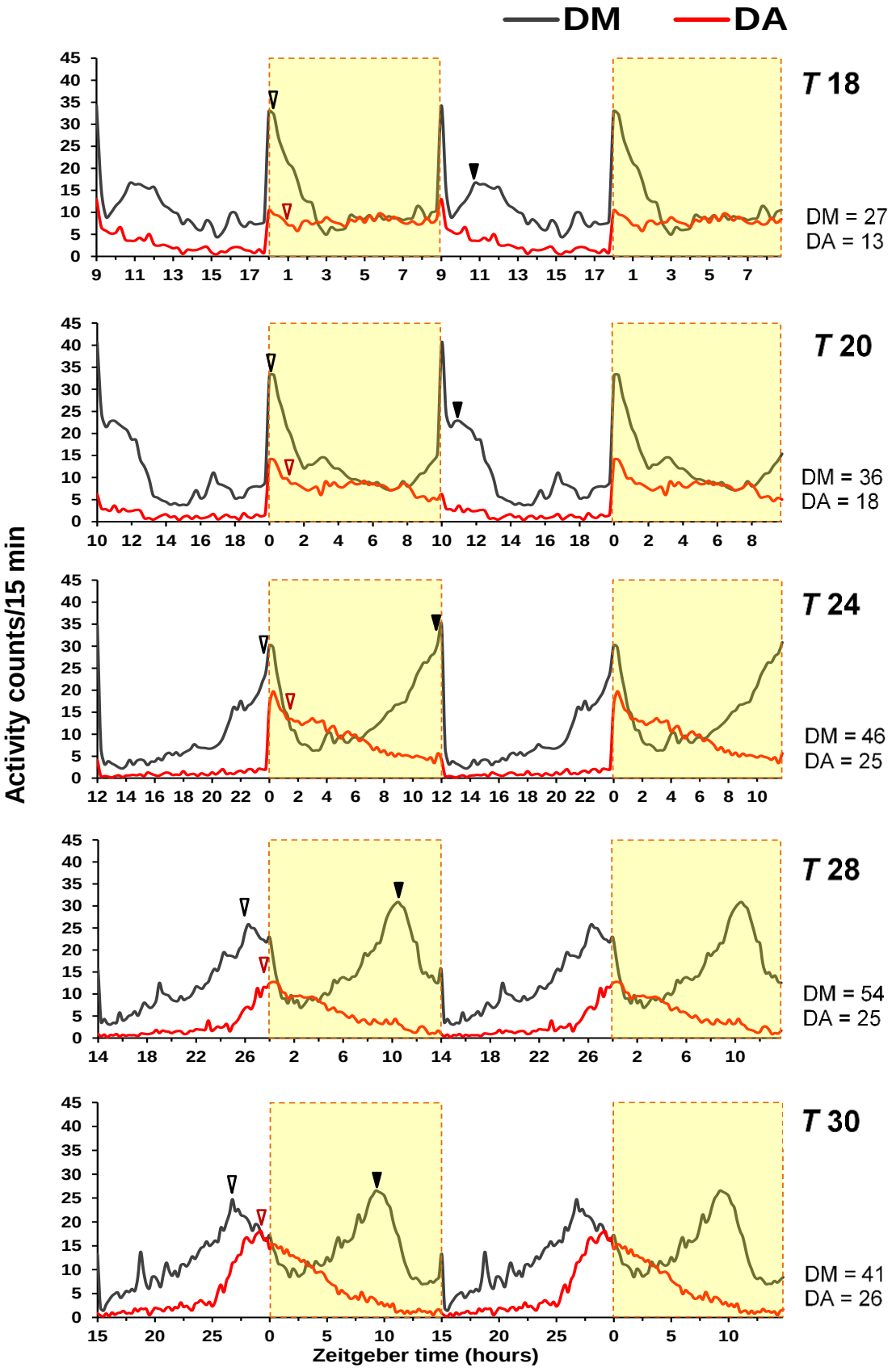

Figure 2.2. Activity profiles of DM and DA under *T* cycles. Yellow shaded region indicates light phase. Black and red unfilled arrows indicate the phase of true morning peak in DM and DA respectively. Filled arrow indicates phase of true evening peak in DM. The number of entrained individuals in each *T* cycle is indicated to the right.

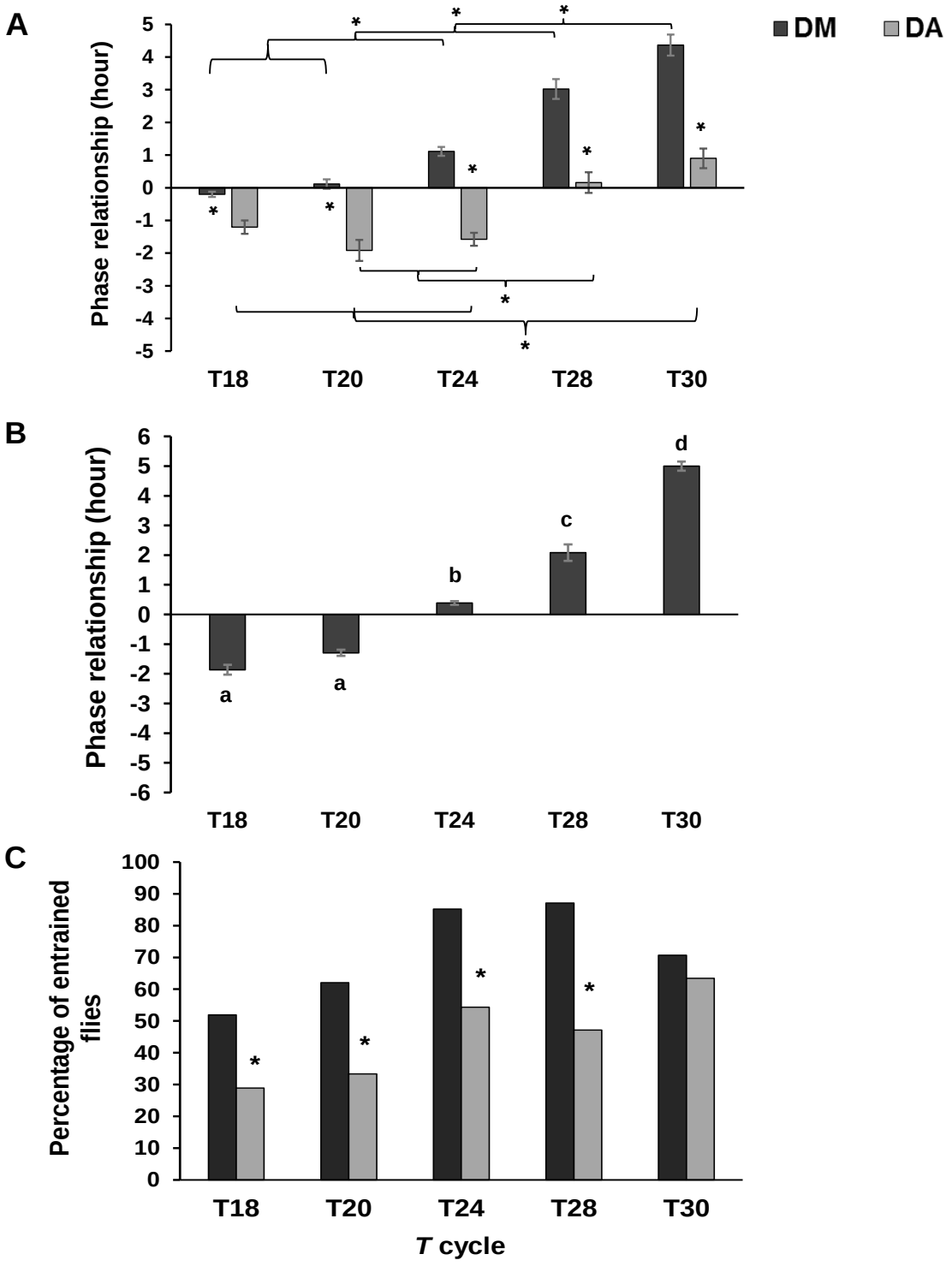

Figure 2.3. Entrainment to *T* cycles (A) The mean phase of morning peak (± SEM) of DM and DA with respect to lights-on under *T* cycles. * indicates significant differences **(B)** Mean phase of evening peak (± SEM) in DM with respect to lights-off, under different *T* cycles; Bars with dissimilar letters are significantly different from each other. (Mann-Whitney test, $p < 0.001$). Positive values indicate phase advance and negative values indicate phase delay **(C)** Percentage of DM and DA flies which entrained to different *T* cycles. * indicates significant difference (Chi-square test for goodness of fit).

The proportion of flies in each species that entrained to different *T* cycles was also different. Under four of the five *T* cycles (*T* 18, *T* 20, *T* 24, and *T* 28), a significantly smaller proportion of DA individuals showed entrained rhythms as compared to the proportion of DM individuals which were able to entrain. Even though a smaller proportion of DA individuals showed entrained rhythm under *T* 30 as compared to DM, the difference was not found to be statistically significant. This could also be due to the smaller sample size considered in case of *T* 30 as larger number of DA flies died in this regime. (Figure 2.3C, Table 2.4).

Overall, it is seen that, when entrained to different *T* cycles, the phase of entrainment in DA does not seem to vary as much as that of DM with change in period of the zeitgeber, and the differences in phasing of activity/rest rhythm of DM and DA cannot be explained by differences in period as the intrinsic period was not found to be different between the two species. Therefore, it is possible that other clock properties that are known to affect differences in phase of entrainment are different between DM and DA. Moreover, a lower proportion of DA flies are able to entrain to *T* cycles shorter or longer than *T* 24, as compared to DM, further suggesting that DM and DA have different clock properties. Therefore, two such clock properties were examined which influence the phase of entrainment and the ability of the clock to entrain to different *T* cycles, namely, circadian photosensitivity and intrinsic amplitude of the clock.

2.3.3 *D. melanogaster* and *D. ananassae* show different proportions of rhythmic individuals under dim constant light

In order to see if the two species differ in their sensitivity to light, the activity/rest rhythm was examined under constant presence of light. Constant light is known to disrupt the circadian clock of *D melanogaster*, rendering flies arrhythmic (Konopka et al., 1989). However, if the light intensity is kept at relatively low levels, some proportion of flies show

free-running rhythms (Konopka et al., 1989). Sometimes, at low light intensities, flies also exhibit complex rhythms (Konopka et al., 1989; Rieger et al., 2006). Individuals are characterized as showing complex rhythms when a pre-existing, single period rhythm splits into a more than one component, each of which free-runs with its own periodicity. The different behaviours of the activity/rest rhythm (arrhythmicity, spitting, or free-running) could arise because of differences in circadian photosensitivity or because of differences in strength of coupling between component oscillators of the clock. Circadian clocks which are less sensitive to light are expected to show free-running rhythms under constant presence of dim light, whereas a more sensitive clock is likely to get disrupted under constant dim light, leading to arrhythmic behaviour. Circadian clocks where the component oscillators are strongly coupled are likely to withstand the disruptive effect of constant light and hence maintain rhythmicity under such conditions. On the other hand, clocks with weakly coupled component oscillators, when subjected to constant presence of dim light, can show complex rhythms. This is could be because the weak coupling among the oscillators are likely to dissociate such that each oscillator driven rhythm now free-runs with its own intrinsic period which may be different from the period that is shown when the oscillators are coupled. I subjected DA and DM flies to low intensity LL to test whether the clocks in the two species are differentially susceptible to splitting by constant dim light (LL). Under 0.1 lux intensity, a greater percentage of DA flies were rhythmic as compared to DM. A significantly greater percentage of DM flies showed complex rhythms as compared to that of DA (rhythmic flies DM = 19.73%; DA = 61.49%, flies showing complex rhythms DM = 35.75% and DA = 1.70%, mean of three replicate experiments) (Figure 2.4). However, the proportion of flies showing arrhythmic behaviour did not differ significantly between DA and DM except for one of the replicate experiments where DM had a significantly higher proportion of arrhythmic flies (arrhythmic flies in DM = 44.51% and in DA = 36.80%, mean of three

