Significance of clock period for clock functions and development in *Drosophila melanogaster*

Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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DECLARATION

I declare that the matter presented in my thesis entitled "**Significance of clock period for clock functions and development in *Drosophila melanogaster"*** is the result of studies carried out by me at the Evolutionary and Integrative Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of late Prof. Vijay Kumar Sharma and Dr. Sheeba Vasu and that this work has not been submitted elsewhere for any other degree.

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

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Place: Bangalore

Date: 29th June, 2019

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CERTIFICATE

This is to certify that the work described in the thesis entitled **"Significance of clock period for clock functions and development in *Drosophilamelanogaster"*** is the result of investigations carried out by Ms. Manishi Srivastava in the Evolutionary and Integrative Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, largely under the supervision of late Prof Vijay Kumar Sharma. I have been closely involved in many of these studies and have taken responsibility of continuing supervision towards completion of the studies and composing of the current thesis. I certify that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Sheeba Vasu, PhD

Supervisor-in-charge

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Synopsis

Circadian (near 24 hours) clocks are internal self-sustained pacemakers which regulate the timing of behavioural and physiological processes in most organisms. They are considered to be adaptive as they have evolved in response to daily environmental cycles which have a period of 24 hours. In absence of time cues the clock runs with its intrinsic period known as free-running period. Previous studies have speculated that major clock functions can be most aptly executed if intrinsic period of the clock is close to 24 hours. Also this value is known to influence the variability of period itself and is also considered to influence certain life-history traits such as the rate of pre-adult development. However, most of the studies through which these conclusions were derived suffered from certain limitations. Different aspects of clocks were studied using different organisms and generalizations were made based on them. In studies where mutants were used to study the role of period, their genetic background was not accounted for and since these mutations are likely to interact with the genetic background, the interpretations could have been confounded by such differences. Also, in most of the previous studies the range of period values was narrow and not distributed symmetrically around 24 hours. Besides, there were other sources of variation like sex and age which were not controlled for in a few studies. In this thesis I have reported results from experiments which were performed to comprehensively examine several aspects of circadian clocks crucial for their accurate functioning, while minimizing such limitations in order to study the importance of free-running period. For this I used mutants of the fruit fly *Drosophila melanogaster* per^{s} and per^{l} which have periodicities of ~18 and ~28 hours respectively along with wild type per^{+} which have been maintained as a large outbred population

and have ~24 h periodicity. The two mutant lines were first backcrossed to the wild type population for a total of ten generations.

In the first chapter of my thesis, I have given introduction to the study I conducted and its relevance for the field of circadian biology. In the second chapter, I have studied the relationships between clock properties and period under constant conditions. I show that precision (a measure of day-to-day stability of period) is not maximum for 24 h clocks as proposed before. However, it is minimum for flies with long period and also the period-precision correlation is dependent on sample size of flies used to conduct the analysis. Two other important clock characteristics regarding which certain speculations were made and which are known to be associated with period are duration of activity referred to as Alpha (α) and duration of rest referred to as Rho (ρ). Our analysis on α and ρ of individual flies shows that differences in τ are brought about by change in α. In the third chapter I have examined the relationships between period and clock properties measured under entrained conditions, i.e, in presence of light dark cycles. I see that both accuracy (a measure of stability of entrainment) and phase relationship depend on periodicities of internal as well as external cycles and accuracy is maximum when both these periods match. Also the relationships observed under entrained conditions cannot be explained by the PRCs obtained for these flies in my study. In the fourth chapter, I have measured the lability of period in face of various factors as a function of period itself. I show that long term stability of period is maintained at extreme high and low temperatures for short and intermediate range flies but not for long period flies. Under constant light only ~24 h period flies can sustain their rhythmicity. When I measured the history dependence of period value in terms of aftereffects or developmental plasticity due to previous regimes I did not observe an advantage of having a 24 h period. In the last chapter, I present the

results of my study of the role of period in rate of pre-adult development and demonstrate that the internal clock and the external zeitgeber cycle both modulate the speed of development.

Introduction

1.1 About circadian clocks

Behavioural and physiological mechanisms have evolved to adapt to the cyclic variations in the environment and adjust to the temporal sequence of events present on earth (Moore-Ede and Sulzman 1981) (Sharma 2003) (Vaze and Sharma 2013). Four geophysical cycles i.e. tidal, daily, lunar and annual cycles are considered to synchronize corresponding biological rhythms that are known to persist even under constant environmental conditions (Aschoff, 1967). The daily or **Circadian** rhythms (term coined by Franz Halberg (Pittendrigh 1965)), are endogenously driven (Aschoff, 1958) near-24 hour rhythms found in almost all life forms examined. They are generated by internal time keeping systems - circadian clocks, and are capable of perceiving local time as well as estimating the progression of time. After the first suggestion by DeMairan (de Mairan 1729) of an internal timing mechanism, many studies aimed to establish the endogenous origins of the same (Kleinhoonte 1929) (Bunning and Stern, 1930, referred in Hamner, 1963) (Pittendrigh 1965) and others). In addition, crossing experiments between individuals with different clock periods established that heritable factors encode an internal clock (Bunning 1932) (Bunning 1973).

The physical environment is characterized by several cycling factors most of which are a result of motion of the earth around the sun. Most pronounced among these are the daily and annual cycles of light and temperature. Natural selection must have required organisms to cope with the challenges raised by these physical periodicities resulting in a need for a program which can anticipate the regularly occurring fluctuations in such factors (Moore-Ede and Sulzman 1981). This explains the development of innate temporal programs in

behaviors and metabolism and also the restriction of these behaviors to a fraction of the external cycle (Daan 1981).

These clocks are characterized by their ability to generate rhythms even in the absence of any external time cue (zeitgeber) with an intrinsic period referred to as Tau (τ) (Pittendrigh and Bruce 1957) (Bunning 1958) (Pittendrigh 1958). The efficiency of this system stems from an important parameter of the clock, namely the absolute value of the **free-running period** or intrinsic period of pacemaker as mentioned above. It has been hypothesized that organisms on earth may have faced selection pressures that have led to the evolution of characteristic period lengths (Sharma 2003). Thus, circadian systems have been thought to evolve self-sustaining oscillators that match the periodicity of the external cycle (Pittendrigh and Bruce 1957) (Pittendrigh 1958). In complex metazoans, multiple such oscillators are thought to exist, governing different rhythms (Pittendrigh 1960).

In absence of external time cues, the internal pacemaker of organisms runs with its own intrinsic period which is referred to as free-running period whereas in presence of time cues or **zeitgebers,** it runs with the period of zeitgeber cycle after adjusting to the external environmental cycle by a process called **entrainment**. Entrained clocks are adjusted to the periodic environment (Daan and Aschoff 1982), and therefore can accordingly time various behavioral and physiological processes of the organism with respect to the environment. By doing so, an appropriate sequence of internal physiology is also ensured. The resultant periodic functioning is also thought to have a survival value for organisms as implied by associations observed between fitness and proper phasing of circadian rhythms (Daan and Tinbergen 1979) (DeCoursey, Krulas et al. 1997) (Hurd and Ralph 1998) (Ouyang, Andersson et al. 1998). For instance, several ecologically relevant behaviors such as

foraging, avoidance of predators, and finding mates, could prove disadvantageous to the organism if inappropriately timed (Daan 2000) (Saunders 2002) (Sharma 2003). Hence the attainment of a stable **phase relationship** with the external cycle with minimal day-to-day variation in timing of behavior is a critical function of circadian clocks and a major feature of entrainment. For a rhythm to be called entrained, the following criteria should be met: a) period match between the entraining (external cycle) and entrained (internal clock) oscillators, b) stable and reproducible phase relationship and c) phase control or continuation of free-running phase from the entrained phase (Hirschie Johnson, Elliott et al. 2003) (Daan and Aschoff 2001). How this adjustment is achieved is still not understood completely. However, there are various theories regarding the same which I will discuss briefly here.

Most circadian phenomena including entrainment have been interpreted in the context of limit-cycle oscillator theory (Hirschie Johnson, Elliott et al. 2003) as these oscillations have been considered to be limit cycle oscillations (Jewett, Kronauer et al. 1991). The **discrete model of entrainment** relies on phase shifts that occur with different magnitudes and in different directions (advance or delay) when the oscillator is perturbed at different times of the day. This phase shift is considered to be equivalent to the difference in periodicities of internal and external oscillations and therefore compensates for the period difference. When the internal oscillation aligns with the external cycle such that the external stimulus induces the appropriate phase-shift required to compensate for the difference in periodicities, a stable phase relationship is attained. The phase shifts obtained by perturbing the rhythm at different time points using a particular time cue are depicted using a **Phase Response Curve** which has been considered extremely useful in explaining entrainment of some organisms (Pittendrigh and Minis 1964) (Pittendrigh and Daan 1976) (Pittendrigh 1965) (Pittendrigh

1981). The **continuous model of entrainment** on the other hand relies on changes in the period of clock as a consequence of tonic effects of light (Aschoff 1960) (Daan and Aschoff 2001) which result in period match and attainment of phase relationship eventually. Circadian entrainment is thought to be more stable when the pacemaker responds to light by changing both its phase and period (Beersma, Daan et al. 1999) as a response to the perturbations it receives due to the zeitgeber. The concept of zeitgebers was introduced by Aschoff (Aschoff, 1958) as the time-givers which are the cues provided to an endogenous rhythm at specific times of the day in order to synchronize it to the external environment (Rawson, 1956). Among various environmental factors, day/night cycle has been considered to be the most pervasive zeitgeber (Moore-Ede, Sulzman et al. 1982) (Dunlap et al., 2003) as light is thought to be the dominant time cue which resets the circadian pacemaker (Edmunds 1988). While light can entrain the pacemaker, circadian output has also been shown to be influenced by direct, clock-independent effects of light (or masking; (Aschoff 1960) (Mrosovsky 1999) (Binkley 1983) (Redlin and Mrosovsky 1999). For example, in *Drosophila*, lights-on is considered to result in a rapid eclosion response with two components, one which is mediated by the clock and the other which is not (McNabb and Truman 2008). Hence, external cycles can influence the expression of rhythmic behaviors through clock-dependent and clock-independent effects. One of the primary parameters of the clock that can determine various properties of the rhythmic output is the free-running period which is the focus of this study.

1.2 Free-running period and its absolute value

The three defining features of circadian rhythms are a) persistence of internal rhythms with their intrinsic periodicity, b) temperature compensation of period and c) entrainability to external cycles. The intrinsic period of the circadian clock is believed to be a major factor influencing the timely occurrence of behaviours and physiological events in organisms. It is one of the major characteristics of the clock and all the functions performed by the pacemaker have a direct or indirect association with it. According to the discrete model of entrainment, entrainment is achieved when light pulses fall at a phase such that a phase shift equal to the difference between the periods of internal (τ) and external (T) oscillators can be induced (Pittendrigh 1965) (Pittendrigh 1981) (Pittendrigh and Minis 1964) (Pittendrigh and Daan 1976). It is assumed thereby that the attained ψ would be a function of τ and T (Von Aschoff and Wever 1962) (Pittendrigh and Daan 1976) (Hoffman, 1963) thus making free-running period important for a major function of the clock i.e. phase-locking of the rhythm with the zeitgeber.

Free-running period does not assume a rigid and invariable value as clocks are constituted by biological processes that are replete with variation at molecular and tissue levels (Barkai and Leibler 2000) (McAdams and Larkin, 1999). These cellular and tissue clocks control different rhythmic behaviors and physiological events within an individual organism which repeat at roughly regular intervals. Pittendrigh (1960) observed that the natural period of any self-sustaining oscillation can exhibit fluctuations either spontaneously or due to effects of external variables (Pittendrigh 1960). While the range of periodicities is rather limited for organisms within a species, there is greater variation observed across species (Shimizu and Masaki, 1997). Corresponding to the periodicity of daily environmental cycles, most

biological rhythms in organisms are expected to have such near 24 h periodicities. However, this value is usually not exactly 24 h, the reason for which is described in chapter 2.

The variation in τ values across individuals maybe attributed to strong genetic components controlling circadian periodicity. As demonstrated by Erwin Bunning, τ values of individuals among progeny are normally distributed around the mean τ value of both parents (Bunning 1973). The study of single gene period mutants has advanced our understanding of circadian rhythms by giving greater insights into the molecular and neural mechanisms involved and by providing more information about period and associated clock properties. Mutations of clock genes resulting in arrhythmicity or altered periodicity have been used to assess association between circadian clocks and fitness (DeCoursey, Krulas et al. 1997) (Kyriacou, Oldroyd et al. 1990) (Sheeba, Sharma et al. 2000) (Beaver, Gvakharia et al. 2002) (Beaver, Rush et al. 2003). Using one such gene (*period*, discussed in detail in a later section) and its mutants, I have conducted studies aimed at shedding light on the significance of circadian period via its effects on other important clock properties and rhythmic outputs:

a) Relationship between free-running period and other clock properties

b) Association of lability of the period with its absolute value

c) Role of clock in mediating the rate of pre-adult development

1.3 Clock properties as a function of free-running period

Circadian clocks, being products of evolutionary pressures, are expected to reliably convey information about time. Thus, several of its properties such as its day-to-day stability, amplitude and phase relationship with time cues are thought to have evolved for maximum

efficiency in performing these functions, therefore making it essential to characterize the relationships between these properties. Precision of the clock is defined as the inverse of standard deviation of free-running period measured across days and therefore indicates its short-term internal stability (Daan and Beersma 2002) and accuracy is defined as the inverse of standard deviation of phase relationship measured across days thereby reflecting the stability of entrainment (Beersma, Daan et al. 1999) (Daan and Beersma 2002). These two characteristics have been proposed to have strong association with the absolute value of free-running period due to the prediction that precision would be higher for clocks with period values close to 24 hours (Pittendrigh and Daan, 1976) (Beersma, 1999). This prediction about precision being higher for 24 h clocks has been extended for accuracy as well as it is intuitive that a clock which shows greater error in the measurement of daily period would also have difficulty in maintaining a stable phase across days. However, this prediction has not been empirically tested. Besides these two clock properties, period has also been thought to be correlated with the entrained phase such that rhythms with longer intrinsic period will have a delayed phase of entrainment compared to those with shorter periods. Also, for rhythms of a given period, phase relationship will get advanced with an increase in the length of the zeitgeber cycle (Pittendrigh and Daan 1976). Similarly, period is also thought to influence activity/rest durations, amplitude and power or robustness of the rhythm under constant as well as entrained conditions as elaborated in chapters 2 and 3 along with the reasons behind such speculations.

Although these speculations have been tested and found to be true in a few empirical studies, there have been instances where either no clear pattern is obtained or the relationships observed do not match the assumptions. For example, precision was not found to be

dependent on period in a recent study on Syrian hamsters (Bittman 2012) though it was shown to be maximum for period close to 24 hours in previous studies on rodents, humans and birds (Pittendrigh and Daan 1976) (Sharma and Chandrashekaran 1999) (Aschoff 1971). Similarly, period and phase are correlated in *per* mutants (Hamblen-Coyle, Wheeler et al. 1992) and several other studies. Indeed, selection for different phases of adult emergence has also shown to result in corresponding changes in circadian period in lab populations of *Drosophila melanogaster* (Kumar, Vaze et al. 2006). However, in *Neurospora crassa,* phase relationship shows inconsistent relationships with period (Lee et al., 2015) i.e. phase has been found to be delayed at some instances and advanced at some others with a change in period in the same direction. Also, certain autosomal mutations in *Drosophila melanogaster* show a lengthened period of emergence rhythm but significantly advanced peak of emergence under light and temperature cycles (Jackson, 2009). Another interesting study examining the effects of *Toki* mutation in *Drosophila* showed that an interaction of this gene with per^s and per^l genes results in a lengthening of period of adult emergence rhythms associated with them but still results in advanced i.e. smaller phase values (Matsumoto et al., 1994). Similarly, the relationships of activity and rest durations with period have been found to be inconsistent across organisms. While Aschoff observed that period length is altered due to a change only in rest duration in chaffinches and humans (Aschoff 1971), in Syrian hamsters this was not found to be true (Bittman, 2012). While I discuss such exceptions to the previously proposed rules and the possible reasons behind the contradictions seen across studies in greater detail in the chapters that follow (Chapters 2 and 3), I also report the findings from my studies which have been done in a more systematic manner under constant and entraining conditions using the same flies. For clarity, I have separated the clock

properties determined under constant darkness and under entraining conditions and written them as two different chapters.

1.4 Influence of external environment on free-running period

Since most organisms live in a rhythmic environment, the circadian system has been proposed to be selected for functioning best in the presence of time cues. However, if the internal pacemaker is not a precise timekeeper under constant conditions, its functions under rhythmic conditions would also be impaired. Two important functions of clocks i.e. conservation of phase angle and estimation of day/night length for regulation of seasonal phenomenon in many organisms, are performed under rhythmic conditions. However, another important function is to appropriately track the passage of time especially in situations where there is no external cue. In order to estimate time across parts of the day, the rate at which the clock runs needs to be constant across days so that it can measure fractions of time accurately. Therefore, there is a need of minimizing spontaneous variations occurring in τ. Apart from variations that occur spontaneously, there are changes in τ observed in response to environmental conditions measured as lability with age, temperature or constant light (Aschoff 1960) (Aschoff 1981) (Page and Barrett 1989) (Eskin 1971) or in terms of history-dependence measured as aftereffects (Aschoff 1960) (Aschoff 1981) or developmental plasticity.

Lability of τ can be defined as the change observed in its value in the face of fluctuations in certain environmental or internal physiological variables (Pittendrigh and Daan 1976) (Aschoff 1979). Certain previous studies (Pittendrigh and Daan 1976) (Aschoff 1979) have examined this aspect of the pacemaker as an important property of the clock though its

relationship with mean internal τ value has not been clearly identified. In addition to internal physiological state, external environmental variables such as light also have effects on clock period. For instance, wild-type and *per* mutant flies exhibit lengthening of τ under constant light with low intensity (Konopka, Pittendrigh et al. 1989) (Matsumoto et al., 1994). Constant light has also been known to cause a single rhythm to **split** into different components each of which begins to run with its own periodicity (Pittendrigh 1960) (Hoffman, 1971). Like mentioned before, different rhythms of an organism may or may not be governed by different oscillators and tend to have different temporal relationships with other internal rhythms and also with the external environmental rhythms. In a given periodic condition therefore, an **internally synchronized** state is maintained. However, under aperiodic environments this internal synchronization may be lost and different rhythms can free-run with their inherent periodicities (Sulzman, Fuller et al. 1979) (Aschoff, 1965). Period as well as amplitude of circadian rhythms is affected by illumination and is a function of its intensity (Aschoff 1960) (Aschoff 1981). Aschoff's rules state that intensity of light affects this value such that τ (LL) < τ (DD) in diurnal animals while the opposite is true for nocturnal animals. This is achieved by lengthening or shortening effects of light on τ. Another crucial property for the clock is temperature compensation which is also critical for conservation of phase angle (Pittendrigh 1993) in the face of day-to-day temperature variations especially in poikilotherms (Zimmerman, Pittendrigh et al. 1968) (Menaker and Wisner 1983) (Underwood 1985) (Chiba, Uki et al. 1993). Temperature compensation has been demonstrated in heterotherms (Menaker 1959) (Lee, Homes et al. 1990) (Grahn, Miller et al. 1994), and obligate homeotherms (Barrett and Takahashi 1995) (Grahn, Miller et al. 1994) (Zatz, Lange et al. 1994). Even in homeothermic vertebrate *Gallus domesticus*, the

rhythms in melatonin were found to be temperature compensated for a range of temperatures (34 to 40) though they were also temperature entrainable.

The state of the pacemaker is often affected by the environment not only during the time of exposure but also after the removal of the environmental regime. The steady state free-run might show an effect of preceding regimes such as length of the entraining cycle or the length of photoperiod (Aschoff 1979). Such an effect can be accumulated during development as well as after development is complete. Presence of light is considered to have an effect on the developing clock as it has been reported that connections between clock and photoreceptors required for photic entrainment are also present during the hatching stage (Sehgal, Price et al. 1992) and the expression of mRNA is affected by light regime (Price et al., 1995). The influence of light on developing clock was also shown by the above-mentioned study on *per* mutants with different photoperiods (Tomioka, Uwozumi et al. 1997) where the effect of light during development was observed to vary in a non-linear mode with the length of the photoperiod as well as another study on cockroaches where rearing the nymphs under non-24 hour light dark cycles resulted in changes in τ of adults (Barrett and Page 1989). The influence that a preceding entraining regime might have on the pacemaker in adult conditions is measured in terms of after-effects i.e. a significant change in τ immediately after transfer from entraining to free-running conditions. Such changes in periodicity of the clock may be mediated by reversible DNA methylation as seen in mice (Azzi et al., 2014). Sometimes, even though τ doesn't change significantly due to the entraining regime, attainment of free-run i.e. the transient stage, is prolonged. Similarly, another factor that might result in a change in τ can be the direction of entrainment i.e. whether the entrainment has been accomplished through advancing or delaying phase shifts.

While after-effects of phase-shifts usually are in the same direction as the phase-shifts (i.e. period lengthening happens after phase delays and vice versa) and thought to help in stable entrainment (Beersma et al., 1999), a study on the antelope ground squirrel did not show such a pattern (Kramm, 1976). Despite reports of such effects on the period, it is not clear how such effects are dependent on the intrinsic period of the individuals.

Table1 summarizes some of the previous results of lability of clocks performed across different organisms. In Chapter 4, I have tested the influence of a few such factors on the period and compared the effects on clocks with different periods.

Model and reference	**Condition tested**	**Effect on τ**
Sparrow ***Eskin, 1971***	**age**	**Steady lengthening**
Rodents ***Pittendrigh and Daan, 1974***	**age**	**shortening**
Lizard ***Hoffmn, 1960***	**Constant light**	**shortening**

Flying squirrel ***DeCoursey, 1961***	**Constant light**	**Lengthening, reduced activity**
Antelope ground squirrel ***Kramer, 1976***	**Constant light**	**Shortening (lasts for many weeks)**
House sparrow ***Eskin, 1971***	**Aftereffects**	**Variations, not in one direction**
White-footed mice ***Pittendrigh and Daan, 1976***	**Aftereffects**	**Variations, not in one direction**
Golden hamster ***Pittendrigh and Daan, 1976***	**Aftereffects**	**No change**
Mice ***Pittendrigh and Daan, 1976***	**Aftereffects**	**shortening after long photoperiod**
Gonyaulux ***Roenneberg and Hastings 1991***	**Aftereffects**	**Lengthening with increasing wavelength of light**

Syrian hamsters ***Reebs and Doucet, 1997***	**Aftereffects**	**Lengthening after long T cycle**
Bulla gouldiana ***Page et al., 1997***	**Aftereffects**	**Lengthening after long T cycle**
Humans ***Scheer et al., 2007***	**Aftereffects**	**Lengthening after long T cycle**
Neurospora crassa ***Schneider et al., 2009***	**Aftereffects**	**Lengthening after shift from LL to DD**
Cockroach ***Page et al., 2001***	**Aftereffects**	**Lengthening after longer T cycle**

Table 1 Table summarizing the extent and direction of variability observed in τ due to different factors across model organisms.

1.5 Pre-adult development and free-running period

In addition to features of behavioral rhythms, circadian period can also affect timings and duration of physiological and developmental events. Development of any organism is a complex process regulated and influenced by several genetic factors. While structural and regulatory genes play an obvious role in development, involvement of two more types of genes has been postulated before. These are architectural genes and temporal genes (Paigen and Ganschow 1965). Among these two, the involvement of temporal genes or a 'genetic clock' has been considered most difficult to imagine (Hirsch 1967). However, even as early as 1961, it was felt that there is ample evidence to suggest existence of these genes

(McClintock, 1961). While the emergence process is well known to be gated by the clock in a manner that allows flies to avoid emerging during that duration of the day when environment is harsh for them (i.e. high temperature and low humidity) (Saunders 2002), the role of this gating in rate of development is not clear. There are several factors affecting developmental rate. Also, these factors do not have similar effects during pupation and emergence. In *Drosophila melanogaster,* males are found to develop later than females. While the timing of formation of puparium is not influenced by the diurnal rhythm, the duration spent in the pupa has been reported to have some influence on the rhythm of emergence (Bakker and Nelissen 1963). Further, while the timing of egg collection has a role in the timing of pupa formation it was not found to have an effect on the overall rate of development (Bakker and Nelissen 1963) (Kumar, Vaze et al. 2006). Additionally, factors determining the weight of the organism are known to play a role in the larval and pupal durations (Bakker and Nelissen 1963). Other than these factors, it has also been shown in *Drosophila simulans* that the rate of development is affected by photoperiods experienced by the parents, which also affect the sensitivity of this rate to larval density (Giesel 1988).

It was recognized as early as in 1960s that "A factor which highly complicates a study of the duration of development is the distinct diurnal rhythm in emergence of the adults from the puparia." This rhythm in emergence was also shown to influence the duration spent in the pupal stage (Bliss 1926) (Poulson 1934) (Powsner 1935) (Sang and Clayton 1957). Whether or not the clock plays an indubitable role in the duration of pre-adult development is the question that I have addressed in chapter 5. Though a few studies have implicated the role of clock, some of them suggested that effects of clock mutations on the rate of development are mediated by pleiotropic effects of clock genes (Kyriacou, Oldroyd et al. 1990) whereas

another study concluded that it is the external environment which has the major influence on developmental rate (Paranjpe, Anitha et al. 2005). While in populations artificially selected to emerge at different times of the day different developmental rates were observed (Kumar, Vaze et al. 2006) external light regime has not been shown to affect the relative differences of populations as they all entrain to these cycles. In chapter 5, I have tried to provide further evidence for a role of clock in the rate of pre-adult development by using flies with different period values which I expected would have different entrainability in light dark cycles of different lengths.

1.6 Role of *period* gene in regulating free-running period

The core clock gene, *period,* was the first gene to be identified and molecularly characterized in *Drosophila melanogaster* (Konopka and Bender, 1971). This discovery paved the way for gradual understanding (as summarized below) of intertwined positive and negative feedback loops which are the central components of circadian pacemakers existing in every cell. This helped in understanding the mechanisms of molecular entrainment of the clocks and how they further regulate the timing of downstream processes.

The protein product of *per* gene controls biological rhythms and abundance of this protein has been considered to regulate the speed at which the clock runs (Bayleis et al., 1987). In mammals, the core molecular clock utilizes several transcriptional and post-transcriptional mechanisms to bring about temporal order in behaviour and physiology (Partch, Green et al. 2014). In *Drosophila,* a feedback loop involving a heterodimer of *per* and *tim* (*timeless,* another core clock gene) that negatively regulates their own transcription is an essential component of the circadian oscillator (Hardin, Hall et al. 1992). Therefore, mutations at the

period locus affect the period of circadian rhythms of *Drosophila melanogaster* by affecting the rate of transcriptional feedback. τ has also been indicated to be a logarithmic function of the product of *per* gene (Coté and Brody 1986). Also, tissues expressing *per* gene have been suggested to have their own intrinsic oscillator activity (Liu, Lorenz et al. 1988). Three chemically induced mutations at *per* locus have provided great insights regarding the role of this gene in biological rhythms. While per^{0} leads to an early translation stop (Amber) resulting in a null mutation, per^{s} and per^{l} are amino acid substitutions that result in shortening and lengthening of period, respectively. It had been suggested that per^{l} mutants produce hypoactive product while per^{s} mutants produce a hyperactive one (Bayleis et al., 1987). Later findings also suggested that the per^{0} mutation is in fact a loss-of-function mutation, which loses the ability to regulate the circadian clock effectively (Rutila, Edery et al. 1992). Another mutation on the same locus, per^{04} was found to have a few behavioral similarities with the null mutant but has slightly different nature of the mutation i.e. it results in the formation of another anomalous product (Hamblen-Coyle, Wheeler et al. 1992).

Studies have reported partial dominance of per^{s} over per^{+} and per^{+} over per^{l} and this has been attributed to the per^{l} gene product being less active while per^{s} gene product is more active compared toper^{+} (Coté and Brody 1986). The gene product is thought to be a multimeric protein as determined through the nature of heterozygotes. It is important to mention that *per* gene expression affects not only the periodicity but also the strength of circadian rhythms in *Drosophila melanogaster* and both these effects are differentially regulated (Lui et al., 1991).

