Behaviour, molecular and life-history trait analyses of *Drosophila melanogaster* populations exhibiting early and late emergence chronotypes

Thesis

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Evolutionary and Organismal Biology

By

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Dedication

This thesis is lovingly dedicated to my parents, and my two dogs

Heidi and Kwick.

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THESIS DECLARATION

I hereby declare that the contents presented in this thesis entitled ‘Behavior, molecular and life-history trait analyses of *Drosophila melanogaster* populations exhibiting early and late emergence chronotypes’ submitted to Jawaharlal Nehru Centre for Advanced Scientific Research for consideration for the award of Doctor of Philosophy 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.

Any part of the presented content if an outcome of collaborative research or if adopted from other studies has been duly acknowledged within the text and in the references. Even though I have ensured my best to make the references and acknowledgements list exhaustive, any omission made in this regard is not deliberate and I sincerely apologize for such oversights.

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CERTIFICATE

This is to certify that the work described in the thesis entitled 'Behavior, molecular and life-history trait analyses of *Drosophila melanogaster* populations exhibiting early and late emergence chronotypes' is the result of investigations undertaken by Mr Nikhil KL 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.

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Glossary

Adaptation: The process by which organisms evolve traits that confer higher fitness advantage in the organisms' habitat. Alternatively, any trait confers higher fitness to organisms in a given environment is termed an adaptation.

Circadian rhythms (Latin *circa* = about/approximately; *diēs* = day): Biological rhythms in behaviour and physiology expressed with a period of ~24 h under constant conditions (absence of external time cues/zeitgebers).

Circadian clocks: Biological time keeping mechanisms that drive circadian rhythms.

Free-running period (τ): The period of the circadian rhythms exhibited under constant conditions.

Clines: Gradual phenotypic variation across a geographical area as a consequence of variation in geophysical features such as latitudes (latitudinal clines) or altitudes (altitudinal clines).

Directional Selection: Selection for a phenotype that constitute the extremes of the phenotype distribution in the population.

Effective population size: The size of an ideal population that would undergo equal amount of genetic drift as that of a non-ideal population of size N is defined as the effective population size (N_e).

Entrainment: Entrainment refers to the process of synchronization of circadian rhythms to external time cues (zeitgeber) such that (a) the period of the entrained rhythm match that of the zeitgeber (b) the rhythms attain a stable and reproducible phase relationship with the zeitgeber (also known as phase of entrainment) and (c) upon removal of the

zeitgeber, the free-running rhythm should start from the phase of entrainment established with the prior zeitgeber.

Fitness: Fitness is the measure of an organism's or a population's ability to survive and reproduce in a given environment.

Inbreeding: Mating among individuals with high genetic relatedness leading to increased homozygosity, isogeny and random fixation of deleterious alleles over generations.

Linkage disequilibrium: Also known as gametic phase disequilibrium; this is the occurrence in members of a population a particular combination of linked alleles in non-random proportions.

Phase Response Curve (PRC): A PRC maps the magnitude of response (measured as phase shifts) to zeitgebers at different phases of the circadian cycle, and therefore is a measure of the circadian clocks' zeitgeber sensitivity.

T-cycle: Zeitgeber cycles of periodicity *T*. For instance *T*-24 indicates a 24 h zeitgeber cycles with the durations of light/dark or thermophase/cryophase summing up to 24 h, *T*-30 a 30 h zeitgeber cycle and so on.

Temperature compensation: Temperature compensation refers to the ability of circadian clocks to maintain a stable and constant τ across different temperatures by compensating for temperature induced changes in the rate of biochemical reactions (Pittendrigh, 1954; Zimmerman et al. 1968).

SYNOPSIS

Timing behaviours to specific times or phases of the day has been hypothesized to be adaptive and consequently driving evolution of the underlying time-keeping mechanisms referred to as circadian clocks. The phase of entrainment (ψ_{ent}) refers to the inherent preference for the time of exhibiting a circadian behaviour, and is usually species-specific. However, individuals in a given population usually exhibit variation in ψ_{ent}, and in terms of human sleep/wake behaviour, individuals exhibiting an advanced ψ_{ent} are termed as 'early chronotypes' and those with a delayed ψ_{ent} as 'late chronotypes'. Among the widely observed circadian clock properties associated with chronotype variation is the clock period (τ) such that early chronotypes exhibit shorter τ while late chronotypes exhibit longer τ. Such ψ_{ent}-τ associations appear to be an evolutionarily conserved feature of circadian rhythms as evidenced by studies on a variety of organisms reporting similar associations. However, such studies till date have either been performed on clock mutants or on natural populations, both of which have their respective shortcomings. For instance, mutant strains generally lack genetic variation as they are highly inbred, and therefore the observed phenotypes may have stemmed from random fixation of alleles including those at non-clock loci. Natural populations on the other hand are generally outbreeding and thus are a rich pool of genetic variation, rendering them as potential model systems for various studies. However, the complex interplay of various biotic and abiotic factors in the environment are known to strongly influence evolution of traits, and therefore the observed phenotypes might not necessarily have evolved as a direct consequence of selection on circadian behaviours but established in the populations due to genetic linkage and/or drift arising due to various reasons including population bottlenecks. Therefore, the unavailability of information regarding the environmental history of the populations'

habitat or their ancestry weakens inferences from studies on natural populations. I have discussed these aspects in detail, along with the evolutionary origins and implications of circadian clocks in chapter 1.

To account for the above discussed shortcomings of previously employed experimental strategies, we intended to establish a laboratory model system for the study of ψ_{ent} and the associated clock properties by driving the evolution of divergent ψ_{ent} under controlled environmental conditions in replicate *Drosophila melanogaster* populations harbouring large genetic variation. In this regard, we employed laboratory artificial selection (discussed in chapter 2) to derive *D. melanogaster* populations exhibiting early and late emergence chronotypes (4 replicate populations each with 1200 individuals per population) which are characterised by advanced (*early* populations) and delayed (*late* populations) ψ_{ent} of emergence rhythm. Over the years we have reported several differences in clock properties of these populations, which primarily include divergence in the τ of emergence and activity/rest rhythms, temporal light sensitivity, phase response curve of emergence rhythm, and enhanced emergence chronotype (ψ_{ent} of emergence rhythm) differences under semi-natural conditions. The studies presented in this thesis are a further extension of the previously reported observations, and can be broadly categorised into 4 sections as discussed below.

a) Role of light and temperature in enhancing emergence chronotype differences between *early* and *late* populations: Previously, we have reported that the emergence chronotype difference between the *early* and *late* populations is considerably enhanced when assayed under semi-natural conditions comprising gradual cycling of light and temperature with some phase-difference between the two zeitgebers. In chapter 3, I wished to further explore what aspects of semi-natural conditions drive such enhancement of emergence chronotype differences, and assayed emergence rhythms of

the two sets of populations under simulated in- and out-of-phase light and temperature cycles (independently and in unison) such that they either followed a square waveform or stepped/ramped cycles mimicking nature. The results demonstrate that light and temperature act antagonistically in driving chronotype differences between the two populations. While light delayed emergence and increased the gate-width of emergence, temperature was observed to advance emergence and reduce gate-width. Additionally, gradual increase and decrease of zeitgebers mimicking the dawn and dusk transitions in nature was observed to further enhance chronotype differences but only by a small magnitude (~30 min). These results highlight the combinatorial role of multiple zeitgebers in nature in determining the ψ_{ent}, and are discussed in light of the possible role of a dominant temperature sensitive B oscillator (Pittendrigh's A-B oscillator model) in driving the delayed ψ_{ent} in the *late* populations.

b) Differences in light sensitivity, oscillator coupling and accuracy of entrainment in *early* and *late* emergence chronotypes: We have previously reported that in response to selection, the *early* and *late* populations evolved shorter and longer τ; however, the magnitude of difference in τ (1.35 h) fails to entirely account for the difference in ψ_{ent} of emergence (4.52 h) between the *early* and *late* populations. Based on this and previously reported differences in temporal light sensitivity between the two populations, I hypothesized that clock properties in addition to τ might be associated with chronotype difference between the *early* and *late* populations. In chapter 4, I further tested light sensitivity of the two populations by assaying their activity/rest rhythm under constant dim light (LL), and observed that a higher proportion of the *late* populations exhibit complex rhythm and behavioural arrhythmia. I reasoned that such phenotypes might arise either due to higher light sensitivity or reduced inter-oscillator coupling in the circadian clock network. To test these two hypotheses, I performed

further set of experiments drawing expectations from predictions by theoretical models of weakly coupled circadian oscillators. In accordance with some of the theoretical predictions, I observed that the *late* populations exhibit wider range of entrainment and reduced rate of re-entrainment to simulated jet-lag suggesting that these populations might have evolved weakly coupled circadian oscillators. The photic dose response curve of the *late* populations did not differ significantly from that of the *early* populations, which suggests that the higher arrhythmicity and wider entrainment range did not stem from higher light sensitivity. Interestingly, while the *late* populations did not exhibit high amplitude PRC (high amplitude advance and delay zones), the magnitude of delay phase-shifts was observed to be significantly higher as assessed by the area under the delay zone, which suggests a possible involvement of continuous/tonic effects of light as opposed to the discrete effects expected from the non-parametric model of entrainment. Furthermore, I also observed that the *late* populations comprise high amplitude circadian oscillators as evidence by the higher amplitude of activity/rest rhythm under entrained as well as free-running conditions. These results highlight the complex interplay of oscillator light sensitivity, amplitude and coupling in driving the delayed phase of ψ_{ent} in the *late* populations.

It has been proposed that the *late* chronotypes exhibit reduced stability of entrainment which is also described as reduced accuracy (higher variation in day-wise ψ_{ent}). In chapter 5, I wished to examine this by estimating accuracy of both emergence and activity/rest rhythms under multiple zeitgeber cycles comprising light/dark, thermophase/cryophase, and semi-natural conditions (SN). Interestingly, I observed that contrary to previous reports, the *late* populations exhibit significantly higher accuracy of entrainment for emergence and activity/rest rhythms under all the environmental regimes tested. I further assessed if higher accuracy of entrainment in the *late*

populations stems from precise circadian clocks, and found that the populations did not differ in the precision of circadian clocks. These results are discussed in the context of the possible roles of higher accuracy of entrainment for organisms exhibiting delayed ψ_{ent} as opposed to their *early* counterparts.

c) Molecular correlates of *early* and *late* emergence chronotypes: The results discussed thus far suggest that multiple clock properties collectively contribute to differential entrainment in the *early* and *late* populations and drive their respective emergence chronotypes. While the neurobiological and molecular bases of circadian clocks have been extensively explored, our knowledge of the molecular mechanisms contributing to ψ_{ent} remains largely elusive, and therefore, as a preliminary study I assessed molecular correlates of the *early* and *late* populations in chapter 6. I studied mRNA profiles of the circadian clock genes in the heads of individuals from the *early* and *late* populations by choosing genes that are input (*cryptochrome*), core clock (*period*, *timeless* and *clock*) and core clock + output (*vrille*) components. Corroborating the chronotype difference between the *early* and *late* populations, the molecular clockwork have also diverged accordingly with mRNA profiles of the three core clock genes (*period*, *timeless* and *clock*) exhibiting an advanced and delayed phase of expression in the *early* and *late* populations respectively. Furthermore, mRNA profiles of some of the genes also exhibit high amplitude oscillations in the *late* populations which partly support the behaviourally observed high amplitude rhythms (Chapter 4) in these populations. The mean phase of *cryptochrome* mRNA expression did not differ between the populations; however, mRNA level was observed to be lower in the *late* populations, which further suggests that higher behavioural arrhythmicity under dim LL observed in the *late* populations (Chapter 4) does not stem from higher light sensitivity. Furthermore, *vrille* mRNA also exhibited similar dynamics as that of the other core

clock genes and was also observed to be expressed at higher levels in the *late* populations. This further corroborates previous reports of longer free-running period and higher activity levels upon *vrille* overexpression. Some of my preliminary data (not presented in this thesis) indicates that difference in phase of emergence between the *early* and *late* populations might be driven by advanced and delayed release of eclosion hormone (EH) thus suggesting the role of ecdysteroid cascade in driving emergence chronotype differences between the two populations. Since VRILLE is known to be one of the direct targets of 20-Hydroxyecdysone (20-HE) a precursor for EH, I hypothesized that selection for *late* emergence which is associated with delayed onset of 20-HE and other ecdysteroids might have driven divergence in *vrille* mRNA oscillation which by virtue of its role in regulating *clock* transcription might have led to the coevolution of divergent clocks in these populations.

In addition, to further explore whether the *late* populations have evolved weakly coupled oscillator network, I estimated cycling and average levels of PIGMENT DISPERSING FACTOR (PDF) in the terminals of small-Ventral Lateral neurons (s-LNv) as PDF is known to function as a coupling factor in the *Drosophila* clock network. PDF levels were observed to exhibit diurnal oscillation in the sLNv terminals with the mean phase of oscillation significantly delayed in the *late* populations as compared to the *early* populations. Interestingly, PDF levels were observed to be significantly higher in the *late* populations which at first sight appears to be contrary to the expectation from a weakly coupled oscillator network in the *late* populations. However, previous studies have reported that overexpression of PDF also renders the circadian clock network susceptible to desynchronization as evidenced by higher degree of complex rhythms and behavioural arrhythmicity in the PDF overexpressed *Drosophila* strains, and similar dynamics has also been observed in mammalian clock network, which can potentially

explain the phenotypes resembling that of a weakly coupled clock network observed in the *late* populations, and is discussed in detail in chapter 6.

d) Analysis of life history traits in *early* and *late* emergence chronotypes: While circadian clocks have been considered to confer adaptive advantages to organisms, majority of the studies aimed at exploring adaptive significance of clocks have relied on the effects of complete abolishment of circadian rhythms either by surgical means or through the use of mutants with dysfunctional circadian clocks. While such studies underscore the relevance of harbouring functional circadian clocks, the consequences of variation in the timing of circadian behaviours on organisms' fitness have not been studied. To test this proposition, in chapter 7, I assessed multiple life history traits in the *early* and *late* populations under both light/dark and constant darkness regimes to be able to dissociate whether the observed differences (if any) are indeed clock driven or not. I observed that in response to selection, the *early* and *late* populations evolved significantly shorter and longer egg-to-pupa and egg-to-adult development times, and further, the difference in development time did not translate into dry-weight differences at either pupation or emergence stages. The egg-to-pupa and egg-to-adult survivorship also did not differ between the two populations. While these results were consistent across LD and DD, light dependent changes were also observed, which suggests that such differences are driven by both clock dependent and independent mechanisms. Interestingly, females of the *late* populations exhibited significantly higher fecundity and reduced median longevity while the longevity of males did not differ across populations. The lack of similar differences in the longevity of males suggests that reduced longevity in females might be due to higher fecundity. Estimation of body-weight differences of females between pre- and post-fecundity assays revealed that the observed increase in fecundity was not associated with increased body-weight or higher

body-weight to egg conversion. These results thus highlight genetic correlation between mechanisms driving ψ_{ent} and multiple life-history traits which further supports the idea that circadian clock driven divergence in ψ_{ent} might influence fitness estimates in natural populations.

In summary, results presented in this thesis underscores the differential role of light and temperature; the complex interplay of multiple circadian clock properties including light sensitivity, amplitude, coupling and accuracy, and the molecular differences in driving emergence chronotype differences in *Drosophila melanogaster*; and also reveals the genetic correlations between mechanisms that time circadian behaviours with that of life-history traits thus highlighting the adaptive roles of circadian clocks.

CHAPTER 1

On the origin and implications of circadian time keeping: An evolutionary perspective

1.1 Introduction

Majority of life forms have been observed to exhibit circadian (Latin: *circa* = about/approximately; *diēs* = day) rhythmicity in behaviour and physiological processes driven by underlying circadian clocks, and has been the topic of study for several decades. Even though the earliest mention of daily rhythms dates back to Androsthenes around 4th century BC, other evidence suggest that such rhythms were extensively noticed and their importance realised much before Androsthenes (Box 1). Today, it is commonplace to encounter statements such as "circadian clocks evolved in response to rhythmic selection pressures imposed by environmental variables as a consequence of earth's rotation about its axis" implying that it probably is a well-established knowledge. Given the prevalence of circadian rhythms in multiple facets of human lives as well as that of other life forms, it may be quite an arduous exercise for chronobiologists to even consider that circadian clocks might not be adaptive. Nevertheless, if one were to critically assess the large number of studies in chronobiology, it would become evident that studies on this topic are relatively few, and the existing body of evidence only demonstrate that circadian clocks provide certain advantages to its bearers under specific contexts. However, from the perspective of an evolutionary biologist's definition of adaptation, being advantageous may not necessarily mean being adaptive. Here we will discuss the evolutionary significance of circadian clocks by reviewing literature on theories and the evidence about their origin and the adaptive value. In later sections, we will discuss the pitfalls of some of the experimental methodologies used and suggest improvements that we believe can fine tune experimental designs for future studies.

Box-1: Pre-Androsthenes mention of circadian rhythms

Majority of the literature discussing daily rhythms invariably refers to Androsthenes, a Greek philosopher and admiral of Alexander III of Macedon who supposedly during his march on India in 4th century BC observed rhythmic movement of tamarind tree leaves. This is followed by another mention of sleep-wake rhythms in bees as observed and written by Aristotle in his book 'History of animals' (Greek: Τῶν περὶ τὰ ζῷα ἱστοριῶν "Inquiries on Animals"; Latin: Historia Animālium "History of Animals" as translated from Greek by D' Arcy Wentworth Thompson) (cf. Daan, 2010) around the same time (350 BC). However, it appears that human knowledge of daily rhythms can be traced further back to the Vedic period predating Androsthenes and Aristotle by almost a thousand years. In fact, unlike the former reports of mere observations of rhythmic behaviours, the relevance of rhythmicity in physiological variables appears to have been extensively studied as can be inferred from the fact that it even formed the basis of the traditional medicinal system 'Ayurveda' whose origin is attributed to the 'Atharva Veda' composed around 1500-1000 BC (Narayanaswamy, 1981). The principles of ayurvedic medicinal treatments resides on three fundamental physiological measures (called 'doshas') - Vata, Pitta and Kapha believed to exhibit 24 h rhythmicity with each of them peaking at specific times of the day (Moritz, 2005; Hankey, 2010 and citations therein). It was observed that sleep-wake cycles when not synchronised with the external day-night cycles would lead to an imbalance between the three doshas resulting in physiological disruption, a concept now acknowledged as circadian misalignment. Thus, even though rigorous scientific analyses of circadian rhythms were initiated relatively recently, the importance of temporal order within the human body seems to have been acknowledged for over 3000 years now. However, extensive and more importantly exact translations of Vedic scriptures or similar ones will help further uncover the rich history of circadian rhythms.

1.2 Adaptation

Since adaptive significance of circadian clocks is our topic of interest, we will briefly revisit some of the popular concepts of adaptation which is essential for rigorous evaluation of methodologies and interpretations of studies aimed at unravelling the adaptive significance of circadian clocks.

Even though adaptation as a concept has enjoyed unanimous acceptance, arriving at its precise definition has remained controversial for over a century (Rose and Lauder, 1996). It is conventional to use the term adaptation in two contexts. One may refer to it as either a continuous process by which organisms adapt to a given environment, or as a character/trait that confers higher fitness to organisms in a given scenario (Chapter 11, Futuyma, 2009). However, we will be using adaptation in the latter context throughout this chapter.

The earliest definitions regarded adaptations as traits 'optimally' designed in an organism for a specific function relevant to its ecology. This idea seemed to have been so convincingly adopted that it even inspired William Paley to propose in his book *Natural Theology* (1802) that such complex designs could have only been shaped by an intelligent designer, and used this argument to support the existence of an 'intelligent deity' or 'god' who functioned as the creator of life. Now pinned under the umbrella term 'the argument from design', such arguments were widely used to explain several phenomena by geologists Sedgwick, Buckland and Murchison, and naturalists like Agassiz which underscores the influence of theological temperament in scientific inquiry (Mayr, 1982). Ironically, even Darwin (as a student) upon reading Paley's work was convinced of the idea of an intelligent 'deity' driving adaptations (The Autobiography of Charles Darwin 1809-1882). However, philosophers like Hume and Kant in their works *Dialogues Concerning Natural Religion* (1779) and *Critique of Judgment* (1790) respectively have been credited to have demolished this argument (Mayr, 1982; Chapter 2, Rose and Lauder, 1996).

While these ideas were finally dismissed by Darwin's theory of natural selection which claimed that traits are shaped by natural selection (Darwin, 1859), and not by an intelligent deity, argument over the exact definition of adaptation continued for over a century. Following debates by GC Williams (1966) and Gould and Lewontin (1979), the definition of 'adaptation' underwent subsequent refinement with a large number of proposed alternatives which can be broadly categorized as 'historical' and 'ahistorical'. Some authors have also discussed another category called as 'teleological definitions' (Reeve and Sherman, 1993) which will not be considered here. A detailed discussion on this topic can be found in Launder et al (1993, and citations therein).

Ahistorical definitions focus on the current utility of the trait considered to be an adaptation without reference to the historical reasons that might have led to its origin. For instance, Bock (1979) proposed that "An adaptation is, thus, a feature of the organisms that interacts operationally with some factor of the environment so that the individuals survive and reproduce", and more recent definition proposed is "An adaptation is a phenotypic variant that results in the highest fitness among a specific set of variants in a given environment" (Reeve and Sherman, 1990). Historical definitions on the other hand highlight the importance of viewing an adaptation (*A*) in the context of the ancestral populations which experienced a given selective regime that eventually drove the evolution of *A*, thus implying adaptation as a relative (to its ancestral state) concept. For instance, Gould and Vrba (1982) proposed that a trait is an adaptation only if the historical selection pressures that led to its evolution for a given utility to the organism are the same selection pressures that currently maintain *A*, and that any trait whose origin was due to a historical selective pressure which currently does not maintain it, then the trait would be called an exaptation. Sober (1984) proposed that "*A* is an adaptation for task *T* in populations *P* if and only if *A* became prevalent in *P* because there was selection for *A*, where the selective advantage of *A* was due to the fact that *A* helped perform task *T*" as also retained by Coddington (1988). The definitions by Sober (1984) and similar versions of it are widely accepted currently.

The above discussed categories even though primarily differ in considering the relevance of the origins of adaptations, it is clear that both define adaptations as traits that confer certain benefits over others in a given environment. The benefits thus conferred would be naturally selected for if they enhanced the ability of organisms/species to survive and reproduce in that environment, referred to as the reproductive fitness. Therefore, individuals who can successfully reproduce and

maximally contribute to the gene pool of the succeeding generation are considered fitter than others, and the traits contributing to the fitness spreads in the population over time (Barker, 2009). Therefore, studying the influence of a trait on the fitness of the organism is the ideal way to assess whether a given trait of interest is an adaptation. But does one measure the fitness of an organism? While the measures of fitness are highly context dependent (as will be discussed later) for practical purposes, fitness is generally partitioned into multiple components. For instance, in a population of sexually reproducing organisms, the ability to reach sexual maturity is dependent on several factors including survivorship of zygotes till birth following which the ability to find mates and successfully reproducing becomes relevant. All these factors collectively influence the reproductive fitness of organisms, and therefore, various components such as survivorship till sexual maturity, mating success, fecundity, fertility and competitive ability (the ability to outcompete others for resources) are used as fitness measures. With this background, we will now discuss in detail the rationale behind the proposition that circadian clock are adaptive, their origins and evolutionary implications.

1.3 Why are circadian clocks considered to be adaptations?

Several features of circadian rhythms may encourage us to speculate about the adaptive significance of the underlying clocks. Firstly, the fact that proximity of the period (τ) of various rhythms observed to that of 24 h day/night cycles is unlikely to be a mere coincidence. Secondly, had circadian clocks originated just by chance and without any selective advantage, then it is unlikely that it could have attained such a wide distribution across most life forms on the earth since natural selection would otherwise have weeded it out. There have also been several examples suggesting convergent evolution of circadian clocks which imply that they might have evolved multiple times

independently which once again would be an unlikely scenario had clocks not been adaptations as will be discussed later. All these evidence are suggestive of the idea that circadian clocks might have been shaped up by natural selection. Therefore, it is logical to hypothesize that circadian clocks probably confer adaptive advantage to organisms.

1.3.1 *How can circadian clocks be advantageous?*

Testing whether circadian clocks are advantageous was among the first exercises to be carried out, and in this regard circadian clocks were believed to confer advantage to organisms in two ways. One by establishing an internal temporal order among various physiological cycles within the organism, referred to as 'intrinsic advantage', and the other by facilitating entrainment of circadian rhythms with the external cycles, known as the 'extrinsic advantage'.

1.3.1 (a) *Intrinsic advantage*

Intrinsic advantage hypothesis proposes that circadian clocks evolved to ensure temporal segregation of cellular and physiological processes within organisms. Since efficient operation of all processes at the same time would require large energy expenditure, timing different processes with appropriate temporal lags may help efficient resource/energy partitioning. Also, biological processes may differ in terms of their requirement for optimal ambient cellular/tissue conditions such as ionic strength, *pH* and so on, and therefore temporal segregation of such processes may help the same tissue regulate multiple processes without calling for the need of multiple tissues. Additionally, the components or products of some of the processes may be incompatible with others (for instance photosynthesis and nitrogen fixation in *cyanobacteria*), and therefore temporal segregation can prevent undesired physiological consequences arising from such incompatibilities. In this regard, Oatley and Goodwin (1971)

proposed that systems with oscillatory components tend to be more stable as compared to their non-oscillatory counterparts, and thus mutual synchronization would enable better homeostatic regulation; however, such proposition remains to be systematically tested. Nevertheless, based on the above arguments it is logical to hypothesize that there probably exists an adaptive advantage for mechanisms that facilitate internal temporal order.

A classic example cited to highlight the importance of temporal segregation of physiological process is that in *cyanobacteria* which exhibit rhythmic but antiphasic N_2 fixation and photosynthesis, with N_2 fixation occurring at night and photosynthesis during the day (discussed in Ditty et al. 2003). It is postulated that since the products of the two processes are toxic to each other, they are segregated so as to avoid interference (Mitsui et al. 1986). This seems to be a convincing explanation; however, it is not the complete story. Based on the above argument, it can be expected that growth of *cyanobacteria* should be retarded under constant light (LL) due to the unavailability of dark phase for N_2 fixation which is the main source of metabolic nitrogen. However, contrary to this, *cyanobacteria* have been shown to exhibit normal, and sometimes even faster growth in LL (discussed in Johnson and Kyriacou, 2005). Additionally, not all *cyanobacteria* fix nitrogen and in some cases individuals of the same genus use both spatial and temporal segregation for these processes while some even balance the rates of the two processes such that the net oxygen produced is zero (Berman-Frank et al. 2001). Therefore, not all of these strategies convincingly support the temporal segregation hypothesis, nevertheless several other evidence do favour the hypothesis.

An interesting study by Harker (1958) highlighted the relevance of circadian timekeeping where she experimentally manipulated cockroaches to disrupt their internal temporal order. Harker transplanted the subesophageal ganglion (SG) known to drive

circadian activity-rest rhythm in cockroaches such that individuals received transplant from a donor entrained either to in phase or antiphasic light/dark (LD) cycles with that of the host. Individuals who received SG transplants from donors entrained to antiphasic LD cycles (resulting in internal desynchrony) developed tumours as compared to those that received transplants from in phase donors suggesting that internal desynchrony might lead to physiological problems. Physiological variables including core body temperature, hepatic degradation, growth hormone, plasma cortisol and potassium levels in humans (Czeisler, 1978), plasma corticosterone, N-acethytransferase activity and serotonin in rats (Gibbs and Van Brunt, 1975), temperature and urinary potassium concentration in squirrel monkeys (Sulzman et al. 1979) are known to exhibit daily variation. Fuller et al (1987a, b) reported that when monkeys are devoid of rhythmicity in the external environment leading to desynchronization of circadian system, reduction in environmental temperature by as little as 8 ^{o}C leads to a 2 ^{o}C reduction in body temperature due to the failure of thermoregulatory homeostasis; whereas when entrained to LD cycles, reduction in ambient temperature had no measurable effect on body temperature. Studies on several circadian clock gene mutants have also highlighted the relevance of temporal order in physiology. Among the studies in recent years, Kondratov et al (2006) reported that *bmal* double knockout mice exhibit reduced lifespan and display symptoms of premature aging including sarcopenia, cataract, reduced subcutaneous fat and organ shrinkage. Similarly, the *clock* mutant mice have been observed to exhibit metabolic dysfunctions including hyperleptinemia, hyperlipidaemia, hepatic steatosis, hyperglycaemia and hypoinsulinemia (Turek et al. 2005), and also develop neurological problems (Barnard and Nolan, 2008). Additionally, *mPer2* deficiency has been observed to result in increased bone mass and higher propensity for cancer (Fu et al. 2002, 2005; Yang et al.

2009) while recent studies on *reverb-α* mutant mice report observing obesity, aberrant lipid metabolism and thermogenesis (Delezie et al. 2012; Gerhart-Hines et al. 2013; Hand et al. 2015).

The advent of genomics has facilitated exploration of circadian clocks' influence on the genome of organisms. Studies have revealed that over a third of the *Arabidposis* genome is under the influence of circadian clocks (Covington et al. 2008), 10-15% of all cellular transcripts exhibiting circadian oscillation in mammals (Akhtar et al. 2002; Panda et al. 2002), and over 43% of all protein coding RNAs cycle in a circadian manner in at least one tissue (Zhang et al. 2014). Regulatory pathways involved in glucose homeostasis and lipid metabolism have also been reported to be under direct circadian control (Akhtar et al. 2002; Panda et al. 2002; Lamia et al. 2008; Zhang et al. 2010). More importantly such rhythms also maintain stable phase relationship with each other thus highlighting the role of circadian clocks in establishing temporal order at the genome level. Collectively these results extend support to the idea that circadian clocks may indeed confer intrinsic adaptive advantages.

1.3.1 (b) *Extrinsic advantage*

Rotation of earth about its own axis with a period of 24 h drives cyclic variation of several abiotic (and consequently biotic) factors most prominent of which include light, temperature, humidity and to some extent barometric pressure as well. A complex interplay of these factors provides an unprecedented rhythmic environment for organisms to rely on and time daily behaviours. While some of the environmental factors may be beneficial to organisms, others may be detrimental. For instance, light during the day may facilitate navigation, search for food, and photosynthesis whereas, high temperature and low humidity may increase the risk of desiccation (especially in

smaller life forms), and furthermore, harmful cosmic rays during the day might increase the probability of errors in DNA replication and cell division (Ravanat et al. 2001). Thus, it is logical to envision that internal time keeping mechanisms which can anticipate such rhythmic changes in abiotic factors and accordingly time behaviour and physiological processes would be advantageous to organisms. Based on this reasoning, it can be hypothesized that circadian clocks may confer adaptive advantages by appropriately timing behaviours so as to avoid harsh environmental conditions, enhance food and mate procurements, promote predator evasion and establishing a temporal niche to reduce interspecific competition (Cloudsley-Thompson, 1960), which is therefore referred to as extrinsic advantage.

Support for the idea of extrinsic advantage of circadian clocks comes from multiple studies. For instance, rhythmic vertical migration is ubiquitously observed in several planktonic species where individuals migrate upward during dawn and dusk, and sink down at other times of the day (Rose, 1925; Russel, 1927; Kikuchi, 1930; Cushing, 1951). Furthermore, most of these species occupy the first 1,000 m from the top which is the depth to which light can penetrate (Welsh et al. 1937). Such diurnal migration has been asserted to daily changes in light which consequently affects other factors such as temperature, salinity and aeration, all of which contribute to photosynthesis. Additionally, Hardy (1956) discussed that water masses move more rapidly at the surface than at lower levels thereby facilitating rapid exchange of nutrients and other factors, and therefore vertical migration provides necessary conditions for the physiological functioning of the organism (similar to foraging). Also, several woodlice and millipede species are known to exhibit diurnal rhythm in activity with preference to wander out mostly at night to avoid harsh desiccative/low humidity conditions during the day. This is supported by experiments which report that when exposed to light,

woodlouse wander aimlessly till they find a dark and damp zone (Gunn, 1937; Fraenkel and Gunn, 1940; Waloff, 1941). Even though this can also be a passively driven response to light, choice chamber experiments demonstrated that the tendency to seek damp regions is lower during the night as compared to the day (Cloudsley-Thompson, 1951, 1952) thereby suggesting that such photic avoidance behaviours are at least partly driven by endogenous time keeping mechanisms.

While the above mentioned studies, in addition to several others (reviewed in Cloudsley-Thompson, 1960) are suggestive of the idea that circadian clocks help in timing behaviours so as to enhance survivability, other aspects of extrinsic advantages conferred by clocks such as facilitating predator evasion has been controversial. Various studies have proposed that temporal partitioning between competing taxa and in predator-prey systems are essential for promoting coexistence in ecological communities (reviewed in Kronfled-Schor and Dayan, 2003). Such partitioning is considered to be driven primarily by biotic factors, and the role of circadian clocks in driving such partitioning remains largely speculative due to the lack of substantial empirical evidence. Schoener (1974) reported that temporal partitioning is significantly less common in comparison to that of habitat or food type partitioning, and used theoretical models to predict that temporal resource partitioning on circadian scale should be relatively rare and would require severe depletion of resources before such behaviours can be observed. This is further supported by relatively limited observation of extreme shift in behaviours from nocturnality to diurnality. Unstriped Nile rats (*Arcicanthis niloticus*) are generally diurnal but become nocturnal when provided with a running wheel (Blanchong et al. 1999). Similar shift from nocturnal to diurnal behaviours is also observed in *Octodon degus* (Stevenson et al. 1968; Kas and Edgar, 1999) and mice (Hut et al. 2011). More importantly, careful analysis of the activity patterns suggested

masked behaviours and that the observed behaviours may be driven by non-clock mechanisms. Do these results suggest that circadian clocks may not mediate predator evasion and timing of foraging in nature? Daan (1981) proposed that since the evolution of nocturnality and diurnality requires different physiological mechanisms (for instance nocturnal and diurnal animals differ in their retinal photoreceptors and adaptations to vision in a given light intensity range reduces the efficiency at other intensities, Jacobs, 1993; van Schaik and Griffiths, 1996), closely related species, competitors or individuals of a predator-prey system generally remain active at a specific phase (day/night/twilight) of the circadian cycle depending on the physiological adaptation to nocturnality or diurnality, and that circadian clocks promote partitioning within this activity phase because adopting reversed or antiphasic activity behaviours can be deleterious. Subsequently DeCoursey and colleagues examined if dysfunctional circadian clocks would perturb the ability of animals to time behaviours at ecologically relevant phases, and whether such mistimed behaviours have any costs. Thus, daily behaviours of SCN lesioned free living diurnal antelope ground squirrels (DeCoursey et al. 1997) and chipmunks (DeCoursey and Krulas, 1998; DeCoursey et al. 2000) were studied in semi natural enclosures. SCN lesioned individuals in DeCoursey et al (1997, 2000), and DeCoursey and Krulas (1998) studies exhibited higher night time restlessness resulting in animals wandering during the night, and consequently incurring higher night time predation as compared to their SCN intact counterparts (Figure 1). However, studies on similarly treated individuals of golden mantled squirrels *Spermophilus lateralis* and Siberian chipmunks *Eutamias sibricus* (Vogelbaum and Menaker, 1993; Ruby et al. 1996) suggested that SCN ablation did not lead to any physiological consequence and such individuals lived for as long as 2.5 years in laboratory conditions. These results suggest that the observed deaths in the SCN

lesioned animals were indeed due to mistimed behaviours and not a physiological consequence of SCN ablation treatment alone. This is among the most convincing field studies till date that support the idea that circadian clocks ensure optimal timing of behaviours in nature to evade risks from predators.

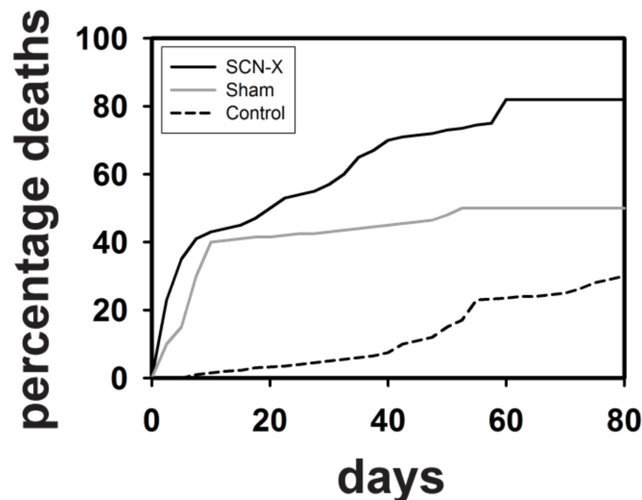


Figure 1: Figure depicting the proportion of SCN ablated (SCN-X), Sham and Control individuals lost due to deaths over 80 days following radio collaring and repatriation. SCN ablation was performed with a stereotaxic lesioning device by drilling a 2 mm (diameter) hole in the skull and passing an electric current using an electrode to lesion the site of interest. 'Sham' controls were also subjected to same procedure but without the use of any electric current while 'control' were not subjected any such procedures. Figure modified after DeCoursey et al (2000).

1.4. Evolutionary origins of circadian clocks

Even though circadian clocks have always intrigued biologists both in terms of their functional complexity, how well do we understand 'when' and 'why' did circadian clocks originate? Here we discuss our current understanding of these two questions.

1.4.1 *When did circadian clocks originate?*

The period of earth's rotation is estimated to have been ~8 h some 3.4 bya (billion years ago) (Turcotte et al. 1977), and was the same until ~3 bya as estimated from the daily growth rings resulting from swimming and settling of algal colonies forming stromatolites (Panella, 1972). Based on these estimations, it has been speculated that the period of earth's rotation was only 4 h when *cyanobacteria* originated (Krasinky, 2002; Lathe, 2004; Johnson and Kyriacou, 2005), and therefore the earliest clocks in *cyanobacteria* probably had a period of ~4 h which gradually extended to 24 h over time as the earth's angular velocity reduced (Tauber et al. 2004). Wells (1963) used annual growth series added to fossilised corals and estimated that the annual duration on earth was ~400 days in the Devonian period (~375 mya). Since time taken for the earth to revolve around the sun has remained constant over the past 4 by (Laskar, 1999), day length during this period was estimated to be ~22 h. Cyanobacteria are currently the oldest life forms whose circadian clocks have been deciphered, and therefore cater as a useful tool to test if its clocks comprising the *kaiABC* (Ishiura et al. 1998) gene cluster were indeed the ancestral clocks.

While *Synechococcus elongatus* has only one copy of the *kaiABC* cluster, multiple copies due to gene duplication have been observed in other species such as *Nostoc linckia* and *Synechocytis*, (Dvornyk et al. 2003). However, not all the three genes appear to have evolved simultaneously. The *kaiC* gene is observed to be

distributed in almost all major taxa of Archaea except the *Methanopyri* and *Thermoplasmata* (Johnson and Golden 1999; Das-Sarma et al. 2001; Dvornyk et al. 2003) but is not that well represented in Eubacteria barring a few major taxa (Dvornyk et al. 2003). *KaiB* on the other hand is distributed in a few proteobacteria including *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* along with *kaiC* (Dvornyk et al. 2003; Larimer et al. 2004), but is observed only in one archaeon, *Methanobacterium thermoauotrophicum* (Dvornyk et al. 2003). The presence of *kaiB* in other prokaryotic domains is speculated to be due to lateral transfer from *cyanobacteria* whereas no reverse transfer from prokaryotes to *cyanobacteria* has been reported. However, *kaiA* seems to be restricted only to *cyanobacteria* thus suggesting that this might be the most recent addition to the cyanobacterial clock network. Therefore, the representation of *kaiC* in *archaebacteria*, *eubacteria* and *cyanobacteria* is suggestive of it being the oldest (over 3.5 by) of the three *kai* genes with the subsequent addition of *kaiB* around 3.5-2.32 bya to form the *kaiBC* cluster. Upon formation of the cluster, both *kaiB* and *kaiC* genes evolved as a unit before the final addition of *kaiA* to the cluster (Figure 2). Studies on the *cyanobacteria Synechococcus* spp. indicate that expression of all three genes is essential for circadian clocks' function and that lack of even one of the three genes renders the clock dysfunctional (Ishiura et al. 1998); however, *kaiA* is not observed in most of the primitive *cyanobacteria* and other photosynthetic proteobacteria (Dvornyk et al. 2003). Does this indicate that even though *kaiBC* genes were present, these organisms did not have a functional clock until the addition of *kaiA*? It is not entirely possible to arrive at such a conclusion since other genes apart from *kaiA* or even *kaiB* might have partnered with *kaiC* to form a functional clock.

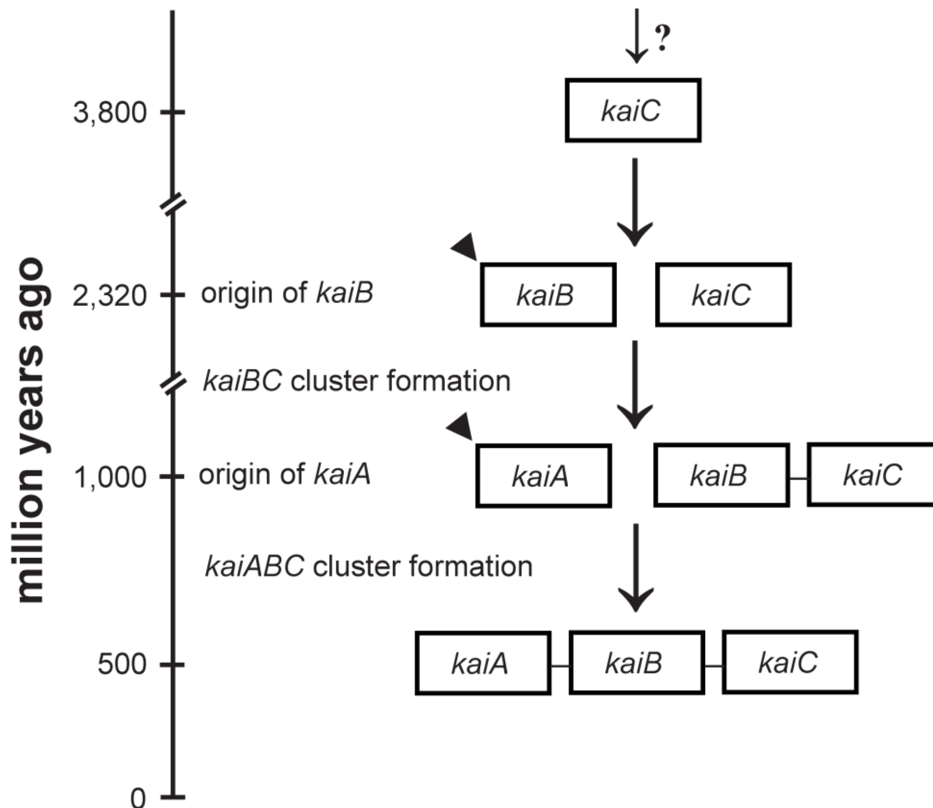


Figure 2: Pictorial timeline representation of major evolutionary events leading to the evolution of *kaiABC* cluster as observe in present day cyanobacteria. Figure modified after Dvornyk et al (2003).

Circadian expression of certain gene transcripts (*Lhcb2*) has also been reported in primitive forms of plants such as the moss *Physcomitrella* (Shimizu et al. 2004) and in gymnosperm *Ginkgo* (Christensen and Silverthrone, 2001). Preliminary data suggests that the *Phsycomitrella* genome shares higher degree of similarity with *Arabidopsis* than *cyanobacteria* (Masashi et al. 2004; Shimizu et al. 2004) suggesting that circadian clocks in higher plants are probably derived from moss genome and not from their earlier ancestors. This is further supported by the observation that the genomes of most modern day plants including rice, tomato and *Arabidopsis* do not share any sequence homology with the cyanobacterial clock genes as will be discussed in the later section.

