A study on the stability of circadian clocks in *Drosophila melanogaster* populations selected for early and late emergence

Thesis submitted in partial fulfillment for the degree of

Master of Science

by:

K. Ratna

Chronobiology Laboratory
Evolutionary and Organismal Biology Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
Bangalore - 560064

Table of contents

DECLARATION

I hereby declare that the contents presented in this thesis entitled 'A study on the stability of circadian clocks in *Drosophila melanogaster* populations selected for early and late emergence' submitted to Jawaharlal Nehru Centre for Advanced Scientific Research for fulfillment of the Master's degree is to the best of my knowledge and belief entirely my original work carried out under the guidance of Prof. Vijay Kumar Sharma in Chronobiology Laboratory, Evolutionary and Organismal Biology Unit of the Centre.

In keeping with 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 error of judgment, is regretted.

Date: 15/04/2016

Place: Bangalore. [K. Ratna]

Evolutionary and Organismal Biology Unit
JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH
P.O. BOX. 6436, JAKKUR, BANGALORE - 560 064, INDIA

VIJAY KUMAR SHARMA, Ph.D., FASc, FNA
Professor and Chairman

Date: 15/04/2016

CERTIFICATE

This is to certify that the work described in the thesis entitled 'A study on the stability of circadian clocks in *Drosophila melanogaster* populations selected for early and late emergence' is the result of investigations undertaken by Ms. K. Ratna under my supervision in the Evolutionary and Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064 (India), and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

(Vijay Kumar Sharma)

Telephone (W): 91-80-22082843; (H) 91-80-23622772; FAX: 91-80-23622766;
E-mail: vsharma@jncasr.ac.in/ vksharmas@gmail.com/ vksharmas@yahoo.com
URL: http://www.jncasr.ac.in/vsharma/

Acknowledgement

I would like to thank my guide, Prof. V. K. Sharma, for giving me the opportunity to work in this laboratory, the option of choosing the project I wanted to work on and for his guidance during the course of this project. I would like to thank Dr. Sheeba Vasu for her course on advanced neurogenetics which I enjoyed a lot, and for her calm presence during seminars and presentations which helped steady the nerves. I would also like to thank Prof. Amitabh Joshi and Dr. T. N. C. Vidya for their interesting courses and for the projects I worked on in their labs.

My work would not have progressed much if not for Rajanna and Muniraju's timely help with the glassware and cages for stock maintenance and all of my experiments. They were extremely helpful and accommodative throughout and for that I am very grateful to them. I would like to thank Nikhil for being an enthusiastic co-guide during my rotation in CBL, it was during this time that I learnt a lot of the chronobiology that I know and also became interested in the 'Gate' populations, and this was mainly due to Nikhil's understanding of this work and his ability to explain it in a simple manner. I would like to thank Manishi for her help with my experiments, without which I am certain this thesis would not have been completed in time. Her company during all those hours spent underground in the basement in darkness was comforting, and I am certain I would have lost my sanity without it. I am also very grateful to her for her constant encouragement when things got very overwhelming. My lab-mates Abhilash and Vishwanath have helped me a great deal by discussing my work and results with me and for this I am thankful to them. I am also extremely grateful to Abhilash and Nikhil for spending an immense amount of time on helping me improve my style of writing and the flow of this thesis. Swathi

was a great companion in the lab and helped me immensely with the stock work and my experiments and her cheerful attitude made the lab a nicer place to work in, and I am grateful to her for this. I would also like to thank Venkat for all the help with the stock maintenance and the entertainment that he provided in the lab. Rutvij helped a lot with stock maintenance, especially when I was ill, and I'm thankful to him for this. Anuj and Radhika have been very fun to be around and I am glad of their company and the morale boost that they gave me when I needed it.

I would like to thank Sheetal from BNL for useful discussion about my work. I would like to thank Avani and Antara, my co-guides from my rotations in EBL and BNL for teaching me how to manage and design experiments. Sunil from EMU stepped in like a knight in shining armor and saved me from weeks of mind-numbing copy-pasting during my analysis with his code and for this I am extremely thankful to him.

I would like to thank Nakul and Anjali for being great friends throughout the duration of my stay in JNC and for making it easier to stay and study here. Without them I would not have retained a positive attitude at the end of the last three years. Goirik has been a great friend and companion right from the time of the interviews and has been the reason for most of the happy moments in the last three years, and I cannot thank him enough for all the support and encouragement, and also all the good food that he has given me throughout. I would like to thank my friends Anisha, Shraddha and Ruchita for their support, encouragement and for all the entertainment they have brought into my life right from the time I met them. My parents have been wonderful throughout this journey, and without their support, encouragement and

willingness to let me try things which they might not be familiar with, I would not have made it this far. Also, the home-made food that they kept bringing over kept me very happy during the last couple of years.

Most of the work involved in stock maintenance, setting up of experiments and parts of my analysis involved a lot of manual labor, and I would like to thank Stephen Fry for his brilliant narration of the Harry Potter audiobooks which kept me entertained and alert during most of the last one year. Lastly, my three years in JNCASR have been quite eventful and instructive, and have taught me a lot of life-lessons and for this I am thankful to everyone who has contributed to my growth as a person and helped train me to be better able to deal with a lot of not-so-pleasant scenarios going forward in life.

Summary

Circadian clocks are thought to confer adaptive advantage to organisms by appropriately scheduling behaviours in accordance with environmental conditions, and it is this feature of the clock (Ψ, phase of entrainment) that is under the purview of natural selection (Vaze and Sharma, 2013). Laboratory selection on different phases of behaviour and subsequent examination of the correlated responses of various clock properties is therefore likely to reveal genetic correlations between such clock properties and the phasing of rhythmic behaviours, and the manner in which they do so. This we think will enable us to understand the manner in which circadian clocks evolve to serve their adaptive functions. In this regard we initiated a long-term laboratory selection experiment wherein flies from four large, outbred populations of *Drosophila melanogaster* were selected for morning and evening emergence (Kumar et al., 2007). Along with a direct response in terms of increased emergence during morning and evening hours in the 'early' and 'late' populations respectively, correlated responses in many other clock properties were observed. The period (τ) of the 'early' and 'late' populations were shorter and longer than the 'control' populations respectively for both the activity-rest and eclosion rhythms, and the phase-shift responses to light pulses for eclosion rhythm among the populations were also different (Kumar et al., 2007), and later studies showed that other clock properties such as zeitgeber (cyclic environmental signal, such as light-dark cycles) sensitivity over prolonged durations, oscillator amplitude, and inter-oscillator coupling were different in these populations as a result of selection on delayed Ψ of emergence (Vaze et al., 2012a; Nikhil et al., 2016a).

Differences in the area under the phase response curve (PRC; a plot of phase-shift due to a single perturbation of the clock by zeitgeber pulses) between the populations indicated that continuous effects of light on the clocks of 'early' and 'late' populations may have also evolved (Nikhil et al., 2016a). Based on these results we hypothesized that differences in clock period lability (area of an abstract period space within which each period can be stably assumed by individuals of the population) in these populations may have evolved to facilitate differences in such continuous effects of light. In order to examine this, we carried out several experiments that are described in my thesis. These experiments include estimation of day-to-day variation in τ (precision) and magnitude of change in τ brought about by changes in environmental conditions, because these are surrogate measures of period stability within and across regimes and may provide evidence for evolved period lability if at all. Results of the experiments are briefly described in the following sections.

Stability of period across different ambient temperatures

A long-term recording of the activity-rest behaviour of the 'early', 'control' and 'late' populations was carried out under different ambient temperatures in constant darkness. The 'late' populations showed a greater magnitude of change in τ across different ambient temperatures as compared to the 'early' and 'control' populations, and showed a higher precision at 25 °C and 28 °C, but not at 18 °C. This was found to be due to a greater change in clock precision in the 'late' populations across different temperatures and was found to be significantly reduced at 18 °C. Therefore, the results of this chapter indicate that the 'late'

populations have evolved greater period lability, and possibly increased temperature sensitivity as compared to the 'early' and 'control' populations.

After-effects of *T*-cycles on the τ of the activity-rest rhythm

History-dependent changes in the τ of the activity-rest rhythm due to entrainment to different *T*-cycles (*T* is the period of the zeitgeber) is known as after-effects and has been documented in many diurnal and nocturnal organisms (Pittendrigh and Daan, 1976b). Circadian clocks with greater lability in τ are expected to be greatly affected by the entraining *T*-cycles, and would perhaps show greater after-effects post-entrainment to these novel conditions. The difference in τ following entrainment to, and prior to the entraining *T*-cycles was used to estimate after-effects and it was found that exposure to *T*30 temperature cycles alone had significant after-effects on the 'late' populations, whereas exposure to any of the LD (Light-Dark) and other TC (Thermophase-Cryophase) cycles did not elicit after-effects in any of the study populations. Studies on after-effects have shown that following exposure to *T*-cycles there is a change in τ, but the organism gradually moves to its steady-state τ over a period of time (Pittendrigh and Daan, 1976b). Therefore, another estimate of after-effects, the rate of regression of τ to its steady-state value, could perhaps highlight after-effects in the study populations.

Developmental plasticity

Results in my thesis so far suggest higher plasticity of period in the 'late' populations when subjected to various environmental conditions as adults. Although the plasticity of circadian clocks during pre-adult development has not been as well studied as the plasticity of the clock

in adults, it can be argued that since clocks in the pre-adult conditions are sensitive to environmental factors they could also show similar responses as seen in the adults. The effect of rearing under different LD and TC *T*-cycles on the τ and precision of the 'early', 'control' and 'late' populations was estimated to ascertain the extent of developmental plasticity in these populations. It was found that neither LD nor TC *T*-cycles had any effect on inter-population differences in period and precision. Therefore, although there could possibly be an increase in period lability of circadian clocks in the 'late' populations in the adult stage, this does not manifest when exposed to such conditions during pre-adult development.

In summary, the results of my thesis provides evidence for differences in the period lability in the 'late' populations as compared to the 'early' and 'control' populations, which seems to be apparent only in the adult stage.

Chapter 1

Introduction

Life forms on earth are believed to have evolved circadian (~24-h) clocks to facilitate optimal timing of behaviours so as to anticipate and avoid harsh environmental conditions, predators or competitors, procure food and mate, all of which are likely to be advantageous to the focal organism in terms of survival and reproduction, and will therefore enhance their fitness (Vaze and Sharma, 2013).

Rotation of the earth about its axis drives periodic changes in environmental factors such as light and temperature, and entrainment is the process by which circadian rhythms synchronize to such cyclic times-cues in the environment (zeitgebers) so as to (a) match the clock period (τ) and period of the external cycle (T), and (b) maintain a stable and reproducible phase-relationship with the zeitgeber cycle (ψ_{ENT}; Johnson et al., 2003). There are two schools of thought which propound different mechanisms through which circadian entrainment could be realized: the discrete, phasic or non-parametric model, and the continuous, tonic or parametric model (Johnson et al., 2003). The non-parametric model postulates that the entraining stimuli induce time-of-day dependent instantaneous phase-shifts in the clock so as to compensate for the mismatch between τ and T, thereby enabling entrainment (Pittendrigh and Daan, 1976a). This model stemmed from the early discovery of PRCs, which are plots of phase-shifts as a function of phase of the rhythm at which the organisms were exposed to the perturbing stimulus (Pittendrigh, 1981; Johnson et al., 2003). The predictions of the non-parametric model

seem to hold good under many scenarios and in many organisms, indicating that this could be the prevalent mechanism through which circadian clocks entrain to environmental cycles of light and darkness (LD; Johnson et al., 2003). The parametric model of entrainment on the other hand assumes that light has a continuous or tonic effect on the circadian clocks, rather than the instantaneous discrete effect as suggested in the non-parametric model (Johnson et al., 2003). It proposes that tonic/continuous effect of zeitgebers would change the τ such that it matches T thereby driving entrainment (Johnson et al., 2003), and has found evidence in some burrow-dwelling organisms (Aschoff, 1960; 1981; Johnson et al., 2003). Beersma et al (1999) proposed that circadian clocks of most organisms would entrain more stably when they use both non-parametric and parametric mechanisms in conjunction thereby indicating that in the wild, use of both mechanisms may be molded by natural selection to give rise to appropriate ψ_{ENT} (Daan, 2000).

Circadian clocks as a network of coupled oscillators

Circadian rhythms were initially conceptualized to stem from a single core clock/oscillator responsible for governing all the properties of the circadian rhythms, and modelling circadian clocks in this fashion has been useful in understanding circadian entrainment (Wever, 1965; Pavlidis, 1978; Kronauer et al., 1982). However, modelling circadian clocks as a single oscillator has not been able to successfully explain multiple experimental data from a range of studies. For instance, it was observed that the locomotor activity rhythm of nocturnal rodents when studied under constant light (LL) show 'splitting', wherein the activity bout splits into two individual components which free-run initially with different periods until they are out of phase

by 180°, and then continued to free-run with similar periodicities. Based on this, Pittendrigh and Daan (1976b) proposed that circadian clocks comprise two oscillators, each having its own τ, thereby laying down the foundation for studies that modelled circadian clocks as a network of two coupled oscillators, where the emergent τ of the rhythm was also dependent upon the extent of coupling between the oscillators (Daan and Berde, 1978). Based on the dual-oscillator model, it was predicted that under LD, each of the two oscillators would be phase-locked with lights-ON and OFF respectively, and the extent of coupling between the two would determine the rate of regression of their periods back to the original values when they are released in DD, thereby explaining the basis for after-effects (history dependence of the entraining stimulus on τ; Daan and Berde, 1978) observed in certain experimental scenarios. It was predicted that if the coupling between the oscillators is strong, the oscillators would synchronize (and attain a steady state phase-relationship with respect to each other) immediately after release into constant conditions, thereby exhibiting a steady-state τ almost immediately. If, however, the coupling between the oscillators is weak, it would take a while for the clock to achieve a steady-state τ and the rhythm would exhibit transient cycles, or after-effects of the previous environmental conditions. Empirical evidence demonstrating the transient behaviour supports the notion that circadian clocks are a network of oscillators, and that the differences in coupling between the constituent oscillators could possibly bring about differences in the transients that are seen (Berde, 1976; Pittendrigh and Daan, 1976b; Daan and Berde, 1978).