There have been several studies where these mutants were used for better understanding of the circadian system. When studied in nature, PER profile has been reported to change with

changing seasons which is not the case for TIM (Menegazzi, Vanin et al. 2013). Also using these lines it was demonstrated that temperature has a stronger influence on the clock than light as PER and TIM profiles were shown to be decoupled under high temperature conditions (summer). In the context of questions that I am addressing, there have been prior studies examining clock properties in these mutants. The *per* mutant flies were found to exhibit changes in their periodicities in opposite directions in response to constant light or changes in temperature (Konopka, Pittendrigh et al. 1989). Further, their responsiveness to rearing regimes of different light/dark ratios (developmental plasticity) were also demonstrated to be different (Tomioka, Uwozumi et al. 1997) as also were their PRCs (Saunders 1994).

1.7 Relevance of this work

In any scientific field that is relatively young, deriving broad conclusions becomes difficult due to the paucity of knowledge. Decades of studies on circadian clocks, while giving us much needed information about the nature and importance of clocks, have also pointed out the confounding effects of certain factors which have not been taken into consideration in several of the early studies from which the major formulations or principles of circadian clockwork were framed (Pittendrigh and Daan, 1976) (Aschoff et al., 1971) (Sharma and Chandrashekharan, 1999).

While most associations between period and other clock related aspects like the ones mentioned in section 1.3 and 1.4 were made based on the existing models of entrainment, their empirical testing was done mostly using organisms from different species, leading to spurious correlations, some of which have later turned to be inconsistent across studies. In

certain studies where a single species was used, genetic background was not controlled for. Similar mutant lines but with different genetic background have been considered causative of the divergent phenotypes across studies (Chandler et al., 2000). Also, most prominent animal models that are commonly used to study and characterize the role of clocks in behavior are derived from inbred lines. Since most behaviors are emergent properties of complex genetic networks, they are characterized by pleiotropy and epistasis. The inheritance and distribution of gene at one locus is not independent of those at other loci and the same holds true for any phenotype that is genetically regulated. Therefore, there are multiple loci contributing to the expression of a quantitative trait and there is substantial variation arising from gene interaction among loci. Also, the allelic effects within loci are not always additive and therefore epistatic interactions are major contributors in the resulting genetic variation. During developmental stages as well as adulthood in *Drosophila*, the behavioral phenotypes are affected by the interacting networks of genes and environment (Sokolowski 2001). During the development of an organism, different genes are expressed at different times and in different tissues and such a spatial and temporal gene expression affects behavioural patterns (Sokolowski 2001). There are numerous examples where genetic background becomes an important determinant of the phenotype in question (Toivonen et al., 2009) (Burgess et al., 2011). Therefore, similarity of genetic background is an essential prerequisite for deriving any conclusion. Also, absence of individuals with period values considerably longer than 24 h in the previous studies restricts us from making conclusion that indeed it is the 24 h clock which performs better.

Another limiting factor in a few previous studies is the analysis of periodicity in the organisms studied which depended to a great extent on the instrumentation available to

measure or record data at appropriate regular intervals for long enough durations. Since absolute value of period is the focus of this study we have compared different estimators of period for accurate predictions of daily period values and other measures that stem from it.

1.8 Approach

The present study is aimed at analyzing the effects of the absolute value of free running period on several aspects related to the clock (summarized in Figure 1.3). *Drosophila melanogaster* has been a model for circadian rhythm research where the underlying molecular and cellular underpinnings have been described to a relatively greater detail and has also been the subject of research on the formal properties of circadian clocks and their inter-relationships (Hardin 2005). It has also been considered a valuable model to study age associated effects on sleep and rhythms (Koh et al., 2006). Studies using this model system have been providing useful insights into cellular, molecular and evolutionary insights in behaviour. I chose this system for all the assessments of functions of circadian period because of the wide range of τ values, almost symmetrically around 24 hours made available by the mutants of *per* gene.

To obtain a broad range of τ values we used wild type and mutant strains of per^s and per^l of *Drosophila melanogaster*. Also, to minimize the effects of varying genetic backgrounds we first backcrossed the mutant lines to a wild type population used as the controls or intermediate range in my studies. As mentioned before, these two mutants are alleles of a single locus on the X chromosome and result in shortening and lengthening of τ (Konopka and Benzer 1971). We made an attempt to bring similarity in the genetic background of fly lines employed to address our question regarding the significance of a near-24 hour period.

For this, we backcrossed the mutant lines with the wild type population which is used as controls in all our studies. For each generation of backcrossing, homozygous mutant females were crossed with wild type males and progeny (F1) obtained were allowed to mate. The progeny from this cross (F2) being a mixture of homozygous and heterozygous females were screened using DAM (Drosophila activity monitor system, (Pfeiffenberger, Lear et al. 2010) monitor system whereby free-running period was determined using periodogram analysis and female flies with period range matching that of the homozygous mutant lines were selected and used as parents for next generation. Scheme for backcrossing has been depicted in Figure 1.1. Experiments performed using 5-10 backcrossed generations of flies have been used to derive conclusions in this thesis. Figure 1.2 shows the frequency distribution of fly lines after 7 generations of backcrossing. Through all generations, the fly lines were maintained on standard banana-jaggery food medium since certain behaviours such as egg-laying are known to change as a result of introduction to a novel food resource (Sheeba, Madhyastha et al. 1998). Thus, I generated lines of fruit flies with distinct τ values in the range of 17-30 h using mutations in a core clock gene *period (per)* introgressed into the background of a wild-type, outbred population. This population has been consistently maintained with a large size (~1000 flies) to minimize the loss of genetic variation or accumulation of mutations due to genetic drift or bottlenecks. Besides speculating about adaptive relationships between different clock properties, my aim was to identify functional correlations with circadian period while minimizing the effects of interactions with genetic background and employing a wide range of τ almost symmetrical around 24 hours. A systematic assessment of all core clock properties with this range and absence of other

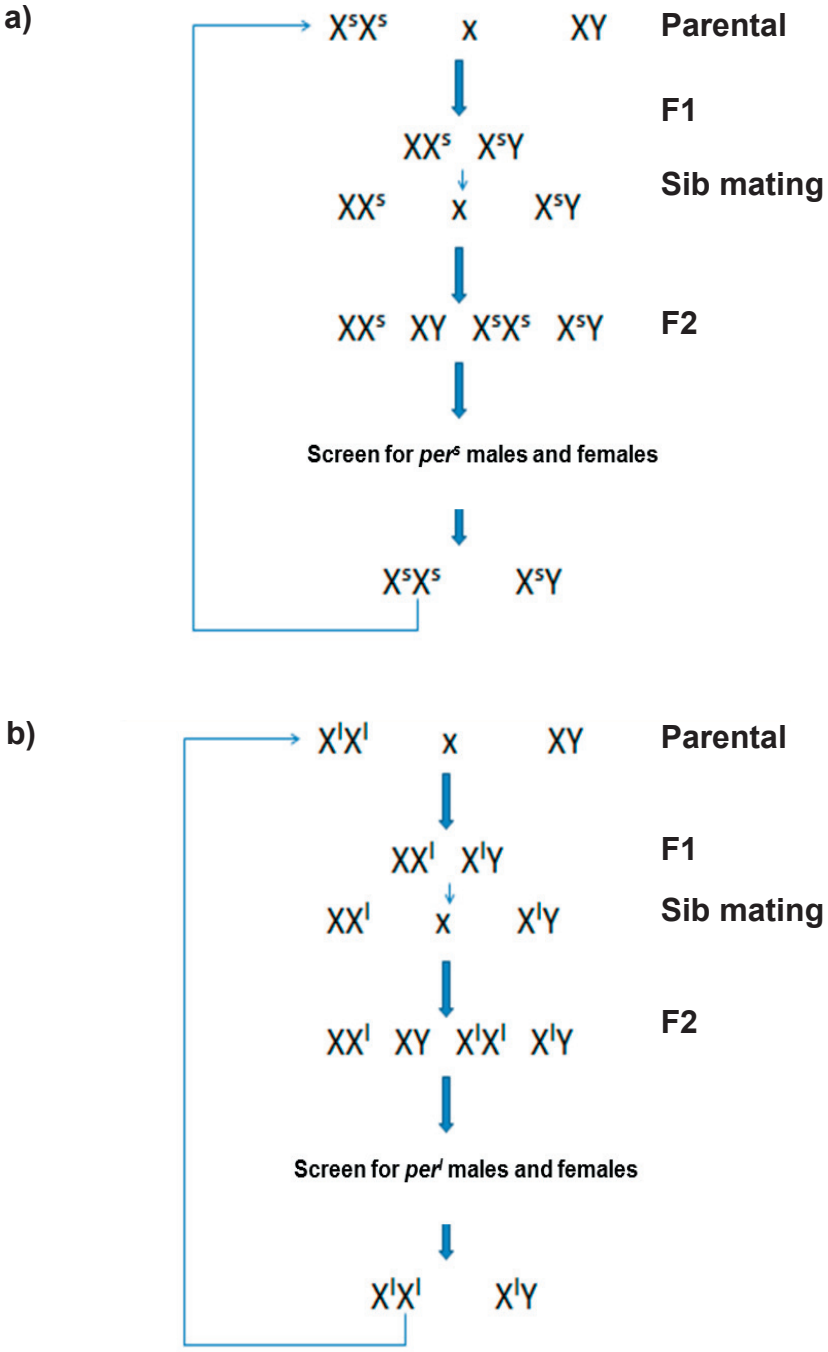


Figure 1.1 Scheme employed for backcross. Females from the mutant line per^s **(a)** and per^l **(b)** were crossed with males sampled from the wild-type population. Individuals from F1 generation were allowed to interbreed. Progeny from this cross were screened for homozygous mutant females by assessing free-running period of individual flies using DAM monitor system. These females (n ranging from 10 to 100) were then utilized for the next generation of backcrossing with wild-type males. Virgin females were collected for setting up each cross. Flies were allowed to mate for 2-3 days and then adults were discarded. This process was continued for 10 generations and experiments were conducted from flies sampled at 5th, 7th or 10th generation (as specified for each study).

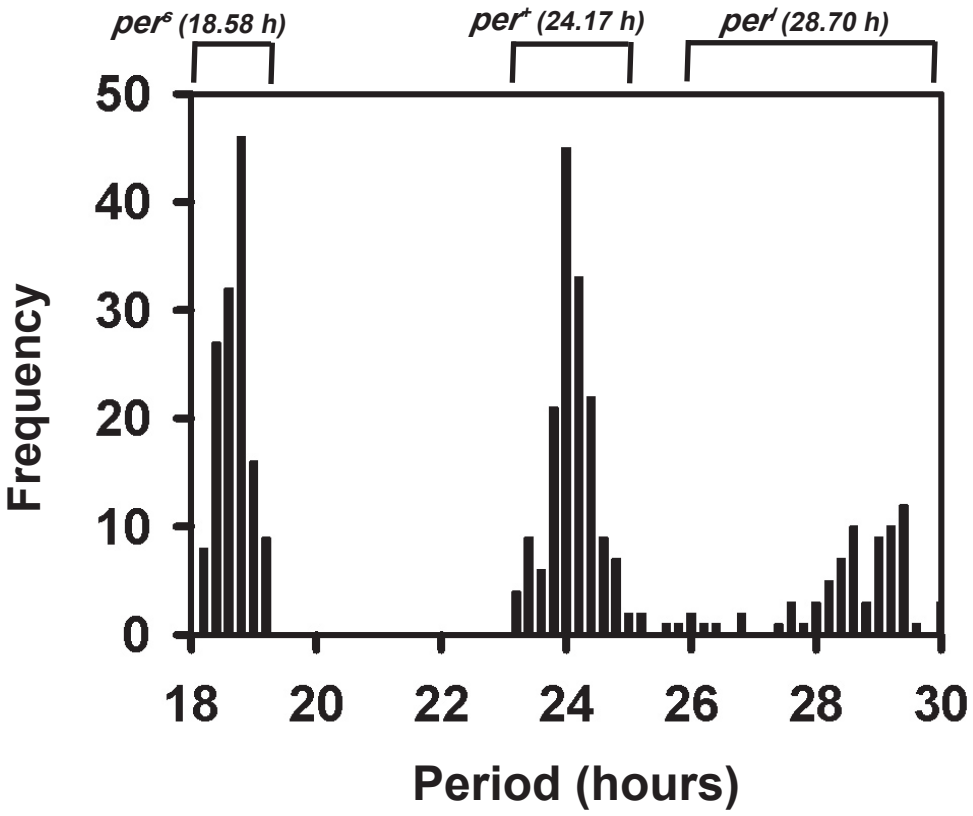
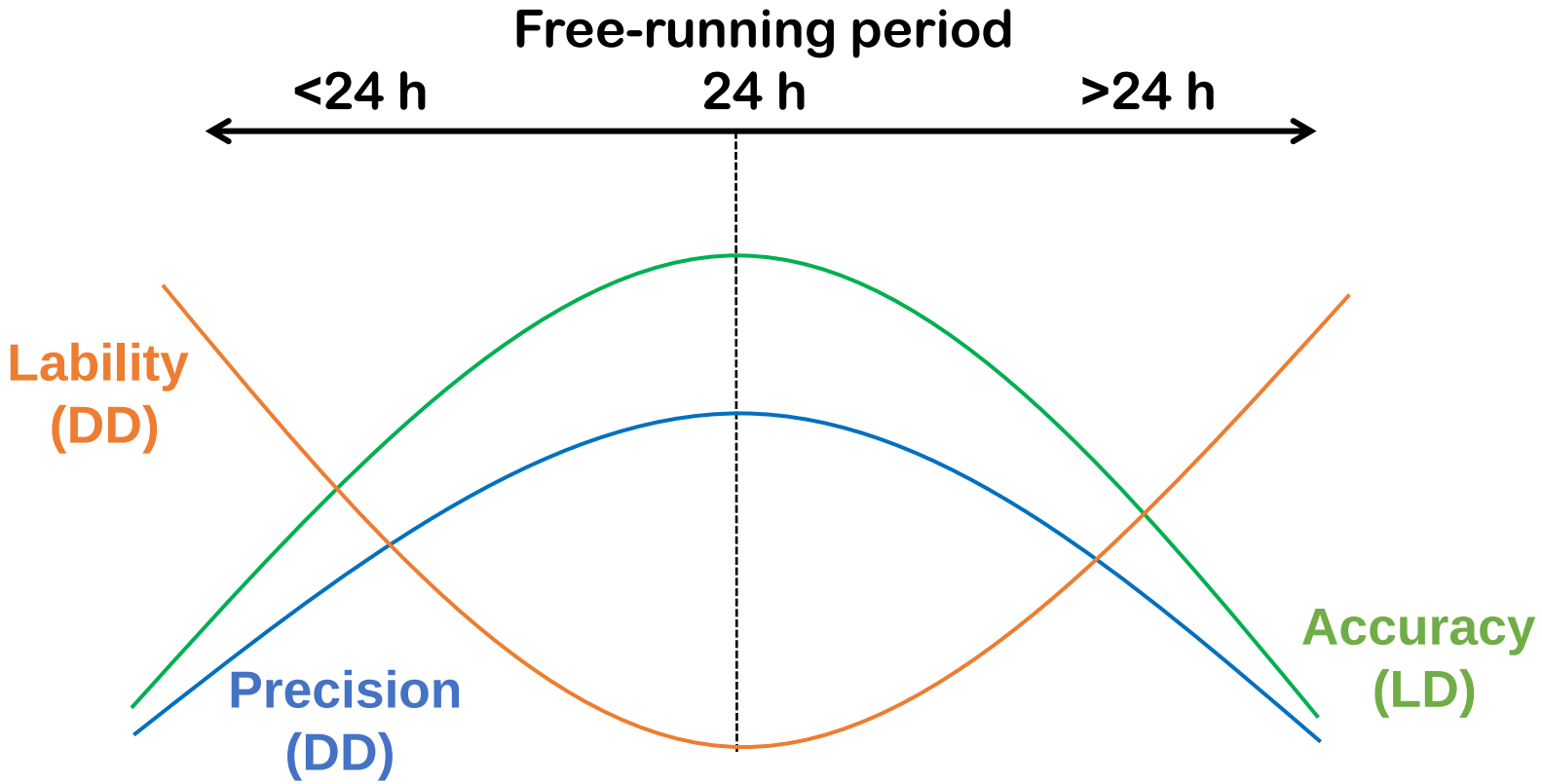


Figure 1.2 Frequency distribution of free-running period. Histogram of free-running period estimated for individual flies after 7 generations of backcrossing using chi-square periodogram. Inverted brackets indicate the range of flies designated as each of the three genotypes. Mean free-running period for each genotype is shown in parentheses.



Limitations of the studies which lead to above conclusions/model:

- Sampling of period variability from across species
- Missing controls for genetic background
- Confounding factors of age and sex
- Asymmetric sampling of period around 24 h

Figure 1.3 Schematic summarizing the basis for the study.

limitations like differences in genetic background, age or sex is exclusive to this study to the best of my knowledge.

In fruit flies, different overt rhythms are known to be controlled by different clocks. Most pioneer studies in *Drosophila* were done using adult-emergence rhythms (rhythms in emergence of adult fly from its pupa). In *Drosophila melanogaster*, adult-emergence rhythms are robust with the potential to entrain to light and temperature cycles (Saunders 2002). Eclosion pattern was also the read-out used for screening for *per* and *tim* mutants in *Drosophila melanogaster* (Konopka and Benzer 1971) (Sehgal, Price et al. 1994). However, the most commonly studied rhythmic behaviour is its locomotor activity/rest pattern which is bimodal in *Drosophila* with peaks around dawn and dusk under light/dark cycles.

In this thesis, I present the results of my studies which aimed to determine the relationships among various clock properties and the lability of free-running period for which I have used locomotor activity/rest rhythms of individual flies. As opposed to eclosion rhythms, which are population-based rhythms and occur once in the lifetime of a fly, activity/rest rhythm can be studied for an individual fly owing to the high-throughput nature of monitoring system used for locomotor recording. Study of individual flies was an essential requirement for my study as I wanted to correlate different traits with intrinsic periodicity which shows considerable inter-individual variation in our fly lines. Since activity/rest patterns of flies are remarkably consistent in different environmental regimes, employing them for assessing different clock properties, which indirectly contribute to fitness and therefore form the basis of circadian resonance, must help in deriving conclusions regarding the relevance of a circadian period for wild-type organisms. Considering light/dark cycle as the dominant cue I have used it to examine all the relationships in my studies. For the part where I examine the

role of clock in rate of pre-adult development I assayed the adult-emergence rhythms as well since the daily phase of emergence is also associated with the overall development time.

Circadian Clock Properties under constant conditions and their Relationships as a function of free-running period

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2.1 Introduction

Organisms exhibit τ (free-running period) close to 24 h under constant environmental conditions, but rarely exactly 24 h, and show remarkable variability in τ across individuals within a species as well as across different species. To explain this observation that very few individuals display τ of exactly 24 h (Pittendrigh and Daan 1976) under the paradigm of the non-parametric model of entrainment, it was proposed that when τ precisely matches the period of external cycle (T), phase relationship (ψ) would be prone to large fluctuations even with small deviations in τ, leading to weak/unstable entrainment (Pittendrigh and Daan, 1976a). This suggests that evolutionary forces might act against individuals exhibiting τ exactly equal to 24 h (Pittendrigh and Daan 1976) unless it is compensated for by high precision. This could partly explain the observation that individuals with τ exactly 24 h are rare. Additionally, the deviation from 24 h has also been proposed to promote seasonal adaptation (Daan and Aschoff 1982) (Daan and Beersma 2002) (Hirschie Johnson, Elliott et al. 2003). While this may suggest that clocks with τ slightly deviating from 24 h would be adaptive, it has been suggested that large deviations from 24 h can also be detrimental (Daan and Beersma 2002). Furthermore, organisms with τ matching T appear to have higher Darwinian fitness compared to those with τ greatly deviant from T (Ouyang, Andersson et al. 1998) (Dodd, Salathia et al. 2005) Emerson, Bradshaw et al. 2008).

Since Pittendrigh and Daan (1976) proposed that reduced accuracy (day-to-day stability of ψ) associated with clocks with τ exactly 24 h is compensated for by their enhanced precision (day-to-day stability of τ), it was hypothesized that individuals with τ approximating 24 h would exhibit higher precision. Some empirical studies have reported evidence of

association between τ and precision, and have found that clocks having τ closer to 24 h are indeed more precise (Aschoff 1971; Pittendrigh and Daan 1976) (Sharma and Chandrashekaran 1999). However, there have been exceptions to the conclusion drawn regarding this previously proposed relationship. For instance, a recent study using mutant hamsters demonstrated that precision is not dependent on τ (Bittman 2012). Such inconsistencies in results could be because of different methodologies or model organisms used in different studies.

In addition to the non-parametric model of entrainment, predictions on the relationship between clock properties have also been proposed based on the parametric model which suggests that τ increases or decreases in order to facilitate entrainment. Two important clock characteristics regarding which certain speculations were made and which are known to be associated with τ are duration of activity referred to as Alpha (α) and duration of rest referred to as Rho (ρ). Aschoff tried to explain certain observations regarding these parameters using a model according to which α or ρ can change with changing levels of threshold, thereby changing the spontaneous frequency of the oscillation (Wever 1960; Aschoff and Wever 1962). α/ρ ratio was also found to be dependent on τ (Eskin 1970; Aschoff 1971). With increase in τ, the ratio decreased and vice versa. An increase in light intensity was thought to shorten τ, increase α and decrease ρ to bring about shortening of τ, thereby increasing the ratio.

In this chapter, I present the results of my experiments aimed to test the hypothesis regarding all these clock properties which can be measured in absence of any time cue while minimizing confounding factors (described in Chapter 1), encountered in the previous studies. Since it has been observed that precision of circadian rhythms varies with the phase-

marker and model system (Daan and Oklejewicz 2003), I first computed precision using multiple phase-markers, namely activity onsets and offsets marking start and end of activity respectively, and Centre of Gravity (CoG; mean daily clock time of all recorded activity impulses and indicates the temporal center of the activity-rest rhythm) (Kenagy 1980). Further, I compared α and ρ values of individual flies across days to examine their relationship with τ. I have therefore tested the following hypothesis:

1. Precision is greater for clocks with periodicities closer to 24 h when compared those with periodicities deviant from 24 h.
2. Change in intrinsic period is a result of changes in rest duration for each cycle.

My results suggest that precision of circadian clocks is significantly decreased when τ is greater than 24 h but not when it is lower than 24 h, as shown previously. Also, my analysis on α and ρ of individual flies shows that differences in τ are brought about by change in α in these flies.

2.2 Materials and Methods

2.2.1 Fly lines and Maintenance:

Using a large outbred population of *Drosophila melanogaster* (Gogna, Singh et al. 2015), individuals carrying either the short or long *period* mutation, per^s or per^l, were backcrossed for a total of ten generations, as described in chapter 1. Experiments described here were performed with 5th, 7th and 10th generation backcrossed individuals. Since my interpretations are not focused on the specific effects of the *period* gene *per se*, henceforth, I have used the term 'Period Range' to denote the three genotypes (period range: 17-19 h or 'short' for per^s;

23-25 h for wild-type 'intermediate'; 26-30 h or 'long' for *per*^{l}). For all analyses I have used only those flies with period values falling in these defined ranges to compare across distinct, non-overlapping segments of the period distribution. However, in the scatter plots I have also included values which did not fall in these ranges. On 9th and 10th days after egg collection, freshly emerging flies were collected, sexed and separated as virgin males and females, within 6 hours of their emergence. To acclimatize flies to assay conditions, all flies were maintained under LD 12:12 cycles at 25 $^{\circ}$C for three to four days after emergence before beginning any assay.

2.2.2 Locomotor Activity Rhythm Recordings

Individual 4-5 days old virgin male flies were introduced into *Drosophila* Activity Monitors (DAM 5, Trikinetics^{TM}, Waltham, MA, USA) at an ambient temperature of 25 $^{\circ}$C in constant darkness (DD). Activity-rest rhythm was recorded in DD (constant darkness) for 8 days to obtain τ of individual flies.

2.2.3 Estimation of Precision

For assessment of various clock characteristics, three phase markers were used: Onset of activity, Offset of activity, and Centre of Gravity. Activity onset and offset phases identified using Clock lab software were verified by visual inspection (Actimetrics, Evanston, USA). ActogramJ was used to estimate the Center of Gravity (CoG) of activity of individual flies for each cycle (Schmid, Helfrich-Förster et al. 2011). τ_{onset} was calculated as the duration between successive activity onsets under constant conditions. Similarly, τ_{offset} and τ_{CoG} were calculated using activity offsets and CoG respectively. Precision was calculated as the inverse of the standard deviation (SD) in τ (see below) across days.

Onset Precision = 1/SD (τ_{onset})

Offset Precision = 1/SD (τ_{offset})

CoG Precision = 1/SD (τ_{CoG})

2.2.4 Alpha and Rho

Activity duration (α) for each cycle was measured as the time difference between the activity onset and offset of that cycle while rest duration (ρ) was the time difference between the offset of one cycle and the onset of the next cycle.

2.2.5 Statistical Analysis

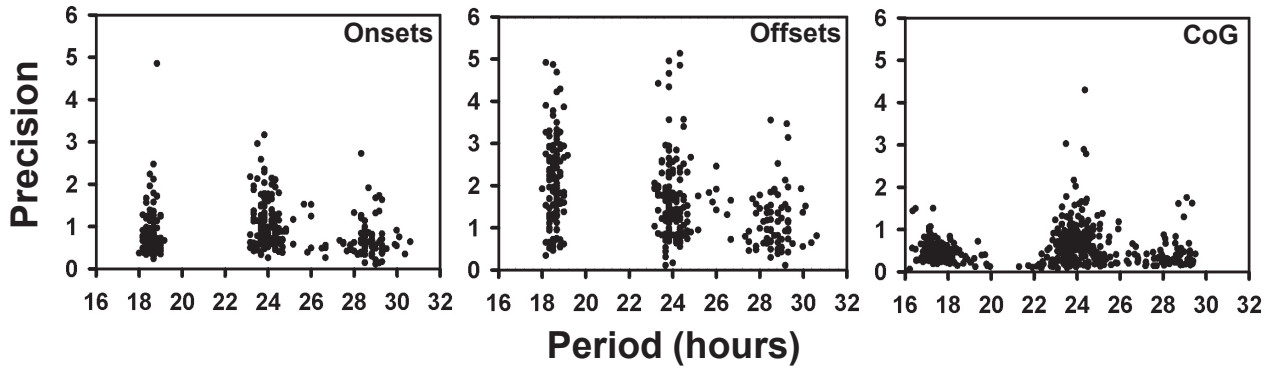
For comparison of mean values of precision across the three period ranges and with different phase markers, repeated measures ANOVA was performed with 'period range' as fixed factor and 'phase marker' as repeated measure. All statistical analyses were performed on STATISTICA, version 7 (Statsoft, 1995).

2.3 Results

2.3.1 Offset is the most reliable phase-marker for estimation of precision

I chose to pool the data across experiments performed over different generations (generations 5, 7 and 10) as the generation number did not seem to be having an effect on precision as well as accuracy (measured in the next chapter); a 2-way anova performed using Generation and Period range as fixed factors did not reveal a significant interaction between the two for (precision : $F_{4,695} = 0.29$, $p>0.05$; accuracy: $F_{4,161} = 1.36$, $p>0.05$). Similarly, I also first

a



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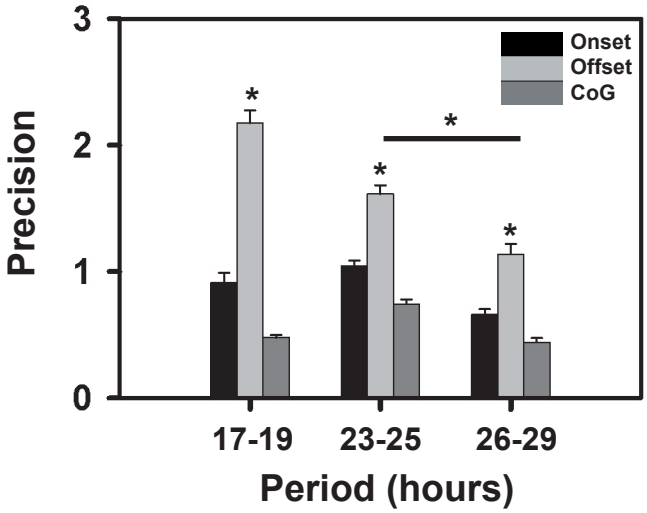

Figure 2.1 (a) Precision and period of individual flies with periods ranging from 17 to 30 h measured using onsets (left), offsets (middle) and CoG (right). Dots represent the precision value on *y*-axis for individual flies (n=368) with the corresponding period value on *x*-axis. **(b) Comparison of precision across the three period ranges measured using onsets (black), offsets (light gray) and CoG (dark gray).** Bars depict the mean precision across flies for each period range (n=138 for short period range, 158 for intermediate period range, 72 for long period range) plotted as a function of the period range. Error bars are SEM. Precision values calculated using offsets were significantly greater compared to those calculated using onsets and CoG. Asterisks above the bars represent significant differences across phase markers at $p<0.05$. Asterisk above the horizontal bar represents difference across period range for offset as a phase

tested the statistical influence of sex on these two measures and chose only males for all the further studies. Females were found to have significantly lower accuracy as well as precision when compared to males (precision $F_{1,132}$=25.63, p<0.05; accuracy $F_{1,95}$=19.93, p<0.05). Considering that females have weaker/less robust rhythms as also observed visually through actograms for all period ranges examined, we chose not to employ them for further studies done under different entraining conditions.