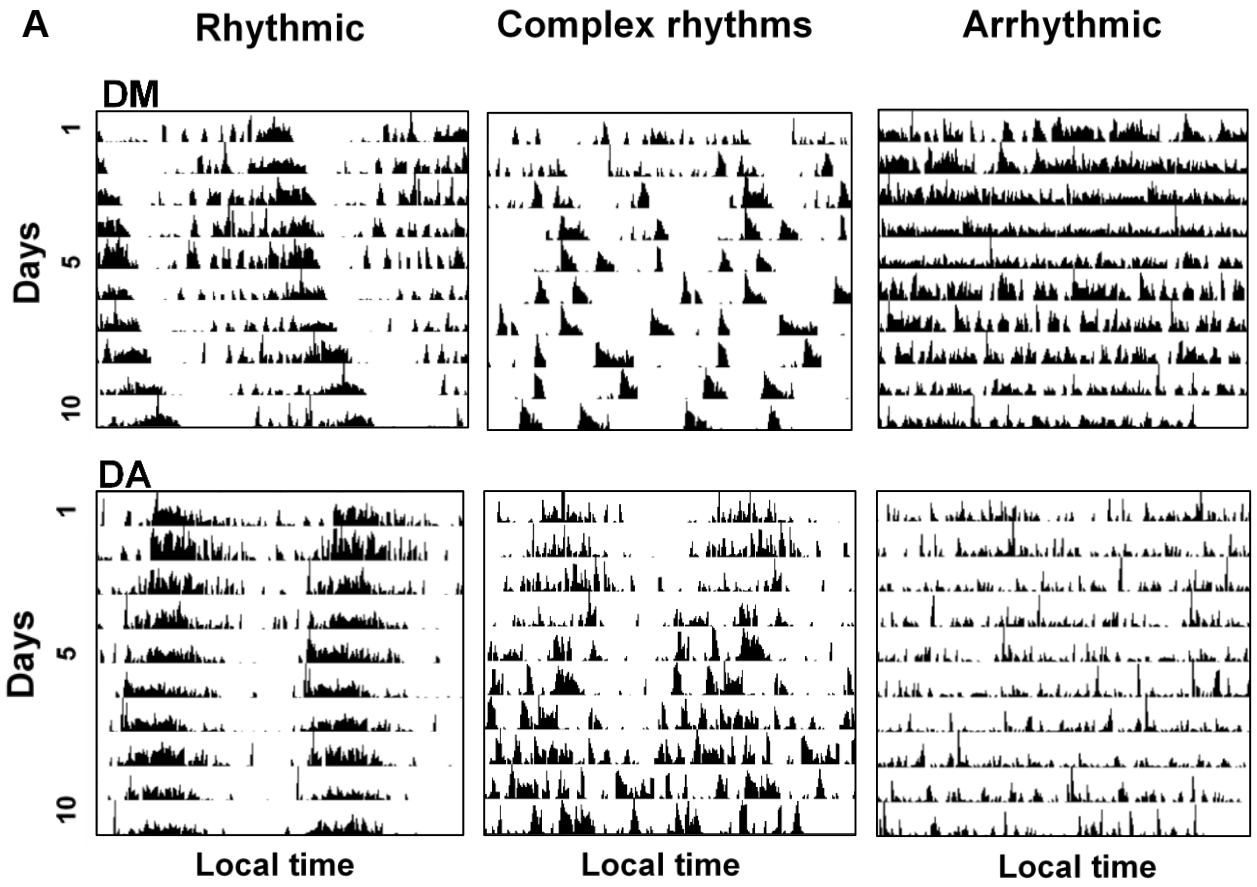

Figure 2.4. Activity/rest rhythm of DM and DA under dim LL.

(A) Representative actograms of DM and DA when activity/rest rhythm is recorded under dim LL.

(B) Percentage of DM and DA flies showing rhythmicity, arrhythmicity, and complex rhythm under dim LL of 0.1 lux. (Chi-square test for goodness of fit) *indicates significant difference.

replicate experiments). Similar results were obtained when the data from all three experiments were pooled and was analyzed using Chi-square test for goodness of fit (Table 2.5, 2.6).

The greater fraction of rhythmic flies in DA suggests that the clock of DA may be less sensitive to constant dim light as compared to that of DM. Given that the proportion of arrhythmic flies did not differ significantly between the two species, it is also possible that the strength of oscillator coupling is higher in DA as compared to DM which enables the DA flies to maintain rhythmicity in constant dim light where most DM flies show complex rhythms.

2.3.4 Amplitude of the rhythm is higher in *D. melanogaster* as compared to *D. ananassae* in both DD and LD 12:12

Apart from its free-running period, amplitude is another intrinsic and characteristic property of the circadian clock. I estimated the amplitude of the overt activity/rest rhythm as a readout of the amplitude of the clock. Overall DM flies showed higher amplitude rhythms under both constant as well as rhythmic environmental conditions (DD and LD 12:12, respectively). Two-way ANOVA with species and regime (DD and LD 12:12) as fixed factors (Table 2.7), showed significant main effect of species ($F_{1, 162} = 20.87$, $p < 0.01$; Two-way ANOVA, Tukey's HSD) and regime ($F_{1, 162} = 15.43$, $p < 0.01$; two-way ANOVA, Tukey's HSD). Species and regime interaction was not significant ($F_{1, 162} = 0.16$, $p > 0.05$; Two-way ANOVA). The amplitude of the rhythm in DD was larger for DM as compared to that for DA (DM = 29.55 ± 3.01 and DA = 22.99 ± 3.01, mean ± 95% CI) (Figure 2.5). The amplitude of the rhythm in LD 12:12 was also higher for DM as compared to that of DA (DM = 35.64 ± 3.01 and DA = 28.58 ± 3.01, mean ± 95% CI) (Figure 2.5). Overall,

these results suggest that circadian clocks of DA have a smaller amplitude when the amplitude of the overt activity/rest rhythm is considered.

2.3.5 *D. melanogaster* and *D. ananassae* have different photic Phase Response Curve (PRC) and Dose Response Curve (DRC)

The sensitivity of the clock to a zeitgeber is reflected in its intrinsic amplitude (see Johnson et al., 2003). Clocks with low amplitude are expected to show greater phase response to a zeitgeber than those with a higher amplitude (Abraham et al., 2010). Based on the results described above, I tested the hypothesis that DA flies will exhibit larger phase response to a light pulse as compared to DM. To do so, a photic phase response curve (PRC) was constructed for both species at 4 different phases of the free running clock using a 5 minute light pulse of 100 lux intensity. In a two-way ANOVA with phase and species as fixed factors (Table 2.8), there was a significant main effect of species ($F_{1, 208} = 28.67$, $p < 0.01$) and phase ($F_{3, 208} = 97.81$, $p < 0.01$), and also a significant interaction of species and phase ($F_{3, 208} = 4.83$, $p < 0.01$). At CT 4, CT 10, and CT 22, the phase-shifts seen in the two species were not different. However, surprisingly, during the early subjective night (CT 16), DA showed smaller delay phase-shift as compared to DM (DM= - 4.18 ± 0.697 and DA= - 1.73 ± 0.697, mean ± 95% CI) (Figure 2.6).

To further examine how the sensitivity of the clock might differ in the two species with changing intensity and duration of zeitgeber, a photic dose response curve (DRC) was constructed where the species were subjected to light pulses of three intensities (1 lux, 10 lux, and 50 lux) with varying durations (1 min, 10 min, and 50 min) falling at two phases of the cycle (CT 14 and CT 21) (Figure 2.7A-F). For each intensity of light pulse and each phase, a two-way ANOVA was done with species and duration as fixed factors (Table 2.9 – 2.14). Both species showed a phase delay for light pulses at CT 14 (early subjective night) and a phase advance for light pulses falling at CT 21 (late subjective night).

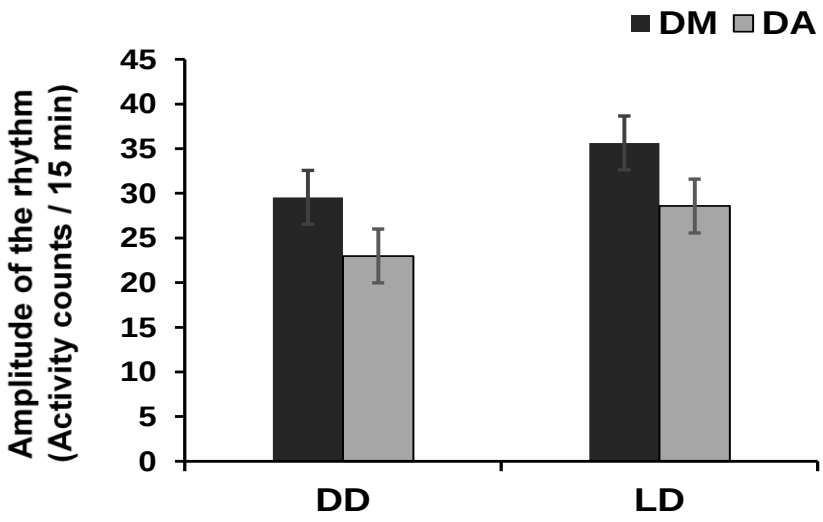

Figure 2.5. Mean amplitude of the activity/rest rhythm in DM and DA under DD, and LD 12:12 (Two-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing.

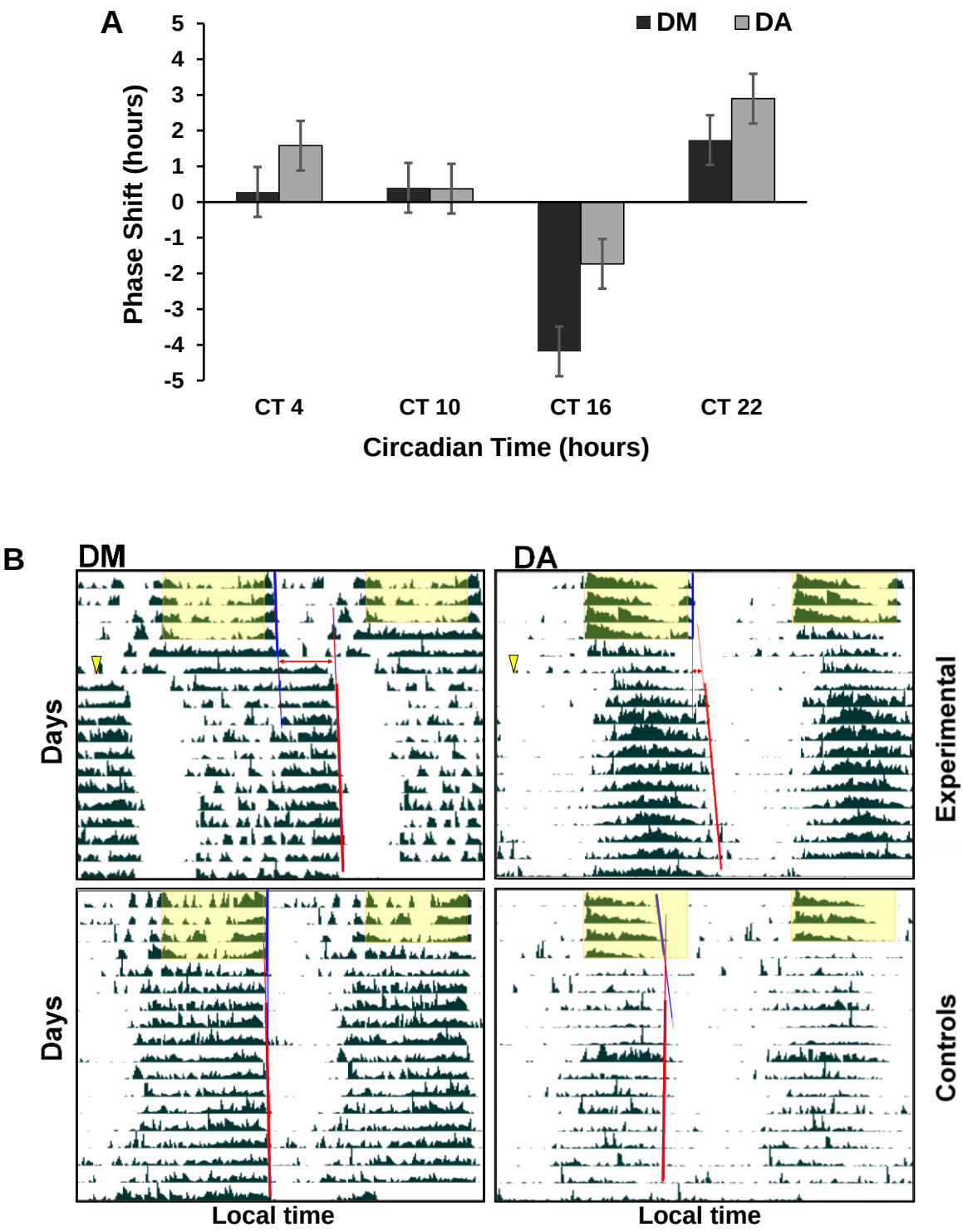

Figure 2.6. Photic phase response of DM and DA. (A) Mean phase-shift values of DM and DA for light pulse at four different circadian times (Two-way ANOVA, Tukey's HSD)**.** Error bars indicate 95% CI for visual hypothesis testing **(B)** Representative actograms of experimental (top panel) flies which received light pulse at CT 16 (indicated by yellow arrows) and their respective controls (bottom panel), showing phase-shift in activity/rest rhythm due to the light pulse (indicated by red two-headed arrow in the top panel). Yellow shaded regions denote the light phase of the LD cycles to which the flies were entrained before being released into DD. The thick blue and red lines denote the phase of offset under LD cycle and DD respectively. Corresponding thin lines are the regressions drawn from them in order to calculate the phase shifts.

From the DRC, it is seen that DA and DM do not differ in their phase response to light pulses of different intensities and duration falling in the late subjective night which results in phase advance of the activity/rest rhythm. One exception is noted where a light pulse of 10 lux intensity for 1 min at CT 21 causes a significantly smaller phase advance in DA as compared to that in DM. Across the three intensities, the phase response of DA and DM are not significantly different for 1 min and 50 min durations of light pulse falling at CT 14 which results in phase delays. One exception is again noted for a 50 lux light pulse of 1 min duration falling at CT 14, where DA shows a significantly smaller phase advance as compared to DM. However, across the three intensities of light pulse, it is seen consistently that DA undergoes significantly smaller phase-shift as compared to DM when a 10 min pulse is given at CT 14. For light pulses of 10 lux and 50 lux intensity, the phase response shown by DM at CT 14 as well as at CT 21 does not vary with duration of the pulse. It appears that the clock of DM is able to get the maximum delay as well as advance phase-shift with the least duration of the light pulse during the early and the late subjective night respectively. However, DA shows increasing magnitude of advance as well as delay phase-shift with increasing duration of light pulse of a 10 lux and 50 lux intensity. DA undergoes smaller phase response to a brief (10 min) light pulse as compared to DM for light pulses falling in the delay zone of its PRC (early subjective night) and this holds true across three different intensities of light pulse.