However, chlamydomonas genome indicates presence of homologues for several kinases and phosphatases such as *casein kinase* and *protein phosphatase* (*ppa1* and *ppa2*; Mittag et al. 2005) which are also ascribed several functions in the circadian clock machinery of *Neurospora*, *Drosophila*, mammals as well as plants (Daniel et al. 2004; Sathyanarayana et al. 2004; Yang et al. 2004). However, since proteins encoded by these genes also perform several non-circadian functions, it is difficult to confirm whether conservation across species is due to their role in circadian clocks or other general cellular functions (Johnson and Kyriacou, 2005).

Collectively, these results suggest that some of the modern day components of circadian clocks in *cyanobacteria* have existed for over ~3.5 by with subsequent addition of other clock genes. If the primitive cyanobacterial clock period was indeed ~4 h as discussed earlier, a clockwork comprising a single gene *kaiC* might have been sufficient to form a feedback loop with a delay of ~4 h which eventually might have evolved to 8 h clocks by the addition of *kaiB* as the day length extended to ~8 h around 3.5 bya, which overlaps with the predicted time of *kaiB* addition (3.5-2.32 bya), and similarly addition of *kaiA* and other associated factors might have led to the evolution of ~24 h clocks. However, mere presence of only one of the three *kai* genes even though suggestive, is not convincing evidence to claim that functional clocks originated over 3.5 bya since other genes might have played preceded *kaiB* and *kaiA* or even *kaiC* and awaits to be discovered.

1.4.2 *Why did circadian clocks originate?*

The great oxygenation event associated with the ozone layer formation that shields the earth from majority of UV radiation occurred around 2.3 bya. If circadian clocks did originate prior to this (as discussed in previous section), organisms inhabiting the earth

are likely to have been subjected to extreme temperatures and harmful solar radiations which might have acted as the potential factors in driving the evolution of mechanisms that helped organisms avoid exposure to such extreme environments (extrinsic advantage). In this regard, we discuss two hypotheses that have been proposed to explain the initial selection pressures that might have driven the origin of circadian clocks.

1.4.2 (a) *Escape from light*

Day night cycles are associated with exposure to harmful solar radiation during the day which is known to increase errors in DNA replication and influence other photochemical reactions in RNA and proteins as well (reviewed in Rajesh et al. 2010), thereby rendering the organisms vulnerable at specific times of the day. Furthermore, even though some of the cellular factors do not function as photoreceptors, are nevertheless sensitive to light probably because of obligatorily associated cofactors such as cytochromes (discussed in Johnson and Kyriacou, 2005). Based on similar premises, Pittendrigh (1965) proposed that organisms might have evolved time keeping mechanisms to anticipate and escape from such harmful effects of light and hence the name 'escape from light' hypothesis. If this is true, given the evolutionary conserved nature of circadian clocks it can be expected that some of the present day life forms may still retain phase specific susceptibility to solar radiation, and indeed *chlamydomonas* have been observed to exhibit rhythmic sensitivity to UV light with enhanced sensitivity around sunset and early night. More importantly, such rhythmic sensitivity to UV light also persists in constant conditions (Nikaido and Johnson, 2000) suggesting a possible role of circadian clocks in driving such responses. Several other observations further support the escape from light hypothesis. For instance, numerous microorganisms exhibit circadian rhythmicity in DNA replication and cell division, with these processes

occurring mostly during the dark phase (Edmunds, 1988). Based on his hypothesis, Pittendrigh (1965) had also anticipated a DNA photolyase type of enzyme to be closely associated with circadian clocks which eventually turned out to be true with studies reporting that molecular circadian clock components involve *cryptochrome* (*cry*) which shares sequence homology with DNA photolyases, a blue light sensitive UV damaged DNA repair enzyme (reviewed in Thompson and Sancar, 2002). Homologues of *cry* have also been identified in cyanobacterium *Synechocytis* spp. (Hitomi et al. 2000) and *Rhodobacter sphaeroides* (Hendrischk et al. 2009); however, their role in circadian function (if any) remains to be elucidated. These results suggest that the ancestral photolyases were probably substrates for natural selection to act upon eventually driving the evolution of *cry* as a core circadian clock component (Nikaido and Johnson, 2000; Gehring and Rosbash, 2003; Johnson and Kyriacou, 2005). Interestingly, the role of *cry* is not entirely conserved in all species; while it is known to function as circadian photoreceptors in some insects (CRY1) (reviewed in Hardin et al. 2011), it acts as a core clock component in mammals (CRY2; Ozturk et al. 2007). This distinction cannot be merely explained on the basis of differences between invertebrate and mammalian clocks as phylogenetic and functional classifications revealed that non Drosophilids such as silk moths, mosquitoes and butterflies express both *cry1* and *cry2* whereas honeybee (*Apis mellifera*) and red flour beetle (*Tribolium castaneum*) possess only *cry2* (Zhu et al. 2005; Rubin et al. 2006). Thus it is not clear as to whether *cry* in circadian clocks originated from its function as photoreceptor or as a core circadian clock component.

1.4.2 (b) *Endosymbiotic coordination theory*

First proposed by Levandowsky (1981) and later elaborated by Kippert (1986), the endosymbiotic coordination theory suggests that since evolution of eukaryotes was

facilitated by endosymbiosis of prokaryotes that formed the precursors for currently observed organelles in eukaryotes, such spatial compartmentation of autonomously functioning organelles might have required a coordination mechanism to temporally regulate processes by interaction among themselves which otherwise would lead to a chaotic cellular systems. Thus, necessity for the establishment of internal temporal order within the cell has been hypothesised to have led to the origin of circadian clocks. If this were to be true, then the circadian clock genes (*KaiABC* cluster) in *cyanobacteria* which are presumed to be the ancestors for chloroplasts in modern day plants must have been transferred to higher plants during endosymbiosis. Unfortunately, no putative homologue of *KaiABC* genes have been observed either in chloroplasts or the nuclear genomes of *Arabidopsis*, rice, tobacco and Medicago (Johnson and Kyriacou, 2005 and citations therein). Eukaryotic alga *chlamydomonas* which is considered to be a closer ancestral relative of plants also does not possess any of the cyanobacterial *KaiABC* genes. Moreover, comparison of *chlamydomonas* and *Arabidopsis* genomes revealed that the former does not have any putative homologue of *Arabidopsis* circadian clock genes including *elf3, gpif3, tej*, while other genes such as *cca1, lhy, col, ztl, ado1, ado 2, ado3, prr1, prr3, prr5, prr7* and *prr9* share minimal homology (Mittag et al. 2005; Johnson and Kyriacou, 2005). Therefore, current evidence barely supports the hypothesis that circadian clocks have evolved to coordinate endosymbiotic process within the cell.

In summary, results from all the studies discussed in this section clearly indicate that we are far from precisely understanding the evolutionary origins of circadian timing systems. However, the lack of clarity in shared sequence homology between genomes of modern and primitive photosynthetic life forms suggests that clocks might have

evolved independently in multiple life forms thus strongly suggesting convergent evolution of circadian clocks.

1.5 Adaptive significance of circadian clocks

It can be noted that majority of the previously discussed studies reported dire consequences of lack of temporal coordination on various aspects of physiology of organisms which highlights that circadian clocks are advantageous. However, barring a few, results from majority of the studies cannot be used as a support to the adaptive nature of circadian clocks, primarily because a trait can be termed adaptive only upon fulfilment of certain criteria that stem from the definition of adaptation as discussed in section 2. In the subsequent sections, we will discuss studies that adopted various experimental strategies to provide relatively direct lines of evidence in support of adaptive significance of circadian clocks.

1.5.1 *Effects of dysfunctional circadian clocks*

One of the straight forward ways to test the functional significance of circadian clocks is to simply devoid the organism of clocks and assess its consequences on its fitness. Studies to test the same have adopted multiple approaches such as the use of mutant strains with non-functional circadian clocks, surgical ablation of the master clock or use of constant light (LL), all of which are known to render most circadian behaviours arrhythmic.

1.5.1 (a) *Effect of circadian dysfunction realised by genetic manipulations*

Molecular components of circadian clocks have been extensively explored in majority of the standard model systems used in chronobiology. Such studies have revealed that the functional principles of circadian clocks are highly conserved across organisms and constitute several interlocking positive and negative feedback loops, and that disruption

of the core clock components renders the clocks dysfunctional leading to arrhythmic behaviours (reviewed in Hardin, 2011). Multiple studies have taken advantage of the ability to genetically manipulate molecular clockwork to abort its functioning, and investigated its influence on organisms' fitness measures such as longevity and fecundity.

Loss of function mutations of core clock genes in *Drosophila* namely *period (per), timeless (tim), clk (clock)* and *cycle (cyc)* have been observed to have deleterious effects on fecundity of *D. melanogaster* with such mutants showing ~40% reduction in egg output (Beaver et al. 2002). Further analysis revealed that such a reduction in fecundity was associated with reduced fertility (as assessed by sperm count) of the mutant males (Beaver et al. 2002). Additionally, clock mutations have also been reported to influence oogenesis in *D. melanogaster* females; however, such effects do not seem to involve circadian clocks but arise from pleiotropic effects of clock genes. Additionally, Hendricks et al (2003) reported that *cyc* null mutants (*cyc*^{o}) of *Drosophila* exhibit reduced lifespan in both LD and DD conditions as compared to their rhythmic wild type controls. Interestingly, such a reduction in lifespan was observed only in males while the lifespan of *cyc*^{o} females did not differ from controls. Thus, it appears that the observed effects of clock gene mutations on life-history traits may not necessarily be mediated through circadian clocks *per se* but by the pleiotropic effects of clock genes. Additional support came from studies on plants. Constitutive expression of the *cca1* (*circadian clock associated 1*) gene in *Arabidopsis* (*CCA1-ox*) results in loss of circadian rhythms under both LL and DD (Wang and Tobin, 1998). Similar arrhythmic behaviour is also observed upon overexpression of another clock gene *elf3-1* (*early flowering 3-1*), but only in LL and not in DD (Hicks et al. 1996; McWatters et al. 2000). Green et al (2002) used these lines to study the effect of loss of circadian

rhythmicity on fitness of *Arabidopsis,* and observed that under very short day conditions *CCA1-ox* plants were less viable than their wild type counterparts. In addition, the two other circadian rhythm mutants of *Arabidopsis*, *LHY-ox* (Schaffer et al. 1998) and *elf3* also have low viability phenotypes. However, the authors quite rightly pointed out that even though their results are suggestive of the role of circadian clocks in determining fitness of *Arabidopsis*, such effects can also arise from pleiotropic influence of the genes under study and may be independent of the circadian defects imposed by the mutations.

1.5.1 (b) *Effect of circadian dysfunction realised by environmental manipulations*

While most organisms exhibit free-running circadian rhythms in DD, majority of them are rendered arrhythmic (both at behavioural and molecular levels) under LL. Several studies have adopted such LL induced arrhythmicity to assess the fitness costs of harbouring dysfunctional circadian clocks as such an approach would help surpass the pleiotropic effects of genetic mutations observed in studies using genetic manipulations. Allemand et al (1973) assayed adult lifespan of *D. melanogaster* maintained under LL, LD and DD, and observed that lifespan of individuals under LL was significantly shorter than that in LD followed by DD which had a significantly higher lifespan (~20% longer for males and 43% for females) thus demonstrating the negative implications of loss of circadian rhythmicity on organisms' longevity. In another study by Kumar et al (2005) who assayed lifespan of spontaneously arrhythmic individuals from inbred and outbred strains of *D. melanogaster* found that the arrhythmic individuals exhibited an ~37% reduction in lifespan in the outbred populations whereas the inbred lines exhibited a more severe reduction (~60%) in lifespan as compared to their rhythmic counterparts. While these studies demonstrate fitness costs of loss of circadian rhythmicity, it is difficult to rule out possible circadian clock independent deleterious effects of LL on the organism's physiology, and therefore such effects may not necessarily be a consequence

of circadian dysfunction. Several other studies have also assessed the effect of LL on circadian behaviours, but since these studies also used other light regime combinations, they will be discussed in the succeeding section.

1.5.2 *Circadian resonance and consequences of circadian mismatch*

If circadian clocks indeed evolved in response to the 24 h periodic selection pressures stemming from the rhythmic rotation of earth about its axis, then individuals with clock periods that are largely deviant from 24 h or those who fail to synchronize to the 24 h environmental cycles thus leading to circadian desynchrony would incur greater fitness costs. This forms the rationale for the 'circadian resonance' hypothesis according to which, organisms are expected to perform at their best when their clock period matches (and thus the clock resonates) with that of the environmental cycles (Pittendrigh and Bruce, 1959). The cost of circadian mismatch with external cycles has been studied in a variety of species spanning unicellular *cyanobacteria*, insects, mammals and plants. The strategies used in such studies can be broadly classified into two categories. One involved the use of genetically manipulated or mutant strains with the clock period deviating from 24 h, and the other strategy involved using light regimes of periods (T) longer than or shorter than 24 h.

1.5.2 (a) Consequence of circadian mismatch on development

Pre-adult development time in insects is one of the commonly studied fitness traits, and multiple studies have tested the proposition that development time is influenced by the period of the environmental cycle and provided further evidence favouring the circadian resonance hypothesis. For instance, when flesh fly *Sarcophaga argyrostoma* were reared under different T-cycles their pre-adult development time was longer under T-cycles closer to 24 h which was approximately the τ of flesh flies, and also under T-

cycles with period which were multiples of 24 h as compared to other *T*-cycles (Saunders, 1972). Similarly, a recent study by Lone et al (2010) reported that pre-adult development was significantly faster when *Camponotus* ants were reared under symmetric *T*-24 cycles as compared to *T*-20 and *T*-28. When growth rate of tomato plants was assessed under different *T*-cycles, the ones growing under *T*-24 outperformed others growing under *T*-12 and *T*-48 and even LL (Withrow and Withrow, 1949; Highkin and Hanson, 1954; Hillman, 1956; Went, 1960). Furthermore, the growth rates under high and low temperatures exhibited a Q_{10} values of about 1.2 suggesting that it was temperature compensated which further substantiates the possible role of circadian clocks in regulation of development time (Pittendrigh, 1954; Zimmerman et al. 1968). However, it appears that the results from tomato plants cannot be generalised. For instance, growth rate of *Arabidopsis* under LL was found to be significantly higher as compared to that in LD12:12 (Johnson and Kyriacou, 2005). Another study on clock disrupted *Arabidopsis* mutants reported that such strains produce fewer viable seeds as compared to wild type strains under LD04:20 (Green et al. 2002). These results further suggest the role of circadian clocks in mediating development time, a fitness measure as will be discussed later.

1.5.2 (b) <u>*Consequence of circadian mismatch on longevity*</u>

Pittendrigh and Minis (1972) observed that when wild type strains of *D. pseudoobscura* were maintained under different *T*-cycles (*T*-21 and *T*-27) or in LL, lifespan of flies maintained under *T*-24 was considerably longer, but these results could not be reproduced in a different laboratory (Johnson and Kyriacou, 2005). A similar trend was also observed in blowflies (*Phormia terraenovae*) which exhibited 22% lifespan reduction (90% deaths occurring by 98 days) in individuals subjected to repeated jetlag as opposed to their controls who lived for almost 125 days (von Saint Paul and Aschoff,

1978). Another study on *D. melanogaster* used wild type (per^+; $\tau \sim 24$ h) and short period (per^T; $\tau \sim 16$ h) and long period (per^L; $\tau \sim 28$ h) strains to examine the cost of circadian mismatch with environmental cycles (Klarsfeld and Rouyer, 1998). The authors assayed lifespan of all three strains under symmetric *T*-16 and *T*-24 cycles, and found that males of per^T and per^L strains lived significantly shorter as compared to wild type males under *T*-24. Moreover, per^T exhibited a larger reduction in lifespan as compared to per^L. The proposed reason for this is per^T whose τ deviated by 8 h in a 24 h LD cycle would incur a greater cost in comparison with per^L whose τ deviated by only 4 h. This is further substantiated by the observation that lifespan difference between per^T and wild type flies was further reduced under *T*-16 (close to the period of per^T) as compared to the difference between them under *T*-24. While these results are partly in accordance with the expectations from the circadian resonance hypothesis, the fact that per^T did not exhibit higher lifespan in *T*-16 is intriguing.

1.5.2 (c) <u>*Consequence of circadian mismatch on competitive ability*</u>

Even though the above discussed studies reported several evidence supporting the circadian resonance hypothesis, it is important to note that most of these studies involved assessment of fitness measures under simple non-competitive conditions. In a more realistic scenario, an organism can be considered to be fit in a given environment only when it can out compete its competitors inhabiting the same environment. Therefore, experiments that can demonstrate the competitive abilities of different individuals/strains are a more rigorous approach to assess fitness of organisms. Here we discuss two such interesting and well-designed experiments that have provided some of the strongest arguments till date in favour of circadian resonance, and thus adaptive significance of circadian clocks.

In a first study of its kind wild type strains of *cyanobacteria S. elongatus* (AMC149 and AMC343; τ = 25 h) were competed with two period mutant strains, SP22 and P28 of τ 23 h and 30 h respectively. The experimental design comprised pairwise competition experiments under multiple *T*-cycles whose period matched with the τ of at least one of the strains subjected to competition. The authors observed that, when equal proportions of two strains were mixed and allowed to compete, the wild type strains outgrew all others under *T*-24 while the SP22 strain performed better under *T*-22. Furthermore, the P28 strain which performed poorly under *T*-22 and *T*-24, were observed to outgrow other strains when competed under *T*-30 (Figure 3; Ouyang et al. 1998). To further test if the observed performance difference between strains were indeed due to period match with the environmental cycles and not a consequence of the mutations harboured, the authors reverse engineered the P28 strain to mimic the wild type strain, and observed that the reverse engineered P28R strain performed similar to that of wild type strain under *T*-24.

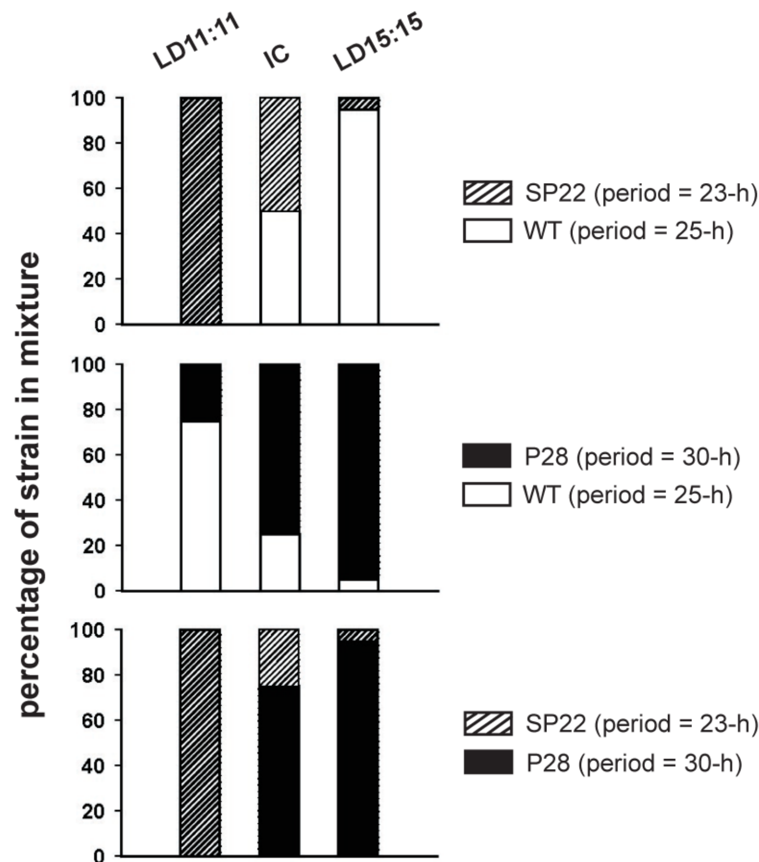


Figure 3: Percentage of each of the short period (SP22), long period (P28) and wild type (WT) under different light regimes after 27 days of competition. Different proportions of strains were mixed as pairs at the beginning of the experiment (IC = Initial Condition) and were let to compete with each other under LD11:11 and LD15:15 for 27 days. The proportion of strains outcompeted the other depending on the proximity of the strains' clock period with that of the LD cycles. Figure modified from Ouyang et al (1998).

In continuation of this study, the authors also performed similar competition experiments for period mutants harbouring point mutations in other core clock genes in *S. elongatus* and reported similar results (Woelfle et al. 2004), thereby ensuring that the observed phenotypes do not stem from pleiotropic effects of genes but is a consequence of circadian misalignment. Dodd et al (2005) used wild type *col-o* (τ = 24 h), long period *ztl-l* (τ = 27.1 h to 32.5 h) and short period *toc1-1* (τ = 20.7 h) strains of

Arabidopsis to study the relevance of circadian resonance. The authors reared all the strains under LD cycles with varying periods (*T*-20, *T*-24, and *T*-28) and assessed photosynthetic activity and growth rates under these conditions. The chlorophyll content was found to be higher in plants reared under *T*-cycles with periods matching their *τ* with *col-o* performing better than other two strains in *T*-24 than in *T*-20 and *T*-28. Similarly, the *ztl-l* and *toc1-1* strains performed better under LD cycles that matched their endogenous periods than in *T*-24. Consequently, all strains exhibited higher CO_2 fixation and greater biomass when reared under *T*-cycles with periods matching their *τ*. In competition between *ztl-l* and *toc1-1* strains which were grown as mixed populations under *T*-20 and *T*-28, each strain exhibited higher chlorophyll content, leaf number and aerial biomass as compared to its competitor when grown under *T*-cycles with periods matching *τ*. These results demonstrated that period match between endogenous clocks and external cycles enhances fitness of individuals and therefore provide strong support for the circadian resonance hypothesis.

1.5.2 (d) *Consequence of circadian mismatch on other traits*

Circadian resonance hypothesis has also gathered support from studies reporting that organisms exhibit multiple physiological disturbances as a consequence of circadian misalignment which has also drawn considerable attention due to its influence on human health (Box 2).

Box-2: Chronotypes and circadian desynchrony in humans

Humans are known to exhibit variation in preferred timing of sleep-wake behaviour with individuals who prefer to wake-up early and sleep early termed as 'early' chronotypes while those who wake-up late and sleep later are termed 'late' chronotypes (Roenneberg, 2012). Furthermore, chronotypes have been observed to be associated with circadian clock period (Roenneberg, 2012). Owing to the societal schedule that generally favors early chronotypes, late chronotypes often struggle to remain in sync with the societal schedule resulting in them experiencing 'social jet-lag' a term used to refer desynchrony between the inherent clock and external cycle. As a result of this, late chronotypes have been reported to experience several psychological, physiological and metabolic disturbances (Roenneberg, 2012). Several studies have also directly examined the impact of circadian desynchrony on human physiology. Such studies adopted protocols that involved placing subjects under dim LD (*T*-20 or *T*-28) cycles with scheduled sleep bouts and mealtimes. Since the human circadian clock cannot entrain to such extreme T-cycles, it tends to free-run thus desynchronizing behavioral cycles (eating and sleeping) with internal clocks and external environment referred to as forced internal desynchrony. Forced internal desynchrony has been reported to be associated with sleep disturbances, decreased vigilance and cognitive performance (Dijk et al. 1992; Dijk and Czeisler, 1995). Also, such desynchrony also results in severe physiological disturbances such as elevated blood glucose, reduced insulin sensitivity and circulating leptin, altered post-prandial insulin release and advent of hypertension (Litinski et al. 2009; Scheer et al. 2009; Morris et al. 2012; Archer et al. 2014; Leproult et al. 2014). Furthermore, analysis of blood transcriptome in desynchronized subjects revealed a strongly attenuated rhythmic gene expression (going from 6.4% of the transcriptome at baseline to only 1.0%) during circadian misalignment (Archer et al. 2014).

A naturally identified autosomal gene mutation (*tau*) in hamsters causes a copy number dependent change in circadian period with the homozygotes exhibiting 20 h and fails to entrain to LD14:10 cycles while the heterozygotes exhibit 22 h rhythms (Ralph and Menaker, 1988). The heterozygous animals were observed to exhibit reduced lifespan under LD14:10 cycles while the homozygous mutants did not show any lifespan deficit (Hurd and Ralph, 1998). Moreover, when housed under *T*-24, heterozygous individuals were observed to exhibit multiple physiological defects such as cardiomyopathy with fibrosis and impaired contractility, and renal dysfunction. The authors speculated that the observed physiological disturbances might stem from circadian misalignment, and interestingly, when housed under *T*-22 which is proximal to the clock period, no such physiological defects were observed in heterozygous individuals.

Another interesting study assessed the importance of circadian desynchrony on reproductive fitness in natural populations of pitcher plant mosquito *Wyeomyia smithii*.

Individuals of this species like many others are known to enter larval diapause under short photoperiods which is terminated under long photoperiods (Bradshaw et al. 2003). Emerson et al (2008) subjected populations of *W. smithii* to LD10:14, LD10:36, and LD10:25 all of which are known to induce diapause in this species. The authors further measured various fitness parameters such as egg-to-pupa survivorship, fecundity, embryonic viability and adult lifespan, and observed that individuals that entered diapause under LD cycles with periods 24 h (LD10:14) or multiples of 24 h (LD10:36) exhibited higher fitness as opposed to individuals under non 24 h cycles (LD10:25).

A recent study on *Arabidopsis* used a novel approach to study adaptive significance of circadian clocks (Yerushalmi et al. 2011). The study made use of crosses between long period (*prr7prr9*, τ = 36.55 h) and short period (*prr5prr7*, τ = 22 h) mutant strains to generate greater variability in τ among the F2 progeny which varied between 20.79 h and 45 h. These strains were then reared under *T*-20 (LD13:07) and *T*28 (LD19:09). After one generation, τ and frequencies of *prr5prr7* and *prr7prr9* alleles were assessed in the F3 populations as a measure of reproductive success of the short and long period variants. Interestingly, τ of the F3 plants was positively correlated with the period of *T*-cycles (*T*-20 or *T*-28) in which their respective parents were reared. Also, the F3 individuals from parents reared in *T*-28 inherited long period *prr7prr9* alleles in higher frequencies. However, surprisingly the frequency of short period allele *prr5prr7* did not differ significantly between the F3 populations coming from F2 parents reared under *T*-20 or *T*-28. Nevertheless, these results suggested that long τ phenotypes are favoured under *T*-28 thus providing further evidence in support of circadian resonance hypothesis. These studies collectively highlight importance of circadian clocks and its alignment with external cycles.

1.5.3 *Studies on natural populations*

1.5.3 (a) Geographical clines

Among the most prominent variation in environmental conditions are changes in day length (photoperiod), light intensity and temperature levels observed across latitudes with such conditions reaching extreme variation as one moves towards the poles (Hut et al. 2013). Such gradation is likely to drive latitude dependent selection pressures on rhythmic behaviours, and therefore, studying how circadian rhythms vary in populations inhabiting different latitudes (clines; see glossary) will be insightful.

The earliest comprehensive analysis of clines in circadian behaviours was undertaken by Lankinen who studied emergence rhythm and seasonal diapause in European *D. littoralis* populations collected from 30º-70ºN. Lankinen assessed phase and τ of emergence rhythm in 12 southern (28ºN) and northern (56º-63ºN) strains of *D. subobscura* and found that phase of emergence was advanced, clock period shortened and amplitude lowered in the northern strains as compared to the southern strains (Lankinen, 1986). Pittendrigh and Takamura (1989) also studied four Japanese strains of *D. auraria* collected from latitudes between 34º-43ºN, and observed that phase of emergence rhythm varied by 2 h and period by 0.5 h. Allemand and David (1976) reported observing a latitudinal cline in circadian oviposition rhythm in *D. melanogaster* collected from equatorial Africa (0ºN) to Scandinavia (62ºN). However, it remains to be tested if the observed cline differences were indeed driven by differences in local environmental conditions and if so, their role in local adaptations. Latitudinal clines in the effects of natural zeitgebers have been studied in birds and mammals but not evolutionary adaptation of their circadian systems to latitude (Hut et al. 2013). Similar studies on humans have also been reported but appear to be controversial. For instance, Ciarleglio et al (2008) proposed that variation in clock genes among the world

populations may primarily be due to genetic drift and not due to directional selection whereas, Cruciani et al (2008) reported that genetic variation in *hper2* gene is under directional selection.

The *Drosophila per* is known to encode a variable length *Thr-Gly* residue repeats with $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$ being the two major variants accounting for 90% of repeat length variation in the European populations (Costa et al. 1992; Rosato et al. 1994). A study on natural populations of *D. melanogaster* collected from different latitudes reported that the two repeat length variants were statistically correlated with latitudes with $(Thr\text{-}Gly)_{17}$ occurring primarily in the southern latitudes while $(Thr\text{-}Gly)_{20}$ was predominant in the northern latitudes (Figure 3; Costa et al. 1992). The authors speculated latitudinal variation in temperature as being the primary driving force and thus assessed the role of *Thr-Gly* repeat lengths in temperature compensation. Indeed it was observed that flies with $(Thr\text{-}Gly)_{20}$ variant exhibited better temperature compensation ability as compared to the $(Thr\text{-}Gly)_{17}$ variant, and therefore it was concluded that the observed latitudinal cline in the frequency of *Thr-Gly* repeat length was shaped by natural selection acting on the ability of circadian clocks to remain stable even at lower temperatures experienced at higher latitudes (Sawyer et al. 1997). A similar but weaker latitudinal cline for $(Thr\text{-}Gly)_{20}$ and $(Thr\text{-}Gly)_{23}$ repeat length variants was observed in *D. melanogaster* populations sampled from Australia where $(Thr\text{-}Gly)_{20}$ positively correlated with latitude (Sawyer et al. 2006). However, this was disputed by another study which failed to detect such a latitudinal cline (Weeks et al. 2006). Nevertheless, observing similar trends in evolutionary footprints across multiple studies on independent populations further strengthens the idea that circadian clocks facilitate adaptation to local environmental conditions.

Natural polymorphism in *tim* (*ls-tim* and *s-tim*) results in two versions of the translated proteins (TIM) with *ls-tim* coding for both long and short variants of TIM while *s-tim* codes for only the short variant (Rosato et al. 1997). Tauber et al (2007) reported a latitudinal cline in the frequencies of the *ls-tim* such that its frequencies were higher in the southern latitudes (Italy) which gradually decreased towards the northern latitudes (Sweden; Figure 4). To further investigate what aspects of the phenotype did the *ls-tim* influence, the authors tested its role in temperature compensation as in Sawyer et al (1997). However, temperature compensation was found to be similar in individuals carrying *s-tim* and *ls-tim.* Northern Europe is characterized by lower temperatures and shorter day lengths, which is known to induce diapause in *Drosophila* (Schmidt et al. 2005; Schmidt and Conde, 2006). Therefore, the authors further tested if the two variants affected the ability of flies to enter into diapause, and found that the *ls-tim* females exhibited higher incidence of diapause as compared to the *s-tim* females. Similarly, higher incidence of diapause in northern latitudes is observed in *D. melanogaster* populations sampled from the eastern United States of America (Schmidt et al. 2005). Furthermore, females with higher incidence of diapause have been reported to show lower stress resistance and enhanced fitness under undesirable conditions suggesting that enhanced diapause confers adaptive advantages (Schmidt et al. 2005; Schmidt and Conde, 2006). Observing a similar trend in the European populations suggests that the observed latitudinal cline was probably due to adaptation to local environmental conditions. However, contrary to this, *ls-tim* which favoured diapause in females was observed to be lower in the northern latitudes as compared to the southern areas. Thus, even though the observed clinal variation may support the idea of local adaptations, it remains unclear as to what factors drove the clinal divergence in the *tim* allele frequencies. Clock genes have been implicated in diapause in a number of insect

species including the pitcher plant mosquito *W. smithii* (Mathias et al. 2007), *Chymomyza costata* (Stehlik et al. 2008), *Riptortus pedestris* (Ikeno et al. 2011), *Drosophila triauraria* (Yamada et al. 2011), and *Sarcophaga bullata* (Han and Denlinger, 2009a, b). While this is suggestive of a causal link between circadian clocks and diapause mechanisms, clock independent pleiotropic effects of clock genes cannot be entirely ruled out. Therefore, the observed results of clinal divergence in *ls-tim* frequencies even though might have been associated with diapause response, does not necessarily imply role of circadian clocks.

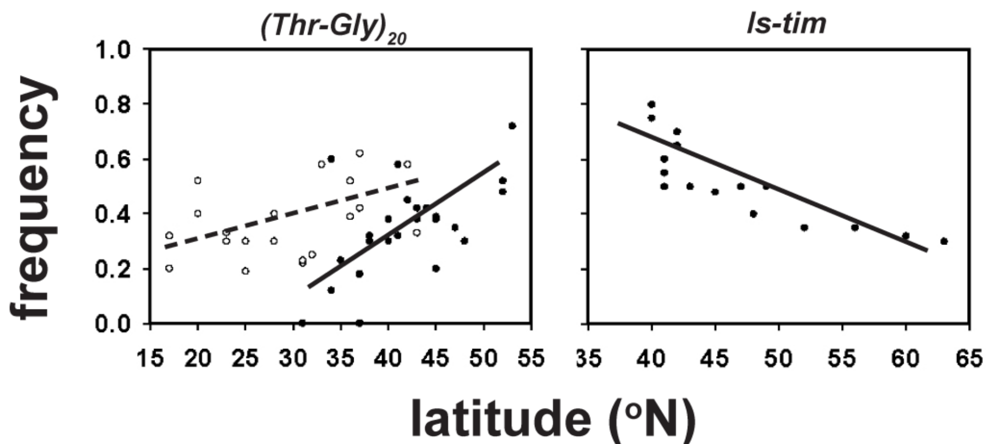


Figure 4: (a) Frequencies of *per* (*Thy-Gly*)$_{20}$ across latitudes in populations of *D. melanogaster* sampled in Australia (open circles) and Europe (closed circles). The dashed and solid regression lines indicate the latitudinal cline of frequencies in Australian and European populations respectively. Both regression lines reveal that the frequencies of per (*Thy-Gly*)$_{20}$ alleles increases towards northward regions which are characterized by reduction in ambient temperature. (b) Frequencies of *ls-tim* across latitudes in populations of *D. melanogaster* sampled in Europe (south-eastern Italy to Sweden). Both the Figures are modified after Kyriacou et al (2007).

Studies reporting latitudinal clines in clock gene polymorphisms in vertebrates have been inconclusive. Fidler and Gwinner (2003) examined sequences of three core clock genes: *bmal1*, *clk,* and *per2* in owls and found the *clk* poly-glutamine (*clock*-polyQ) region to be polymorphic within and among species. In support to this, non-migratory

Blue Tit *Cyanistes caeruleus* and the migratory Bluethroat *Luscinia svecica* populations showed considerable polymorphism in *clock*-polyQ (*clock*-polyQ)$_9$ and (*clock*-polyQ)$_7$, and displayed a positive association between allele length and breeding latitude in the Blue Tit; whereas Bluethroat populations failed to show such correlations (Johnsen et al. 2007). Several other studies on the swallow genus *Tachycineta* studied across North, South and Central America (Dor et al. 2012), barn swallow *Hirundo rustica* (Dor et al. 2011) and Pacific salmon species (genus *Oncorhynchus*) (O'Malley and Banks, 2008; O'Malley et al. 2010) failed to show consistent patterns of latitudinal clines in *clock*-polyQ frequencies.

Among the studies in plants, Mayer (1966) observed that τ of circadian leaf movement rhythm of various plant species were generally shorter further north which is observed to hold true for within genus comparisons in two dandelion (*Taraxacum*) species. When circadian leaf movements were monitored in a collection of 150 natural accessions of *A. thaliana*, period length of their circadian clocks was found to correlate positively with day length at their latitude of origin (Michael et al. 2003). In many plants, circadian clocks have been attributed to synchronize flowering with favourable conditions of the day depending on the latitude. The circadian expression of GmCRY1a which functions in the input pathway of circadian timing system has been observed to be correlated with latitude, and also with flowering time in *Glycine max* (soybean) (Zhang et al. 2008). Therefore, latitudinal clines in clock gene polymorphisms are yet to be well established in plants and vertebrates, and further studies may help shed more light on these aspects.

In addition to latitudinal clines, studies aimed at exploring the functional relevance of circadian rhythms in organisms that live in similar day lengths but different

ambient temperatures (altitudes) even though are minimal, show considerable potential to help explore interesting aspects of circadian clocks. For instance, Sorensen and Loeschcke (2002) examined heat tolerance in populations of *Drosophila buzzatii* originating from different altitudes in North Western Argentina. Interestingly, the authors found that heat tolerance varied across the day in an altitude dependent manner with heat tolerance in the afternoon and early evening being significantly lower in the higher altitude populations as compared to their lower altitude counterparts. Although the authors did not examine differences in circadian rhythmicity between the high and low altitude populations, the timing of heat tolerance was controlled by light phase suggesting a possible involvement of circadian clocks. As a further support to this, other studies have also reported circadian regulation of temperature stress resistance in plants (Rikin et al. 1993; Fowler et al. 2005), and additionally, majority of circadian clock regulated genes in *Arabidopsis* genome are known to be involved in stress resistance pathways (Covington et al. 2008). In addition to studies on longevity and developmental rates, these results suggest that circadian regulation of stress responses may be another substrate for natural selection to act upon, thus underscoring the adaptive role for circadian clocks.

1.5.3 (b) *Populations evolving in constant conditions*

A corollary to the hypothesis that circadian clocks evolved in response to rhythmic selection pressures imposed by cycling environmental conditions is that, if organisms are devoid of cyclic conditions for several generations, their circadian clocks would regress since there would be no obvious advantage of timing behaviours in an otherwise aperiodic environment.

Several species inhabiting constant conditions show considerable changes in morphological features which corroborates the idea of regression of traits that are of little utility in their respective habitats. One prominent example is that of the visual sensory system which is known to be considerably regressed in such species. Based on quantitative assessment of reduction in visual systems, such species have been sorted into three categories: (1) Macrophthalmic species in which the visual system is not substantially reduced in comparison with that in allied surface dwelling species. (2) Microphthalmic species characterized by reduction of the eye ranging from mild to almost complete absence. (3) Anophthalmic species are the extreme types defined by complete lack of any visual system (Friedrich, 2013). However, it is also essential to identify species in which visual sensory systems never evolved *versus* those in which such systems evolved and eventually regressed. Nevertheless, one of the best ways to test such a hypothesis would be to study circadian rhythms in natural populations that have evolved in constant environments for several thousands of generations. In this regard, deep underwater caves and similar deep sea habitats have served as excellent laboratory setups (Poulson and White, 1969) with organisms inhabiting such areas being instrumental in shedding more light on the evolution of circadian clocks. Even though several studies have assessed circadian rhythms in all three macro, micro and anaphthalmic species, we will restrict our discussion to the studies on the microphthalmic and anaphthalmic species which show relatively extreme features of visual system regression. A detailed review on other categories can be found elsewhere (Friedrich, 2013).

Fish: Several studies have assessed circadian rhythm properties in natural populations of troglofauna and stygofauna (land and water dwelling trogbolites respectively). One of the earliest studies in this line tested for temporal activity patterns

in the cave crayfish *Orconectes pellucidus* and reported absence of any rhythmic activity behaviour in constant laboratory conditions (Park et al. 1941). However, 20 years later Brown (1961) analysed the same data with improved statistical tools and surprisingly detected significant circadian rhythmicity in the activity of this species. In continuation, another set of studies not only confirmed the findings by Brown (1961) but also reported circadian rhythmicity in oxygen consumption in *O. pellucidus* (Jegla and Poulson, 1968). This, however, might not be surprising since *O. pellucidus* has been observed to harbour vestiges of the eyes (Hobbs and Barr, 1972) and has also been documented to exhibit photo negative responses (Park, 1941) suggesting that they probably have functional light input pathways which might underlie the observed weak circadian behaviour. Among the vertebrate microphthalmic troglobionts whose circadian behaviours have been studied is the catfish *Rhamdia enfurnada*. Morphological assessments indicate that the visual system of *R. enfurnada* is highly reduced but is never completely missing; however, this species is characterized by extensive population genetic variation of troglomorphic traits. Consistent with this, individuals when tested for rhythmic behaviours in DD exhibited large variation in periodicities ranging from circadian to ultradian values (Trajano et al. 2009), a possible indication of circadian clock regression in progress.

Studies on anophthalmic species have reported several interesting features of circadian clock evolution under constant environments. The anophthalmic cyprinid Somalian cave fish *P. andruzzii* is endemic to a completely enclosed cave habitat (Cavallari et al. 2011), and is estimated to have diverged from its common ancestor and subsequently evolved in constant cave environment for around 1.4–2.6 my (Colli et al. 2008). The effect of such long term evolution is evident from the loss of scales, eyes and pigmentation that are presumed to have regressed in due course. However, *P.*

andruzzi have been observed to exhibit negative phototactic behaviour (Ercolini and Berti, 1975; Tarttelin et al. 2012) at four different wavelengths (480, 539, 615, and 692 nm) and most pronounced to blue light which coincidentally (or not) is also the wavelength that circadian clocks are most sensitive to. Interestingly, the genome of *P. andruzzii* possesses homologs of major core clock genes (*clk1a*, *clk2*, *per1,* and *per1b*; Cavallari et al. 2011), suggesting that their clocks might have been functional earlier but regressed over time. Laboratory studies revealed that neither the central nor the peripheral clocks of *P. andruzzii* are light entrainable. However, non-visual zeitgebers such as periodic feeding have been reported to entrain free-running circadian activity rhythm as well as cycling of the peripheral clocks in various tissues (Cavallari et al. 2011). Interestingly, the circadian clock of this species does not seem to be temperature compensated. Partial exhibition of circadian rhythms and loss of temperature compensation suggests that *P. andruzzii* is probably in the course of evolutionary regression of circadian clocks. However, this can also be reasoned alternatively. Temperature compensation has been hypothesized to be a key adaptive mechanism to ensure that changes in environmental temperatures do not alter τ. Cave environments unlike terrestrial habitats do not experience any temperature changes across the year, in which case a temperature compensated clock may not necessarily be adaptive, and therefore, it is plausible that only mechanisms underlying temperature compensation might have regressed in these populations while functional circadian clocks might still be under selection. Another cave fish, the Indian loach *Nemacheilus evezardi*, which exhibit extreme variation across populations including both micro and anaphthalmic forms (Pradhan et al. 1989) has also served as a model for various circadian rhythm studies (Pati, 2001). The microphthalmic forms have been documented to exhibit time dependent phototactic behaviour (Agrawal, 2008), and also exhibit circadian rhythmicity

in burrowing and air gulping (Biswas et al. 1990a, b; Pradhan and Biswas, 1994; Pati, 2001). Furthermore, scheduled feeding has been speculated to entrain rhythmic activity in *N. evezardi* similar to that observed in the Somalian cave fish (Biswas and Ramteke, 2008). There have been examples of virtual absence of rhythmic behaviours in some of the anophthalmic fish species such as the characiform *Stygichthys typhlops* which do not exhibit any rhythmic activity behaviour either in LD or DD (Trajano et al. 2012). In a set of studies on catfishes from Brazil, it was reported that a majority of individuals, exhibit ultradian and infradian patterns in activity while no significant circadian component was observed (Trajano and Menna-Barreto, 1996 and citations therein).

Arthropods: The cave dwelling arthropod amphipod *Niphargus puteanus*, exhibits reduction of both peripheral and central visual systems (Blume et al. 1962; Günzler, 1964), but appears to be nocturnal under LD conditions. While this may also be due to photic avoidance behaviour and not necessarily endogenous, exhibition of weak free-running rhythm following LD entrainment suggests presence of a functional circadian clocks which probably is degenerative. Studies that tested for persistence of circadian rhythmicity include those on the ground beetle species *Laemostenus navaricus* and *Typhlochoromus stolzi* which have reported that majority of individuals of *L. navaricus* exhibit arrhythmic activity behaviour in DD (Lamprecht and Weber, 1977) similar to those in the *T. stolzi* species (Lamprecht and Weber, 1977, Bartkowiak et al. 1991). A relatively recent study by Koilraj et al (2000) which investigated locomotor activity of millipede *Glyphiulus cavernicolus sulu* under LD, DD and LL conditions found that majority of the individuals were rhythmic in LD, but close to half of them exhibited circadian rhythmicity in DD while others were arrhythmic, and a negligible proportion of them were rhythmic even in LL. The most convincing evidence thus far supporting the idea of regression of circadian timing systems in organisms adapted to

constant conditions comes from the eyeless species of ground beetles and small carrion beetle (Wiley, 1973; Weber, 1980). None of the individuals in these species were observed to exhibit rhythmic activity patterns either in LL, LD, or DD. More importantly, these data seem to be consistent across multiple independent studies on Nearctic and Palearctic species indicating that loss of behavioural rhythmicity due to convergent evolution is plausible, and further substantiates the hypothesis discussed earlier.