Apart from a dual oscillatory architecture, one could possibly imagine a scenario where circadian clocks comprise not just two predominant oscillatory constituents, but a network of a

large number of oscillators, each with an inherent τ and amplitude (Pavlidis, 1978). Indeed in mammals and a few higher organisms, the master clock comprises several thousands of neurons, each of which is capable of acting independently to determine the final output of the clock. For example, the Suprachiasmatic Nucleus (SCN) of mammals comprises a network of ~20,000 neurons, each one of them being an independent oscillator which is coupled with others, and together this network serves as a platform to examine the effect of inter-oscillator coupling on the output of the network (Welsh et al., 2010). Theoretical studies have examined the consequences of differences in the strength of coupling based on the SCN, and these have been validated to some extent by experiments where the period and phase of the molecular rhythm were studied both *in vivo* and *in vitro* (Indic et al., 2002 and citations therein; Abraham et al., 2010). It is believed that coupling between constituent oscillators is mediated through neuropeptides and gap junctions (Tokuda et al., 2015). Among the well-studied neurotransmitters which possibly act as coupling agents between the constituent SCN oscillators are VIP (Vasoactive-Intestinal Peptide) and GABA (gamma amino butyric acid) (reviewed in Welsh et al., 2010). Mutant mice lacking VIP show weak behavioural rhythms often with multiple periodicities (Colwell et al., 2003; Aton et al., 2005), but daily application of VIP agonist was able to restore synchrony among the constituent SCN oscillators (Aton et al., 2005). Neonatal SCN from VIP knockout mice exhibit reduced synchrony of neuronal spike-frequency rhythm (Aton et al., 2005), and when the circadian gene expression in neurons and networks in VIP deficient mice was studied, it was found that the loss of VIP is correlated with reduced synchrony in the network (Ciarleglio et al., 2009). Although GABA is known to primarily have an inhibitory function on SCN neurons, its role has also been implicated in

excitatory functions, which mediate synchronization of circadian neurons (reviewed in Welsh et al., 2010). Coupling between neurons can also be mediated through metabolites, synapses, or gap junctions, and the final output of circadian clocks is greatly dependent on the coupling agents and the nature of synchronization/coupling they bring about, which may include mean field coupling, diffusive coupling, or both (Welsh et al., 2010). Similarly, in *Drosophila*, the core circadian clock is believed to be located in the brain, and comprise two main groups of cells, the dorsal neurons and the lateral neurons, and the neuropeptide pigment dispersing factor (PDF) is known to couple the neuronal subsets driving circadian behaviors, as lack of PDF leads to behavioral arrhythmicity in constant darkness possibly due to mutual desynchronization of constituent oscillators (Helfrich-Föster, 2005). Thus, it appears that the *Drosophila* circadian clock architecture is similar to mammals, and it also comprises a network of coupled oscillators that in unison function to regulate rhythmic output.

In summary, circadian clocks can be conceived of as a network of oscillators, with each constituent oscillator having different period and amplitude, the final output of the clock and several of its properties being dependent on the level and type of coupling between these neurons (Bordyugov et al., 2011; Tokuda et al., 2015). Theoretical studies have predicted the characteristics of circadian clocks with different types and levels of coupling, and this has been validated to some extent by empirical studies on mammalian pacemaker neurons. There is, however, a paucity of studies on this topic when it comes to *Drosophila*, which could be viewed as an excellent model organism to study circadian behaviors. Progression of work concerning the circadian oscillator network and its properties will enable us to better understand the

manner in which circadian clocks carry out their most important and ecologically relevant function, which is to synchronize rhythmic behaviors with the cyclic environment.

Motivation for the current study

As discussed previously, circadian clocks are believed to have evolved as adaptations to enable organisms to schedule functionally relevant behaviours at favorable times of the day, thereby facilitating enhanced survival and reproduction (Cloudsley-Thompson, 1960; Vaze and Sharma, 2013). Therefore, it is intuitive that natural selection may act on the timing of behaviors and this may result in the evolution of key circadian clock properties such as τ, intrinsic oscillator amplitude and zeitgeber sensitivity (Daan and Aschoff, 2001). An example of the role of circadian clocks in restricting rhythmic behaviours to ecologically relevant phases can be drawn from the adult emergence rhythm in *Drosophila*, where emergence is restricted to early morning hours presumably to avoid conditions of high temperature and low humidity later in the day (Pittendrigh, 1993). The activity/rest rhythm in *D. melanogaster* under 12:12-h LD cycles is bimodal with peaks close to dawn and dusk, and is believed to reflect food procurement and courtship related behaviors (De et al., 2013). The two peaks are interrupted by a period of quiescence during the afternoon which is believed to have evolved to facilitate avoidance of harsh temperatures prevalent during the mid-day (Pittendrigh, 1993). Appropriate phasing of behaviors, therefore, appears to be extremely important to organisms, and the study of circadian clock properties that might govern phasing of rhythmic behaviors might provide insights into the manner in which circadian clocks have evolved.

Laboratory selection studies have contributed to an understanding of the evolution of various phenotypes, and the selection pressures acting upon them to give rise to the said phenotypes (Garland and Rose, 2009). Carrying out laboratory selection on timing of behaviors may therefore, help us determine the selection pressures governing that behavior, circadian clock properties that evolve in response to them, and the manner in which they do so.

In this regard, there are a few studies which selected for different timing of emergence in *Drosophila* and other insects, and assessed the direct and correlated responses to the imposed selection pressures on circadian clock properties. In one such study, selection for 'early' and 'late' emergence was carried out on *D. pseudoobscura* and *Pectinophora gossypiella* strains. After 50 generations of selection, although emergence during the selection windows showed an increase, early strains were found to have longer period as compared to late strains, and the light-induced PRCs of the two selected lines did not differ (Pittendrigh, 1967). Selection on *P. gossypiella* also yielded similar results (Pittendrigh and Minis, 1971), however, these results cannot be completely relied upon since details of selection protocol and experimental design are not available in any of these studies, and hence interpretations from such studies are limited by the lack of details pertaining to selection regime, replicates and population size induced random genetic drift (Vaze and Sharma, 2013). Another study attempted to estimate the differences in the responses to selection for early and late emergence in lab-reared and wild-caught strains of *Drosophila* (*Oregon-R* and *W2* strains, respectively; Clayton and Paietta, 1972). The study revealed that the lab-reared strains respond with greater percentage of flies emerging during the selection windows and interpreted this as a reduction in variability in the

wild-caught flies due to strong selection pressures in nature, ignoring the fact that the wild-caught strains were being assayed under novel conditions, and could show effects of linkage disequilibrium. However, this study did not examine the correlated responses in other traits that may characterize or govern clock properties such as period of circadian rhythms (Clayton and Paietta, 1972).

Although several studies have attempted to address the role of circadian clocks in determining the phase of adult emergence, they are fraught with limitations such as lack of population level replicates, use of small population sizes and inbred lines, and studies being carried out on populations which were freshly caught from the wild without allowing for the effects of linkage disequilibrium to dissipate. Most studies do not provide the details of the selection protocol used, thus making it difficult to arrive at any firm conclusion on whether the observed responses were due to the imposed selection pressures alone, or due to other factors such as random genetic drift or linkage disequilibrium (Vaze and Sharma, 2013).

In order to study the consequence of selection on the phase of emergence, and to understand the underlying circadian clock properties governing this, we initiated a long-term laboratory selection study on populations of *D. melanogaster* by subjecting them to selection for different timing of adult emergence and by examining correlated evolution of circadian clock properties, accounting for most limitations of the previous studies. We initiated these selection lines ensuring that they were large, outbred populations (adult density of about 1200-1500 flies with roughly equal number of males and females) with four replicate blocks that were reproductively isolated from each other, ensuring population level replication. Any correlations

between circadian clock properties that will be seen would most likely be due to the selection on timing of adult emergence, due to the elimination of the confounding factors that the previous studies entail. The methods utilized in population maintenance, assaying behaviours, statistical analyses, results of selection on timing of emergence, and the motivation for this study are discussed in the following sections.

Fly population maintenance and selection protocol

Four replicates each of 'early' ($early_{i = 1..4}$), and 'late' ($late_{k = 1..4}$) populations were initiated from four replicate large outbred ancestral stocks ($control_{j = 1..4}$). These 'control' populations were maintained independent of each other for at least 700 generations before the initiation of the 'early' and 'late' populations (Kumar et al., 2007). Every generation, ~300 eggs were collected, dispensed into glass vials and reared in cubicles maintained at constant temperature (25 ± 0.5 ^{o}C; mean ± SD) and relative humidity (75 ± 5%) under alternating 12:12-h light/dark cycles (LD). The morning and evening selection windows, from which 'early' and 'late' populations were collected, comprised 4-h durations each, ZT21-01 (Zeitgeber Time; morning window) and ZT09-13 (evening window; where ZT00 is lights-ON and ZT12 is lights-OFF). In order to select for 'early' populations, flies that had emerged prior to the morning selection window were discarded and, those that emerged during the morning window were collected and maintained in plexi-glass cages. Similarly, for 'late' populations, flies emerging during the evening window were selected after discarding those that emerged at other times of the day. Flies emerging during the selection windows for the first four days formed the breeding adults for the next generation. The breeding adults for 'control' populations comprised flies emerging during the

first four days of emergence. Therefore, 'control' populations were not under any conscious selection pressure for timing of emergence. All populations were maintained as independent breeding entities in plexi-glass cages (25 × 20 × 15 cm^3) on a 21-day generation cycle with no gene flow between them, with each population comprising 1200-1500 adult individuals and sex ratio close to 1. The populations were provided with Banana-Jaggery (BJ) medium in a Petri-dish, which was replenished every alternate day. For the initiation of next generation, flies from the study populations were provided with yeast paste for 3-days followed by a fresh plate of food to lay eggs on the 21^{st} day and from this plate, ~300 eggs were collected and dispensed into glass vials (1.5 cm diameter × 19 cm height) containing ~6 ml BJ medium. Twenty-four, 16 and 48 such vials were used every generation for each of the four 'early', 'control' and 'late' populations, respectively.

Standardization of selected populations:

It is known that non-genetic factors such as altered physiology of the parents due to environmental conditions that they experience, or maternal effects can influence the phenotypes of their progeny (Vaze and Sharma, 2013). At least one generation of common rearing for the selected populations is known to eliminate such non-genetic parental effects. Therefore, all populations were subjected to a common rearing condition for one generation. This was achieved by relaxing selection and collecting adults emerging throughout the day (similar to the 'controls') to make the required population size of 1200-1500 adults for both 'early' and 'late' populations; and are henceforth referred to as the 'standardized' populations. All experiments were performed on the progeny of standardized populations.

Activity/rest rhythm assays

Since majority of the assays discussed in this thesis involves activity/rest recording, I will briefly discuss here the basic protocol common for all the assays, and details pertaining to minor differences in the assay protocols will be described in the respective chapters.

Activity/rest rhythm assay on individuals from the different populations was carried out on 3-4 day old virgin flies (*n* = 32/population/block). Recording was done in 5-min bins in individual locomotor activity tubes containing sucrose-yeast media using Drosophila activity monitor system (Trikinetics, Waltham, MA). The phase of activity offset was determined for every individual over the duration of recording using CLOCKLAB (Actimetrics, Evanston, IL) and this was used to calculate day-wise period (duration between two consecutive offsets). Day-wise period was used to estimate precision of period (inverse of standard deviation of day-to-day period). The period and precision values were calculated over multiple age windows of 5-days each for the entire duration of recording.

To test for significant differences among the populations, mean period and precision during each of the age windows was estimated for all the four replicate blocks of selection lines and analyzed using a mixed model repeated measures Analysis of Variance (ANOVA) with population and age-window as fixed, and block as random factors.

Results thus far

Following several generations of selection, the proportion of flies emerging during the morning and evening selection windows increased for the 'early' and 'late' populations as compared to

the ‘control’ populations (Kumar et al., 2007). The period of their circadian rhythms was also found to have changed, with the period of the ‘early’ and ‘late’ populations being significantly shorter and longer respectively than the ‘controls’ for both adult emergence and activity/rest rhythms (Kumar et al., 2007).