When a light pulse of 1 lux intensity was given at CT 21, there was no significant main effect of species or duration, and the two-way interaction effect of species and duration was also not significant (Table 2.9). 1 lux light pulses given at CT 21 did not elicit significantly different phase responses in the two species for any of the three durations. Within a species also, across different durations of 1 lux light pulses, the phase response did not vary significantly at CT 21 (Figure 2.7A, Table 2.15).

For a 1 lux light pulse given at CT 14, there were significant main effects of species ($F_{1, 136}$ = 14.42, p < 0.01) and duration ($F_{2, 136}$ = 32.41, p < 0.01), and a significant two-way interaction effect of species and duration ($F_{2, 136}$ = 36.73, p < 0.01) (Table 2.10). When a 1 lux light pulse was given for 10 min at CT 14, DA had significantly smaller delay phase-shift as compared to DM. 1 lux light pulse at CT 14 for durations of 1 min and 50 min did not elicit different phase responses in DA and DM. Considering the phase response of DA to a light pulse of 1 lux intensity at CT 14, maximum delay phase-shift was seen for a pulse duration of 50 min, as compared to those for pulse durations of 1 min and 10 min. However, in case of DM, maximum delay phase-shift was seen for pulse durations of 50 min and 10 min. The phase response in DM was not different for pulse durations of 50 min and 10 min, whereas, the phase response for pulse duration of 1 min was significantly smaller as compared to that of both 50 min and 10 min duration (Figure 2.7B, Table 2.16).

For a light pulse of 10 lux intensity given at CT 21, there was a significant main effect of duration ($F_{2, 126}$ = 11.72, p < 0.01) and a significant two-way interaction effect of species and duration ($F_{2, 126}$ = 8.41, p < 0.01) (Table 2.11). 10 lux light pulse at CT 21 did not cause significantly different phase-shifts in DA and DM when the duration was 10 min or 50 min. However, for a 10 lux light pulse of 1 min duration given at CT 21, DA had significantly smaller phase-shift as compared to DM. Considering the phase response shown by DM for a 10 lux pulse, across durations of 1 min, 10 min, and 50 min, the phase-shifts were not significantly different. In case of DA, however, phase-shift due to light pulse of 1 min duration at CT 21 was significantly smaller than those due to pulses of 10 min or 50 min durations (Figure 2.7C, Table 2.17).

For a 10 lux light pulse given at CT 14, there were significant main effects of species ($F_{1, 134}$ = 7.79, p < 0.01) and duration ($F_{2, 134}$ = 28.73, p < 0.01), and a significant two-way interaction of species and duration ($F_{2, 134}$ = 7.54, p < 0.01) (Table 2.12). Similar to the

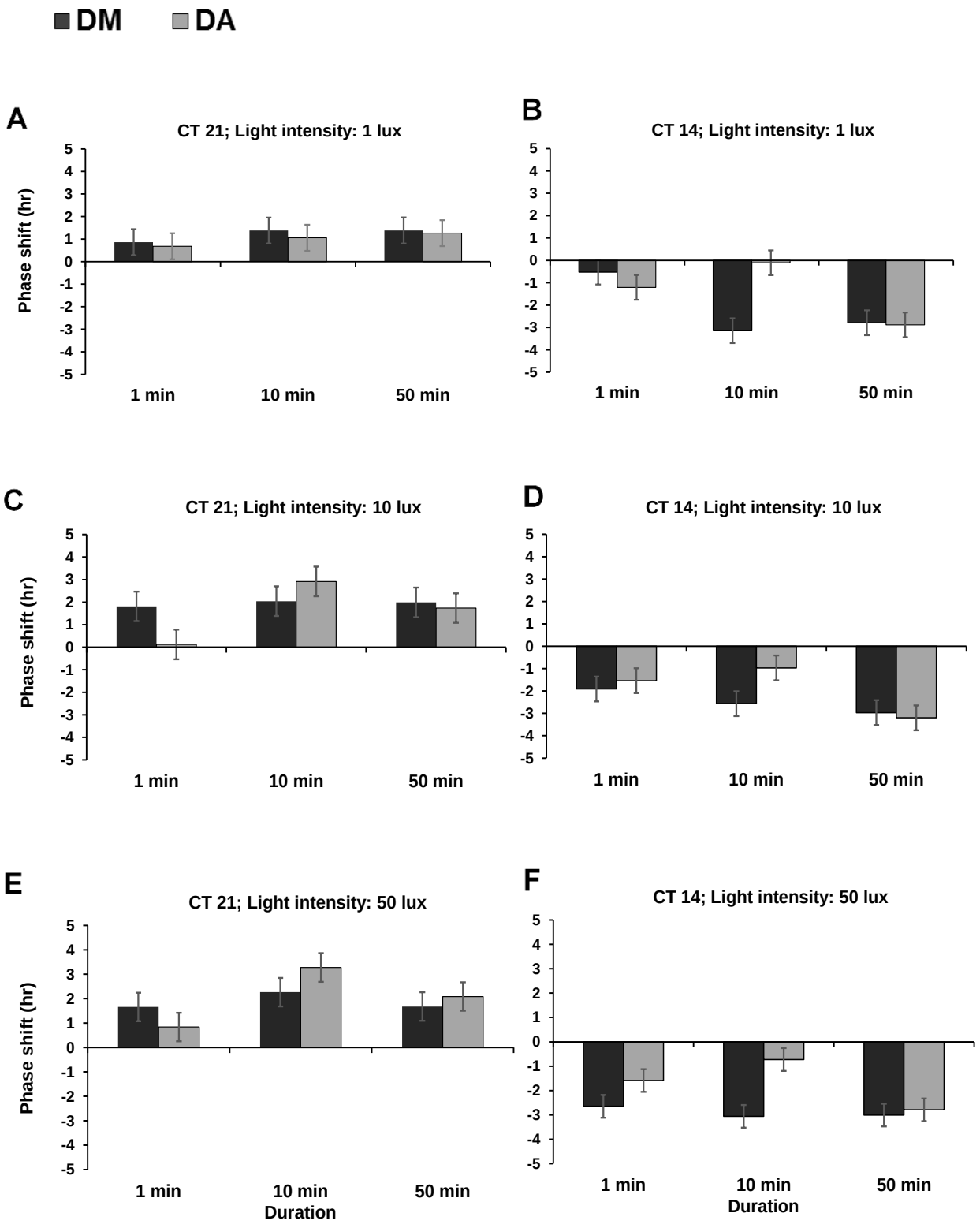

Figure 2.7. Photic duration response curve of DM and DA (Two-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing.

effect of 1 lux light pulse at CT 14, in case of a 10 lux light pulse at CT 14, DA had significantly smaller phase-shifts than DM when the pulse duration was for 10 min. Considering the phase-shift of DM across three durations of 10 lux light pulse, the phase-shift due to a 1 min pulse was smaller than that due to a 50 min pulse. In DA, similar to the effect of 1 lux pulse, with a 10 lux pulse also, the phase-shift for 1 min and 10 min duration of pulse was not significantly different from each other but they were both smaller in magnitude than the phase-shift seen due to a pulse of 50 min (Figure 2.7D, Table 2.18).

When a light pulse of 50 lux intensity was given at CT 21, there was significant main effect of duration ($F_{2, 132} = 13.72$, $p < 0.01$), and a significant interaction effect of species and duration ($F_{2, 132} = 5.04$, $p < 0.01$) (Table 2.13). As in case of a light pulse of 1 lux and 10 lux intensity, a 50 lux pulse did not cause significantly different phase-shifts between DA and DM across different durations at CT 21. DM did not show different phase-shifts for different durations of 50 lux pulse at CT 21. Unlike in DM, the phase response of DA to a 50 lux light pulse varied with duration of the pulse (Figure 2.7E, Table 2.19).

For a 50 lux pulse given at CT 14, there were significant main effects of species ($F_{1, 140} = 43.75$, $p < 0.01$), and duration ($F_{2, 140} = 13.99$, $p < 0.01$), and a significant interaction effect of species and duration ($F_{2, 140} = 12.39$, $p < 0.01$) (Table 2.14). DA showed a significantly smaller phase-shift as compared to DM when the pulse durations were for 1 min and 10 min. At CT 14, DA showed a significantly larger phase-shift for a pulse duration of 50 min as compared to that for 1 min as well as 10 min (Figure 2.7F, Table 2.20).

The results, taken together, suggests that the circadian clock of DA is less sensitive to light pulses in the early subjective night as compared to that of DM. The PRC and the DRC also contradicts the expectation that DA would show larger phase response to a light pulse than DM, given that the amplitude of the clock is lower in DA as compared to DM. It is possible

that this is because the amplitude of the activity/rest rhythm is not truly reflective of the intrinsic amplitude of the circadian clock.

2.3.6 Entrainment to single light pulses of brief duration in *D. melanogaster* and *D. ananassae*

Given that the PRCs of DM and DA differed, I wanted to test whether the entrainment of the activity/rest rhythm in the two species can be explained by the non-parametric model of entrainment. In order to do so, the two species were subjected to a single light pulse of five minutes occurring every 24 hours. If the individuals who entrain to this light pulse are using phase-shifts to do so, then the phase of entrainment will be such that the pulse occurs at a phase of the circadian rhythm where the clock can get the required phase-shift. Assuming that onset of activity occurs at CT 0, the circadian time at which the light pulse occurred for the entrained individuals was calculated from the phase relationship of the onset of activity with the zeitgeber. For both DA and DM, the onset of activity was phase advanced with respect to the light pulse and the phase of entrainment (Ψ_{ent}) was significantly different between DA and DM (Ψ_{ent} for DA = 9.2 ± 0.64 hours, Ψ_{ent} for DM = 11.02 ± 0.64 hours; mean ± 95% CI; Figure 2.8, Table 2.21). Thus, for DA, the light pulse occurred about CT 9.2, and for DM, at CT 11.02. From the PRCs, it can be seen that for both DM and DA the light pulse occurred at a phase of the circadian rhythm which would phase advance the clock. Light pulse at CT 10 induces negligible phase advance in both DM and DA. However, in order to entrain to a zeitgeber of 24 hour period (in this case, a single light pulse occurring every 24 hours), both DM and DA need phase delays as they have an intrinsic period which is shorter than 24 hours. Thus, the results suggest that the non-parametric model of entrainment cannot explain entrainment in these species.

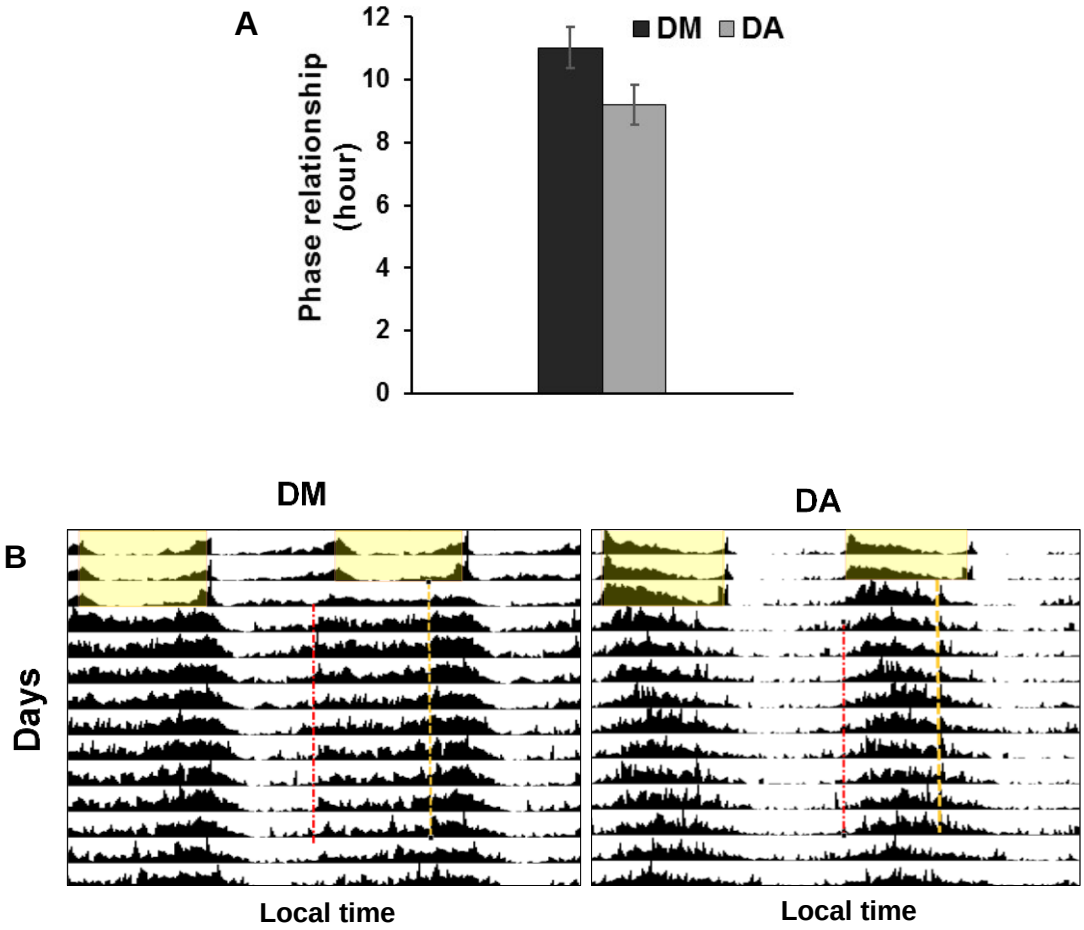

Figure 2.8. Entrainment of DM and DA to single light pulses occurring every 24 hours. (A) Phase relationship of onset of activity with time of occurrence of light pulse (One-way ANOVA). Error bars indicate 95% CI for visual hypothesis testing. **(B)** Batch actograms of flies that entrained to the single light pulse cue. Yellow shaded regions denote the light phase of the LD cycle. The dotted yellow line denotes the phase at which the light pulse occurred in every cycle. The dotted red line shows the average phase of onset of activity with respect to the phase of the light pulse.