Chordata: Cave salamander *Proteus anguinus* comprises the subspecies *P. anguinus anguinus* which has degenerated visual system unlike its epigean counterpart *Proteus anguinus parkelj* (Kos et al. 2001). Studies aimed at assessing circadian rhythms in *P. anguinus anguinus* have reported mixed results. While one study demonstrated the presence of circadian and ultradian rhythms in activity (Briegleb and Schatz, 1974), another study that recorded activity for over 350 days could only gather tentative evidence supporting the presence of circadian rhythms with mild incidences of ultradian and infradian rhythms (Schatz et al. 1977). In addition, studies in DD did not observe any significant behavioural rhythm (Hervant et al. 2001). However, it is believed that the reported discrepancies might be due to low activity levels in *P. anguinus anguinus* because of their inherently lower metabolic rates (Friedrich, 2013) which generally complicates time series analysis to identify rhythmic trends in data.

Even though we discussed selected examples of troglobionts and other organisms known to have evolved in constant environments for several generations, Friedrich (2013) extensively compared studies across 40 vertebrates and arthropod species revealing a compelling trend suggestive of the regression of visual system being correlated with clock regulated rhythmic behaviours (Figure 5). It can be observed that

among the 9 micropthalmic species studied, not surprisingly, a large portion (88%) exhibit rhythmic behaviours which includes circadian, ultradian and infradian periodicities, whereas one species studied thus far was arrhythmic (Figure 5). Nevertheless, anophthalmic species which represent more extreme phenotypes in terms of adaptation to constant conditions provide strong support with over 60% of the species studied exhibiting arrhythmic behaviours (Figure 5). The higher incidence of rhythmic behaviours in microphthalmic species might not be surprising as light input through the incompletely degenerated visual systems might drive rhythmic behaviours. It is also possible that the visual systems might not be used for navigation, food and mate procurement, and other behaviours; however, its regression might have been constrained by the necessity of photic input pathways for circadian clocks' functioning. This further substantiates the adaptive significance of circadian clocks in these species.

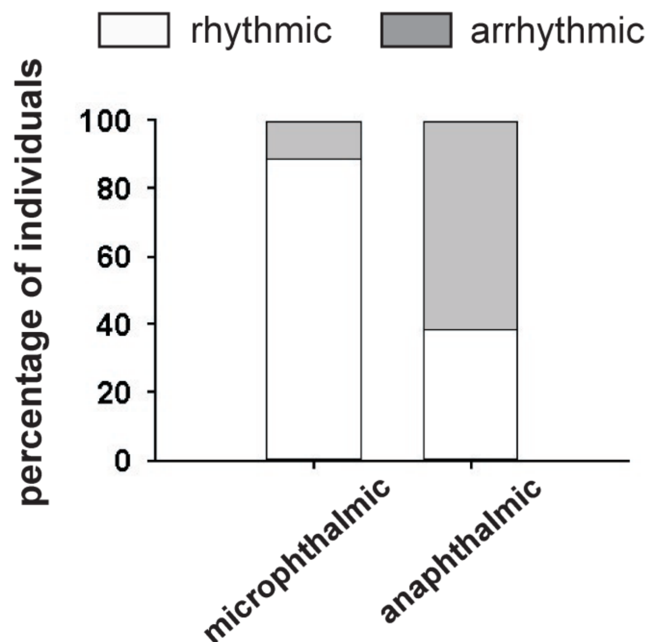


Figure 5: The graph depicts percentage of microphthalmic and anaphthalmic species (among the ones tested) observed to exhibit rhythmic (including circadian, ultradian, and infradian) and arrhythmic behaviours. Figure modified after Friedrich (2013).

In addition to studies on natural populations, a couple of laboratory selection studies have also provided interesting insights on the evolution of circadian clocks under constant conditions. In a study the authors' laboratory studies *D. melanogaster* populations that were maintained under LL for over 600 generations in constant temperature and humidity. Interestingly, even after 600 generations, these populations were observed to exhibit circadian rhythms in emergence, activity-rest and oviposition behaviours (Sheeba et al. 1999, 2001a, b, 2002). Additionally, these populations also entrained to several non 24 h LD cycles including 22 h and 28 h (Paranjpe et al. 2005). Similarly, another recent study on *D. melanogaster Oregon R* strains reared under DD for over 1340 generations also reported persistence of rhythmic activity-rest behaviour (Imafuku and Haramura, 2011). From calculations based on the mutation and fixation rates in *Drosophila*, and considering Kimura's neutral theory of evolution, the authors roughly estimated that based on the effective population size (N_e) in their populations , it would require close to 3000 generations from the occurrence to fixation of an arrhythmic mutant allele in their populations (Imafuku and Haramura, 2011). In light of the results from the above mentioned study, it is not surprising that the LL flies discussed previously continued to exhibit rhythmic circadian behaviours. However, the two studies differ in a critical aspect that is the population size used in the studies. The population size in the Imafuku and Haramura (2011), study largely fluctuated from 50 to 200 individuals while that in Sheeba et al (1999, 2001a, b, 2002) were kept constant to ~1200 flies. Additionally, no details of population replicates are provided by the former while Sheeba et al (1999, 2001a, b, 2002) used 4 replicate populations. Also, Imafuku and Haramura (2011) used *Oregon R* strain which to begin with is an inbred line and therefore, a combination of small population size and isogenic background of the founding populations would increase chances of random fixation of alleles which might

underlie the observed phenotypes. Alternatively, in addition to the rationale presented by Imafuku and Karamura based on the neutral theory of evolution suggesting that the evolutionary time scales are considerably smaller for observing any loss of circadian rhythmicity, lack of regression of circadian rhythms in both the above mentioned studies as well as those from natural populations has also been reasoned taking support from the intrinsic advantage hypothesis. While circadian clocks in organisms under constant environments might not require synchronization of behavioural rhythms with the environment, the same cannot be said for maintenance of internal temporal order within the organisms. Therefore, it is not surprising that circadian clocks may continue to persist even under arrhythmic environmental conditions by virtue of the intrinsic advantages conferred by them.

1.5.4 *Evidence from laboratory selection experiments*

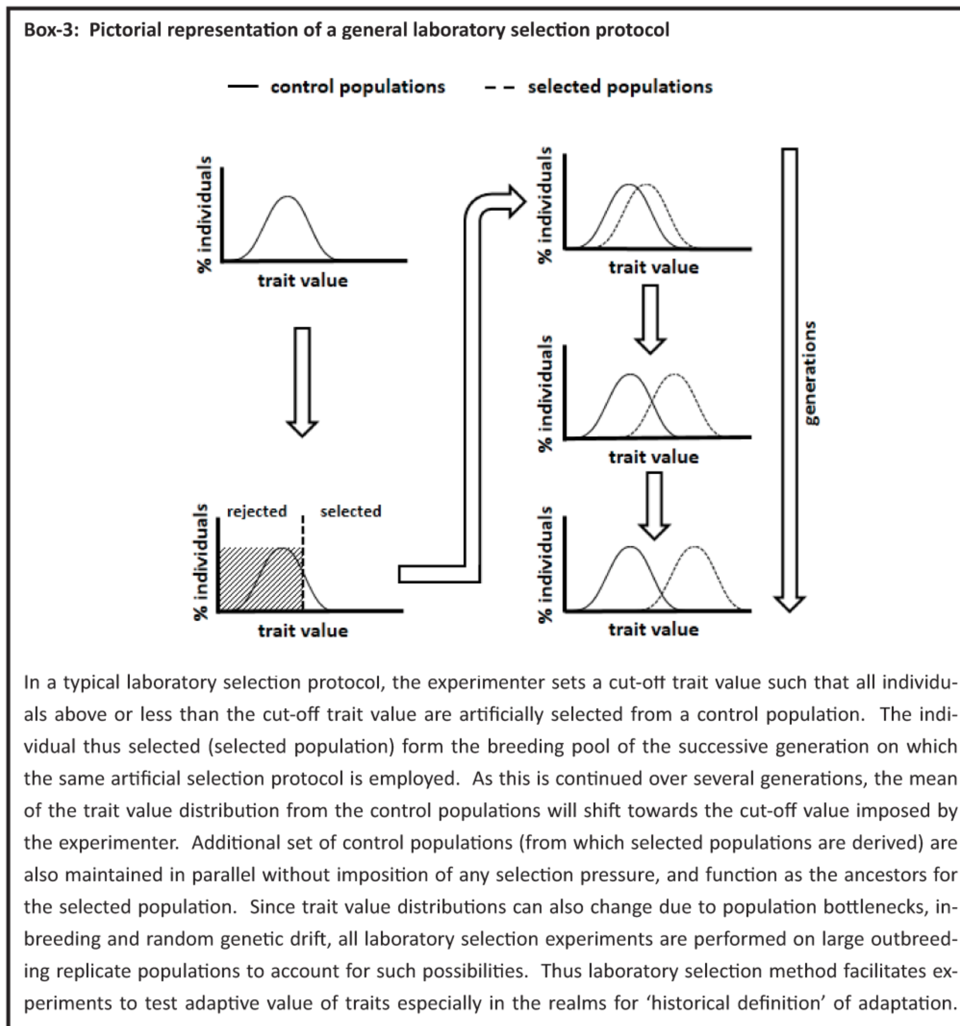
Laboratory selection is an ideal strategy for the study of fitness traits that are ultimate measures to test if the evolved traits are indeed adaptations to the selection regime imposed (Box 3), but is amongst the relatively less adopted strategies to study the evolution of circadian clocks.

Among the earliest studies of this kind was by Clayton and Paietta (1972) who imposed artificial selection for morning and evening emergence in *Oregon R* strain and a wild caught strain (W2) of *D. melanogaster*. After over 16 generations of selection, the populations responded by evolving higher emergence in the morning and evening hours respectively. However, the study did not test for the coevolution of circadian clock properties and therefore, it is difficult to assert the evolved differences in phase-of-emergence to the underlying circadian clocks. Pittendrigh (1967) and Pittendrigh and Minis (1971) selected for "early" and "late" emergence under LD12:12 in populations of

D. pseudoobscura and *Pectinophora gossypiella*. Subsequently, Pittendrigh and Takamura (1987) also imposed a similar selection in *Drosophila auraria* but under a short photoperiod of LD01:23. In all cases the populations responded to selection pressure with early and late emerging populations exhibiting differences in phase-of-emergence. The *D. pseudoobscura* diverged by 4 h, *P. gossypiella* by 5 h and the *D. auraria* populations diverged by 6 h. Additionally, correlated changes in their τ were observed in all three cases, albeit in different directions. The early flies of *D. pseudoobscura* and *P. gossypiella* evolved longer τ and late flies evolved shorter τ, whereas early and late flies of *D. auraria* evolved shorter and longer τ, respectively. Although Pittendrigh explained this paradox in the realms of the master-slave oscillator model, and to differential coupling between pacemaker and zeitgeber arising from the differences in light regimes used during selection, we believe that such a discrepancy could have arisen from other factors which pose to be the considerable limitations of the experimental design. For instance, the selection protocols used in these studies did not appear to have (at least not mentioned in the respective publications) (a) population replicates within a selection regime, (b) any details of population ancestry and (c) details of population size and employed selection protocol. Even moderately small differences in the above mentioned factors have been shown to considerably influence the response to selection since the evolutionary trajectories of populations greatly differ depending on the standing genetic variation harboured by the baseline populations and micro environmental conditions (reviewed in Vaze and Sharma, 2013). Therefore, even though these studies demonstrate the coevolution of circadian clocks in response to selection, whether such changes were indeed responses to selection or cannot be clarified. Furthermore, since these studies also did not assess the effect of selection on

fitness measures, whether timing of emergence does confer adaptive advantages remained unaddressed.

Box-3: Pictorial representation of a general laboratory selection protocol



In a typical laboratory selection protocol, the experimenter sets a cut-off trait value such that all individuals above or less than the cut-off trait value are artificially selected from a control population. The individual thus selected (selected population) form the breeding pool of the successive generation on which the same artificial selection protocol is employed. As this is continued over several generations, the mean of the trait value distribution from the control populations will shift towards the cut-off value imposed by the experimenter. Additional set of control populations (from which selected populations are derived) are also maintained in parallel without imposition of any selection pressure, and function as the ancestors for the selected population. Since trait value distributions can also change due to population bottlenecks, inbreeding and random genetic drift, all laboratory selection experiments are performed on large outbreeding replicate populations to account for such possibilities. Thus laboratory selection method facilitates experiments to test adaptive value of traits especially in the realms for 'historical definition' of adaptation.

With such shortcomings in consideration, a long term laboratory selection study was initiated in the author's laboratory to examine the evolutionary trajectories of circadian clocks in response to selection for different phase of emergence (emergence chronotypes) and subsequently circadian clocks' role in mediating chronotype differences. In contrast to studies on inbred, so called wild type strains which may provide an inaccurate picture of the underlying genetic architecture of the phenotypes

under study, Kumar et al (2007a) utilized 4 independent replicate outbreeding populations *D. melanogaster*, and reported that after 55 generations of selection, *early* populations exhibited an increased emergence of ~60% during morning as compared to ~45% emergence in the control populations, whereas *late* populations exhibited an enhanced emergence of ~25% in the evening as opposed to ~15% observed in the control populations. Consequently the two populations evolved divergent emergence waveforms with the mean phase of emergence differing by 4.5 h (Kumar et al. 2007a). Furthermore, contrary to the previous studies by Pittendrigh (1967) and Pittendrigh and Minis (1971), *early* and *late* populations in Kumar et al (2007a) evolved shorter and longer τ differing by ~50 min, divergent photic phase response curves (PRCs; see glossary) for emergence rhythm (Kumar et al. 2007a), and the 2 populations differentially utilized light in the morning and evening hours to exhibit their respective emergence chronotypes (Vaze et al. 2012a). This is in accordance with the earlier studies that have reported correlations between τ and chronotypes in humans (Duffy et al. 1999; Roenneberg et al. 2003a). Also, the morning and evening chronotypes in humans are observed to be correlated with the nature and duration of light exposure (Duffy et al. 2001; Roenneberg et al. 2003a, b; Goulet et al. 2007) which can partly explain the observations reported by Vaze et al (2012a). The evolution of similar circadian phenotypes in independently evolving populations strongly suggests that *early* and *late* emergence chronotypes can be attributed to differences in the underlying circadian clocks. *late* populations also evolved high amplitude circadian clocks as compared to *early* populations (Nikhil et al. 2016a), which is in accordance with the latitudinal cline studies that reported that southern strains of *Drosophila* exhibiting delayed phase of emergence are associated with high amplitude circadian clocks (Pittendrigh and Takamura, 1989). These results collectively suggest that laboratory

selection can help reproduce several aspects of natural populations, and with the ability to precisely control the environmental conditions the selection pressures that can drive the evolution of various clock properties can be accurately studied thereby highlighting experimental evolution a strong experimental platform for the study of "evolutionary chronobiology". Furthermore, in a recently concluded set of studies, various life-history measures including pupation and development times, egg-to-pupal and egg-to-adult survivorships, dry-weight at pupation and emergence, fecundity and longevity were assayed in *early* and *late* populations (*Nikhil et al. submitted manuscript*). As correlated responses to selection, *late* populations evolved significantly longer pupation and development times, higher fecundity and reduced female lifespan as compared to *early* populations. Thus, evolution of various life-history traits in response to selection further underscores the possible genetic correlations between the mechanisms governing timing of emergence (probably circadian clocks) and various life-history traits.

Precision in circadian clocks have been hypothesized to be an essential property for efficient time keeping (Pittendrigh and Daan, 1976a). To study the evolution of precise circadian clocks and associated properties, another laboratory selection study was initiated in the authors' laboratory that involved imposing selection on *D. melanogaster* populations for higher precision of emergence within a very short window of 1 h every day. It was observed that the selected CP (chrono precise) populations showed a gradual increase in emergence during the 1 h selection window and reduced emergence outside the window as compared to the control populations (Kannan et al. 2012a). The CP populations consequently evolved higher accuracy for both emergence and activity-rest rhythms which persisted under multiple environmental conditions suggesting that the evolved mechanisms driving higher accuracy constitute a common unit (circadian clock) governing both the rhythms (Kannan et al. 2012a, b, c). This was

substantiated by correlated evolution of τ, with the CP populations evolving a shorter τ as compared to the controls (Kannan et al. 2012a). Furthermore, it was observed that the CP population exhibit reduced variance in development times, and the females of these populations exhibited higher midlife fecundity and reduced lifespan as compared to the controls (Varma et al. 2014). Collectively, results from Nikhil et al (*submitted manuscript*), and Varma et al (2014) strongly suggest that natural selection acting on timing of rhythmic behaviours can be associated with correlated changes in life-history traits that the fitness is partitioned into, and thus highlights the possible role of circadian clocks in mediating fitness of organisms in specific environments.

Other selection studies have also implicated circadian clock's role in one of the life-history measures - the development time. When two lines of melon fly *B. cucurbitae* were subjected to selection for faster and slower pre-adult development under LD14:10, the lines responded by evolving faster and slower development times respectively. The faster developing lines after 21 generations of selection exhibited about 3 days reduction in development time as compared to the controls, while the slower developing lines took about 5 days longer after 16 generations of selection (Shimizu et al. 1997). Interestingly, the selection response was also associated with correlated changes in the τ of activity-rest rhythm with the mean τ of faster developing lines having reduced by ~2 h as compared to the controls (24.7 h) while that for the slower developing lines was lengthened by 3.5 h (Shimizu et al. 1997). Such correlation between development time and τ of activity-rest rhythm was also reported in the *per* mutants of *D. melanogaster* with homozygous individuals of the short period allele of *per* (*per*^{s}) exhibiting shorter development time as compared to the wild type flies, and individuals homozygous for the long period allele *per*^{l} had longer development time (Kyriacou et al. 1990). However, whether the genetic basis for observed difference in τ

in the selected lines from Shimizu et al (1997) can be attributed to *per* remains unanswered since the authors did not undertake further genetic analyses of the populations. The results of Shimizu et al (1997) may seem quite intuitive since biological timing mechanisms which time several processes can alter the rate of such processes just by change in the τ. In other words, slower running clocks would drive developmental processes at slower rates and consequently delay development time, and the same would be true for faster running clocks as well. Another study on melon flies reported that circadian clocks may in fact be associated with the timing several other life-history events as well. Miyatake (2002) observed that *B. cucurbitae* when selected for different ages of reproduction, coevolve divergent mating phases and τ. The set of strains selected for early age (10-15 days) of reproduction (Y-lines) for 65 generations, and later age (55-60 days) of reproduction (O-lines) for 24 generations showed correlated response to selection such that the Y-lines were observed to mate earlier in the day as compared to the O-lines that mated later. Also, τ of Y-lines was significantly shorter (males: 22.8 h; females: 23.05 h) while that for the O-lines was longer (males: 26.5 h; females: 27.6 h). Even though the observed coevolution of τ strongly suggests their role in timing life-history events, there is one critical aspect in the study that needs to be considered before arriving at any conclusion. Due to the nature of selection protocol employed, the O-lines selected for reproduction at 55-60 days old would have incurred greater mortality therefore reducing the effective population size (N_e) in these populations as compared to the 10-15 days at reproduction in the Y-lines. Over several generations, this might have led to higher degree of inbreeding depression in the O-lines and therefore, the change in τ cannot be entirely attributed to selection but can also be an artefact of inbreeding. This is further substantiated by the large variation in phenotypes observed among the three replicate populations used in the study (Miyatake, 2002).

The broader picture that arises from all of the above discussed studies can be summarized as follows. Selection on specific timing of an overt behaviour leads to the coevolution of various clock properties such as τ, precision, zeitgeber sensitivity and amplitude of underlying circadian clocks thus emphasizing the role of such properties in timing rhythmic behaviours. Furthermore, such evolutionary responses are also associated with changes in multiple life-history traits spanning the pre-adult and adult stages which demonstrate the underlying genetic correlations between circadian timing mechanism and life-history evolution. Therefore, it is reasonable to infer that circadian clocks by virtue of genetic correlations with life-history traits can confer adaptive advantages to the organisms by appropriately timing rhythmic behaviours so as to enhance the organism's fitness in a given environment.

1.6 A critique on currently used methodologies, and the way forward

At the beginning of the chapter, we briefly discussed the concept of adaptation and criteria that are considered essential to test the adaptive nature of a trait. However, if one were to critically compare these criteria in the context of studies discussed thus far, several obvious experimental design and methodological shortcomings. While we appreciated the enormous efforts invested by a large number of researchers, we also take the privilege of critically assessing the methodologies and shortcomings (even though some of them may be unavoidable) of some of the above discussed studies, and suggest pointers for improving the previously adopted experimental designs. In this regard, we may at certain places choose examples from actual studies, but we would like to clarify that such examples will be chosen purely to elaborate on our arguments, and does not reflect our intentions to specifically target the study or personnel involved.

a) *Assessment of fitness*: Majority of the studies that assess fitness under various experimental setups implicitly assume that the measures used truly reflect the overall fitness of organisms or sometime is even synonymous with the term fitness. For instance, several studies reported the clock manipulation either by genetic or environmental means severely reduce longevity thus concluding that reduced longevity is indeed maladaptive and therefore circadian clocks are adaptive in nature. However, life-history theory highlights that the overall fitness of an individual can be partitioned across multiple life stages and among different fitness measures. For instance, all else being equal, if an individual *K* lives longer but eventually produces lower number of viable offspring as compared to another individual *L* that lives considerably shorter than *K* but has a higher offspring output, it is obvious that *L* would be fitter than *K*. However, if lifespan alone is used as a fitness measure, then one is bound to disastrously conclude *K* to have higher fitness than *L*. Even though estimating the overall fitness is practically impossible due to several constraints, we believe that it is essential for experimenters to assess a large number of fitness measures before arriving at a conclusion regarding the fitness benefits of harbouring circadian clocks.

b) *Choosing appropriate fitness measures*: In addition to assessing multiple fitness measures, it is equally critical to ensure that the fitness measures chosen are ecologically relevant to the organism. A classic example of this can be discussed using experimental evolution studies. For instance, *D. melanogaster* populations used in Kumar et al (2007a) were maintained on a 21 day discrete generation cycle which means that eggs for succeeding generation were collected on day 21 post-egg collection from the previous generation. In this regard, if parents in a given population were to ensure the representation of their progeny to the next generation, survival till day 21 and highest fecundity on day 21 are the only critical fitness components as eggs laid post or pre day

21 will not contribute to the succeeding generation. Therefore, how long the parental populations survive post day 21 is irrelevant as their realised fitness beyond it is essentially zero. In this regard, if one were to estimate fitness in such populations, only traits that would influence the populations' survival till day 21 such as pre-adult development time, egg-to-adult survivorship, starvation and desiccation resistance, and fecundity until day 21 are adequate fitness measures, whereas other measures such as lifetime fecundity and longevity even though would be relevant to address other unrelated questions, might not be critical measures of fitness in this scenario. Therefore, it is important to bear in mind that the measures of fitness are relevant to the ecology of the organism under study.

c) *Use of genetic manipulations*: Several studies reported fitness consequences of circadian clock disruption in conventional laboratory strains such as *Oregon R* (as in *Drosophila*) or other mutants of core clock genes. It is well known that life-history traits have complex genetic architecture involving a large number of genes and multiple allelic combinations (Garland and Rose, 2009). However, conventional laboratory wild type and mutant strains are generally severely inbred, and might have accumulated multiple random mutations within their genomes. Inbreeding is known to have several dire consequences on the genetic architecture such as increased homozygosity, reduction in effective population size and recombination rates, and also increased linkage disequilibrium in comparison with those of the outbreeding natural populations. Such factors might reduce the organism's fitness (Charlesworth, 2003) independent of the trait in question, and therefore, use of such strains even though might be useful in certain experimental designs, should be avoided to the maximum possible extent especially in the assessment of fitness.

d) *Studies on natural populations*: Most studies testing the evolutionary benefits of circadian clocks in natural populations use organisms that have either evolved in constant conditions, or are latitudinal or altitudinal clines. Inferential arguments drawn from such studies are often rendered weak primarily due to the lack of knowledge about the environmental history of the geographical area in which the organisms evolved. Considering the complex interplay of several biotic and abiotic factors in an ecosystem, there can be a plethora of reasons that might have led to the evolution of observed traits in a given environment. Moreover, floods and earthquakes, or epidemics might have led to severe population bottlenecks in the past consequently changing the evolutionary trajectories of the traits being studied. In this regard, we propose that rather than relying on evidence from restricted sampling of member of a given clade, a comprehensive comparison across members of the clade or closely related clades occupying both similar and dissimilar geographical areas might provide a more reasonable estimate of how and why the trait in question might have arose. This information along with the knowledge of environmental conditions around that time would assist identification of possible selection pressures that might have driven evolution of the trait in question thus reducing the chances of wrongly interpreting results from studies on natural populations.

e) *Studies relying on environmental manipulations*: We also discussed several studies that assessed the consequences of arrhythmic circadian clocks or induce circadian desynchrony induced by light regime manipulations. While such studies have provided interesting insights into the role of circadian clocks, not all such results can be generalised; primarily because such studies fail to distinguish whether the observed phenotypes are actually clock mediated or are direct consequences of manipulated light regime, especially given that light apart from being a zeitgeber also influences several clock independent processes. An example for this is the decreased growth rates

observed in tomato plants when reared under LL as compared to that in LD12:12. This even though is suggestive of the detrimental effects of clock disruption (due to LL) on growth rate, *Arabidopsis* on the other hand is observed to grow considerably faster in LL as compared to LD12:12 which questions the plausibility of the interpretations from tomato studies (discussed in Johnson and Kyriacou, 2005). Therefore, it is essential that studies aimed at assessing the effects of clock through multiple approaches which would further strengthen the claims of adaptive significance of circadian clocks.

f) *From lab to nature and back*: It is notable that majority of the studies discussed so far have been performed either entirely under controlled laboratory conditions or on natural populations. Stochasticity in multiple unavoidable factors in nature make it difficult to attribute the observed phenotype to a given environmental variable. Contrary to this, laboratory studies are performed under relatively simplistic environmental conditions where organisms are generally maintained at optimal population size, constant light or temperature regimes with *ad libitum* food and water. Therefore, even though the phenotypes observed in laboratory conditions may sometimes be quite and can be attributed to few of the environmental factors, they may not necessarily reflect the dynamics in nature. For instance, one possible reason for observing clear differences in competitive ability between strains in Ouyang et al (1998) may be due to the seemingly simplistic environmental conditions used in the experiment. These results, even though are suggestive of the adaptive nature of circadian clocks, whether the same is observed under a more naturalistic environment remains to be addressed? Therefore, as a further extension to existing methodologies, studies aiming to assess the adaptive nature of circadian clocks in controlled laboratory setups should try and reproduce the same under more realistic natural setups which would provide a stronger support to the hypothesis being tested.

While we have tried to review majority of the studies over the past few decades that have made phenomenal contributions to our understanding of these marvellous circadian time keeping mechanisms, we sincerely apologise for failure in citing any studies, and acknowledge that such oversights were purely unintentional. This being said, a comprehensive analysis of the current state of knowledge pertaining to the evolutionary origins and implications of circadian clocks leaves an impression that even though we have substantial evidence that strongly underscores the relevance of circadian clocks across all life forms, we still have a long road to take before arriving at undisputable conclusions regarding the origin of circadian clocks and their adaptive significance. Nevertheless, advances in technologies combined with continued collaborative efforts from researchers across disciplines to address such questions in future is bound to be an exciting and intellectually enlightening journey for the field of chronobiology.

CHAPTER 2

Introduction to *early* and *late* populations

2.1 Introduction

As discussed in the previous chapter, appropriate timing of rhythmic (circadian) behaviours (also referred to as phase-of-entrainment or ψ_{ent}), including activity-rest, mating, feeding and adult emergence in insects have direct implications on the survival and reproductive success of organisms as they ensure maximal accession of food, procurement of mates, avoidance of predators, and so on (Cloudsley-Thompson, 1960; Aschoff, 1967; Pittendrigh, 1993; Fleury et al. 2000). Therefore, ψ_{ent} can be thought to be under strong natural selection, consequently driving the evolution of the underlying circadian clocks. The idea that behaviour evolves first and drives the evolution of the underlying mechanisms is not new to evolutionary biology (Mayr, 1958; Blomberg et al. 2003), a classic instance of which is the domestication of wolves into dogs (Savolainen et al. 2002).

Among the widely observed circadian clock properties to be associated with chronotype variation is the clock period (τ) such that early chronotypes exhibit a shorter τ while *late* chronotypes exhibit a longer τ. Such ψ_{ent}-τ associations appear to be an evolutionarily conserved feature as evidenced by studies on other organisms reporting similar associations (Aschoff and Pohl, 1978; Roenneberg, 2012). However, studies reporting such observations till date have either been performed on clock mutants or on natural populations both of which have their respective shortcomings (discussed in detail in Chapter 1). Briefly, for instance, the mutant strains generally lack genetic variation as they are highly inbred, and therefore the observed phenotypes might stem from random fixation of alleles including those at non clock loci. Natural populations on the other hand are generally outbreeding and thus are a rich pool of genetic variation rendering them as potential model systems for various studies. However, the complex interplay of various biotic and abiotic factors in the environment are known to strongly influence

evolution of traits, and therefore the observed phenotypes might not necessarily have been a direct consequence of selection but established in the populations due to genetic linkage and/or drift arising due to various reasons such as population bottlenecks and so on. Therefore, the unavailability of information regarding the environmental history of the populations' habitat or their ancestors makes inferences from studies on natural populations debatable.

The waveform of adult emergence in *D. melanogaster* comprises a primary peak of emergence at dawn (in nature) or around night-day transition (in lab) while fewer individuals emerge at dusk or around day-night transition with almost no emergence at night (Figure 1), a phenomenon known as gating (Skopik and Pittendrigh, 1967). To account for the above discussed shortcomings of other experimental strategies (see also Chapter 1), we intended to establish a laboratory model system for the study of ψ_{ent} and the associated clock properties by adopting the laboratory selection approach to drive the evolution of divergent ψ_{ent} in replicate *Drosophila melanogaster* populations under controlled environmental conditions. Laboratory selection involves subjecting large outbreeding laboratory populations of individuals to artificial selection for a phenotype of interest, and studying the evolution of the focal and related phenotypes (reviewed in Vaze and Sharma, 2013). The major strength of laboratory selection is the ability to use replicate populations harbouring sufficient genetic variation under controlled laboratory conditions which provides a strong platform for the study of evolutionary dynamics under well-defined environments (Garland and Rose, 2009 and citations therein). It also aids the analysis of multiple evolutionary trajectories that might lead to the evolution of the same phenotype (Garland and Rose, 2009 and citations therein). Additionally, it facilitates the study of behavioural, physiological and genetic correlates of various life-history traits and associated trade-offs which are of particular interest in the study of life-

history evolution (Garland and Rose, 2009 and citations therein). In this regard, we employed laboratory selection to derive *D. melanogaster* populations exhibiting early and late emergence (also referred to as early and late emergence chronotypes in the following chapters) which are characterised by advanced (*early* populations) and delayed (*late* populations) ψ_{ent} of emergence rhythm.

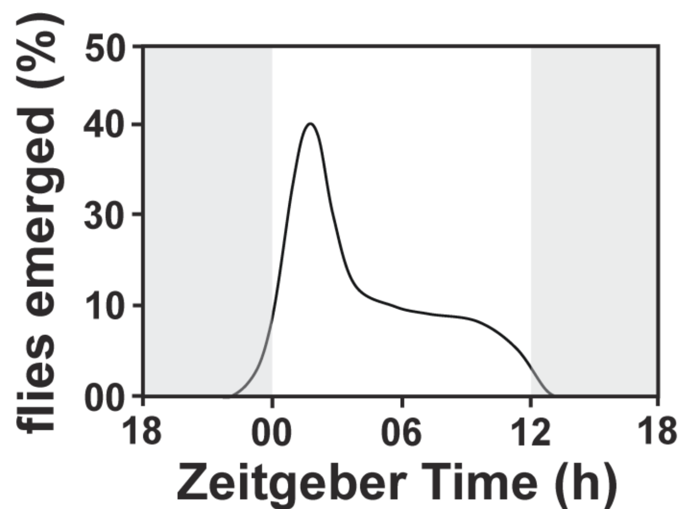


Figure 1: Schematic of adult emergence profile of *D. melanogaster* under laboratory LD12:12 (12 h of light and dark each) cycles at 25 ºC. The shaded area depicts night and the unshaded area depicts day. Zeitgeber Time depicts the time of the day with ZT00 indicating lights-ON and ZT12 representing lights-OFF.

Here, I will introduce the experimental protocol employed to derive *early* and *late* populations and discuss the direct and correlated responses of circadian clocks in these populations to the selection imposed.

2.2 Materials and Methods

(a) ***Selection protocol employed to derive early and late populations*****:** Figure 2 presents a schematic of the selection protocol employed to derive *early* and *late* populations from *control* populations. Populations selected for early morning and late evening emergence comprised four replicates each of $early_i$ and $late_j$ ($i = j$ = 1-4) initiated from four replicates of $control_k$ (k = 1-4), whose ancestry details are provided elsewhere (Sheeba et al. 1999). Briefly, *early* and *late* populations with a given subscript were derived from *control* population with the same subscript, and therefore share a common ancestry. For example, $early_1$ and $late_1$ populations were initiated from $control_1$ population, and similarly for the other three replicates. All 12 populations (four replicates each for *early*, *control* and *late*) were maintained on a 21 day discrete generation cycle, and flies were housed in plexi glass cages of dimension 25 × 20 × 15 cm with ~1200 individuals per cage (sex ratio ~1:1), and were provided with *ad libitum* banana-jaggery (BJ) medium. The parental populations were provided with food supplemented with yeast paste (to boost their fecundity) for three days prior to egg collection, and ~300 eggs were collected and dispensed into each culture vial (16 vials for *control,* 24 for *early* and 48 for *late* populations) containing ~6 ml BJ medium. From the initiation of emergence, which is generally on day 9 (at 25 ^{o}C) post egg collection, flies that emerged early in the morning between Zeitgeber Time (ZT) 21-01 (ZT00 and ZT12 represents time of lights-ON and lights-OFF respectively under LD12:12) for 3-4 consecutive days were collected to form *early* populations, while flies that emerged late in the evening between ZT09-13 formed *late* populations. For *control* populations, flies were collected once every 24 h for the same 3-4 days and thus, comprised individuals emerging throughout these 3-4 days without any selection imposed on timing of emergence. The days of initiation and termination of fly collection within the respective selection windows was kept constant

for all populations. In other words, if collection of flies for *early* populations was started on day x and terminated on day y, collection of flies for *control* and *late* populations was also initiated and terminated on days ‘x’ and ‘y’ respectively so as to ensure that the populations are selected only for emergence at different gate/time of the day and to avoid any unintended selection for faster and slower pre-adult development. The implementation of selection protocol and regular maintenance of populations was performed under LD12:12 with ~0.4 Wm^{-2} light intensity during the light phase,
25 ± 0.5 °C temperature, and 75 ± 5% relative humidity. Fly handling and experiments in the dark were performed under dim red light ($\lambda > 650$ nm).

To minimize the effects of non-genetic inheritance (reviewed in Garland and Adolph, 1991) due to different selection regimes, all populations were subjected to one generation of standardization with the maintenance protocol same as that used for *control* populations, referred to as ‘standardized populations’. This was achieved by relaxing selection on timing of emergence by collecting all flies that emerged throughout the first 4 days similar to that for *control* populations, and the population size was kept constant at ~1200 flies per replicate population. All experiments presented henceforth were performed on the progeny of standardized populations.

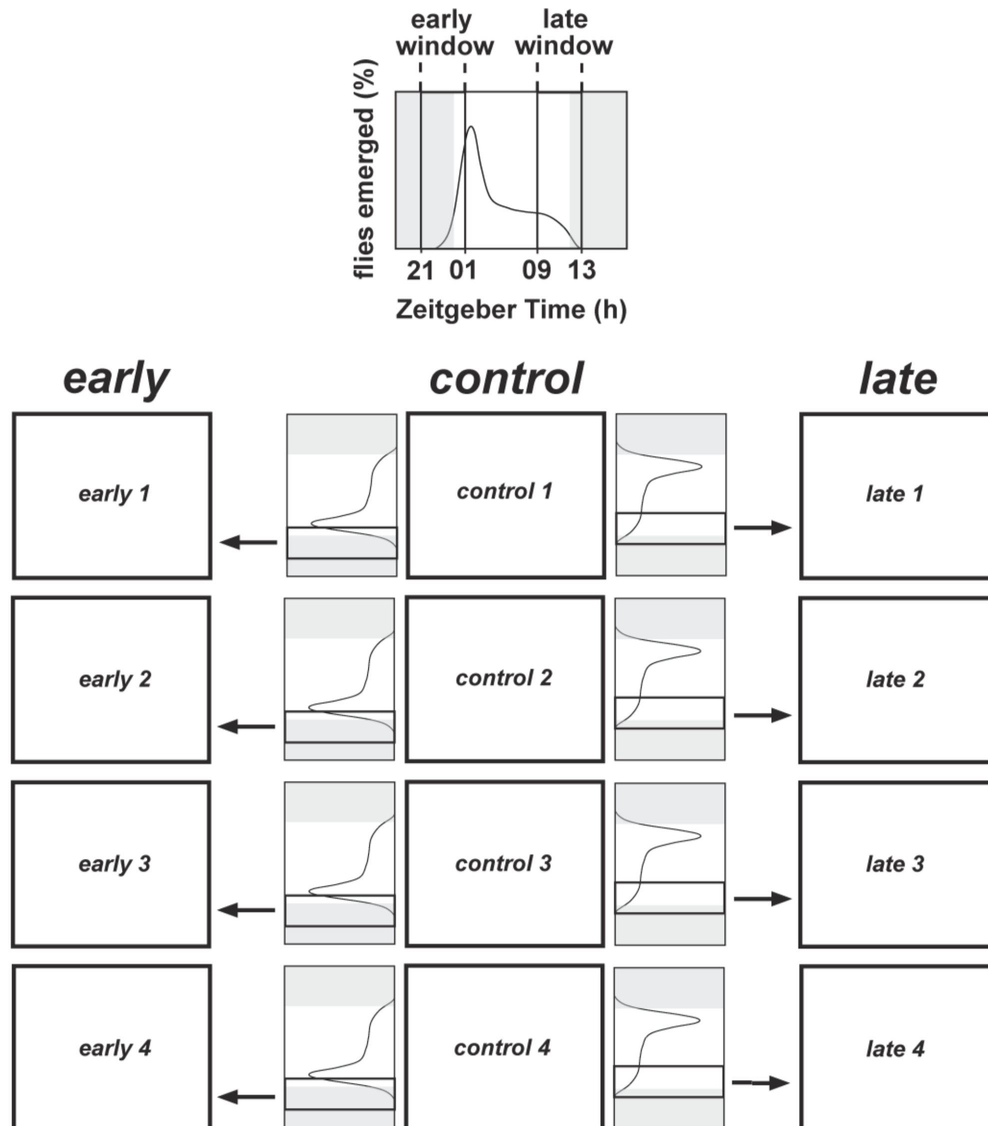


Figure 2: Schematic of laboratory selection protocol employed to derive *early* and *late* populations. Zeitgeber Time 00 or ZT00 represents the time of lights-ON under a LD12:12 regime. ZT21-01 represents the morning window during which flies for *early* populations are collected and ZT09-13 represents the evening window during which flies for *late* populations are collected.

(b) ***Adult emergence rhythm assay*****:** Adult emergence rhythm assay was performed on all the 12 populations under LD12:12 (12 h of alternating light and dark phases each) cycles at 25 °C with light-intensity during the photo phase maintained at ~70 lux. Approximately 300 eggs were collected and dispensed into 6 replicate vials (per population) containing BJ medium, and the vials were transferred to the respective light regime for the assay. From the initiation of emergence, flies emerging from each vial were recorded at 2 h interval for 4 days, and only vials in which at least 30 flies emerged every day were considered for analyses.

The mean phase of entrained rhythm for each population was calculated as has been described previously (Nikhil et al. 2014). This is considered as a more reliable measure of the phase of emergence rhythm as it encompasses the overall emergence waveform as opposed to just the peak or onset of emergence.

Assay to estimate the period (τ) of emergence rhythm was also performed similarly with the only exception that for the first 8 days after egg collection, all vials (n = 10 vials/population) were maintained under LD12:12 and then transferred to constant darkness (DD) for the next 6 days. The τ of emergence rhythm was estimated using COSINOR analysis (modified from Refinetti et al. 2007) using MATLAB. The proportion of flies emerging per day was used to fit cosine curves with varying amplitude and period, the combination that yielded the best fit as assessed by minimum sum of squared differences between the expected and experimental data was chosen to compute τ of the rhythm. The τ values were computed separately for each replicate vial of all the 12 populations and then averaged to obtain the block means.

(c) ***Activity-rest rhythm assay*****:** Activity-rest rhythm assay was performed on 3-5 day old males (n = 32 per population) under the same set of light regimes as described for emergence assay. Activity data from the first 7 days of recording was used to plot averaged activity profiles, and to estimate the mean phase of entrained rhythm by circular statistics (described in the 'Statistical analyses' section). The mean phase of activity-rest rhythm was estimated the same way as that for emergence rhythm, but since the daily activity profile of *Drosophila* is bimodal, a transformation referred to as "angle doubling" was performed as described in Zar (2009). The results were back transformed and rescaled to 24 h. Data from flies recorded similarly in DD for 10 consecutive days was used to estimate τ of activity-rest rhythm using Chi-square periodogram. Data recording and analyses were performed using Drosophila Activity Monitors (Trikinetics, MA) and Clocklab (Actimetrics, IL) respectively.

(c) ***Statistical*** **analyses:** The number of flies emerged or activity counts (whichever applicable) at every phase was calculated separately for each of the 4 blocks and served as data points for a mixed model randomised block design Analysis of Variance (ANOVA) to test for statistically significant differences among populations. All post hoc comparisons were performed using Tukey's HSD at a significance level $\alpha = 0.05$.

The mean phase (time of the day measured in Zeitgeber Time - ZT), and length of the mean polar vector for emergence and activity-rest rhythms were calculated as described in Zar (2009). In the circular time scale used for analysis, 0° refers to the Zeitgeber Time 00 (ZT00) and therefore, 15° represents 1 h. While mean phase (θ) indicates mean time of the day around which emergence is concentrated, length (r) of the polar vector serves as a measure of coherence, and thus higher r values are indicative of

greater coherence in emergence rhythm, and *vice versa*. As a first step, Rayleigh test (α = 0.01) was used to test for the null hypothesis that emergence of a population is randomly distributed throughout the day and therefore has no mean direction (Batschelet, 1981; Jammalamadaka and SenGupta, 2001; Zar, 2009). Rejection of null hypothesis favours the alternate hypothesis that there exists a mean direction of emergence, facilitating further statistical analyses.

The mean phase and τ of emergence and activity-rest rhythms were estimated separately for all the 12 populations, and served as data for a mixed model randomised block design Analysis of Variance (ANOVA) to test for statistically significant differences among populations. All post hoc comparisons were performed using Tukey's HSD at a significance level $\alpha = 0.05$.

Calculation of the mean phase of expression, Rayleigh Test, Rao Test and NPTD were implemented on R statistical language platform (R Development Core Team, 2011) using custom written codes with the aid of "CircStats" (Jammalamadaka and SenGupta, 2001) and "Circular" packages (Agostinelli and Lund, 2013) while ANOVA and Tukey's HSD were implemented on Statistica (Statsoft, USA).