I first measured precision of individual flies (shown in Figure 2.1a) to determine its relationship with the intrinsic period. Repeated measures ANOVA performed to determine the optimal marker for assessment of rhythm properties showed a significant main effect of 'phase-marker' ($F_{2,348}$=24.862, p<0.05) and 'period range' ($F_{2,696}$=195.05, p<0.05). Post-hoc tests performed using Tukey's HSD showed that precision calculated using activity offsets was significantly higher when compared to that using onsets or CoG (Figure 2.1b) within each period range (p<0.05) and long period range flies had significantly lower precision compared to short and intermediate range flies. Moreover, 'phase-marker' × 'period range' interaction was also found to be significant ($F_{4,696}$=15.058, p <0.05) where precision values obtained using offsets (versus onset or CoG) were greatest in flies in the short period range as compared to the other period ranges (p<0.05) (Figure 2.1b). Therefore, I did not find precision to be highest for the intermediate period range while offset appeared to be the most precise phase marker.

2.3.2 α and ρ correlations

A significant negative correlation was found between α and succeeding ρ (Figure 2.2 a right) as well as between α and preceding ρ (Figure 2.2 a left) for all period ranges in DD (Table

2.1). ANOVA results for comparison of the summed standard deviation of α and ρ with that of τ showed that the combined variation of α and ρ was significantly greater than the variation in τ for all the period ranges which were examined (Figure 2.2 b) (17-19h ($F_{1,56}$= 24.547, $p < 0.05$); 23-25h ($F_{1,46} = 19.332, p < 0.05$); 26-30 h ($F_{1,56}$ = 20.445, $p < 0.05$)).

2.4 Discussion

Daily precision and persistence of the rhythm have been one of the most striking features of circadian rhythms (Pittendrigh 1965). In this regard, Pittendrigh and Daan's approach to understanding the properties of the entrainment mechanisms of a complex circadian timing system had utilized the genetic diversity in natural populations of different species (Pittendrigh and Daan 1976). However, absence of a wide range of τ as well as the fact that major differences in τ were primarily between species were limitations to interpreting the relationship between τ and other clock properties. I conducted a comprehensive study that largely negates this limitation by the use of mutant lines which have been reared in laboratory conditions and backcrossed to a wild-type, outbred fly stock derived from a natural population.

Previous studies had suggested that clocks with τ close to 24 hours would show higher precision (Aschoff 1971; Pittendrigh and Daan 1976; Sharma and Chandrashekaran 1999), which I did not find when data from multiple independent experiments were analyzed separately (Figure 2.1b). However, certain methodological limitations or differences across model organisms in the previous studies could be responsible for the inconsistencies in the results. For instance, the study by Pittendrigh and Daan involved rodents of different species with different ranges of τ which were pooled together to obtain a correlation between

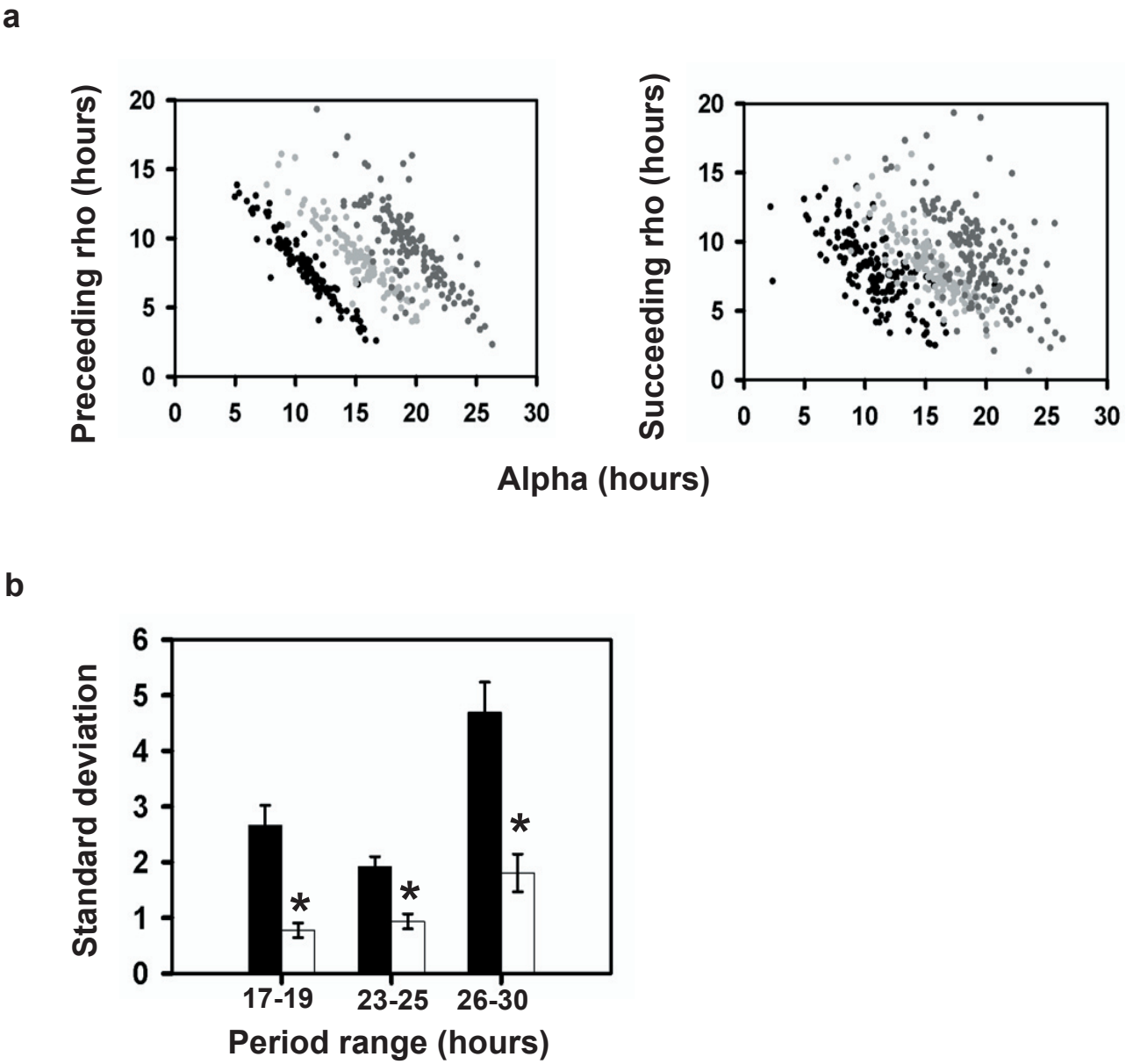

Figure 2.2 Activity/rest durations and intrinsic period. (a) Correlation of α with preceding (left) and succeeding (right) ρ of individual flies of the three period ranges (black dots: short; light grey dots: intermediate; dark grey dots: long) under DD. **(b)** Bar graph showing comparison of summed deviation of α and ρ with deviation in free-running period. Error bars are SEM. Asterisks denote significant difference at $p<0.05$.

precision and τ (Pittendrigh and Daan 1976). A wide variety of loci have been known to influence clock properties (Shimomura, Low-Zeddies et al. 2001; Takahashi, Shimomura et al. 2008) and therefore, comparison of these clock properties across species may not be ideal since several factors including genetic backgrounds and ancestry, ecological niches, etc. could be confounding factors in interpreting the results. Similarly, in another study where this correlation was observed, a larger range of τ was generated for the comparison of period and precision in birds by exposure to constant light (Aschoff 1971). This approach might have produced effects on the clock apart from change in τ since constant light is known to induce after-effects, decoupling of oscillators and arrhythmia at higher intensities (Pittendrigh and Daan 1976). Indeed, Aschoff and colleagues noted that constant light may induce changes in activity durations as well as phase variability by changes in the amplitude level of oscillator (Aschoff 1971). Hence, the correlations observed in the study on birds may not reflect the true relationship between endogenous τ and precision. While the study on *Mus booduga* was conducted on mice of similar genetic background (Sharma and Chandrashekaran 1999) and avoided artificial manipulations of τ, the range of τ obtained across individuals was narrow and asymmetric across 24 h, with few individuals showing τ greater than 24 h. Hence, these results suggest that the correlation between τ and precision is somewhat inconsistent.

The finding from my results that precision is not always greater when τ approximates 24 h does not support the notion that individuals with τ close to 24 h are selected for increased precision. Indeed, phase relationship of the rhythm with external cycle and its accuracy are the characteristics that likely confer adaptive advantage to the organism (Roenneberg and

Merrow, 2015) and hence, I have tested these properties and the relationship between precision and accuracy in the next chapter.

I also do not see any sudden dip in frequency of individuals at 24 h in my data (Chapter 1, Figure 1.2) which would be expected according to the proposed hypothesis. It has been speculated previously that variation in τ has no direct correlation with fitness and therefore τ is susceptible to random variation within a circadian limit (Shimizu, Miyatake et al. 1997). Moreover, precision was also observed to be not related to τ in homogenized lines of closely related Syrian hamsters with τ ranging from 17.8 to 24.2 hours (Bittman 2012). My results provide evidence in favour of a weak correlation between period and precision. I also note here that even on normalizing the precision values with the mean period values for each genotype I obtained a higher value of precision for the near-24 h flies compared to short and long period flies. Thus, it can be inferred that the different relative deviation of period across days across the genotypes is not the reason behind the three genotypes having different precision values. While the precision of offsets in this species (coefficient of variation is roughly 1 part in 25 in individuals with $\tau \sim 24$ h) is somewhat lower than the remarkable precision seen in the activity rhythms of birds and rodents (upto 1 part in 500) but comparable to other rhythms such as chirping of crickets and spontaneously firing neurons (upto 1 part in 50; Enright, 1980). However, it is important to remember here that the measure of precision in this case is the behavior (activity rhythms) which does not directly correspond to the precision of the core pacemaker. For instance, the precision of the core circadian pacemaker is thought to be much greater than that of phase markers of the overt rhythm (Pittendrigh and Daan, 1976; Welsh et al., 1986). Although individual pacemaker neurons are less precise, the interactions between neurons results in precise tissue rhythms

and behavioural output (Enright, 1980; Herzog et al., 2004). The circadian synchrony finally achieved is a result of multiple signals during the ontogeny of organism as studied in SCN of mammals (Carmona-Alcocer et al., 2018).

In my study, activity and rest durations were found to be interdependent and negatively correlated under constant conditions for all period ranges examined. However, the correlation of α seems to be stronger with the preceding ρ for all three period ranges (Figure 2.2). This observation was similar to that from a previous study on Syrian hamsters, where the correlation of α and succeeding ρ was found to be stronger across the full range of τ examined (Bittman 2012). Therefore, the correlation between α and ρ does not appear to be dependent on τ which is inconsistent with Aschoff's predictions (Aschoff 1971). Instead, it appears that the relationships vary across different model organisms used in the different studies. The importance of α-ρ interdependence in maintaining stability in τ was further shown by comparing the summed variation of α and ρ and the variability in τ itself. I found that variation in τ measured using offsets was consistently lower than the summed variability of α and ρ. Our data shows that both α and ρ vary across days in a manner so as to minimize the variation in period. These results support the findings from the previous study on birds and humans (Aschoff 1971) and the one on Syrian hamsters (Bittman 2012). Chaffinches which have roughly equal α and ρ also showed highest precision in the former study, however this was not observed in our study since most flies showed a higher α compared to ρ.

Aschoff's study suggested that the increase or decrease in τ is mostly due to change in the ρ in each cycle (Aschoff 1971). It was however later reported that short period mutants of female *Drosophila* change their activity duration while the longer period mutants change

their rest duration (Saunders, 1994). A proportionate reduction of both α and ρ was shown in tau and *duper* mutants of Syrian hamsters (Bittman 2012). I have found that α changes, to bring about change in the intrinsic period, and not ρ (data not shown). The inconsistencies in the findings from different studies could be because the trends are specific to the model organisms. Such changes could also be determined by which of the two, α or ρ, is flexible to bring about change in period. Under LD 12:12, α increased and ρ decreased for individuals with increasing periods. These results indicate that both activity and rest durations are adjusted for entrainment to different light-dark conditions.

The model (according to which, the level of any oscillation passes through a threshold twice during every cycle and as long as the level is above the threshold, the organism is active) which predicts that α should be correlated with both succeeding and preceding ρ supports our findings. However, the hypotheses based on the extension of the model, which assumed that the shape of the oscillation (thought to represent propensity for activity which is gated by the clock according to the model) would change with τ, were not found to hold true in our case. According to this assumption, an oscillation with a short τ would be left-skewed and therefore have a higher precision for onset of activity compared to offset and vice versa. However, we found offsets to be more precise for all three period ranges (Figure 2.1). It may be inferred; therefore, that waveform in our case is right-skewed for all τ values. It can be speculated therefore that the skewness of the oscillation does not change with τ as hypothesized previously. The skewness of the oscillation explains our results about alpha being more strongly correlated with preceding rho for all three period ranges. This can also explain why offsets are more precise for rhythms of fruitflies. This specific skewness seems

to be species specific and might be dependent on the ecological factors in their evolutionary history.

It is important to note that while making the predictions about the change in α-ρ relationship with τ, it was assumed that the change in the level of oscillation was caused by changing the intensity of light which therefore changed τ and α (Aschoff et al., 1971). However, in my study and in some other previous studies, individuals with distinct τ values were obtained using mutations (Saunders, Gillanders et al. 1994; Bittman 2012). The effect of varying environmental conditions on τ could be quite different from that of a mutation in a core-clock gene as in my case. Moreover, the effects of mutations also may be different under natural environments compared to laboratory conditions as has been observed (Vanin, Bhutani et al. 2012). Therefore, it is important to keep in mind these considerations before making conclusions about clock properties and their relationships with τ.

In summary, my results from this chapter show weak association between period and precision of the clock but not in the manner previously proposed. I also find some evidence in support of the threshold model to explain the patterns of activity and rest durations observed in my study but did not find my results to be entirely consistent with the non-parametric model of entrainment.

Circadian Clock Properties and their Relationships as a function of free-running period under entraining conditions

The contents of this chapter have been published as the following research article:

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3.1 Introduction

Entrainment of biological rhythms is crucial for organisms, enabling ecologically appropriate and stable phasing of near 24 h internal clocks with the 24 h external environmental cycles. The phase-relationship with the environment is thought to be adaptive to organisms due to appropriate timing of behavioural and physiological processes that may increase their reproductive fitness. For this phasing of behavior to be adaptive, it must be consistent across days. This consistency or lack of variability of entrained phase across days is measured as accuracy (Daan and Beersma 2002). While τ is considered a core parameter of the clock, and accuracy a trait that may confer adaptive advantages to organisms, there are several clock properties such as range of entrainment, response to environmental cues, etc., that are related to both τ and ψ. Various studies have examined the relationships between them and certain facts regarding clock properties have been established over years. However, this consensus has emerged based primarily on the non-parametric model of entrainment (Daan and Pittendrigh 1976) (Daan and Aschoff 2001) (Hirschie Johnson, Elliott et al. 2003), and there are some inconsistencies in the results.

Since recognizing local time is one of the clock's major functions, previous studies also investigated how τ influences phase relationship (ψ). Association between ψ and τ has been observed using fruit flies with altered τ (Hamblen-Coyle) (Wheeler et al. 1992) and with those selected for early or late adult emergence (Kumar, Kumar et al. 2007). Similar correlations between these two variables have been observed in humans and mice among other organisms (Pittendrigh and Daan, 1976; Duffy et al., 2001). The periodicities of external cycle (T) to which the circadian clock can entrain (range/limits of entrainment) is

also known to be a function of τ in addition to the magnitude of phase resetting (Pittendrigh 1965). Additionally, it is observed that ψ of entrained behavior changes with the period of T-cycles such that entrainment to increasing length of zeitgeber cycle is associated with advancing ψ (Aschoff and Pohl 1978) suggesting that ψ, in addition to being dependent on τ, is also a function of T. However, each of these previous studies suffered from certain limitations such as, non-uniform genetic background among individuals with contrasting τ (Pittendrigh and Daan 1976), use of environmental manipulations to generate a range of τ (Aschoff 1971), or comparison across a small range of τ (Sharma and Chandrashekaran 1999).

Moreover, there have been exceptions to the conclusion drawn regarding several of the above-mentioned relationships. τ- ψ relationships which do not follow the proposed rule of delayed phase with increasing τ have also been observed in a study with *Neurospora crassa* (Lee, Shiva Kumar et al. 2017). In the field mouse, *Mus booduga*, it was seen that the τ-ψ relationship holds true under gradual transitions of light and dark but not under abrupt transitions (Sharma, Chandrashekaran et al. 1998). These inconsistencies in results could be because of different methodologies or model organisms in different studies.

Here I attempted to test the hypothesis regarding the above mentioned clock properties which are measured during entrained conditions while minimizing confounding factors encountered in the previous studies. Therefore, following hypothesis were tested:

1. Accuracy of phase relationship is greater for near 24 h clocks.
2. An increase in intrinsic period results in a delay of phase relationship.

3. An increase in period of external cycle results in an advancement of phase relationship.
4. Phase period relationships observed can be explained through the phase shifts measured across the day.

For this, I first computed accuracy using multiple phase-markers like in chapter 2. I also asked to what extent my observations can be explained by the phase response curves (PRCs) of the fly lines employed.

My results from correlation analysis suggest that accuracy of entrained phases does vary with τ and was found to be higher when τ was closer to T. ψ was found to be correlated with τ and T, consistent with predictions from the non-parametric model of entrainment whereby shorter τ flies are found to be phase advanced relative to longer τ flies. Thus, I demonstrate that the relationships between intrinsic period, accuracy and phase relationship of entrained rhythm appear to be governed by endogenous period as well as period of the external cycle.

3.2 Materials and Methods

3.2.1 Fly lines and Maintenance:

Using a large outbred population of *Drosophila melanogaster* (Gogna, Singh et al. 2015), individuals carrying either the short or long *period* mutation, per^s or per^l, were backcrossed for a total of ten generations like described in chapter 1. Experiments described here were performed with 5^{th}, 7^{th} and 10^{th} generation backcrossed individuals. Since my interpretations are not focused on the specific effects of the *period* gene *per se*, henceforth, I have used the term 'Period Range' to denote the three genotypes like in chapter 2 and also used similar

range for analysis as described in chapter 2. On 9th and 10th days after egg collection, freshly emerging flies were collected, sexed and separated as virgin males and females, within 6 hours of their emergence. To acclimatize flies to assay conditions, all flies were maintained under LD 12:12 cycles at 25 °C for the three to four days after emergence before beginning any assay.

3.2.2 Locomotor Activity Rhythm Recordings

Individual 4-5 days old virgin male flies were introduced into *Drosophila* Activity Monitors (DAM 5, Trikinetics™, Waltham, MA, USA) at an ambient temperature of 25 °C either in constant darkness (DD) or in light-dark cycles with light intensity of 1 lux (obtained by using filters over LEDs which were locally sourced and emitted cool white light consisting primarily of wavelengths in the 450 nm, 550 nm and 600 nm range) during light phase. I employed a low intensity of light to estimate stability of entrainment because higher light intensities could result in similar levels of high accuracy across genotypes due to masking. Activity-rest rhythm was first recorded in DD (constant darkness) for 8 days to obtain τ of individual flies (data used for chapter 2), after which different subsets of flies were transferred to different T-cycles (T20, T24, or T28 symmetric regimes with10:10 h, 12:12 h or 14:14 h light-dark cycles) for 10 cycles. This was followed by a few days of recording in DD to verify if entrainment has occurred. To obtain PRCs, for each period range, circadian times (CTs) were separately calculated and light pulses (15 min, 1 lux) were given at six different circadian times after four days of entrainment under LD 12:12. Along with the experimental set, I used one set of flies (disturbance controls) of each period range which were disturbed at each of the CT points but not given any light pulse. Since my main aim was to check if my observations under T24 can be explained by PRCs, I chose to keep the

intensity and duration of light similar to what was used to estimate phase relationship and stability of entrainment under T24.

3.2.3 Estimation of Accuracy

Modulo-T plots of actograms were plotted, and the activity onset and offset phases identified using Clock lab software were verified by visual inspection (Actimetrics, Evanston, USA). ActogramJ was used to estimate the Center of Gravity (CoG) of activity of individual flies averaged across 10 cycles (Schmid, Helfrich-Förster et al. 2011). Phase relationship (ψ) under entrained conditions was calculated by measuring the duration between the phases of entraining zeitgeber and entrained rhythms (for example ψ (offset) is difference in timings of lights-off and activity offset). Phase shifts were calculated by measuring the difference between the predicted and obtained phases after light pulse extrapolated to the day of the light pulse (Phase-shift = Pre-pulse phase – post-pulse phase). Delays in phase relationships as well as phase-shifts were given negative values while advances were given positive values by convention similar to Pittendrigh and Daan (1976). The difference of phase shifts between disturbance controls and experimentals for each time-point was used as the real estimate of the phase shift occurring due to the light pulse.

3.2.4 Statistical Analysis

For accuracy and ψ, I compared mean values across T-cycles using ANOVA where 'regime' was used as fixed factor and individual flies were taken as replicates for the dependent variables, accuracy and ψ. To measure differences in phase shifts across time of the day, two-factor ANOVA testing the effect of period range and time point on phase shifts was

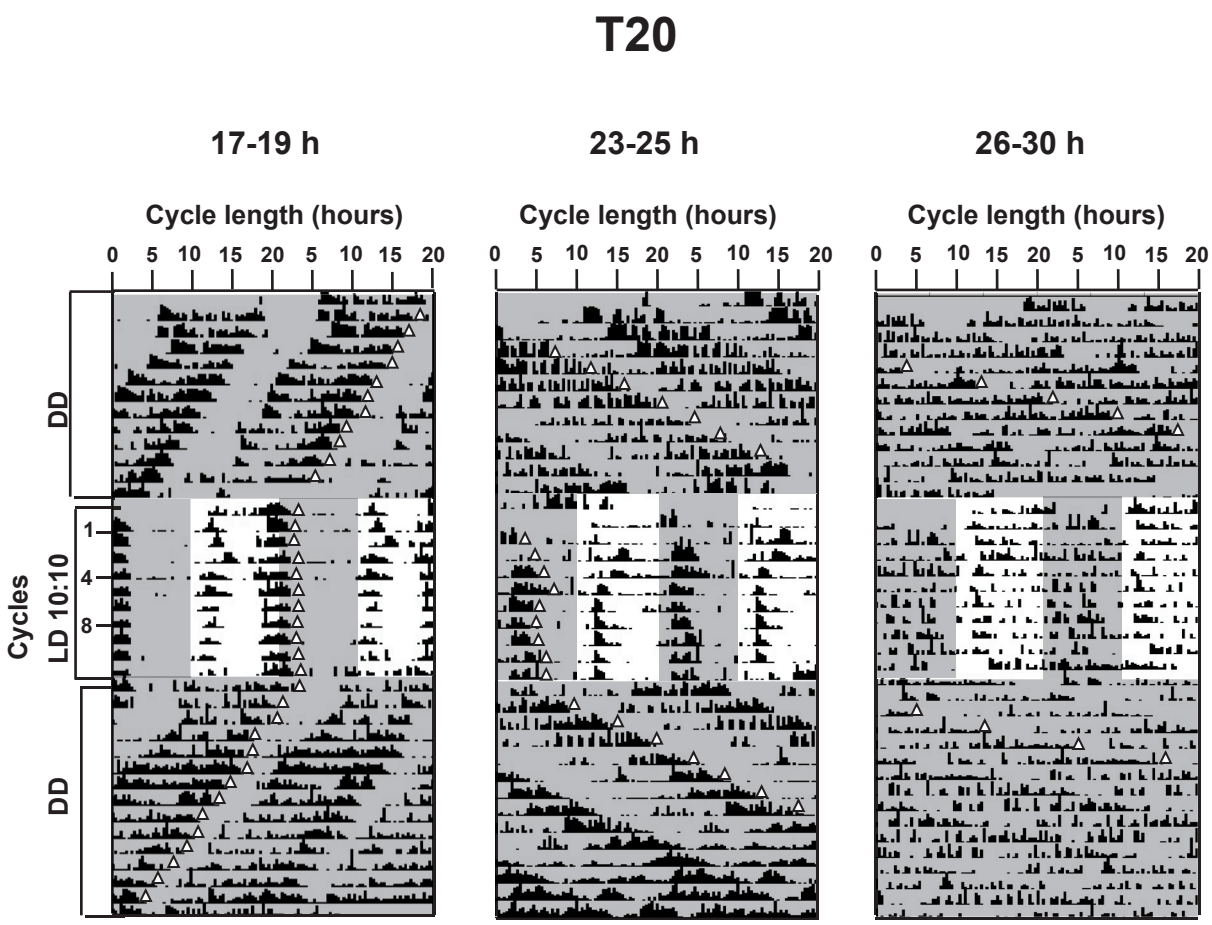

Figure 3.1 Representative actograms under T20. Actograms for an individual fly of each of the three period ranges under T20 (cycle number shown by values on *y*-axis) preceded and followed by DD as indicated on *y*-axis. Here, scale on *x*-axis is adjusted to 20 h. Triangles depicting the offset phase for each cycle are placed to qualitatively follow the free-running and entrained rhythms. Shaded areas in the actograms depict the dark phase in each cycle.

T24

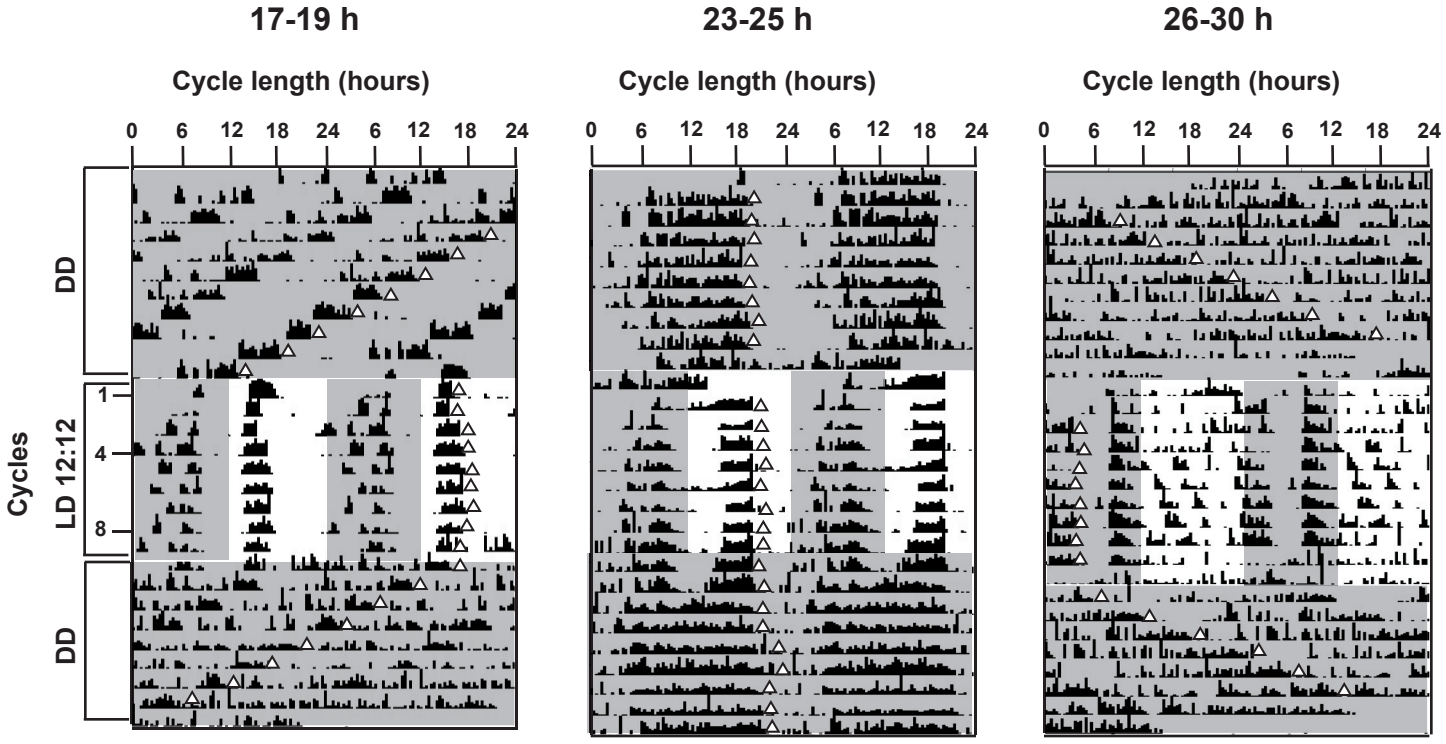

Figure 3.2. Representative actograms under T24. Actograms for an individual fly of each of the three period ranges under T24 (cycle number shown by values on *y*-axis) preceded and followed by DD as indicated on *y*-axis. Here, scale on *x*-axis is adjusted to 24 h. Triangles depicting the offset phase for each cycle are placed to qualitatively follow the free-running and entrained rhythms. Shaded areas in the actograms depict the dark phase in each cycle.