2.3.7 Aftereffects of entrainment to *T* cycles on the period of the clock are not different for *D. melanogaster* and *D. ananassae*

It is possible that the clock entrains to zeitgebers by changing its period to match the period of the zeitgeber (parametric model of entrainment). In such a case, if entrained individuals are released into constant conditions following entrainment, the clock may free-run with the altered period length for a short duration – an aftereffect of the entrained state. Thus, the period of the clock following entrainment is different from the intrinsic period before entrainment. The presence of such aftereffects of entraining conditions is one way of examining if entrainment had occurred by period changes. In order to test for existence of aftereffects of entraining conditions on the period of the clock, DM and DA flies were subjected to five different *T* cycles *T* 18, *T* 20, *T* 24, *T* 28, and *T* 30 which had period lengths of 18 hr, 20 hr, 24hr, 28hr, and 30 hr respectively. The period of the clocks of DM and DA were analyzed in DD following entrainment to *T* cycles and the difference in period was calculated. For a given *T* cycle, the change in period was compared between flies of each species with their respective controls which were not subjected to *T* cycles and were recorded in constant darkness. Significant aftereffects were not observed in the period of DM and DA under any of the *T* cycles (Figure 2.9).

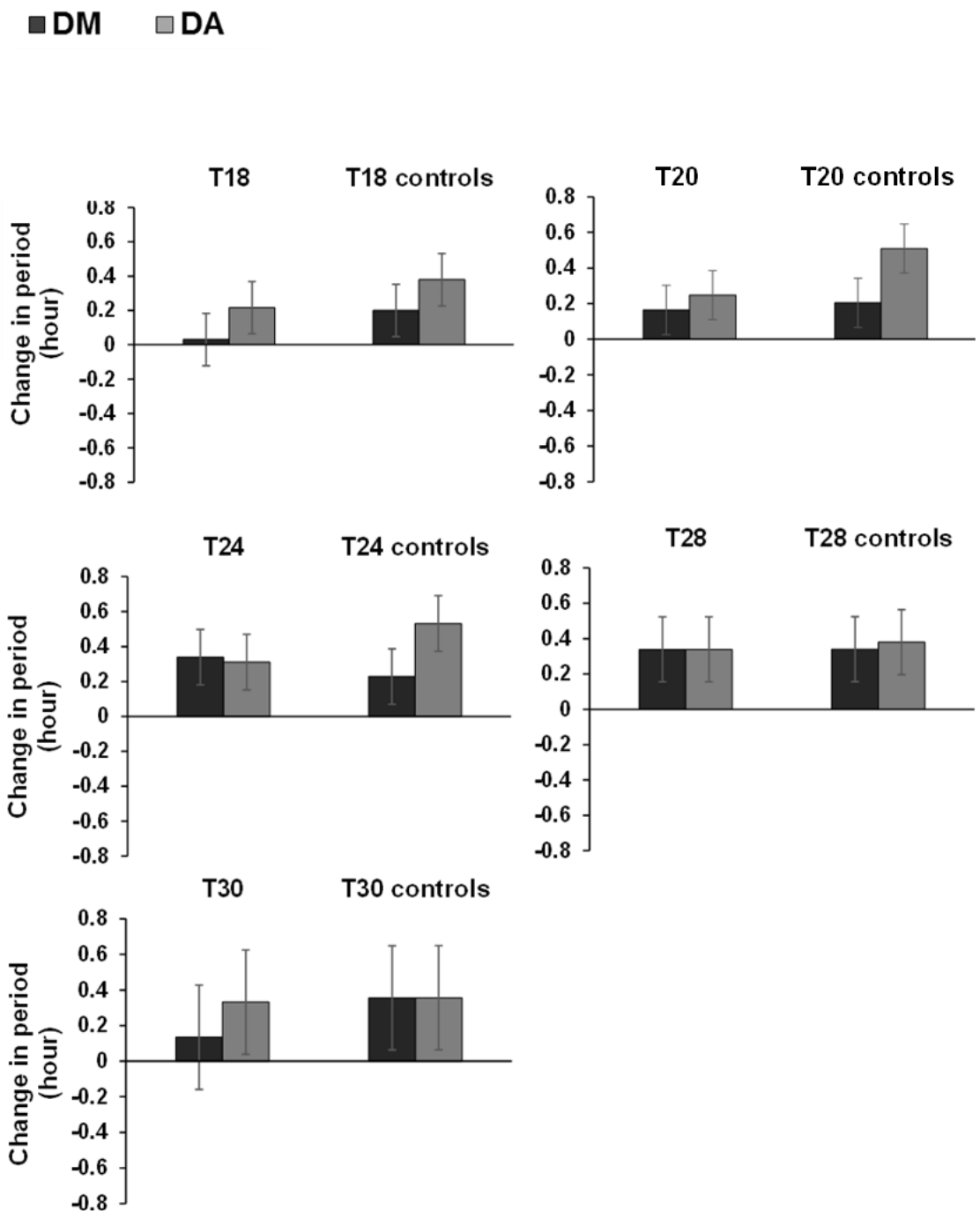

Figure 2.9. Aftereffects of different *T* cycles on free-running periods of DM and DA. Error bars indicate 95% CI for visual hypothesis testing (Two-way ANOVA, Tukey's HSD)..

Table 2.1 Results of ANOVA done on FRP values of DM and DA.

Effect	***df***	**MS**	***F***	***p***
Experiment	5	0.2	1	0.34
Species	1	0.5	4	0.10
Experiment * Species	5	0.1	1	0.67

Table 2.2 Results of ANOVA performed on amplitude of Chi-square periodogram of DM and DA.

Effect	***df***	**MS**	***F***	***p***
Experiment	5	9215	2.045	0.22
Species	1	388831	87.135	0.000152
Experiment * Species	5	4507	1.251	0.28

Table 2.3 Mean phase of morning peak with respect to lights-on under *T* cycles in DM and DA. Positive and negative values indicate advanced and delayed phase respectively.

T cycle	DM		DA	
	Mean (hour)	SEM	Mean (hour)	SEM
T 18	-0.20	0.08	-1.20	0.20
T 20	0.11	0.14	-1.92	0.32
T 24	1.11	0.14	-1.58	0.20
T 28	3.02	0.30	0.16	0.31
T 30	4.37	0.32	0.90	0.30

Table 2.4 Number and percentage of DM and DA flies that showed entrainment to different *T* cycles.

T cycle	Species	% entrained	% not entrained	Number entrained	Number not entrained	Total numbers
T 18	DM	51.92	48.08	27	25	52
	DA	28.89	71.11	13	32	45
T 20	DM	63.16	36.84	36	21	57
	DA	33.33	66.67	18	36	54
T 24	DM	85.18	14.81	46	8	54
	DA	54.35	45.65	25	21	46
T 28	DM	87.09	12.90	54	8	62
	DA	47.17	52.83	25	28	53
T 30	DM	70.67	29.31	41	17	58
	DA	63.41	36.58	26	15	41

Table 2.5 Number of DM and DA flies showing rhythmic, arrhythmic, and complex rhythmic behaviour in dim LL in the three replicate experiments and results of Chi-square test.

		Rhythmic flies		Flies showing complex rhythms		Arrhythmic flies		Critical value (df = 1, α = 0.05)
Experiment	Species	Number	χ^2	Number	χ^2	Number	χ^2	
1	DA	49		1		11		3.814
	DM	28	5.73	18	15.21	17	1.28	
2	DA	23		1		33		
	DM	0	23	38	35.10	23	1.78	
3	DA	37		1		20		
	DM	9	17.04	10	7.36	42	7.81	

Table 2.6 Percentage of DM and DA flies showing rhythmic, arrhythmic, and complex rhythmic behaviour in dim LL in the three replicate experiments.

Experiment	Species	Rhythmic	Complex rhythms	Arrhythmic
1	DA	80.33	1.64	18.03
	DM	44.44	28.57	26.98
2	DA	40.35	1.75	57.89
	DM	0	62.29	37.70
3	DA	63.79	1.72	34.48
	DM	14.75	16.39	68.85

Table 2.7 Results of ANOVA performed on amplitude of the activity/rest rhythm of DA and DM under DD and LD.

Effect	***df***	**MS**	***F***	***p***
Regime	1	1445.8	15.429	< 0.001
Species	1	1955.6	20.869	0.00001
Regime * Species	1	14.9	0.16	0.69

Table 2.8 Results of ANOVA performed on phase-shift values as obtained from the PRC.

Effect	***df***	**MS**	***F***	***p***
Phase	3	274.9132	97.81458	< 0.00001
Species	1	79.1643	28.16679	< 0.00001
Phase * Species	3	13.5896	4.83519	< 0.005

Table 2.9 Results of ANOVA performed on phase-shift values as obtained from the DRC with 1 lux intensity light pulse at CT 21.

Effect	***df***	**MS**	***F***	***p***
Species	1	1.2853	0.85607	0.35
Duration	2	3.2077	2.13654	0.12
Species * Duration	2	0.1118	0.07444	0.92

Table 2.10 Results of ANOVA performed on phase-shift values as obtained from the DRC with 1 lux intensity light pulse at CT 14.

Effect	***df***	**MS**	***F***	***p***
Species	1	19.0002	14.425	< 0.001
Duration	2	42.695	32.4141	< 0.0001
Species * Duration	2	48.3838	36.7331	< 0.0001

Table 2.11. Results of ANOVA performed on phase-shift values as obtained from the DRC with 10 lux intensity light pulse at CT 21.

Effect	***df***	**MS**	***F***	***p***
Species	1	4.0944	1.9937	0.16042
Duration	2	24.0863	11.7282	< 0.0001
Species * Duration	2	17.2879	8.4179	< 0.001

Table 2.12 Results of ANOVA performed on phase-shift values as obtained from the DRC with 10 lux intensity light pulse at CT 14.

Effect	***df***	**MS**	***F***	***p***
Species	1	11.3955	7.7929	< 0.01
Duration	2	28.732	19.6486	< 0.00001
Species * Duration	2	11.0314	7.5439	< 0.001

Table 2.13 Results of ANOVA performed on phase-shift values as obtained from the DRC with 50 lux intensity light pulse at CT 21.

Effect	***df***	**MS**	***F***	***p***
Species	1	1.3382	0.7166	0.3988
Duration	2	25.629	13.7243	< 0.0001
Species * Duration	2	9.4189	5.0438	< 0.01

Table 2.14 Results of ANOVA performed on phase-shift values as obtained from the DRC with 50 lux intensity light pulse at CT 14.

Effect	***df***	**MS**	***F***	***p***
Species	1	51.9055	43.7513	< 0.0001
Duration	2	13.9906	11.7928	< 0.0001
Species * Duration	2	14.7046	12.3945	< 0.0001

Table 2.15 Mean values of phase-shifts in DM and DA for a light pulse of 1 lux intensity at CT 21.

Duration	Species	Mean (hour)	95% CI
1 min	DM	0.86	0.58
	DA	0.68	
10 min	DM	1.38	
	DA	1.06	
50 min	DM	1.38	
	DA	1.26	

Table 2.16 Mean values of phase-shifts in DM and DA for a light pulse of 1 lux intensity at CT 14

Duration	Species	Mean (hour)	95% CI
1 min	DM	-0.52	0.55
	DA	-1.20	
10 min	DM	-3.14	
	DA	-0.10	
50 min	DM	-2.79	
	DA	-2.88	

Table 2.17 Mean values of phase-shifts in DM and DA for a light pulse of 10 lux intensity at CT 21.

Duration	Species	Mean (hour)	95% CI
1 min	DM	1.81	0.67
	DA	0.12	
10 min	DM	2.04	
	DA	2.92	
50 min	DM	1.99	
	DA	1.74	

Table 2.18 Mean values of phase-shifts in DM and DA for a light pulse of 10 lux intensity at CT 14.

Duration	Species	Mean (hour)	95% CI
1 min	DM	-1.91	0.55
	DA	-1.54	
10 min	DM	-2.57	
	DA	-0.97	
50 min	DM	-2.97	
	DA	-3.2	

Table 2.19 Mean values of phase-shifts in DM and DA for a light pulse of 50 lux intensity at CT 21.

Duration	Species	Mean (hour)	95% CI
1 min	DM	1.66	0.58
	DA	0.84	
10 min	DM	2.27	
	DA	3.28	
50 min	DM	1.68	
	DA	2.08	

Table 2.20 Mean values of phase-shifts in DM and DA for a light pulse of 50 lux intensity at CT 14.

Duration	Species	Mean (hour)	95% CI
1 min	DM	-2.65	0.46
	DA	-1.59	
10 min	DM	-3.05	
	DA	-0.73	
50 min	DM	-3.01	
	DA	-2.8	

Table 2.21 Result of ANOVA performed on phase relationship of onset in DA and DM when entrained to single light pulse occurring every 24 hours.