2.3 Results

(a) ***early and late populations evolved higher emergence within the respective selection windows***

In response to selection over several generations, *early* populations which exhibited ~45% emergence by generation 10 evolved higher percentage of emergence in the morning window reaching ~62% by generation 243, while the percentage of emergence for *late* populations reduced over generations from ~40% at generation 10 to ~24% by generation 243 (Figure 3; $F_{36,72} = 21.09$; $p < 0.001$). Similarly, *late* populations which

initially exhibited ~16% emergence in the evening window gradually evolved significantly higher percentage reaching ~41% emergence in evening window by generation 243, while that for *early* populations reduced from ~15% in generation 10 to ~2% in generation 243 (Figure 3; $F_{36,72} = 28.56$; $p < 0.001$) whereas emergence for *control* populations remained unchanged across generations with ~42% and 16% emergence within the morning and evening windows respectively (Figure 3).

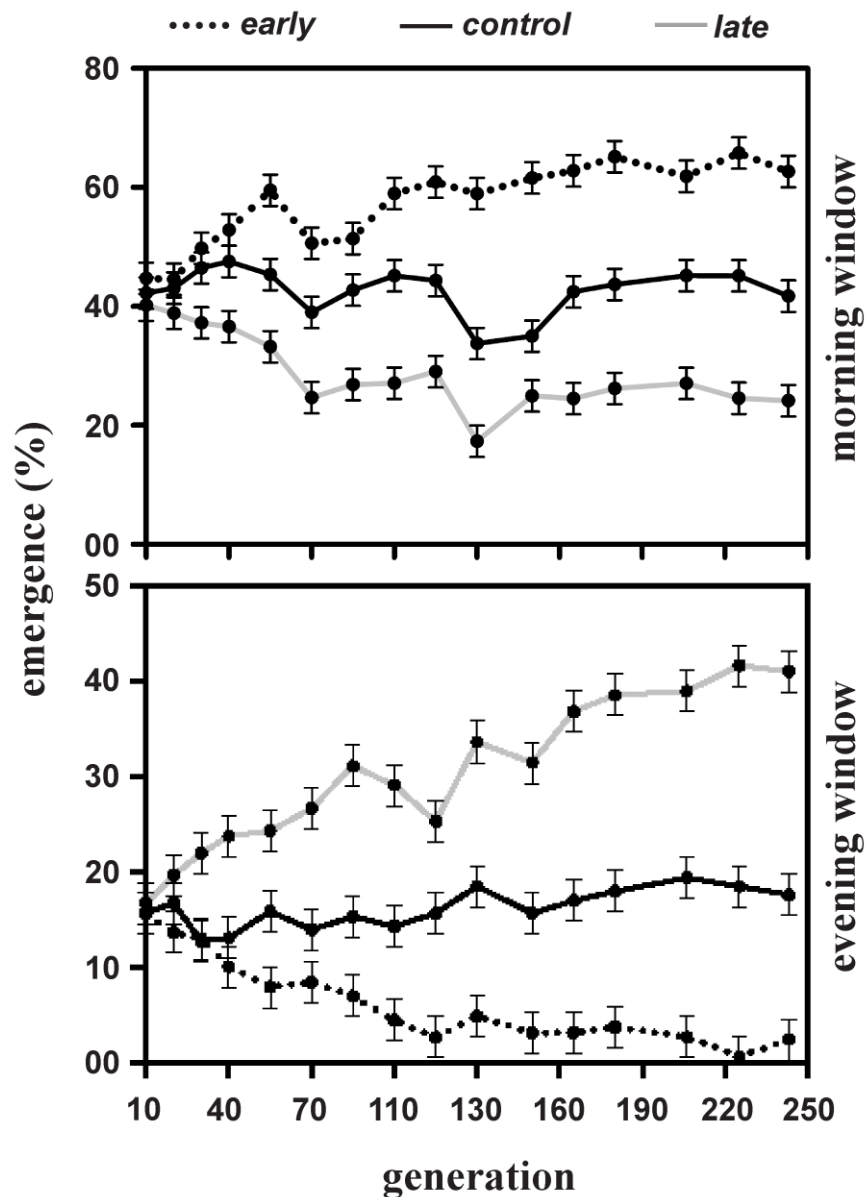


Figure 3 (previous page): The percentage of emergence of all three populations observed within the morning (ZT22-02) window (top panel) and evening (ZT10-14) window (bottom panel) over 243 generations of selection. Since the assay to estimate emergence is performed every two hours, the morning and evening windows were chosen such that they encompass the selection widows for *early* (ZT21-02) and *late* (ZT09-13) populations. In response to selection imposed, the emergence within the morning window increased over generation for *early* populations while that for *late* populations decreased. The emergence of *late* populations increased within the evening window while *early* populations gradually evolved decreased emergence within this window. Error bars depict 95% CI (Confidence Interval) calculated by Tukey's HSD following randomized block design ANOVA with 'population' and 'generation' as fixed and 'block' as random factors.

(b) ***early and late populations evolved divergent emergence waveforms but not activity-rest rhythm***

Since we observed that *early* and *late* populations evolved significantly higher emergence in their respective selection windows, we further assessed how this was associated with changes in emergence waveforms under LD12:12. Also, as will be discussed later, several previous studies have suggested that a common central circadian clock governs both emergence and activity-rest rhythms the circadian clocks, we also assessed if divergence in emergence waveform was associated with coevolution of activity-rest rhythm.

As reported earlier (Kumar et al. 2007a), emergence waveforms of *early* and *late* populations under LD12:12 differed such that *early* populations exhibited advanced onset of emergence with higher morning emergence, while *late* populations exhibited a delayed onset and higher evening emergence, and *control* populations exhibited an intermediate emergence pattern (Figure 4a-top panel). This is reflected in their mean phase of emergence, with *early* (θ_e = 1.84 h) and *late* (θ_l = 8.39 h) populations being significantly advanced and delayed respectively as compared to *control* (θ_c = 5.01 h) populations ($F_{2,6}$ = 193.92; $p < 0.05$; Figure 4a-bottom panel).

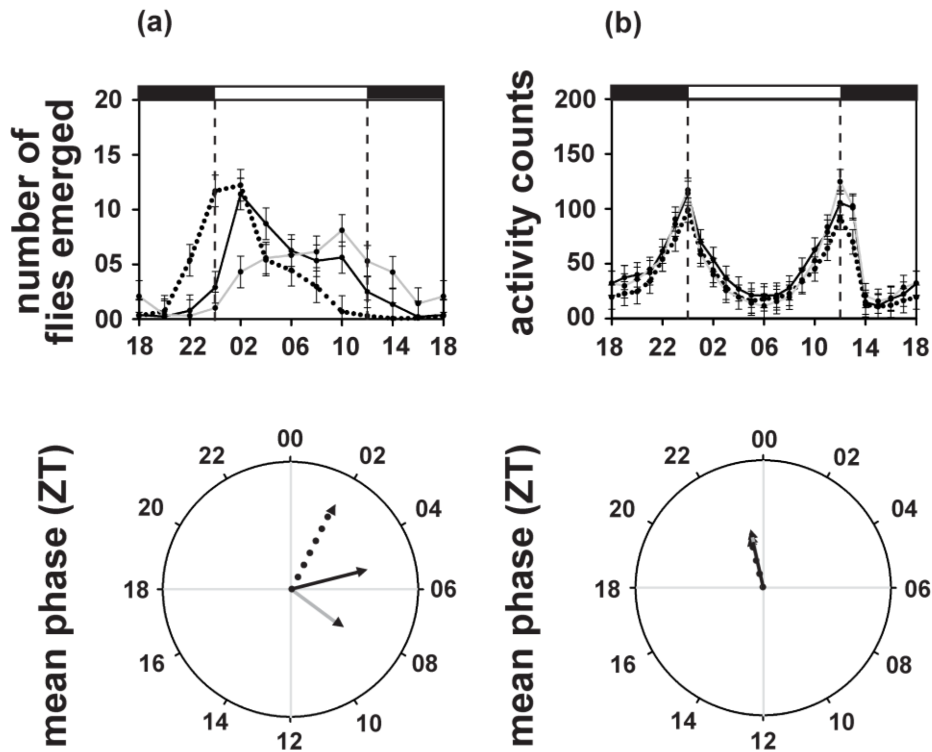


Figure 4: (a) Adult emergence profiles (top panel) and mean phase of emergence rhythm (bottom panel), and (b) activity-rest profiles and mean phase of activity-rest rhythm of *early*, *control* and *late* populations under LD12:12. The dark and light bars depict night and day respectively with the dashed vertical lines depicting transitions between day and night. Error bars depict 95% CI calculated by Tukey's HSD following randomized block design ANOVA with 'population' as fixed and 'block' as random factors.

When assayed under LD12:12, the profile of activity-rest rhythm did not differ much between the populations as also indicated by their mean phase of activity-rest rhythm (θ_e = 5.55 h; θ_c = 5.50 h; θ_l = 5.58 h) which did not differ statistically either ($F_{2,6}$ =1.38; $p >$ 0.05; Figure 2a). However, activity during the evening peak was significantly higher in *late* populations as compared to *early* populations, the possible reasons for which is extensively discussed in chapter 6 (Figure 4a-top panel).

Thus, selection for morning and evening emergence led to coevolution of divergent emergence waveforms without any associated changes in the waveforms of activity-rest rhythm of the two populations.

(c) *early and late populations evolved divergent clock periods for both emergence and activity-rest rhythms*

To further test if *early* and *late* populations evolved divergent circadian clocks, we also assessed the τ of emergence and activity-rest rhythms.

τ of emergence rhythm was found to be significantly shorter in *early* (22.51 h) populations compared to *late* populations which exhibited a longer τ of 23.86 h ($F_{2,6}$ = 30.10; $p < 0.001$). *Control* populations exhibited an intermediate τ of 22.94 h which was significantly shorter than *late* but did not differ from *early* populations (Figure 5a). Interestingly, we observe that the robustness of emergence rhythm in *late* populations appears to diminish overtime in DD with the gate width of emergence increasing by day 4, and consequently higher subjective night emergence is observed over days 5-6 leading to an apparent disruption of emergence overtime in DD (Figure 1d), and will be discussed later in chapter 6.

Furthermore, we observed a similar trend for the activity-rest rhythm with *early* (23.28 h) populations exhibiting a significantly shorter τ as compared to *control* (23.63 h) populations, which in turn was significantly shorter than that of *late* (24.03 h) populations ($F_{2,6}$ = 29.71; $p < 0.0001$; Figure 5b).

Thus, in response to selection for morning and evening emergence, *early* and *late* populations evolved shorter and longer clock periods for both emergence and activity-rest rhythms.

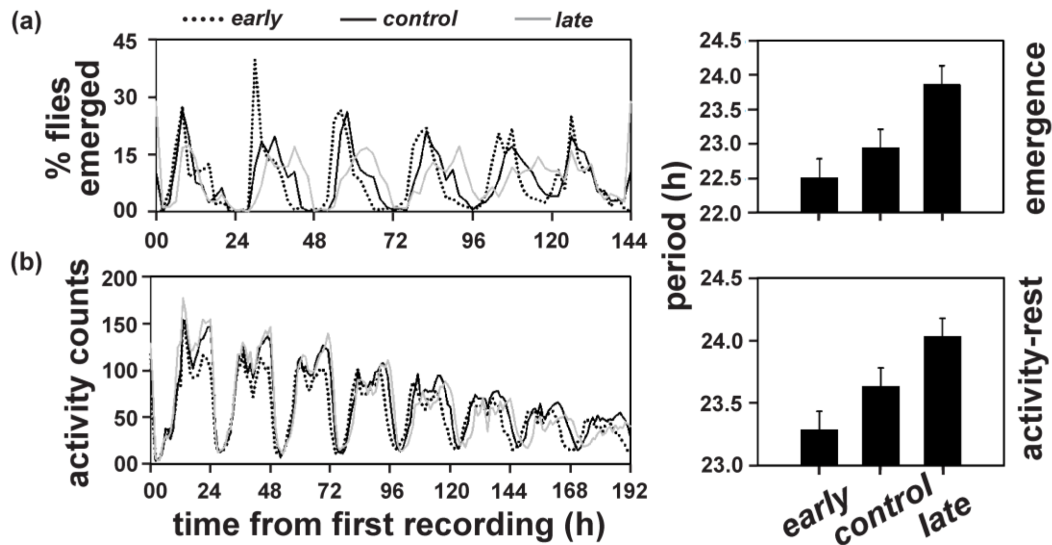


Figure 5: Time series depicting adult emergence (top panel) and activity-rest (bottom panel) rhythm of all the three populations under DD (left), and their respective circadian periods (right). Error bars depict 95% CI calculated by Tukey's HSD following randomized block design ANOVA with 'population' and 'time' (whichever applicable) as fixed and 'block' as random factors.

2.4 Discussion

We had previously reported the coevolution of divergent emergence waveforms and circadian clock periods in *early* and *late* populations by generation 55 (Kumar et al. 2007a). To examine if the circadian phenotypes of *early* and *late* populations persisted or have further diverged over 250 generations, we assayed the emergence rhythm of the three sets of populations under LD12:12, and observed that the mean phase of emergence rhythm of *early* and *late* populations has diverged by ~4 h while that for activity-rest rhythm did not differ between the populations (Figure 3). A closer look at the emergence in morning and evening windows reveals that the emergence of *early* populations in the morning window increased till approximately generation 115 following which the emergence within the morning window has remained constant while *late* populations continued to exhibit reduction in emergence within the morning window till generation

150 before stabilising at ~24% since generation 150 (Figure 2a). Unlike that for the morning window, emergence within the evening window continued to increase till generation 180 for *late* populations while that for *early* populations reduced to ~2% by generation 115 after which no significant reduction in emergence was observed (Figure 2b). Thus, it appears that the further divergence in mean emergence between *early* and *late* populations observed after generation 55 is primarily due to continued response of *late* populations until generation 180. Consistent with the generally observed τ-ψ_{ent} associations (Aschoff and Pohl, 1978; Roenneberg, 2012), the τ of emergence rhythm in *early* and *late* populations diverged by 1.35 h (~0.1 h greater than that reported in Kumar et al. 2007a) with *early* populations exhibiting shorter τ and *late* populations a longer τ. Also, the τ of activity-rest rhythm appears to have diverged by a small magnitude 0.7 h (~0.2 h greater than that reported in Kumar et al. 2007a).

Thus, while the phase of emergence has drastically diverged between *early* and *late* populations, their circadian periods exhibit a relatively smaller divergence which suggest that other clock properties in addition to τ differences might be associated with the chronotype divergence observed between the two populations, which also forms the basis for some of my studies presented in the forthcoming chapters. Since the populations were selected for divergent timing of emergence, it is not surprising that the activity-rest rhythm did not exhibit divergence between the two populations. Interestingly, the τ of activity-rest rhythm appear to have diverged between the two populations which further suggest that the differences in evolved clock properties might pertain to the core circadian oscillator driving both the rhythms. However, in spite of divergence in τ of activity-rest rhythm, the mean phase of *early* and *late* populations did not differ under LD12:12 (Figure 3). While one possible explanation for this discrepancy might be that the magnitude of difference in τ might not be large enough to promote

divergent phase of activity-rest rhythm, this observation also provides an interesting clue for further studies on the widely observed τ-ψ_{ent} association.

Thus, in response to selection for morning and evening emergence for over 250 generations, *early* and *late* populations evolved divergent emergence waveforms characterized by an advanced and delayed phase of entrainment (ψ_{ent}) for emergence rhythm, and therefore will henceforth be referred to as 'early emergence chronotype' and 'late emergence chronotype'. Furthermore, the two populations also evolved divergent clock periods for both emergence and activity-rest rhythms. In the subsequent chapters, I will further explore other clock properties and the underlying molecular correlates of *early* and *late* populations.

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CHAPTER 3

Differential role of light and temperature in driving emergence chronotypes of *early* and *late* populations

The contents of this chapter has been published as the following research article:
KL Nikhil, G Goirik, K Ratna and VK Sharma (2014) Role of temperature in mediating morning and evening emergence chronotypes in fruit flies *Drosophila melanogaster*. *Journal of Biological Rhythms*. 29(6):427-441.

3.1 Introduction

Rhythmic regulation of behavioural and physiological processes ensuring their occurrence at appropriate times of the day (or life stages), thus enhancing survival, and ultimately fitness of organisms is believed to have led to the evolution of circadian clocks (and other such biological timing systems) (Dunlap et al. 2004). Circadian clocks govern such rhythmic processes at both population (e.g. adult emergence rhythm in insects) and individual (e.g. activity-rest rhythm) levels (Saunders, 2002; Dunlap et al. 2004).

The pattern of adult emergence in *Drosophila melanogaster* under 12:12 h laboratory light/dark cycles (LD12:12) assumes a shouldered unimodal waveform with a prominent primary peak around "lights-ON" and a less prominent shoulder close to "lights-OFF" (Sheeba et al. 1999). Landmark studies by Pittendrigh and co-workers on emergence rhythm in *Drosophila* and other insects (Pittendrigh, 1967, 1981; Pittendrigh and Minis, 1971; Skopik and Pittendrigh, 1967) have helped establish some of the conceptual foundations of circadian rhythms. Two core circadian clock genes *period* and *timeless*, were also identified in mutagenesis screens for arrhythmic adult emergence phenotypes (Konopka and Benzer, 1971; Sehgal et al. 1994) thus highlighting the contribution of emergence rhythm in studying circadian clocks. In spite of being a key readout, emergence rhythm has received little attention especially in the domains of exploring molecular and neuronal mechanisms underlying circadian rhythms.

The nature of emergence waveforms varies across insect species. The primary peak of emergence occurs close to dawn in the yellow dung fly *Scopeuma stercoraria* (Lewis and Bletchley, 1943), Queensland fruit fly *Dacus tryoni* (Myers, 1952; Bateman,

1955), in moths *Pectinophora gossypiella* (Pittendrigh and Minis, 1964), and *Heliothis zea* (Callahan, 1958). Flour moth *Anagasta kuhniella* primarily emerge in the post-afternoon and early evening hours (Bremer, 1926; Scott, 1936; Moriarty, 1959), while in some *Chironomids,* emergence is observed during the night (Palmen, 1955). Irrespective of the differences in the waveform of emergence across insect species, one of its noticeable aspects is the restriction of emergence to specific times of the day, a phenomenon referred to as 'gating' (Skopik and Pittendrigh, 1967). Gating results in a specific duration becoming an "allowed zone" for emergence (gate width) during which a substantial proportion of flies emerge out of their pupae, and another interval becoming a "forbidden zone", during which little or no emergence occurs (Pittendrigh, 1966). This gating is so stringent that flies which complete their development after closure of the emergence gate remain inside the puparium until the next gate opens on the following day (Skopik and Pittendrigh, 1967). Depending on the ecology, emergence at unfavourable hours of the day may be maladaptive to individuals either due to the presence of predators, lack of food availability or unfavourable environmental conditions like high temperature and low humidity resulting in desiccation, all of which reduce survivability of the newly emerged individuals. Thus, gating is likely to be an adaptation to selection pressures imposed by various environmental factors but the specific roles of different zeitgebers in nature in modulating gating remain poorly understood.

There have been previous attempts to use laboratory selection approach to study evolution of circadian clock's phasing of adult emergence. Pittendrigh (1967) and Pittendrigh and Minis (1971) selected for "early" and "late" emerging strains of *D. pseudoobscura* and moth *Pectinophora gossypiella* under LD12:12. Several years later, Pittendrigh and Takamura (1987) imposed a similar selection on *D. auraria*, but under a

very short photoperiod of LD01:23. In all cases, the populations responded to selection with early and late emerging populations exhibiting phase divergences (difference in mean emergence phase) of about 4 h in *D. pseudoobscura*, 5 h in *Pectinophora gossypiella*, and about 6 h in *D. auraria*. All three species also showed correlated changes in their circadian clock periods albeit in different directions. In *D. pseudoobscura* and *P. gossypiella*, early flies had longer clock periods and late flies shorter clock periods, while early and late flies of *D. auraria* had shorter and longer clock periods respectively. Although Pittendrigh attributed this paradox to a slave oscillator (driven by a master oscillator) controlling emergence rhythm, and to differential coupling between pacemaker and light cycle arising from the differences in light regimes used during the selection process, the selection protocols used in these studies suffered several methodological shortcomings such as lack of (a) replication at the population level within a selection regime, (b) information on population ancestry, and (c) precise details of selection protocols employed. Over the decades, the field of experimental evolution has demonstrated that even moderately small differences in the above mentioned factors may considerably affect the response of selected populations (evolutionary trajectories of populations greatly differ depending on the standing genetic variation harboured by the baseline populations, and microenvironment differences), thus leading to misinterpretation of data (reviewed in Sharma and Joshi, 2002). Improvising on the methodological aspects of previous studies, and incorporating the basic prerequisites of experimental evolution, we initiated a long term laboratory selection study aimed at examining the evolutionary trajectory of circadian clocks in response to selection for different phases of adult emergence, and subsequently their role in mediating chronotype differences. The populations used in our study comprise four independent replicates of large random mating populations, and therefore harbour

sufficient genetic variation thus providing an ideal platform for various kinds of evolutionary studies. This is in contrast to studies performed on inbred, so called 'wild type' strains which may provide an inaccurate picture of the underlying genetic architecture of the phenotypes under study.

Contrary to previous studies by Pittendrigh (1967) and Pittendrigh and Minis (1971), *early* and *late* populations in our study evolved shorter and longer circadian periods (τ) (Kumar et al. 2007a). This ambiguity can be attributed to the possible shortcomings in the selection protocol employed in former studies (discussed earlier). Also, *early* and *late* populations evolved dominant morning and evening oscillators (Kumar et al. 2007b) respectively, divergent photic phase response curves (PRCs) for emergence rhythm (Kumar et al. 2007a), and under laboratory 12:12 h LD cycles, the two populations differentially utilized light in the morning and evening hours to exhibit their respective emergence chronotypes or phase divergence (Vaze et al. 2012a). Previous studies have reported correlation between circadian periods and chronotypes in humans (Duffy et al. 1999; Roenneberg et al. 2003). Also, morning and evening chronotypes in humans are correlated with the nature and duration of light exposure (Duffy et al. 2001; Roenneberg et al. 2003; Goulet et al. 2007). Taken together, these studies suggest that chronotypes of *early* and *late* populations can be attributed to differences in the underlying circadian clocks and its interaction with environmental cycles.

Several recent studies under semi natural conditions have explored the effect of multiple zeitgebers on emergence and activity rhythms of *Drosophila* (De et al. 2012, 2013; Menegazzi et al. 2012, 2013; Vanin et al. 2012; Prabhakaran et al. 2013). Although these studies have revealed several interesting and unexplored aspects of such rhythms that remained masked under standard laboratory conditions, they have only

managed to convince us about the complexity of circadian clock-zeitgeber interaction, failing to provide further insights into the nature of such interactions. Vaze et al (2012a, b) reported that environmental cycles in nature considerably enhanced chronotype differences between the morning (*early*) and evening (*late*) selected populations of *D. melanogaster* compared to standard laboratory condition. Adding to the enhanced chronotype differences, emergence rhythm of *early* and *late* populations also exhibited significant reduction in gate widths of emergence in SN, suggesting a causal role of multiple zeitgebers in modulating gate widths.

Following up on the reported observations of Vaze et al (2012b), by simulating various zeitgeber profiles in the laboratory, we intended to test the following hypotheses: (a) stepwise change in light cycles mimicking twilight conditions in nature are sufficient, and more effective than abruptly changing light cycles to promote phase divergence between *early* and *late* emergence chronotypes, (b) stepwise change in temperature cycles mimicking nature are sufficient, and more effective than abruptly changing temperature cycles to promote phase divergence, (c) combined cycling of light and temperature promotes phase divergence better than light and temperature independently, and (d) the phase difference between light and temperature observed in nature plays a role in promoting phase divergence.

3.2 Materials and methods

(a) *Experimental* populations: Details of experimental populations and maintenance protocol are provided in chapter 2.

(b) *Adult emergence rhythm assay:* The protocol for adult emergence rhythm assay is described in chapter 2, and details of different experimental regimes used in the study are provided in Table 1. Briefly, experimental regimes primarily comprised abrupt and

stepwise changes in light and temperature either individually, or in combination. Abruptly changing light cycles (ALC) employed rectangular waveforms of light with a sudden upshift from 0 Wm^{-2} to 0.4 Wm^{-2} marking lights-ON (ExT06) and a sudden down shift to 0 Wm^{-2} at lights-OFF (ExT18). Similarly, abruptly changing temperature cycles (ATC) employed rectangular waveforms of temperature cycling between 18 ^{o}C and 28 ^{o}C with sudden temperature upshift and downshift occurring at Ext06 (ExT00 indicates the midpoint of cryophase) and ExT18 respectively. Stepwise changing light cycles (SLC) also involved cycling of light between 0 and 0.4 Wm^{-2}, but in gradual steps of 0.04 Wm^{-2} every 20 min, and therefore comprised 10 steps spanning 3 h 20 min in the morning and evening. Similarly, stepwise changing temperature cycles (SLC) employed a rate of 1 ^{o}C change in temperature every 20 min thus comprising 10 steps spanning 3 h 20 min in the morning and evening. In both SLC and STC, ExT06 marks the onset of stepwise light/temperature increase and offset of stepwise light/temperature decrease in the evening is marked by ExT18. Further, combined cycles of light and temperature comprised two types of experimental regimes; abruptly, and stepwise changing light + temperature in-phase (ALTC1 and SLTC1), and out-of-phase (ALTC2 and SLTC2). ALTC1 and SLTC1 employed the combined waveforms of light and temperature as discussed above (ALC and ATC for ALTC1; SLC and STC for SLTC1) with low temperature (18 ^{o}C) coinciding with the dark phase and high temperature (28 ^{o}C) with light phase. Out-of-phase cycles of light and temperature (ALTC2 and SLTC2) also employed the combined waveforms of light and temperature as in ALTC1 and SLTC1, but the temperature cycles lagged the light cycles by 4 h. The rate of increase and decrease of light and temperature, and the 4 h phase difference between them were set to resemble conditions in nature during the month of March, 2011 in Bangalore (12°59'N 77°35'E), India as reported in Vaze et al (2012b). Emergence assays were performed in

Percival Drosophila chambers (Percival, USA) programmed to simulate the different experimental regimes discussed above.

Experimental Regime	Light Condition	Temperature Condition	Phase-Difference
Abruptly changing light cycles (ALC)	Abrupt transitions during lights-ON and lights-OFF	NA	NA
Stepwise changing light cycles (SLC)	Stepped transitions in light intensity at a rate of 0.04 Wm^{-2}/20 min	NA	NA
Abruptly changing temperature cycles (ATC)	NA	Abrupt transitions between high (28 °C) and low (18 °C) temperatures	NA
Stepwise changing temperature cycles (STC)	NA	Stepped change in temperature at a rate of 1 °C/20 min	NA
Abruptly changing light + temperature cycles (in-phase) (ALTC1)	Abrupt transitions during lights-ON and lights-OFF	Abrupt transitions between high (28 °C) and low (18 °C) temperatures	0 h
Stepwise changing light + temperature cycles (in-phase) (SLTC1)	Stepped transitions in light intensity at a rate of 0.04 Wm^{-2}/20 min	Stepped change in temperature at a rate of 1 °C/20 min	0 h
Abruptly changing light + temperature cycles (out-of-phase) (ALTC2)	Abrupt transitions during lights-ON and lights-OFF	Abrupt transitions between high (28 °C) and low (18 °C) temperatures	4 h
Stepwise changing light + temperature cycles (out-of-phase) (SLTC2)	Stepped transitions in light intensity at a rate of 0.04 Wm^{-2}/20 min	Stepped change in temperature at a rate of 1 °C/20 min	4 h

Table 1: Light and temperature conditions of different experimental regimes used in the study. For experimental regimes involving light cycles, the minimum and maximum light intensities used were 0 Wm^{-2} and 0.4 Wm^{-2} respectively, and that for temperature were 18 ºC and 28 ºC respectively. Light always preceded temperature by 4 h in experimental regimes where light and temperature cycles were provided out-of-phase (NA - not applicable).

(c) ***Analyses of emergence characteristics:*** The mean phase (θ) measured as External Time (ExT) of emergence, and length of the mean polar vector (r) were calculated as described in chapter 2. In the circular time scale used for analysis, 00° refers to the external time 00 (ExT00) and therefore, 15° represents 1 h (Daan et al. 2002). The gate width (gw) of emergence refers to the duration during which 90% of the total emergence occurs.

Under any given experimental regime, Rayleigh test ($\alpha = 0.01$) was used to test for the null hypothesis that emergence of a population is randomly distributed throughout the day and therefore has no mean direction (Batschelet, 1981; Jammalamadaka and SenGupta, 2001; Zar, 2009). Rejection of null hypothesis favours the alternate hypothesis that there exists a mean direction of emergence, facilitating further statistical analyses. Employing parametric tests in directional statistics requires that the data approximately follow 'Von Mises' distribution (a circular equivalent of 'Gaussian' distribution) with high values of the concentration parameter 'κ' (Jammalamadaka and SenGupta, 2001). Furthermore, such tests require low scatter and unimodality in data to facilitate high statistical power. Since not all of these criteria were met under certain experimental regimes, we adopted non-parametric methods for all statistical analyses unless specified.

Non-parametric test for dispersion (NPTD) (Batschelet, 1981) and Rao's test for Homogeneity were used to test if mean phase of emergence (θ) between the populations differed significantly from each other (Jammalamadaka and SenGupta, 2001) under a given experimental regime. All pairwise comparisons were carried out at $\alpha = 0.01$ (99% confidence level) following Bonferroni corrections to ensure that the total family wise error rate does not exceed $\alpha = 0.05$ (95% confidence level).

(d) ***Test for phase divergence between populations:*** To quantify the extent of phase divergence between two populations, we used the measure 'angular distance (β)'. Angular distance is the shortest distance in degrees between two points located on a circular scale (Zar, 2009), and thus the difference between mean angles of emergence (θ) of any two populations would indicate the extent of phase divergence between them. The angles were later expressed as ExT in h by simple linear transformation. Since, our intention was to study the role of different zeitgebers in enhancing chronotype differences between *early* and *late* populations to the extent observed under semi natural conditions (SN) (Vaze et al. 2012b), we compared phase divergences between the populations in any given experimental regime to SN using Wilcoxon Rank Sum test or Wilcoxon Mann Whitney test (Zar, 2009) at $\alpha = 0.05$ (95% confidence level). The experimental regime being tested is considered to mimic SN in enhancing chronotype differences if the phase divergence between the populations in that regime is either equal to or greater than that observed under SN.

(e) ***Analyses of gate width of emergence:*** The population (block) means of *gw* values when subjected to Shapiro Wilk test for normality ($\alpha = 0.05$), were found to be normally distributed, and were analysed by the method of Analysis of Variance (ANOVA). Differences among the populations within an experimental regime were analysed by a two way mixed model ANOVA with population as fixed, and block as a random factor, whereas a three way ANOVA model was used to test for differences among populations across experimental regimes with population and experimental regime as fixed factors, and block as a random factor. Post hoc multiple comparisons in both cases were performed using Tukey's HSD method at $\alpha = 0.05$. ANOVA and other linear statistical analysis were executed on Statistica for Windows, Release 5.0B (Statsoft, 1995).

3.3 Results

The mean emergence phase (θ expressed as ExT) values for all populations were calculated only after subjecting the emergence data to Rayleigh test ($\alpha = 0.01$) following which the null hypothesis that emergence is randomly distributed across the day, was rejected. All tests for within population differences for both mean emergence phase (θ) (NPTD and Rao's test for Homogeneity) and gate width (*gw*) (ANOVA) were performed as described in materials and methods, and unless specified, differences between populations under a given experimental regime were found to be significant.

In view of the hypothesis being tested (see introduction), the results will primarily focus on (a) whether the extent of phase divergence under the experimental regime being tested is equal to or greater than that observed under SN (tested by Wilcoxon Rank Sum test), and (b) gate widths under a given experimental regime being tested is equal to or lesser than that observed under SN (tested by ANOVA).

(a) *Emergence under semi natural environmental cycles (SN) and abruptly changing light cycles (ALC)*

For the ease of comparison across experimental regimes, data from Vaze et al (2012b) were reanalysed by the method of circular statistics (see materials and methods for details of analysis). As reported by Vaze et al (2012b), emergence of *early* populations was restricted to early morning (θ_e = 4.46 h; *control* populations around 3 h later following *early* populations (θ_c = 7.47 h), and *late* populations emerging significantly later (θ_l = 10.26 h), 3 h after *control,* and 6 h after *early* populations (Figure 1a-top and middle panels; Table 2). The mean emergence phases under ALC were delayed compared to SN with *early* populations emerging at 8.72 h and *controls* 10.84 h (Figure 1b-top and middle panels; Table 2). *late* populations on the other hand had a delayed

mean emergence phase of 13.24 h with emergence extending into the dark phase (Figure 1b-top and middle panels; Table 2).

The phase divergence between *early* and *late* populations ($\beta_{e\text{-}l}$) was significantly reduced from 5.76 h in SN to 4.51 h under ALC (Wilcoxon test, $p < 0.05$). Similarly, divergence between *early* and *control* populations ($\beta_{e\text{-}c}$) was reduced from 3.1 h in SN to 2.12 h under ALC (Wilcoxon test, $p < 0.05$) and that between *control* and *late* populations ($\beta_{c\text{-}l}$) reduced from 2.75 h in SN to 2.4 h in ALC (Figure 1a, b-top and middle panels; Table 2).

The gate widths of *control* (gw_c = 11.5 h) and *late* (gw_l = 14.5 h) populations was significantly wider under ALC compared to SN ($F_{2,6} = 25.8$, $p < 0.01$), while that of *early* populations (gw_e = 9 h) did not differ from that in SN (Figure 1a, b-bottom panel; Table 2).

SN thus promotes greater divergence in chronotypes of the three populations by (a) increasing phase divergences (β) between the populations, and (b) by tightening the gate widths of emergence thereby reducing the extent of overlap between the emergence waveforms of *early* and *late* populations (Figure 1a, b; Table 2). Since the extent of phase divergence between the populations was less pronounced in ALC compared to that under SN, we tested if stepped cycles of light resembling gradual dawn and dusk transitions might improve the phase divergence.

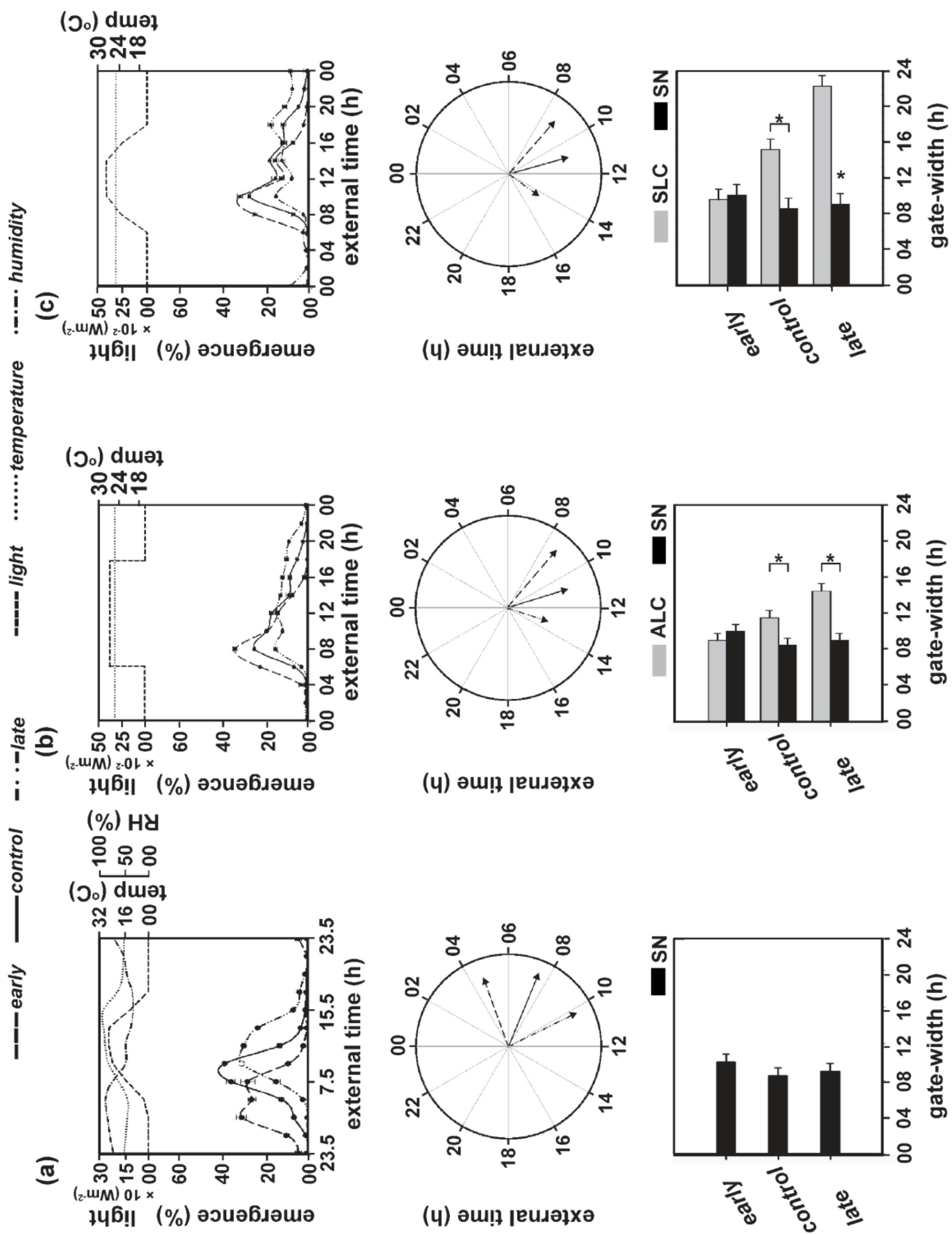
(b) *Emergence under stepwise changing light cycles (SLC)*

The mean emergence phases of the three populations under SLC (θ_e = 8.74 h, θ_c = 10.98 h, θ_l = 14.26 h) were similar to that in ALC (Figure 1b, c-top and middle panels; Tables 1, 2). While, divergence between *early* and *late* populations in SLC ($\beta_{e\text{-}l}$ = 5.51 h) was similar to SN (Wilcoxon test, $p > 0.01$), that between *early* and *control* ($\beta_{e\text{-}c}$ = 2.22 h), and *control* and *late* ($\beta_{c\text{-}l}$ = 3.3 h) populations were significantly lower

compared to SN (Wilcoxon test, $p < 0.01$; Figure 1c-top and middle panels; Table 2). In comparison to ALC, phase divergence between populations in SLC tended to be greater but not statistically significant (Table 2).

early and *late* populations exhibited narrower ($gw_e = 9.5$ h) and wider ($gw_l = 22$ h) gate widths of emergence respectively compared to *controls* ($gw_c = 15$ h). In comparison with SN, gate widths of *control* and *late* populations ($F_{2,6} = 12.72$, $p < 0.01$; Figure 1c-botom panel), but not *early* populations were significantly wider in SLC.

Therefore, SLC was not effective in reproducing the effects of SN either in terms of phase divergence or gate width reduction. But in comparison with ALC, phase divergence between the populations, even though not statistically different, was marginally greater in SLC.



early
control
late
light
temperature
humidity
(a)
(b)
(c)
light
× 10 (Wm-2)
× 10-2 (Wm-2)
emergence (%)
temp (°C)
RH (%)
external time (h)
early
control
late
gate-width (h)
SN
ALC
SLC

Figure 1 (previous page): Emergence waveforms (top panel) and respective polar plots (middle panel) (NPTD and Rao's test for Homogeneity) of *early*, *control,* and *late* populations under (a) semi natural conditions (SN), (b) abruptly changing light cycles (ALC), and (c) stepwise changing light cycles (SLC). External time 00 (ExT00) (top and middle panels) indicates midpoint of the dark phase (Daan et al. 2002). Bottom panel: Gate width of emergence of *early*, *control,* and *late* populations under (a) SN, (b) ALC, and (c) SLC regimes in comparison with that of SN. Asterisk indicates significant difference (ANOVA followed by Tukey's HSD). Error bars represent 95% CI for visual hypothesis testing.

Experimental regime	Population	Mean Polar Vector Length (r)	Mean Angle (θ in h)	Gate-Width (h)
Semi-natural (SN)	*early*	0.75	4.46	10
	control	0.81	7.47	8.5
	late	0.78	10.26	9
Abruptly changing light cycles (ALC)	*early*	0.76	8.72	9
	control	0.63	10.84	11.5
	late	0.41	13.24	14.5
Stepwise changing light cycles (SLC)	*early*	0.70	8.74	9.5
	control	0.60	10.98	15
	late	0.37	14.26	22
Abruptly changing temperature cycles (ATC)	*early*	0.81	4.59	8
	control	0.77	8.06	9
	late	0.74	9.79	8.5
Stepwise changing temperature cycles (STC)	*early*	0.80	2.87	8
	control	0.78	6.18	9
	late	0.80	9.13	6.5
Abruptly changing light + temperature cycles (in-phase) (ALTC1)	*early*	0.93	7.53	4
	control	0.89	9.17	4.5
	late	0.86	9.24	6
Stepwise changing light + temperature cycles (in-phase) (SLTC1)	*early*	0.92	7.56	4
	control	0.84	8.67	5
	late	0.82	10.05	6
Abruptly changing light + temperature cycles (out-of-phase) (ALTC2)	*early*	0.93	7.06	3.5
	control	0.87	9.16	6
	late	0.84	11.64	8
Stepwise changing light + temperature cycles (out-of-phase) (SLTC2)	*early*	0.92	8.48	4
	control	0.90	10.25	6
	late	0.85	12.78	6.5

Table 2 (previous page): Polar coordinates describing mean phase of emergence (θ) (hours) and lengths of polar vectors (r) for emergence of *early, control,* and *late* populations under different experimental regimes. Gate width (gw) of emergence rhythm indicates the duration during which 90% of total emergence occurs.

(c) *Emergence under abruptly changing temperature cycles (ATC)*

Having observed that neither abrupt nor gradual changes in light could reproduce the effects of SN, we assessed the effect of temperature which is another robustly cycling zeitgeber in nature, using abruptly cycling temperature cues (Table 1).

The mean emergence phases of the three populations (θ_e = 4.59 h, θ_c = 8.06 h, θ_l = 9.79 h) in ATC were advanced (compared to ALC and SLC) and comparable to that observed under SN (Figure 2a-top and middle panels; Table 2). Also, phase divergence between *early* and *late* populations ($\beta_{e\text{-}l}$ = 5.2 h) was significantly higher in ATC than under SN (Wilcoxon test, $p < 0.01$; Figure 2a-top and middle panels), while phase divergence between *early* and *control* populations ($\beta_{e\text{-}c}$ = 3.48 h), and *control* and *late* populations ($\beta_{c\text{-}l}$ = 2.93 h) did not differ significantly from that in SN (Wilcoxon test, $p > 0.01$; Figure 2a-top and middle panels). Also, gate widths of the three populations (gw_e = 8 h, gw_c = 9 h, gw_l = 8.5 h) under ATC did not significantly differ from SN ($F_{2,6} = 2.28$, $p > 0.05$; Figure 2a-bottom panel).

Thus, abruptly changing temperature cycles were highly effective in reproducing the emergence chronotypes of *early* and *late* populations as observed in SN, in all aspects including enhanced phase divergence between populations, reduction in gate widths, and also overall advancement in emergence. Additionally, narrow gate widths of all the populations under ATC suggested that temperature plays a key role in tightening gate width of emergence in the absence of light.

(d) ***Emergence under stepwise changing temperature cycles (STC)***

Even though ATC effectively reproduced the effects of SN, since abrupt transitions in temperature are not realistic in terms of what is observed in nature, we further tested if stepwise increase and decrease in temperature can also reproduce or further enhance phase divergence between the populations.