The laboratory conditions in which the study populations are reared ensured that the only cyclic time-cue experienced by these populations is light, and it was under LD conditions that the adult emergence behaviour was assayed. Therefore, the differences in emergence reported by Kumar et al (2007) were under the influence of light as the solitary zeitgeber. Natural conditions present a myriad of zeitgebers such as light, temperature and humidity, all of which gradually change in their intensity across the day. To test if the emergence chronotypes of the ‘early’ and ‘late’ flies persist even under natural conditions, the adult emergence rhythm was assayed in nature and the differences were found to be considerably enhanced under these conditions (Vaze et al., 2012b). To understand the role of temperature and light in mediating the enhanced differences in emergence chronotypes, adult emergence rhythm was assayed under conditions of in-phase and out-of-phase light and temperature cycles. The study revealed that light had a delaying effect on the phase of the rhythm and increased the gate-width of emergence (the duration of the day during which adults emerge) whereas temperature advanced the phase of the rhythm and reduced the gate-width, thus highlighting the complex interplay between light and temperature in driving the observed difference in emergence chronotypes in nature (Nikhil et al., 2014). These results suggest the evolution of a

fundamental property of circadian clocks in response to selection on timing of emergence that may be common to light and temperature entrainment.

Furthermore, the 'early' and 'late' populations were found to utilize light for different durations during different parts of the day to entrain their emergence rhythms to LD cycles (Vaze et al., 2012a). A skeleton photoperiod comprising two brief light pulses of 15-min each, one given during the dark to light transition and the other during the light to dark transition to mimic dawn and dusk was unable to mimic the emergence waveforms of the study populations under complete photoperiod, suggesting that longer durations of light might be needed for entrainment of adult emergence rhythm in these populations. These results indicate a possible difference in phase dependent light sensitivity between the two populations, and are indicative of a difference in the circadian photosensitivity in these populations. A recent study by Nikhil et al (2016a) revealed that when assayed under constant dim light condition (LL), the 'late' populations show a higher incidence of arrhythmicity as compared to the 'early' and 'control' populations, further indicating that the 'late' populations could have evolved higher photosensitivity. Intriguingly, the three populations did not differ in the magnitude of light-induced phase-shifts even across multiple light intensities (Dose Response Curve; Nikhil et al., 2016a). Assay of activity/rest rhythm under phase-shifted LD cycles revealed that the 'late' populations take significantly longer to re-entrain as compared to the 'early' and 'control' populations, indicating that 'late' populations have evolved reduced re-entrainment rate as compared to the 'early' and 'control' populations (Nikhil et al., 2016a). The DRC and re-entrainment rate studies failed to support the hypothesized increase in photosensitivity in the

'late' populations. However, the 'late' populations were found to exhibit a wider range of entrainment (they can entrain to LD cycles of periods deviating from 24-h). Therefore, it was hypothesized that higher incidence of behavioral arrhythmicity in 'late' populations might not be due to higher photo-sensitivity but due to lower inter-oscillator coupling, and since coupling is known to influence the period of the oscillator network (Abraham et al., 2010), weaker coupling in the 'late' populations might further facilitate wider range of entrainment. Weakly coupled oscillators are known to exhibit large magnitude phase-shifts and consequently faster re-entrainment to phase-shifted zeitgeber cycles (Abraham et al., 2010). However, this holds true only within the realms of the non-parametric model of entrainment, and based on the lower re-entrainment rate and lack of difference in dose responses between the populations, it was speculated that the phenotypes of the 'early' and 'late' populations might stem from differences in the tonic responses to light in these populations. As preliminary support for this, Nikhil et al (2016a) analyzed the area under the curve of the PRC as a proxy measure of the integrated effect of light over longer durations (parametric model of entrainment), and it was observed that the area under the PRC of the delay zone was significantly higher in the 'late' populations as compared to the 'early' populations suggesting that these populations differ in their ability to integrate light over longer durations. In summary, the results so far indicate that the 'late' populations could differ from the 'early' and 'control' populations in terms of their intrinsic oscillator amplitude, circadian photosensitivity to prolonged durations of light, and possibly inter-oscillator coupling.

In this thesis, I intend to study how the above mentioned differences between the circadian clocks of the 'early' and 'late' populations influence two aspects of the activity-rest behaviour, a) the ability to ensure constancy in period across days (precision), and b) stability of period across multiple environments involving light and temperature changes. These properties would in general help us assess the manner in which oscillator amplitude, coupling and zeitgeber sensitivity differences are related to the stability of the circadian clock network. I aim to study short and long-term stability of period in the 'early' and 'late' populations. As discussed previously, the ability to resist changes in period and to accurately predict local time are essential features of the circadian clock. The ability of circadian clocks to entrain to different *T*-cycles is mediated by the above-mentioned factors such as inter-oscillator coupling and ability to integrate light over longer durations, and these factors also determine the extent to which circadian clocks are modulated by zeitgebers. In order to probe the extent to which zeitgebers affect clocks, the current study estimates period and precision of the activity/rest rhythm after being subjected to *T*-cycles of LD and thermophase/cryophase (TC), either at the pre-adult stage, or at the adult stage. Studying period changes after exposure to such cycles would enable us to estimate the extent to which circadian clocks are affected by these zeitgebers, and precision would determine the day-to-day stability of τ upon being exposed to zeitgebers, and both these measures combine to give us an understanding of the stability of the circadian clock. *T*-cycles that are far removed from 24-h provide ideal conditions to test for the effect of zeitgebers on circadian clocks as the ability of the clocks to entrain to these conditions will be determined by differences in the previously discussed circadian clock properties, and will reflect differences in those properties. Indeed, Nikhil et al (2016a) showed that differences in

entrainability were pronounced when the ‘early’, ‘control’ and ‘late’ populations were subjected to different LD *T*-cycles. Therefore, a study of these properties following entrainment to *T*-cycles would be informative.

Therefore, by employing LD and TC *T*-cycles at pre-adult and adult stages, and different ambient temperatures at the adult stage, this study would attempt to estimate the stability of clock period and the possible mechanisms which govern it in the ‘early’, ‘control’ and ‘late’ populations.

Chapter 2

Introduction

Circadian clocks are a network of oscillators which generate rhythms with ~24-h periodicities, and one of its essential features is the ability to actively compensate for changes in period (τ) brought about by change in ambient temperature, a phenomenon known as temperature compensation (Pittendrigh, 1954; 1993). The effect of temperature on circadian clocks was initially documented by Kalmus (1935), but a comprehensive and systematic study on the effect of different ambient temperatures on *Drosophila* circadian clocks was undertaken much later by Pittendrigh (1954), who found that although change in the ambient temperature brings about an immediate change in the phase of the circadian rhythm, its τ remains largely unaffected (temperature compensated) as assessed by the Q_{10} value (ratio of τ before and after 10 ^{o}C increase in temperature), which for the *Drosophila* eclosion clock was found to be 1.02 (Pittendrigh, 1954). Subsequently it was shown that circadian clocks are not temperature insensitive but actively compensate for changes in τ caused by increase/decrease in temperature (Hastings and Sweeney, 1957). Given that τ of circadian clocks is an aspect of the clock output that is altered to some extent by temperature change, the stability (reduced day-to-day variation in τ and increased ability of the clock to resist changes in τ brought about by changes in environmental conditions; see chapter 1) of τ is a feature that can be investigated under different ambient temperatures. Synchrony between constituent circadian oscillators is thought to be brought about through neurotransmitters such as PDF in *Drosophila* (Shafer and

Yao, 2014) and VIP in mammals (Aton et al., 2005; Ciarleglio et al., 2009), and it is possible that the levels of these coupling factors in the circadian oscillator network, or their effectiveness could be affected by change in ambient temperature. This is expected to affect the synchrony of the oscillator network and therefore in turn would alter its output.

A previous study on the 'early' and 'late' populations of fruit flies *Drosophila melanogaster* has led to the hypothesis that the 'late' populations might have evolved higher τ lability (area of an abstract period space within which each period can be stably assumed by individuals of the population) possibly due to weaker coupling in their circadian clock network (Nikhil et al., 2016a). Also, the 'late' populations were reported to exhibit higher accuracy (lower day-to-day variation in the entrained phases) of entrainment for both activity-rest and adult emergence rhythms across multiple environmental conditions (Nikhil et al., 2015). Moreover, this increased accuracy does not appear to stem from inherently more precise circadian clocks in the 'late' populations (Nikhil et al., 2015). However, the precision of circadian rhythms in this study was assessed only during the first few days of transfer from LD (Light-Dark cycles) to DD (Constant darkness), and therefore, the observed lack of difference in precision might be due to the after-effect of exposure to LD cycles. Therefore, a long-term assessment of precision would be ideal to examine if 'late' populations have indeed evolved higher precision of circadian clocks that may contribute to the stability of τ.

In order to assess the stability of τ across age and ambient temperatures, we performed long-term recordings of the activity-rest rhythm in flies from the 'early', 'control' and 'late' populations under three ambient temperatures vis-à-vis 18, 25 and 28 °C, and assessed period,

precision and temperature compensation abilities of these populations with the hypothesis that the 'late' populations may have evolved higher period lability.

Materials and methods

Activity/rest assay

Flies from the three sets of populations were reared under 12:12-h LD cycles at 25 °C and used for the assays. In order to study long-term stability of the clock period, the activity-rest rhythms of flies from the three sets of populations were recorded for the entire life-span of the flies under 18 and 28 °C, and τ and precision was estimated as described in the first chapter. Due to time constraints the recording in 25 °C was carried out for only for the first 20-days. Flies were transferred into activity tubes containing fresh food medium on every 8^{th} day of the recording. The rest of the details of the assay protocol can be found in the methods section of the first chapter.

Statistical Analysis

The total duration of recording was divided into multiple 5-day age-windows, and τ as well as precision of activity-rest rhythm in DD was calculated across each of these age-windows. Repeated measures ANOVA was carried out to test for statistically significant difference in τ and precision, with 'population' and 'age-window' as fixed, and 'block' as random factors. The Q_{10} value for different populations was computed as the ratio of the τ at lower temperature (τ_{18}) to τ at higher temperature (τ_{28}), and a mixed model randomized block design ANOVA was carried out on the block means with 'population' as a fixed factor and 'block' as a random factor.

Results

'late' populations display poor temperature compensation ability as compared to the 'control' and 'early' populations

ANOVA on the Q_{10} values revealed a statistically significant effect of 'population' ($F_{2,6}$=46.36, p<0.01). While the 'early' and 'control' populations did not differ from each other, and showed Q_{10} values closer to 1 (0.985 and 0.968 respectively), the 'late' populations were significantly weakly compensated as compared to both the 'early' and 'late' populations, and showed a Q_{10} value of 0.939 (Figure 1.1).

'late' populations show longer τ and higher precision at 25 °C

A repeated measures ANOVA on the τ values at 25 °C revealed a statistically significant effect of 'population' ($F_{2,6}$=62.94, p<0.01), with the 'late' populations exhibiting longer τ values across all the age-windows (τ=24.3-h) followed by 'control' (τ=24.04-h) and 'early' (τ=23.6-h) populations (Figure 1.2a). There was no statistically significant effect of 'age-window' ($F_{3,9}$=0.94, p>0.01) or 'population' × 'age-window' interaction ($F_{6,18}$=0.90, p>0.01), suggesting that the τ of free-running activity-rest rhythm of the three sets of populations does not change in an age-dependent manner.

There was a statistically significant effect of 'population' ($F_{2,6}$=19.74, p<0.01) and 'age-window' ($F_{3,9}$=5.23, p<0.02) on precision. Post-hoc multiple comparisons using Tukey's HSD revealed that the 'late' populations show significantly higher precision (4.71-h^{-1}) as compared to the 'control' (4.14-h^{-1}) populations, whereas the 'early' (3.73-h^{-1}) populations show significantly

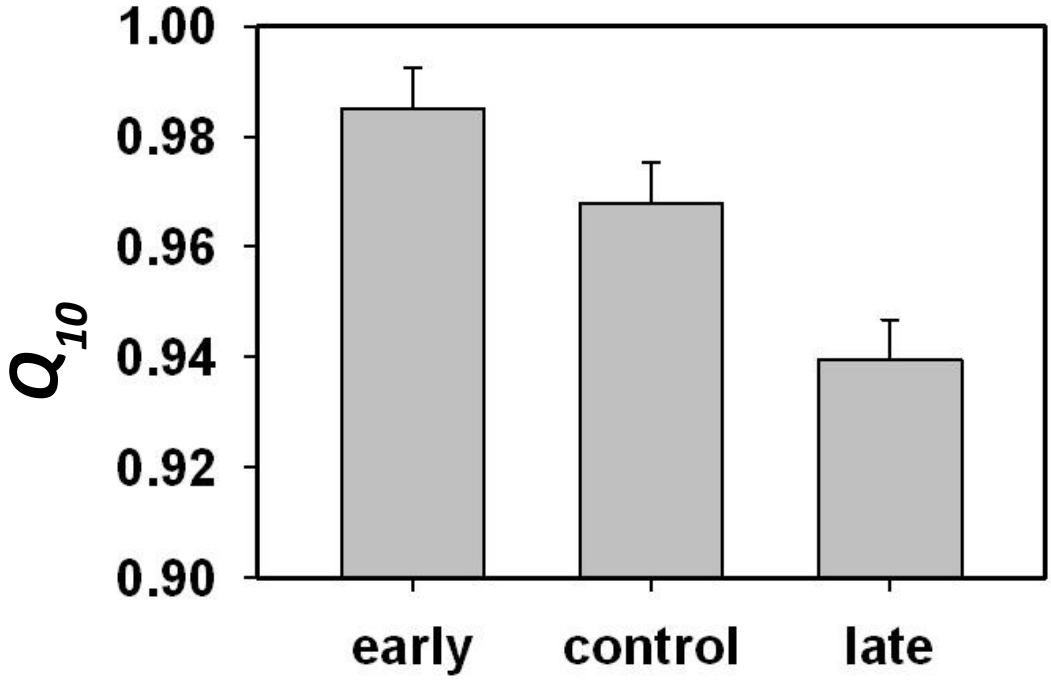

Figure 1.1: Q_{10} values (see text) of the 'early', 'control' and 'late' populations. Error bars are 95% CI.