Effect	***df***	**MS**	***F***	***p***
Species	1	5.7171	4.921	< 0.05

2.4 Discussion

Two sympatric Drosophilid species, *D. melanogaster* and *D. ananassae*, were previously shown to have different temporal pattern of activity/rest rhythm (Prabhakaran and Sheeba, 2012). DM shows a crepuscular behaviour with activity peaks at dawn and dusk, while DA shows a diurnal behaviour with one peak of activity in the morning, and little or no activity in the evening and night. The diurnal nature of activity/rest rhythm in DA was shown to persist in natural conditions as well as when natural conditions of light and temperature were simulated in the laboratory (Prabhakaran and Sheeba, 2013b, 2014). The phase of activity was also found to be delayed in DA as compared to that in DM. Phasing of rhythm under entrained conditions is affected by the free running period (FRP) of circadian clock (see Moore-Ede et al., 1982). In the present study, I find that, despite having distinctly different temporal pattern of activity/rest rhythm under cyclic conditions, the free-running period (FRP) of the circadian rhythm is not significantly different in the two species. The amplitude of the periodogram, however, was higher in DA than in DM, which suggests that the FRP in DD is possibly more precise in DA as compared to DM. Given that DM and DA have similar FRPs, it was interesting to find that the phase of morning peak in DA was delayed as compared to that in DM under different *T* cycles of periodicities both longer and shorter than 24 hours. This is consistent with the results of a previous study which found that DA has a delayed phase of morning peak as compared to DM even under long and short photoperiods (Prabhakaran and Sheeba, 2012). Thus, clock properties other than the FRP are likely to be bringing about the differences seen in activity/rest rhythm in the two species.

When T < FRP, it is expected that the phase of the rhythm will be delayed with respect to the zeitgeber, whereas the phase of the rhythm is advanced with respect to the zeitgeber when T > FRP (see Moore-Ede et al., 1982). Thus, phase of the rhythm should change systematically with varying period of *T* cycle. Even though the FRPs of DM and DA are

not significantly different, in DA, the extent of change in phase of morning peak with changing length of *T* cycles is smaller than what is seen in DM. In DA, the phase of morning peak did not differ among *T* 18, *T* 20 and *T* 24, and among *T* 28 and *T* 30. For DM, however, the phase of both morning and evening peak showed an expected variation with changing *T* cycle period. Thus, the morning activity in DA appears to be more tightly phase-locked to the dark-light (dawn) transition as compared to that in DM. Moreover, a lower fraction of DA individuals were found to entrain to the different *T* cycles as compared to DM, suggesting that the ability to entrain to *T* cycles of varying periodicities is lower in DA. Taken together, it seems like the clock of DA is more robust to changing under entraining conditions than that of DM. As suggested by Prabhakaran and Sheeba (2012), the results of the present study also indicate that the morning oscillator in DA is more strongly coupled to the dark-light transition as compared to that in DM, which might be also involved in bringing about the robust phasing of the morning activity. Given the ancestral distribution of DA in tropical and sub-tropical regions (Das et al., 2004; Tobari, 1993; Dobzhansky and Dreyfus, 1943), they are not likely to face drastic changes in environmental cycles around the year. In such a case, it is possible that the clock of DA does not need to be as labile as that of DM. However, a less robust clock in DM would be helpful for scheduling activity in the temperate regions that the species is known to inhabit (David and Tsacas, 1981; David and Capy, 1988), as the environmental conditions (eg. photoperiods) in the temperate regions show more variation around the year.

The differences in phasing of activity between DM and DA was found to be accompanied by differences in clock properties like circadian photosensitivity and amplitude of the clock. A greater proportion of DA individuals showed persistent rhythmicity under dim LL of 0.1 lux intensity as compared to DM, suggesting that circadian photosensitivity of DA is lower than that of DM. In this light regime, a larger fraction of DM individuals showed complex

rhythms as compared to DA individuals. Complex rhythms are seen when the otherwise coupled component oscillators of a clock decouple and each oscillator-driven rhythm free-runs with its inherent periodicity which may be different from the overall period that is seen when the oscillators are coupled (Pittendrigh, 1960; Hoffman, 1971). A clock with weaker inter-oscillator coupling is likely to dissociate more at a particular light intensity, whereas, a clock with stronger inter-oscillator coupling is likely to withstand the decoupling effect of light. Thus, it seems that, along with lower circadian photosensitivity, DA is likely to have a clock with stronger inter-oscillator coupling as compared to DM, since a lower proportion of DA individuals showed complex rhythms in dim LL when compared to DM. Differences in strength of inter-oscillator coupling can also be examined by comparing the number of transient cycles required by the species when re-entraining to an LD cycle whose phase is shifted (advanced or delayed) with respect to a prior LD cycle that the flies have been entrained to.

In constant (DD) as well as under rhythmic (LD 12:12) conditions, the amplitude of the activity/rest rhythm in DM was found to be larger than that of DA, suggesting that the circadian clock of DA has a lower amplitude as compared to that of DM. In this study, for the lack of a protocol for measuring the amplitude of the oscillator directly, the amplitude of the overt activity/rest rhythm was used as a measure of the amplitude of the oscillator. Previously, for DM, this measure has been used to examine the amplitude of the clock (Nikhil et al., 2016). Also, a study on *Drosophila pseudoobscura* has used the amplitude of the overt eclosion rhythm to construct an amplitude response curve of the oscillator showing that the amplitude of the overt rhythm is, in fact, related to the amplitude of the oscillator (Winfree, 1973).

Intrinsic amplitude of the circadian clock affects the phase response of the clock to the zeitgeber such that, for the same stimulus strength, a low amplitude oscillator is expected to

undergo larger phase-shifts as compared to a high amplitude oscillator (Pittendrigh et al., 1991; Abraham et al., 2010, discussed in Introduction of this chapter). However, the photic PRC shows that DA undergoes smaller phase delay as compared to DM, and does not differ from DM with respect to magnitude of phase advances, even though DA has a low amplitude rhythm in DD. It is possible that the amplitude of the overt activity/rest rhythm is not an appropriate reflection of the amplitude of the oscillator in this species. Nevertheless, the results suggest that the clocks of DM and DA show phase-dependent differences in sensitivity to brief light pulses of 100 lux. From the DRC, it is seen that this trend holds true across different intensities and duration of light pulses. DM and DA do not differ in their phase responses to light when the pulse falls in the late subjective night. However, light pulses within a range of intensities falling on the clock in the early subjective night results in greater phase delay in DM as compared to that in DA. Apart from differences in this phasic effect, light also seems to produce different tonic effects in the two species. This is evident from the DRC, where, DM shows faster saturation of phase response with increasing duration of light pulse as compared to DA. The light pulses were given at CTs that were calculated from the mean FRP values of DM and DA as described in Prabhakaran and Sheeba (2012), which are slightly different from what has been found in the present study. Thus, it is possible that the light pulses fell at approximately the same CTs, but not exactly. However, the magnitude of deviation is small, and the phase responses of DM or DA are not likely to vary significantly within that range.

Overall, there seems to be differences in tonic as well as phasic effects of light on the circadian clocks of DM and DA. So which of these effects can explain entrainment of the activity/rest rhythm in the two species? In order to examine the mode of entrainment in DM and DA, two more preliminary experiments were done. From the phase of entrainment of the two species when entrained to single light pulses occurring every 24 hours, it can be

seen that the light pulse occurred at about CT 09 and CT 11 for DA and DM respectively, assuming that the onset of activity is at CT 0. From the PRC, it is seen that if entrainment is occurring solely by phase resetting, light pulses falling at these time points cannot result in the phase-shift required for entrainment in either of the two species. Thus, it appears that the discrete model of entrainment cannot explain the entrainment of the clock in DM and DA. However, this inference is based on the assumption that the onset of activity is in alignment with CT 0 of the PRC. In order to examine if entrainment is occurring by parametric changes, the aftereffects of entrainment to *T* cycles on the FRP was examined in the two species and no significant aftereffects were observed in the FRP of either species. This may be because while entraining to *T* cycles with periods very different from the FRP, clocks can attain a large portion of the period change required (i.e. FRP – *T*) by phase resetting alone, and thus, the parametric changes that are contributing to entrainment are too small to detect. However, future studies may examine aftereffects of entrainment to different photoperiods in DM and DA. When entraining to *T* 24 cycles with different photoperiods, FRP is very close to *T*, and it is expected that if entraining by phase resetting, the clock will undergo phase-shifts much larger in magnitude than what is necessary to entrain. In such a case, parametric changes, instead, may be contributing predominantly in attainment of period matching. Indeed, DM populations are seen to have pronounced aftereffects post entrainment to different photoperiods (Lakshman, personal communication).

Overall, the present study shows that, the differences in temporal pattern of activity/rest rhythm in two sympatric Drosophilid species with similar intrinsic clock periods are accompanied by differences in the amplitude, phase response, and possibly, inter-oscillator coupling of the underlying circadian clock.

Chapter III

Sleep characteristics of *D. melanogaster* and *D. ananassae*

3.1 Introduction

3.1.1 Sleep and Arousal: circadian and homeostatic regulation and functional significance

Sleep is a physiological state in animals that is primarily characterized by a state of inactivity during which the organism shows decreased sensory and motor function (Campbell and Tobler, 1984). Arousal is another physiological state which is, in a sense the opposite of sleep, characterized by increased sensory and motor function (van Swinderen and Andretic, 2003). Sleep and arousal occur rhythmically. This sleep-wake cycle exhibits a circadian rhythm, and has been shown to be under the control of a circadian clock (Daan et al., 1984; reviewed in Franken and Dijk, 2009). However, sleep is also regulated by a homeostatic mechanism (Borbely, 1982; reviewed in Franken and Dijk, 2009) such that severe sleep deprivation results in compensatory sleep occurring at times when the animal is normally awake, a phenomenon called sleep rebound. While circadian control determines the *timing* of sleep and wakefulness, homeostatic mechanisms regulate the *quality* of sleep i.e. the amount, duration, and distribution of sleep bouts (Saper et al., 2005).

The phenomenon of sleep is seen in most animal taxa studied, ranging from insects, reptiles, birds, to terrestrial and aquatic mammals, and there is inter-species variation in the average duration of sleep (reviewed in Allada and Siegel, 2008; Cirelli and Tononi, 2008). It would seem that, by being in a state of sleep, which is characterized by non-responsiveness to

external stimuli, and immobility, organisms are not only vulnerable to external threats but they are also losing precious time that could otherwise be utilized in more beneficial activities like mate searching, foraging, etc. So what is the functional significance of sleep? Observations like sleep being conserved across a wide range of animal taxa, presence of sleep rebound, and deleterious effects of sleep deprivation has led to the proposition of various hypotheses regarding the functional significance of sleep. It has been suggested that sleep is necessary for maintaining synaptic functions, information processing, and memory formation. The energy reduction hypothesis suggests that sleep has a role in regulating the energy demands of the organism. Sleep is also thought to be important for replenishing the energy stores of cells which are depleted during wakefulness, and thus serve a restorative function. Numerous studies on vertebrate as well as invertebrate model systems have provided evidence supporting these hypotheses, even though they have not successfully reached a consensus as to why animals sleep (reviewed in Cirelli and Tononi, 2008; Mignot, 2008; Potdar and Sheeba, 2013).

3.1.2 Sleep in *D. melanogaster*

Sleep has been extensively studied in the past two decades using *Drosophila melanogaster* as a model system, ever since the species was found to exhibit a rest-like state which is similar to sleep in mammals (Hendricks et al., 2000; Shaw et al., 2000). This state in *D. melanogaster* shows many of the characteristics that have been used to define sleep in other organisms (Campbell and Tobler, 1984). Sleep in *D. melanogaster* is characterized by a species-specific sleep posture at a preferred site, immobility, increased arousal threshold, and circadian as well as homeostatic regulation (Hendricks et al., 2000; Shaw et al., 2000). The electrophysiological correlates are also similar to that of sleep in other species (Nitz et al., 2002). In *D. melanogaster*, an individual showing continuous inactivity for five minutes

is considered to be sleeping because at this stage, higher intensity stimuli is required to elicit activity from the fly, distinguishing it from a state of simple inactivity (Shaw et al., 2000; Huber et al., 2004). When the sleep pattern is considered, this species shows a bimodal sleep profile with increase in sleep level around midday and after dark. Arousal threshold during the day time was found to be lower as compared to that during night time sleep (Huber et al., 2004), indicating that night time sleep is deeper than day time sleep. Moreover, sleep in *D. melanogaster* depends on the sex and mating status, with mated female flies showing lower day time sleep (Huber et al., 2004; Isaac et al., 2010).

This well-characterized behaviour of sleep in *D. melanogaster*, along with the modern molecular and genetic manipulation methods available for the species, has helped unravel some of the neurogenetic correlates of the behaviour, of which the circadian clock genes and the clock neurons were found to be important players (reviewed in Tomita et al., 2017). As has been elucidated in the previous chapters, various circadian behaviours have been found to show a wide range of variation among different Drosophilid species along with correlated differences in their underlying machinery, and examining the inter-species variations seen in these behaviours would be useful for understanding the general principles that govern the behaviours. However, there are few studies on comparison of sleep among different species of *Drosophila*.