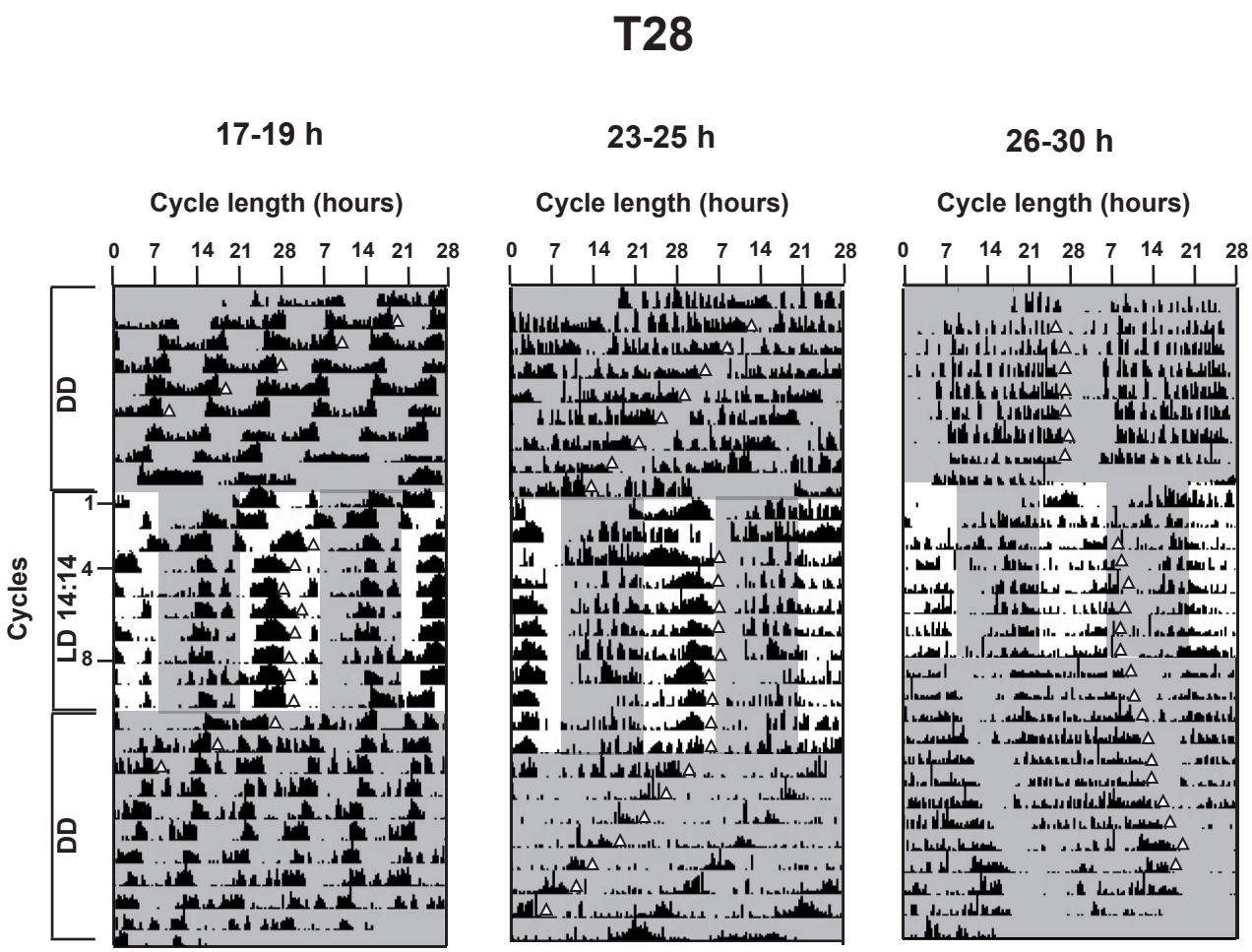

Figure 3.3. Representative actograms under T28. Actograms for an individual fly of each of the three period ranges under T28 (cycle number shown by values on *y*-axis) preceded and followed by DD as indicated on *y*-axis. Here, scale on *x*-axis is adjusted to 28 h. Triangles depicting the offset phase for each cycle are placed to qualitatively follow the free-running and entrained rhythms. Shaded areas in the actograms depict the dark phase in each cycle.

performed. All statistical analyses were performed on STATISTICA, version 7 (Statsoft, 1995).

3.3 Results

3.3.1 Accuracy is maximum for clocks with τ close to that of external cycle

I tested the relationship between τ and accuracy of entrainment in presence of LD cycles of 24 h (T24) while also testing the hypothesis that accuracy is enhanced when τ is closely matched with T. For this, I used two other regimes, T20 and T28 with symmetric LD durations and checked if flies with different τ entrained under these regimes (each fly was assessed for entrainment based on whether it showed periodicity ±0.5 hours of entraining regime and phase of its rhythm following transfer to DD could be extrapolated back to the phase on the last day of entrainment. Flies of a particular period range were included for analysis only if >75% of them fulfilled these criteria. Representative actograms showing activity patterns for each period range under the three T-cycles have been shown here (Figures 3.1-3.3). Under T20, flies in short and intermediate τ range were found to entrain but not those with long τ. Under T28, flies entrained irrespective of their intrinsic period. I tested whether for a given τ range, absolute value of accuracy (shown for individual flies in a T-cycle in Figure 3.4a) itself varies across T-cycles. Separate one-way ANOVA (a composite ANOVA was not feasible in this case as the long period flies did not entrain under T20) for each period range testing the effect of T-cycles on accuracy revealed a significant effect of 'T-cycle' on accuracy for the short ($F_{2,53}$=53.00, p<0.05) and intermediate ($F_{2,62}$=28.71, p<0.05) period ranges but not for longer period range (p>0.05). Individuals in short period range showed higher accuracy in T20 compared to other T-cycles (Figure 3.4 b)

whereas those in intermediate range showed higher accuracy in T24 (Figure 3.4 b). I have shown the comparison made across T-cycles in Figure 3.1 b by replotting the data shown for individual flies in Figure 3.4 a to highlight that for a given period range, accuracy is significantly lower in the two T-cycles which are deviant from the mean τ. I also show accuarcy for each period range under varying photoperiods and do not see any consistent trend (Figure 3.5). Thus, the notion that accuracy would be greater when T is closest to τ is upheld for short and intermediate period ranges.

3.3.2 Phase and period are related

I tested the widely held notion that τ is strongly associated with ψ. To do so, I examined ψ for flies belonging to the three period ranges under three T-cycles(phase relationship is shown for individual flies in a T-cycle in Figure 3.6a). ANOVA on ψ for each of the period ranges across different T-cycles revealed a significant main effect of regime across period ranges (17-19 h: $F_{2,53}$=1515.12, p<0.05; 23-25 h: $F_{2,61}$=783.41, p<0.05; 26-30 h: $F_{1,20}$=12.46, p<0.05; Figure 3.6 b). Post-hoc comparisons showed that ψ under T28 was significantly advanced relative to T24 and T20 for period ranges of 17-19 h and 23-25 h (Figure 3.6 b). Flies of period range 26-30 h largely did not entrain to T20, however under T28, ψ was more advanced compared to that under T24 (Figure 3.6 b)

3.3.3 Phase response curves for the three period ranges

I constructed light pulse PRCs for individual flies of the three period ranges (Figure 3.7 a). Two-factor ANOVA testing the effect of period range and time point on phase shifts showed significant main effects of period range ($F_{2,431}$=3.89, p<0.05), time point ($F_{5,431}$=11.34, p<0.05) as well as their interaction ($F_{10,431}$=11.34, p<0.05). Interestingly, the extent of phase

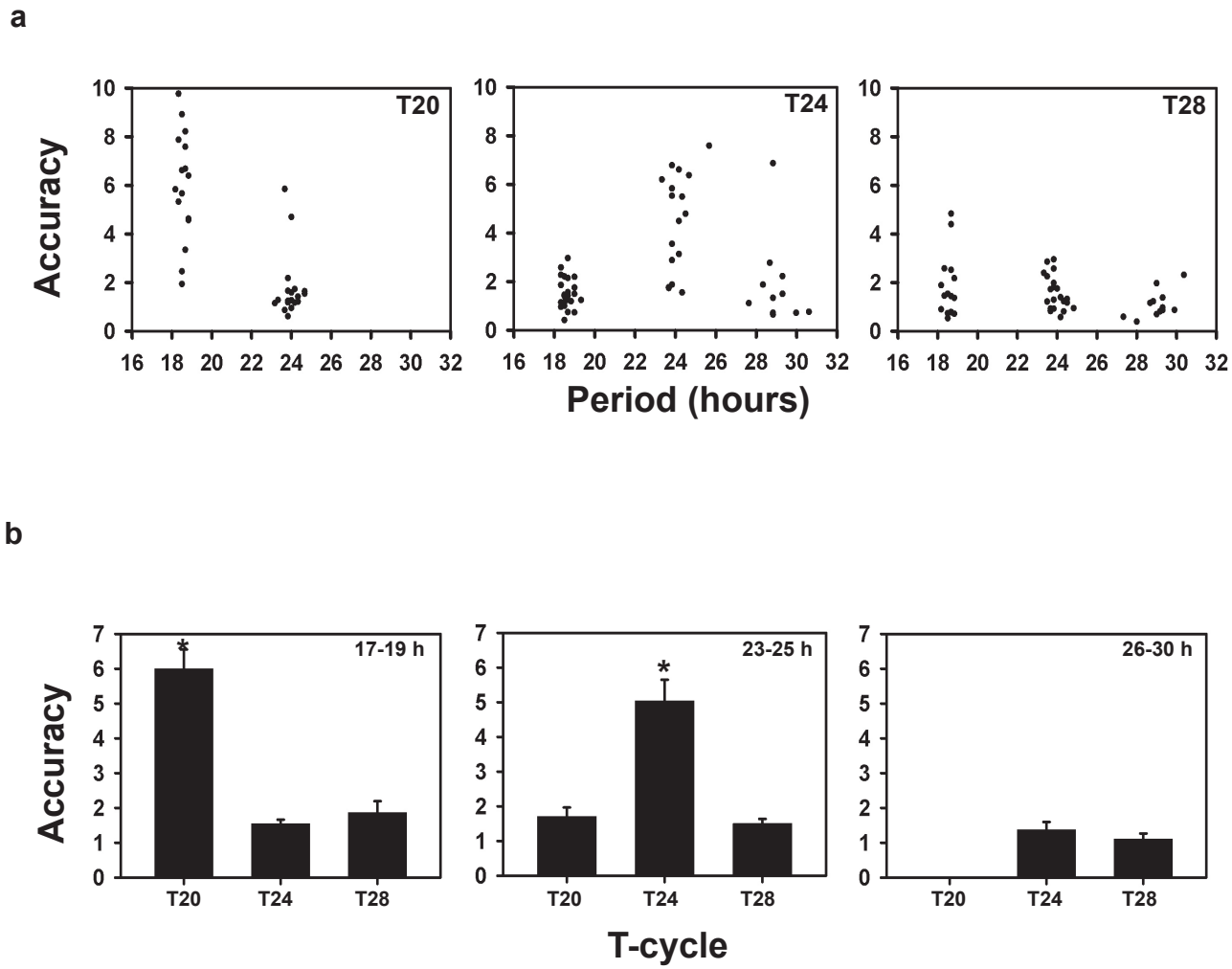

Figure 3.4 (a) Accuracy and period of individual flies with periods ranging from 17 to 30 h measured under different T-cycles: T20 (left), T24 (middle) and T28 (right). Dots represent the accuracy values on *y*-axis for an individual fly with the corresponding period value on *x*-axis. Correlation coefficients are given in Table 3. **(b) Comparison of accuracy across T-cycles for the three period ranges**: Bars depict mean accuracy across flies for each period range under each T-cycle. Error bars are SEM. Asterisks above the bars represent significant differences across T-cycle at $p<0.05$.

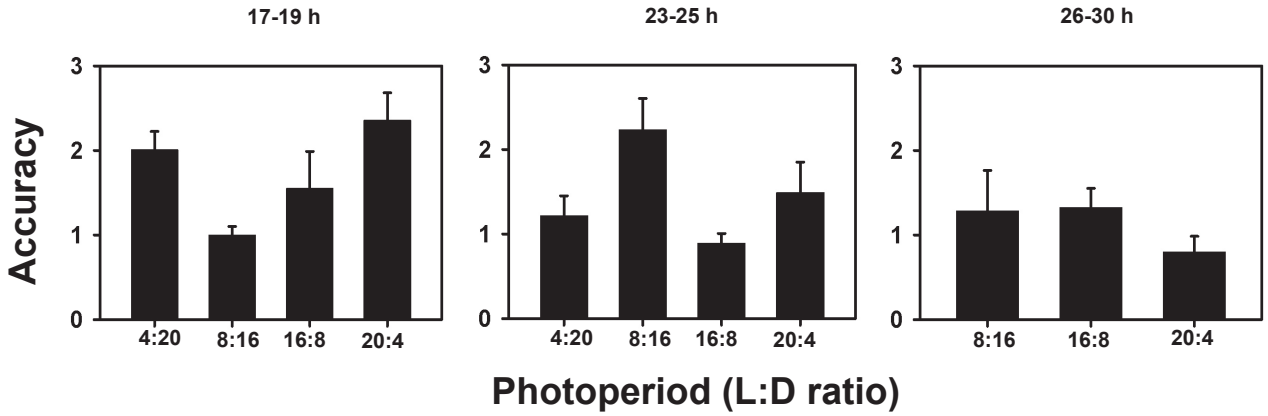

Figure 3.5 Accuracy under different photoperiods. Accuracy of flies of the three period ranges under different photoperiods. Error bars are SEM.

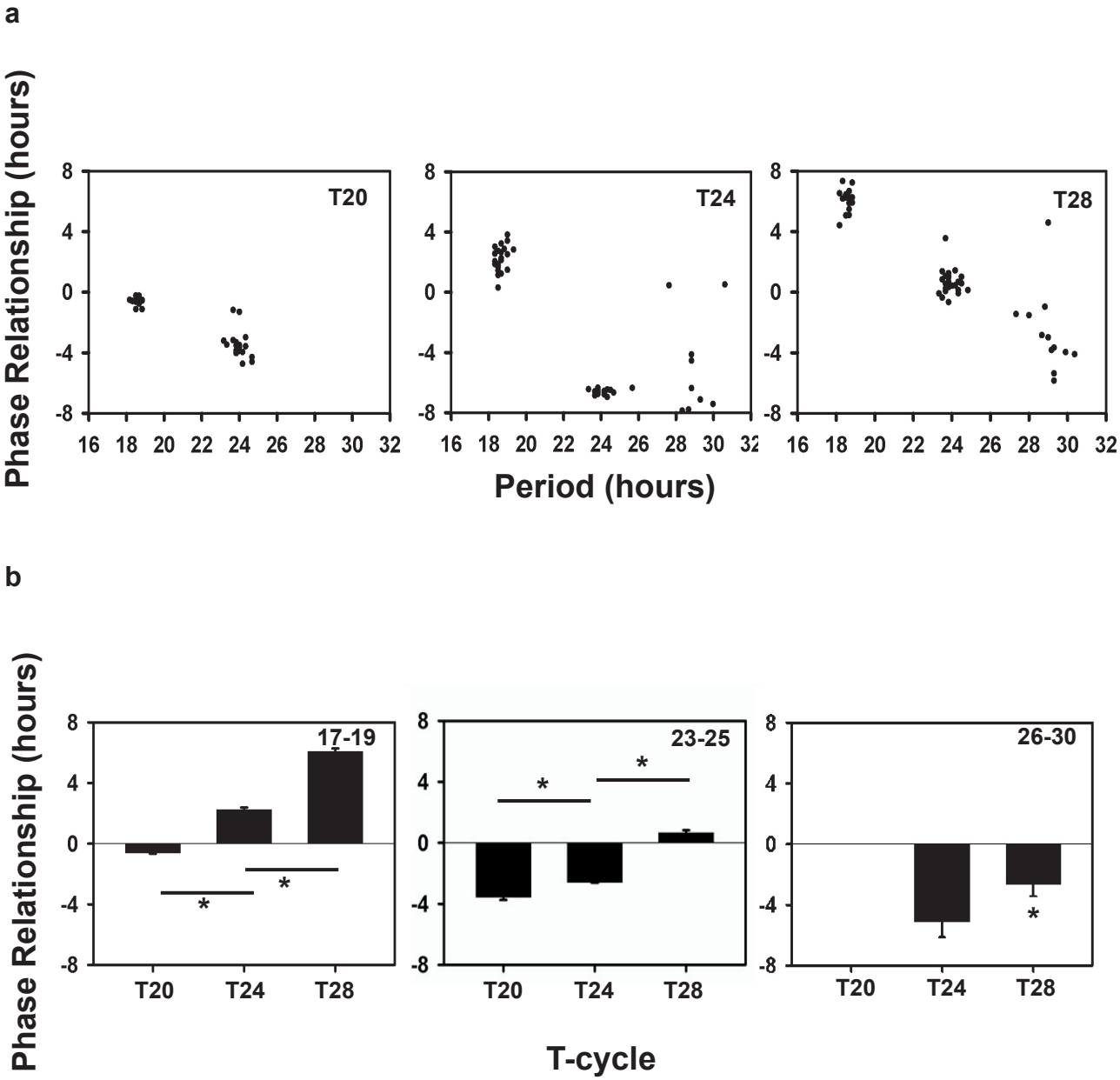

Figure 3.6 (a) Phase relationship and period of individual flies with period values ranging from 17 to 30 h measured under different T-cycles: T20 (left), T24 (middle) and T28 (right). Dots represent the phase value on *y*-axis for an individual fly with the corresponding period value on *x*-axis. Negative values imply a delayed phase relationship, and positive values imply an advanced phase relationship. **(b) Comparison of phase relationship across T-cycles for the three period ranges:** Bars depict mean phase-relationship across flies for each period range under each T-cycle. Error bars are SEM. Asterisks denote significant differences across T-cycles at $p<0.05$.

shift did not differ significantly across the different circadian time points tested for short τ individuals (Figure 3.7 a top). While intermediate τ range showed significantly greater advances at CT20 and delays at CT16 (Figure 3.7 a middle), long τ range showed maximum advances at CT4 and delays at CT16 (Figure 3.7 a bottom). For clarity, I have shown bar graphs where comparisons across period ranges for each time point are depicted in a combined manner (Figure 3.4 b) while comparison across time points are shown separately for short (Figure 3.7 c top), intermediate (Figure 3.7 c middle) and long (Figure 3.7 c bottom) period ranges. I also estimated delay/advance ratios (D/A) for each period range (D/A for short period range:1.12; intermediate period range:0.74; long period range:0.63) and find that it reduces with lengthening period.

3.4 Discussion

Earlier attempts to use mutant fly lines to test circadian resonance in *Drosophila* with different light regimes have yielded negative results (Klarsfeld and Rouyer 1998), despite its success in cyanobacteria, Arabidopsis, and mice (Ouyang, Andersson et al. 1998; Dodd, Salathia et al. 2005; Wyse, Coogan et al. 2010). However, this may be attributed to the lack of a common genetic background and/or effects of inbreeding in mutant fly lines which are typically critical for interpreting fitness traits. Since activity-rest patterns of flies are remarkably consistent in different environmental regimes, employing them for assessing different clock properties, which indirectly contribute to fitness and therefore form the basis of circadian resonance, must help in deriving conclusions regarding the relevance of a circadian period for wild-type organisms.

In our study, accuracy under LD 12:12 was higher for individuals lying in the intermediate period range compared to those in short and long period range (Figure 3.1). This demonstrates that individuals with τ closer to 24 h can entrain to 24 h light-dark cycles with greater stability of entrained phase relative to clocks with τ deviating from 24 h. The trends in accuracy across period ranges, were seen to differ in altered periodicities of the external cycle (T). To some extent, the accuracy values were found to be higher when T matched τ. For example, flies with shorter τ showed higher accuracy under T20 (Figure 3.4). Also, accuracy for flies in this range is reduced under both T28 as well as T24 (Figure 3.4) which indicates that the enhanced accuracy observed under T20 is indeed because T matched τ and not just because it is within the range of entrainment. Similarly, individuals with intermediate τ exhibited reduced accuracy under shorter and longer T.

It is interesting that long period range individuals do not show greater accuracy under T28 than other period ranges. This may be attributed to the nature of the long period mutant gene which produces a PER protein which is defective in its interaction with TIM. The interaction between TIM and PER is thought to determine the nuclear entry of PER (Gekakis, Saez et al. 1995) and regulated timing of nuclear translocation of PER has been considered a critical determinant of τ (Vosshall, Price et al. 1994). Moreover, it is argued that the presence of inter-locked feedback loops is essential for increased rhythm stability and robustness in the face of random perturbations or noise (Brown et al., 2012; Partch et al., 2014); therefore, reduced accuracy and precision of long period range flies may be attributed to this defect in one component of the molecular feedback loop. However, recent studies under natural-like conditions have shown that nuclear entry of PER and TIM are not always synchronous under summer conditions mainly due to long photoperiods (Menegazzi, Vanin et al. 2013). Hence,

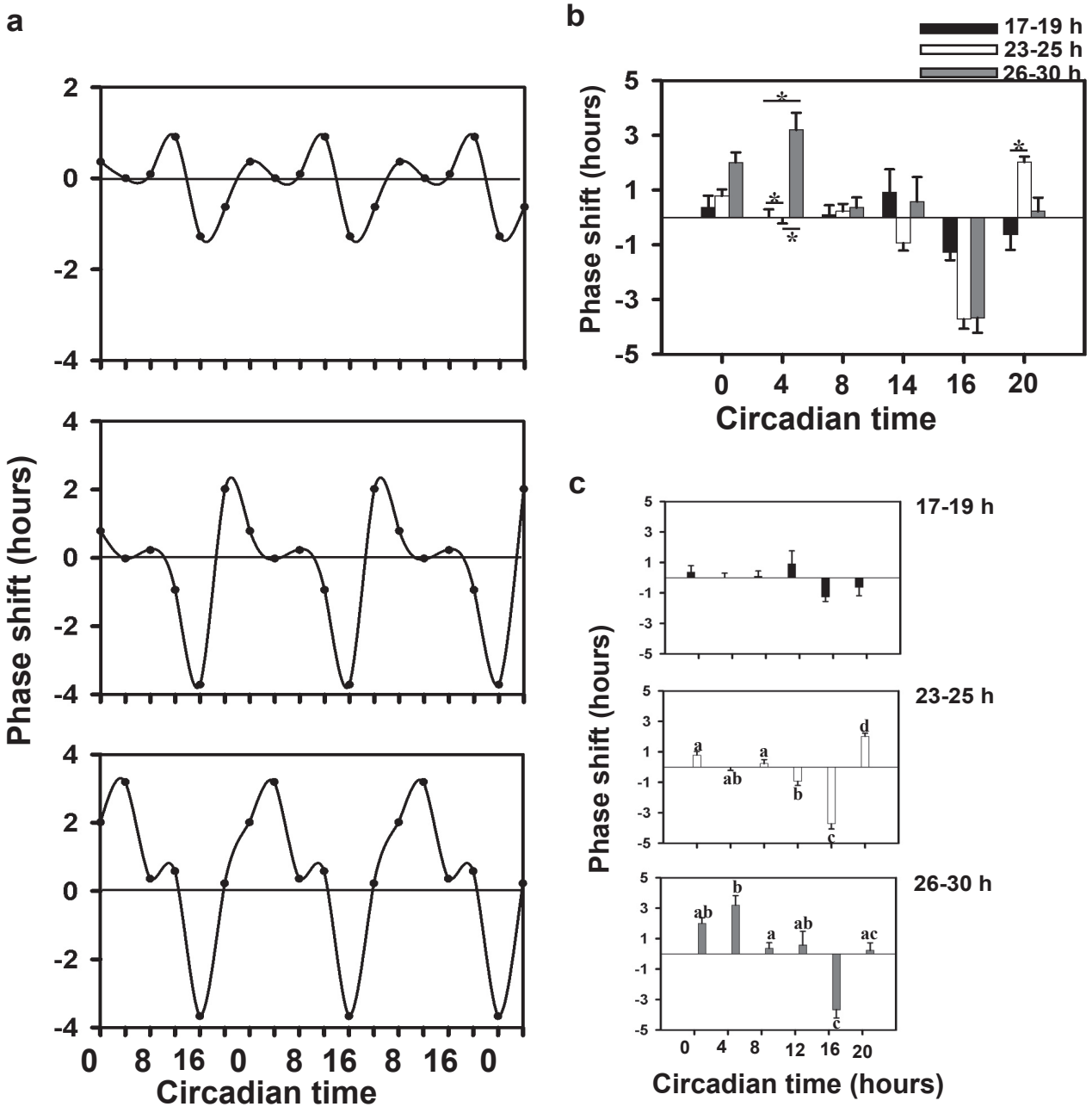

Figure 3.7 Phase response curves of flies from the three period ranges. (a) Phase shifts in response to light pulses of 15 min duration and 1 lux light intensity plotted against circadian time for 17-19 h (top), 23-25 h (middle) and 26-30 h (bottom) (n~32 for each time point for each period range). Negative values imply a delayed phase relationship, and positive values imply an advanced phase relationship. The obtained curves have been tri-plotted for clarity. (b) Bar graphs showing comparisons across period ranges for each time point. (c) Bar graphs showing comparison across time points for each period range17-19 h (top), 23-25 h (middle) and 26-30 h (bottom). Error bars are SEM. Asterisks and different alphabets denote significance at $p<0.05$.

although I acknowledge that inferences from the experiments described here are limited to laboratory conditions with low strength of zeitgeber and symmetric photoperiod, the role of differences in the interlocked feedback loops in rhythm stability can be thought of as a more general phenomenon affecting different period range individuals to different extents. I also see that the trends in accuracy varies under different photoperiods, however there is no consistent trend with increasing photoperiods for any period range (Figure 3.5).

Since precision is also not correlated to accuracy of entrained phase in our study *(r*=-0.02; *p*>0.05*),* other properties of the clock may be important for such resonance dependent increase in accuracy and therefore, be under indirect selection. These results suggest that accuracy is not a function of the variability of clock's intrinsic period alone but varies depending on the external conditions. These results also suggest that accuracy of phase may be affected by sensitivity of the clock to zeitgebers as individuals with similar levels of precision show differences in accuracy. However, based on the PRCs I obtained, reduced accuracy exhibited by long period range flies cannot be explained by the relatively large amplitude PRCs, since large magnitude phase shifts are expected to contribute to greater accuracy (Beersma et al., 1999). While molecular clocks consisting of cycling proteins of core clock genes of period and clock would be expected to determine intrinsic clock period and its precision (Gonze, Halloy et al. 2002), accuracy of entrained rhythms would be also affected by activity of cryptochrome and degradation of timeless in response to light (Emery, So et al. 1998; Busza, Emery-Le et al. 2004). This could also explain why I do not find strong correlations between precision and τ since accuracy does not seem to be dependent on precision and therefore, selection for greater accuracy may not entail concomitant selection for precision. Moreover, theoretical studies have suggested that factors affecting accuracy

under naturalistic light-dark cycles where continuous, fluctuating zeitgebers are present may be significantly different from those under square-wave, laboratory light-dark cycles (Beersma, Daan et al. 1999). Thus, I must qualify my interpretations as being limited to observations made under laboratory conditions alone.