When assayed under stepwise changing temperature cycles (STC; Table 1), mean phases of emergence of all *early* and *control* populations but not *late* were advanced compared to ATC (θ_e = 2.87 h, θ_c = 6.18 h, θ_l = 9.13 h; Figure 2b-top and middle panels). Also, as in ATC, phase divergence of *early* populations from both *late* and *control* populations ($\beta_{e\text{-}l}$ = 6.26 h, $\beta_{e\text{-}c}$ = 3.31 h) was significantly greater under STC than in SN (Wilcoxon test, $p < 0.05$; Table 2) while divergence between *control* and *late* populations was not different from SN (Table 2).

All three populations exhibited narrower gate widths of emergence (gw_e = 8 h, gw_c = 9 h, gw_l = 6.5 h) under STC compared to SN (Figure 2b-bottom panel). While gate widths of *control* populations were not significantly different from that in SN, gate widths of *early* and *late* populations were significantly narrower under STC than in SN ($F_{2,6} = 18.6$, $p < 0.05$; Figure 2b-bottom panel).

Thus, stepwise changing temperature cycles also effectively enhanced chronotype differences between the three populations similar to, and in some cases more than that observed under SN. Reduced gate widths of emergence in STC further strengthen the idea that temperature plays a key role in the reduction of gate widths. Additionally, phase divergence was further enhanced by a small magnitude in STC, suggesting that, similar to light, stepwise increase and decrease in temperature also contribute to the enhancement of phase divergence.

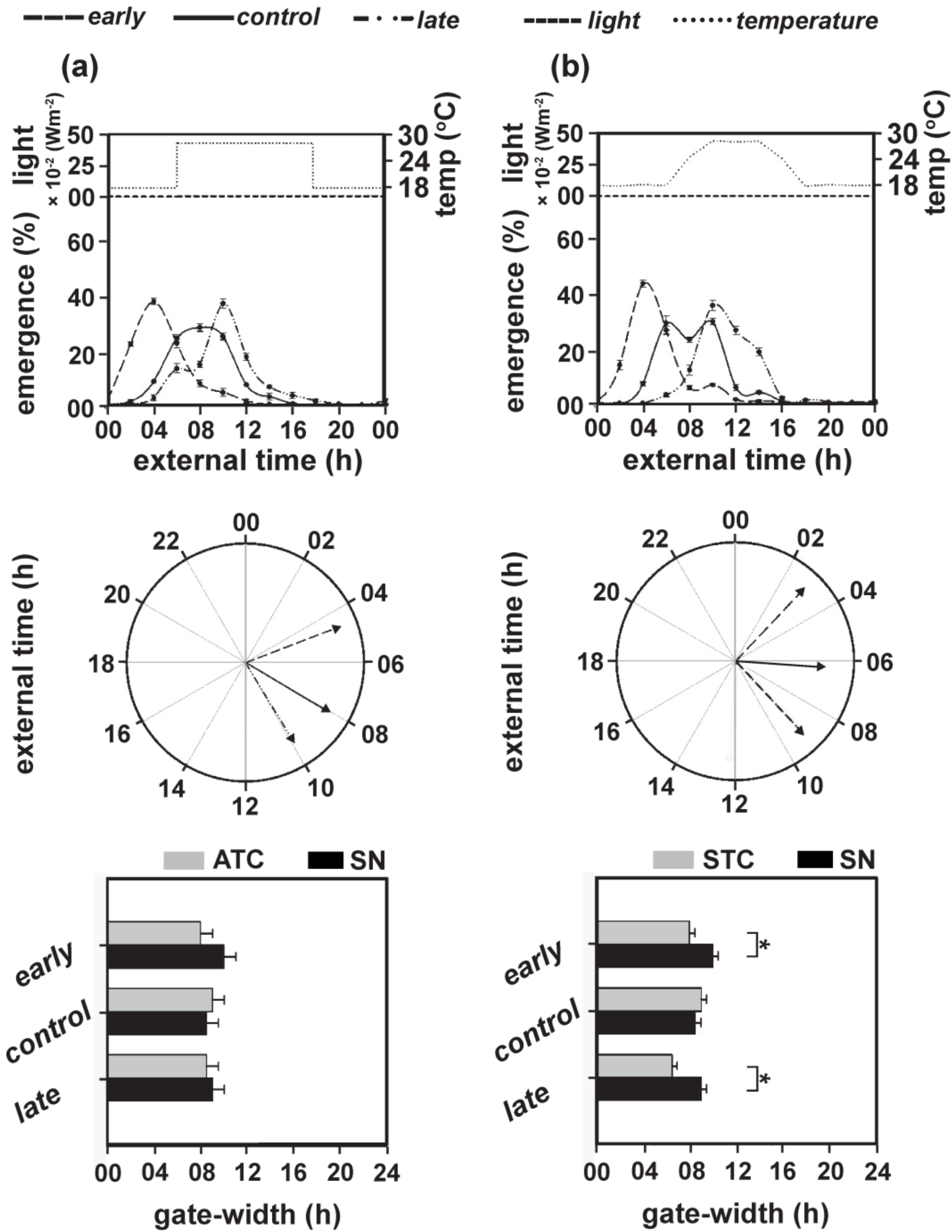


Figure 2: Emergence waveforms (top panel) and respective polar plots (middle panel) (NPTD and Rao's test for Homogeneity) of *early*, *control,* and *late* populations under (a) abruptly changing temperature cycles (ATC), and (b) stepwise changing temperature cycles (STC). External time 00 (ExT00) (top and middle panels) indicates midpoint of the cryophase (Daan et al. 2002). Bottom panel: Gate width of emergence of *early*, *control,* and *late* populations under (a) ATC, and (b) STC regimes in comparison with that of semi natural conditions (SN). Asterisk indicates significant difference (ANOVA followed by Tukey's HSD). Error bars represent 95% CI for visual hypothesis testing.

(e) ***Emergence under abruptly changing in-phase light and temperature cycles (ALTC1)***

Further, to test the combined effect of light and temperature, and the role of phase difference between the two (as seen in nature), we assayed emergence under cycles of abruptly changing in-phase and out-of-phase light and temperature (ALTC1, ALTC2; Table 1).

While *early* populations emerged earlier (θ_e = 7.53 h), and *late* populations emerged later than *controls* (θ_l = 9.24 h, θ_c = 9.17 h) in ALTC1, there was a marginal advancement in emergence of all the three populations greater than ALC and SLC but not to the extent observed in ATC, STC and SN (Figures 1, 2, 3a-top and middle panels). Wilcoxon test revealed that phase divergence between the three populations (β_{e-l} = 1.71 h, β_{e-c} = 1.64 h, β_{c-l} = 0.84 h) under ALTC1 was completely disrupted and lower compared to all other previous experimental regimes including that of SN ($p < 0.05$).

Interestingly, gate widths of all the three populations (gw_e = 4 h, gw_c = 4.5 h, gw_l = 6 h) were significantly narrow under ALTC1 compared to their respective values in SN ($F_{2,6} = 21$, $p < 0.01$; Figure 3a-bottom panel).

While chronotype differences between the three populations were greatly attenuated and instead phase convergence was observed under ALTC1, it could only partially reproduce the effects of SN by considerably reducing the gate widths of emergence rhythm.

(f) ***Emergence under stepwise changing in-phase light and temperature cycles (SLTC1)***

We assayed emergence under stepwise changing cycles of light and temperature in-phase (SLTC1; Table 1) to test if such cycles would minimize or rescue the phase converging effects brought about by ALTC1.

With mean phases - θ_e = 7.56 h, θ_c = 8.67, and θ_l = 10.05 h, emergence of all the three populations was phase advanced in SLTC1, compared to light cycles but not to the extent observed under ATC, STC and SN (Figures 1, 2, 3b-top and middle panels; Table 2). Phase divergence between the three populations (β_{e-l} = 2.48 h, β_{e-c} = 1.10 h, β_{c-l} = 1.37 h) was also significantly smaller than that observed under SN (Wilcoxon test, $p <$ 0.05; Table 2).

The gate widths of emergence (gw_e = 4 h, gw_c = 5 h, gw_l = 6 h) under SLTC1 were reduced, and significantly narrower than that in SN ($F_{2,6}$ = 19.19, $p < 0.01$; Figure 3b-bottom panel; Table 2).

Thus, SLTC1 partially reproduced the effects of SN failing to enhance chronotype differences between the three populations but significantly reducing gate widths. Contrary to our previous observations, we did not observe any further enhancement of phase divergence under SLTC1 in comparison with ALTC1. This indicates that phase divergence promoting effect of stepwise cycles can be overridden when the two zeitgebers are in-phase and highlights the importance of phase difference between light and temperature in nature.

(g) ***Emergence under abruptly changing out-of-phase light and temperature cycles (ALTC2)***

With ALTC1 and SLTC1 highlighting the importance of phase difference between light and temperature in enhancement of phase divergence, we tested if abruptly changing

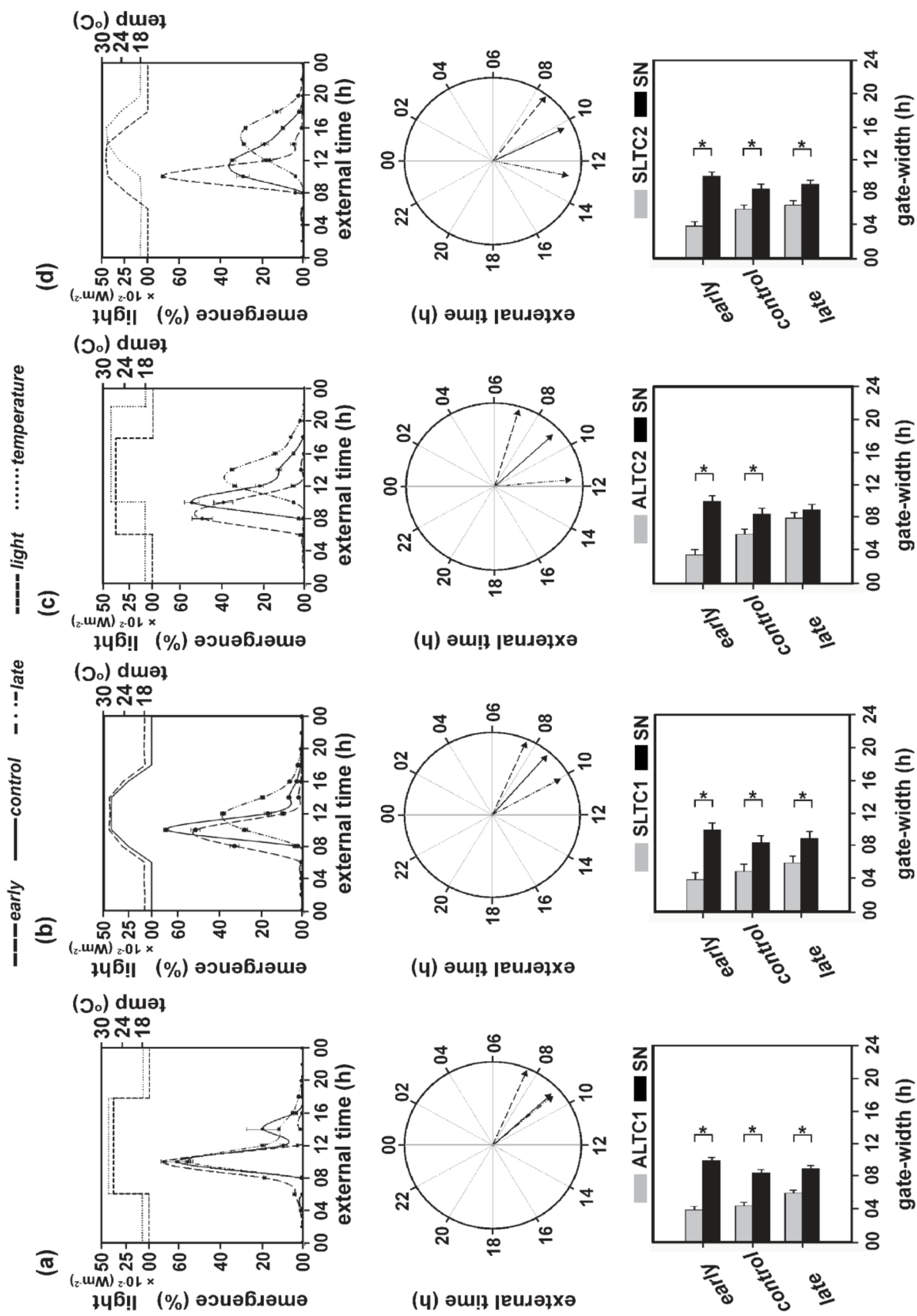
out-of-phase (light cycles phase leading temperature cycles by 4 h) cycles of light and temperature can mimic the effects of SN (ALTC2; Table 1).

With the mean emergence phases of 7.06 h, 9.16 h, and 11.64 h in *early, control,* and *late* populations respectively, no phase advance in emergence was observed under ALTC2 (Figure 3c-top and middle panels; Table 2). Comparisons by Wilcoxon's test revealed that phase divergence between *early* and *late* populations (β_{e-l} = 4.58 h) under ALTC2 did not differ from SN (Wilcoxon test, $p > 0.05$), but that between *early* and *control* (β_{e-c} = 2.09 h), and *control* and *late* populations (β_{e-c} = 2.48 h) were lower than SN (Wilcoxon test, $p < 0.05$).

Furthermore, gate widths of *late* (gw_l = 8 h) populations under ALTC2 did not differ significantly from SN, whereas those of *early* (gw_e = 3.5 h), and *control* (gw_c = 6 h) populations were significantly narrower than SN ($F_{2,6}$ = 26.45, $p < 0.01$; Figure 3c-bottom panel; Table 2).

Therefore, with marginal increase in phase divergence and narrower gate widths of emergence of all three populations, ALTC2 was even though more effective than the in-phase cycles (ALTC1 and SLTC1) in enhancing phase divergence, was partially effective in reproducing the effects of ATC, STC, and SN.

Figure 3 (next page): Emergence waveforms (top panel) and respective polar plots (bottom panel) (NPTD and Rao's test for Homogeneity) of *early*, *control,* and *late* populations under in-phase (a) abruptly changing light and temperature cycles (ALTC1), (b) stepwise changing light and temperature cycles (SLTC1), and out-of-phase (c) abruptly changing light and temperature cycles (ALTC2), (d) stepwise changing light and temperature cycles (SLTC2). External time 00 (ExT00) (top and middle panels) indicates midpoint of the dark phase (Daan et al. 2002). Bottom panel: Gate width of emergence of *early*, *control,* and *late* populations under (a) ALTC1, (b) SLTC1, (c) ALTC2, and (d) SLTC2 regimes in comparison with that of semi natural conditions (SN). Asterisk indicates significant difference (ANOVA followed by Tukey's HSD). Error bars represent 95% CI for visual hypothesis testing.


early
control
late
light
temperature
(a)
(b)
(c)
(d)
emergence (%)
light
× 10-2 (Wm-2)
temp (°C)
external time (h)
gate-width (h)
ALTC1
SLTC1
ALTC2
SLTC2
SN

(h) ***Emergence under stepwise changing out-of-phase light and temperature cycles (SLTC2)***

Finally, we assayed emergence under stepwise changing out-of-phase light and temperature cycles (SLTC2) which resembled SN in terms of the zeitgeber profiles, with both gradual increase and decrease of light and temperature levels, and also a phase difference of 4 h between the two zeitgeber cycles (Table 1).

With no observable phase advance, the mean emergence phases of the three populations (θ_e = 8.48 h, θ_c = 10.25 h, θ_l = 12.78 h) in SLTC2 were found to be similar to that under ALTC2 (Figure 3d-top and middle panels; Table 2). Interestingly, analysis revealed that phase divergences between the three populations under SLTC2 (β_{e-l} = 4.29 h, β_{e-c} = 1.76 h, β_{c-l} = 2.53 h) were lower than in SN (Wilcoxon test $p < 0.05$; Table 2).

The gate widths of all the three populations (gw_e = 4 h; gw_c = 6 h; gw_l = 6.5 h) under SLTC2 were found to be significantly narrower than their respective values in SN ($F_{2,6} = 21$, $p < 0.01$; Figure 3d-bottom panel; Table 2).

Thus, even though SLTC2 enhanced phase divergence between the three populations, surprisingly it failed to mimic SN. Similar to that under ATC, STC and SN, the gate widths of emergence were significantly lower under SLTC2.

3.4 Discussion

While recent studies have reported several interesting features of circadian rhythms in nature and attributed it to the cycling of multiple zeitgebers (De et al. 2012, 2013; Menegazzi et al. 2012, 2013; Vanin et al. 2012; Prabhakaran et al. 2013), what remains unclear is the relative contribution of different zeitgebers independently, and in unison, to the regulation of rhythmic behaviours. In addition to addressing the hypotheses (see introduction), the experimental regimes we used also allowed us to test the role of (a) independent and combined effect of light and temperature, (b) gradual increase and decrease of zeitgeber levels simulating nature, and (c) phase difference between light and temperature profiles (as observed in nature) in enhancing phase divergence between *early* and *late* emergence chronotypes, and modulation of gate widths of emergence rhythm.

Since the fly populations used in this study have been reared under LD12:12 cycles at constant temperature, and based on previous reports of differential temporal photosensitivity in these populations (Vaze et al. 2012a), we speculated that light might be a primary regulator of *early* and *late* chronotypes. However, when assayed under conditions of abrupt light transitions (ALC), the phase-divergence in emergence was significantly lower, and gate widths higher compared to semi natural conditions (SN) (Figure 1a, b; Table 2). Even under stepwise cycling of light (SLC), the shape of emergence profiles and other characteristics of the rhythm did not match that of SN (Figure 1a, c; Table 2). Thus, cycles involving abrupt or stepwise changes in light could not reproduce the effects of SN, suggesting that light cannot independently promote phase divergence, and might require the presence of other zeitgebers. Interestingly enough, we observed that phase divergence between *early* and *late* populations in SLC was enhanced by an hour more than that under ALC (Table 2) indicating that stepwise

increase and decrease in light, mimicking twilight transitions contribute to the enhancement of phase divergence although only by a small magnitude. Such twilight transitions have been previously reported to enrich entrainment of circadian clocks in mammals (Boulos et al. 1996; Sharma et al. 1998; Boulos et al. 2002), although there have been contradicting arguments by others (Comas and Hut, 2009).

Since, temperature is another important zeitgeber that robustly cycles in nature, we tested the effect of abruptly (ATC) and stepwise (STC) changing temperature cycles. Interestingly, both ATC and STC were highly effective in enhancing phase divergence between the populations, and also in significantly reducing gate widths to the extent observed in SN (Figures 1a, 2). These results suggest that most features of the emergence rhythm observed under SN are likely to be influenced by temperature cycles. Furthermore, similar to light cycles, phase divergence between *early* and *late* populations in STC was enhanced by about an hour more than ATC (Figure 2; Table 2), suggesting that stepwise changing temperature mimicking twilight conditions, additionally contribute to promoting phase divergence.

The cycling of light and temperature in nature is interdependent, and there often exists some phase difference between light and temperature with light preceding temperature in a season dependent manner. Therefore, to study the role of combined cycles of the two zeitgebers, and also the phase difference between them in promoting phase divergence, we assayed emergence under cycles of both light and temperature in-phase (ALTC1 and SLTC1) and out-of-phase (ALTC2 and SLTC2). When in-phase, neither abrupt nor gradual cycles of light and temperature were effective in enhancing phase divergence between the populations, and instead reduced it leading to phase convergence (Figure 3a, b; Table 2) but the gate widths of all the three populations were significantly reduced, and in some cases even lower than that in SN. The phase

divergence between the populations under ALTC2 and SLTC2 was greater than ALTC1 and SLTC1, but not comparable to SN (Figures 1a, 3; Table 2) and the gate widths of emergence of all three populations were reduced to the extent observed in SN (Figure 3). Therefore, ALTC2 and SLTC2 were only partially effective in reproducing the effect of SN (ATC and ALC as well). Nevertheless, both cycles were more effective than ALTC1 and SLTC1 in the enhancement of phase divergence thus highlighting the importance of phase difference between the zeitgebers in mediating *early* and *late* emergence chronotypes.

Since SLTC2 resembles SN better than any other experimental regimes in terms of zeitgeber profiles, we expected the features of emergence profiles observed under SN to be best expressed under SLTC2. Quite surprisingly, both ALTC2 and SLTC2 could not entirely reproduce the effects of SN. One possible reason for this discrepancy may be the restricted flexibility in programming of experimental fly chambers used due to which the light and temperature profiles observed in nature could not be perfectly reproduced. In nature, light intensity changes in orders of 0.1 lux, but due to technical limitations, our study employed a rate of 10 lux (approximately 0.04 Wm^{-2}) every twenty min which was not identical to nature. Therefore, the first light that the pupae experienced is a direct up-shift from 0 lux to 10 lux unlike that of a 0.1 lux rise in SN. Adult emergence in *D. melanogaster* has been shown to be mediated by (a) circadian clocks that time emergence, and (b) a direct masked response to lights-ON (McNabb, 2008). Thus, we speculate that the first exposure to 10 lux when lights are turned ON could initiate such masked response leading to a burst of emergence immediately following lights-ON. This can be clearly seen in Figure 3 as a sudden increase in emergence in both *early* and *control* populations within the first two hours following lights-ON, and might partially contribute to reduced phase divergence thus highlighting

the inadequacy of stepwise cycles of light and temperature employed to mimic SN profile. Additionally, we did not incorporate daily changes in the light quality (changes in composition of different wavelengths) and humidity which are likely to promote phase divergence between *early* and *late* populations in nature, and thus, lack of these factors also partially explains the inability of SLTC2 to entirely reproduce the effects of SN.

We find the maximal phase divergence observed under ATC and STC to be interesting as it represents a completely novel environment for the populations which have evolved under LD12:12 and constant temperature for over 230 generations. The enhanced phase divergence under ATC and STC can be explained under the framework of the dual oscillator model proposed by Pittendrigh (Pittendrigh and Bruce, 1959) according to which the circadian clock comprises a pace making A oscillator which is primarily light sensitive and temperature insensitive while the B oscillator (coupled and driven by A but also feeds back onto A) is primarily temperature sensitive and light insensitive. Under light cycles (ALC and SLC), the A oscillator dominates over B and utilizes light to phase *early* and *late* emergence whereas, under ATC and STC in the absence of light, the A oscillator being temperature insensitive, receives feedback from the temperature sensitive B oscillator which in turn drives enhanced phase divergence under these conditions. If this is true, it is reasonable to speculate that evolved divergence between *early* and *late* populations might be driven by differences in the B oscillator (this does not negate the possibility of differences in A oscillator) which in combination with the A oscillator drives chronotype differences, the maximal expression of which is restricted by the dominance of A oscillator under light cycles. When subjected to cycles of both light and temperature (ALTC2 and SLTC2), the combined effect results in chronotypes intermediate to that seen under light, and temperature

cycles alone (ATC and STC), suggesting antagonistic effects of light and temperature (Figure 4). Even though coupled oscillators have postulated to control morning and evening activity in *Drosophila* (Grima et al. 2004; Stoleru et al. 2004), the above explanation remains largely speculative since there are no empirical evidence of the differential sensitivity of the two oscillators to light and temperature. Another noticeable aspect is that phase advances in emergence waveform in presence of temperature. We speculate that phase advances might be driven by thermo sensitive splicing of *period* transcripts which has earlier been shown to play an important role in timing the activity-rest rhythm in a temperature dependent manner (Majercak et al. 2004; Low et al. 2012) but needs to be validated in future experiments.

Furthermore, enhanced gate widths of emergence under ALC and SLC indicate that light widens gate widths of emergence rhythm whereas comparisons with temperature regimes suggest that temperature has an opposing effect compared to light (Figures 1-4). In addition to significantly reducing gate widths, temperature cycles considerably phase advanced emergence thus causing emergence to occur during *early* morning hours (Figures 2, 4). When in combination, the phase advancing effect of temperature is reduced by light and gate widening effect of light is reduced by temperature, as a consequence of which, emergence during early morning hours is phase delayed and restricted to the morning and day as observed in nature (De et al. 2012; Vaze et al. 2012b). Such antagonistic effects of the light and temperature in mediating circadian rhythms have been reported earlier. A recent study reported that *cryptochrome*, which promotes light entrainment also antagonizes entrainment of *Drosophila* circadian clocks to temperature cycles (Gentile et al. 2013) suggesting that molecular mechanisms underlying such antagonistic interactions of zeitgebers with circadian clocks can exist. Additionally, evolution of gated emergence in *Drosophila*

that restricts emergence to morning and day has been hypothesized to be an adaption to enhance survivability of newly emerged individuals (Pittendrigh, 1966). Therefore, it is plausible that such antagonistic interaction of light and temperature with circadian clocks is essential to restrict emergence during the day in *Drosophila*.

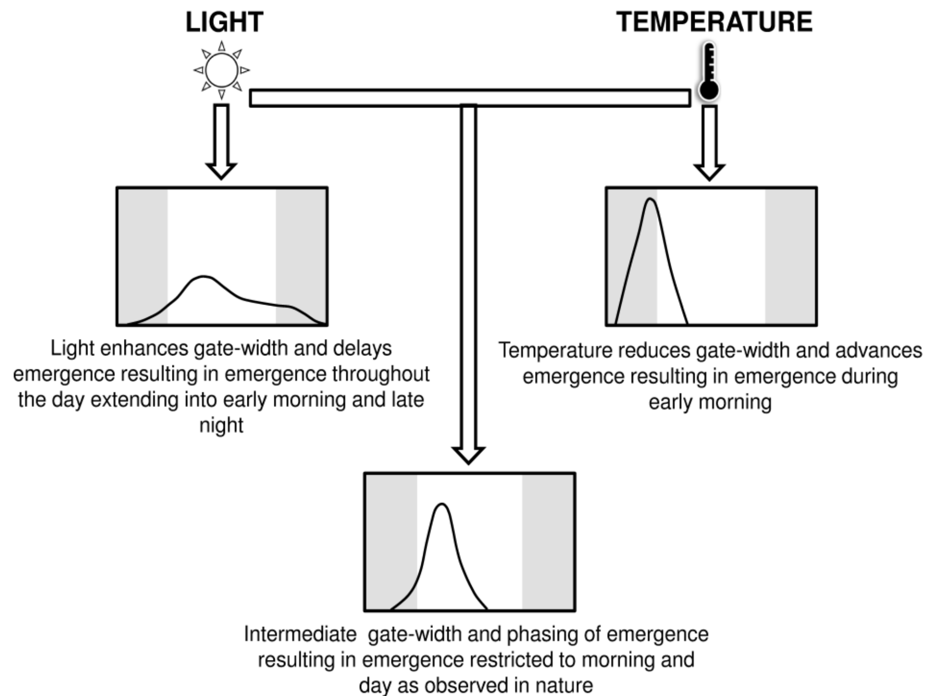


Figure 4: Schematic of the influence of light and temperature cycles on the timing adult emergence rhythm in *D. melanogaster*.

Results from in- and out-of-phase cycles suggest that the phase difference between light and temperature also serves as an essential cue for promoting phase divergence between chronotypes. Coupling of PERIOD and TIMELESS oscillations in *Drosophila* clock neurons has been shown to vary across seasons (Menegazzi et al. 2013). Taken together, these results suggest that information about the phase difference between light and temperature might be critical for circadian clocks in mediating chronotype differences across seasons; variation of chronotypes across seasons and the relative roles

of light and temperature can only be tested by employing combined cycles of varying magnitude of phase differences between light and temperature.

Summarising our results, we find that that when provided independently, temperature is more effective than light in enhancing chronotype differences but when together, the two zeitgebers interact antagonistically. Stepwise changing zeitgeber cycles mimicking twilight transitions of nature additionally contribute to enhanced phase divergence albeit by a small magnitude. Also, information of phase difference between the two zeitgebers serves as an essential cue for circadian clocks to mediate chronotype differences. Thus, our study sheds light on the importance of clock-zeitgeber interaction, and the role of different zeitgebers in nature to time behaviour and mediate chronotype divergence.

CHAPTER 4

Light sensitivity, amplitude and oscillator coupling differences in circadian clocks of *early* and *late* populations

<u>The contents of this chapter has been published as the following research article:</u>

KL Nikhil, KM Vaze, K Ratna and VK Sharma (2016) Circadian clock properties of fruit flies *Drosophila melanogaster* exhibiting early and late emergence chronotypes. *Chronobiology International.* (*In press*)

4.1 Introduction

Entrainment of circadian rhythms to environmental cycles (zeitgebers) is characterized by the establishment of a stable and reproducible phase relationship (ψ_{ent}) with the zeitgebers, and chronotypes are characterized by the variation in ψ_{ent} which is widely observed across a variety of species (Daan and Aschoff, 1975; Duffy et al. 2001; Rémi et al. 2010). Clock period (τ) is correlated with ψ_{ent} such that individuals with shorter τ exhibit an advanced ψ_{ent} ('early' chronotypes) and those with longer τ exhibit a delayed ψ_{ent} ('late' chronotypes) (Aschoff, 1965; Aschoff and Pohl, 1978; Wright et al. 2005; Rémi et al. 2010; Roenneberg, 2012). However, attempts to explain this association using classical models of entrainment (Aschoff, 1979; Pittendrigh, 1981b) have only been partially successful thus invoking a possible role of other clock properties. Furthermore, even though several physiological and behavioural differences have been found to be associated with morningness-eveningness (chronotypes) in humans (Dijk and Lockley, 2002), unlike the assessment of ψ_{ent} in other species which rely primarily on controlled experiments, the same in humans is highly variable due to a plethora of reasons, and thus correlations of chronotypes with other circadian clock properties are generally weak (reviewed in Levandovski et al. 2013) rendering them less reliable for the assessment of circadian regulation of ψ_{ent}.

Previously, laboratory selection and latitudinal cline studies have assessed strains of *Drosophila pseudoobscura* (Pittendrigh, 1967), *Pectinophora gossypiella* (Pittendrigh and Minis, 1971), *Drosophila auraria* (Pittendrigh and Takamura, 1987) and *Drosophila subobscura* (Lankinen, 1993) differing in ψ_{ent}. These studies have greatly motivated conceptualization of circadian clocks as a network of coupled oscillator (reviewed in Bell-Pederson et al. 2005), and extensive studies by Pittendrigh and co-

workers, and several others have been instrumental in exploring properties of circadian clocks associated with ψ_{ent} variation, some of which are discussed below.

Among the properties known to influence ψ_{ent} are the strength or amplitude of the zeitgeber (A_z) and intrinsic amplitude of the clock (A_o) (Pittendrigh 1981a; Pittendrigh et al. 1991; Vitaterna et al. 2006; Brown et al. 2008), such that higher A_z/A_o leads to larger phase shift incurred by the clock, and consequently a wider entrainment range. In other words, low amplitude circadian oscillators would have higher zeitgeber sensitivity. Additionally, magnitude of coupling between the constituent oscillators of circadian clock also influence ψ_{ent} (Aschoff, 1978; Pittendrigh, 1981a; Pittendrigh et al. 1991; Abraham et al. 2010; Granada et al. 2013). "Coupling" refers to the interactions between individual neurons or oscillators that form a network referred to as the circadian clock, and not to that between clock and zeitgeber, input or output pathways. Another clock property studied in this regard is the relaxation rate, which refers to the propensity of the clock or oscillator to converge to its intrinsic limit cycle amplitude (A_o) following perturbations. Oscillators with higher relaxation rates recover faster, and are therefore termed rigid, while those with lower relaxation rates take longer to relapse to their intrinsic limit cycle, and are termed weak oscillators (Granada and Herzel, 2009; Abraham et al. 2010; Granada et al. 2013). Furthermore, mathematical models based on generic Poincaré, Hopf and Becker-Weimann-Bernard oscillators proposed that relaxation rate differences among oscillators can influence amplitude, rates of re-entrainment, entrainment range and ψ_{ent} (Guckenheimer and Holmes, 1983; Becker-Weimann et al. 2004; Bernard et al. 2007; Abraham et al. 2010; Bordyugov et al. 2011; Granada et al. 2013).

In this study, we used fruit fly *D. melanogaster* populations which are products of a long term (over 14 years) laboratory selection study for morning (early) and evening (late) adult emergence (Kumar et al. 2007a) to explore the circadian clock properties underlying ψ_{ent}. In response to the selection imposed, *early* populations evolved shorter τ (of emergence rhythm) and advanced ψ_{ent}, while *late* populations evolved longer τ and delayed ψ_{ent}, and also shorter and longer τ of activity-rest rhythm respectively (Kumar et al. 2007a), implying that the diverged ψ_{ent} stems from a common central circadian clock governing the two rhythms. Based on studies under different photoperiods, we had reported that *early* and *late* populations might have evolved dominant morning (M) and evening (E) oscillators respectively (Kumar et al. 2007b). A closer look at the same data revealed that across photoperiods ranging from light/dark (LD) 08:16 to LD16:08, ψ_{ent} of *early* populations changed by 2 h, while that of *late* populations by 4 h (twice the magnitude) indicating phase sensitivity differences, as also substantiated by ~42% greater standard deviation in ψ_{ent} across photoperiods in *late* as compared to *early* populations. In another study, we observed that when T = 24 h, and therefore for a period mismatch of 0.4 h for *early* (τ = 23.6 h) and 0.2 h for *late* (τ = 24.2 h) populations (Kumar et al. 2007a), the difference in ψ_{ent} between the two varied considerably from 1.71 h to 5.8 h depending on zeitgeber conditions (Nikhil et al. 2014). Even though it is well-known that small variation in τ can lead to large variation in ψ_{ent} (Ralph and Menaker, 1998; Merrow et al. 1999; Ouyang et al. 1999), high phase sensitivity and large variation in the difference of ψ_{ent} between populations for same τ–T mismatch was intriguing, and suggested differential entrainment, as also highlighted by Vaze et al (2012a). Therefore, in light of the propositions by Pittendrigh (1981a) regarding association of clock properties with ψ_{ent} variation, and that from the above discussed observations by Kumar et al (2007b), Vaze et al (2012a) and Nikhil et al (2014), we

hypothesized that zeitgeber sensitivity and amplitude of circadian oscillators might be associated with ψ_{ent} differences between *early* and *late* populations, in addition to differences in τ.

In this regard, we initially tested if *early* and *late* populations have evolved differences in light sensitivity (the populations were selected under LD12:12 at constant temperature and humidity, rendering light as the only zeitgeber) by assaying activity-rest rhythm under constant light (LL), and further explored the possible involvement of other above mentioned clock properties as well, the results of which will be discussed later.

4.2 Materials and methods

(a) *Experimental* populations: Details of experimental populations and maintenance protocol are provided in chapter 2.

(b) *Activity-rest rhythm assay*: The basic protocol for activity-rest rhythm assay is described in chapter 2.

***Recording in LL*:** Recording in LL was performed under light intensities of 4×10^{-3} Wm^{-2} and 4×10^{-4} Wm^{-2}. Flies were initially subjected to 4 days of LD12:12 with light intensity during the day same as that under the LL regime, and then recorded in LL for 12 days. Block wise percentage of flies exhibiting free-running rhythm (presence of a single statistically significant period), complex rhythm (presence of two or more statistically significant periods) and arrythmicity (absence of any statistically significant period) in LL were then calculated. Statistical analyses of percentage values were implemented using a randomised block design mixed model Analysis of Variance (ANOVA) with 'population' as fixed and 'block' as random factors following Shapiro Wilk test (Shapiro and Wilk, 1965) for normality. Unless otherwise specified, block wise averages for all measures used henceforth were analysed by ANOVA with

population and light regime or phase (whichever applicable) as fixed factors and block as random factor. Post hoc multiple comparisons were performed using Tukey's HSD method (Tukey, 1949) at α of 0.05 in Statistica (StatSoft, USA).

***Recording in T-cycles*:** Since pre-adult rearing and virgin collection was in LD12:12, flies were initially exposed to *T*-cycles (*T*-18: 9 h light and 9 h darkness; *T*-20, *T*-28, and *T*-30, with light intensity during the light phase being 4×10^{-4} Wm^{-2}) for 7 days, loaded into activity tubes, and recorded for 7 cycles in the respective *T*-cycle followed by 5 days in constant darkness (DD) to assess phase control during entrainment. Activity-rest behaviour in *T*-cycles was categorized as **1) free-run**: if neither morning nor evening activity components were phase locked to the LD cycles, **2) weakly entrained**: if one of the two components was phase locked to the LD cycles while the other exhibited free-run and **3) entrained**: if both activity components were phase locked to the LD cycles (Figure 1). For individuals that did not survive till recording in DD, phases of activity onsets and offsets were assessed, and the rhythm was considered entrained only if phase markers were either advanced or delayed with respect to ZT00. Flies with activity onsets coinciding with ZT00 were not considered as it might be masked to light. The percentage of free-running, weakly entrained and entrained flies were not found to qualify the assumptions of ANOVA, and hence were analysed by the non-parametric Kruskal Wallis test (Kruskal and Wallis, 1952) followed by post hoc multiple comparisons using 'multiple comparisons of mean ranks for all groups' test. This test is based on the Mann Whitney test and is detailed in Siegel and Castellan (1988). All the above mentioned tests were implemented in Statistica (Statsoft, USA).

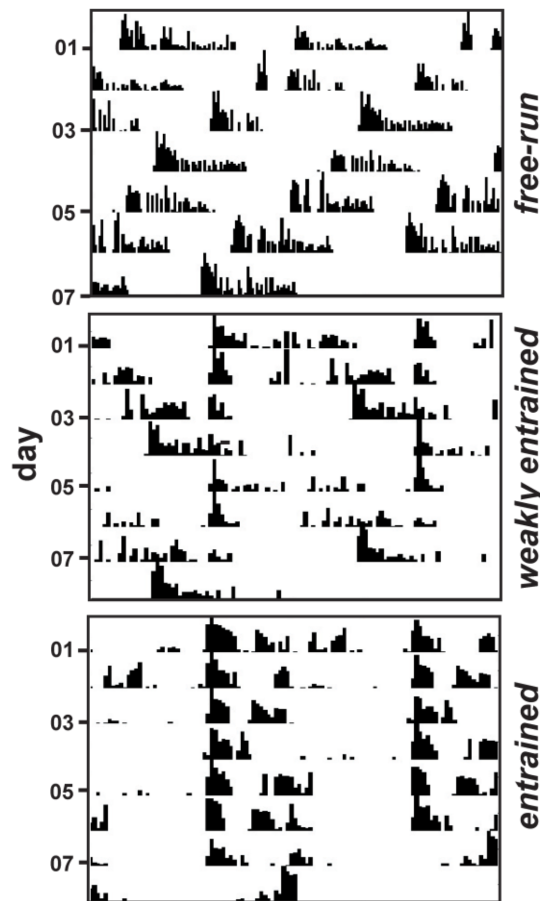


Figure 1: Representative actograms of flies that exhibit free-run (neither morning nor evening activity components phase locked to the LD cycle) in *T*-30, weakly entrained (one of the two components phase locked to the LD cycles while the other exhibited free-run) in *T*-18, and entrained (both activity components phase locked to the LD cycle) rhythm in *T*-18.

(c) *Adult emergence rhythm assay*: The basic protocol for adult emergence rhythm assay is described in chapter 2. Briefly, adult emergence assay was performed in LD12:12 in high (4×10^{-1} Wm^{-2}) and low (4×10^{-4} Wm^{-2}) light intensities in 10 replicate vials (~300 eggs per vial) for every block. Following egg collection, vials were transferred to respective light regimes for development, and upon initiation of emergence, numbers of flies emerging from every vial was recorded at 2 h intervals for

5 consecutive days. Only those vials that exhibited rhythmic emergence (with a minimum of 15 flies in a day) for at least 3 consecutive days were considered, and the number of flies at each time point was averaged over multiple days independently for each block. Due to differences in modality and gate width of emergence (Kumar et al. 2007a) between populations, a single reference point could not be used to measure the rhythm amplitude. Instead, the net difference in number of flies emerging across the day between the two regimes (*number of flies at low – high light intensities*) was used as a measure.

(d) *Assessing rhythm amplitude*: Due to lack of a standard procedure to assess the intrinsic amplitude of the circadian clock directly, we measured the amplitude of activity-rest rhythm as a proxy.

Amplitude in LD (entrained amplitude: A_{ent}): The activity profile of *D. melanogaster* in LD12:12 is bimodal comprising morning (M) and evening (E) activity peaks. To estimate amplitude of entrainment (A_{ent}), activity-rest data for at least 7 days recorded under LD12:12 (light intensity = 4×10^{-4} Wm^{-2}) was used to plot activity profile at 1 h bins (activity counts/h) using Microsoft Excel (Microsoft, USA). From this profile, total activity in the morning (ZT22-02) and evening (ZT10-14) was calculated and served as the amplitude of morning and evening peaks respectively.

Amplitude in DD (Intrinsic amplitude: A_o): Data for activity-rest rhythm recorded in DD for 10 days was binned into 1 h intervals, and used to plot the activity profile as activity counts/h using Table Curve 2D (Systat Software Inc., USA). From the profiles thus obtained, the highest activity count/h was identified and used to calculate activity count/min. Since activity/min values were obtained from data binned at 1 h intervals, highest value indicates that the individual exhibited highest activity for the day in that 1

h duration representing the peak of activity, and thus served as a measure of intrinsic amplitude (A_o). The day wise highest activity count/min values were first averaged across days for a given individual, and then across replicate individuals of a given block. We observed dramatic reduction in amplitude for the first 3 days following transfer from LD (in which the flies were initially reared) to DD, and therefore data from the first three days in DD was excluded from analysis. Normalized actograms (modulo τ), were used to estimate activity onsets and offsets using ClockLab (Actimetrics, USA) from which the total activity during the subjective day and night was calculated.

(e) *Re-entrainment to phase shifted LD cycles*: Activity-rest was recorded in LD12:12 for the first 5 days following which the flies were subjected to either a 9 h advance or 9 h delay on day 6, and recorded for next 10 days. The days taken to re-entrain (transients) were estimated by a method similar to that in Sharma and Daan (2002). The phases of activity offsets from day 6 were regressed over the next 10 days to estimate the slope and R^2 of the regressed line. R^2, also known as the coefficient of determination, is a measure of goodness of fit of the regressed line to the data, and indicates percentage variation in the data that can be explained by the regression line. For instance, $R^2 = 0.9$ indicates that 90% of variability in the data can be explained by the regressed line, and therefore higher R^2 value would indicate better fit. After the first regression, data from day 6 was removed and those from day 7 were regressed over next 9 days, followed by 8 days and so on. This was continued till slope and R^2 values of the regressed line were closest to zero (complete lack of regression) indicating steady state attainment, and therefore complete re-entrainment to new LD cycles. The number of days taken to reach steady state was considered as transients.

Since re-entrainment rate is known to be a function of the phase of the circadian rhythm (limit cycle) at the time of phase shift (Granada and Herzel, 2009), we estimated

block wise re-entrainment rates as follows: ($\psi_{before\text{-}shift} - \psi_{after\text{-}shift}$)/transients, and the block means were then log transformed and analysed by ANOVA.

(f) *Estimating photic dose response curve (DRC)*: The activity of 3 day old flies was recorded in LD12:12 (light intensity = 16×10^{-2} Wm^{-2}) for 6 days and transferred to DD on day 7. During the first cycle in DD, different sets of flies were subjected to 5 min light pulses of intensities 0, 4×10^{-3}, 4×10^{-2}, 4×10^{-1} or 4 Wm^{-2} (0-1000 lux) at Circadian Time (CT) 14 and CT22 which represent the phases of maximum phase delay and phase advance respectively (Dunlap et al. 2004). Recording was then continued in DD for the next 12 days. During exposure to light pulse, the activity monitors had to be displaced from the recording incubator to the light chamber. Therefore, to account for phase shifts due to physical disturbance during light pulse, additional sets of ‘disturbance controls’ were maintained, and each set was only physically disturbed at the respective phases similar to the experimental flies but were not exposed to light. Activity offset of each cycle was marked, a regression line drawn through the activity offsets for days 1-6, and extrapolated to predict the phase of offset on day 7 (day of light pulse). Similarly, another regression line was drawn through offsets from days 10-16 and extrapolated backwards to identify the phase of offset on day 7. The time difference between the phases extrapolated from both the regression lines indicates the magnitude of phase shift. All ‘disturbance controls’ were also analysed in the same way, and the phase shift differences between the experimental and disturbance controls were considered as the phase shift due to light pulse alone. This procedure was implemented on data from each fly and averaged across flies of a given block to obtain block average.