3.1.3 Comparative studies of sleep in Drosophilid species and rationale for the present study

To the best of my knowledge, comparative studies of sleep in Drosophilid species are few. A previous study from the lab involved a preliminary investigation into the sleep characteristics of *D. melanogaster* (DM) and *D. ananassae* (DA) (Prabhakaran and Sheeba, 2012). The study found that the sleep patterns in virgin males of the two species under LD

12:12 were distinct, with DA showing significantly higher amount of night-time sleep as compared to DM, which is consistent with the preference for diurnal activity seen in DA. The number of night time sleep bouts (i.e. continuous stretches of uninterrupted sleep) was lower in DA as compared to DM and the trend was reversed for day time sleep bout. This implies that DA showed more consolidated sleep during night than that seen in DA, and even though the day time sleep level was not different between the species, DA showed less consolidated sleep during day time. Moreover, it appears that the day time sleep peaks in DA at a later phase as compared to DM. Thus, both the timing and the quality of sleep in DM and DA showed distinct variation, indicating that the underlying circadian and homeostatic control are likely to be different. Many of the neuronal clock components of the activity/rest rhythm are known to overlap with neuronal circuits regulating sleep in DM. This, along with the findings discussed in Chapter II regarding the differences in circadian clock properties of DM and DA, further suggests that the mechanism of sleep regulation in the two species may be different.

In order to examine the differences in regulation of sleep, a systematic comparison of the sleep-wake behaviour in the two species is required at first. Sleep characterization in DA has previously been done only for virgin males of the species (Prabhakaran and Sheeba, 2012). However, in DM, many features of sleep are influenced by sex and mating status. Therefore, the aim of the present study was to characterize and compare the features of sleep in DA and DM for mated and virgin individuals of each sex.

3.1.4 The present study

The present study addressed the following questions: (1) What are the characteristics of sleep-wake behaviour in *D. ananassae* and how do they vary with mating status and sex? (2) How do the sleep characteristics of *D. ananassae* differ from those of *D. melanogaster*?

In order to address the above questions, the activity of DM and DA individuals was recorded under laboratory conditions of LD 12:12 and 25°C using DAM system (refer to Chapter II). From the activity data, the following measures of sleep were analyzed: sleep level, level of day time and night time sleep, number and duration of sleep bouts during day and night. In this study, I also examined whether the two species show differences with respect to how deeply they sleep at different times of the day. To that end, virgin and mated females of the two species were disrupted at two time points (one during day time sleep and one during night time sleep) using the same strength of stimulus, in this case, a mechanical perturbation that lasted one second. The percentage of sleeping flies that were aroused in the two species at the two time points were then compared.

3.2 Materials and methods

3.2.1 Fly strains and stock maintenance: Same as discussed in Chapter II.

3.2.2 Activity recording: Activity of the flies were recorded using the Trikinetics *Drosophila* Activity Monitors (DAM) system (Trikinetics, Waltham, MA, USA) (refer to Chapter II). Activity was recorded for 5 day old flies. In each species 32 flies of each mating status (mated or virgin) and each sex was recorded in LD 12:12 at 25°C and ~500 lux light intensity. Since mated female flies would lay fertilized eggs which would interfere with the recording, all the flies were supplied with agar-sucrose medium food that prevents hatching of eggs.

3.2.3 Sleep analysis: Activity count data was obtained at 1 minute intervals and the following was calculated for each individual and each day by using the software, Pysolo v1.0 (Gilestro and Cirelli, 2009; http://www.pysolo.net/): the mean of total sleep level, day time and night time sleep, number and duration of day time and night time sleep bouts. As

mentioned previously, a minimum sleep bout is defined as a period of continuous inactivity (zero beam breaks / minute) lasting for 5 minutes. The values were averaged across days for an individual and these mean values were used for statistical analysis. A factorial ANOVA was done using these values with species, mating status, and sex as fixed factors. Pairwise comparisons were made by post-hoc test (Tukey's HSD). The statistical analyses were done in Statistica 7 (Statsoft.inc). The sleep profiles of flies of each sex, mating status, and species showing the sleep amount / 30 minutes across the time of the day was obtained using Pysolo.

3.2.4 Analysis of percentage of flies that were aroused by mechanical stimuli: The activity of 5-6 day old virgin and mated females of the two species were recorded under LD 12:12 at 25°C and ~500 lux light intensity. The activity monitors were placed on a vortexer which can be programmed to provide mechanical perturbation. Flies were subjected to a mechanical perturbation which lasted for one second at zeitgeber time (ZT) 08 (ZT 0 = lights on; ZT 12 = lights off) and at ZT 16 in two different experiments. Each experiment was repeated three times. Control sets of flies were recorded at the same time but they did not receive the mechanical perturbation. The activity count data was obtained at 1 minute intervals. The number of flies that were sleeping before the initiation of the stimulus was calculated by counting those channels in the monitor that showed five consecutive activity counts of zero (i.e. they were inactive for at least five minutes at a stretch before the stimulus was given). The number of flies that were aroused was calculated by counting those flies which showed five minutes of inactivity before the stimulus and showed a non-zero activity count after the stimulus. The percentage of sleeping flies that were aroused was calculated for flies of each mating status and each species. There were 32 flies in each set. The percentage values were transformed by arcsine square root transformation. The calculations were done in Microsoft Excel (Microsoft, USA). A factorial ANOVA was performed on

these transformed values with species, mating status, and time point as factors which was followed by Tukey's HSD for pair-wise comparisons. The statistical analyses were done in Statistica 7 (Statsoft.inc).

3.3 Results

3.3.1 *D. melanogaster* and *D. ananassae* have different sleep patterns

Sleep patterns were compared between the two species taking into account the sex (male or female) as well as the mating status (virgin or mated). Results from two replicate experiments have been reported here, each of which was analyzed separately due to lack of more replicate experiments. In both the experiments, it was seen that DM and DA had differences in their sleep patterns when sex and mating status were taken into account (Figure 3.1). Overall, DA showed less sleep during the day as compared to DM, and during the night DA slept more than DM.

In order to quantify the amount of sleep, the following were analyzed: average of total sleep in a day (mean sleep), mean day time and night time sleep, and mean number and length of sleep bouts during the day and the night. For each of these quantities, a factorial ANOVA was done with species, mating status, and sex as fixed factors (Table 3.1 – 3.14). Across both the experiments, mean sleep was significantly different between mated females of DM and DA, with mated DA females showing greater amount of mean sleep as compared to mated DM females (Figure 3.2A, Table 3.15). Mean sleep in DA was not found to be significantly different across the two sexes and mating status. However, in DM, as reported previously (Huber et al., 2004; Isaac et al., 2010), mating status and sex affected the amount of mean sleep, with mated female files showing lower amount of mean sleep as compared to mated males (Figure 3.2A).

Day time sleep: Overall, as compared to DM, DA showed less mean day time sleep with lower mean length of sleep bout and greater mean number of sleep bouts (Figure 3.2B, Table 3.16). Mean day time sleep level did not differ among the two sexes and mating status of DA flies, but mating status and sex did affect mean day time sleep in DM. Unlike mean total sleep, neither the mean day time sleep levels nor the mean number and length of day time sleep bouts was significantly different between mated females of DM and DA (Figure 3.2B, D, E). DA males showed a shorter mean length of sleep bout during the day as compared to DM males (Figure 3.2D).

Night time sleep: DA showed higher mean night time sleep with lower number of sleep bouts and larger mean length of sleep bout as compared to DM (Figure 3.2C, F, G, and Table 3.17). Similar to day time sleep, night time sleep in DA was not different between the sexes or for different mating status. In DM, also, no consistently significant differences were seen in night time sleep levels among the mated and virgin flies of the two sexes. Mated females and mated males of DA showed a significantly lower mean number of night time sleep bouts as compared to mated females and mated males of DM. There was no consistently significant difference in mean number of night time sleep bout between DA and DM with respect to virgin males and females.

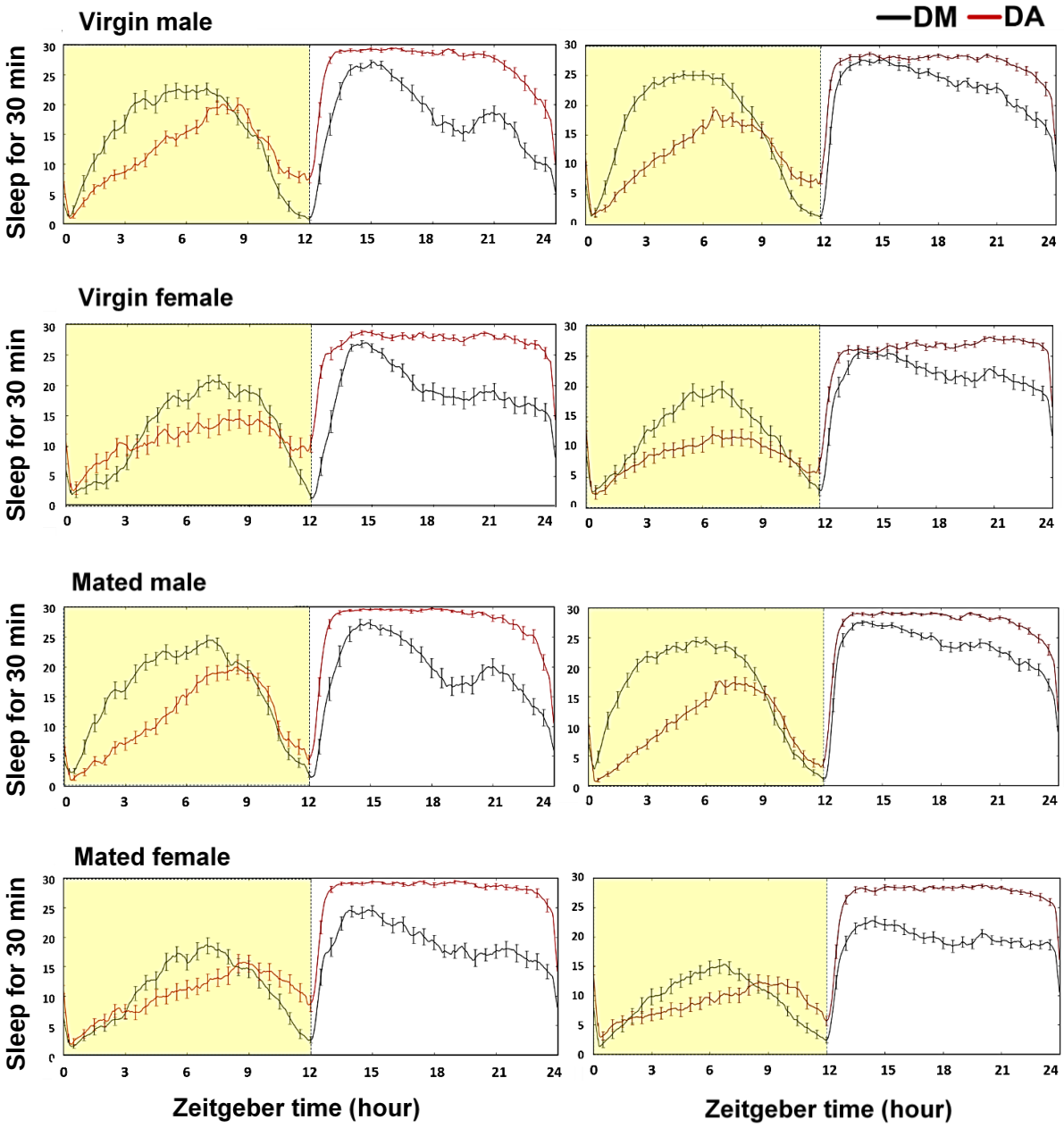

Figure 3.1. Sleep profiles of DM and DA. The results from experiment 1 (left panel) and experiment 2 (right panel). Yellow shaded region denotes the light phase.