Our results supported the predictions based on the discrete model of entrainment (Daan and Pittendrigh 1976) (Pittendrigh and Daan, 1976) that ψ would be delayed with an increase in τ as this was found to be true under all T-cycles examined (Figure 3.3). In earlier studies, the trend of phase being delayed for greater τ was seen even when period range was considerably narrower (Daan and Pittendrigh 1976; Sharma and Chandrashekaran 1999) (Pittendrigh and Daan, 1976b:23.6-24.8 h; Sharma et al., 1999:23-24 h). However, in my study when similar narrow ranges of period were considered (0.5 to 2h), correlation between τ and ψ was not found to be significant *(r* = 0.3, -0.08 and 0.17 for short, intermediate and long period ranges respectively $p>0.05$). This could be limited by the methodology of our measurement of ψ and τ within such narrow ranges since in such cases, the intra-individual variability in ψ and τ is greater than the variability between individuals (almost twice in case of short and intermediate period ranges). The relationship of ψ with different T has been considered as a criterion for entrainment as opposed to masking (Aschoff 1965) where phase of a rhythm would immediately follow a transition from dark to light or light to dark irrespective of T. Supporting the findings of a previous study (Aschoff and Pohl 1978) where a relatively small range of τ was examined, I show that even across a wide range of τ, with increasing length of T-cycles, ψ was advanced.

Based on the D/A ratios, the pattern of phase shifts obtained across time points for flies with different τ meets the expectations of the non-parametric model of entrainment for long τ flies

as they exhibit larger advance regions and short τ flies show larger delay regions (Figure 3.7). However, the range of entrainment that I obtain for the three genotypes does not completely conform to expectations based on the observed phase shifts since flies with longer τ would be expected to show larger range of entrainment compared to those with shorter τ since they exhibit larger amplitude PRCs. Perhaps non-parametric mode of entrainment, based on which most of our hypotheses were made, is not sufficient to explain the relationships among clock properties for a broad range of τ. However, the trend in D/A ratios imply period range-dependent differences in parametric effects of light which may contribute to the observed differences. In case of short period flies, since the shape of the PRC is similar to the other period ranges, I speculate that the lack of significant phase shifts could be due to large inter-individual variability in phase shifts that I obtained. Overall, PRCs of the *period* mutants are largely comparable to previously reported PRCs (using higher light intensity and duration) on these mutants (Saunders, Gillanders et al. 1994). However, I obtain relatively small amplitude PRCs and especially in case of per^s, I do not detect any significant phase shifts. Overall, I show that the results from phase shift experiments do not completely explain my observations regarding phase relationships and accuracy under entrained conditions. This is not surprising as the entrainment phenomenon is considered too complex to be explained by only PRCs as also demonstrated in a recent study where multiple modulations of internal and external periods along with photoperiods were used to show the limitations of PRC based entrainment as various endogenous and exogenous components seem to be affecting it (Remi et al., 2010).

In summary, our results show that phase relationship and its accuracy under entrained conditions is a result of the interaction between intrinsic period and periodicity of the

external cycle. While it is likely that several factors such as the strength of the zeitgeber, duration of the photic phase and even rearing conditions can affect accuracy, I acknowledge that inferences from the experiments described here are limited to low strength of zeitgeber, mostly symmetric photoperiod and fixed rearing regime. Further studies accounting for different photoperiods and natural-like environments may help make these conclusions more general.

Influence of the free-running period on lability of the circadian pacemaker

4.1 Introduction

In absence of daily time cues, the endogenous circadian pacemaker present in living organisms runs with its intrinsic free-running period (τ). Since the endogenous clock is composed of physiological processes, environmental variables can affect the functioning and expression of the behavioural rhythms that it controls. Despite such vulnerability, rhythmicity is sustained for prolonged durations of time and the intrinsic period of the clock remains relatively stable. Such homeostasis of frequency has been previously proposed to be an evolved mechanism which allows τ to remain largely invariant in the face of internal or external changes within physiologically tolerable limits (Pittendrigh and Caldarola 1973) (Daan and Pittendrigh 1976).

While individuals show small daily fluctuations in τ around its mean value, there are several factors that can lead to long-term, directional changes in τ. For instance, different levels of environmental factors such as temperature or light can potentially bring about a change in the free-running period of these rhythms in addition to entraining circadian rhythms when they are cycling. Relative independence of τ from both light and temperature was first reported for humans by Aschoff (Aschoff 1963). While circadian rhythms show some changes in their periodicity under different temperatures, the magnitude of such changes is rather small. This relative independence from temperature is referred to as temperature compensation and is considered to be important for reliably measuring the passage of time under changing temperature conditions experienced by organisms across the day and year (Pittendrigh 1954) (Hastings and Sweeney 1957) (Pittendrigh 1993). In contrast, different levels of constant light are known to have far greater effects on circadian period as demonstrated by Aschoff

with early studies reporting shortening of period under increasing intensities of constant light in diurnal animals and lengthening in nocturnal organisms (Aschoff, 1960; 1981). However, later studies have shown that these trends in the direction of change in period are not always based on diurnality or nocturnality as seen in invertebrates such as insects. Additionally, circadian rhythms were demonstrated to split into multiple components, each with a different period in the presence of constant light in certain organisms (Daan and Berde 1978). Fruit flies are known to become arrhythmic under constant light due to the continuous degradation of an integral component of the core transcriptional-translational feedback loop protein TIMELESS, by light, due to the action of the photoreceptor CRYPTOCHROME (Emery, So et al. 1998) (Stanewsky et al. 1998). However, flies can resist breakdown of the activity/rest rhythm under constant light of low intensity and continue to show free-running rhythms (Konopka, Pittendrigh et al. 1989). Moreover, cryptochrome-deficient mutants do not exhibit arrhythmicity at levels of constant light at which wild-type flies become arrhythmic. Therefore, the propensity of rhythm persistence under increasing intensity of constant light has been taken as a measure of the sensitivity of the core clock to light and can be regarded as an indicator of the robustness of the clock (Rieger et al., 2004; 2007).

Other than effects of ambient conditions like the ones mentioned above, previous exposure to a time cue might also result in small changes in τ immediately after the organism is transferred to constant darkness. These effects of entrainment history can persist for several days and are called after-effects. Such after-effects on τ due to previous entraining regimes such as T-cycles (environmental cycles of periodicity different from 24 h) or photoperiods (environmental cycles of photophase different from 12 hours) have been reported in mice, hamsters, and in certain species of insects (Pittendrigh 1960) (Daan and Pittendrigh 1976)

(Page and Block 1980) with long T-cycles resulting in lengthening of period. Apart from conditions experienced as an adult, an organism's clock may also be modulated by the external environment during its pre-adult stages. *Drosophila* clock has been shown to be functional as early as at egg hatching (Sehgal, Price et al. 1992). Developmental plasticity of τ has been reported in a few studies in cockroaches and fruit flies where rearing under different T-cycles or photoperiods resulted in a change in τ (Barrett and Page 1989) (Page and Barrett 1989) (Sheeba, Chandrashekaran et al. 2002). Also, a lack of entraining stimulus during development has been shown to result in an increase in percentage of arrhythmicity (Hurd and Cahill 2002). However, there have been few attempts at addressing whether such after-effects depend upon the intrinsic value of the period. I had summarized the effects of above-mentioned factors which can potentially affect the period in chapter 1.

Early studies on lability of the pacemaker suggested that clocks with period close to 24 hours show greater stability of τ (Pittendrigh and Daan, 1976). The rodent species *Peromyscus maniculatus* with average τ deviant from 24 hours showed larger changes in period following exposure to exotic T-cycles as well as greater shortening of period with age compared to the species *Peromyscus leucopus* which had an average period closer to 24 hours (Pittendrigh and Daan, 1976). Since Aschoff (1971) showed a similar affinity for greater daily precision in individuals with period close to 24 hours, they proposed a general homeostasis of frequency against all possible factors that can affect the stability of period in such individuals or species (Pittendrigh and Caldarola, 1973). However, the influence of period on the long-term stability of period has not been investigated on individuals within a species across a large range of periods.

Using our fly lines with τ values ranging from 17 to 30 h, I observed greater accuracy of entrained phase for clocks with close to 24 h under LD 12:12 conditions (Chapter 3). In this chapter, I tested whether and to what extent the lability of τ (change in intrinsic period of the pacemaker), due to aging, environmental temperature, susceptibility to after-effects and constant light, is a function of τ being deviant from 24 h and basically tested the hypothesis that clocks with period values closer to 24 h are less labile compared to those with period deviant from 24. We find that clocks with τ closer to 24 h show greater persistence of rhythmicity under constant light but do not show minimum lability under other conditions that we examined.

4.2 Materials and Methods

4.2.1 Fly lines and maintenance

Mutant lines per^s and per^l were backcrossed to a wild-type population and maintained as described in chapter 1 (Srivastava et al., 2018). Briefly, they have been maintained as cage populations with banana-jaggery medium as the food source under LD 12:12 at a constant temperature (25 °C) and humidity (~80 %). Flies from these three populations have been categorized as the three 'period ranges' with mean values of the free-running period (τ) being short (17-19 h), intermediate (23-25 h) and long (26-30 h). The experiments in this study were performed after 7 generations of backcrossing.

4.2.2 Lability with age and temperature

~70 eggs were collected from the three cage populations into vials with ~30 ml food and were maintained under symmetric light-dark cycles of 24 hours (LD 12:12) with constant

temperature (25 °C) and constant humidity (~80 %). Freshly emerged adult flies were separated within 6-8 hours of their emergence and 4-5 day old virgin males were introduced into activity tubes (5 mm × 65 mm) with corn food at one end and cotton plug at the other end. Long-term recording of the activity rhythms of 32 flies/ period range/ temperature regime was performed under constant darkness using Drosophila Activity Monitors (DAM 5, Trikinetics^{TM}, Waltham, MA, USA) at two different temperatures i.e., 18 °C and 28 °C. At both the temperatures, flies were transferred to tubes with fresh food every 8th day of the recording which was continued till more than 75% flies of each strain were dead. τ for each cycle was estimated by marking activity offsets for each cycle on actograms using Clock lab software (Actimetrics, Evanston, USA) and average τ for each age-window (7 days) was calculated since flies were transferred to tubes with fresh food every 8th day as the minimum number of cycles for effective determination of τ is considered to be 7 days. However, at the higher temperature, the total number of days for which flies were rhythmic was not sufficient to be categorized as separate age windows and therefore for that regime we made comparisons across age (days) instead of age windows. Days to arrhythmicity was estimated by examining the number of days for which the flies remained rhythmic (day of onset of arrhythmicity was subjectively determined as the day from which consolidated activity bouts could not be discerned). Additionally, we also calculated the difference in τ estimated at the two temperatures as a measure of temperature compensation (τ measured at 18 °C – τ measured at 29 °C).

4.2.3 Lability under constant light

~70 eggs were collected from the three cage populations into vials with ~30 ml food and were maintained under symmetric light-dark cycles of 24 hours (LD12:12) with constant

temperature (25 °C) and constant humidity (~80 %). Freshly emerged adult flies were separated within 6-8 hours of their emergence and 4-5 day old virgin males were introduced into activity tubes (5 mm x 65 mm) with corn food at one end and cotton plug at the other end. Recording of the activity rhythms of 64 flies/ period range was performed under constant light of 0.1 lux intensity (obtained by using filters over LEDs as described in section 3.2.2) with Drosophila Activity Monitors (DAM 5, Trikinetics™, Waltham, MA, USA) at 25 °C.

4.2.4 Lability in terms of History Dependence

4.2.4.1 Assay to test after-effects

Freshly emerged adult flies derived from the three populations were separated within 6-8 hours of their emergence and virgin males were introduced into activity tubes for recording locomotor activity. Flies were allowed to free run under constant darkness for 7 days and exhibit their intrinsic period under DD (DD1). Different subsets of flies (32 in each subset) were then transferred to different light regimes i.e. LD 12:12, LD 10:10 (T20), LD 14:14 (T28), LD 8:16 (short photoperiod or spp) and LD 16:8 (long photoperiod or lpp) for a minimum of 7 cycles. For each of these light regimes, the cubicles housing the flies were illuminated by high light intensity white compact fluorescent lamps (Philips 8 watts, Phillips India limited. Gurugram India with intensity adjusted to ~100 lux) were used to ensure that entrainment is strong enough to alter the intrinsic period. In parallel, one subset of flies were recorded in DD throughout and were considered as controls. Subsequently, they were transferred to DD again (DD2) for estimating the after-effect on period. τ values were measured before and after the cyclic conditions (DD1 and DD2 respectively) using daily

estimation of the difference between two consecutive offsets and the average across days was computed for each fly. For each individual fly the change in period between DD1 and DD2 was estimated and a two-way ANOVA was performed across regimes and period range.

4.2.4.2 Assay for determination of development plasticity

Roughly 70 eggs were collected from the three cage populations into vials with ~30 ml food. For each strain, different subsets of vials containing eggs were maintained under different light regimes (i.e. DD, LD 12:12, LD 10:10, LD 14:14, LD 8:16 and LD 16:8; high light intensity bulbs (~100 lux) used during the light phase) with constant temperature (25 °C) and constant humidity (~80 %). Freshly emerged adult flies were separated within 6-8 hours of their emergence and virgin males were introduced into activity tubes. Activity rhythms were recorded for 7 days under constant darkness and free-running period was estimated for this duration. Comparisons were made on the average τ measured for flies reared in different light regimes.

4.2.5 Statistical analysis

To compare time taken for onset of arrhythmicity (in days) at different temperatures, ANOVA was performed with 'period range' as the independent variable and 'days to become arrhythmic' as dependent variable. Similarly, to quantify the effect of constant light on the rhythm, ANOVA was performed with 'period range' as independent variable and 'days to exhibit complex rhythms' as dependent variable. To measure after-effects, one-way ANOVA was performed for each period range with entraining regime as independent variable and period difference (DD1-DD2) as the dependent variable. For examination of developmental plasticity on τ, one-factor ANOVA was performed for each period range with

'rearing regime' as independent variable and τ as the dependent variable. Post-hoc comparisons were done using Tukey's HSD test. All statistical analyses were conducted on STATISTICA^{TM} for windows release 7.0 (Statsoft Inc 1995).

4.3 Results

4.3.1 τ of long period flies is most labile to temperature

Long term recording of activity rhythm under two contrasting temperatures of 18 and 29 ºC enabled us to obtain measures of intrinsic period over the lifespan of individual flies. Representative actograms for flies from the three period ranges at the two temperatures are shown (Figure 4.1, Figure 4.2). As expected, overall the lifespan of flies was shorter under warm temperature – mean lifespan of <30 days as compared to >60 days under low temperature. Flies show rhythmic locomotor activity for a major part of their lifespan under cold temperatures (Figure 4.3). Two-way ANOVA to test the effects of period range and temperature on days to arrhythmicity showed a significant main effect of period range ($F_{5,111}$=2.14; p<0.05), temperature($F_{5,111}$=2.14; p<0.05) and the interaction between the two($F_{2,180}$=8.10; p<0.05). Post-hoc results using Tukey's HSD showed that flies falling in the long range (~28 hours) took significantly lesser number of days to become arrhythmic when compared to short and intermediate period range flies (p<0.05) under high temperature, thus demonstrating that these flies are less capable of sustaining their free-running rhythms (Figure 4.3 right) under constant high temperature. Mean τ of individuals measured across 7-day age windows throughout the lifespan, at the two temperatures is depicted in Figure 4.4 for the three period ranges. I also tested whether the absolute value of τ has an impact on the extent to which ambient temperature affects its value. I estimated the change in the value of

τ for one age window (the first) at the two temperatures ($\tau_{18\,°C}$ - $\tau_{29\,°C}$) and found that τ of flies with short and intermediate period did not differ much (0.26-0.62 h respectively), whereas mean τ was longer by 4.01 h for flies with long period under the cooler temperature (Figure 4.4). Thus, while short or intermediate τ appear to be temperature compensated in the range we examined, clocks with longer values of τ show overcompensation at the cooler temperature. These results reveal that intrinsic period influences long-term stability of period under temperatures that are cooler or warmer than standard rearing conditions of 25 °C.

4.3.2 Clocks with ~24 h τ sustain rhythmicity under constant light

While temperature compensation of the circadian clock period is well established, another important environmental factor that affects the τ value and the stability of free-running rhythms of many organisms is constant light. I examined the effects of constant light with very low light intensity on the ability to sustain rhythmicity in flies across the three period ranges. Assessment of free-running rhythms under constant dim light (representative actograms shown in Figure 4.5) showed that while flies from intermediate period range continued to free-run with their intrinsic periodicities, those in short and long period ranges showed complex rhythms within a few days of recording (Figure 4.6). However, the number of days taken for the complex rhythms to appear was not significantly different among the short and long period range flies (p>0.05) (Figure 4.6 left). Thus, ~24 h clocks are more resistant to the disruptive effects of constant light compared to those with deviant τ.

4.3.3 Greater after-effects for long period clocks

The circadian period can also be altered as a consequence of prior history of different environmental regimes such as external cycles with period different from 24 h (or T-cycles)

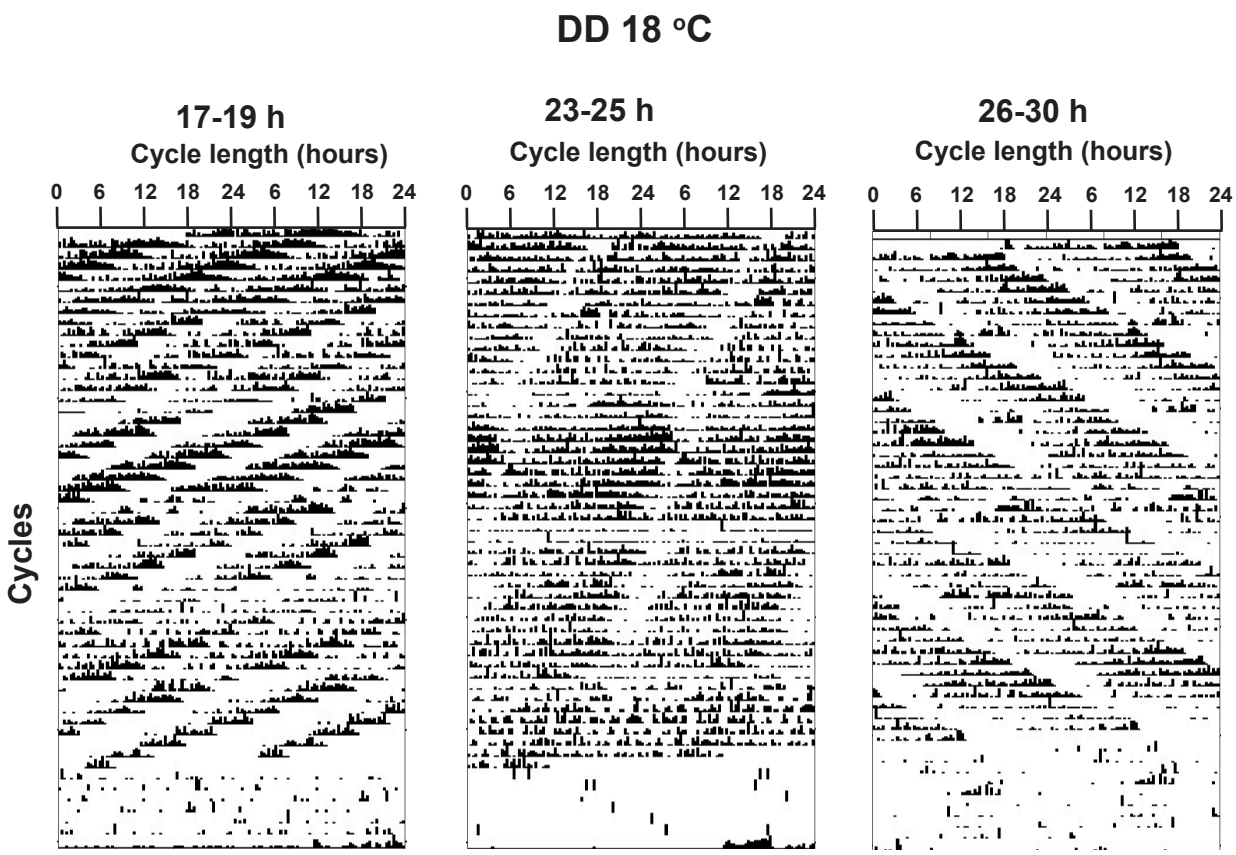

Figure 4.1 Free running rhythms under low temperature Represntative actograms showing rhythms under low temperature of 18 °C for short (left), intermediate (middle) and long (right) period ranges.

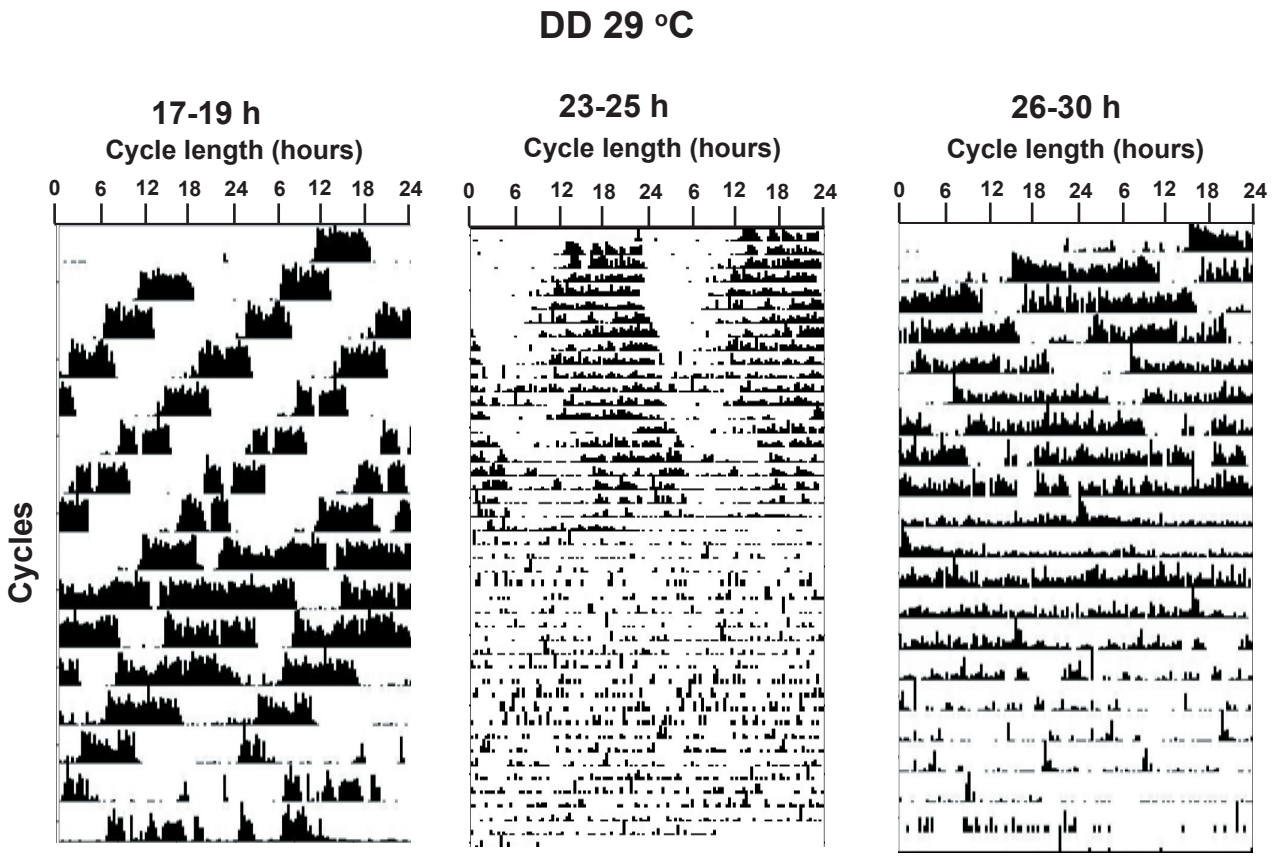

Figure 4.2 Free running rhythms under high temperature Represntative actograms showing rhythms under high temperature of 29 °C for short (left), intermediate (middle) and long (right) period ranges. Number of cycles vary for the three period ranges depending on the onset of arrythmicity.

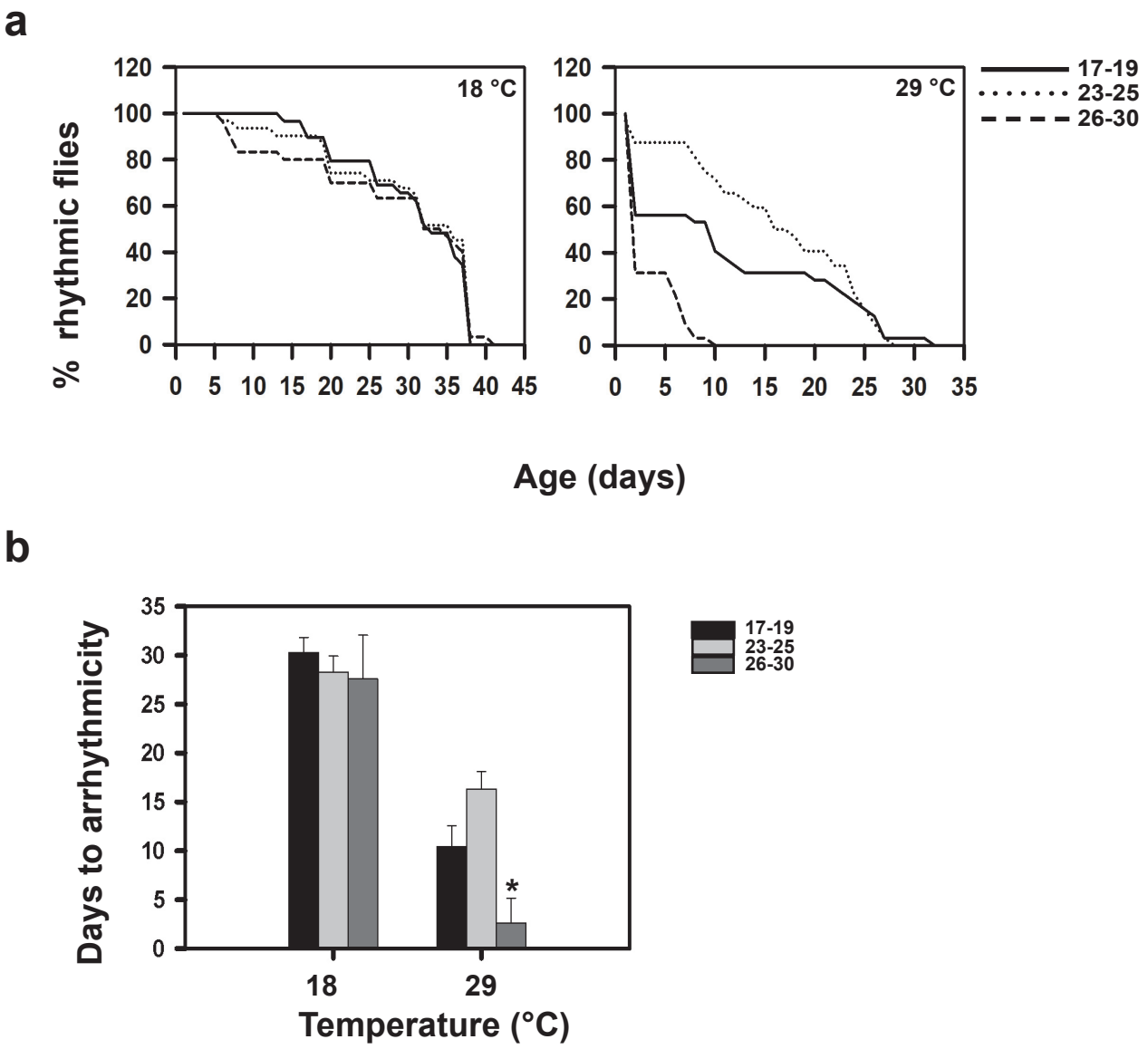

Figure 4.3 Lability with age at two different temperatures. (a) Percentage rhythmic flies plotted against age for the short (solid line), intermediate (dotted line) and long (dashed line) period ranges (n~32) at low (left) and high (right) temperatures. **(b)** Number of days for which flies of the three period ranges remained rhythmic at the two temperatures. Error bars are SEM. Asterisk denotes significant difference at $p<0.05$.