Since bright light saturates *Drosophila* photoreceptors (Juusola and Hardie, 2001), we reasoned that the light intensity required to elicit maximum phase shift might not serve as a reliable measure of photosensitivity, and thus IPS50 was chosen, however,

one may also choose IPS25 or IPS75 which represents the first and third quartiles of the phase shift distribution. To estimate the light intensity eliciting 50% of maximum phase shift (IPS50 - median of the underlying phase shift distribution), the maximal phase shift value for a given block was set to 1, and all other lower phase shift values for that block were expressed as the proportion of the maximum phase shift between 0-100% with 100% being the maximum phase shift. A nonlinear dose response equation of the form Y = 100/(1+10^((LogEC50 – X)*hill – slope)) was fit to the phase shift data using simple least squares method in Prism 5 (GraphPad, USA) with phase shift and log (light intensity) as ordinate and abscissa respectively. From this plot, the abscissa corresponding to the ordinate value of 50% of maximal phase shift was estimated and served as the IPS50. This procedure was implemented separately for each of the 4 blocks at two phases (CT14 and CT22). Details of hill slope calculations can be found in Prism 5.

(g) *Estimating area under photic phase response curve (PRC)*: Flies were recorded for 6 days in LD12:12 and on the first day in DD (day 7), they were subjected to a brief 5 min light pulse of intensity 16×10^{-2} $Wm^{-2}s^{-1}$ (70 lux) every 4 h starting at CT02, after which they were recorded in DD for 12 days. Phase shift estimation for PRC analysis was same as that used for DRC.

Several nonlinear polynomials were fit to the phase shift data using TableCurve (Systat, USA), and from the list of polynomials thus obtained, the polynomial with a combination of least number of coefficients and highest R^2 (explained earlier) was considered (Table 1), and one such polynomial was obtained for every block. Since we were interested in estimating the area under the curve (AUC) and the experimental data was obtained at 4 h intervals which is a poor resolution to calculate AUC, we used the best fit polynomial to obtain predicted phase shifts at intermediate CT with a resolution

of 0.04 h by interpolation. In other words, from the best fit polynomial of the form $y = f(x)$ we calculated the ordinate (y = phase shift) for every increase in abscissa (x = CT) by 0.04 h, and the interpolated phase shift values were then used to estimate AUC by simple integral under the curve method. To ensure that the polynomials accurately reflected the experimental data, we estimated the sum of squares (SS) values between the phase shifts values obtained from the experiment at every 4 h intervals with those of the interpolated data from the polynomial. We found that the SS values were very low and did not differ significantly from 0 (*t*-test at $\alpha = 0.01$; Figure 2) suggesting that the polynomials effectively represented the experimental data. Block wise AUC values thus obtained were subjected to ANOVA and post hoc comparisons.

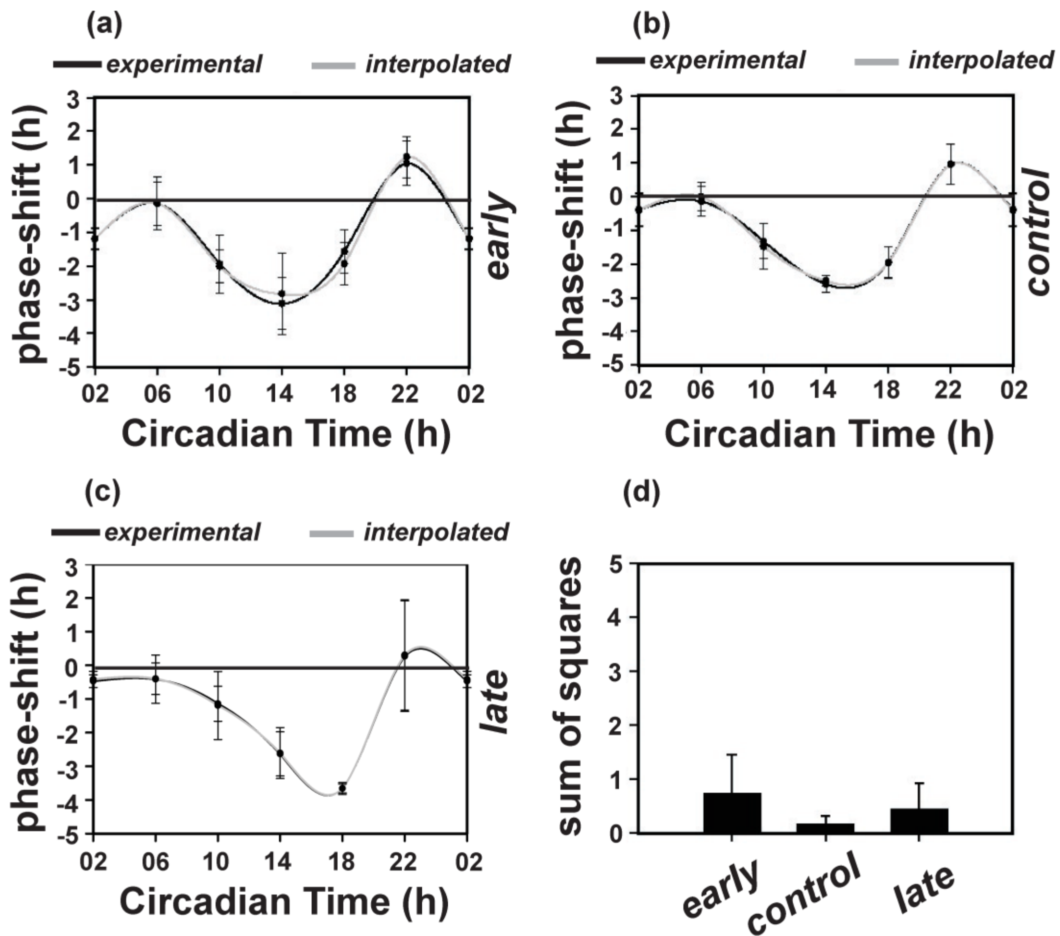


Figure 2: (a)-(c) represent the PRCs plotted from experimental (black line) and interpolated (grey line) data from polynomials used to estimate area under the curve for the (a) *early* (b) *control* and (c) *late* populations. (d) Sum of squares values indicating the goodness of fit between the polynomial and experimental data. All sum of square values reported here did not differ significantly from 0 thus indicating the polynomials used for AUC analysis reflects the experimental data accurately.

Table 1 (next page): Details of polynomials obtained by curve fitting to experimental PRC data. The selection of polynomials was based on highest R^2 values indicating goodness of fit and least number of coefficients.

		block 1		block 2		block 3		block 4
early	$y=a+bx+cx^{2.5}+dx^3+ee^x$		$y=a+bx+cx^3+de^x+e/x^2$		$y=a+bx^2+cx^4+dx^6+ex^8+fx^{10}$		$y=a+bx+cx^3+de^x+e\ln x/x^2$	
	R^2	0.955317674	R^2	0.837365931	R^2	0.999277944	R^2	0.936109756
	a	-8.11602602	a	9.163518506	a	-2.38160908	a	2.351191599
	b	9.851402678	b	-4.38186548	b	1.373449817	b	-3.42333506
	c	-3.98625742	c	0.122330037	c	-0.24467596	c	0.113796245
	d	1.502223689	d	-0.01997385	d	0.014092844	d	-0.017344
	e	-0.0548885	e	-6.46107614	e	-0.000321	e	16.31920159
					f	2.53E-06		
control	$y=a+bx^2+cx^4+dx^6+ex^8+fx^{10}$		$y=a+bx+cx^2+dx^3+ex^4+fx^5$		$y=a+bx+cx^{2.5}+de^x+e/x^2$		$y=a+bx+cx^{2\ln x}+dx^3+ee^x$	
	R^2	0.977749981	R^2	0.987934691	R^2	0.983689312	R^2	0.975602135
	a	-1.43497958	a	11.32164822	a	14.55127251	a	-1.99351764
	b	0.620452214	b	-24.689396	b	-7.10829061	b	1.050497192
	c	-0.09867596	c	18.65207431	c	0.390503898	c	-1.71092602
	d	0.004373099	d	-6.30150412	d	-0.01385338	d	0.54198498
	e	-6.61E-05	e	0.94927054	e	-7.73199016	e	-0.02615298
	f	2.61E-07	f	-0.05178496				
late	$y=a+bx+cx^2+dx^3+ex^4+fx^5$		$y=a+bx+cx^2+dx^3+ex^4+fx^5$		$y=a+bx^2+cx^4+dx^6+ex^8$		$y=a+bx+cx^3+d(\ln x)^2+e/x^2$	
	R^2	0.999950352	R^2	0.96120615	R^2	0.905906231	R^2	0.998725481
	a	2.418326646	a	-12.9238362	a	0.722655957	a	232.197012
	b	-4.43362575	b	17.8179283	b	-0.45664792	b	-153.013914
	c	2.382178875	c	-9.84501063	c	0.035403632	c	0.394368112
	d	-0.49683413	d	2.738486331	d	-0.00123564	d	186.5907559
	e	0.022850698	e	-0.38102054	e	1.43E-05	e	-7.83E+01
	f	0.00162637	f	0.020441504				

4.3 Results

(a) *late populations exhibit higher arrythmicity under dim constant light (LL)*

To test if *early* and *late* populations evolved differential light sensitivity, we assayed activity-rest behaviour under dim LL which is known to render the behaviour arrhythmic (Konopka et al. 1989).

Under high light intensity (4×10^{-3} Wm^{-2}), majority of the individuals in all populations were arrhythmic (*early* = 78.29%, *control* = 90.44%, *late* = 57.36%; Figure 3a) and did not differ significantly from each other ($F_{2,6} = 4.97$, $p > 0.05$). Percentage of individuals exhibiting either complex (*early* = 7.84%, *control* = 5.83%, *late* = 20.30%; $F_{2,6} = 1.81$, $p > 0.05$; Figure 3a) or free-running rhythm (*early* = 13.85%, *control* = 3.72%, *late* = 22.32%; $F_{2,6} = 2.62$, $p > 0.05$; Figure 3a) did not differ across populations either. This prompted us to assay the activity-rest rhythm in low light intensity (4×10^{-4} Wm^{-2}).

Under dim LL of 4×10^{-4} Wm^{-2}, significantly higher percentage of flies from *late* populations (58.48%) were arrhythmic as compared to *early* (24.98%) and *control* (21.83%) populations ($F_{2,6} = 22.34$, $p < 0.001$; Figure 3b), while the latter two did not differ from each other. Percentage of individuals exhibiting complex rhythm did not differ significantly across *early* (42.22%), *control* (43.18%), and *late* (33.46%) populations ($F_{2,6} = 1.19$, $p > 0.05$; Figure 3b), but a significantly lower percentage of *late* populations (8.05%) exhibited free-running rhythm as compared to *early* (32.75%) and *control* (34.98%; $F_{2,6} = 74.82$, $p < 0.01$; Figure 3b), while the latter two did not differ from each other.

Interestingly, proportion of flies from *late* populations exhibiting arrhythmic behaviour did not differ across the two light intensities but that for *early* and *control* increased

significantly at high light intensity. This suggests that besides enhanced light sensitivity *late* populations might have also evolved an oscillator network marked by weak coupling, and will be discussed in detail later.

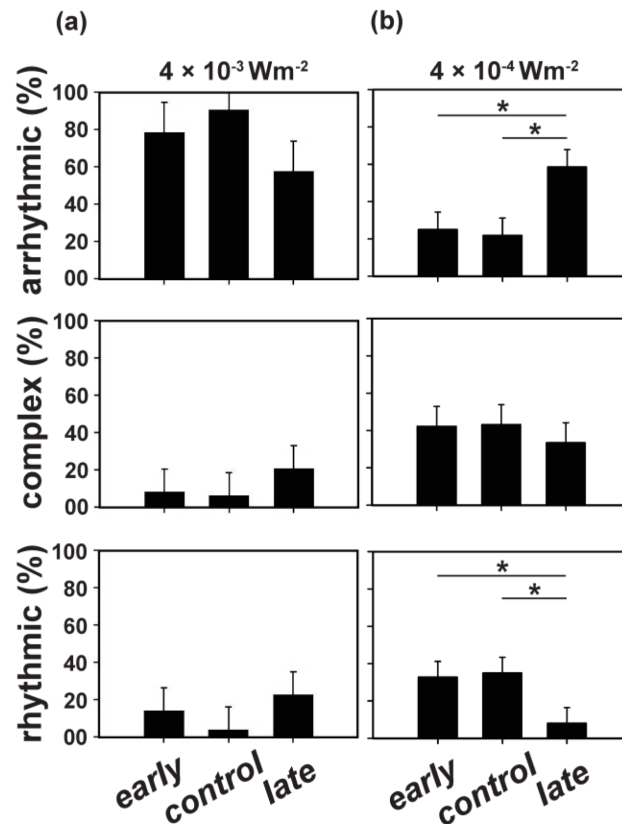


Figure 3: Percentage of *early*, *control* and *late* flies exhibiting arrhythmic (row 1), complex (row 2), and rhythmic (row 3) activity-rest behaviour under constant light (LL) of high (4×10^{-3} Wm^{-2}; left) and low (4×10^{-4} Wm^{-2}; right) light intensities. The actograms were analysed by Chi-square periodogram and the behaviour was categorized as follows. Free-running rhythm: presence of a single statistically significant period; complex rhythm: presence of two or more statistically significant periods and arrythmicity: absence of any statistically significant period). Error bars represent 95% CI with asterisks indicating significant differences between populations ($p < 0.05$). A significantly higher proportion of *late* populations exhibit behavioural arrythmicity under dim LL suggesting that they might have evolved higher light sensitivity.

(b) ***late* populations exhibit high amplitude of entrainment (A_{ent})**

To further confirm if *late* populations indeed exhibit enhanced light sensitivity, we also assessed activity-rest rhythm in LD12:12, the rationale being that higher light sensitivity would promote robust entrainment with higher amplitude and power of rhythm.

ANOVA on activity/h revealed a statistically significant effect of 'population × phase' interaction ($F_{46,138} = 11.74$, $p < 0.01$), and activity levels of *late* populations was significantly higher at most phases (Figure 4a). As an additional measure of A_{ent} we calculated total activity around the M and E peaks as these are known to be clock controlled (Stoleru et al. 2004). M activity was significantly higher for *late* populations as compared to the other two populations ($F_{2,6} = 9.65$, $p < 0.05$) while the latter two did not differ (Figure 4a, b). Activity around the E peak was also significantly higher for *late* populations ($F_{2,6} = 5.89$, $p < 0.05$) as compared to *early* populations (Figure 4a, b). We also calculated the ratio of daytime/night time activity and found it to be significantly higher for *late* (2.15) populations as compared to that for *early* (1.72) and *control* (1.93) populations ($F_{2,6} = 20$, $p < 0.01$; Figure 4c). Further, power of the rhythm as estimated by amplitude of the Chi-square periodogram was also significantly higher for *late* (211.64) populations followed by *control* (202.76) and *early* (183.34) populations ($F_{2,6} = 5.89$, $p < 0.05$; Figure 4d).

These indicators highlight a relatively robust entrainment in *late* populations even under low intensity LD cycles further suggesting that these populations might have evolved higher light sensitivity.

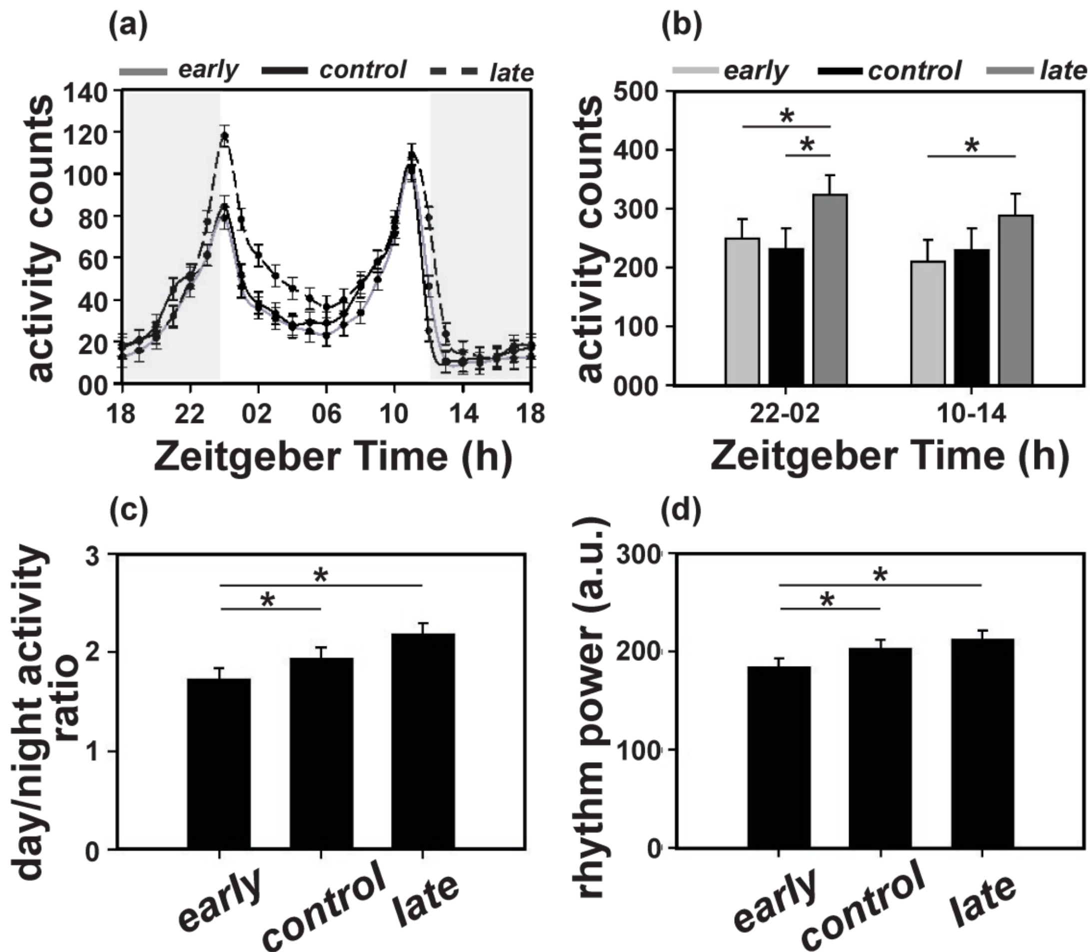


Figure 4: (a) Activity profile expressed as activity counts/h for *early*, *control,* and *late* populations under 12:12 h light/dark (LD12:12) cycles. The shaded region represents night phase while the unshaded region represents day. (b) Total activity around the morning (ZT22-02) and evening (ZT10-14) activity peaks for all three populations obtained by summing activity counts across the respective windows which span the morning and evening peaks respectively. (c) Ratio of total activity during the day (ZT00-12) to that during the night (ZT12-00) for all three populations under LD12:12. (d) Amplitude of Chi-square periodogram of activity-rest rhythm for all three populations recorded under LD12:12. Light intensity during the day for all the experiments was 4×10^{-4} Wm^{-2}. Error bars represent 95% CI with asterisks indicating significant differences between populations ($p < 0.05$). *late* populations exhibit significantly higher amplitude and robust entrainment even under very low light intensity.

(c) ***Emergence rhythm in late populations is highly light sensitive***

We further wished to test if the observed effects are restricted to activity-rest rhythm alone or if it is manifested in emergence rhythm as well. Observing similar effects in two independent rhythms would strengthen the idea that such differences might stem from a common central oscillator governing both the rhythms, and therefore we assayed adult emergence of all populations under high and low light intensity LD12:12. We decided to use LD cycles instead of LL because emergence rhythm being a population rhythm, it is not meaningful to analyse incidence of arrhythmic and complex phenotypes unlike activity rhythm which is studied at the individual level.

ANOVA on emergence data revealed a statistically significant effect of 'phase × light intensity' interaction for all three populations (*early:* $F_{11,33} = 6.85$, $p < 0.01$; *control*: $F_{11,33} = 16.22$, $p < 0.01$; *late*: $F_{11,33} = 15.81$, $p < 0.01$). Interestingly, amplitude of emergence in *early* and *control* populations decreased with reduction in light intensity while that for *late* populations increased significantly ($F_{2,6} = 8.51$, $p < 0.05$; Figure 5a, b).

In addition, Anticipation Index (AI) for *late* populations increased by 0.33 units with decreasing light intensity, significantly higher than *early* (0.0002) populations which exhibited almost no response while *control* populations responded intermediately (0.10) (Figure 5c).

Similar trends in the differences between population for activity as well as emergence rhythms indicate that the observed phenotypes stem from components pertaining to a common central clock. Therefore, we restricted further studies to only the activity-rest rhythm.

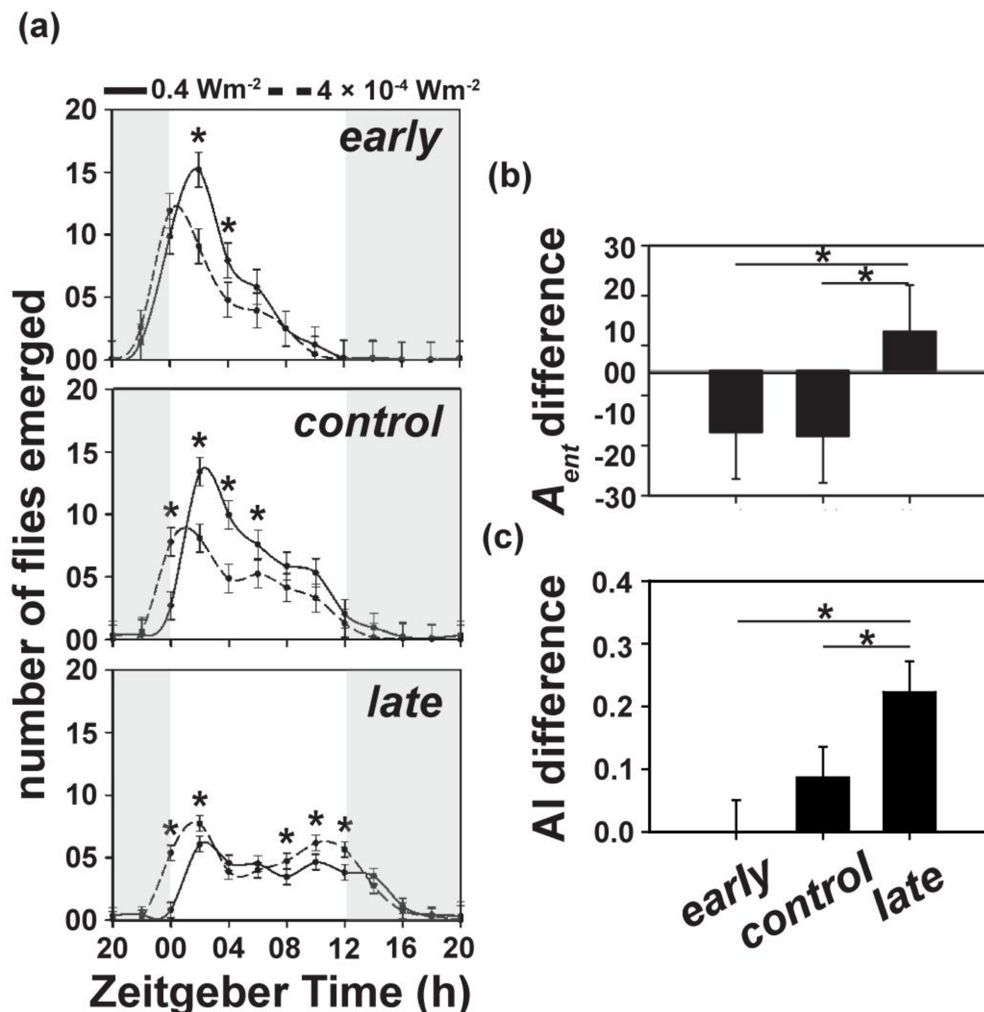


Figure 5: (a) Adult emergence profile of *early, control,* and *late* populations depicting number of flies emerging from the pupal case across different times of the day (Zeitgeber Time) under two different 12:12 h light/dark (LD12:12) cycles with high (4×10^{-3} Wm^{-2}) and low (4×10^{-4} Wm^{-2}) light intensities during the day. (b) Difference in entrainment amplitude (A_{ent}) calculated as *number of flies at low – high light intensities* and (c) anticipation index (AI) calculated as (AI at low light intensity – AI at high light intensity) of emergence under high and low light intensity LD12:12 for the three populations. AI was calculated as the ratio 'number of flies emerged 2 h prior to ZT00/number of flies emerged 4 h before lights-ON'. Error bars represent 95% CI with asterisks indicating significant differences between populations ($p < 0.05$). *late* populations exhibit significantly higher responses to changes in light intensity as can be observed in A_{ent} and AI differences.

(d) ***late populations exhibit high intrinsic amplitude*** **(A_o)**

We also reasoned that higher amplitude of entrainment in *late* populations might not necessarily be due to higher light sensitivity but also due to higher intrinsic clock amplitude (A_o). Additionally, previous studies (see introduction) have also reported that the ψ_{ent} is correlated with A_o. To test for these possibilities we estimated the amplitude of free-running rhythm (A_o) in all the three populations.

A_o was found to be significantly higher for *late* populations (3.02 activity counts/min) as compared to *early* (2.42 activity counts/min) and *control* (2.41 activity counts/min) populations ($F_{2,6} = 24.81$, $p < 0.01$; Figure 6a). Also, the ratio of activity in the subjective day to that of the subjective night was significantly higher in *late* (5.91) populations as opposed to that for *early* (4.05) and *control* (3.77) populations ($F_{2,6} = 13.06$, $p < 0.01$; Figure 6b), while *early* and *control* populations did not differ between each other for both the measures (Figure 6a, b). Taken together, these indicators suggest that *late* populations have evolved high amplitude circadian oscillations.

We further calculated difference in amplitudes of entrained and free-running rhythm (amplitude expansion = $A_{ent} - A_o$). Amplitude expansion can be influenced by both light sensitivity and nature of inter oscillator coupling, and therefore might reveal interesting properties of clocks in these populations.

To facilitate comparison of amplitudes of entrained and free-running rhythm, we computed the activity/min values in LD similar to that in DD. As expected, activity/min was found to be highest at the two phases corresponding to the M and E peaks. Concordant with earlier results, ANOVA on average activity/min in LD revealed a statistically significant effect of population ($F_{1,3} = 31.9$, $p < 0.001$) but not of ‘peak’ or ‘population × peak’ interaction indicating that the M and E peaks did not differ for a given population, and therefore, we averaged the activity/min values across M and E

peaks (Figure 7). The averaged values were then considered as measures of amplitude under LD to calculate amplitude expansion.

While there was a trend of increase in amplitude expansion from *early* to *late,* ANOVA on 'amplitude expansion' values did not reveal any significant effect of population ($F_{2,6} = 2.88$, $p = 0.13$) suggesting that the three populations exhibit similar amplitude expansion (*early* = 2.44 activity/min; *control* = 2.67 activity/min; *late* = 2.77 activity/min; Figure 6c). Therefore, it appears that high amplitude of entrainment in *late* populations is probably driven by high A_o and not necessarily higher light sensitivity.

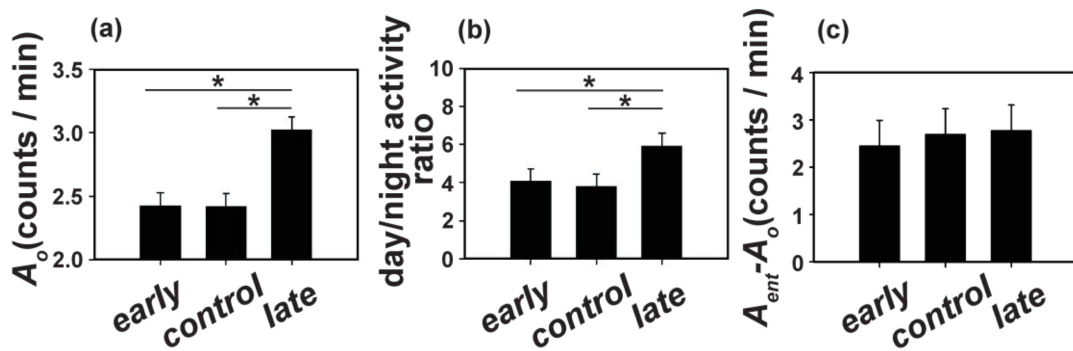


Figure 6: (a) Intrinsic amplitude (A_o) of *early*, *control,* and *late* populations estimated as the highest activity counts/min from 1 h binned average activity profiles across 7 days. (b) Ratio of activity during the subjective day to that of the subjective night used as an additional measure of amplitude of the rhythm. (c) Magnitude of amplitude expansion calculated as the difference between A_{ent} and A_o of activity-rest rhythm for all three populations. Error bars represent 95% CI with asterisks indicating significant differences between populations ($p < 0.05$). *late* populations exhibit significantly higher amplitude of free-running rhythm but did not differ in magnitude of amplitude expansion.

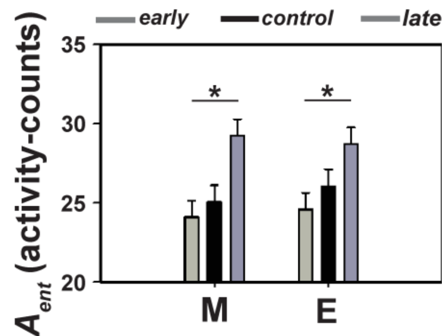


Figure 7: Amplitude of entrainment (A_{ent}) estimated as the sum of activity counts around the morning (M) and evening (E) peaks averaged across 7 days for all three sets of populations under LD12:12 with 4×10^{-4} Wm^{-2} light intensity during the day. *late* populations exhibited significantly higher A_{ent} for both M and E peaks as compared to the other populations while the M and E peaks did not differ significantly from each other for a given population. Asterisks indicate statistically significant differences ($p < 0.05$).

(e) ***late populations exhibit reduced rate of re-entrainment***

A_o is inversely related to the phase resetting ability of the clock (see introduction), and therefore we tested this proposition by estimating the re-entrainment rates of all populations to 9 h phase advance or delay in LD cycles with the rationale that higher A_o in *late* populations would reduce their re-entrainment rate.

ANOVA on re-entrainment rates revealed a statistically significant effect of population for both advance ($F_{2,6} = 10.03$, $p < 0.05$) and delay ($F_{2,6} = 13.03$, $p < 0.01$) phase shifts. The re-entrainment rate of *late* populations was significantly lower for both advance (2.5 h/day) and delay phase shifts (2.5 h/day) as compared to those for *early* (advance = 4.50 h/day; delay = 6.71 h/day) and *control* (advance = 2.80 h/day; delay = 6.30 h/day) populations (Figures 8, 9).

Had *late* populations evolved enhanced light sensitivity as suggested by some of the earlier results, they would be expected to exhibit high re-entrainment rate as well, which is contrary to what we observed. Therefore, it appears that reduced rates of re-entrainment in *late* populations might be driven by their reduced ability to undergo large phase shifts due to high A_o value.

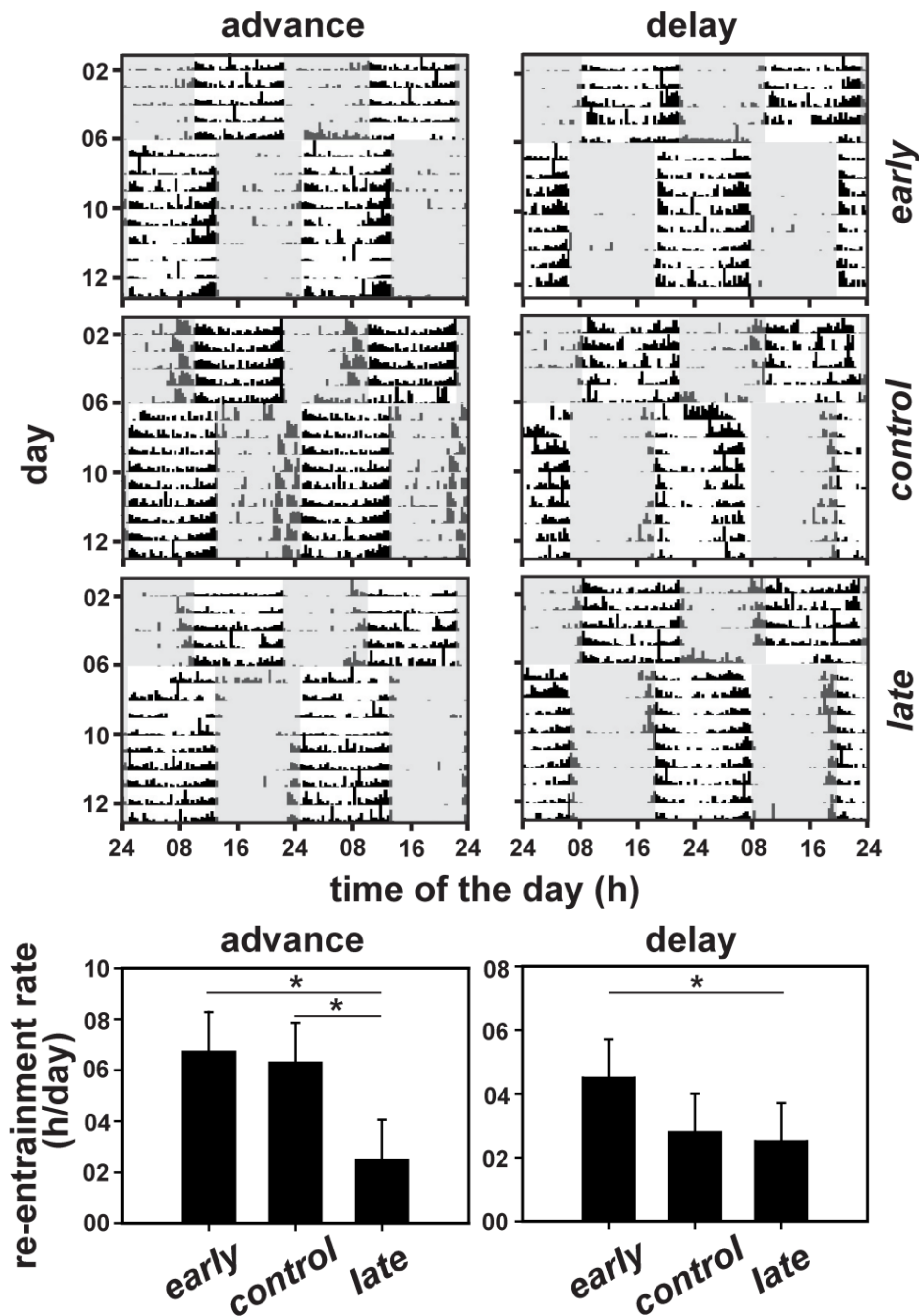


Figure 8: Representative actograms depicting re-entrainment of *early*, *control* and *late* populations to 9 h phase advanced (column 1) and delayed (column 2) 12:12 h light/dark (LD12:12) cycles. The shaded regions represent night phase. Bottom panel depicts the re-entrainment rates to 9 h phase advanced (left) and delayed (right) LD12:12 cycles. Re-entrainment rates were calculated as '9 h (magnitude of phase shift)/number of transients. Error bars represent 95% CI with asterisks indicating significant differences between populations ($p < 0.05$). The figures indicate that *late* populations take significantly longer to re-entrain to both advance and delay phase shifts.

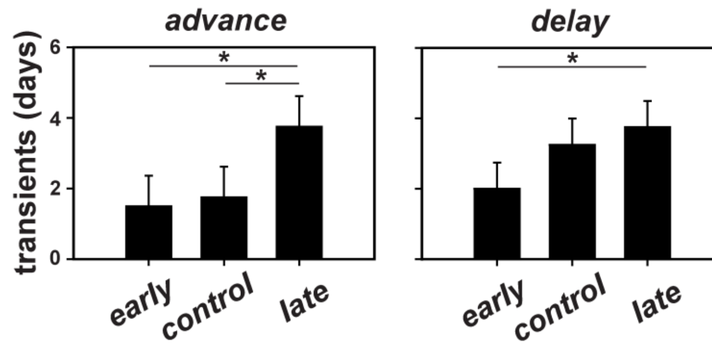


Figure 9: Number of days (transients) required for the three sets of populations to re-entrain to 9 h phase advance and 9 h phase delay in light/dark12:12 cycles. ANOVA on transients revealed a significant effect of population for both advance ($F_{2,6} = 9.52$, $p < 0.05$) and delay ($F_{2,6} = 6.88$, $p < 0.05$). In both cases *late* populations took significantly longer to re-entrain as compared to *early* populations while it significantly differed from *control* for phase advance and not phase delay. Asterisks indicate statistically significant differences ($p < 0.05$).

(f) ***late populations exhibit wider entrainment range***

Additionally, we also assessed the entrainment range to test if high A_o in *late* populations would curb its phase resetting ability, and consequently, restrict their entrainability to non 24 h *T*-cycles.

In *T*-18, a significantly higher percentage of *early* (59.85%) populations failed to entrain compared to *late* (20.15%) (Kruskal Wallis $H = 8$, $df = 2$, $p < 0.05$) whereas *control* (30.08%) populations did not differ from either (Figure 10-row 1). The percentage of individuals exhibiting entrainment was almost negligible while significantly higher percentage (77.57%) of *late* populations weakly entrained as opposed to 37.86% in *early* and 69.82% in *control* populations (Figure 10-row 1).

In *T*-20, the percentage of individuals which failed to entrain (free-running) was low (*early* = 8.25%, *control* = 29.33%, *late* = 10.01%; Figure 10-row 2) and the

populations did not differ significantly (Kruskal Wallis $H = 2.59$, $df = 2$, $p > 0.05$), and the same was observed for weakly entrained individuals as well (Figure 10-row 2).

The percentage of flies exhibiting free-running rhythm in *T*-28 was 27.30% for *early*, 29.26% for *control,* and 11.95% for *late* populations (Figure 10-row 3). A higher percentage of flies weakly entrained (*early* = 55.00%, *control* = 59.48%, *late* = 66.51%) as compared to those that entrained (*early* = 17.70%, *control* = 11.25%, *late* = 21.53%; Figure 10-row 3), while the populations did not differ in the percentage of flies exhibiting free-run (Kruskal Wallis $H = 0.58$, $df = 2$, $p > 0.05$), weak (Kruskal Wallis $H = 1.65$, $df = 2$, $p > 0.05$) or complete entrainment (Kruskal Wallis $H = 1.82$, $df = 2$, $p > 0.05$; Figure 10-row 3).

When assayed under *T*-30, a significantly higher proportion of *early* (70.70%) populations failed to entrain as compared to 15.29% of *late* (Kruskal Wallis $H = 6.59$, $df = 2$, $p < 0.05$) whereas close to half of *control* (48.02%) populations failed to entrain but did not significantly differ from the other two (Figure 10-row 4). The percentage of individuals showing weak entrainment did not differ across populations (*early* = 25.96%, *control* = 45.34%, *late* = 56.25%; Figure 10-row 4), but those that entrained was significantly higher for *late* (28.44%) populations as compared to *early* (3.33%) and *control* (6.62%) populations (Figure 10-row 4).

Surprisingly, contrary to our expectation of reduced entrainment range in *late* populations, while both *control* and *late* populations entrained to all *T*-cycles, *late* populations consistently showed higher incidence of entrainment probably driven by high zeitgeber sensitivity.

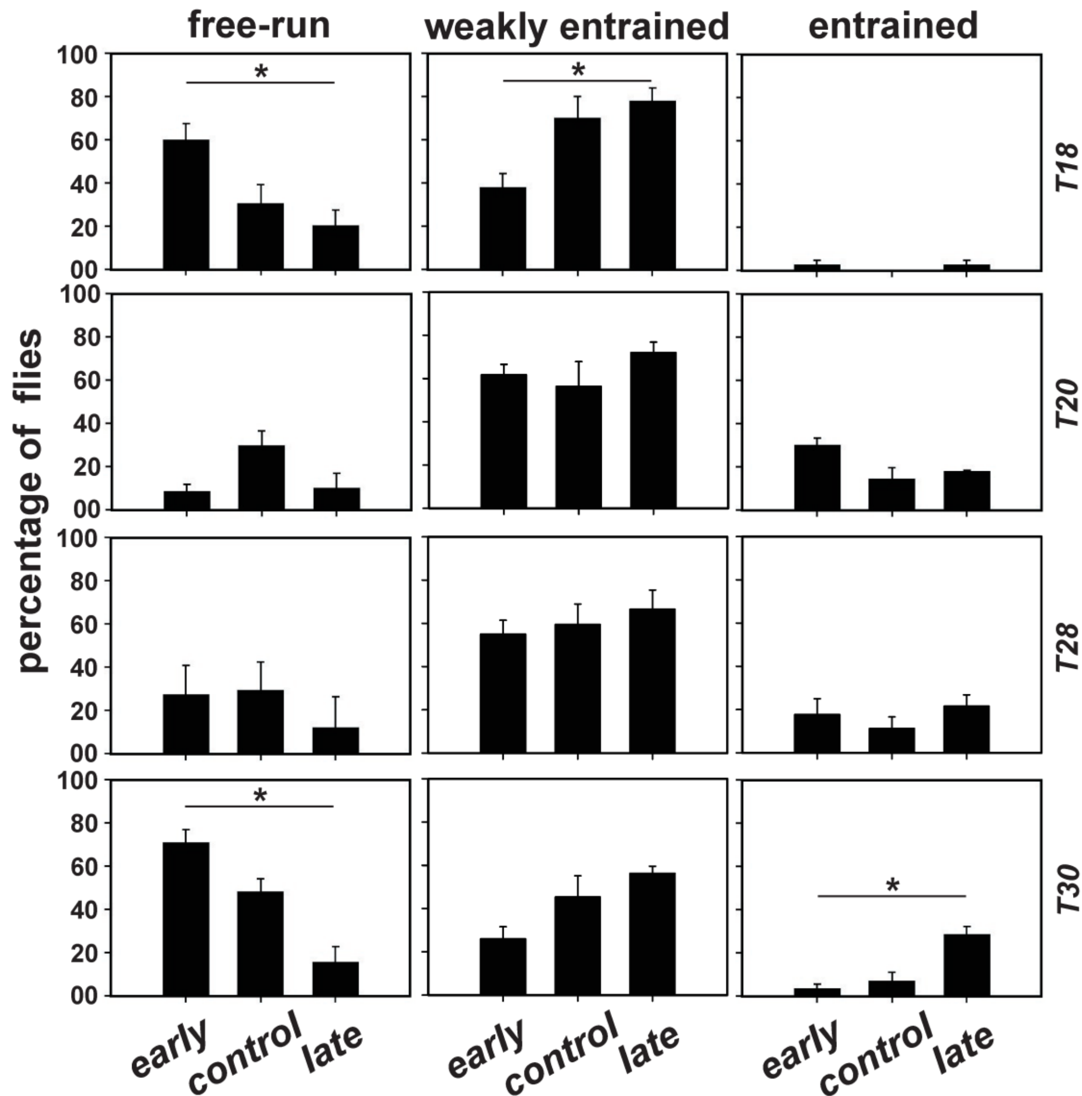


Figure 10: Percentage of *early*, *control,* and *late* populations exhibiting free-run (column 1), weakly entrained (column 2) and entrained (column 3) activity-rest rhythm in *T*-18 (row 1), *T*-20 (row 2), *T*-28 (row 3) and *T*-30 (row 4) regimes with light intensity during the day being 4×10^{-4} Wm^{-2}. All actograms were visually analysed and the entrained behaviour was categorized based on the following criteria. **Free-run**: if neither morning nor evening activity components were phase locked to the LD cycles; **weakly entrained**: if one of the two components was phase locked to the LD cycles while the other exhibited free-run; **entrained**: if both activity components were phase locked to the LD cycles (Figure 1). Error bars represent SEM across blocks with asterisks indicating significant differences between populations ($p < 0.05$). *late* populations appeared to exhibit a wider entrainment range as opposed to the other two populations in which lower proportion of individuals entrained to extreme *T*-cycles.