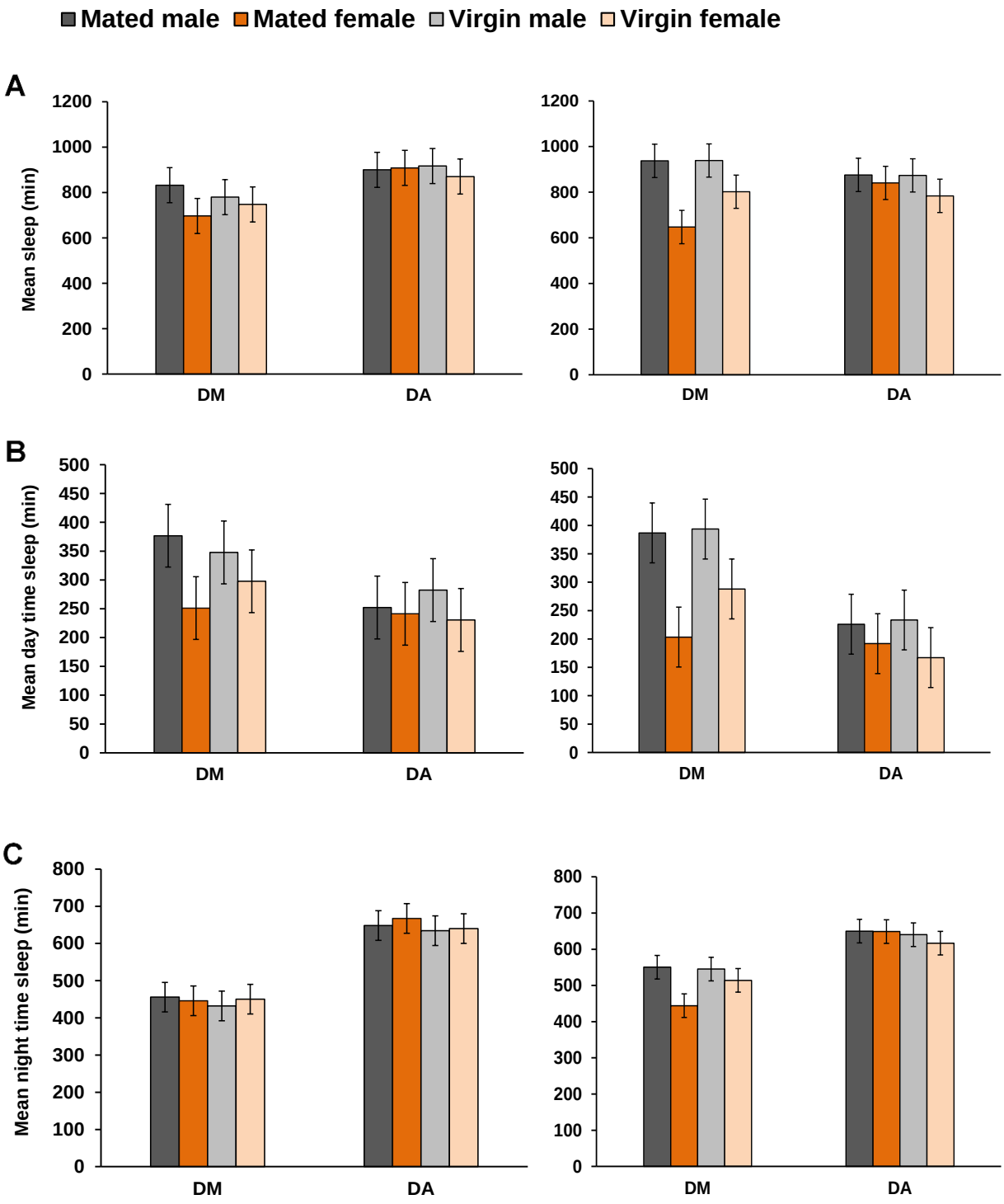

Figure 3.2. Sleep characteristics of DM and DA. **(A)** Mean sleep, **(B)** mean day time sleep **(C)** mean night time sleep. Results from experiment 1 and experiment 2 are shown in the left and the right panel respectively (Three-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing. Continued to next page.

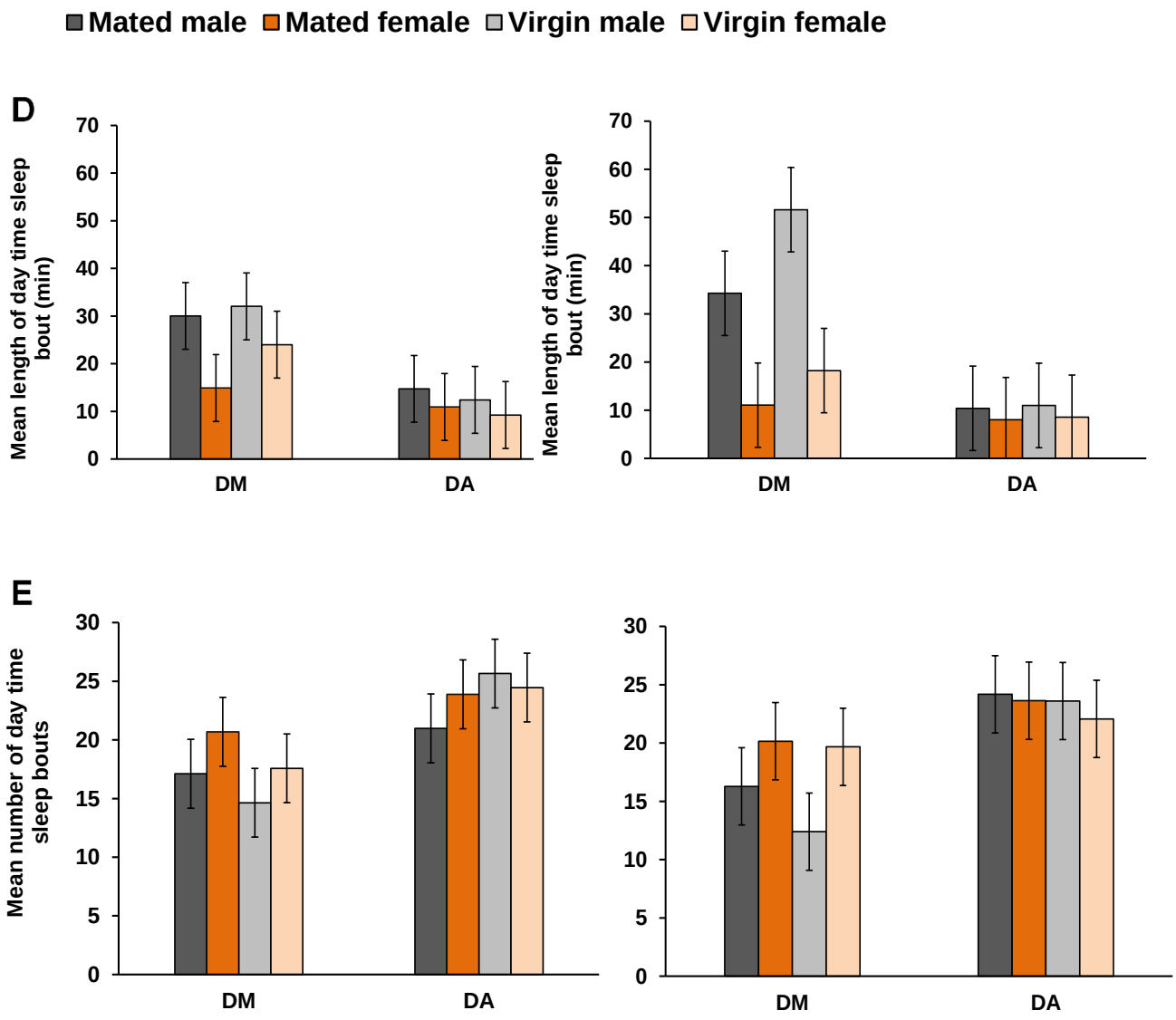

Figure 3.2. Sleep characteristics of DM and DA. Continued from previous page. Mean length **(D)** and number **(E)** of day time sleep bout. Results from experiment 1 and experiment 2 are shown in the left and the right panel respectively (Three-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing. Continued to next page.

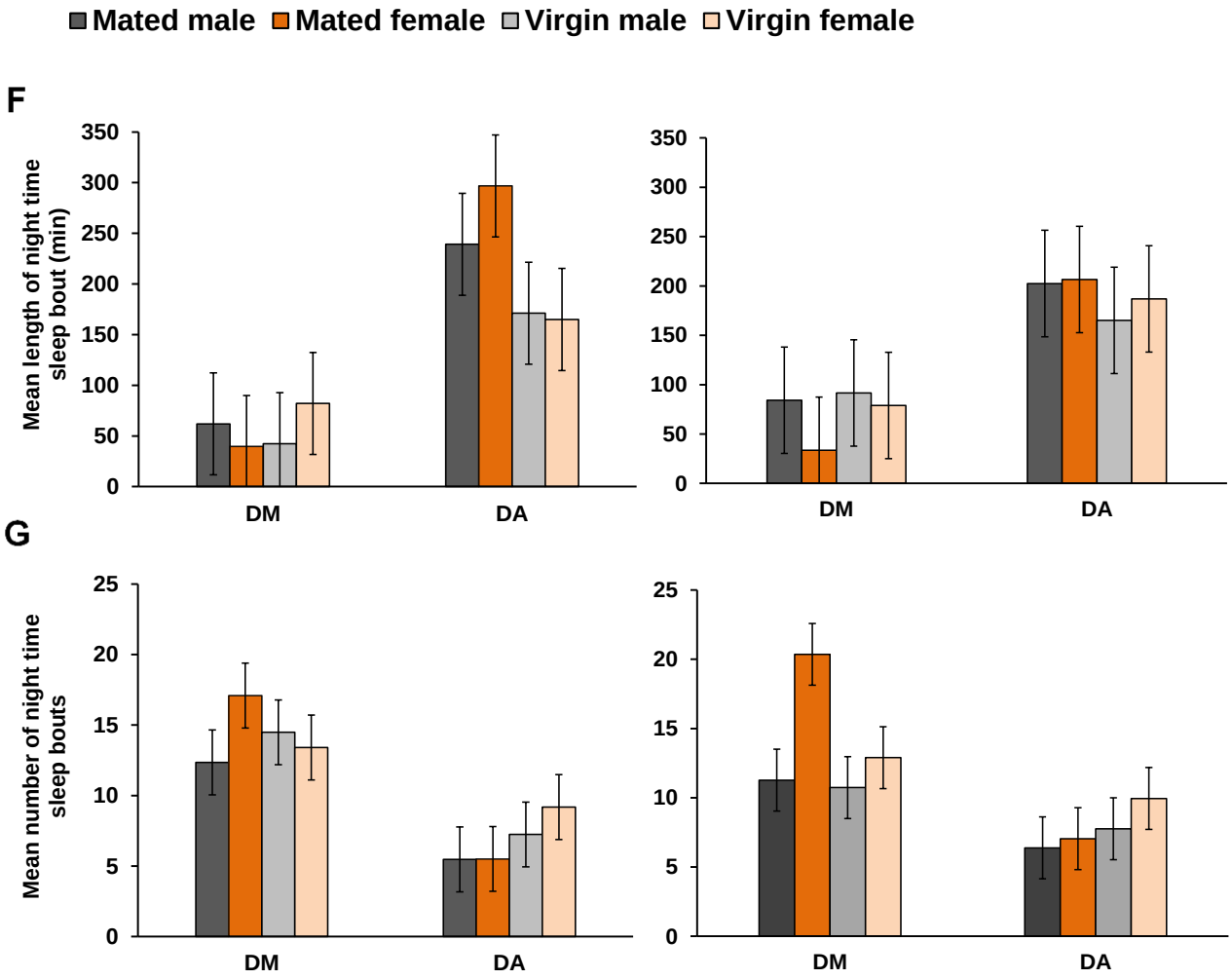

Figure 3.2. Sleep characteristics of DM and DA. Continued from previous page. Mean length **(F)** and number **(G)** of night time sleep bout. Results from experiment 1 and experiment 2 are shown in the left and the right panel respectively (Three-way ANOVA, Tukey's HSD). Error bars indicate 95% CI for visual hypothesis testing.

3.3.2 Percentage of flies aroused during day time and night time sleep in *D. melanogaster* and *D. ananassae*

DM and DA showed different amounts of sleep during different times of the day. Do the two species also differ with respect to how deeply they sleep during different times of the day? In order to address this question, the percentage of DM and DA flies, which were aroused by a mechanical stimulus at two times of the day, was compared (Figure 3.3). The percentage of sleeping flies that were aroused following a mechanical stimulation of 1 second was calculated and a three way ANOVA was done with species, phase (day or night), and mating status as fixed factors (Table 3.18). There was a significant main effect of species ($F = 34.57$, $p < 0.01$) and of phase ($F = 17.41$, $p < 0.01$). At both ZT 08 (day) and ZT 16 (night), a significantly greater percentage of mated DM females were aroused as compared to mated DA females. However, a significantly greater number of virgin DM females were aroused as compared to virgin DA females at only ZT 08, but not at ZT 16. The percentage of sleeping flies that were aroused in DM did not depend on mating status or time of the day. On the other hand, a significantly lower percentage of mated DA females were aroused at ZT 08 as compared to that at ZT 16. No such difference was seen for virgin DA females. Overall, during the day, lower percentage of DA females were woken up by the same stimulus as compared to that of DM females i.e. DA sleep appears to be deeper than that of DM during the day. At night, however, only mated DA females seem to be sleeping more deeply than mated DM females, as percentage of virgin females aroused were not different between the two species. Interestingly, mated females of DA appear to be sleeping more deeply during the day than during the night, whereas virgin DA females do not show such a difference.

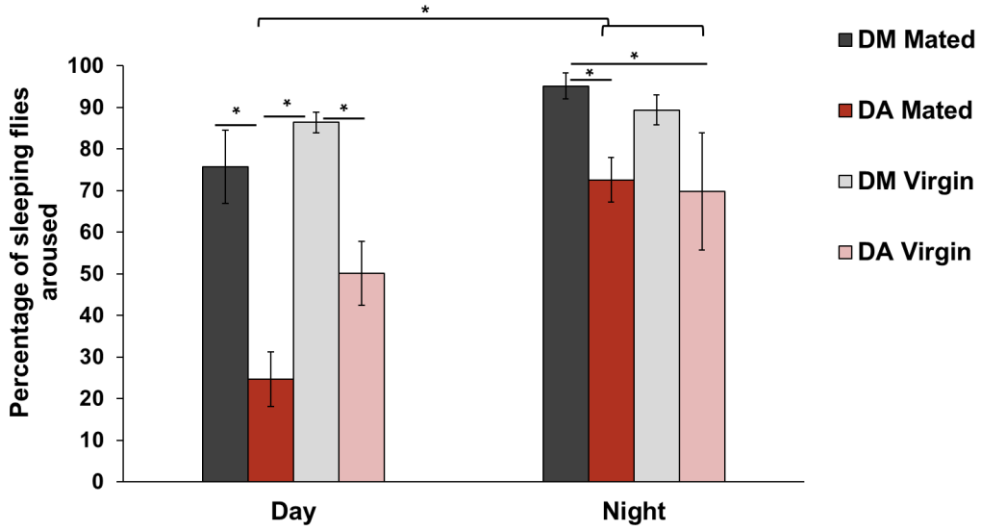

Figure 3.3. Percentage of sleeping flies in DM and DA that were aroused by a mechanical stimulus of one second duration during the day (ZT 08) and the night (ZT 16) (Three-way ANOVA, Tukey's HSD). * indicates significant differences. Error bars are SEM across three replicate experiments.