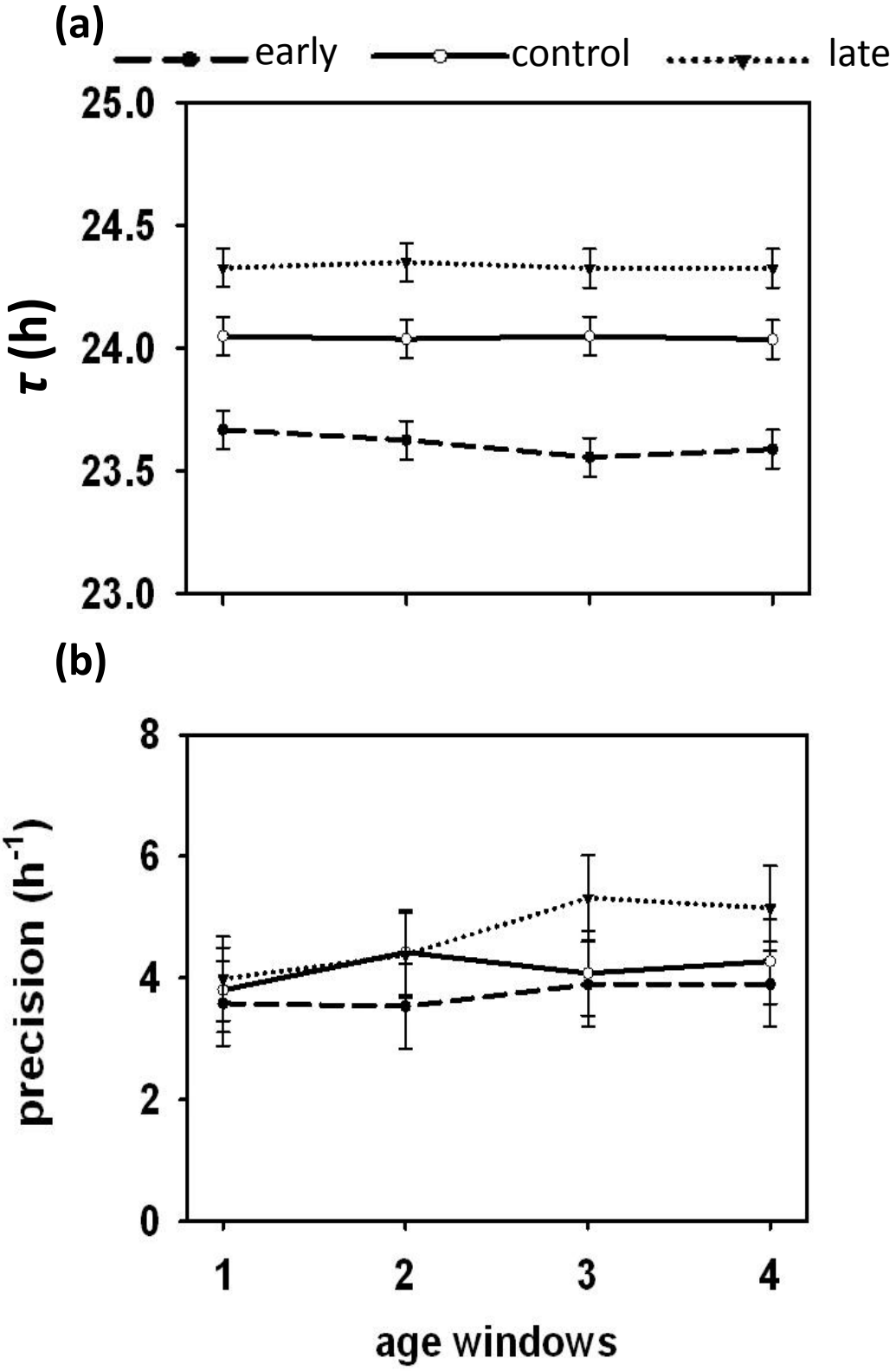

Figure 1.2: (a) Period (τ) and (b) precision across age windows for the 'early', 'control' and 'late' populations at 25°C. Error bars are 95% CI.

lower precision as compared to 'control' populations (Figure 1.2b); however, there was no significant difference in precision across different age-windows. The effect of 'population' × 'age-window' interaction ($F_{6,18}$=1.59, p>0.01) on precision was also statistically not significant, suggesting that the precision of free-running activity-rest rhythm does not change across populations in an age-dependent manner.

'late' populations show a longer τ and higher precision at 28 °C

ANOVA on the τ values at 28 °C revealed a statistically significant effect of 'population' ($F_{2,6}$=246.95, p<0.01) and 'age-window' ($F_{3,9}$=5.023, p=0.026) with the 'late' populations exhibiting a significantly longer τ across all age-windows (τ=24.82-h) followed by the 'control' (τ=24.09-h), and 'early' populations (τ=23.52-h, Figure 1.3a). Post-hoc multiple comparisons did not reveal any difference in period across age-windows. The effect of 'population'× 'age-window' interaction was also statistically not significant ($F_{6,18}$=1.19, p>0.05).

A statistically significant effect of 'population' ($F_{2,6}$=10.35, p=0.011) and 'age-window' on precision ($F_{3,9}$=34.77, p<0.01) was observed at 28 °C. Post-hoc multiple comparisons using Tukey's HSD revealed that the 'late' populations show greater precision (2.47-h^{-1}) as compared to 'early' (1.89-h^{-1}) populations whereas they do not differ from 'control' (2.56-h^{-1}) populations (Figure 1.3b). Post-hoc multiple comparisons using Tukey's HSD revealed that the average precision across the first age-window (2.69-h^{-1}) was significantly greater than that of the second (1.93-h^{-1}) and fourth (2.25-h^{-1}) age-windows, but was not different from the third age-window (2.35-h^{-1}) and the second, third and fourth age-windows also did not differ from each other.

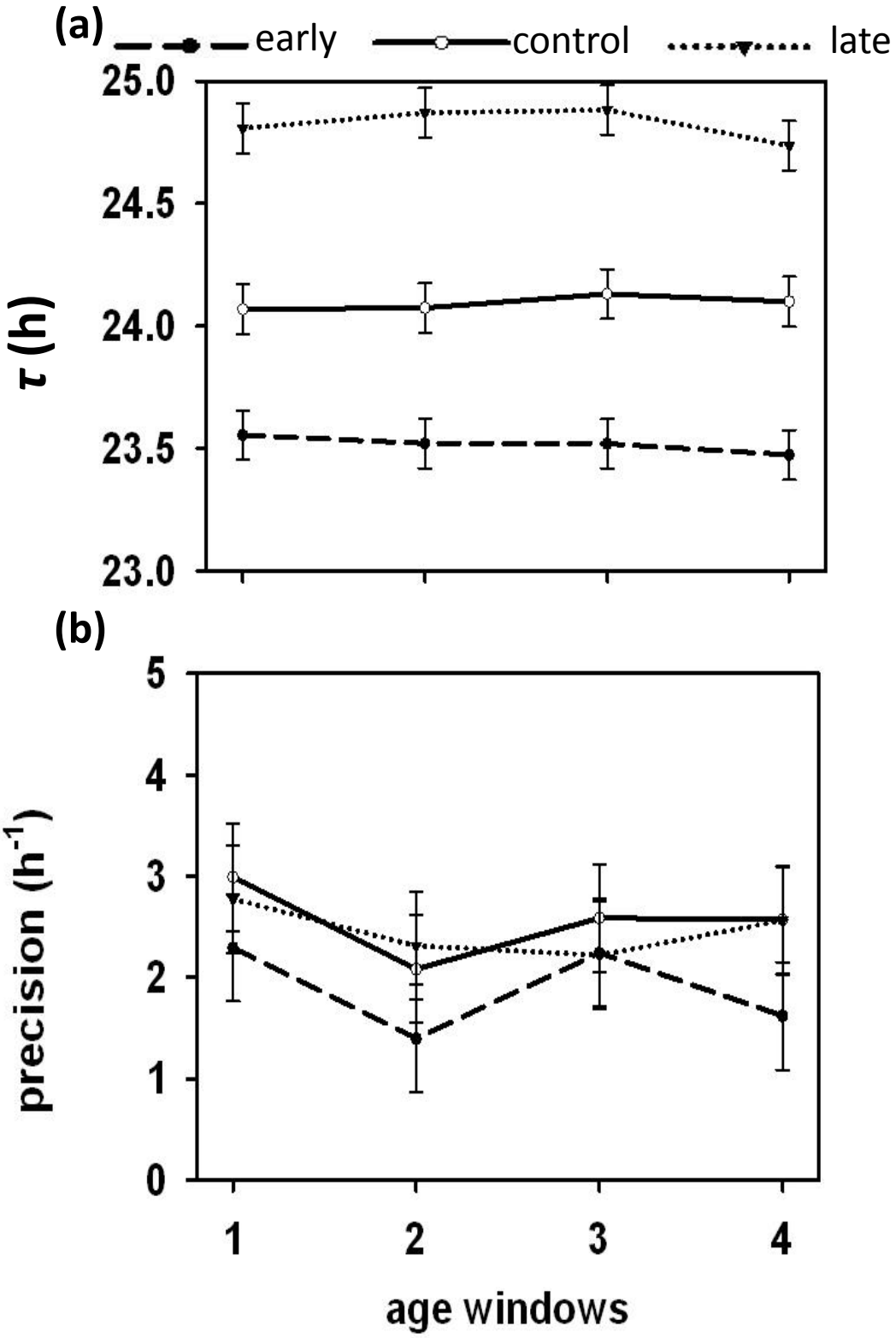

Figure 1.3: (a) Period (τ) and (b) precision across age windows for the 'early', 'control' and 'late' populations at 28°C. Error bars are 95% CI.

There was no statistically significant effect of 'population' × 'age-window' interaction on both τ ($F_{6,18}$=1.19, p>0.05) as well as precision ($F_{6,18}$=1.37, p>0.05) at 28 °C, suggesting that population level differences in τ and precision seen under 25 °C is maintained even at a higher temperature.

'late' populations show longer τ under 18 °C

ANOVA on the τ values at 18 °C revealed a statistically significant effect of 'population' ($F_{2,6}$=12.44, p<0.01) and 'age-window' ($F_{3,16}$=19.98, p<0.01) with the 'late' populations exhibiting a significantly higher τ across all age-windows (τ=23.44-h) as compared to the 'early' (τ=23.32-h) populations but did not differ from the 'control' (τ=23.48-h) populations (Figure 1.4a). Post-hoc multiple comparisons using Tukey's HSD revealed that the τ of activity-rest rhythm across age-windows 8 (23.6-h), 9 (23.57-h) and 10 (23.54-h) was greater than that seen across the first five age-windows, the former and the latter did not differ among each other. There was, however, no statistically significant 'population' × 'age-window' interaction ($F_{32,96}$=0.50, p>0.01) on τ at 18 °C.