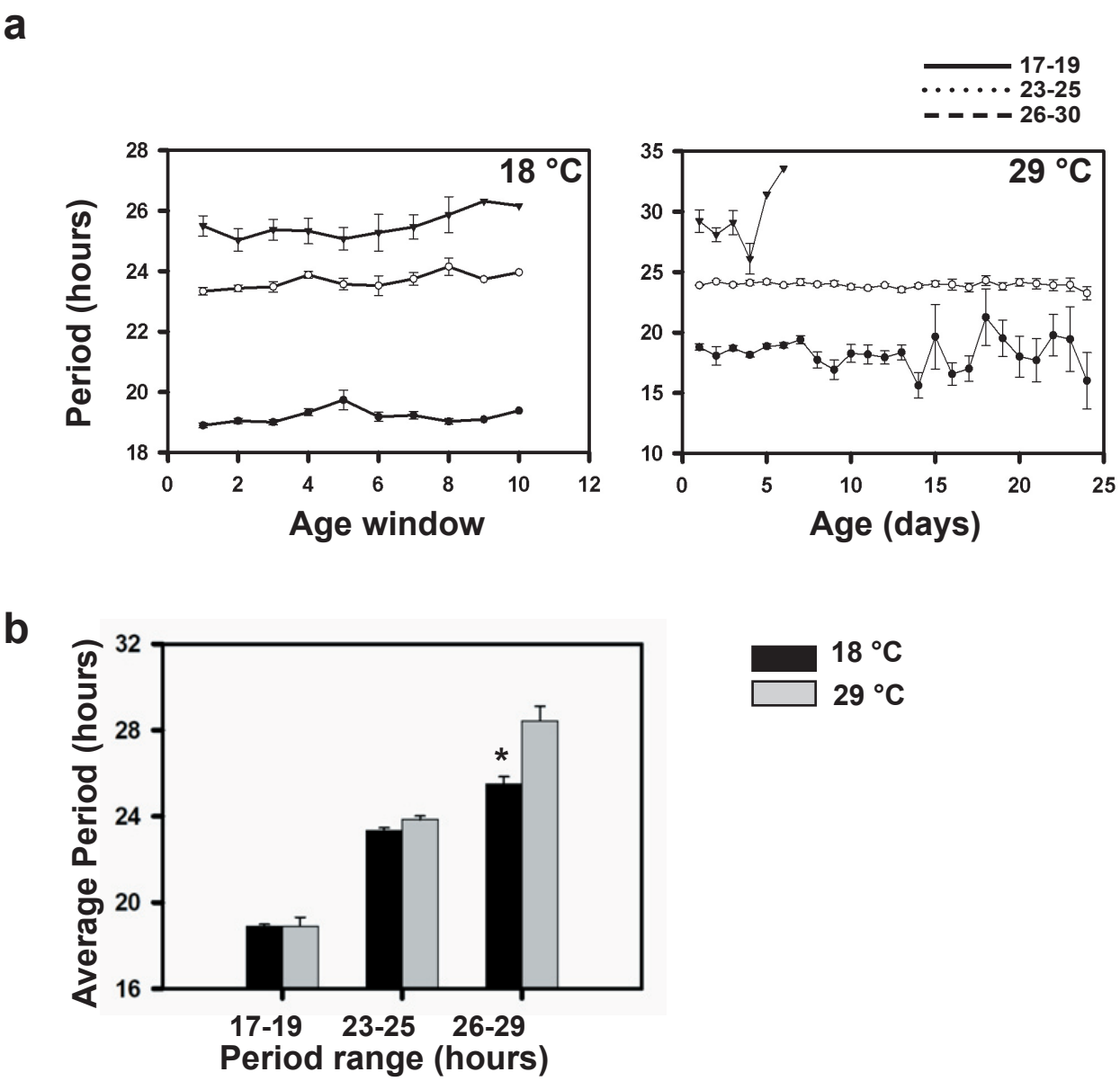

Figure 4.4 Lability of period with to temparature. (a) Free running period plotted against age window (left; comprising 7 consecutive days used to analyse period) or age (right) for the three period ranges (n~32) at low (left) and high (right) temperatures. **(b)** Average Period measured at the two temperatures for the three period ranges. Error bars are SEM. Asterisk denotes significant difference at $p<0.05$.

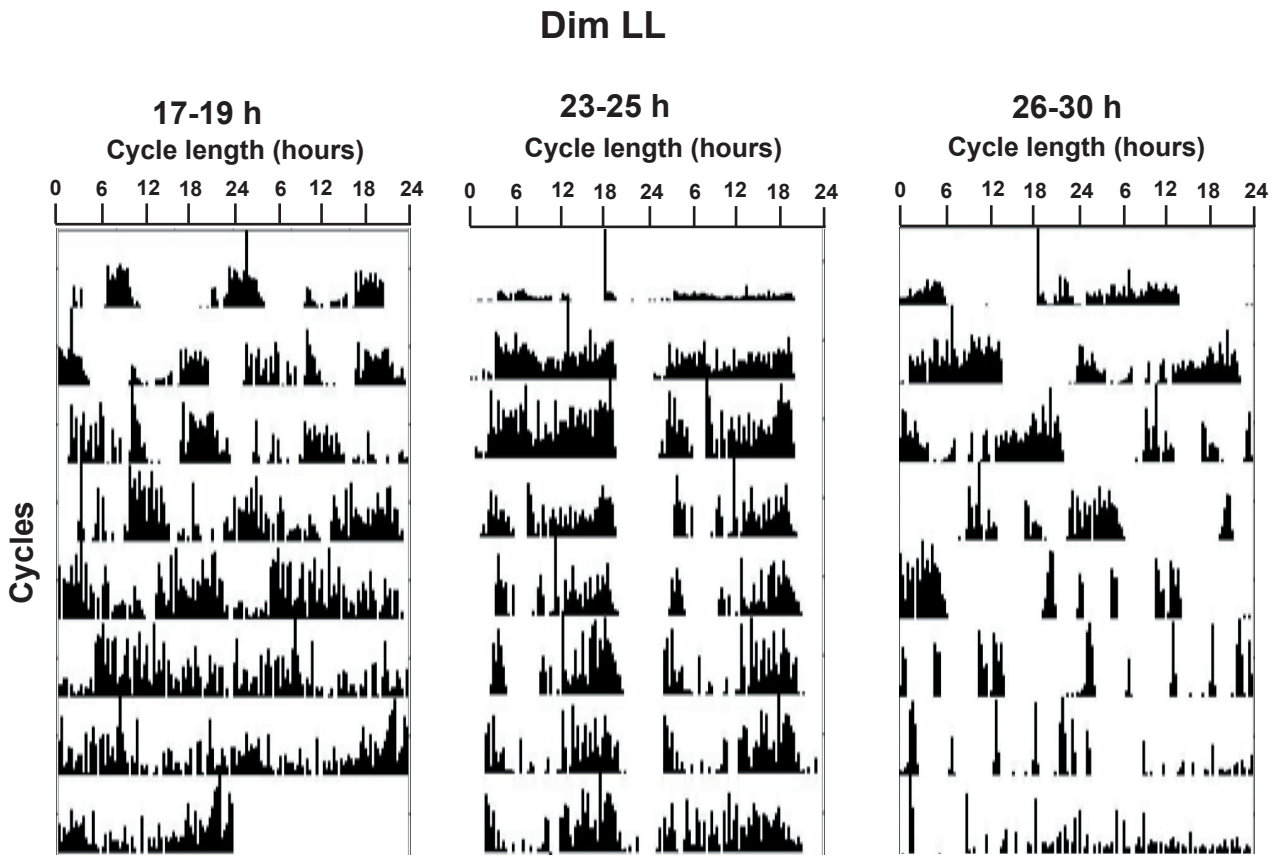

Figure 4.5 Free running rhythms under Dim LL Representative actograms showing rhythms under constant light of 0.1 for short (left), intermediate (middle) and long (right) period ranges.

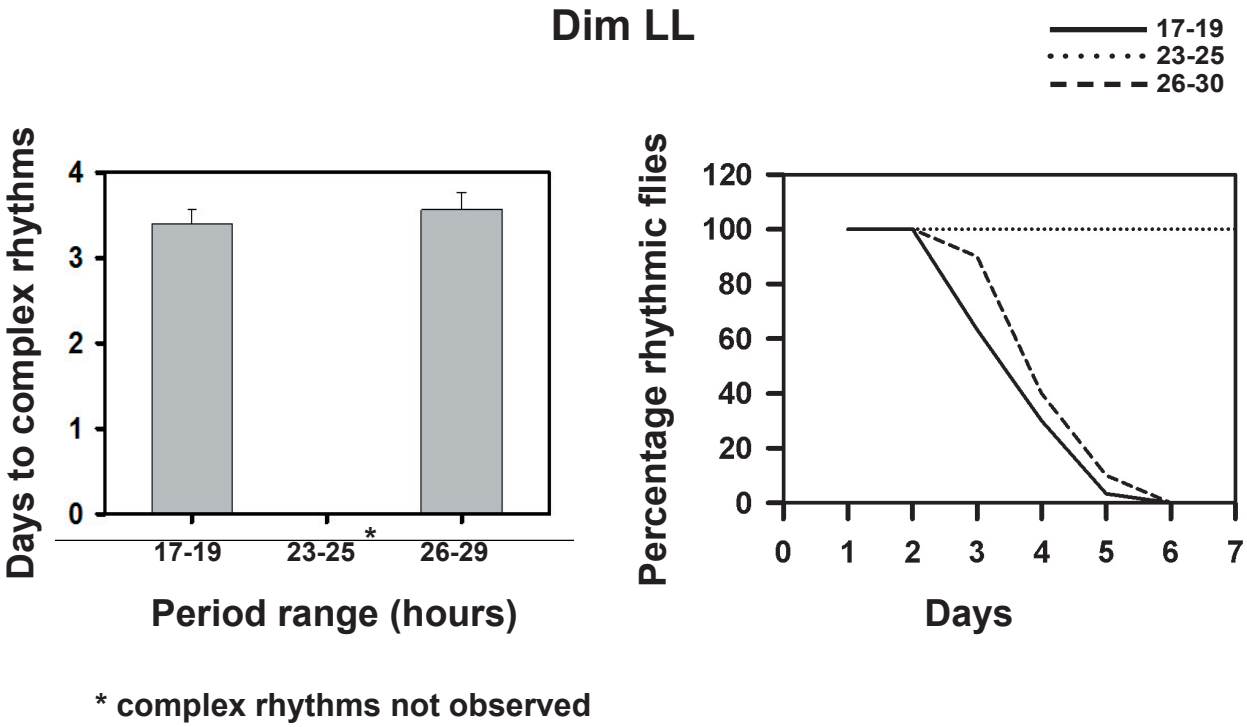

Figure 4.6 Lability under constant light. (left) Number of days taken for rhythms to become complex for the three period ranges when recorded under low intensity constant light (n~ 64 for each range). The intermediate period range did not show complex rhythms. Error bars are SEM. (right) Percentage rhythmic flies plotted against days of recording for short (solid line), intermediate (dotted line) and long (dashed line) period ranges.

or unequal photophase and scotophase durations (or photoperiods) (Aschoff, 1960). To examine whether the extent of such aftereffects differs based on the intrinsic period, I first compared the difference in period seen before and after subjecting flies to a variety of environmental regimes across the three period ranges. Two-way ANOVA with period-difference as the dependent variable showed a significant effect of interaction between period range and entraining regime ($F_{10,353}$=4.16; p<0.05). Post-hoc results using Tukey's HSD showed that flies in the long period range showed a significant change in period after being entrained under T28 and short and long photoperiods as compared to DD controls (p<0.05) (Figure 4.7 a). Hence, I found that only long period range flies show after-effects of prior entraining conditions.

4.3.4 Greater developmental plasticity for ~24 h clocks

Having seen that the adult circadian clock period can be modified by the entraining regime based on whether its intrinsic period falls in the short, intermediate or long range, we asked whether such effects may be more intense if pre-adult stages of development were subject to such regimes. To estimate whether the speed of the clock is a factor in determining how clock properties are affected by time cues during developmental stages, we reared flies from all the three period ranges in light-dark cycles of two different period lengths (T-cycles) and two varying durations of light phase (photoperiods). I then estimated τ of adult fly locomotor activity upon release into DD. Two-way ANOVA with τ as dependent variable showed a significant effect of interaction between period range and entraining regime on τ ($F_{10,416}$=3; p>0.05). Post-hoc using Tukey's HSD showed that τ measured after rearing under T20 and T24 regimes was significantly smaller compared to that after DD (p<0.05) for the intermediate period range while τ after rearing under other regimes (T28, lpp and spp) was

not significantly different from the DD controls (p>0.05). For the short and long period ranges no such differences were observed (Figure 4.7b). Therefore, I see that clocks with τ ~24 h show developmental plasticity in their period whereas those deviant from 24 h do not.

4.4 Discussion

In a comprehensive attempt at examining the significance of the absolute value of τ in the long-term stability of circadian rhythms, I assessed the effect of different ambient environmental conditions and rearing or entraining regimes on the value of τ and compared clocks with distinct range of τ values using mutant flies. To the best of my knowledge, this is the first analysis of effects of temperature, age, constant light and prior entraining conditions on the period of organisms of the same species on a common genetic background with a wide range of intrinsic period values.

At the two temperatures which I used to measure the life-long stability of clock, namely cool 18 ˚C and warm 28 ˚C, I observed reduced stability of τ in flies with longer τ when compared to those with shorter or ~24 h τ since rhythmicity was sustained for lowest durations in these flies. Also, while flies from all the three period ranges were able to conserve their inherent periodicity at the higher temperature, at the lower temperature, flies in the long period range exhibited overcompensation as their τ was shorter at this temperature whereas those in the short and intermediate period ranges did not show much change in their internal period (Figure 4.4). These results do not completely match the earlier observations on per^s and per^l flies where reciprocal change in τ was observed for the two mutants and both per^s and per^l had inefficient temperature compensation when compared to the wild-type flies (Konopka, Pittendrigh et al. 1989). The direction of change observed for per^l flies at the lower

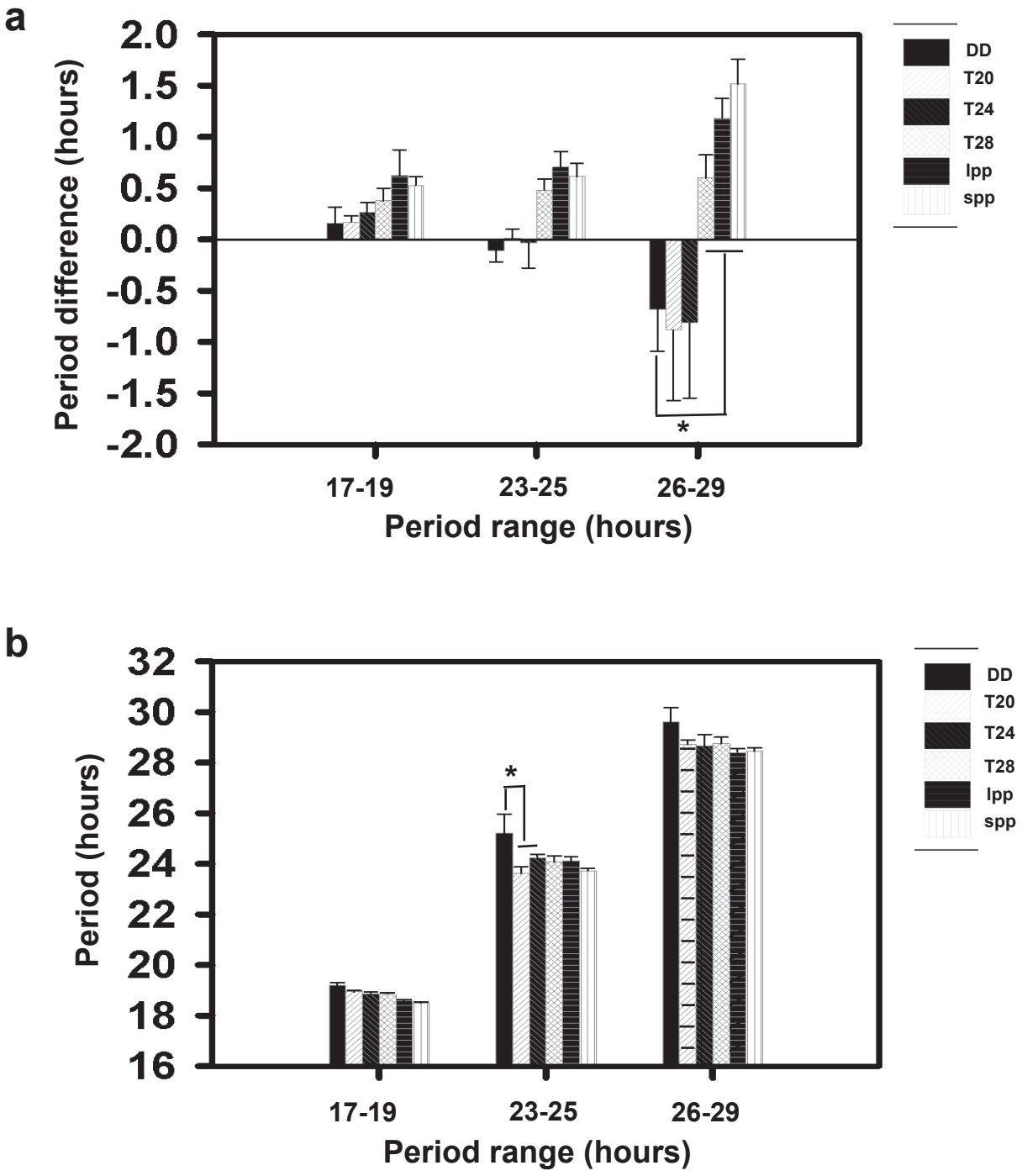

Figure 4.7 (a) History dependence in terms of aftereffects. Difference in free-running period of flies from the period ranges before (DD1) and after (DD2) being recorded in different T-cycles and photoperiods (n~32). **(b) History dependence in terms of developmental plasticity.** Free-running period of flies after being reared in different T-cycles and photoperiods. Error bars are SEM (n~32). Asterisks denote significant difference at $p<0.05$.

temperature in this study was the same as observed in the present study i.e. τ was significantly shorter at the low temperature. This inefficiency of per^l clocks of not showing temperature compensation has been attributed to the defective interaction between TIM and PER proteins in these mutants (Gekakis et al., 1995). However, the study by Konopka and coworkers also reported longer τ for per^s flies at the low temperature compared to that at the high temperature which is not observed in my study (Figure 4.4). This discrepancy in the behaviour of these mutant flies across studies can possibly be explained by the difference in their genetic background. Although flies in both the studies have the same mutation which is responsible for the altered τ, the genetic background might be significantly different among the different lines which might be affecting various phenotypes including temperature compensation due to epistatic interactions of the mutant alleles with the other genes (Chandler, Chari et al. 2013).

Age-dependent changes on activity have been observed in previous studies (Richter 1922; Darnell and Meierotto 1965). However, the effect of age on the sustenance of rhythmicity is not very well characterized. While examining the effect of age on flies with different τ, I observed that although most flies start becoming arrhythmic with advancing age, this loss of rhythmicity occurred at an earlier age at the higher temperature for all flies irrespective of their period range (Figure 4.3). However, even at this temperature, the number of days for which the free-running rhythms were sustained was lowest for long period range flies (Figure 4.3). Hence, I find that while ambient temperature affects the ability to sustain the rhythms across age in all flies, this effect is greatest in long period range flies. It is important to mention here that since we have used locomotor activity to determine periodicity, which is

bound to deteriorate with age, the lability of period observed cannot be considered as the lability of pacemaker only.

In addition to age and temperature, constant light is also known to affect the expression of rhythmicity in several organisms. While constant light can result in changes in period or splitting of rhythms in mammals and birds (Aschoff, 1979; Daan and Berde, 1978), even relatively low intensities of constant light results in arrhythmicity in *Drosophila melanogaster*. Enhanced effects of constant light on per^s flies compared to their wild-type controls have been reported previously in a study which examined the effects of light on pacemaker function (Marrus, Zeng et al., 1996). Light was also shown to affect phosphorylation and accumulation of PER and TIM proteins and also the cycling of *per* and *tim* RNA. Another behavioral study demonstrated that per^s and per^l flies show changes in τ in opposite directions when assayed under increasing intensities of illumination (Konopka et al., 1989). Comparison of studies conducted across different organisms with different illumination of constant light suggests that there exists no single rule for the direction in which τ changes with changing light intensities. For example, it was shown that Tau mutant hamsters which have shorter τ exhibit lower proportion of splitting of their rhythms under constant light when compared to their wild type controls (Bittman et al 2007) whereas opposing trends for changes in τ have been reported for closely related nocturnal and diurnal species (Aschoff, 1979). Moreover, the extent of difference across different intensities of constant light was considered to be influenced by intrinsic τ measured under constant darkness under this study (Aschoff, 1979). I show that at very low intensity constant light, flies with ~24 h τ maintain free-running rhythms with no significant change in τ whereas flies with τ deviant from 24 h begin to exhibit complex rhythms within a few days such that either

multiple periodicities or arrhythmicity are detectable for these flies (Figure 4.6). These results support the notion that clocks with τ closer to 24 h are less labile when compared to those deviant from 24 h. However, like mentioned in previous chapters the use of mutation to obtain different period values is a limitation important to be considered while making this inference. Since the effects of constant light observed on the rhythm of the fly are due to light affecting the PER protein accumulations and *per* gene cycling, the mutation in this gene might itself be responsible to an extent for the differences observed.

However, these results are specific to the model organism and the range of period values I have examined. For example, late human chrono types have been shown to be associated with greater amplitude in behavior (Baehr et al., 2001). Hence, it would be inappropriate to extrapolate these findings on fruit flies to other organisms.

History-dependence of the pacemaker can be interpreted in two ways 1) an effect of certain rearing or entraining regimes on τ can be considered indicative of lability of the pacemaker 2) such a change is also reflective of the flexibility that the pacemaker has in order to entrain to these regimes. I measured the history-dependence of our flies with distinct values and checked for its association with the lability that we observed under conditions mentioned above. I did not observe an after-effect of any of the entraining regimes that I examined on short and intermediate period range flies. However, long period range flies were found to significantly alter τ as a consequence of entrainment to varied photoperiods and long duration T cycles. A previous study on mice and hamsters reported an increase in τ with the increase in zeitgeber length (T-cycles) (Daan and Pittendrigh 1976). Also, an increasing length of the photoperiod led to lengthening in τ for diurnal organisms and shortening in nocturnal ones (Daan and Pittendrigh 1976). Bittman's study using mutant Syrian hamsters with a common

genetic background also yielded similar results where TAU mutation which results in a significantly shortened τ for these hamsters did not affect the level of after effects in these mutants (Bittman et al 2007). Previous studies have also reported an influence of length as well as the ratio of light dark cycle on the developing circadian clock (Barret and Page 1989; Page and Barret 1989). My results (where only an effect of absence of light seems to increase the period in case of the intermediate range, Figure 4.7) are also in contradiction with what was observed previously with these mutants in terms of developmental plasticity (Tomioka et al. 1998) where it was reported that rearing under different photoperiods significantly affects τ recorded immediately after adult emergence. The effect was also found to be different for per^{l} flies when compared to per^{s} and wild type flies. However, unlike my experiments, in the previous study, flies were exposed to various light regimes as adults as well and therefore the changes in free-running periods observed could be a result of after effects of these regimes rather than developmental effects. Based on my study with no exposure of flies to any regime other than the rearing regime during development, I infer that intermediate period range flies do not show minimum plasticity of τ (Figure 4.7). Thus, my observations of developmental plasticity as well as after effects do not provide evidence for 24 h period conferring advantage to the clock in terms of lower lability. Further, I want to highlight that genetic background is an important consideration while making such conclusions. Since it is difficult to minimize the effect that mutation has on various related phenotypes it is important to reduce all the other possibilities of different factors affecting the target phenotype.

Overall, I show that clocks with intrinsic period close to the natural period of the environmental cycle do not always show minimum lability. In fact, it is only flies with very

long intrinsic period which show greater susceptibility to most factors examined. It is possible that this higher lability of longer period flies is associated with their lower precision as shown in chapter 2.

Environmental cycles regulate development time via circadian clock mediated gating of adult emergence

The contents of this chapter have been published as the following research article:

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5.1 Introduction

In fruit flies *Drosophila melanogaster,* the most commonly studied circadian behaviors are locomotor activity/rest rhythms and adult emergence rhythms. While activity/rest rhythms can be studied for individual flies, adult emergence, which is a one-time occurrence in the life of an individual fly, shows rhythmicity at the population level and is known to be gated by circadian clocks (Pittendrigh 1966; Kronopka and Benzer 1971).

Adult emergence in *Drosophila* shows circadian rhythmicity (Brett 1955) with a large proportion of emergence occurring during daytime and the emergence peak occurring shortly after dawn (Scott 1936). Early studies on the circadian control of eclosion in *Drosophila melanogaster* showed that even though the intermediate steps of development are not under circadian control, the phenomenon of adult emergence is rhythmic (Harker 1965) (Skopik and Pittendrigh 1967) (Pittendrigh and Skopik 1970). The circadian control of eclosion was further elaborated through studies which showed an interaction between prothoracic gland which regulates developmental state and the circadian clock (Myers 2003). Prothoracic gland secretes ecdysone, a steroid hormone which regulates molting and metamorphosis and activity of this gland can also influence the periodicity of emergence rhythm (Palacios-Muñoz and Ewer 2018). Oscillations of clock proteins observed in prothoracic gland under light/dark cycles have been found to require inputs from clock neurons in the brain in order to persist under constant conditions (Myers 2003). Such input from the circadian clock to the prothoracic gland maybe via Prothoracicotrophic Hormone (PTTH) which also sets the duration of feeding interval as demonstrated by delayed larval development and increased size of adults that emerge in absence of PTTH (McBrayer, Ono et al 2007). Additionally, PTTH plays an important role in coordinating environmental cues and the developmental

process and therefore has an influence on adaptive plasticity of the developmental program (Shimell, Pan et al. 2018). Furthermore, core clock genes such as *period* and *timeless* are required for rhythmicity in eclosion (Konopka and Benzer 1971; Palacios-Muñoz and Ewer 2018) and are also essential for development as they are required for transcriptional up-regulation of enzymes needed for production of steroids that play an important role in larval development (Di Cara and King-Jones 2016). Thus, there are multiple, intricate connections between circadian clock, steroidal hormones and developmental processes which interact to regulate timing of adult emergence.

Apart from governing the rhythm in emergence of an adult fly from its pupa by gating it as discussed above, clocks are also considered to have an effect on the overall rate of pre-adult development. Circadian clocks are known to be functioning from early developmental stages in other organisms like zebrafish and mice (McBrayer, Ono et al. 2007; Shimell, Pan et al. 2018) and also affect differentiation and proliferation of adult stem cells (Di Cara and King-Jones 2016) though there is not much clarity in their role in embryonic development (Ebisuya and Briscoe 2018). In *Drosophila,* clocks are known to be functional even during hatching (Sehgal, Price et al. 1992) and a relationship between clock and pre-adult development was first established by a study on the *period* gene mutants where a positive correlation was seen between clock period and pre-adult development time (Kyriacou, Oldroyd et al. 1990). It was demonstrated that while short period (per^s) mutants tended to show shorter overall pre-adult development, long period mutants (per^l) showed a longer development time in terms of time taken for pupation as well as emergence (with slight differences between the pupation and emergence profiles). However, since the correlation between period and development time persisted even under constant light (LL, where many behavioural patterns of flies are

rendered arrhythmic and core clock oscillations are expected to be disrupted), it is not possible to conclude that the differences in developmental rate are mediated by the circadian clock per se. Hence, the contribution of circadian clocks in determining development time differences remains ambiguous. Additionally, since mutant fly lines are typically highly inbred and could generate spurious genetic correlations between fitness components, they are not ideal for examining relationships between critical life-history traits like development time and the circadian clock (Vaze and Sharma 2013). Another study using wild type flies (per^{+}) and per^{s} mutants established that the clock assesses developmental state a few hours prior to eclosion, which results in determination of the gate chosen for eclosion as well as the timing of emergence within that gate (Qiu and Hardin 1996). Moreover, in *Drosophila melanogaster* populations subjected to T-cycles of increasing period lengths, a positive correlation was seen between the rate of pre-adult development and the length of the external light-dark cycle (Paranjpe, Anitha et al. 2005). Besides suggesting a possible role for the clock in gating of eclosion and in the regulation of pre-adult development, this study also concluded that differences in development time between clock mutants may not be due to pleiotropic effects since flies with similar free-running period (~24 h) exhibited different rates of pre-adult development under external cycles of different periodicities. However, it can also be speculated from these results that the period of external cycle is a more important determinant of the rate of pre-adult development than the internal clock period. Moreover, these results were not in concordance with the previous study using *period* gene mutants (Kyriacou, Oldroyd et al. 1990) where it was shown that irrespective of external conditions, the positive correlation between the free-running period of the clock and the mean

development time is maintained therefore implying that the external conditions have minimal influence on the regulation of development by the circadian clock.