Table 3.1 Results of ANOVA performed on mean sleep levels in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	1061538	35.587	0
Mating status	1	1962	0.066	0.79
Sex	1	154898	5.193	< 0.05
Species * Mating status	1	1478	0.05	0.82
Species * Sex	1	61282	2.054	0.15
Mating status * Sex	1	8578	0.288	0.59
Species * Mating status * Sex	1	91122	3.055	0.08

Table 3.2 Results of ANOVA performed on mean sleep levels in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	8943	0.336	0.56
Sex	1	1154342	43.329	< 0.0001
Mating status	1	35773	1.343	0.24
Species * Sex	1	345915	12.984	< 0.001
Species * Mating status	1	175694	6.595	< 0.05
Sex * Mating status	1	36743	1.379	0.24
Species * Sex * Mating status	1	163924	6.153	0.01

Table 3.3 Results of ANOVA performed on mean day time sleep levels in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	260383	17.534	< 0.0001
Mating status	1	4993	0.336	0.56
Sex	1	207759	13.99	< 0.001
Species * Mating status	1	13	0.001	0.97
Species * Sex	1	46422	3.126	0.07
Mating status * Sex	1	4342	0.292	0.58
Species * Mating status * Sex	1	49472	3.331	0.06

Table 3.4 Results of ANOVA performed on mean day time sleep levels in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	780309	56.128	< 0.00001
Sex	1	575836	41.42	< 0.00001
Mating status	1	20920	1.505	0.22
Species * Sex	1	134884	9.702	< 0.01
Species * Mating status	1	44693	3.215	0.07
Sex * Mating status	1	7902	0.568	0.45
Species * Sex * Mating status	1	45953	3.305	0.07

Table 3.5 Results of ANOVA performed on mean length of day time sleep bout in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	10513.4	42.779	0
Mating status	1	183.37	0.7461	0.38
Sex	1	3318.6	13.5034	< 0.001
Species*Mating status	1	834.4	3.3952	0.06
Species*Sex	1	955.74	3.8889	< 0.05
Mating status*Sex	1	216.12	0.8794	0.34
Species*Mating status*Sex	1	151.35	0.6158	0.43

Table 3.6 Results of ANOVA performed on mean length of day time sleep bout in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	22602.4	58.9592	< 0.00001
Sex	1	14308.79	37.325	< 0.00001
Mating status	1	2489.19	6.4931	< 0.05
Species*Sex	1	10175.72	26.5437	<0.00001
Species*Mating status	1	2076.4	5.4164	< 0.05
Sex*Mating status	1	399.67	1.0425	0.30
Species*Sex*Mating status	1	387.67	1.0112	0.31

Table 3.7 Results of ANOVA performed on mean number of day time sleep bout in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	2274.8	52.961	< 0.0001
Mating status	1	0.39	0.009	0.92
Sex	1	246.52	5.739	< 0.05
Species*Mating status	1	428.93	9.986	< 0.01
Species*Sex	1	84.24	1.961	0.16
Mating status*Sex	1	81.95	1.908	0.16
Species*Mating status*Sex	1	43.62	1.015	0.31

Table 3.8 Results of ANOVA performed on mean number of day time sleep bout in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	2364.55	43.14	< 0.0001
Sex	1	311.43	5.682	< 0.05
Mating status	1	160.69	2.932	0.08
Species * Sex	1	664.82	12.129	< 0.001
Species * Mating status	1	18.67	0.341	0.55
Sex * Mating status	1	22.1	0.403	0.52
Species * Sex * Mating status	1	72.93	1.331	0.24

Table 3.9 Results of ANOVA performed on mean night time sleep in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	2373408	299.231	< 0.0001
Mating status	1	13214	1.666	0.19
Sex	1	3873	0.488	0.48
Species * Mating status	1	1766	0.223	0.63
Species * Sex	1	1030	0.13	0.71
Mating status * Sex	1	714	0.09	0.76
Species * Mating status * Sex	1	6311	0.796	0.37

Table 3.10 Results of ANOVA performed on mean night time sleep in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	956323	179.99	< 0.0001
Sex	1	99581	18.74	< 0.0001
Mating status	1	1980	0.37	0.54
Species * Sex	1	48788	9.18	< 0.01
Species * Mating status	1	43161	8.12	< 0.01
Sex * Mating status	1	10567	1.99	0.15
Species * Sex * Mating status	1	36293	6.83	< 0.01

Table 3.11 Results of ANOVA performed on mean length of night time sleep bout in DM and DA in experiment 1.

Effect	*df*	MS	*F*	*p*
Species	1	1523611	120.2719	< 0.0001
Mating status	1	114213	9.0158	< 0.01
Sex	1	17304	1.3659	0.24
Species * Mating status	1	181326	14.3136	< 0.001
Species * Sex	1	4229	0.3338	0.56
Mating status * Sex	1	13	0.001	0.97
Species * Mating status * Sex	1	57812	4.5636	< 0.05

Table 3.12 Results of ANOVA performed on mean length of night time sleep bout in DM and DA in experiment 2.

Effect	*df*	MS	*F*	*p*
Species	1	847932	58.4007	< 0.0001
Sex	1	5321	0.3665	0.54
Mating status	1	77	0.0053	0.94
Species * Sex	1	30123	2.0747	0.15
Species * Mating status	1	45699	3.1475	0.07
Sex * Mating status	1	11671	0.8039	0.37
Species * Sex * Mating status	1	1565	0.1078	0.74

Table 3.13 Results of ANOVA performed on mean number of night time sleep bout in DM and DA in experiment 1.

Effect	***df***	**MS**	***F***	***p***
Species	1	3267.9	123.8493	< 0.0001
Mating status	1	55.52	2.1043	0.14
Sex	1	115.72	4.3857	< 0.05
Species * Mating status	1	178.65	6.7706	< 0.01
Species * Sex	1	10.35	0.3921	0.53
Mating status * Sex	1	55.74	2.1126	0.14
Species * Mating status * Sex	1	217.29	8.235	< 0.01

Table 3.14 Results of ANOVA performed on mean number of night time sleep bout in DM and DA in experiment 2.

Effect	***df***	**MS**	***F***	***p***
Species	1	2209.18	88.509	< 0.001
Sex	1	753.38	30.183	< 0.001
Mating status	1	52.36	2.098	0.14
Species * Sex	1	266.93	10.694	< 0.01
Species * Mating status	1	572.08	22.92	< 0.0001
Sex * Mating status	1	110.5	4.427	< 0.05
Species * Sex * Mating status	1	270.75	10.848	< 0.01

Table 3.15 Mean values of total sleep for virgin and mated males and females of DM and DA in two replicate experiments.

Expt. No.	Species	Mating status and sex	Mean sleep (min)	± 95% CI
1	DM	Virgin male	780.05	77.24
		Virgin female	747.77	
		Mated male	832.44	
		Mated female	696.91	
	DA	Virgin male	916.96	
		Virgin female	870.46	
		Mated male	900.40	
		Mated female	908.68	
2	DM	Virgin male	938.8	73.00
		Virgin female	801.97	
		Mated male	937.29	
		Mated female	647.33	
	DA	Virgin male	873.61	
		Virgin female	783.83	
		Mated male	875.77	
		Mated female	840.72	

Table 3.16 Day time sleep in virgin and mated, males and females of DM and DA in two replicate experiments

DS: day time sleep; LDS: length of day time sleep bout; NDS: number of daytime sleep bout

Expt. No.	Species	Mating status and sex	Mean DS (min)	95% CI	Mean LDS (min)	95% CI	Mean NDS	95% CI
1	DM	Virgin male	347.8	54.50	32.04	7.01	14.64	2.93
		Virgin female	297.68		23.99		17.57	
		Mated male	376.75		30.02		17.11	
		Mated female	251.17		14.90		20.69	
	DA	Virgin male	282.4		12.41		25.66	
		Virgin female	230.46		9.23		24.46	
		Mated male	252.2		14.73		20.98	
		Mated female	241.23		10.92		23.88	
2	DM	Virgin male	393.56	52.73	51.61	8.76	12.4	3.31
		Virgin female	287.95		18.22		19.67	
		Mated male	386.79		34.27		16.28	
		Mated female	203.32		11.06		20.16	
	DA	Virgin male	233.42		10.99		23.60	
		Virgin female	167.05		8.547		22.06	
		Mated male	225.89		10.4		24.18	
		Mated female	191.73		8.03		23.63	

Table 3.17 Night time sleep in virgin and mated, males and females of DM and DA.

NS: night time sleep; LNS: length of night time sleep bout; NNS: number of night time sleep bout

Expt. No.	Species	Mating status and sex	Mean NS (min)	95% CI	Mean LNS (min)	95% CI	Mean NNS	95% CI
1	DM	Virgin male	432.25	39.83	42.38	50.34	14.48	2.29
		Virgin female	450.09		82.08		13.41	
		Mated male	455.69		61.88		12.35	
		Mated female	445.74		39.58		17.09	
	DA	Virgin male	634.56		171.14		7.24	
		Virgin female	640.01		164.94		9.18	
		Mated male	648.21		239.17		5.47	
		Mated female	667.45		296.82		5.51	
2	DM	Virgin male	545.24	32.60	91.55	53.89	10.74	2.23
		Virgin female	514.03		78.85		12.9	
		Mated male	550.51		84.18		11.27	
		Mated female	444.01		33.60		20.36	
	DA	Virgin male	640.2		165.10		7.76	
		Virgin female	616.78		186.81		9.95	
		Mated male	649.89		202.46		6.38	
		Mated female	648.99		206.58		7.04	

Table 3.18 Results of ANOVA performed on percentage of sleeping flies in DM and DA that were aroused by a mechanical stimulus of one second duration.

Effect	***df***	**MS**	***F***	***p***
Phase	1	0.46702	17.4113	< 0.001
Species	1	0.92741	34.5756	< 0.0001
Mating status	1	0.02256	0.8411	0.372686
Phase * Species	1	0.05029	1.875	0.189823
Phase * Mating status	1	0.11231	4.1872	0.057528
Species * Mating status	1	0.03019	1.1255	0.3045
Phase * Species * Mating status	1	0.00006	0.0022	0.963481

3.4 Discussion

The present study shows that the differences in sleep pattern observed previously between virgin males of two sympatric Drosophilid species, DM and DA (Prabhakaran and Sheeba, 2012) hold true even in females and regardless of the mating status. Consistent with the previous study, DA showed less day time sleep with higher number of day time sleep bouts and smaller length of sleep bout as compared to DM. During the night, DA showed higher amount of sleep with lower bout number and higher bout length. Thus, DA shows less consolidated day time sleep and more consolidated night time sleep as compared to DM.

In DM, mating affected the day time sleep in females, with mated females showing less amount of sleep and smaller length of sleep bouts as compared to mated males. Number of night time sleep bouts was also higher in mated females of DM as compared to that of mated males. The results are consistent with previous studies showing sexual dimorphism in sleep pattern (Huber et al., 2004), and the effect of mating status on sleep of female DM (Isaac et al., 2010). During mating, the transfer of sex peptide from the males was reported to play a role in the post-mating sleep pattern observed in DM females (Isaac et al., 2010). Neuropeptide F (NPF), which is known to have sex-specific expression pattern (Lee et al., 2006), and is known to promote sleep in DM (He et al., 2013), has been implicated to be involved in bringing about a sexually dimorphic sleep pattern in DM. Moreover, NPF is expressed in a sexually dimorphic manner in a subset of the LNd neurons, which is also a part of the neuronal network regulating circadian behaviours in DM (discussed in Chapter I).

Interestingly, neither amount of sleep nor number and length of sleep bouts during the day and night were affected by mating status or sex in DA. Taken together, the results indicate that the neuronal network underlying sleep regulation is likely to be different between DM and DA. Previous studies have not found major differences in the neuronal architecture of

circadian neurons in these species (Hermann et al., 2013; Prabhakaran, 2014, PhD thesis). Thus, it is possible that the expression pattern of neuropeptides, the number of neurons in the different subsets, or the nature of communication among the neurons is different in the two species. Examining the role of sex peptide in DA, and the expression pattern of NPF in the DA brain may be useful in this regard.

So far, the only effect of mating status on sleep of females in DA seems to be on how deeply the females sleep during different times of the day. The present study finds that for a stimulus of same strength, a greater proportion of mated females of DA are aroused during the night as compared to that during the day. This trend is not seen for virgin females of DA. Thus, it appears that mated females of DA may be sleeping more deeply during the day than during the night. Overall, a lower proportion of DA females were aroused by the mechanical perturbation during the day as well as during the night when compared to DM females, suggesting that DA females may be showing deeper sleep in general than DM females.

In conclusion, the results suggest that the differences in sleep pattern observed across mating status and sexes of the two species are brought about by differences in circadian and homeostatic mechanism. In order to study the underlying mechanisms, a holistic view of the differences in sleep characteristics of DM and DA is required. Further studies examining other features of sleep like, nature of sleep rebound and arousal threshold at different times of the day, would be the way forward.

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