ANOVA on precision values at 18 °C revealed a statistically significant effect of 'age-window' ($F_{3,16}$=23.01, p<0.01) and 'population' × 'age-window' interaction ($F_{32,96}$=1.85, p<0.01). Post-hoc multiple comparisons using Tukey's HSD revealed that on an average precision in age-windows 12 and 14-17 was greater than that seen in age-windows 1-5 and 7, and the remaining windows did not differ from either of these windows. Precision values were found to increase in 'control' populations after the first half of the life as compared to 'early' and 'late' populations (Figure 1.4b). There was no significant effect of 'population' ($F_{2,6}$=1.66, p>0.05). These results

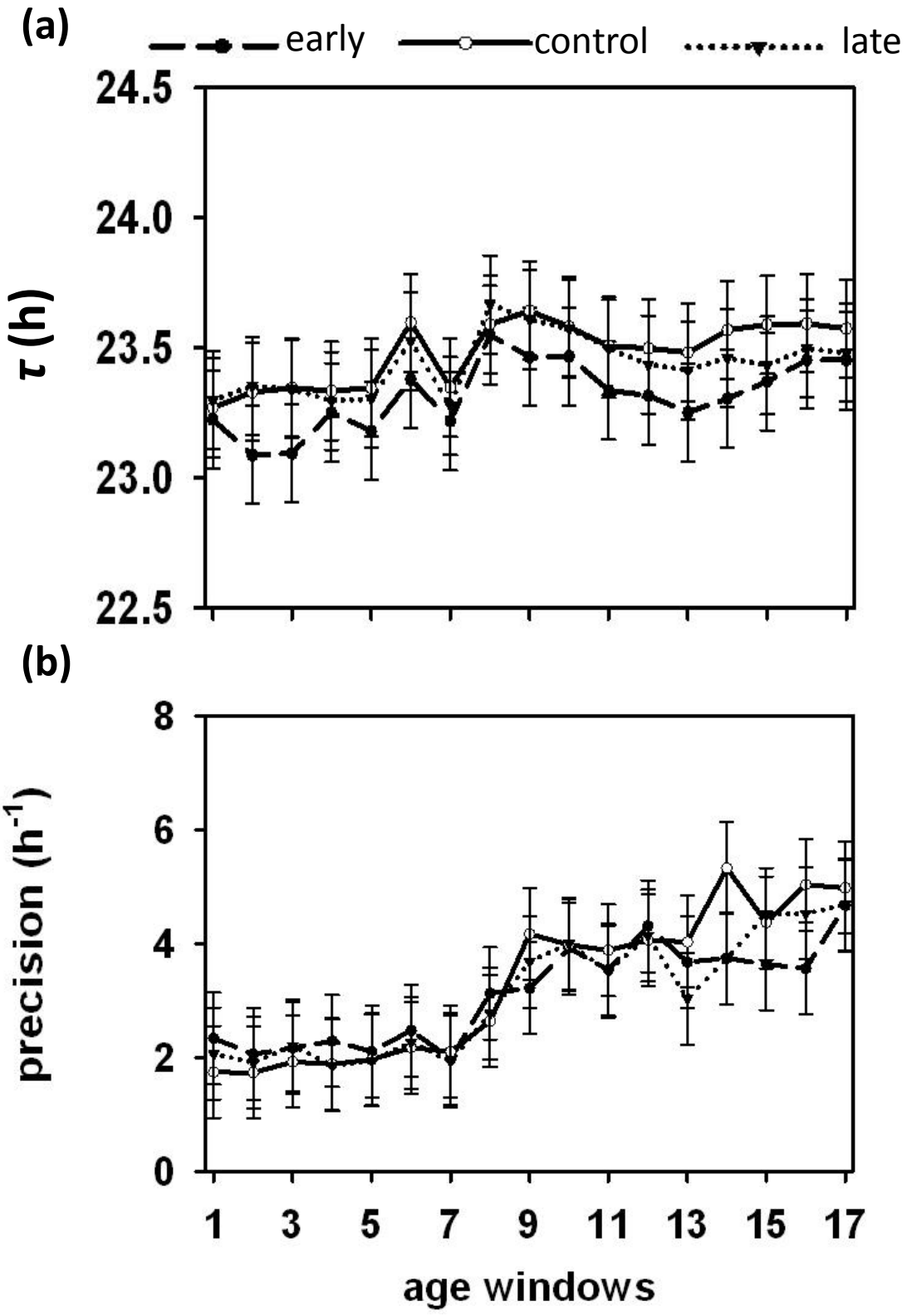

Figure 1.4: (a) Period (τ) and (b) precision across age windows for the 'early', 'control' and 'late' populations at 18°C. Error bars are 95% CI.

indicate that although the differences seen in τ under both 25 °C and 28 °C persists, the differences observed in precision between the populations is lost at 18 °C.

Discussion

Following results from Nikhil et al (2016a) we hypothesized that the 'late' populations may have evolved higher period lability, and decided to test if this manifests in terms of temperature compensation or Q_{10} values, period and precision in the 'early', 'control' and 'late' populations across different ambient temperatures. The Q_{10} value was found to be significantly lower for the 'late' populations as compared to the 'early' and 'control' populations, while no significant differences were observed in the Q_{10} values between the latter two populations, suggesting that the 'late' populations have evolved weakly temperature compensated circadian clocks (Figure 1.1). Furthermore, the greater change in τ brought about by change in temperature corroborates the hypothesis that the 'late' populations indeed have a greater inherent lability in τ.

We further wished to examine if the stability of τ differs across the study populations in a temperature dependent manner. The rationale being that the 'late' populations by virtue of having evolved a weakly coupled network of circadian oscillators would exhibit lower precision across temperatures as compared to the other two populations. In this regard we observed that the 'late' populations indeed exhibit higher precision as compared to the 'early' and 'control' populations, across all the different ambient temperatures studied (Figures 1.2-1.4). This is quite surprising as weakly coupled oscillator networks would be expected to exhibit

lower precision in free-running rhythms due to inherent noise and other associated factors (Aschoff, 1981). However, the results of my study do not conform to these expectations and it is likely that higher precision in the 'late' populations might stem from two other properties of the oscillator network. One is that the 'late' populations exhibit higher intrinsic amplitude (Nikhil et al., 2016a), which is known to be associated with higher precision of circadian clocks. Furthermore, a recent study by Webb et al (2012) proposed that weakly coupled cells tend to resynchronize better as compared to strongly coupled networks, which might explain the observed higher precision in the 'late' populations as it could also stem from either higher amplitude or higher mutual re-synchrony of the circadian clock in these populations. Interestingly, Nikhil et al (2015) showed that the 'late' populations exhibited higher accuracy in both activity-rest and emergence rhythms under multiple environments, which was not associated with higher precision of the underlying circadian clocks. However, as discussed in the introduction, the precision in Nikhil et al (2015) study was assessed only during the first few days after transfer to DD. Therefore, it is possible that the observed lack of differences in precision between the three sets of populations might be masked or altered due to after-effects of the exposure to LD cycles just before the transfer to DD, and long-term recordings under constant darkness might reveal the underlying differences in clock precision, which is seen from the results of this study.

Another interesting aspect that we noticed was that even though the 'late' populations exhibit higher precision under both the higher temperatures, these differences were not seen at 18 °C. A closer look at the data revealed that this is mostly due to a greater reduction in precision of

the ‘late’ populations as compared to the ‘early’ and ‘control’ populations (Figure 1.4b). This suggests that the ability of the ‘late’ populations to maintain higher precision is not robust across temperatures. Furthermore, at 18 °C, where there is a drastic reduction in τ, the decrease in precision is so much that the difference seen in precision at both 28 °C and 25 °C is lost (Figures 1.2b, 1.3b, 1.4a, b), it is likely that 18 °C is below the range of temperatures at which the ‘late’ populations can compensate for changes brought about in τ, and this could explain the drastic reduction in their precision. Therefore, whenever there is a change in the environmental conditions due to which τ is affected, there also seems to be a reduction in precision in ‘late’ populations.

In conclusion, while the differences in τ among the ‘early’, ‘control’ and ‘late’ populations are maintained across multiple temperatures, the same does not seem to be the case for precision. It appears that the ability to maintain higher precision at a given temperature reduces the robustness of the clock network in the ‘late’ populations resulting in large magnitude changes in precision as temperature decreases thus highlighting a less robust clock network in the ‘late’ populations.

Chapter 3

Introduction

After-effects or history-dependent effects in the context of circadian rhythms are the effects of previous environments on the free-running period (τ) of the rhythm, and such effects are found to manifest immediately after the organism is moved into constant darkness (DD; Pittendrigh, 1960; Pittendrigh and Daan, 1976b). The environments inducing after-effects may include light-dark (LD) and/or thermophase-cryophase (TC) cycles with varying photo- and thermo-periods, or LD and TC *T*-cycles (period of the entraining cycles; Pittendrigh and Daan, 1976b). It has been observed that in nocturnal as well as diurnal animals, τ of the locomotor activity rhythm shortens or lengthens after an exposure to short or long *T*-cycles respectively, however, τ after exposure to long photoperiods shortens in nocturnal animals and lengthens in diurnal animals, and the reverse is seen for short photoperiods (Pittendrigh and Daan, 1976b). After-effects of τ have been observed across vertebrates and invertebrates, with most studies being carried out on mice and hamsters (Pittendrigh and Daan, 1976b), cockroaches (Page and Block, 1980) and a few other insects (Christensen and Lewis, 1982).

A previous study on 'early', 'control' and 'late' populations of fruit flies *Drosophila melanogaster* reported that a greater percentage of flies from the 'late' populations entrain to both *T*18 and *T*30 LD cycles as compared to the other two populations, and that the 'late' populations are likely to entrain to such deviant *T*-cycles via period changes (as indicated by the greater area under the PRC; Nikhil et al., 2016a).

In light of the above result we hypothesized that the 'late' populations may have evolved higher period lability that may facilitate successful entrainment to a wider range of *T*-cycles, and some evidence supporting this hypothesis is also provided in the previous chapter. To further test this hypothesis, we studied the effect of exposure to LD and TC *T*-cycles with *T* far removed from 24-h, on the τ of activity/rest rhythm of 'early', 'control' and 'late' populations with the expectation that if 'late' populations have evolved higher period lability then they would also show greater after-effects.

Materials and Methods

Activity/rest rhythm assay: The basic protocol has been described in detail in the materials and methods section of the first chapter. Following are the details pertaining to the specific experiments performed in this chapter.

The activity-rest behavior of 3-4 day old virgin males was recorded using DAM monitor (Trikinetics, Waltham). The activity-rest behavior of flies from 'early', 'control' and 'late' populations was recorded for a minimum of 7-days in DD at 25 °C and the τ values (τ_1) were calculated, following which the flies were exposed to the respective LD or TC *T*-cycles (*T*18, *T*24 and *T*30). The light intensity during the light phase of LD cycles was 100 lux and temperature was maintained constant at ~25 °C, whereas in the TC cycles the temperature cycled between 18 °C and 28 °C in DD. After being subjected to the *T*-cycles, activity-rest behaviour of flies was monitored for another 7-days in DD at 25 °C and the τ values (τ_2) were estimated. A set of flies from each of the populations was also recorded in DD at 25 °C for the entire duration of the

experiment, and these flies served as age-matched controls. Flies were transferred into activity tubes containing fresh food media on the first and last days of the *T*-cycles during the respective photo- and thermo-phases.

Statistical analysis: After-effect was defined as the difference in τ of activity-rest rhythm before and after being subjected to the *T*-cycles (τ_2-τ_1). A mixed model ANOVA was carried out to test for statistically significant difference in after-effects with 'population' and '*T*-cycle' as fixed factors and block as random factor. To estimate the extent of after-effects that could be attributed to the *T*-cycles alone, and not to age-related changes in τ, after-effects of each regime was compared to that of the respective age-matched controls.

Results

After-effects of Light-Dark *T*-cycles

Although ANOVA on after-effects of LD *T*-cycles showed a statistically significant effect of '*T*-cycle' ($F_{3,9}$=20.82, p<0.05), post-hoc comparisons using Tukey's HSD did not reveal any statistically significant difference between different *T*-cycles (*T*30: 0.24-h, *T*24: 0.17-h, *T*18: -0.10-h, and DD: -0.02-h; Figure 2.1). Moreover, the effect of 'population' ($F_{2,6}$=2.78, p>0.05) and 'population' × '*T*-cycle' ($F_{4,12}$=0.469, p>0.05) interaction was statistically not significant.

After-effects of Temperature *T*-cycles

ANOVA on after-effects revealed a statistically significant effect of 'population' ($F_{2,6}$=7.41, p<0.05) and '*T*-cycle' ($F_{3,9}$=10.26, p<0.05), and post-hoc multiple comparisons using Tukey's HSD

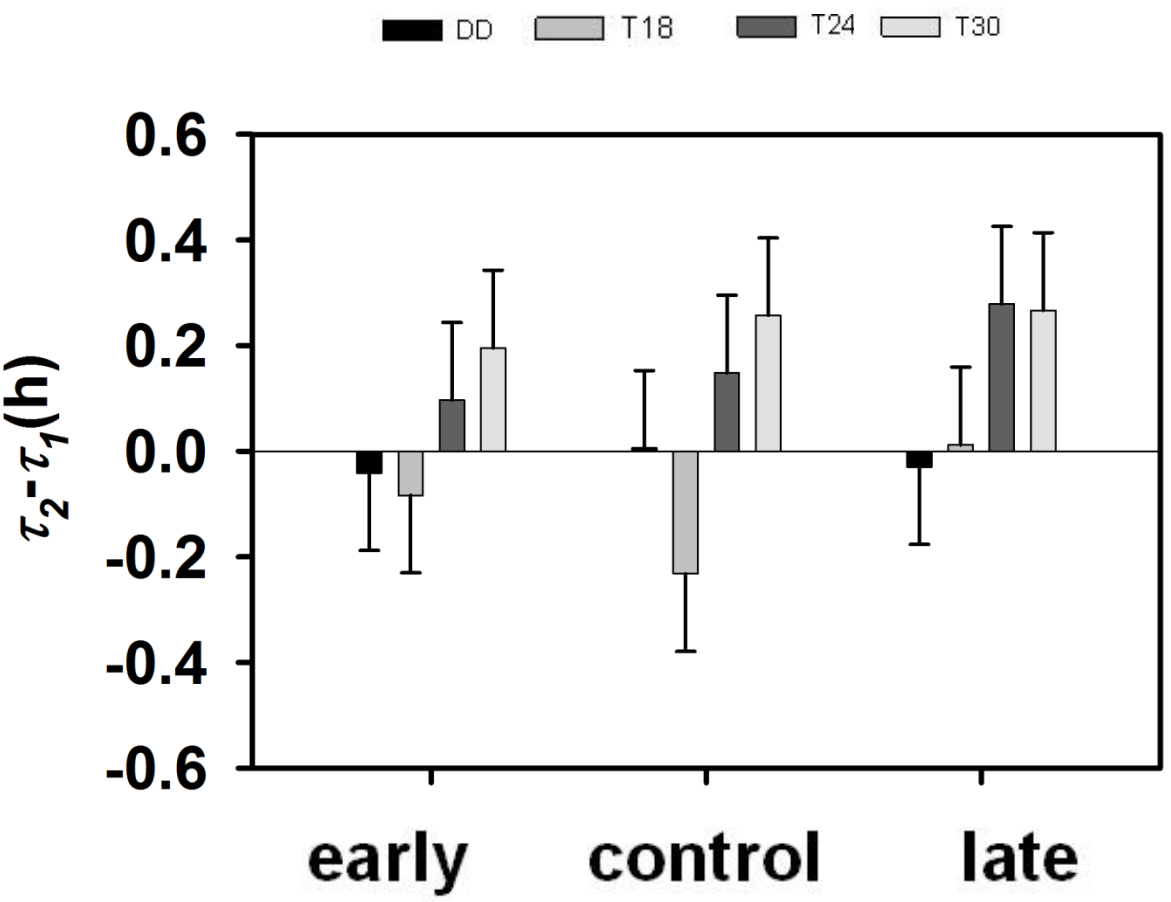

Figure 2.1: After-effects (τ_2-τ_1) of LD (Light-Dark) *T*-cycles of the 'early', 'control' and 'late' populations. Error bars are 95% CI.