The above studies lead to contradictory conclusions but are not directly comparable due to the difference in their approaches, the former used mutant fly lines with divergent intrinsic period while the latter used wild type flies and subjected them to extreme T-cycles. Therefore to examine the relative roles of circadian clock and external cyclic environment on the speed of pre-adult development and the gating of emergence in *D. melanogaster* I conducted a systematic study using the fly lines with distinct periods that I have described in previous chapters. To verify whether pre-adult development time (measured as total time taken from egg collection to emergence) may be altered by merely harboring a certain allele of the *period* gene, I first assayed the three fly lines in constant darkness (DD) and constant light (LL) where external cycles cannot play a role. Further, in order to understand the influence of periodicities of internal and external rhythms in determining development time, I assayed time to emergence across different T-cycles (length of external light-dark cycle) as well. Using this approach, I had combinations of different internal and external periods. As seen in chapter 2, flies from short and long period ranges show differences in the range of entrainment of their activity rest rhythm where per^{l} long period flies do not entrain to a light-dark cycle of 20 hours duration, while both short and intermediate range flies entrain to a wide range of T-cycles. Therefore, I first assessed the entrainability of adult-emergence rhythms of these three strains under three different T-cycles. I speculated that if the internal clock has a major role to play in regulating time to emergence, the trend across strains would further vary depending on whether a strain can entrain to that regime i.e. the strains which entrain will show similar time to emergence whereas the one that does not might have a

different time to emergence. Therefore, I tested the hypothesis that the internal clock regulates the rate of pre adult development in fruit flies.

In this chapter I show an association between intrinsic period and time to emergence, and further demonstrate that even under the influence of external cycles the variation in time to emergence is dependent on the intrinsic period of the clock as flies which do not entrain are not bound by the eclosion gate imposed by the light/dark cycle and therefore emerge as they would in absence of any external cycle.

5.2 Materials and Methods

5.2.1 Fly lines and Maintenance

Mutant lines per^s (~18 h) and per^l (~28 h) (maintained as inbred laboratory lines, originally obtained from Jeffrey Hall's lab, Brandeis university, USA) were backcrossed for five to seven generations to a wild type outbred population, per^+ (~24 h). Using a large outbred population of *Drosophila melanogaster* (Gogna, Singh et al. 2015), individuals carrying either the short or long period mutation, per^s or per^l, were backcrossed for seven generations to create two lines each carrying a short or long period allele of the *period* gene in an otherwise similar genetic background under the assumption that five generations of back crossing should result in similarity of the background (Hospital 2005). The backcrossing scheme is depicted in chapter 1. Another arrhythmic strain i.e. per^0 was backcrossed for five generations to per^+ and compared with the latter under T24. per^+ was a population obtained by mixing four replicates of an outbred population used as controls [25]. One of these control populations (CP1) (Kannan, Vaze et al. 2012) were also employed in this study to examine rhythmicity in pupation. The backcrossed lines have been maintained in cages on a

21 day generation cycle under LD 12:12 at constant temperature of 25 ˚C and controlled humidity with banana-jaggery food provided ad libitum on alternate days. The frequency distribution of free-running period under DD at 25˚C in the three strains is shown in Additional File 2. Cages were provided with yeast paste for two days prior to egg collection for the experimental setups. Two kinds of experiments, one to assay time to emergence/pupation (two different experiments) and another to examine rhythm in emergence were conducted separately with different egg densities as described below.

5.2.2 Development-time Assay

Prior to egg collection, a dummy food plate was placed in the cage populations for 1 h which allowed flies to lay any eggs that may have been retained within the female ((Sellier, 1955, referred in (Markow, Beall et al. 2009). This dummy plate was replaced by another food plate on which flies laid eggs for two hours and these eggs were collected for the assays (similar to (Yadav and Sharma 2014)). Exactly 30 eggs were collected from cage populations (which were maintained under LD12:12) and placed into long glass vials (19 cm x 2.5 cm) and for each strain, 10 replicate vials were prepared. Though we started with 10 vials and 30 eggs in each of them, during analysis, vials with very low survivorship (<33%) were removed. Therefore, 'n' as mentioned in Figure Legends represents the number of vials used for analysis. Five separate experimental regimens were used namely: T20 (LD10:10), T24 (LD12:12), T28 (LD 14:14), DD and LL. For all regimes, eggs were collected at ZT 4 under LD12:12 where ZT 0 is the time of lights-on for all experiments.

Pupation time was estimated only under T20, T24 and T28 regimes, where the vials were inspected once every two hours after the first indication of pupation and the number of pupae were counted. Time to pupation was calculated as the duration between the mid-point of egg

collection and the formation of puparium for each individual fly. Further, for all the five regimes examined, once the pigmentation stage for all pupae was complete, vials were inspected every two hours and the freshly emerged adults (over the past 2 hours) were separated and counted giving the total number of emerged flies (without distinguishing between sexes). Time to emergence was calculated as the duration between the mid-point of egg collection and the emergence of an adult fly from the pupa.

5.2.3 Pupation Rhythmicity Assay

Approximately 300 eggs were collected from cage populations of a wild type strain (intermediate period range) which is an ancestor of the control *per*$^{+}$ flies, CP1, (Kannan, Vaze et al. 2012) into long glass vials (19 cm x 2.5 cm) and for each strain, 10 replicate vials were placed under T24 (LD 12:12). Once larvae began to pupate, vials were observed manually every two hours to count the number of pupae. A dim far red (>650 nm) lamp was used during the dark phase. Pupation profiles were plotted by averaging the number of larvae pupated across vials across days for each time point.

5.2.4 Adult-emergence rhythm Assay to determine periodicity of emergence

Approximately 300 eggs were collected from cage populations into long glass vials (19 cm x 2.5 cm) and for each strain, 10 replicate vials were placed in each of the T-cycles i.e., T24 (LD 12:12), T20 (LD 10:10) and T28 (LD 14:14). For the light phase in each of these regimes, light intensity of the bulbs used was adjusted to 75-100 lux (as described in section 4.2.4.1). Although we started with 10 vials and ~300 eggs in each of them, during analysis, vials in which the number of flies dropped to less than 20 per cycle were not considered. Therefore ‘n’ as mentioned in Figure Legends represents the number of vials used for

analysis. Once the pigmentation stage for all pupae was complete, observations were made every two hours to count the number of emerged flies (males+females). Adult-emergence profiles were plotted by averaging the number of flies emerged across vials across days for each time point. We were able to obtain a maximum of 3-4 cycles for our flies and therefore it was not possible to have free-running rhythm data.

5.2.5 Statistical Test

Since the development profiles for all strains were skewed, we chose median rather than mean to assess the differences. Median time to emergence was estimated for each vial for each strain by marking the time at which 50% or more emergence had occurred. Similarly, median pupation time was estimated for each vial for each strain by marking the time at which 50% or more pupation had occurred. Kruskal-Wallis test was performed for comparison of medians obtained from the three strains in each regime examined as all the data sets did not conform to normality as determined by Shapiro-Wilk test. For pupation rhythm and adult emergence rhythm assays, percentage pupation and emergence respectively were calculated at every time point for each cycle and one-way ANOVA was performed over mean values across vials to examine the effect of time point. Further, we estimated periodicity over 3-4 cycles for each vial using COSINOR analysis in Microsoft Excel (version 2010). The time series obtained for a given vial was fitted with a cosine curve [M-(Acoswt + ψ), M being the mesor for time series for the vial, ψ being the phase and A being the amplitude] with a specific period, phase and amplitude. That combination of period and amplitude giving minimum mismatch value (least sum of square deviations) was considered as the periodicity of that vial. After estimating period for all 10 vials, it was determined whether the period of that strain was significantly different from period of entraining regime

using the one-sample t-test at $p<0.05$. For example, to test whether eclosion rhythm of per^{+} flies in vials 1-10 were entrained to T20, one-sample t-test was done between the period values for the 10 vials (obtained as described above) against the value of 20. For all analyses, statistically significant differences were determined at $p<0.05$. It must be noted that if flies of a given vial did not entrain to a regime, it may not necessarily mean that they were free-running as they could sometimes adopt a period far from their own intrinsic period, and that it could also include phenomena such as relative co-ordination.

5.3 Results

5.3.1 Flies with different free-running periods exhibit different development time under constant conditions

I first compared the average pre-adult developmental time (measured as time from egg collection to emergence) across the three strains under 25 ºC in absence of external cycles. Under DD, median emergence time across strains was ~250 hours. Kruskal-Wallis test for medians showed that time to emergence for long period range flies was significantly longer when compared to that for short and intermediate range ($p<0.05$; Figure 5.1a) while there was no statistically significant difference between short and intermediate range. These results differ from that in a previous study (Kyriacou, Oldroyd et al. 1990) where all three fly strains showed significantly different time to emergence as well as pupation from each other under constant as well as entraining conditions.

Under LL, as previously reported (Paranjpe, Anitha et al. 2005), the median time to emergence was much shorter (~185 hours) than what was observed under DD for all the three strains. The trend observed was similar to that under DD i.e., significantly larger time

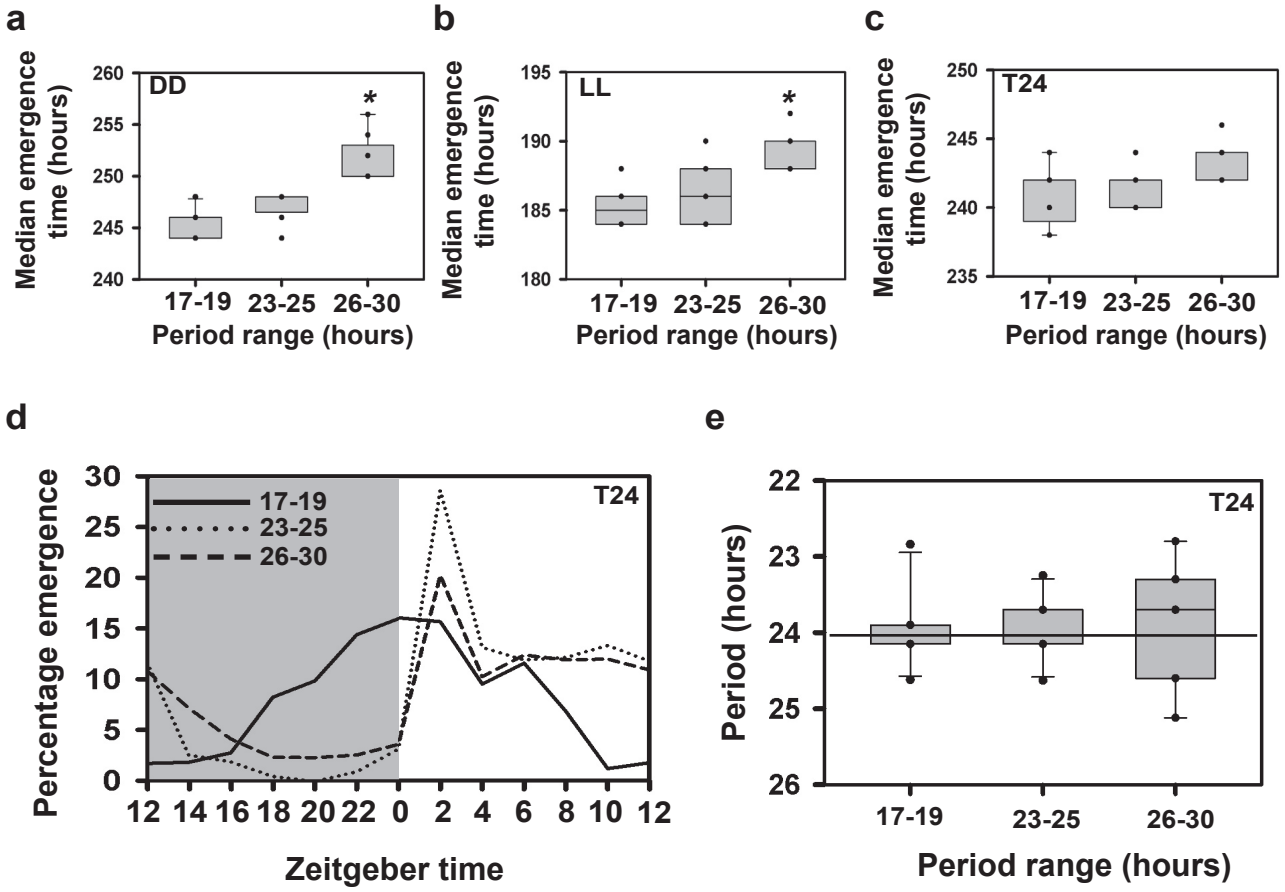

Figure 5.1 Emergence time under constant conditions and LD 12:12. (a-c) Box plots show the median time to emergence for each vial and strain (n>8 vials; 30 eggs/ vial) under three regimes. Whiskers extend up to highest and lowest values and dots show the individual data. Under **(a)** constant darkness (DD) at 25 ˚C and **(b)** constant light (LL) at 25 ˚C asterisks show that long period range differs significantly from other strains (p<0.05) based on Kruskal-Wallis test for multiple independent samples. **(c)** LD 12:12 (T24) at 25 ˚C. **(d)** Adult-emergence profiles of the three strains (n=10 vials; 300 eggs/vial) under T24 where percentage emergence is plotted against zeitgeber time, 0 being the time of lights-on. Shaded region represents duration of the LD cycle during which lights were off. **(e)** Box plots show period values obtained by COSINOR analysis for each strain under T24. Whiskers extend up to highest and lowest values and dots show the individual vial median data points. Black line depicts the period of T-cycle.

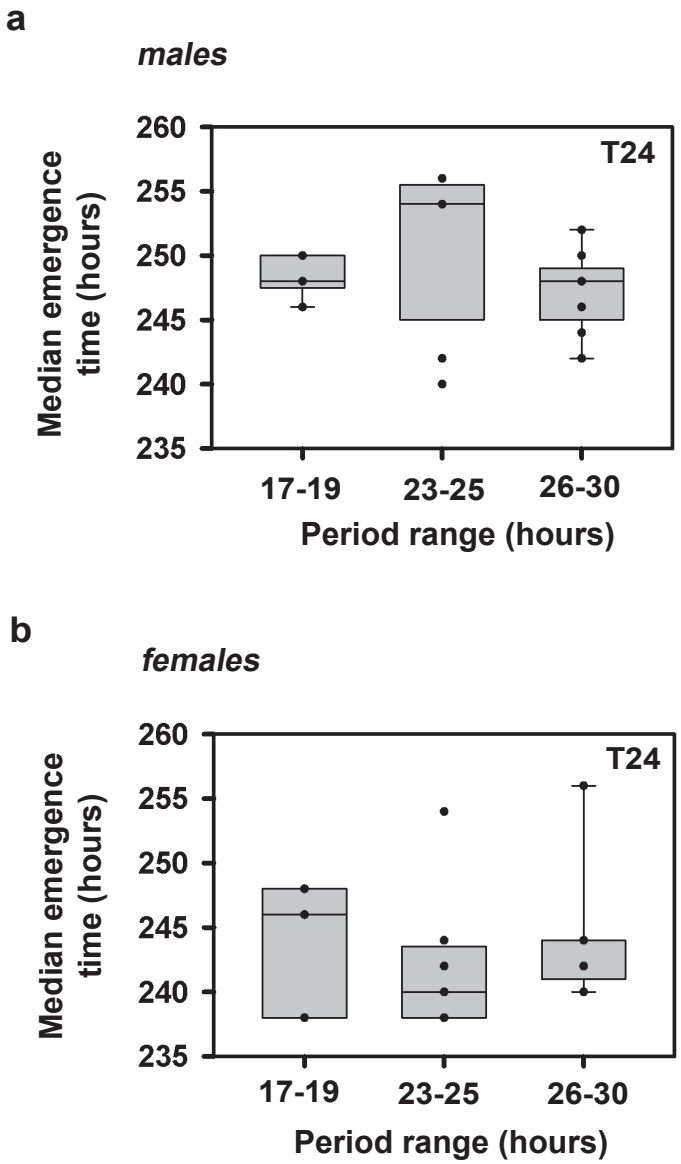

Figure 5.2 Emergence time for male and female flies. Box plots of median time to emergence for each strain, for males **(a)** and females **(b)** (n>8 vials) for the three strains assayed under T24. All other details are similar to Figure 5.1.

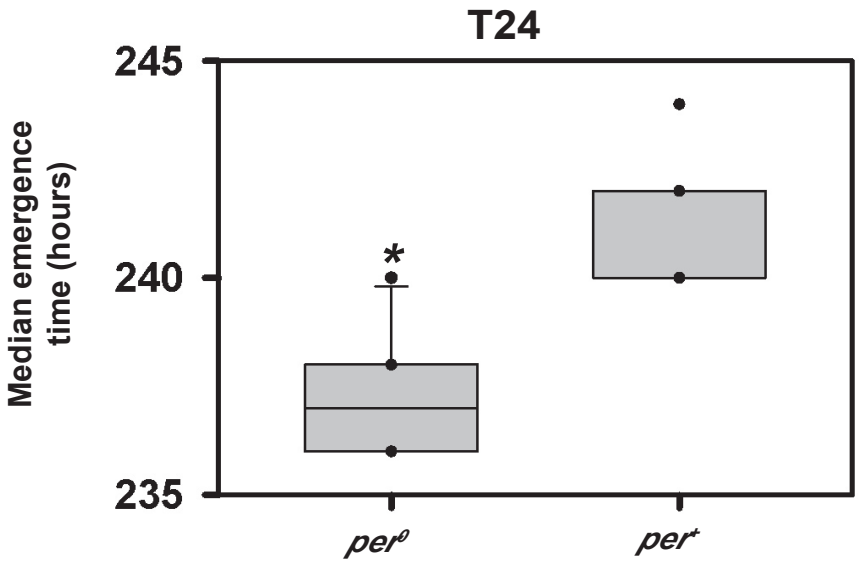

Figure 5.3 Emergence time for per^0 flies. Box plots for median time to emergence for *per^0* and *per$^+$* flies (n=10 vials; 300 eggs/vial) when assayed under T24. All other details are similar to Figure 5.1. Asterisk shows that *per^0* differs significantly from *per$^+$* (p<0.05) based on Kruskal-Wallis test for multiple independent samples.

to emergence for long period range flies when compared to short and intermediate range (p<0.05; Figure 5.1b) while there was no statistically significant difference seen between short and intermediate range. Thus, I found that in the absence of daily time cues, irrespective of DD or LL conditions, time to emergence of long period range flies is longer than the other two strains suggesting that the per^{l} allele causes a delay in the completion of the developmental process.

5.3.2 Time taken for adult emergence varies depending on entrainability to light dark cycle

Next, I asked how environmental cycles might alter developmental rate and whether interactions between entraining conditions and internal clock affect developmental rate. I assessed time to emergence under symmetric light-dark cycles of different cycle lengths (T-cycles) along with adult emergence rhythms of the three strains under the same conditions. Pairwise comparison using Kruskal-Wallis test for medians showed no significant difference in time to emergence among the three strains under T24 (p>0.05; Figure 5.1c) when assayed at a density of 30 eggs/vial. Under this regime, I also separately analyzed time to emergence of males and females and found that both males and females of the three strains also do not show significant differences in time to emergence (p>0.05; Figure 5.2 a, b) like the combined data shown in Figure 5.1c. I also compared time to emergence of arrhythmic per^{0}mutants with wild type per^{+} (intermediate range flies) in this regime and found that per^{0} flies had significantly shorter time to emergence compared to per^{+}(p<0.05; Figure 5.3) i.e., they emerged sooner than controls suggesting that in wild-type flies, *period* gene mediated gating of developmental processes delays adult emergence.

Adult emergence rhythms of all the three strains were assayed by collecting eggs at a density of 300 eggs/vial (in order to obtain multiple cycles of emergence), to determine whether a given regime was capable of entraining the circadian clock. I found that all three strains entrained under T24 (Figure 5.1 d) as their periodicities were not significantly different from 24 h ($p>0.05$; Figure 5.4, Figure 5.1 e). Under T20, long period range flies did not entrain (Figure 5.5 a) i.e., they exhibited periodicity significantly different from 20 h ($p<0.05$; Figure 5.6, Figure 5.5 b) whereas the other two strains entrained with periodicities not significantly different from 20 h ($p>0.05$). Similarly, under T28, short period range flies did not entrain (Figure 5.5 c), showing periodicity significantly different from 28 h ($p<0.05$) which was not the case for other two strains ($p>0.05$; Figure 5.7, Figure 5.5 d).

In accordance with the results obtained for emergence rhythms, under T20, time to emergence of long period range flies was significantly longer when compared to short and intermediate range ($p<0.05$) while short and intermediate range flies did not differ from each other ($p>0.05$; Figure 5.5 e). Under T28, short period range flies took significantly lesser time to emergence compared to long and intermediate range ($p<0.05$) while long and intermediate range did not differ significantly from each other ($p>0.05$; Figure 5.5 f). Across T-cycle comparisons for total time to emergence using Kruskal-Wallis test showed a significant main effect of regime on median emergence time for each strain ($p<0.05$), when assayed for time to emergence, with a trend of increase in time to emergence with increase in length of T-cycle. Thus, I show that rate of pre-adult development is dependent on the length of the external light-dark cycle and flies which are able to entrain to a given T-cycle have comparable time to emergence.

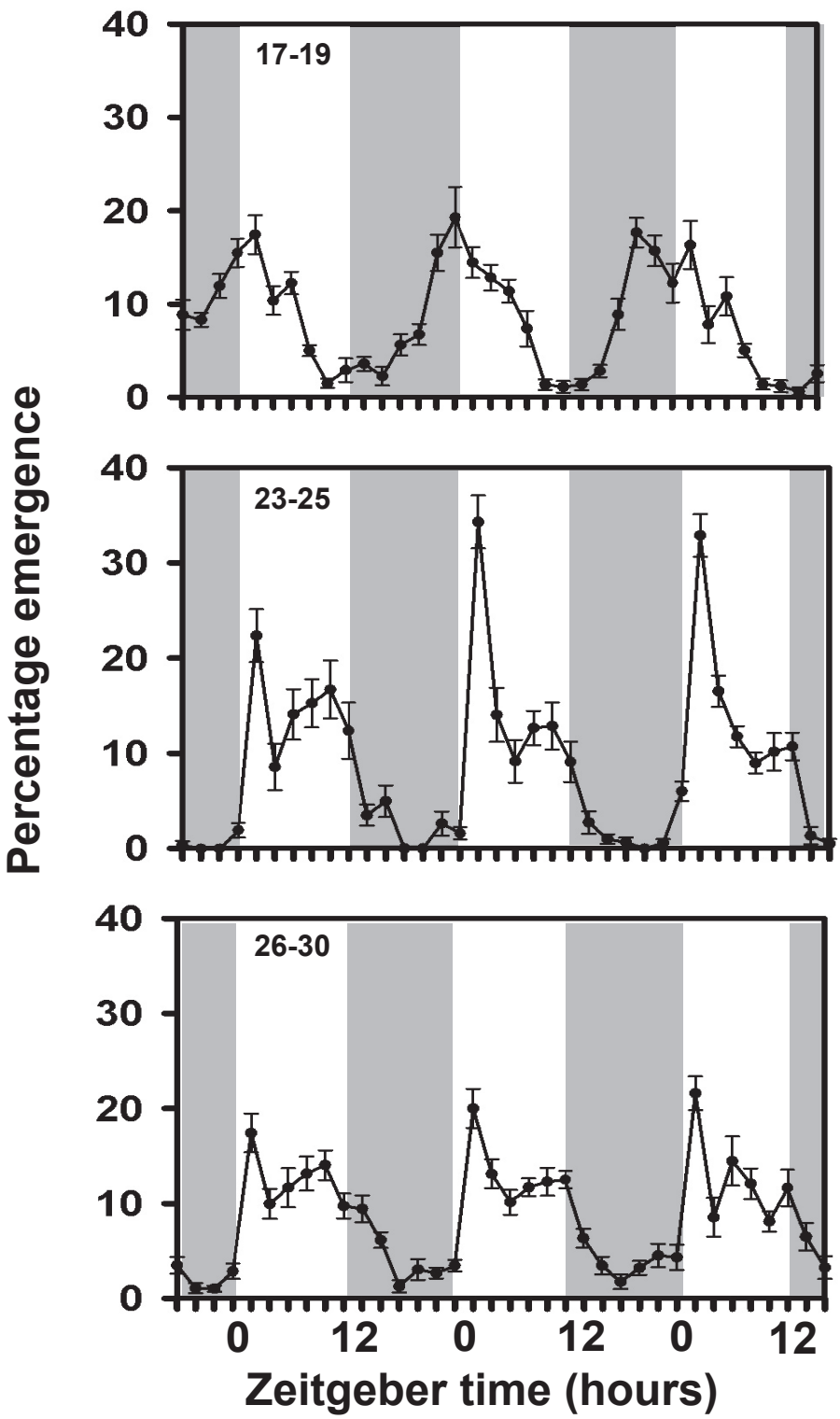

Figure 5.4 Adult emergence time series under T24. Adult-emergence profiles of the three strains (n=10 vials; 300 eggs/vial) under T24 across consecutive cycles where percentage emergence is plotted against Zeitgeber time, 0 being the time of lights-on for each cycle. Shaded regions represent duration of the LD cycle during which lights were off. Error bars are SEM measured across replicate vials (n=10).