(g) ***Dose response curves (DRCs) indicate no difference in light induced phase shifts***

In light of the seemingly contradicting results from re-entrainment rate and entrainment range assays, we further decided to confirm if *late* populations actually differ in phase resetting ability, and therefore generated DRCs by measuring phase shifts elicited by light pulses of increasing intensities at CT14 and CT22.

As expected, the phase shifts increased with light intensity at both CT14 and CT22 (Figure 11a), and ANOVA on IPS50 values reveal a statistically significant effect of 'population' neither at CT14 ($F_{2,6}$ = 0.48, p > 0.05; Figure 11a, b) nor CT22 ($F_{2,6}$ = 0.78, p > 0.05; Figure 11a, c).

Therefore, even though high A_o can partly account for reduced re-entrainment rate, lack of difference in the DRCs further complicate the interpretability of lower re-entrainment rate and wider entrainment range in *late* populations. Therefore, we speculated that the observed differences probably arise from mechanisms apart from mere light induced phase resetting.

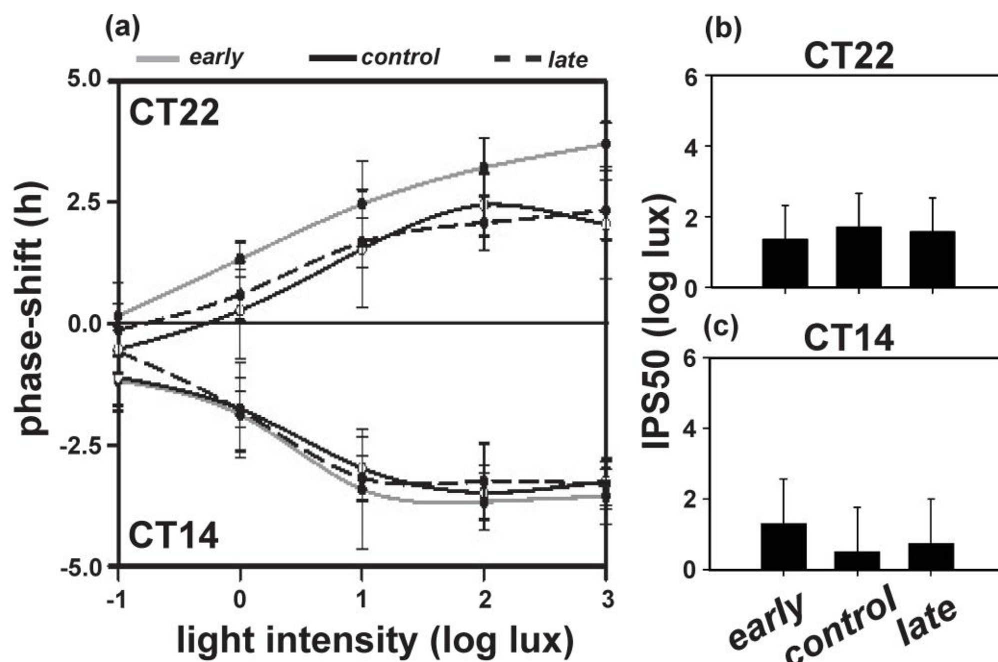


Figure 11 (previous page): (a) Dose Response Curves depicting phase shifts plotted as a function of light intensity (in log units) at CT14 and CT22. (b) IPS50 (light intensity required to elicit 50% of maximal phase shift) values for light exposures at CT14 and (c) CT22. Procedure for estimating IPS50 is detailed in materials and methods. Briefly, the phase shift data was used to fit a nonlinear dose response curve of the form Y = 100/ (1+10^ ((LogEC50 – X)*hill – slope)) from which the abscissa value corresponding to 50% of the maximal phase shift was estimated and served as the IPS50 value. Error bars represent 95% CI. DRC and IPS50 analysis indicate that the three populations do not differ in magnitude of instantaneous phase shifts even across multiple light intensities.

(h) ***Area under phase response curves (PRCs) suggests continuous effects of light***

Based on the results from previous section, we speculated that wide entrainment range might be driven by continuous or tonic effect of light, and not by phasic effects as assessed by the DRC. To test this, we estimated AUC for photic PRCs of all populations which is a measure of overall phase shift accumulated over a longer duration.

ANOVA on phase shift values reported a statistically significant effect of 'population × phase' interaction ($F_{10,30} = 2.58$, $p < 0.05$). *late* populations exhibited larger phase shifts at CT18 as compared to *early* and *control* populations (Figure 12a). ANOVA on AUC values reported a statistically significant effect of 'population' for delay phase shifts ($F_{2,6} = 6.22$, $p < 0.05$) and was marginal for advance phase shifts ($F_{2,6} = 4.48$, $p = 0.06$). Post hoc comparisons revealed that area under the advance zone was considerably smaller for *late* (19.47 h^2) populations as compared to *early* (33.41 h^2) and *control* (169.98 h^2) but did not differ statistically (Figure 12b), whereas area under the delay zone was significantly greater for *late* (943.62 h^2) populations as compared to *early* (735.76 h^2) and *control* populations (689.66 h^2; Figure 12c).

These results partly suggest that the populations might have evolved differences in their ability to integrate light over prolonged durations.

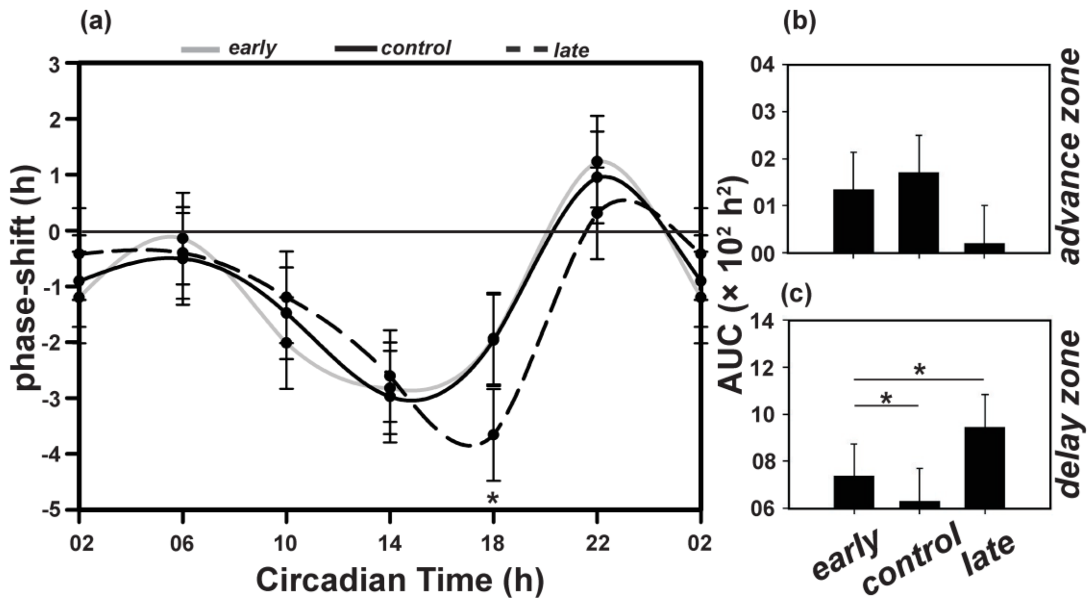


Figure 12: (a) Phase Response Curves depicting magnitude of phase shift elicited by 5 min light pulse of intensity 16×10^{-2} Wm^{-2} across different circadian times of the day for *early*, *control,* and *late* populations. (b) and (c) represent the total area under the curve (AUC) for advance and delay zones respectively in the three populations. To estimate AUC, the polynomial that best fit to the experimental PRC data was used to interpolate phase shift values at intermediate circadian times every 0.04 h. The interpolated phase shift values were then used to estimate the area under the advance and delay zones by integral under the curve method. Error bars represent SEM across blocks with asterisks indicating significant differences between populations ($p < 0.05$). It can be observed that the AUC under the delay zone was significantly higher for *late* populations suggesting a possible role of tonic effects of light on entrainment.

4.4 Discussion

Motivated by the results from our previous studies, in conjunction with those from others as discussed in the introduction, we employed populations of *D. melanogaster* selected for early and late emergence to explore the association of circadian clock (network) properties with the ψ_{ent}.

We first assessed if *early* and *late* populations might have evolved light sensitivity differences. Significantly higher proportion of *late* populations were observed to exhibit behavioural arrythmicity under dim LL as compared to the other two populations which exhibited free-run (Figure 3b), suggesting that *late* populations might have evolved higher light sensitivity. This was further substantiated by robust high amplitude entrainment of both emergence and activity-rest rhythms in *late* populations under low intensity LD cycles (Figures 4, 5). Alternatively, we reasoned that the high amplitude of entrainment in *late* populations may not necessarily be due to enhanced light sensitivity but can also be driven by a high amplitude circadian oscillation, or in other words, high amplitude circadian clocks, which turned out to be the case when we observed that *late* populations exhibited high amplitude activity-rest rhythm in DD. As discussed earlier, sensitivity of circadian clocks to zeitgeber is inversely proportional to its amplitude. Therefore, based on the observed higher arrythmicity in *late* populations under dim LL, one would expect circadian clock amplitude in these populations to be lower than that of the other populations which is clearly not the case, thus implying that higher arrythmicity in *late* populations might not entirely be due to light sensitivity differences but may involve other mechanisms as will be discussed later. Nevertheless, higher A_o in *late* populations clarifies their reduced re-entrainment rates to both advance and delay jetlag (Figure 8). In other words, higher A_o in *late* populations would lead to

lower A_z/A_o thus resulting in small magnitude phase shifts, and consequently reduced re-entrainment rate which is in accordance with the observed results.

Having observed divergent clock amplitudes and re-entrainment rates to simulated jetlag, we further assessed if such properties were intertwined with phase resetting ability of the underlying clocks to gain further insights into how entrainment differences might drive *early* and *late* emergence chronotypes. Intriguingly, contrary to the observations by Pittendrigh (1967, 1981a) and Pittendrigh and Takamura (1987, 1989), despite A_o differences, *early* and *late* populations did not differ in their phase resetting ability even across light intensities spanning orders of 10^4 (Figures 11, 12); whereas *late* populations exhibited wider entrainment range (Figure 10) which appears counterintuitive under the realms of the discrete entrainment model (Pittendrigh, 1960). However, the underlying assumption of this model is that light instantaneously shifts the clock phase, which even though successfully tested, has been reconsidered multiple times motivating the proposal of a modified discrete entrainment model involving continuous (tonic) effect of light (Pittendrigh and Daan, 1976b). Therefore, even though *late* populations do not differ in instantaneous phase shifts, the wider entrainment range in these populations might additionally be facilitated by tonic effects of light over longer durations. Previously, Vaze et al (2012a) reported that when entrained to skeleton photoperiod comprising 15 min light pulses in the morning and evening, none of the populations exhibited their respective ψ_{ent} as observed in LD12:12, but when entrained to asymmetric skeleton photoperiods, *late* populations required longer duration of light (6 h) in the morning while *early* populations required longer light duration in the evening to exhibit their respective ψ_{ent}, indicating that these populations integrated light information over prolonged durations. This proposition is further supported by the observation that the accumulated phase shifts over longer duration (as estimated by the

AUC) in the delay zone of the PRC of *late* populations is significantly greater as compared to the other populations thereby suggesting the role of both phasic and tonic effects of light in *late* populations.

Previously, we had reported that *early* and *late* populations might have evolved dominant M and E oscillators or neurons (Kumar et al. 2007b), whose coupling and consequently dominance is known to change with photoperiod thus driving seasonal adaptations (Grima et al. 2004; Stoleru et al. 2004, 2007). As a further extension of Kumar et al (2007b), most if not all of the results of our study can be explained in the framework of dominant E neurons in *late* populations. E neurons primarily contribute to delay phase shifts (Stoleru et al. 2005), and thus can account for larger AUC in delay zone of *late* populations. Alternatively, enhanced AUC in the delay zone might also be facilitated by higher CRY expression in the E neurons. This proposition also explains several of our observations in *late* populations. CRY in E neurons considerably reduces the ability of the M neurons to dominate over the E neurons (Zhang et al. 2009) thus rendering the latter relatively independent. Also, E neurons alone have been implicated to maintain rhythmicity in LL (Stoleru et al. 2007). Therefore, higher CRY levels in the E neurons of *late* populations would render them more sensitive to LL thus abolishing molecular oscillation, and consequently drive arrythmicity. Furthermore, dominant E neurons might underlie higher evening activity in *late* populations while PDFR only in E neurons can also drive high morning activity (Lear et al. 2009) which might explain high amplitude of M peak in *late* populations (Figures 4, 7). Alternatively, the morning activity can also be due to higher light induced activity or masking. Either ways, the high A_{ent} in *late* populations can be accounted for by dominant E neurons.

As discussed earlier, arrhythmic phenotypes observed in *late* populations even though are suggestive of higher light sensitivity, other results do not entirely support this

idea. Furthermore, arrythmicity in *late* populations did not increase with increasing light intensity (Figure 1). Therefore, it appears that other factors apart from light sensitivity might underlie the observed LL phenotypes. This can alternatively be explained in terms of coupling of constituent neuronal oscillators in *late* populations. In principle light induced decoupling of constituent oscillators can facilitate mutual desynchrony and consequently behavioural arrythmicity. Observations in *pdf^{01}* flies (Yoshii et al. 2009) suggest that PDF or PDFR driven reduction in coupling can also desynchronize individual clock neurons leading to behavioural arrythmicity. Additionally, reduction in PDF or PDFR levels can reduce M neurons' influence over E neurons in *late* populations. Therefore, reduced coupling strength driven by decrease in PDF or PDFR levels can underlie the observed LL phenotypes in *late* populations.

Re-entrainment to simulated jetlag in *Drosophila* is known to involve the E neurons which undergo phase shift followed by resynchronization by the M neurons, and this resynchronization is weakened when the two sets are rendered independent (Lamba et al. 2014). While enhanced CRY in the E neurons might facilitate large magnitude phase resetting in the E neurons, reduced coupling might attenuate resynchronization rate of the M neurons, resulting in lower re-entrainment rates in *late* populations.

Can the hypothesized reduction in coupling in *late* populations account for the observed amplitude changes? The effect of coupling on amplitude is dependent on the nature of coupling. Pure mean field coupling enhances amplitude by facilitating resonance between constituent oscillators, whereas diffusive coupling is known to have an opposite effect (Bordyugov et al. 2011). Moreover, heterogeneous nature of coupling rather than pure mean field or diffusive coupling is believed to drive synchronization among the circadian clock neurons (reviewed in Welsh et al. 2010), thus making it

difficult to attribute the observed amplitude differences in *late* populations to any particular coupling mechanism. Nevertheless in light of previously reported evolution of dominant M and E oscillators in *early* and *late* populations (Kumar et al. 2007b), and both theoretical and experimental studies reporting coupling strength to be intertwined with oscillator amplitude (reviewed in Helfrich-Förster et al. 2005; Welsh et al. 2010; Bordyugov et al. 2011), ψ_{ent} and entrainment range (Abraham et al. 2010; Bordyugov et al. 2011) all of which differ between *early* and *late* populations, it is highly likely that coupling differences might underlie the observed *late* phenotypes, but awaits experimental validation. We acknowledge that the above discussed bases for the observed phenotypes are speculative and built entirely on the available information. Nevertheless, they provide interesting testable hypotheses that might shed light on the M-E oscillator system.

To summarize, we report that selection for delayed ψ_{ent} of emergence results in the coevolution of large amplitude circadian clocks characterized by low relaxation rates (weak oscillators), and altered zeitgeber sensitivities (tonic effects of light), and drawing inferences from ours as well as from previous studies (Pittendrigh, 1981b; Abraham et al. 2010; Bordyugov et al. 2011; Granada et al. 2013) we further hypothesize that ψ_{ent} differences can also stem from different coupling strengths between the constituent neurons or oscillators. In addition to these core clock properties, differences in downstream pathways are also highly likely to further contribute to ψ_{ent} variation, and needs to be addressed. Having observed that amplitude, zeitgeber sensitivity and relaxation rates are associated with emergence chronotype; it would be intriguing to explore how these properties influence other aspects of entrainment such as rhythm stability and adaptation to seasonal changes in nature.

CHAPTER 5

late populations exhibit higher accuracy of entrainment

The contents of this chapter is due to be published as the following short communication article:

KL Nikhil, KM Vaze and VK Sharma (2016) Late emergence chronotypes of fruit flies *Drosophila melanogaster* exhibit higher accuracy of entrainment. *Chronobiology International*. (*In press*).

5.1 Introduction

Circadian clocks schedule physiology and behavioural processes at favourable times of the day via the process of entrainment which is characterized by the establishment of a stable and reproducible phase-of-entrainment (Ψ_{ent}; Daan and Aschoff, 2001). The ability to consistently exhibit high accuracy (low day to day variability) in Ψ_{ent} in the face of stochastic zeitgeber fluctuation in nature is considered to be adaptive, and therefore genetic mechanisms underlying accuracy of entrainment is likely to experience natural selection (Sharma, 2003). Among the factors presumed to facilitate accuracy of entrainment are precision (low cycle to cycle variability in free-running), organisms' sensitivity to zeitgebers, and day to day variation in zeitgebers (Beersma et al. 1999). Theoretical studies have revealed that linear response characteristics of phase response curve (PRC) and gated response to light also facilitate stable entrainment (Stelling et al. 2004; Rand et al. 2006; Thommen et al. 2010; Pfeuty et al. 2011), but await critical empirical validation.

The term ‘Chronotype(s)’ (Ehret, 1974) refers to inter individual variation in Ψ_{ent} with some individuals exhibiting advanced and others delayed Ψ_{ent}, generally in the context of human sleep-wake cycles (Roenneberg, 2012). However, such variation in Ψ_{ent} is also widely observed in several other species (Aschoff and Pohl, 1978). Chronotype has gained considerable attention due to its predominant influence on factors that affect the 'quality of life' such as psychological and neurological well-being (Mecacci and Rocchetti, 1998; Giannotti et al. 2002), substance abuse (Adan, 1994; Mecacci and Rocchetti, 1998; Taillard, 1999) and cognitive functions (Roenneberg, 2012). Late chronotypes in humans with inherent preference to wake up late in the day and sleep late at night thus establishing a delayed Ψ_{ent} have generally been associated with poor quality of life due to their struggle to remain in sync (although misaligned)

with the societal schedule; termed as 'social jetlag' (Roenneberg, 2012). Consequently, large differences in the duration and phase of sleep-wake cycles between weekdays and weekends are observed in late chronotypes resulting in reduced stability of entrainment (Roenneberg et al. 2003; Wittman et al. 2006).

Chronotype variation has been found to be associated with clock properties such as period, amplitude and light sensitivity in several species including humans (Aschoff and Pohl, 1978; Pittendrigh and Takamura, 1987; Wright et al. 2005; Rémi et al. 2010) thus proposing a causal role for clock properties and entrainment mechanisms in chronotype variation. However, inferences drawn about associations between circadian clock properties and chronotypes in humans cannot be generalized considering several confounding factors such as inter population genetic differences, age, sex, social environment and questionnaires used (reviewed in Levandovski et al. 2013), thus calling for a suitable model system for such studies under controlled laboratory setting which would also facilitate dissection of the molecular genetic bases of chronotype variation. Recently, genetic and molecular bases of chronotype variation have been investigated in mouse (reviewed in Pfeffer et al. 2015a; Pfeffer et al. 2015b) and *Drosophila* (Pegoraro et al. 2015). The study on *bmal*$^{-/-}$ mice displaying *late* chronotype in spontaneous locomotor activity reported reduced rhythm stability (Pfeffer et al. 2015b). Such individuals also recovered significantly faster from advance and delay jetlag, but did not differ in their retinal response to light as compared to controls (Pfeffer et al. 2015b). In a separate study, Pegoraro et al (2015) identified differentially expressed candidate genes between two isogenic strains of Drosophila Genetic Reference Panel (DGRP) exhibiting *early* and *late* chronotypes. Inferences on molecular genetic bases of chronotypes primarily drawn on the basis of association between chronotype and rhythm stability (Pfeffer et al. 2015b) or transcriptional landscape variation (Pegoraro et al.

2015) may not be appropriate because individuals representing early and late chronotypes were not sampled from the same parental strain but belonged to two separate highly inbred strains. Consequently, one cannot rule out the possibility that such associations are not spurious genetic correlations arising from (a) unknown genetic differences between the ancestral strains, or (b) inbreeding induced random fixation of alleles at different loci due to small population sizes during maintenance, and the lack of sufficient genetic variation in the founding populations.

Considering the above discussed concerns we initiated a long term laboratory selection on *Drosophila melanogaster* populations to study the evolution of circadian clock properties in response to selection for morning (*early*) and evening (*late*) emergence, which consequently evolved divergent emergence phases and clock periods in addition to several other properties (Kumar et al. 2007a; Nikhil et al. 2014). Interestingly, contrary to the findings of Pfeffer et al (2015a), results from our recently concluded experiments suggest that *late* populations have reduced ability to recover from advance and delay jetlags. In light of the above discussed reports, we further asked if *early* and *late* populations differ in two key clock properties - accuracy and precision (Daan and Beersma, 2002; Kannan et al. 2012a), and whether such associations are specific to any particular rhythm and environmental condition.

We report that under light/dark (LD) cycles, accuracy of both emergence and activity-rest rhythms is significantly higher in *late* populations as compared to *early* populations, a trend which is consistently observed across multiple environments including thermophase/cryophase (TC) cycles and semi natural (SN) conditions. Furthermore, we report that enhanced accuracy does not stem from precision of circadian clocks but from differential genotype × environment interaction. Our results thus demonstrate a genetic correlation between accuracy of entrainment and chronotype,

suggesting that difference in entrainability and associated clock properties may contribute to chronotype variation.

5.2 Materials and methods

(a) ***Experimental populations***: Details of experimental populations and maintenance protocol are described in chapter 2.

(b) ***Adult emergence rhythm assay*****:** The basic protocol for adult emergence rhythm assay is described in chapter 2. Briefly, following egg collection, the vials were transferred to respective environmental regimes for pre-adult development. LD constituted of 12 h each of light and darkness at 25 ^{o}C and TC comprised 12 h each of 18 ^{o}C and 28 ^{o}C in constant darkness (DD). SN comprised an outdoor enclosure in JNCASR, Bangalore (12°59′ N 77°35′ E). Light intensity varied between 1×10^{-3} Wm^{-2} at night and ~230 Wm^{-2} during the day, and temperature varied between 12 ^{o}C and 28 ^{o}C. Detailed description of the same can be found in Vaze et al (2012b). To account for the possibility that the observed accuracy of entrainment does not stem from light induced masking, assays were performed at high (4×10^{-1} Wm^{-2}) and low (4×10^{-4} Wm^{-2}) light intensities.

(c) ***Activity-rest rhythm assay*****:** The basic protocol for activity-rest rhythm assay is described in chapter 2. The activity-rest assay was performed under LD, TC, and SN, the data thus obtained was used to calculate day wise phase of activity similar to that for emergence rhythm. Phases of emergence and activity-rest rhythms were computed using custom written codes in R (R Development Core Team, 2011) and Matlab (Matlab R2011a, The MathWorks Inc., Natick, MA, 2011). For estimating the precision, period of each individual was calculated, and modulo τ actograms were plotted by normalizing the free-running rhythm of each individual by its period in ClockLab (Actimetrics,

USA). This modulo τ plot was then used to mark day wise phases of activity onsets and offsets using ClockLab, which was then used to calculate precision of respective phase markers. Only individuals whose activity-rest data could be obtained for a minimum of 4 days were considered for analysis. With the same rationale as described above for emergence, activity-rest rhythm assays were performed at high (4×10^{-1} Wm^{-2}) and low (4×10^{-4} Wm^{-2}) light intensities.

(d) ***Statistical analyses*****:** The reciprocal of standard deviation (SD) of day wise phase of activity and phase of emergence under different environmental cycles (LD, TC, and SN) was used as an estimate of accuracy [accuracy = (SD of daily phases of entrained rhythm)$^{-1}$] while that of activity onset and offset phases in DD were used as an estimate of precision [precision = (SD of daily phases of free-running rhythm)$^{-1}$]. Accuracy and precision estimated for each individual was then averaged across individuals of a given block, and the block wise mean accuracy and precision thus obtained were analysed by randomized block design Analysis of Variance (ANOVA) followed by post hoc multiple comparisons by Tukey's HSD method at a significance level of 0.05.

5.3 Results

(a) ***late populations exhibit higher accuracy in light/dark (LD) cycles***

Accuracy of mean emergence phase in LD did not reveal a statistically significant effect of population at high light intensity of 4×10^{-1} Wm^{-2} ($F_{2,6} = 1.08$, $p > 0.05$) suggesting that the populations did not differ in accuracy of emergence rhythm. We speculated that high light intensity induced masking might reduce inter population differences, and therefore we estimated accuracy of the rhythm under low light intensity of 4×10^{-4} Wm^{-2}. At low light intensity, we observed a statistically significant effect of population ($F_{2,6} = 64.74$, $p < 0.0001$) with *late* populations exhibiting significantly higher accuracy as

compared to both *early* and *control* populations which did not differ among each other (Figure 1-left panel).

Similarly, difference in accuracy of the activity-rest rhythm under high light intensity was statistically not different between populations ($F_{2,6} = 0.61$, $p > 0.05$), but *late* populations exhibited a significantly higher accuracy ($F_{2,6} = 59.37$, $p < 0.001$) under low light intensity as compared to both *early* and *control* populations which did not differ among each other (Figure 1-right panel).

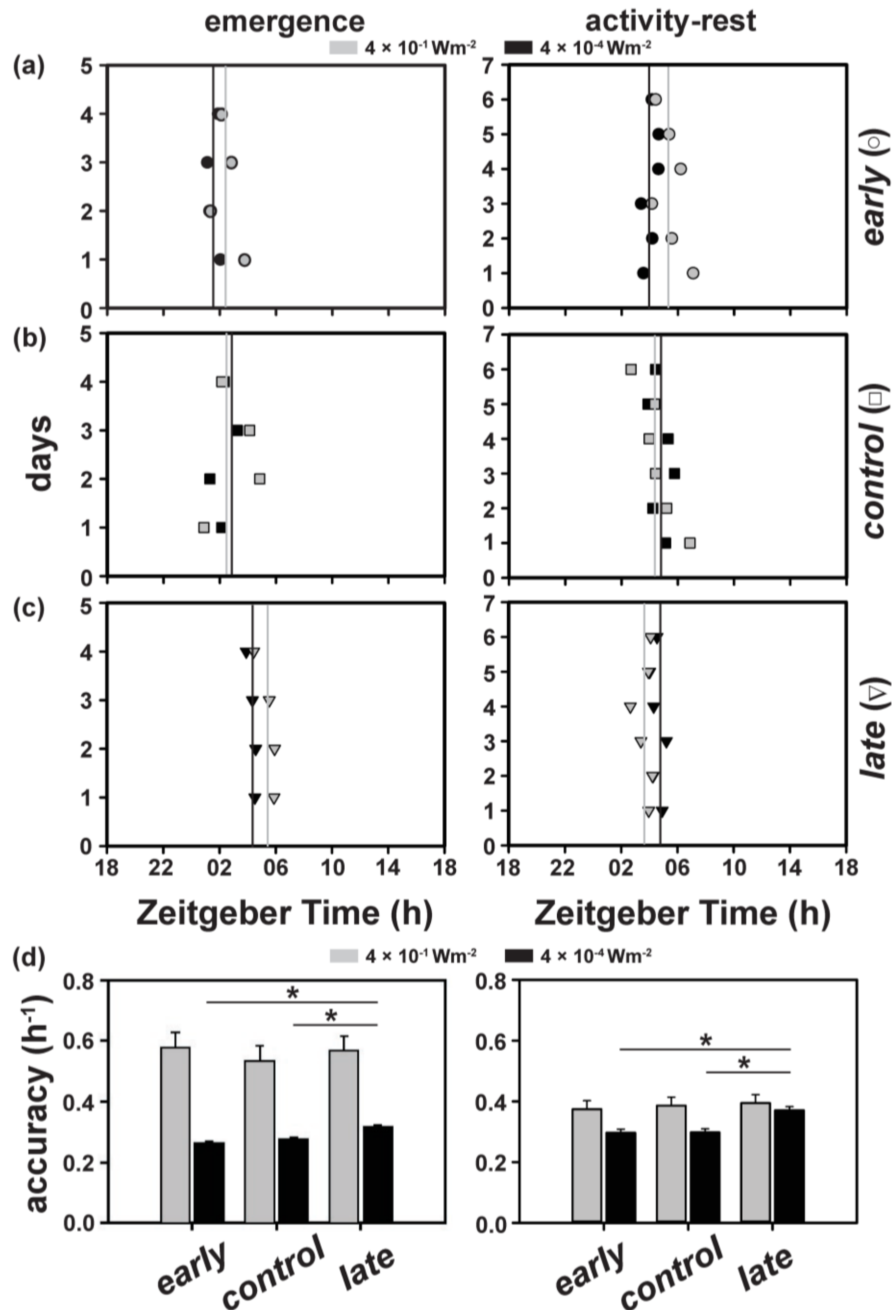


Figure 1: Daily phases in ZT (ZT00 indicates time of lights-ON) of emergence (left panels) and activity-rest (right panels) rhythms in (a) *early* (circles), (b) *control* (squares), and (c) *late* (inverted triangles) populations under 12:12 h light/dark (LD) cycles. Grey and black symbols indicate phases under high (4×10^{-1} Wm^{-2}) and low (4×10^{-4} Wm^{-2}) light intensity LD cycles respectively while grey and black lines indicate mean phases across all days of recording under high and low light intensities. d) Accuracy of emergence (left panel) and activity-rest (right panel) rhythms for all three populations under high (grey bar) and low (dark bar) light intensity LD cycles. Error bars represent 95% CI, and asterisks indicate statistically significant difference ($p < 0.05$).

(b) ***late populations exhibit higher accuracy in thermophase/cryophase (TC) cycles***

We further assayed both the rhythms in TC to examine if enhanced accuracy observed in *late* populations was restricted to LD alone or if it persists under novel environments as well.

A statistically significant effect of population for both emergence ($F_{2,6} = 30.59$, $p < 0.001$) and activity-rest ($F_{2,6} = 20.56$, $p < 0.01$) rhythms was observed with *late* populations exhibiting higher accuracy for both the rhythms as compared to *early* and *control* populations while the latter two did not differ statistically (Figure 2).

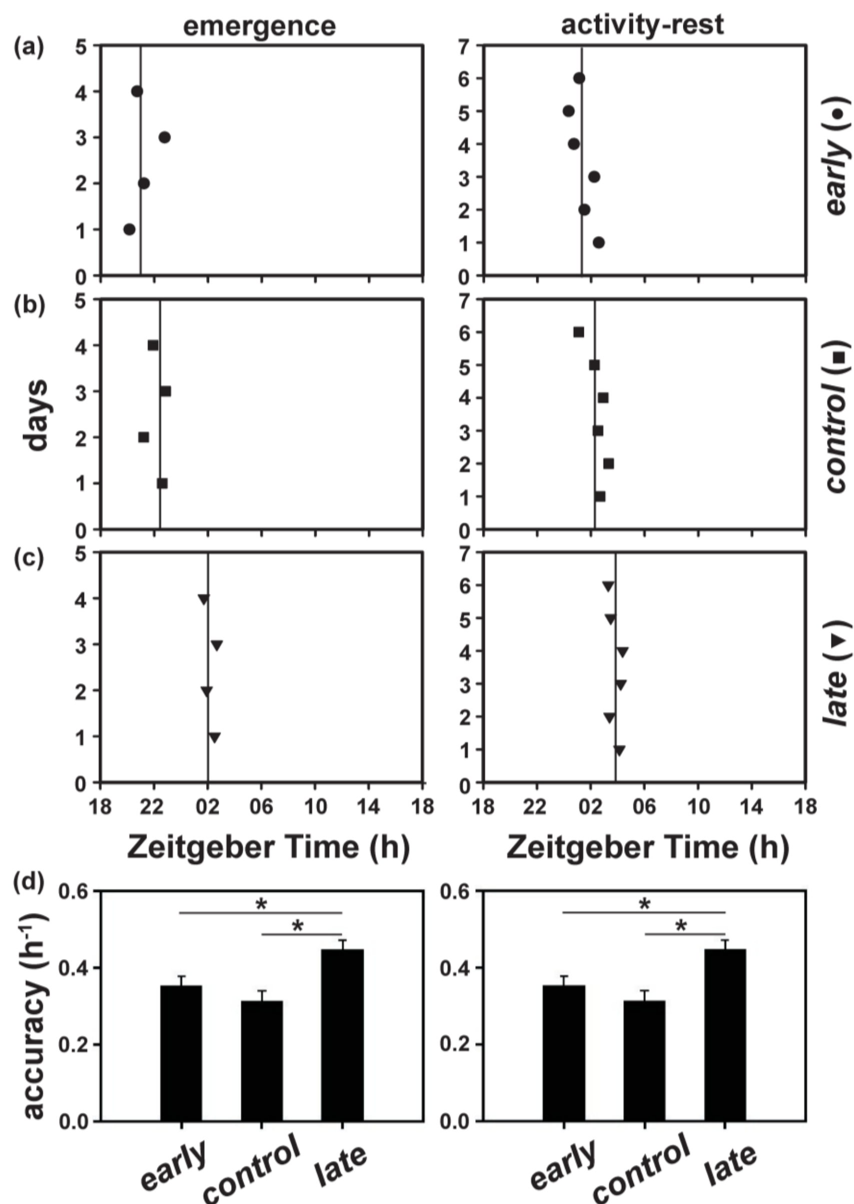


Figure 2 (previous page): Daily phases in ZT (ZT00 indicates time of temperature upshift from 18 ºC to 28 ºC) of emergence (left panels) and activity-rest (right panels) rhythms in (a) *early*, (b) *control,* and (c) *late* populations under 12:12 h thermophase/cryophase (TC) cycles. (d) Accuracy of emergence (left panel) and activity-rest (right panel) rhythms for all three populations under TC. All other details are same as that described in Figure 1.

(c) ***late populations exhibit higher accuracy in semi natural (SN) conditions***

We then tested if higher accuracy in *late* populations persists even in SN where organisms in addition to experiencing gradual changes in zeitgebers, also face day to day stochastic fluctuations in zeitgebers.

A statistically significant effect of population for both emergence ($F_{2,6} = 11.67$, $p < 0.01$) and activity-rest rhythms ($F_{2,6} = 10.00$, $p < 0.05$) was observed, and accuracy of emergence rhythm for *late* populations was significantly higher than *early* but not *control* populations (Figure 3-left panel), while that of activity-rest rhythm was higher in *late* populations than both *early* and *control* populations (Figure 3-right panel).

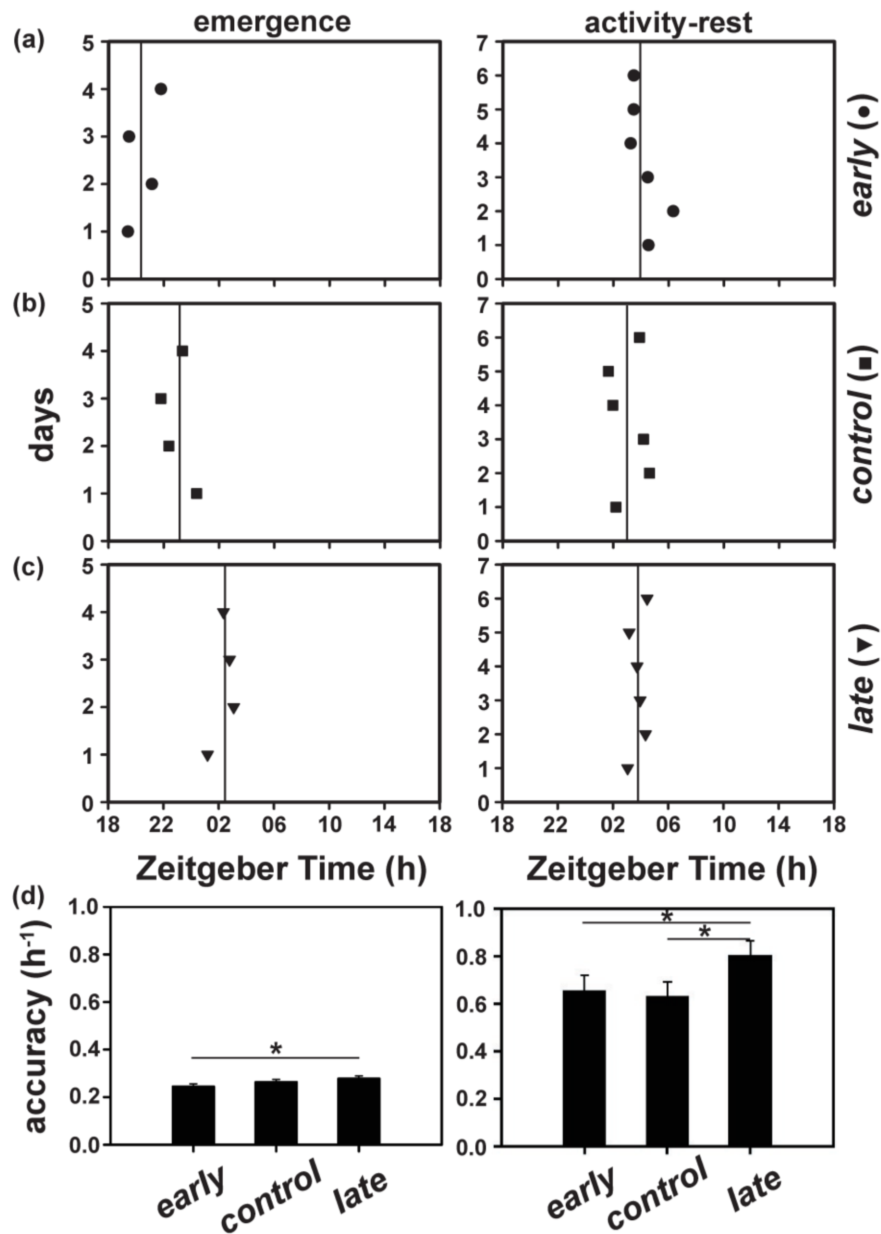


Figure 3: Daily phases in ZT (ZT00 indicates time at which light intensity crossed 0 Wm^{-2}) of emergence (left panels) and activity-rest (right panels) rhythms in (a) *early*, (b) *control,* and (c) *late* populations under semi natural (SN) conditions. (d) Accuracy of emergence (left panel) and activity-rest (right panel) rhythms for all three populations under SN. All other details are same as that described in Figure 1.

(d) ***early and late populations do not differ in precision of circadian clocks***

We asked if the accuracy differences observed between the populations are attributable to differences in precision of their circadian clocks and therefore assayed activity-rest rhythm under DD.

We did not observe a statistically significant effect of population neither for phase of onset ($F_{2,6}$ = 4.30, p > 0.05) nor phase of offset ($F_{2,6}$ = 4.23, p > 0.05) suggesting that circadian clock precision does not mediate differences in accuracy of entrainment between *early* and *late* populations (Figure 4).

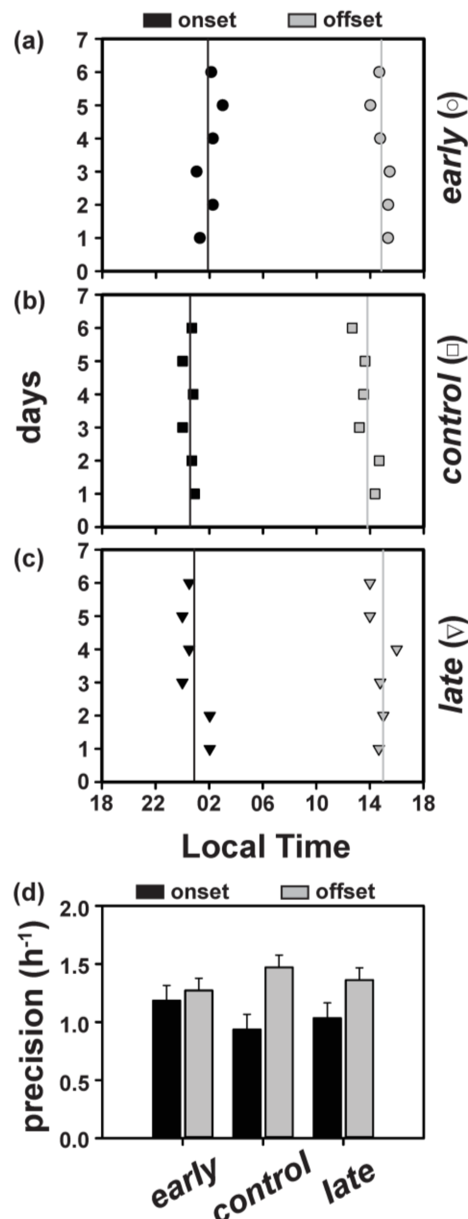


Figure 4: Daily phases (normalized by the clock period) of activity onset (dark symbols) and offset (grey symbols) for (a) *early*, (b) *control,* and (c) *late* populations in constant darkness (DD at 25 ºC). The black and grey lines indicate mean onset and offset phases respectively across all days of recording. (d) Precision of activity onset (dark bars) and offsets (grey bars) for all three populations in DD. All other details are same as that described in Figure 1.

5.4 Discussion

Results derived from mathematical models (Beersma et al. 1999; Tommen et al. 2010; Pfeuty et al. 2011) partly supported by experimental observations (Pittendrigh and Daan, 1976; Kannan et al. 2012a, b) suggest that accuracy of entrainment depends on the nature of entraining signals, zeitgeber sensitivity, PRC response characteristics, clock period and precision. Since some of these properties are also associated with chronotype variation, we hypothesized that such differences might influence accuracy and precision of rhythms in *early* and *late* populations. We observed that *late* populations exhibit higher accuracy as compared to *early* and *control* populations for emergence as well as activity-rest rhythms only under low but not high intensity LD cycles (Figure 1). A closer examination of the data to reason the lack of difference at high intensity revealed that accuracy of both the rhythms in *early* and *control* populations increases at high intensity as compared to low intensity whereas that for *late* populations remains higher under both the intensities. Thus, *late* populations exhibit higher accuracy regardless of strength of the zeitgeber (Figure 1), whereas the enhanced accuracy in the other two populations at high light intensity is probably due to light induced masking. Interestingly, *late* populations exhibit higher accuracy even under TC (Figure 2) thus suggesting that higher accuracy in *late* populations is unlikely to be driven by light sensitivity differences alone but may also involve other mechanisms. We further examined if *late* populations exhibit higher accuracy under SN, which, in addition to gradual changes and phase difference between light and temperature cycles, is also characterized by daily stochastic fluctuations in environmental variables thus posing to be considerably challenging for organisms as compared to the controlled laboratory conditions. Once again, *late* populations exhibited higher accuracy in both the rhythms as compared to *early* populations (Figure 3). Previously, Kannan et al

(2012b) had reported that populations selected for higher accuracy of emergence rhythm in LD also exhibit higher accuracy in TC and SN, similar to that of ours. Observing similar results from multiple studies further suggest that mechanisms governing accuracy of entrainment are conserved across rhythms and are not restricted to light input pathways alone but constitute core clock mechanisms that integrate information from multiple zeitgebers to facilitate stable entrainment to various environmental cycles. However, the contribution of clock input and clock output components cannot be entirely disregarded.