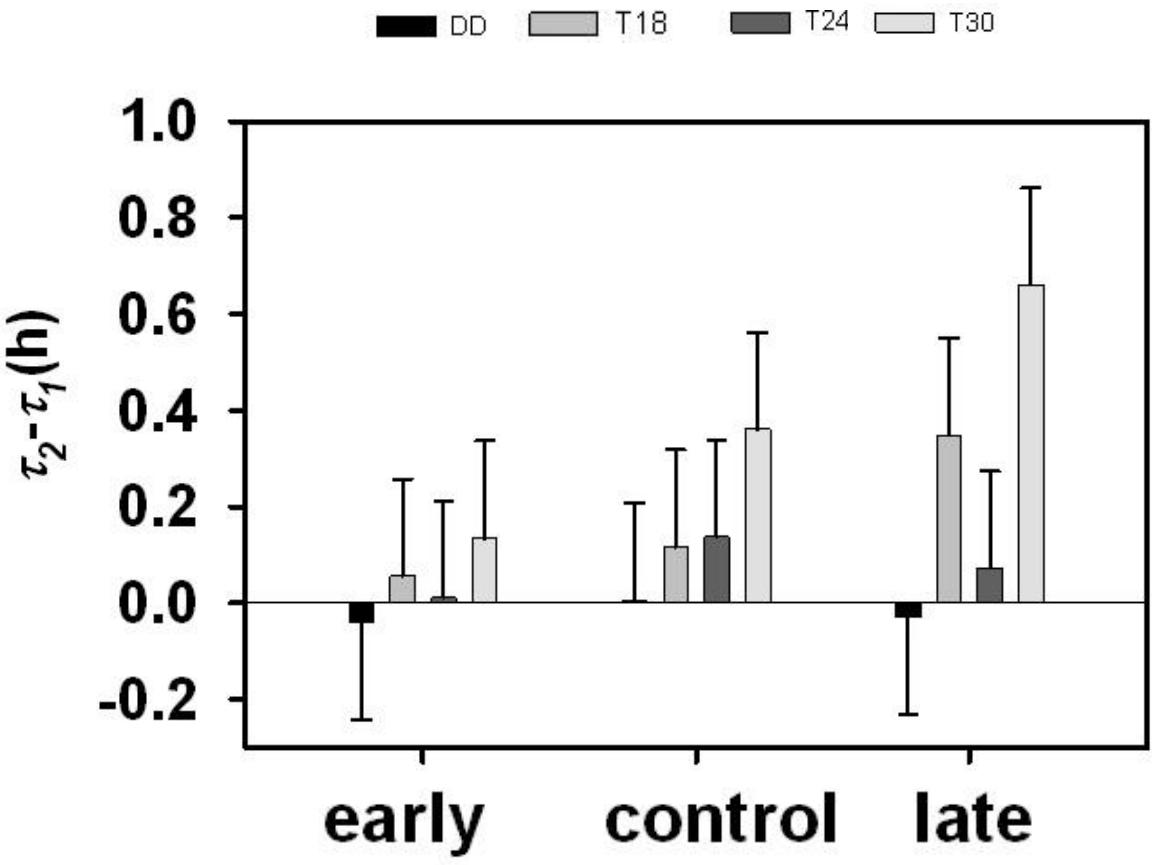

Figure 2.2: After-effects (τ_2-τ_1) of TC (Thermophase-Cryophase) *T*-cycles of the 'early', 'control' and 'late' populations. Error bars are 95% CI.

revealed that none of the populations differed from each other ('early': 0.04-h, 'control': 0.15-h, 'late': 0.26-h). However, *T*30 had statistically significant after-effects (DD: -0.02-h, *T*18: 0.07-h, *T*24: 0.07-h, *T*30: 0.17-h). Additionally, ANOVA showed a statistically significant effect of 'population' × '*T*-cycle' interaction ($F_{6,18}$=2.76, p<0.05), and post-hoc multiple comparisons revealed that 'late' populations show statistically significant after-effects but only after being entrained to *T*30 (Figure 2.2).

Discussion

In order to test the hypothesis that the 'late' populations have evolved greater period lability as a correlated response to selection on timing of emergence, we estimated the effect of exposure to several LD or TC *T*-cycles on the τ of activity-rest rhythm of the 'early', 'control' and 'late' populations and found that while population-level differences in τ (τ_{early}<$\tau_{control}$<τ_{late}) persisted across all the regimes, the 'late' populations showed a statistically significant after-effect when exposed to *T*30 TC cycles (Figures 2.1, 2.2). However, none of the populations showed any differences in after-effects following entrainment to LD *T*-cycles. One possible reason for this might be that temperature may be a more potent zeitgeber as compared to light. Alternatively, the strength of LD cycles (100 lux during the light phase) might not be sufficient to elicit detectable after-effects in these populations.

Although the results from Nikhil et al (2016a), and those discussed in the previous chapter indicate that 'late' populations may have evolved greater period lability, this does not seem to manifest in terms of after-effects of most of the entraining *T*-cycles and there could be a few

possibilities as to why this may the case. Although differences in the level and nature of coupling could drive period lability, it might not be the only clock property which could be thought to do so. Regardless of the manner in which period lability is brought about, one can imagine that a greater sensitivity of the circadian clocks to the zeitgebers would influence τ to a greater extent, and this effect would persist for a longer time as compared to clocks which have inherently lower sensitivity to the zeitgebers. The 'late' populations have earlier been shown to have evolved reduced levels of the circadian photoreceptor CRYPOCHROME (CRY), and hence reduced circadian photosensitivity (Nikhil et al., 2016b), which might be the reason for them not showing significant after-effects to any of the LD *T*-cycles.

On the other hand, it has typically been observed that as a result of after-effects, organisms exhibit τ close to *T*, at least for an initial duration in DD, after which their τ gradually approaches the steady-state values in due course of time (Pittendrigh and Daan, 1976b). For instance, following entrainment to *T*22 LD cycles, the τ of locomotor activity rhythm of hamsters was initially found to be much shorter than that prior to entrainment, and was eventually found to lengthen to attain the steady-state τ (Pittendrigh and Daan, 1976b). Estimating the rate at which circadian clocks return to their steady-state τ following after-effects would therefore be an additional measure of period lability and might highlight differences between the populations, and could provide evidence in favor of increased period lability in the 'late' populations.

Although previous studies indicate a possible difference in period lability between 'early' and 'late' populations, an estimation of after-effects of the entraining *T*-cycles in terms of the

difference between τ values before and after entrainment does not show a difference in the extent to which the 'early', 'control' and 'late' populations are affected. Other methods of estimation of after-effects (rate of regression back to steady-state τ) might serve to elicit such differences between the populations. Therefore the hypothesis that the 'late' populations may have evolved higher period lability which manifests itself in terms of after-effects awaits conclusive validation.

Chapter 4

Introduction

Phenotypic plasticity is the ability of organisms to show altered trait values as a result of environmental (ecological) effects without changing the genotype. This is a complex trait that is itself subject to evolution and is also seen during development (West-Eberhard, 2003). Phenotypic plasticity of circadian clocks can manifest due to environmental conditions experienced during pre-adult developmental stages (developmental plasticity) as well as adult-stages (adult plasticity). Circadian clocks show a range of τ values which are largely determined by the genotype, however, a few studies have reported developmental plasticity in circadian period τ (Barrett and Page, 1989; Page and Barret, 1989; Sheeba et al., 2002), while others have studied the after-effects of different environmental regimes on the adult circadian clocks (Pittendrigh et al., 1958; Pittendrigh, 1960; Pittendrigh and Daan, 1976b).

The handful of studies that do assess the developmental plasticity of circadian clocks have only estimated the effects of varying light-dark (LD) *T*-cycles and photoperiods (different light and dark duration) on τ in some insect species since light is known to affect the circadian clocks even at the developmental stage. In a series of experiments on cockroaches Barrett and Page (1989) assessed the extent of developmental plasticity due to LD *T*-cycles by estimating the clock properties at the adult stage. They found that τ of the activity-rest rhythm of adult cockroaches was determined by the LD cycles that they were reared in as pre-adults, such that τ of individuals reared under *T*22 was significantly shorter than those reared under *T*24, while the

τ of individuals reared under *T*26 was significantly longer than both *T*22 and *T*24 reared individuals, and they found that the altered τ values remained stable for over 7-months in constant darkness (DD), and did not change even when subjected to varying photoperiods (Barrett and Page, 1989). LD cycles were found to play an important role in the development of circadian clocks in zebra fish as the lack of entraining stimulus during rearing stages resulted in a higher percentage of arrhythmic adults (Hurd and Cahill, 2002). Therefore, developmental plasticity of circadian clocks is a conserved aspect of circadian timekeeping, and occurs across a diverse range of taxa despite there being a drastic difference in their developmental processes.

In *Drosophila*, despite it being holometabolous, light pulses administered during the early first instar larval stage have significant effects on the adult circadian clocks such that the time of administration of pulse determines the phase of the adult activity-rest rhythm (Sehgal et al. 1992; Malpel et al,. 2004). Subsequently in *Drosophila*, it was shown that τ is also influenced by the pre-adult rearing environment such that τ_{LL} (LL: constant light) is shorter than τ_{DD} (Tomioka et al., 1998). This study also showed that the ability of circadian clocks to entrain to LD cycles as larvae was dependent on the extent of mismatch between τ and *T* such that per^{L} flies showed free-running rhythm (τ ~28-h) after being subjected to extremely short photo-periods (Tomioka et al., 1998). Another study which estimated the developmental plasticity of circadian clocks in *Drosophila melanogaster* populations reared in LL for over 600 generations found that rearing under LD, DD and LL for one generation alone affected the τ of the activity-rest rhythm of the adults such that $\tau_{DD} < \tau_{LL} < \tau_{LD}$ (Sheeba et al., 2002). A study on *D. melanogaster* populations selected for faster pre-adult development revealed that the pre-

adult developmental rate of flies was affected under different ambient and cyclic temperature conditions, and found that the magnitude of difference in τ between the selected and control populations varied with different temperature regimes (Yadav and Sharma, 2013). These studies conclusively demonstrate that the circadian clocks of *Drosophila* exhibit developmental plasticity to different pre-adult rearing environments in a manner similar to that seen in other organisms.

Previous studies have shown that as adults, the 'late' populations of *D. melanogaster* have a wider range of entrainment as compared to the 'early' and 'control' populations, as a greater proportion of individuals from the 'late' populations were found to entrain to extreme *T*-cycles (Nikhil et al., 2016a). The 'late' populations were also found to have a higher intrinsic amplitude of the clock and could possibly be different from the 'early' and 'control' populations in their ability to integrate light over longer durations, indicating that both these factors, as well as a possible difference in the inter-oscillator coupling in the 'late' populations could be responsible for the wider entrainment range and lower relaxation rates that were observed (Nikhil et al., 2016a).

Studying the effects of varying cyclic environmental cues and constant ambient temperatures on the periodicity and precision of the adult activity-rest rhythm (adult plasticity in these clock properties) has revealed differences in the extent of responses in the 'early', 'control' and 'late' populations (Chapters 2 and 3). The 'late' populations tend to show greater variability in τ and increased precision relative to the other two populations under different constant ambient temperatures (Chapter 2). In addition, the 'late' populations show significant after-effects

under *T*30 TC cycles (Chapter 3), thereby collectively suggesting increased ability of the 'late' populations to mold their τ in response to different environmental stimuli. In order to test if this ability to mold the τ in response to different environmental stimuli is also present during the pre-adult stages, we assayed the effects of such environmental stimuli on the period and precision of flies exposed to different LD and TC (Thermophase-Cryophase) *T*-cycles during their pre-adult developmental stages.

Materials and methods

Experimental populations: Refer to details of selection protocol and maintenance regime in the first chapter.