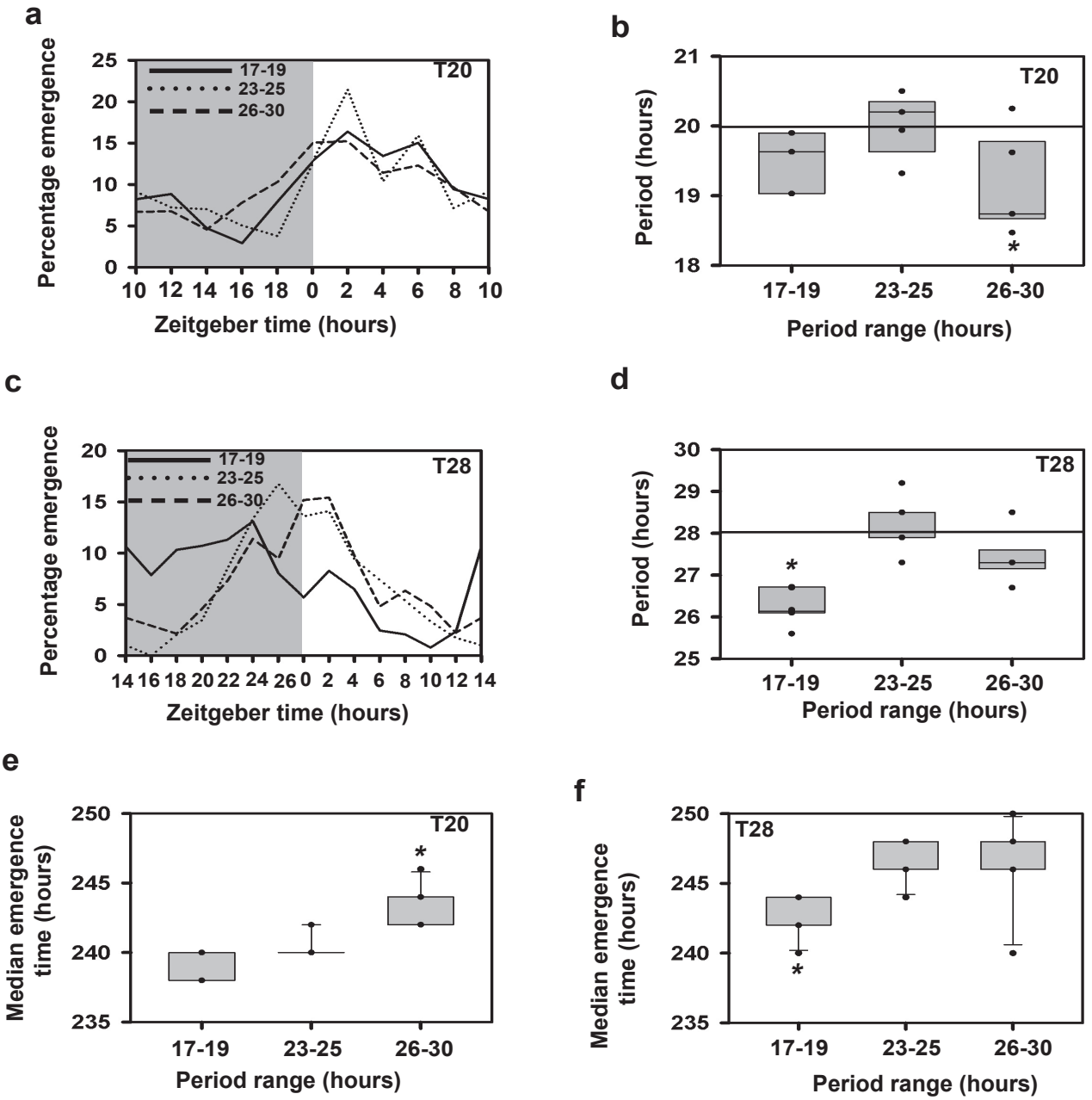

Figure 5.5 Emergence time under deviant T-cycles. (a) Average adult-emergence profiles of the three strains (n>8 vials; 300 eggs/vial) under T20 averaged across cycles where percentage emergence is plotted against zeitgeber time. **(b)** Box plot shows period values obtained by COSINOR analysis for each strain under T20. **(c)** Adult-emergence profiles of the three strains (n>8 vials; 300 eggs/vial) under T28 averaged across cycles where percentage emergence is plotted against zeitgeber time. **(d)** Box plot shows period values obtained by COSINOR analysis for each vial under T28. **(e)** Median pre-adult emergence time for each strain (n>9 vials; 30 eggs/vial) calculated as average of medians across vials when assayed under LD 10:10 (T20) at 25 °C. Asterisk shows that perl differs significantly from other strains (p<0.05) based on Kruskal-Wallis test for multiple independent samples. **(f)** Median pre-adult emergence time for each strain (n>9 vials; 30 eggs/vial) calculated as average of medians across vials when assayed under LD 14:14 (T28) at 25 °C. Asterisk shows that pers differs significantly from other strains (p<0.05) based on Kruskal-Wallis test for multiple independent samples. All other details are similar to Figure 5.1.

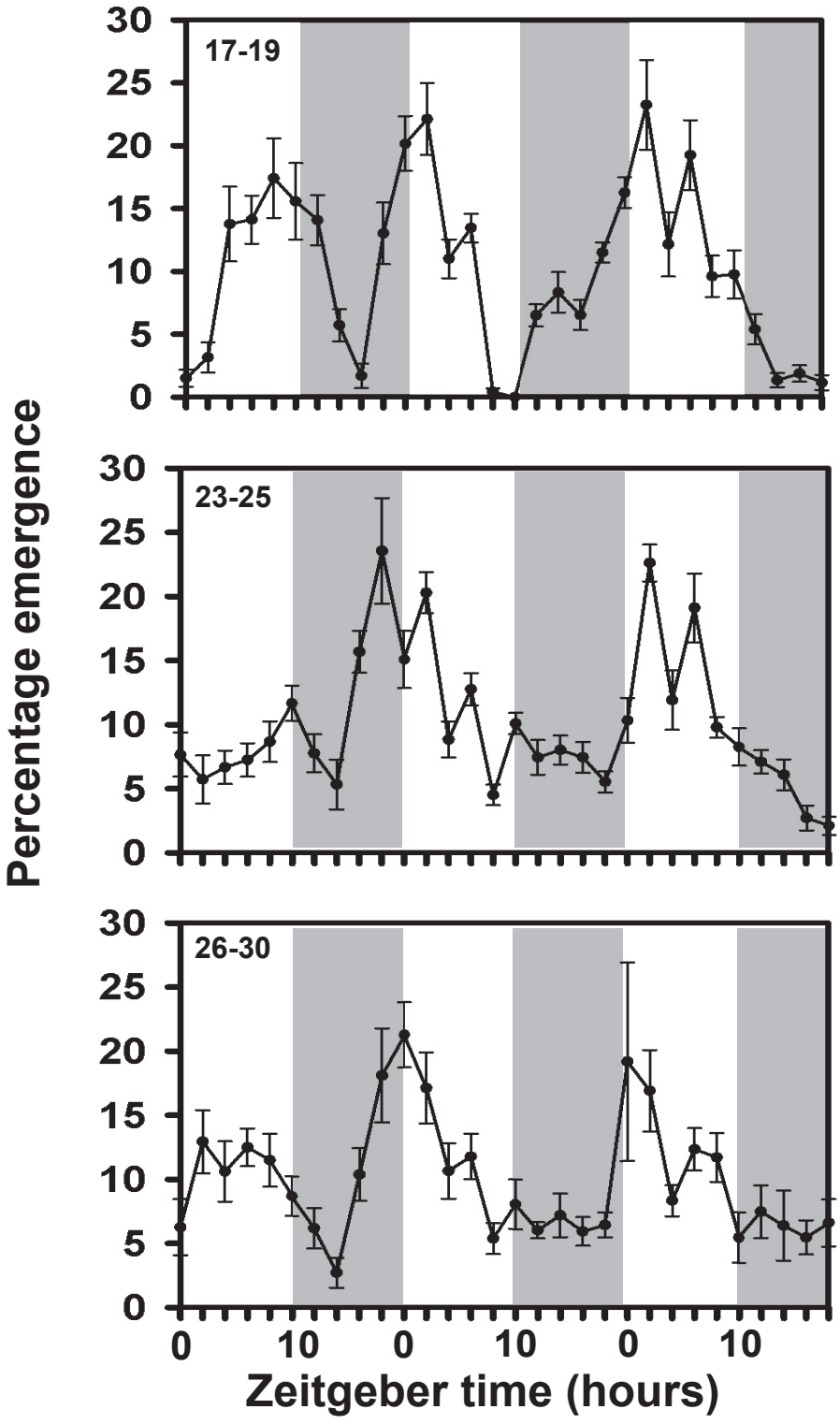

Figure 5.6 Adult emergence time series under T20. Adult-emergence profiles of the three strains (n=10; 300 eggs/vial) under T20 across consecutive cycles where percentage emergence is plotted against Zeitgeber time, 0 being the time of lights-on for each cycle. Shaded regions represent duration of the LD cycle during which lights were off. Error bars are SEM measured across replicate vials (n=10).

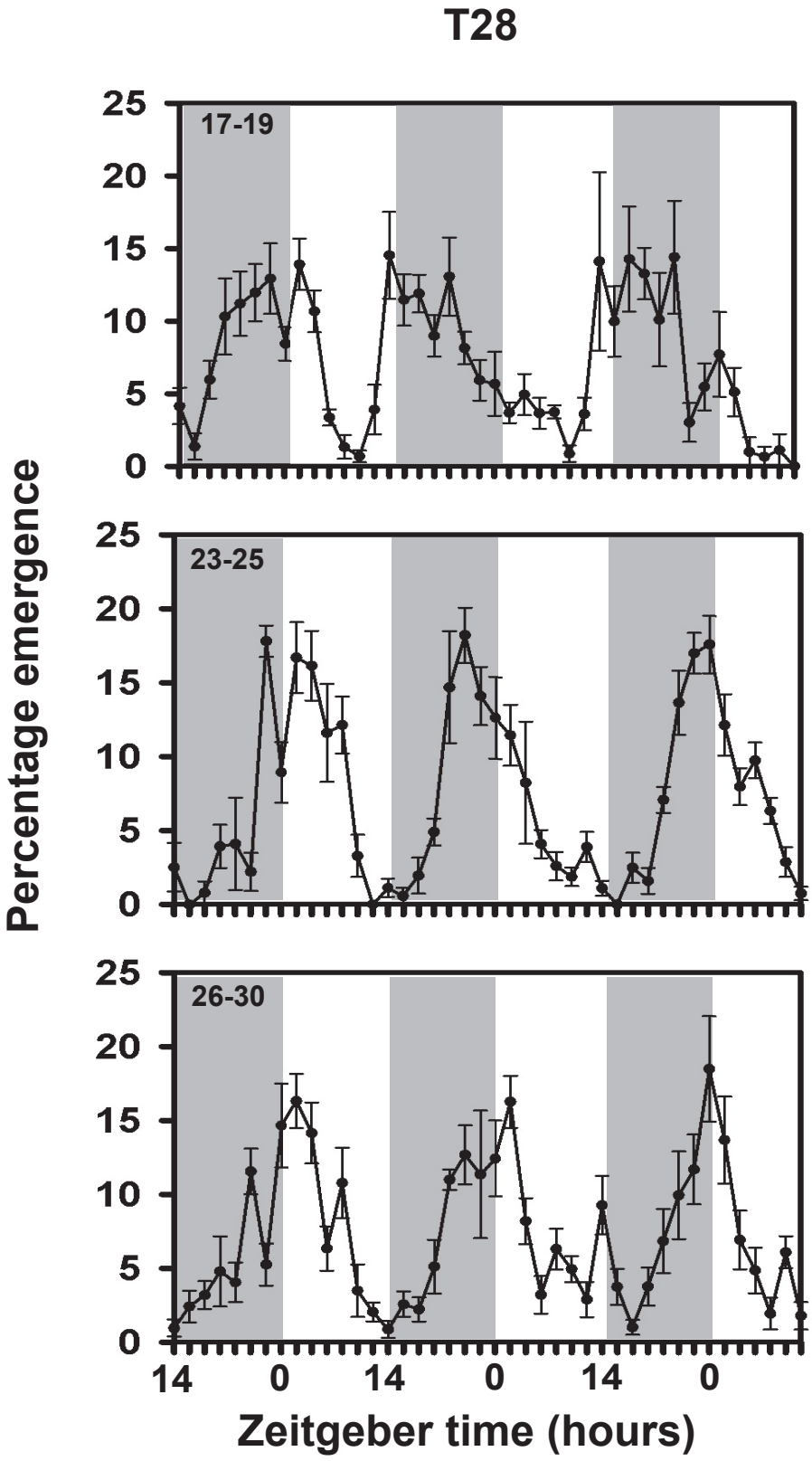

Figure 5.7 Adult-emergence time series under T28. Adult-emergence profiles of the three strains (n=8; 300 eggs/vial) under T28 across consecutive cycles where percentage emergence is plotted against Zeitgeber time, 0 being the time of lights-on for each cycle. Shaded regions represent duration of the LD cycle during which lights were off. Error bars are SEM measured across replicate vials (n=8).

5.3.3 Strain differences exhibit divergent trends in pupation and emergence time

Since it is possible that environmental cycles and/or the circadian clock may act on some prior developmental stages such as pupariation, and the final act of emergence may be simply a reflection of gating at earlier stages, I compared time to pupation for the three strains under the three T-cycles examined and compared the trends among strains with time to emergence. Kruskal-Wallis test for medians showed that pupation time under T20 and T24 for long period range flies was significantly longer when compared to short and intermediate range ($p<0.05$; Figure 5.8 a, b) while there was no significant difference between short and intermediate range ($p<0.05$; Figure 5.8 a, b). Under T28, all three strains were significantly different from each other ($p<0.05$; Figure 5.8 c). I find that although the trends among strains for pupation and emergence are similar under T20 (compare figures 5.5 e and 5.8 a), the trends were not consistent among strains across the two stages of pupation and development under T28 (compare figures 5.5 f and 5.8 c) and T24 (compare figures 5.1 c and 5.8 b). Since I hypothesized that the presence or absence of differences between strains in emergence time depends on gating by the external cycle under entrained conditions, I asked whether pupation also is gated by the external cycle. In a separate study assaying the pupation rhythm of a related ancestral population, I found that wild-type flies do not pupate rhythmically under T24. One-way ANOVA on pupation profile across cycles revealed no significant effect of time point ($p>0.05$; Figure 5.9). Thus, I find that pupation is not rhythmic and the patterns of differences among strains does not correspond with time to emergence in all regimes.

5.4 Discussion

Rate of development is an important life-history trait that may be a critical contributor to reproductive fitness in insects (Vaze and Sharma 2013). Since the circadian clock gates adult emergence and influences developmental rate, an understanding of the role of clocks in this regulation of pre-adult development is important. Although a few studies have shown an effect of clock period on development time, there is no clarity on the extent to which this regulation is imposed by the internal clock/external cycle and to what characteristics of the clock this role can be attributed.

I saw that, under DD, long period range flies had significantly longer time to emergence when compared to short and intermediate range flies (Figure 5.1 a). However, short and intermediate range flies did not differ from each other. This is in contradiction to what was seen in the previous study with *period* mutants, where all the three strains differed significantly from each other (Kyriacou, Oldroyd et al. 1990). This difference in the observation across the two studies can be attributed to differences in genetic backgrounds of the flies used for the two studies. While the previous study was done on mutant and wild-type lines that were backcrossed with a line carrying a deficiency on X chromosome, which was likely to be highly inbred, in our study all the *period* alleles have been backcrossed to a highly outbred, wild-type population which is therefore less likely to have undergone genetic drift and hence fixation of alleles at large number of loci in the genome. Since any phenotype affected by a mutation is also likely to have effects of the interactions of that mutation with other genes (Chandler, Chari et al. 2013), there is a possibility that the difference which was observed previously between short and intermediate range flies was a consequence of interactions of the mutated clock genes with the specific genetic background.

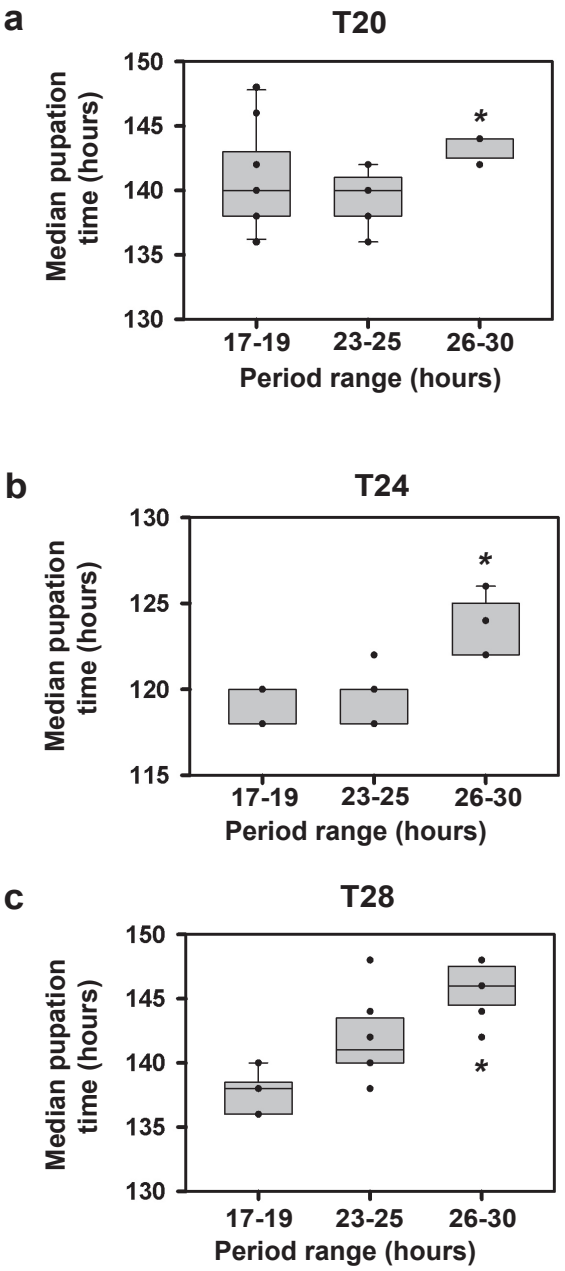

Figure 5.8 Pupation time under deviant T-cycles. Box plots depict the median pupation time for each strain (n>9 vials; 30 eggs/vial) calculated as average of medians across vials when assayed under **(a)** LD 10:10 (T20) **(b)** LD12:12 (T24) and **(c)** LD 14:14 (T28) at 25 °C. Asterisks show that long period range differs significantly from others (p<0.05) based on Kruskal-Wallis test for multiple independent samples. All other details are similar to Figure 5.8.

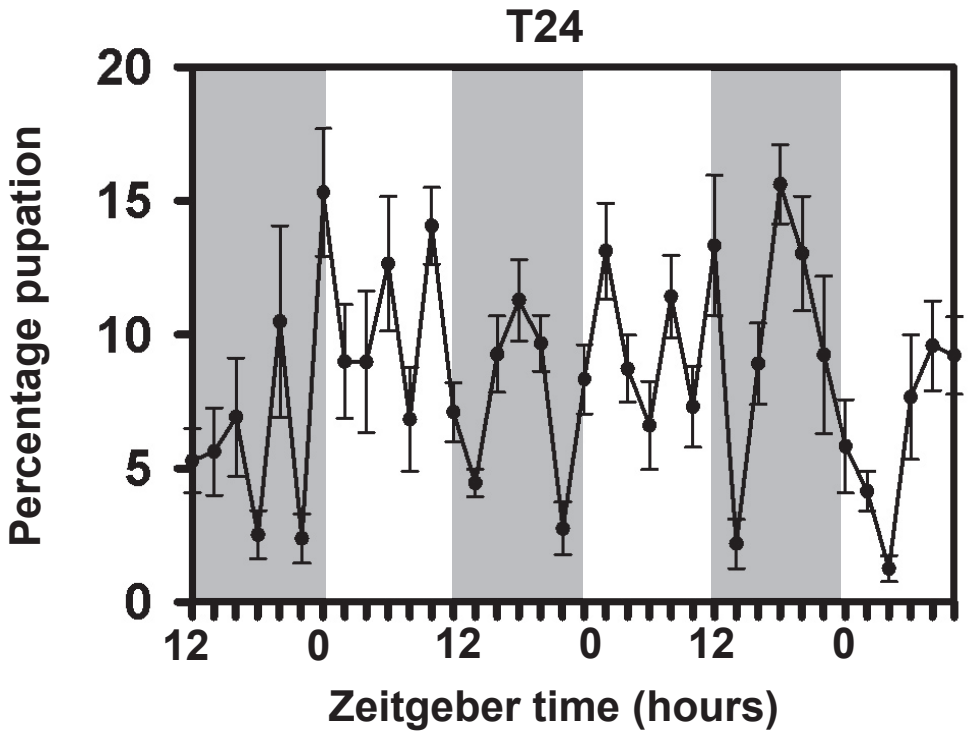

Figure 5.9 Pupation profile under T24. Pupation profiles of wild type flies (n=8; 300 eggs/vial) under T24 where percentage pupation is plotted against Zeitgeber time across cycles, 0 being the time of lights-on for each cycle. Shaded regions represent duration of the LD cycle during which lights were off. Error bars are SEM measured across replicate vials (n=8).

I find that under LL, although time to emergence was relatively shorter compared to DD, the differences across three strains were similar to that under DD i.e. long period range flies had a slower developmental rate (Figure 5.1b). Therefore, my observation of faster development under LL compared to DD is in concordance with the previous studies on mutants as well as on the other wild-type populations (Paranjpe, Anitha et al. 2005; Kumar, Vaze et al. 2006). I also concur with previous reports (Paranjpe, Anitha et al. 2005) that under LL, an absence of gating for eclosion results in this acceleration. Thus, I can say that the inherent differences observed across the three strains are maintained when there are no time cues present and even when the clock may have been rendered dysfunctional and therefore there is a dependence of time to emergence on the free-running period, genetic background and the limitations imposed by the gates available for adult-emergence. Correlations between period and rate of development have also been observed in studies where large, outbred, replicate laboratory populations with sufficient genetic variation were used for imposing artificial selection. Selection on phase of emergence as well as on pre-adult development time has been shown to result in correlated responses in the clock's period. Selection on early and late phase of adult emergence yielded concomitant decrease and increase in pre-adult development time of *Drosophila* populations (Kumar, Vaze et al. 2006). Clock period and development time were also found to be correlated in some other laboratory selection studies where fly populations were selected for faster or slower development. An increase in rate of pre-adult development as a result of artificial selection also resulted in the shortening of the free-running period in most cases (Miyatake 1995) (Shimizu, Miyatake et al. 1997) (Yadav and Sharma 2013). Thus, correlations between period and pre-adult development have been observed through mutant lines as well as artificially selected populations.

The pupation results from my study show a general delay in rate of pupation with increase in period in each of the three T-cycles (Figure 5.8) which does not correspond to the trends observed in time to emergence. However, this is not surprising as assay of percentage pupation across multiple cycles did not reveal a significant main effect of time point which indicates that gating by external cycle does not influence the rate of pupation but occurs only later during emergence, thereby altering the total time taken to emergence. Hence, it appears that the internal clock does have an effect on developmental rate, though the timing of emergence is determined by the circadian gate imposed by the external cycle when the fly is entrained to it.

To verify that these differences are clock mediated, we modified the period of the clocks by subjecting them to light-dark cycles of different durations. Since all clock-regulated processes are believed to be accelerated with an increase in the speed of the clock, development time should also conform to such expectations if it is under circadian control. Hence, we used an approach of altering the period of the clock as well as the zeitgeber to study the relationship between developmental rate and clock. Time to emergence increased with an increase in the length of zeitgeber. This observation is in concordance with a previous report of flies with ~24 h period showing an increase in duration of development time with increase in length of light-dark cycle (Paranjpe, Anitha et al. 2005) and thus demonstrates that influence on rate of development by clock genes is not mediated by pleiotropic effects.

In the adult emergence assay conducted across T-cycles of different durations, I see that short period range flies entrain under T20 and T24 regimes but not under T28 whereas long period range flies entrain under T24 and T28 conditions but not under T20 (Figure 5.5 a-d). On the

other hand, wild type flies (per^{+}), which are employed as controls, show entrainment under all the three T-cycles (Figure 5.1e) as also previously reported discussed (Refinetti 2016). I see that difference in time to emergence which is observed across flies with different free-running periods under constant conditions is absent under LD 12:12 (Figure 5.1c). Also, per^{0} flies differed significantly when compared with per^{+} in time to adult-emergence and emerged sooner than controls (Figure 5.3). The adult-emergence rhythms of these loss-of-function mutants have been shown to be arrhythmic even under laboratory LD 12:12 conditions (De, Varma et al. 2012). Therefore, it is possible that due to an absence of gating by the internal clock, per^{0} flies were able to emerge earlier compared to wild-type flies which entrained to LD 12:12 and therefore were bound to emerge only within the gates imposed by their entrained clocks.

My finding under T24 also differs from what was previously reported in *Drosophila* populations subjected to an artificial selection for timing of emergence where close association between phase of emergence and mean development time in the early and late chronotypes (which have different free running periods) was observed. However, the evolved differences in development time of early and late populations in that study could not be attributed to circadian gating since this difference persisted not only under entrained but also LL conditions. In the present study, I have used an approach to minimize the influence of any factor other than gating imposed by the internal and external cycle on the rate of development. Therefore, I attribute the absence of a difference in the pre-adult development time among the three strains in LD 12:12 to the fact that all flies could entrain to this particular regime and thus they were bound to emerge in the same gate. Under short T-cycle (T20), I show that the developmental rate of long period range flies is slower compared to

short and intermediate range whereas under long T-cycle (T28) that of short period range flies is faster compared to long and intermediate range (Figure 5.5 e, f). This is in concordance with our expectations that flies which entrain to a particular regime would be bound to emerge in the same gate whereas those that do not entrain could emerge according to gates of their free-running eclosion rhythm or could be subject to phenomena such as relative co-ordination. Thus, these sets of experiments confirm that the gating of eclosion, which depends on the ability of a fly to entrain to a given T-cycle is the major determinant of total time to emergence. It shows that development time is dictated by the environment only if the environmental cycle is able to entrain the circadian clock. Further experiments which examine other loss of function mutants of the circadian clock using a similar approach would add credence to the above conclusion.

Future Directions

The results from studies conducted for this thesis should help us delineate the role of internal clock under constant versus entrained conditions. While results of chapter 2 do not support the hypothesis that close to 24 h clocks perform the function of keeping time in constant conditions better than clocks with deviant period as precision is not maximum in such individuals, results from chapter 3 strongly indicate that these clocks are most stably entrained under a light/dark cycle of 24 h which is the period of our naturally occurring environmental cycles. Results from chapter 4 where lability is found to be higher for long period range flies match with the lower precision observed for these flies and again ~24 h clocks do not show any advantage when it comes to maintaining the inherent periodicity in face of change in external factors or history-dependence. Therefore I conclude that possessing a 24 h period gives a significant advantage to the organism only in the presence of time cues. However, my study has certain limitations which I briefly summarize here. Despite using a range of period values it is difficult to make conclusive statements regarding the dependence of the clock properties on the intrinsic period. This is primarily because of the use of mutant lines to generate organisms with distinct period values. It is difficult to attribute less robust rhythms observed in a mutant line to its deviant period as the mutation could have multiple pleiotropic effects independent of the clock which could result in an overall weak clock phenotype. An important consideration with the three backcrossed lines that I have studied is that the range of values of periodicities is different across the lines. While the intermediate and short period lines have lower inter individual variation in period the long period fly lines have strikingly large variation in the period values across individuals. This could be considered a confounding factor in this study as it would have been ideal to have fly lines with similar inter individual variation in the focal trait i.e., the

intrinsic period. The reason for this larger variation in these lines is also not clear. However, it is an indication of the vulnerability of complex traits such as period to mutations and could be attributed to the reduced robustness of rhythms in long period mutants. Secondly, I have tested all the clock properties which can be measured under entrained conditions only with light as the time cue. Since the mechanisms of entrainment to temperature cycles and other time cues in the environment are somewhat different from that to light, our conclusions regarding relationships between clock properties may not hold across other zeitgebers. It is therefore important to assess the clock related aspects studied in chapters 3, 4 and 5 with a different zeitgeber, especially temperature as it is perhaps the second most dominant cue for the clock of fruitflies after light. Therefore, entrainment of these lines under temperature cycles of different lengths and durations of the thermoperiod could be used to examine the correlations between different circadian clock properties under such conditions. Also, constructing PRCs using temperature pulses of different strengths should be useful in concluding whether the non-parametric model of entrainment can explain the entrainment in the individuals. Assessment of pre-adult development rate under different temperatures and also under different temperature regimes is essential before concluding about the role of clock in this physiological process as there is sufficient evidence to suggest the sensitivity of developmental rate to temperature. A limiting factor in such studies of temperature on development is the emergence of most or all flies within a single cycle inhibiting the possibility of measuring clock properties such as period.

Some of my results from chapter 4 and 5 also are not in concordance with what was observed previously using these mutant lines. While I can attribute this mainly to the differences in genetic background in the lines used previously, to confirm this, same lines in a few other

backgrounds (but similar across the three strains) can be employed and these studies should be performed again with them.

Also, since the long period range flies that I have used exhibit weaker rhythms compared to the other it should be first confirmed whether such poor rhythms observed are because of the extreme lengthening of period or it is the effect of per^{l} allele only. For this, mutations in other genes such as *Doubletime* (dbt^{s} and dbt^{l}) or the overexpression of *shaggy* which can lengthen the period of the clock should be employed and after bringing several such mutant lines with varying periodicities on a common genetic background, the conclusions regarding the absolute value of period can be derived.

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List of Publications

- Srivastava M, Varma V, Abhilash L, Sharma VK, Sheeba V. Circadian Clock Properties and Their Relationships as a Function of Free-Running Period in *Drosophila melanogaster*. Journal of biological rhythms. 2019 Apr:0748730419837767.
- Srivastava M, James A, Varma V, Sharma VK, Sheeba V. Environmental cycles regulate development time via circadian clock mediated gating of adult emergence. BMC developmental biology. 2018 Dec;18(1):21.