Activity-rest rhythm assay: The basic protocol has been described in the materials and methods section of the first chapter. Following are the details pertaining to the experiments performed in this chapter alone.

Rearing conditions and activity-rest behaviour assay: ~300 eggs were collected from the standardized populations and were dispensed into glass vials containing banana-jaggery medium. These populations were then reared under *T*-cycles of *T*18, *T*24, *T*30 LD and TC cycles, and one set of each of the above mentioned populations was also reared in DD. Virgin males were collected from the different developmental regimes soon after emergence and transferred into DD at 25 °C for activity recording as soon as sufficient number of flies had emerged. Virgin collection was done a few hours before, after, and once during the photo- and thermo-phase. Care was taken to ensure that flies from different populations did not

experience any cyclic time-cue post emergence. The light intensity during the light phase of the LD cycles was 100 lux with a constant temperature of 25 °C, and the temperature cycled between 18 °C and 28 °C in the TC cycles in constant darkness. The activity-rest recording was carried out for ~22-days and flies were transferred into activity tubes containing fresh sucrose-yeast medium on the 11th day of recording.

Statistical analysis

A mixed model randomized block design ANOVA was carried out to test for statistically significant effects of different rearing conditions on τ and precision (dependent variables) of activity-rest rhythm in the 'early', 'control' and 'late' populations with 'population' and 'developmental regime' as fixed factor and 'block' as a random factor.

Results

Comparison of the effect of development under different LD regimes on the activity-rest rhythm of the three sets of populations

ANOVA on the period values showed a statistically significant effect of 'population' ($F_{2,6}$=15.73, p<0.01) and 'developmental regime' ($F_{2,6}$=24.22, p<0.01). Post-hoc comparisons using Tukey's HSD revealed that the 'early' (23.52-h) and 'late' (24.22-h) populations had significantly shorter and longer periods as compared to the 'control' (23.89-h) populations, and on an average period following development in *T*18LD (23.74-h) was shorter than that following *T*24LD (24.01-h), but did not differ from any of the other regimes (DD: 23.86-h; *T*30: 23.88-h). There was no significant effect of 'population' × 'developmental regime' interaction ($F_{6,18}$=0.45, p>0.05),

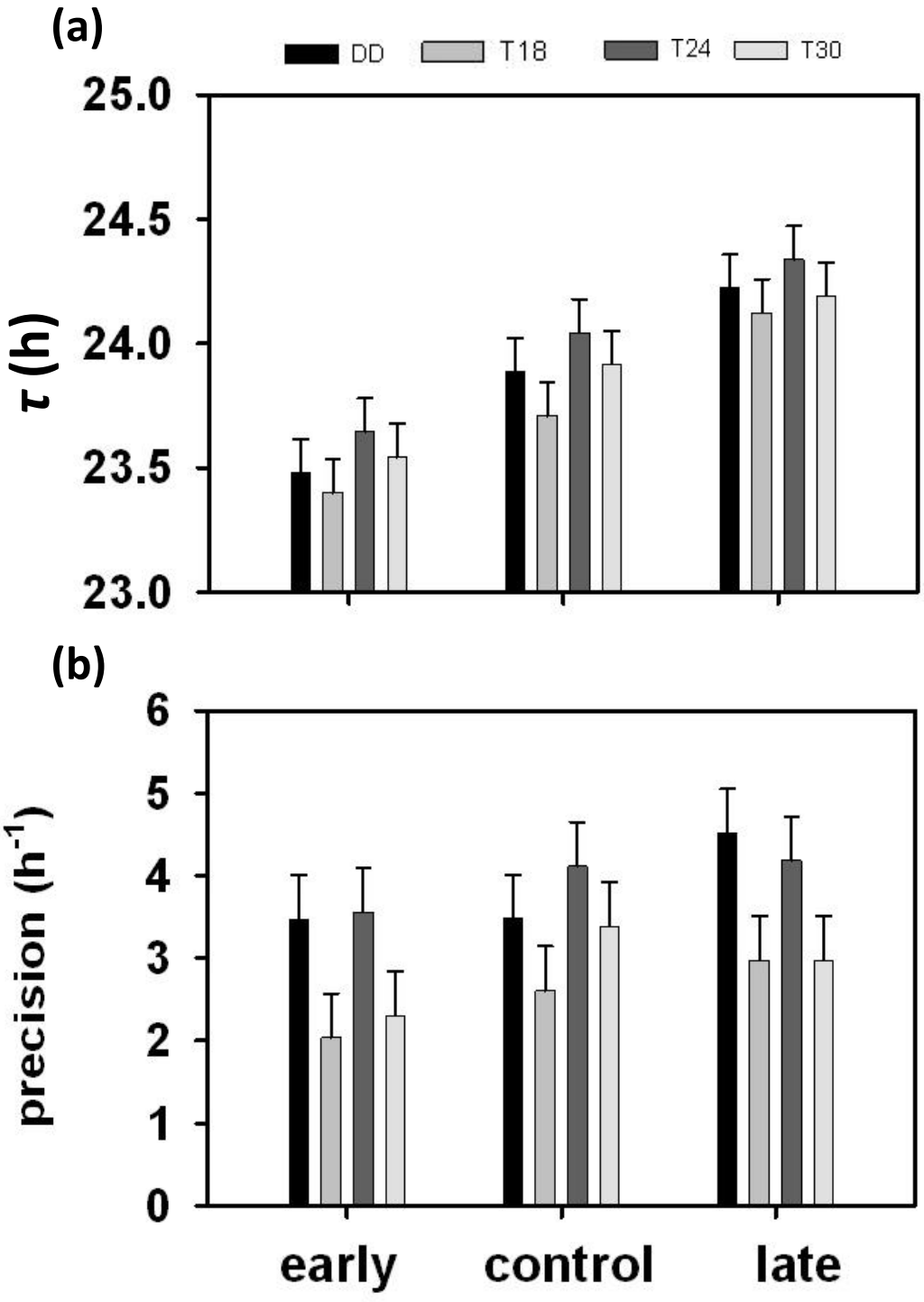

Figure 3.1: (a) Period (τ) and (b) precision of the 'early', 'control' and 'late' populations after development under LD (Light-Dark) *T*-cycles. Error bars are 95% CI.

indicating that the different developmental regimes had a similar effect on the period values of the three sets of populations (Figure 3.1a).

Similarly, ANOVA on the precision values showed a statistically significant effect of 'population' ($F_{2,6}$=138.18, p<0.01) and 'developmental regime' ($F_{2,6}$=9.24, p<0.01; Figure 3.1b). Post-hoc multiple comparisons using Tukey's HSD revealed that the 'early' (2.84-h^{-1}), 'control' (3.40-h^{1}) and 'late' (3.66-h^{-1}) populations did not differ from each other. However, post-hoc comparisons using Tukey's HSD revealed that on an average, clock precision after development in *T*18 LD (2.54-h^{-1}) cycles was significantly lower than that after development in DD (3.82-h^{-1}) and *T*24 LD (3.95-h^{-1}) but did not differ from *T*30 LD (2.89-h^{-1}), and precision after development in DD, *T*24 LD and *T*30 LD did not differ from each other. There was no statistically significant effect of 'population' × 'developmental regime' interaction ($F_{6,18}$=2.43, p>0.05), indicating that the magnitude of differences in precision between the study populations did not change across regimes.

Comparison of the effect of development under the different TC regimes on the activity-rest rhythm of the three sets of populations

ANOVA on the period values showed a statistically significant effect of 'population' ($F_{2,6}$=276.38, p<0.01) and 'developmental regime' ($F_{2,6}$=10.55, p<0.01). Post-hoc multiple comparisons using Tukey's HSD revealed that the 'late' (24.33-h) populations showed a significantly longer period as compared to the 'control' (23.97-h) populations, and the 'early' (23.56-h) populations showed a significantly shorter period as compared to the 'control' populations, and that τ following development in *T*30 (24.04-h) was significantly longer than that following DD (23.86-

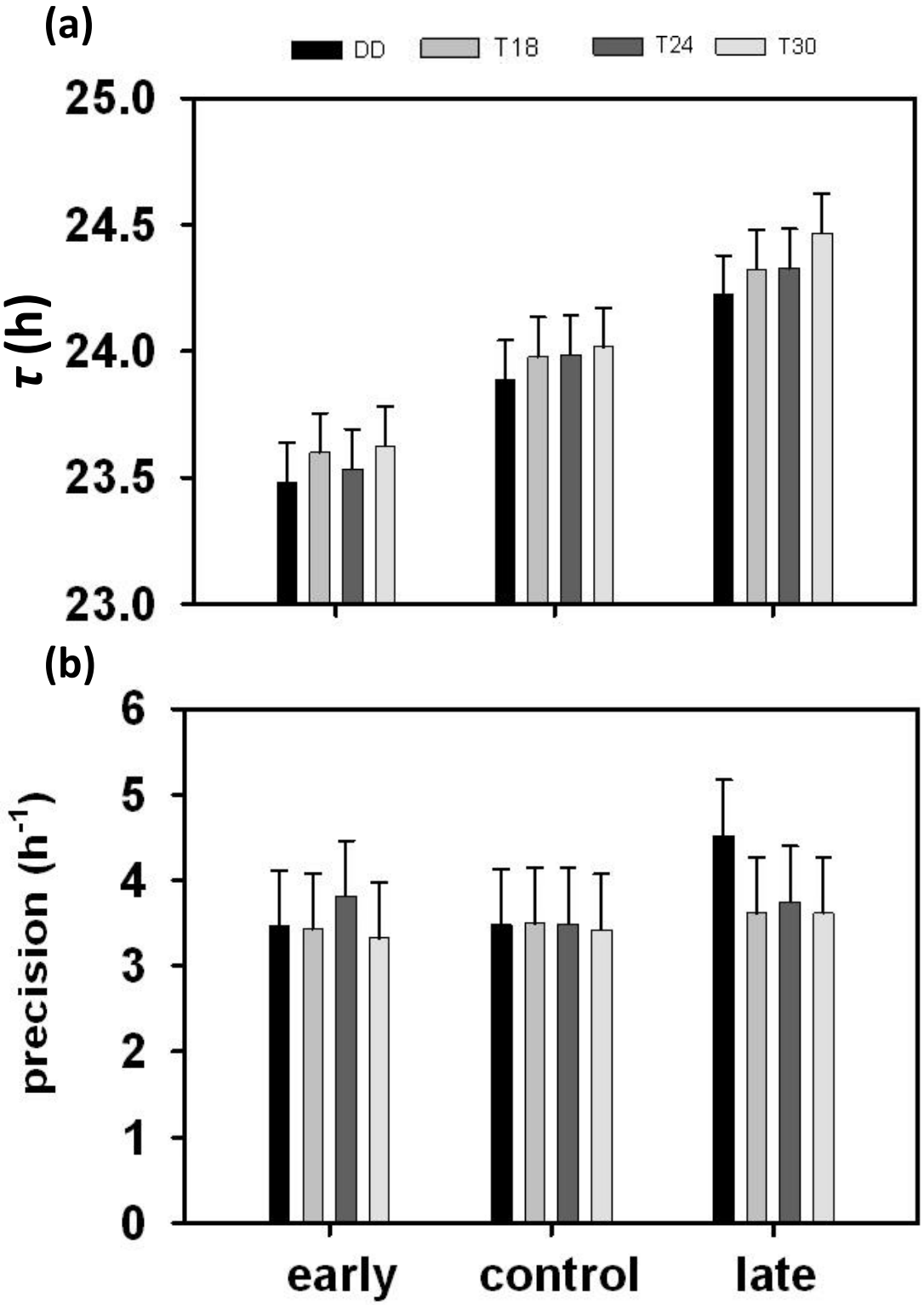

Figure 3.2: (a) Period (τ) and (b) precision of the 'early', 'control' and 'late' populations after development under TC (Thermophase-Cryophase) *T*-cycles. Error bars are 95% CI.

h), but was not different from τ following T18 (23.97-h) and T24 (23.95-h), τ after development in DD was not significantly different from that following T18 and T24 (Figure 3.2a). There was no significant effect of 'population' × 'developmental regime' interaction ($F_{6,18}$= 0.33, p>0.05), indicating that different developmental regimes had a similar effect on the period values of the different populations.

ANOVA on the precision values did not show a statistically significant effect of 'population' ($F_{2,6}$= 3.40, p>0.05), 'developmental regime' ($F_{2,9}$= 1.83, p>0.05) or of 'population' × 'developmental regime' interaction ($F_{6,18}$= 1.23, p>0.05; Figure 3.2b).

Discussion

Previous results from the 'early', 'control' and 'late' populations have shown that as adults these populations differ in intrinsic clock period and amplitude, zeitgeber sensitivity and possibly inter-oscillator coupling (Nikhil et al., 2016a). This could in turn affect the extent of plasticity of the clock in the adults, as is indeed seen to some extent from the results of the previous chapters. To investigate whether such effects persist even at the pre-adult developmental stages we studied the effect of rearing under different LD and TC T-cycles on the τ and precision in the adults of the study populations. The results show that the τ of activity-rest rhythm is determined by the genotype as well as the rearing conditions of the flies as there was a statistically significant effect of 'population' and 'developmental regime' on τ across all the LD and TC T-cycles (Figures 3.1a, 3.2a).

After rearing under each of the LD and TC *T*-cycles, the effect of genotype is evident as the population-level differences in τ continues to persist as seen in the previous chapters, however, there appears to be change in the absolute τ under the different developmental regimes (Figures 3.1a, 3.2a). This is also the case with precision across developmental regimes of LD cycles, but not TC cycles (Figures 3.1b, 3.2b). After rearing in LD *T*18 cycles, the τ of all the populations is significantly shorter as compared to that following rearing *T*24LD regime (Figure 1a). Rearing under LD *T*18 cycles appears to reduce precision across all the populations as compared to that when they complete development in DD (Figure 3.1b). This could stem from the increased change in τ following rearing under this novel condition, as seen in the previous chapter.

Following rearing under TC *T*-cycles there was no change in τ as compared to that following development in DD for any of the study populations (Figure 3.2a). Although there was no significant effect of developmental regime on precision, separate comparisons of the precision values of the 'late' populations showed that the lack of differences in precision seen between the 'early', 'control' and 'late' populations after rearing under any of the TC cycles appears to be due to the reduction in precision of the 'late' populations following rearing in DD. This indicates that exposure of the 'late' populations to any temperature *T*-cycle is sufficient to bring about a reduction in precision in the 'late' populations, and could be a result of increased sensitivity to temperature in these populations. The results of Chapter 2 could also be interpreted in this manner.

Therefore, as seen in previous studies (Page and Barrett, 1989; Barret and Page, 1989) subjecting the 'early', 'control' and 'late' populations to LD and TC *T*-cycles affects the τ, however, there appears to be no difference in the developmental plasticity between the populations as the effect of change in τ is similar across all populations. Although the results of Nikhil et al (2016a) and that of the previous chapters points towards greater period lability in the 'late' populations, it does not seem to manifest itself when individuals are reared under different environmental conditions.

References

Abraham, U., Granada, A.E., Westermark, P.O., Heine, M., Kramer, A. and Herzel, H., 2010. Coupling governs entrainment range of circadian clocks. *Molecular Systems Biology*, *6*(1), pp.438-451.

Aschoff, J., 1981. Der tagesgang der Körpertemperatur von vögeln als funktion des körpergewichtes. *Journal für Ornithologie*, *122*(2), pp.129-151.

Aton, S.J., Colwell, C.S., Harmar, A.J., Waschek, J. and Herzog, E.D., 2005. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nature Neuroscience*, *8*(4), pp.476-483.

Barrett, R.K. and Page, T.L., 1989. Effects of light on circadian pacemaker development. *Journal of Comparative Physiology A*, *165*(1), pp.41-49.

Beersma, D.G., Daan, S. and Hut, R.A., 1999. Accuracy of circadian entrainment under fluctuating light conditions: contributions of phase and period responses. *Journal of Biological Rhythms*, *14*(4), pp.320-329.

Berde, C., 1976. Nonmonotonic transients and some mathematical models of circadian rhythms. *Journal of Theoretical Biology*, *56*(2), pp.297-313.

Bordyugov, G., Granada, A.E. and Herzel, H., 2011. How coupling determines the entrainment of circadian clocks. *The European Physical Journal B*, *82*(3-4), pp.227-234.

Christensen, N.D. and Lewis, R.D., 1982. The circadian locomotor rhythm of *Hemideina thoracica* (Orthoptera; Stenopelmatidae): the circadian clock as a population of interacting oscillators. *Physiological Entomology*, *7*(1), pp.1-13.

Ciarleglio, C.M., Gamble, K.L., Axley, J.C., Strauss, B.R., Cohen, J.Y., Colwell, C.S. and McMahon, D.G., 2009. Population encoding by circadian clock neurons organizes circadian behavior. *The Journal of Neuroscience*, *29*(6), pp.1670-1676.

Clayton, D.L. and Paietta, J.V., 1972. Selection for circadian eclosion time in *Drosophila melanogaster*. *Science*, *178*(4064), pp.994-995.

Cloudsley-Thompson, J.L., 1960. Adaptive functions of circadian rhythms. In *Cold Spring Harbor symposia on quantitative biology* (pp. 345-355). Cold Spring Harbor Laboratory Press.

Colwell, C.S., Michel, S., Itri, J., Rodriguez, W., Tam, J., Lelievre, V., Hu, Z., Liu, X. and Waschek, J.A., 2003. Disrupted circadian rhythms in VIP-and PHI-deficient mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *285*(5), pp.R939-R949.

Daan, S. and Aschoff, J., 2001. The entrainment of circadian systems. In *Circadian Clocks* (pp. 7-43). Springer: US.

Daan, S. and Berde, C., 1978. Two coupled oscillators: simulations of the circadian pacemaker in mammalian activity rhythms. *Journal of Theoretical Biology*, *70*(3), pp.297-313.

Daan, S., 2000. Colin Pittendrigh, Jurgen Aschoff, and the natural entrainment of circadian systems. *Journal of Biological Rhythms*, *15*(3), pp.195-207.

De, J., Varma, V., Saha, S., Sheeba, V. and Sharma, V.K., 2013. Significance of activity peaks in fruit flies, *Drosophila melanogaster*, under seminatural conditions. *Proceedings of the National Academy of Sciences- U. S. A.*, *110*(22), pp.8984-8989.

Garland, T. and Rose, M.R., 2009. *Experimental evolution: concepts, methods, and applications of selection experiments* (p. 752). Berkeley: University of California Press.

Hastings, J.W. and Sweeney, B.M., 1957. On the mechanism of temperature independence in a biological clock. *Proceedings of the National Academy of Sciences- U. S. A.*, *43*(9), pp.804-811.

Helfrich-Förster, C., 2005. PDF has found its receptor. *Neuron, 48(2)*, pp. 161-163.

Hirschie Johnson, C., Elliott, J.A. and Foster, R., 2003. Entrainment of circadian programs. *Chronobiology International*, *20*(5), pp.741-774.

Hurd, M.W. and Cahill, G.M., 2002. Entraining signals initiate behavioral circadian rhythmicity in larval zebrafish. *Journal of Biological Rhythms*, *17*(4), pp.307-314.

Indic, P., Schwartz, W.J., Herzog, E.D., Foley, N.C. and Antle, M.C., 2007. Modeling the behavior of coupled cellular circadian oscillators in the suprachiasmatic nucleus. *Journal of Biological Rhythms*, *22*(3), pp.211-219.

Kalmus, H., 1935. Periodizität und autochronie (ideochronie) als zeitregelnde eigenschaffen der organismen. *Biologia Generalis*, *11*, pp.93-114.

Kronauer, R.E., Czeisler, C.A., Pilato, S.F., Moore-Ede, M.C. and Weitzman, E.D., 1982. Mathematical model of the human circadian system with two interacting oscillators. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *242*(1), pp.R3-R17.

Kumar, S., Kumar, D., Paranjpe, D.A., Akarsh, C.R. and Sharma, V.K., 2007. Selection on the timing of adult emergence results in altered circadian clocks in fruit flies *Drosophila melanogaster*. *Journal of Experimental Biology*, *210*(5), pp.906-918.

Malpel, S., Klarsfeld, A. and Rouyer, F., 2004. Circadian synchronization and rhythmicity in larval photoperception-defective mutants of *Drosophila*. *Journal of Biological Rhythms*, *19*(1), pp.10-21.

Nikhil, K.L., Goirik, G., Ratna, K. and Sharma, V.K., 2014. Role of temperature in mediating morning and evening emergence chronotypes in fruit flies *Drosophila melanogaster*. *Journal of Biological Rhythms*, *29*(6), pp.427-441.

Nikhil, K.L., Vaze, K.M. and Sharma, V.K., 2015. Late emergence chronotypes of fruit flies *Drosophila melanogaster* exhibit higher accuracy of entrainment. *Chronobiology International*, *32*(10), pp.1477-1485.

Nikhil, K.L., Vaze, K.M., Ratna, K. and Sharma, V.K., 2016a. Circadian clock properties of fruit flies *Drosophila melanogaster* exhibiting early and late emergence chronotypes. *Chronobiology International*, *33*(1), pp.22-38.

Nikhil, K.L., Abhilash, L. and Sharma, V.K., 2016b. Molecular correlates of circadian clocks in fruit fly *Drosophila melanogaster* populations exhibiting early and late emergence chronotypes. *Journal of Biological Rhythms*, *31*(2), pp.125-141.

Page, T.L. and Barrett, R.K., 1989. Effects of light on circadian pacemaker development. *Journal of Comparative Physiology A*, *165*(1), pp.51-59.

Page, T.L. and Block, G.D., 1980. Circadian rhythmicity in cockroaches: effects of early post-embryonic development and ageing. *Physiological Entomology*, *5*(3), pp.271-281.

Pavlidis, T., 1978. Qualitative similarities between the behavior of coupled oscillators and circadian rhythms. *Bulletin of Mathematical Biology*, *40*(6), pp.675-692.

Pittendrigh, C., Bruce, V. and Kaus, P., 1958. On the significance of transients in daily rhythms. *Proceedings of the National Academy of Sciences- U. S. A.*, *44*(9), p.965.

Pittendrigh, C.S. and Minis, D.H., 1971. The photoperiodic time measurement in *Pectinophora gossypiella* and its relation to the circadian system in that species (Coincidence model tests of photoperiodic time measurement relation to circadian system in moth *Pectinophora gossypiella*, using induction by skeleton photoperiods and light cycles). In *Biochronometry*, pp.247-250. National Academy of Sciences Press: Washington.

Pittendrigh, C.S., 1954. On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proceedings of the National Academy of Sciences- U. S. A.*, *40*(10), pp.1018-1029.

Pittendrigh, C.S., 1960. Circadian rhythms and the circadian organization of living systems. In *Cold Spring Harbor Symposia on Quantitative Biology* (Vol. 25, pp. 159-184). Cold Spring Harbor Laboratory Press.

Pittendrigh, C.S., 1967. Circadian systems. I. The driving oscillation and its assay in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences- U. S. A.*, *58*(4), pp.1762-1767.

Pittendrigh, C.S., 1981. Circadian systems: entrainment. In *Biological Rhythms* (pp. 95-124). Springer US.

Pittendrigh, C.S., 1993. Temporal organization: reflections of a Darwinian clock-watcher. *Annual Review of Physiology*, *55*(1), pp.17-54.

Pittendrigh, C.S., and Daan, S., 1976a. A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents IV. Entrainment: Pacemaker as Clock. *Journal of Comparative Physiology*, *106*(4), pp.291-331.

Pittendrigh, C.S. and Daan, S., 1976b. A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *Journal of Comparative Physiology*, *106*(3), pp.223-252.

Sehgal, A., Price, J. and Young, M.W., 1992. Ontogeny of a biological clock in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences- U. S. A.*, *89*(4), pp.1423-1427.

Shafer, O.T. and Yao, Z., 2014. Pigment-dispersing factor signaling and circadian rhythms in insect locomotor activity. *Current Opinion in Insect Science*, *1*, pp.73-80.

Sheeba, V., Chandrashekaran, M.K., Joshi, A. and Sharma, V.K., 2002. Developmental plasticity of the locomotor activity rhythm of *Drosophila melanogaster*. *Journal of Insect Physiology*, *48*(1), pp.25-32.

Tokuda, I.T., Ono, D., Ananthasubramaniam, B., Honma, S., Honma, K. and Herzel, H., 2015. Coupling controls the synchrony of clock cells in development and knockouts. *Biophysical Journal*, 109, pp.2159-2170.

Tomioka, K., Sakamoto, M., Harui, Y., Matsumoto, N. and Matsumoto, A., 1998. Light and temperature cooperate to regulate the circadian locomotor rhythm of wild type and period mutants of *Drosophila melanogaster*. *Journal of Insect Physiology*, *44*(7), pp.587-596.

Vaze, K.M. and Sharma, V.K., 2012a. Early-and late-emerging *Drosophila melanogaster* fruit flies differ in their sensitivity to light during morning and evening. *Chronobiology International*, *29*(6), pp.674-682.

Vaze, K.M., Kannan, N.N., Abhilash, L. and Sharma, V.K., 2012b. Chronotype differences in *Drosophila* are enhanced by semi-natural conditions. *Naturwissenschaften*, *99*(11), pp.967-971.

Vaze, K.M. and Sharma, V.K., 2013. On the adaptive significance of circadian clocks for their owners. *Chronobiology International*, *30*(4), pp.413-433.

Webb, A.B., Taylor, S.R., Thoroughman, K.A., Doyle III, F.J. and Herzog, E.D., 2012. Weakly coupled circadian cells improve resynchrony. *PLoS Computational Biology*, *8*(11), p.e1002787.

Welsh, D.K., Takahashi, J.S. and Kay, S.A., 2010. Suprachiasmatic nucleus: cell autonomy and network properties. *Annual Review of Physiology*, *72*, p.551.

West-Eberhard, M.J., 2003. *Phenotypic plasticity and evolution.* Oxford University Press: Oxford.

Wever, R., 1965. *A mathematical model for circadian rhythms.* Circadian Clocks (Vol. 47, pp.47-63). Northern Holland Publishing Company: Amsterdam.

Yadav, P. and Sharma, V.K., 2013. Correlated changes in circadian clocks in response to selection for faster pre-adult development in fruit flies *Drosophila melanogaster*. *Journal of Comparative Physiology B*, *183*(3), pp.333-343.