Kannan et al (2012a) had reported that *D. melanogaster* populations selected for higher accuracy of emergence rhythm coevolved precise circadian clocks suggesting that clock precision might drive enhanced accuracy of entrainment. Additionally, studies have reported correlations between clock period and precision such that clocks with periods close to 24 h are more precise (Pittendrigh and Daan, 1976; Lakin, 1985; Sharma and Chandrashekaran, 1999; Oklejewickz, 2001), however, other studies fail to observe such correlations (Bittman, 2012). Pittendrigh and Daan (1976) reported that accuracy of entrained rhythm is lowest when clock period matches that of the external cycle and proposed that natural selection would favour individuals with clock period close to but not exactly 24 h. Therefore, it appears that a collective interplay between higher precision and near 24 h clocks facilitates enhanced accuracy of entrainment. However, we observe that clock period of *early* (τ = 23.6 h) and *late* (τ = 24.2 h) populations even though are statistically different (Kumar et al. 2007a), their clock precision does not differ (Figure 4). Furthermore, both *control* (τ = 23.8 h) and *late* (τ = 24.2 h) populations deviate equally from 24 h but *control* populations do not exhibit accuracy comparable to *late* populations. These results collectively suggest that higher

accuracy in *late* populations is driven neither by precise circadian clocks nor by the period proximity to that of the zeitgeber.

Previous studies have acknowledged that accuracy of entrainment requires both phase and period responses of circadian clocks (Beersma et al. 1999). Vaze et al (2012a) have shown that even though *early* and *late* populations entrain to short recurrent light pulses during morning and evening (skeleton photoperiods), they could not entirely achieve their characteristic LD emergence waveform. Instead, the populations required light for different durations in the morning and evening to achieve their representative emergence waveforms (Vaze et al. 2012a). Additionally, studies in progress in our laboratory suggest that the two populations do not differ considerably in instantaneous phase responses to light pulses but exhibit differences in integrating light over longer durations. Thus it appears that light sensitivity differences along with both phase and period responses underlie accuracy of entrainment in *late* populations. While all of the above mentioned studies have assessed responses to light alone, observing higher accuracy of entrainment in *late* populations under both TC and SN regimes suggests that such response characteristics are conserved across multiple zeitgebers.

Two interrelated questions that arise from our results are 'why have *late* populations evolved higher accuracy?' and 'what is the ecological relevance of such a chronotype-accuracy association?' The evolution of emergence waveforms in *early* and *late* populations has shown some clear trends with *early* populations evolving advanced phase and narrow emergence gate while *late* populations evolved delayed phase and wider emergence gate (Kumar et al. 2007a). Consequently, the narrow gate of emergence in *early* populations may allow the emergence of relatively less accurately entraining genotypes within *early* selection window whereas, the wider gate of emergence in *late* populations would allow emergence of only accurately entraining

genotypes in *late* selection window. Additionally, the window of selection for *early* populations (ZT21-01) spans the night-day transition while that for *late* populations is 12 h later (ZT09-13). Recent experiments have revealed that a large proportion of flies from *early* populations exhibit enhanced response (masking) to night-day transition suggesting that lower accuracy in these populations can probably be compensated by masking response. Additionally, we also observe that area under the delay zone of the PRC for *early* and *late* populations is significantly greater as compared to that of their advance zones. Therefore, *early* populations with clock period of 23.6 h would entrain by undergoing larger phase delays whereas *late* populations with 24.2 h period cannot stably entrain due to its inability to undergo large phase advances, and thus would require additional compensatory mechanisms to facilitate higher accuracy of entrainment. Therefore, since a combination of factors such as reduced gate width and masking responses to light, and PRC can facilitate *early* emergence, *early* populations might not have experienced a strong selection pressure for evolving higher accuracy. The onset of rhythmic behaviours in late chronotypes generally occurs post-sunrise (in case of diurnal organisms) or post-sunset (in case of nocturnal organisms). If such organisms harbour a less accurate clock, small errors in time keeping in addition to daily stochastic variation in environment might considerably advance or delay the behaviour either too early or too late which may be maladaptive. This might further facilitate a mismatch between external cycles and behavioural and metabolic cycles which might prove detrimental to the organism. Early chronotypes on the other hand schedule behaviours pre-sunrise or sunset depending on diurnality or nocturnality respectively, and even if the internal clock fails to accurately track local time, organisms can still manage to exhibit rhythmic behaviours during the day or night by masking. Since rhythmic processes in *late* chronotypes generally occur after sunrise or sunset which

does not provide any environmental factors for masking, late chronotypes probably require an accurate time keeping mechanism. While our results suggest that evolving mechanisms that ensure higher accuracy is probably critical for late chronotypes, and that the more delayed the Ψ_{ent} is, the less likely is the possibility that the organisms will be aided by masking to dawn and dusk transitions therefore requiring a more accurate time keeping mechanism; the same proposition will also hold true for early chronotypes as well. But due to the nature of selection window imposed, which is relatively closer to morning (ZT21-01), the selection pressure on *early* populations is not as strong as that for *late* populations whose selection window is almost 12 h later (ZT09-13), and therefore it is not surprising that accuracy of daily rhythm in *early* populations is similar to *control* populations.

Even though it is tempting to assume that robustly observed high accuracy in *late* populations might be advantageous over their *early* counterparts, it is essential to consider that such enhanced features might be associated with trade-offs, a universal aspect of biological systems (Kitano, 2007). In principle, while enhanced zeitgeber sensitivity and feedback control mechanisms (to correct for day to day variation in zeitgebers) can help attain stable entrainment, the same can also be achieved by evolving mechanisms that facilitate phase insensitivity. Nevertheless, evolution of either higher zeitgeber sensitivity or phase insensitivity can potentially be associated with other trade-offs. For instance, when organisms are faced with seasonal transitions involving large daily changes in environment, higher zeitgeber sensitivity and strong feedback mechanisms might lead to phase shift overshoots rendering the system unstable. Alternatively, high phase insensitivity might reduce the ability of the organism to reentrain to seasonally changing environment cycles. This corroborates the observed reduction in reentrainment rates to both advance and delay simulated jetlag in

late populations as discussed earlier. Such observations have also been reported in humans with extreme chronotypes experiencing higher travel direction dependent jetlag induced fatigue but have been attributed to clock period difference alone (Roenneberg, 2012) whereas the above discussed factors can also underlie such observations. The results from our study can be further extended to understand the possible contributory roles of clock precision, PRCs and accuracy of entrainment to better evaluate differences in entrainment and its consequences on several factors including the ability to recover from jetlag in human chronotypes. Furthermore, such trade-offs associated with the evolution of higher accuracy and possible consequences of phase stability due to minor time keeping errors might be maladaptive and can be one of the reasons for observing low proportion of individuals with extreme chronotypes in natural populations.

Thus, in addition to earlier studies that reported clock properties such as period and PRC to be associated with chronotype variation, our study provides evidence for the existence of genetic correlations between mechanisms underlying accuracy of entrainment and that of chronotype variation further suggesting that the establishment of a delayed phase-of-entrainment is probably facilitated by or requires a relatively more accurate circadian rhythms which may not necessarily stem from inherently precise clocks. Even though period and PRC differences are generally associated with chronotype differences, our results suggest that accuracy of entrainment may additionally contribute to chronotype variation thus highlighting a complex interplay of several clock properties in the determination of Ψ_{ent}. Further studies might help elucidate such mechanisms and possible trade-offs associated with the evolution of higher accuracy in *late* populations.

CHAPTER 6

Molecular correlates of circadian clocks in *early* and *late* populations

The contents of this chapter is due to be published as the following research article:

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6.1 Introduction

Circadian clocks time rhythmic behaviours in consultation with environmental (zeitgeber) cycles by establishing a stable and reproducible phase relationship, a trait known to vary considerably across species spanning invertebrates to humans (Aschoff and Pohl, 1978). Such variation in ψ_{ent} is also observed across conspecific individuals, a well-known example of which is the variation for preferred sleep-wake timing in humans (termed as chronotypes; Roenneberg, 2012). Chronotypes are found to be associated with circadian clock period (τ) such that *early* chronotypes have shorter and *late* chronotypes longer τ (Duffy et al. 1999; Roenneberg, 2012). Such τ-ψ_{ent} associations have been observed in a wide variety of species (Aschoff and Pohl, 1978) thus highlighting its evolutionarily conserved nature.

While it is encouraging that recent studies have proposed the use of mouse models for examining circadian clock-chronotype associations (reviewed in Pfeffer et al. 2015a), such models do not conform to the widely observed τ-ψ_{ent} associations. For instance, in Wisor et al (2007), mice which exhibited *early* chronotypes did not have shorter τ. Similarly, *cry1* and *cry2* KO mice, which in spite of having large differences in τ, did not show chronotype divergence (Spoelstra et al. 2004). In yet another study (Pfeffer et al. 2015b) it was reported that *bmal* KO mice resemble *late* chronotypes but were arrhythmic under constant darkness (DD). In a recent study on inbred strains of *Drosophila* (DGRP), large scale differences in transcriptional landscapes were reported between the strains exhibiting *early* and *late* emergence chronotypes (Pegoraro et al. 2015). Surprisingly, contrary to the widely reported circadian clock-chronotype associations, the authors did not report differences in the expression of any of the circadian genes. While these results raise

serious doubts regarding the role of circadian clocks in the regulation of chronotype, it is also important to note that in several of these studies (Spoelstra et al. 2004; Wisor et al. 2007; Pegoraro et al. 2015) individuals exhibiting *early* and *late* chronotypes were derived from genetically different strains and therefore were of different genetic background, which is known to considerably modify circadian behaviours (Shimomura et al. 2001; Bittman, 2012 and citations therein). Furthermore, some of the mice strains were also deficient in one or more core circadian genes, an unlikely scenario in natural populations. In addition, since in many cases the strains were highly inbred (Spoelstra et al. 2004; Pegoraro et al. 2015; Pfeffer et al. 2015b) and therefore lacked genetic variation, they do not mirror natural populations which are likely to be outbred and harbour large genetic variation (reviewed in Ellegren and Sheldon, 2008). Therefore, inferences drawn regarding the molecular genetic bases of circadian clock-chronotype associations from such studies cannot be generalized.

With an intention to study the circadian clock properties underlying ψ_{ent} variation, we initiated a long term study wherein 4 populations each of *early* (selected for emergence early in the morning) and *late* (selected for emergence late in the evening) populations were raised by imposing artificial selection on four large (~1200 individuals each) *control* populations of *D. melanogaster*. The use of replicate, large populations would negate the possibility of the observed phenotypes arising due to random genetic drift and is likely to ensure maintenance of large genetic variation mirroring natural populations. In a previous study (Kumar et al. 2007a), we had shown that at generation 55 the populations had diverged from each other such that they exhibited relatively higher emergence in the morning (*early* emergence chronotype) or in the evening (*late* emergence chronotype), and in addition, *early* and *late* populations evolved shorter and longer τ respectively for both

adult emergence and activity-rest rhythms, which is in agreement with the τ-ψ_{ent} associations discussed earlier. Over the years, we have documented evolutionary divergence in several circadian clock properties such as photic phase response curves (PRCs) for both emergence and activity-rest rhythms, temporal light sensitivity, accuracy of entrainment and circadian network coupling between the two populations (Kumar et al. 2007a; Vaze et al. 2012a; Nikhil et al. 2016a, b). These results strongly suggest that in addition to evolving divergent core circadian oscillators, *early* and *late* populations have also evolved differences in input and output pathways. In this study, we intended to explore molecular correlates of chronotype differences in *early* and *late* populations, and studied mRNA expression profiles of some of the core circadian genes - *period (per)*, *timeless (tim)* and *clock (clk)*. We also assessed mRNA profiles of *cryptochrome (cry)* and *vrille (vri)*, which can be broadly classified as clock input and output components (reviewed in Hardin, 2011). Furthermore, in light of earlier studies suggesting the evolution of a weakly coupled circadian oscillator network in *late* populations (discussed in Nikhil et al. 2016b), we also examined expression of the neuropeptide pigment dispersing factor (PDF) which is known to function as a coupling factor in the *Drosophila* circadian clock network (reviewed in Yoshii et al. 2012). Since *early* and *late* populations have continued to experience selection for over 250 generations (14 years), we reassessed their circadian phenotypes to be able to better correlate the observed molecular clock differences with the expectation that the divergent behavioural phenotypes would be reflected in divergence in molecular components as well.

6.2 Materials and Methods

(a) ***Experimental populations***: Other details of experimental populations and maintenance protocol are described in chapter 2.

(b) ***Adult emergence rhythm assay*****:** Adult emergence rhythm assay was performed under three photoperiods - LD12:12, LD06:18 (winter type), and LD18:06 (summer type) at 25 ^{o}C with the light intensity being ~70 lux. Other details of the assay protocol are same as that described in chapter 2.

(c) ***Activity-rest rhythm assay*****:** All activity-rest rhythm assays were performed under light regimes same as described for adult emergence rhythm assay. Other details of the assay protocol are same as that described in chapter 2.

(d) ***mRNA extraction and quantitative real-time polymerase chain reaction*****:** The progeny of standardized populations were collected and housed in plexi glass cages under LD12:12 for 3-4 days post-emergence. On day 5, ~90 flies/block/population were collected and frozen every 2 h (starting at ZT00) across a 24 h day from all 12 populations following which the 90 flies collected at every time point were randomly distributed into 3 extraction replicates (a total of 432 replicates for all the 12 populations) with approximately equal number of males and females. The flies were decapitated using liquid nitrogen and total mRNA from the isolated heads was extracted using TRIzol reagent (Invitrogen, USA) as per manufacturer's instruction. 1 µg of the total mRNA was then treated with DNase I for the removal of genomic DNA accumulated during the extraction and the DNAse treated mRNA served as template for the first strand cDNA synthesis using Maxima first strand cDNA synthesis kit (Fermentas, USA). 1 µl of this served as the template for quantitative

real time polymerase chain reaction (qRT-PCR) carried out in StepOnePlus Real Time PCR system using SYBR Green Real-Time PCR master mix (ABI, USA) with standard reaction conditions and primer specific annealing temperatures. All extraction replicates were treated independently and qRT-PCR reactions were set in triplicates from each of the 3 extraction replicates therefore making a total of 1296 reactions per gene for all the 12 populations. The same protocol was used to estimate fold mRNA estimation for the *per*, *tim*, *clk, vri* and *cry* genes relative to the house keeping gene *rp49*. 3-5 primer sets were designed for every gene using NCBI Primer-BLAST with default parameter values barring amplicon size, which was restricted to 150 bp. Annealing temperature and concentration of all the primer sets were optimized to ensure minimal primer dimer formation and nonspecific amplification, and only the primer sets (sequences provided in Table 1) with amplification efficiency of > 90% were selected.

gene	sequence	length
period	forward: GAGCTGAGTGACATATAGCC	20
	reverse: TTGACTAGTGCGAGATTACAC	21
timeless	forward: CACTTCTTTTGGCTGGTAAC	20
	reverse: AGAATAGTGTCGATGTGCTC	20
clock	forward: GGATGCCAATGCCTACGAGT	20
	reverse: ACCTACGAAAGTAGCCCACG	20
cryptochrome	forward: CCACCGCTGACCTACCAAAT	20
	reverse: GGTGGCGTCTTCTAGTCGAG	20
vrille	forward: TTCAGCCTTCTTTCGAAGCG	20
	reverse: CTCACTTGCTTATCGCGGTTG	21
rp49	forward: CTTCAAGGGACAGTATCTGA	20
	reverse: CACCAGGAACTTCTTGAATC	20

Table 1. Details of primer sequences used for qRT-PCR. Up to 5 different sets of primers (per gene) were tested for amplification efficiency, and the ones with greater than 90% efficiency were selected.

(e) ***Immunocytochemistry*****:** 5 day old adult male flies were collected at each time point (every 2 h between ZT22-10 and at ZT14 and ZT18; n = 6-10 fly brains per time point), immediately dissected in cold Phosphate Buffered Saline (PBS) and fixed in 4% Paraformaldehyde (PFA) solution for 30 min. The fixed brains were then washed in 0.5% PBT (PBS with 0.5% Triton-X), blocked using blocking solution (10% Horse serum in PBT) for 1 h at room temperature (RT) followed by 5 h at 4 ºC and incubated with anti-PDF

antibody (generated by Nitabach et al. 2006) at 1:30,000 dilution for 24 h at 4 ºC. This was followed by six 15 min washes in PBT and incubation with appropriate secondary antibody for 1 h at RT and 16 h at 4 ºC. The brains were then washed similarly and mounted onto glass slides for imaging. Brains were imaged using Observer.Z1Axio (Zeiss, Germany) microscope, and staining intensity in the small ventral-lateral neuron (sLNv) terminals was quantified using ImageJ. Intensity measurements were performed for each hemisphere separately and statistically analysed by ANOVA to test for the effects of population and time point. Quantification and analysis of amplitude and average PDF expression levels were performed similar to that for amplitude and average mRNA expression of the core clock genes. However, unlike the mRNA expression experiments, which were performed on individuals collected separately from every replicate population, individuals for ICC experiments were collected from a pool of all four replicate populations for each of the three (*early*, *control,* and *late*) populations. Due to this reason, data from this experiment were bootstrapped (described in the 'Statistical analyses' section) in order to facilitate statistical comparisons. ICC staining intensity profiles plotted from the raw experimental data closely resembled the bootstrapped data.

f) *Statistical analyses*: The mean phase and τ of emergence and activity-rest rhythms were estimated separately for all the 12 populations, and served as data for a mixed model randomised block design Analysis of Variance (ANOVA) to test for statistically significant differences among populations. All post hoc comparisons were performed using Tukey's HSD at a significance level $\alpha = 0.05$.

The threshold cycle (C_T) values obtained by qRT-PCR were used to estimate fold mRNA expression at a given time point relative to *rp49* using comparative C_T method

(Schmittgen and Livak, 2008). The mean phase of mRNA expression was also calculated similar to that for emergence and activity-rest rhythms. Prior to calculating the mean phase of expression, mRNA levels across the day were subjected to Rayleigh Test ($\alpha = 0.05$), the results of which would help reject the null hypothesis that 'expression is randomly distributed across the day' thus indicating that there is a mean directionality/phase of expression (Batschelet, 1981; Jammalamadaka and SenGupta, 2001; Zar, 2009). Following Rayleigh test, statistical differences in the mean phase of expression between the populations were tested by the non-parametric test for dispersion (NPTD) (Batschelet, 1981) and Rao's test for homogeneity (Jammalamadaka and SenGupta, 2001), as has previously been used to analyse mean phase of the emergence (Nikhil et al. 2014). To ensure that probability of the total family wise error rate does not exceed $\alpha = 0.05$, all pair wise comparisons for differences in the mean phase of expression were performed at $\alpha = 0.01$ (99%CI) following Bonferroni corrections.

In addition to the mean phase, we also estimated the amplitude and average mRNA expression levels. The amplitude of mRNA oscillation was calculated as the difference between the peak and trough mRNA levels while the average mRNA level was calculated by averaging expression levels across the day. Both measures were calculated separately for each of the 12 populations and analysed as described for emergence rhythm.

For the analysis of PDF staining intensity data, since the individuals were pooled across four replicate populations, the data could not be subjected to a randomized block design ANOVA as used for all other experiments. Therefore, the staining intensity data was bootstrapped using R statistical language platform (R Development Core Team, 2011) to obtain 500 replicate values that were drawn from the experimental data. The

bootstrapped data was then used to statistically analyse the difference in the mean phase, amplitude and average levels of PDF expression across populations using factorial ANOVA at $\alpha = 0.01$ (99% CI).

Calculation of the mean phase of expression, Rayleigh Test, Rao Test and NPTD were implemented on R statistical language platform (R Development Core Team, 2011) using custom written codes with the aid of "CircStats" (Jammalamadaka and SenGupta, 2001) and "Circular" packages (Agostinelli and Lund, 2013) while ANOVA and Tukey's HSD were implemented on Statistica (Statsoft, USA).

6.3 Results

(a) *Emergence chronotypes of early and late populations persist robustly under different photoperiods*

As reported earlier (Kumar et al. 2007a), emergence waveforms of *early* and *late* populations under LD12:12 differed such that *early* populations exhibited advanced onset of emergence with higher morning emergence, while *late* populations exhibited a delayed onset and higher evening emergence, and *control* populations exhibited an intermediate emergence pattern (Figure 1a-top panel). This is reflected in their mean phase of emergence, with *early* (θ_e = 1.84 h) and *late* (θ_l = 8.39 h) populations being significantly advanced and delayed respectively as compared to *control* (θ_c = 5.01 h) populations ($F_{2,6}$ = 193.92, $p < 0.05$; Figure 1a-bottom panel).

We further wished to test if the emergence chronotypes of *early* and *late* populations persist under winter type (LD06:18) and summer type (LD18:06) photoperiods. Under winter type photoperiod, the mean phase of emergence of *early* populations was significantly advanced (θ_e = 21.98 h) and that of *late* populations significantly delayed (θ_l =

3.48 h) as compared to *control* populations (θ_c = 0.77 h; $F_{2,6}$ = 10147.09, $p < 0.05$; Figure 1b). The emergence chronotypes of the two populations were further diverged under summer type photoperiod, with the mean phase of emergence for *early* (θ_e = 4.52 h), *control* (θ_c = 8.66 h), and *late* (θ_l = 17.60 h) populations being significantly different from each other ($F_{2,6}$ = 173.16, $p < 0.05$; Figure 1c).

Thus, *early* and *late* populations exhibit phase advanced and phase delayed emergence waveforms as compared to *control* populations and this difference in timing of emergence robustly persists under winter and summer type photoperiods.

(b) *The period of emergence rhythm differ between early and late populations*

ANOVA on τ of emergence rhythm revealed a statistically significant effect of 'population' ($F_{2,6}$ = 30.10, $p < 0.001$), with *early* (22.51 h) and *control* (22.94 h) populations exhibiting significantly shorter τ as compared to *late* (23.86 h) populations (Figure 1d), while τ of *early* and *control* populations did not differ. Interestingly, we observed that the robustness of emergence rhythm of *late* populations diminished over time in DD with the gate width of emergence increasing by day 4, and consequently higher subjective night time emergence over days 5-6 leading to an apparent disruption of emergence rhythm over time in DD (Figure 1d), and will be discussed later.

Thus, in agreement with the τ-ψ_{ent} associations (see Introduction), selection for *early* and *late* emergence chronotypes results in the coevolution of divergent τ of emergence rhythm.

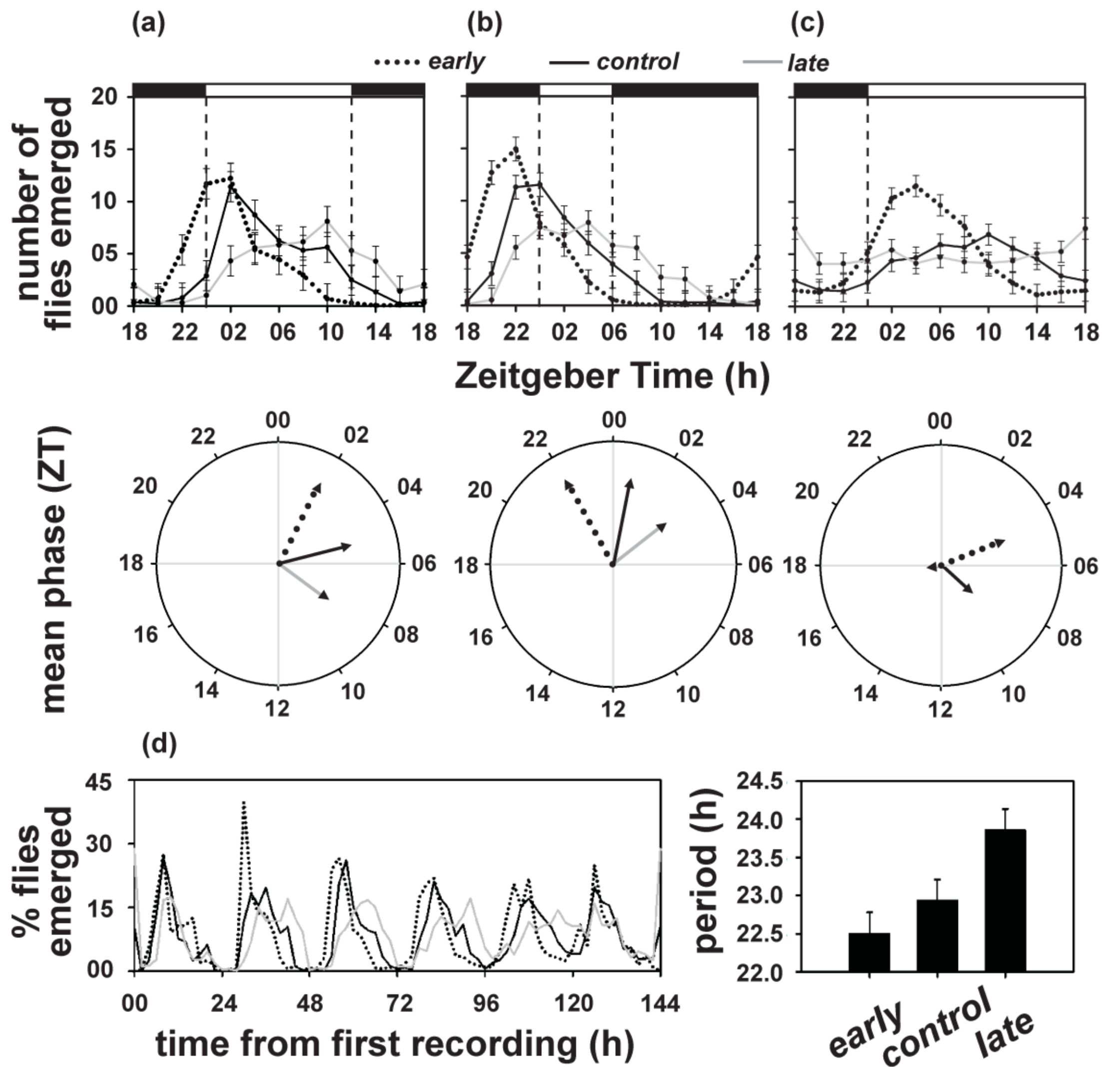


Figure 1: Adult emergence profiles (top panel) and mean phase of emergence rhythm (bottom panel) of *early*, *control,* and *late* populations under a) LD12:12, b) LD06:18, and c) LD18:06. d) Time series depicting adult emergence rhythm of all the three populations under DD (left) and their respective circadian periods (right). Error bars depict 95% CI calculated by Tukey's HSD following ANOVA and asterisks (*) indicate statistically significant differences ($p < 0.05$).

(c) ***Activity-rest patterns of early and late populations differ under different photoperiods***

Since common circadian oscillators have been attributed to regulate both emergence and activity-rest rhythms, we further wished to test if selection for divergent ψ_{ent} of emergence has led to the coevolution of divergent activity-rest rhythm.

When assayed under LD12:12, the mean phase of activity-rest rhythm of the three populations (θ_e = 5.55 h; θ_c = 5.50 h; θ_l = 5.58 h) did not differ statistically ($F_{2,6}$ = 1.38, p > 0.05; Figure 2a). However, activity during the evening peak was significantly higher in *late* populations as compared to *early* populations (Figure 2a-top panel).

Under winter type photoperiod, the mean phase of activity-rest rhythm differed statistically between the three populations, with *early* populations (θ_e = 4.73 h) exhibiting a significantly advanced phase as compared to *late* populations (θ_c = 5.47 h), while that for *control* populations (θ_c = 5.13 h) was intermediate ($F_{2,6}$ = 14.54, p < 0.05; Figure 2b). This probably is a consequence of advanced morning activity in *early* populations, which was delayed in *late* populations (Figure 2b-top panel). Activity around the evening peak was significantly higher in *late* populations as compared to *early* populations (Figure 2b-top panel).

Interestingly, under summer type photoperiod, the mean phase of activity-rest rhythm for *late* (θ_l = 17.47 h) populations was significantly advanced as compared to *early* populations (θ_e = 18.03 h), while that for *control* (θ_c = 17.75 h) populations was intermediate ($F_{2,6}$ = 24.13, p < 0.05; Figure 2c-bottom panel). A closer examination of the activity profiles revealed that this surprising result stems from a marginally advanced evening peak in *late* populations around the time when activity levels were significantly higher in *late* populations (Figure 2c-top panel).

In summary, the mean phase of activity-rest rhythm of *early* and *late* populations differ significantly under winter and summer type photoperiods but the evening peak of activity of *late* populations was consistently higher than *early* populations under all the three photoperiods, the possible reasons of which will be discussed later.

(d) *The period of activity-rest rhythm differ between early and late populations*

ANOVA on τ of activity-rest rhythm revealed a statistically significant effect of 'population' ($F_{2,6}$ = 29.71, $p < 0.0001$), with *early* (23.28 h) populations exhibiting a significantly shorter τ as compared to *control* (23.63 h) populations, which in turn had a significantly shorter τ than *late* (24.03 h) populations (Figures 2d, 3).

Thus, consistent with the difference in τ of emergence rhythm, selection for *early* and *late* emergence chronotypes results in the coevolution of divergent τ of activity-rest rhythm.

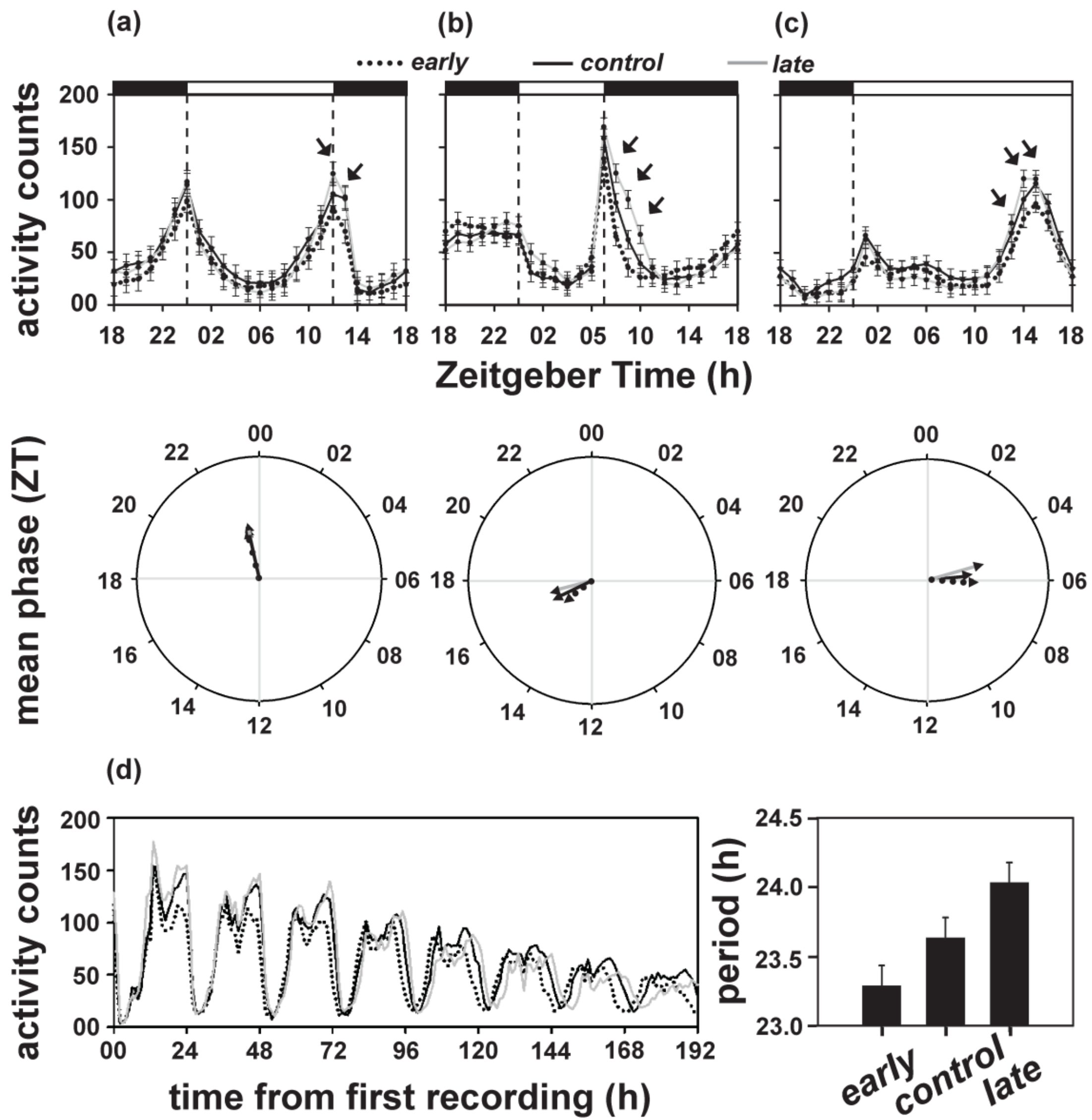


Figure 2: Average activity-rest profiles (top panel) and mean phase of activity-rest rhythm (bottom panel) of *early*, *control,* and *late* populations under a) LD12:12, b) LD06:18, and c) LD18:06. The arrow marks in (a) (c) indicate significantly higher evening activity observed in *late* populations compared to *early* populations. d) Time series depicting activity-rest rhythm of all the three populations under DD (left) and their respective circadian periods (right). All other details are same as in Figure 1.

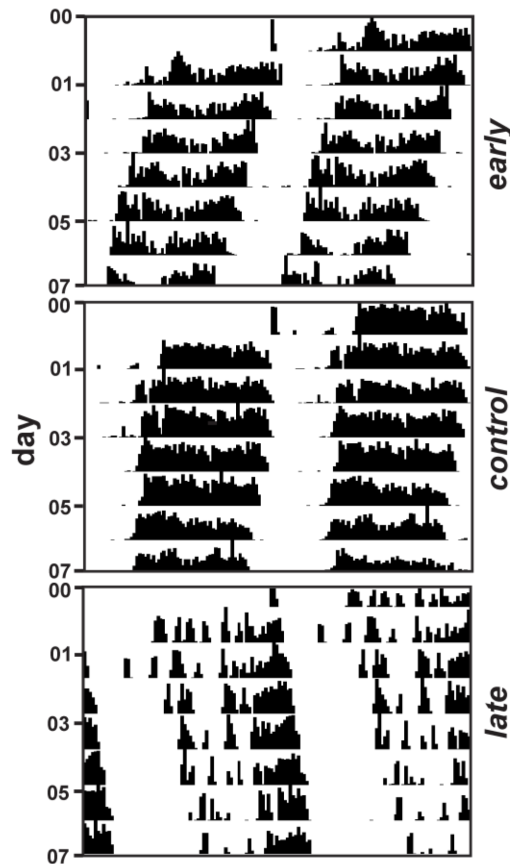


Figure 3: Representative actograms of male flies (3-5 days old) from *early*, *control,* and *late* populations recorded in constant darkness at 25 ºC. As a correlated response to selection for divergent phase of emergence, *early* and *late* populations evolved shorter and longer clock periods.

(e) ***early and late populations differ in phase and level of per mRNA expression***

The *per* mRNA exhibited rhythmic expression in all the three sets of populations with relative mRNA levels in both *early* and *control* populations rising earlier than that in *late* populations (Figures 4a-top panel, 5). The mRNA levels in *early* populations peaked at ZT14 followed by *control* populations at ZT16 and then *late* populations at ZT18 (Figures 4a-top panel, 5). Circular analysis revealed that with θ_l = 16.73 h, the mean phase of *per*

mRNA expression in *late* populations was significantly delayed by ~2.5 h as compared to *early* populations (θ_e = 14.50 h) and by ~1.5 h as compared to *control* (θ_e = 15.19 h) populations (Figures 4a-bottom panel, 5; Table 2), while the mean phase in *early* and *control* populations did not differ.

ANOVA on the amplitude of mRNA oscillation did not differ between the three populations (*early* = 80.98; *control* = 75.09; *late* = 80.51; $F_{2,6} = 0.61$, $p > 0.05$; Figure 4b; Table 2).

ANOVA on average mRNA levels revealed a statistically significant effect of 'population' ($F_{2,6} = 6.87$, $p < 0.05$) with *late* (25.17) populations exhibiting ~16% higher levels as compared to *early* (21.55) and *control* (21.01) populations while *early* and *control* populations did not differ (Figure 4c; Table 2).

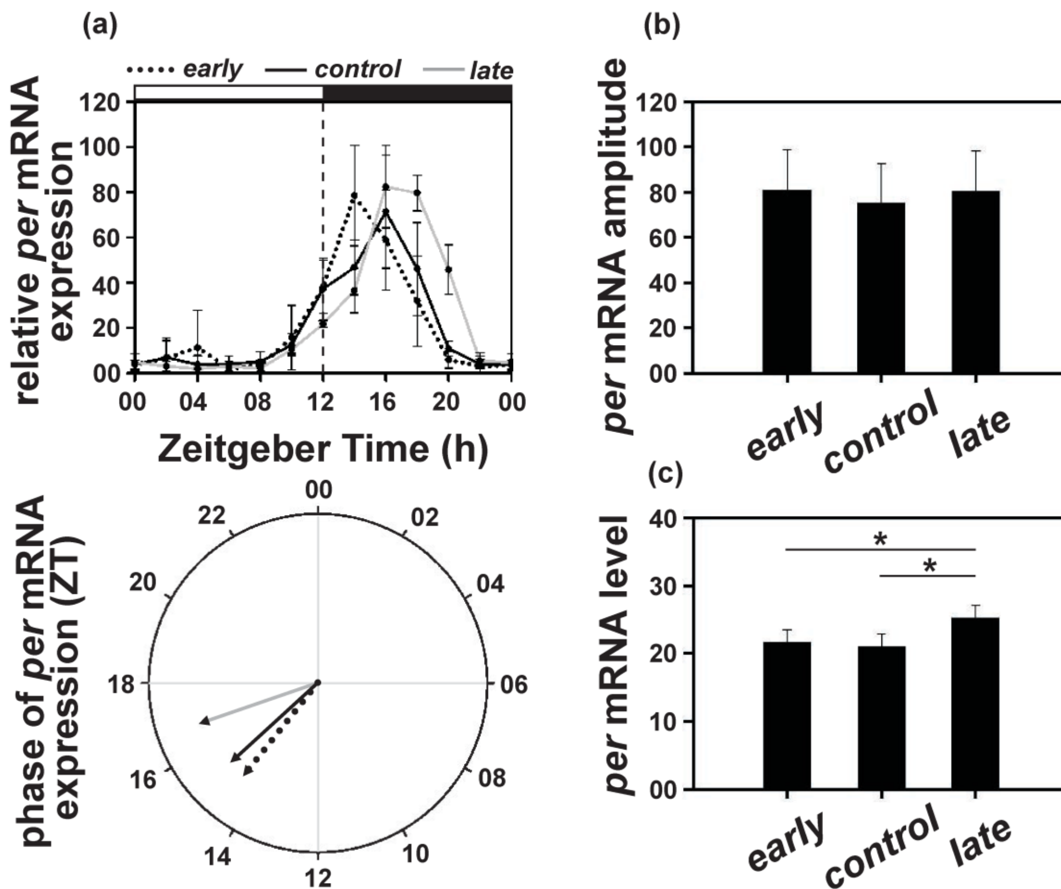


Figure 4: (a) Top panel: *period* (*per*) mRNA levels (relative to *rp49*) under LD12:12 in *early*, *control,* and *late* populations across the day. Error bars depict SEM across the four replicate blocks. The shaded and unshaded regions represent dark and light phases, and dashed lines depict lights-ON and OFF. Bottom panel: The mean phase (in ZT) of *per* expression (relative to *rp49*) in *early* (14.47 h), *control* (15.19 h), and *late* (16.73 h) populations. (b) Amplitude depicting difference between peak and trough, and (c) average levels depicting mean *per* mRNA (relative to *rp49*) levels across the day in *early*, *control,* and *late* populations. All other details are same as in Figure 1.

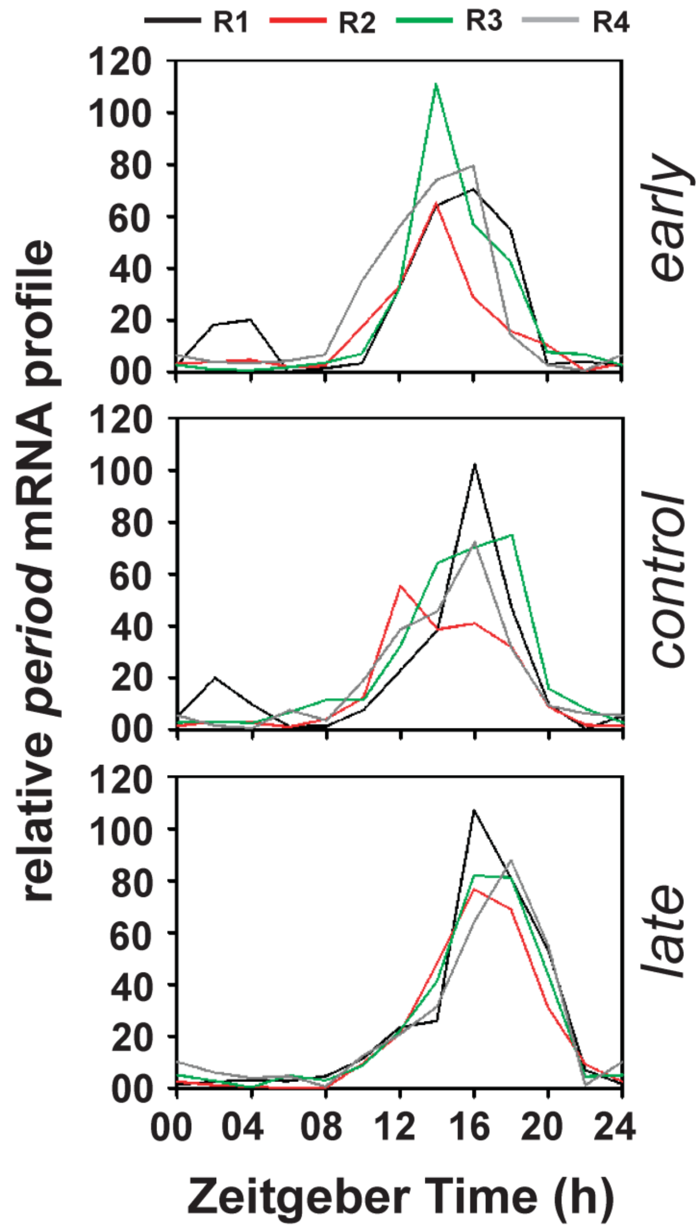


Figure 5: *period* mRNA expression (relative to *rp49*) profiles for each of the 4 replicate blocks (R1-R4) of *early*, *control,* and *late* populations. The values at each Zeitgeber Time represent an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

population	replicate	phase	amplitude	mRNA level
early	*1*	15.47	70.21	24.13
	2	14.08	64.53	12.25
	3	14.96	110.37	22.75
	4	13.78	78.87	23.82
control	*1*	15.96	101.95	20.04
	2	14.04	54.30	16.77
	3	15.58	72.45	25.22
	4	14.84	71.67	20.01
late	*1*	16.76	76.58	26.88
	2	16.41	76.49	22.34
	3	16.64	81.68	26.70
	4	17.11	87.19	24.77

Table 2: Mean phase (in ZT), amplitude and levels (relative to *rp49*) of *period* mRNA expression across 4 replicate blocks of *early, control,* and *late* populations. The values for each of the measures are an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

(f) ***early and late populations differ in phase of tim mRNA expression***

The *tim* mRNA levels also exhibited an earlier increase as well as decrease in *early* and *control* populations as compared to *late* populations, with mRNA levels peaking at ZT14 and ZT16 in *early* and *control* populations while that in *late* populations peaked at ZT18 (Figures 6a-top panel, 7). Accordingly, the mean phase of *tim* mRNA in *late* populations (θ_l = 16.50 h) was significantly delayed by ~2.5 h as compared to *early* (θ_e = 14.08 h) and *control* (θ_c = 14.35 h) populations, while the latter two did not differ (Figures 6a-bottom panel, 7; Table 3).

ANOVA revealed that the three populations did not differ in amplitude (*early* = 92.28; *control* = 85.13; *late* = 94.82; $F_{2,6}$ = 0.58, $p > 0.05$; Figure 6b; Table 3) and average *tim* mRNA levels (*early* = 23.25; *control* = 23.04; *late* = 26.99; $F_{2,6}$ = 1.21, $p > 0.05$; Figure 6c; Table 3).

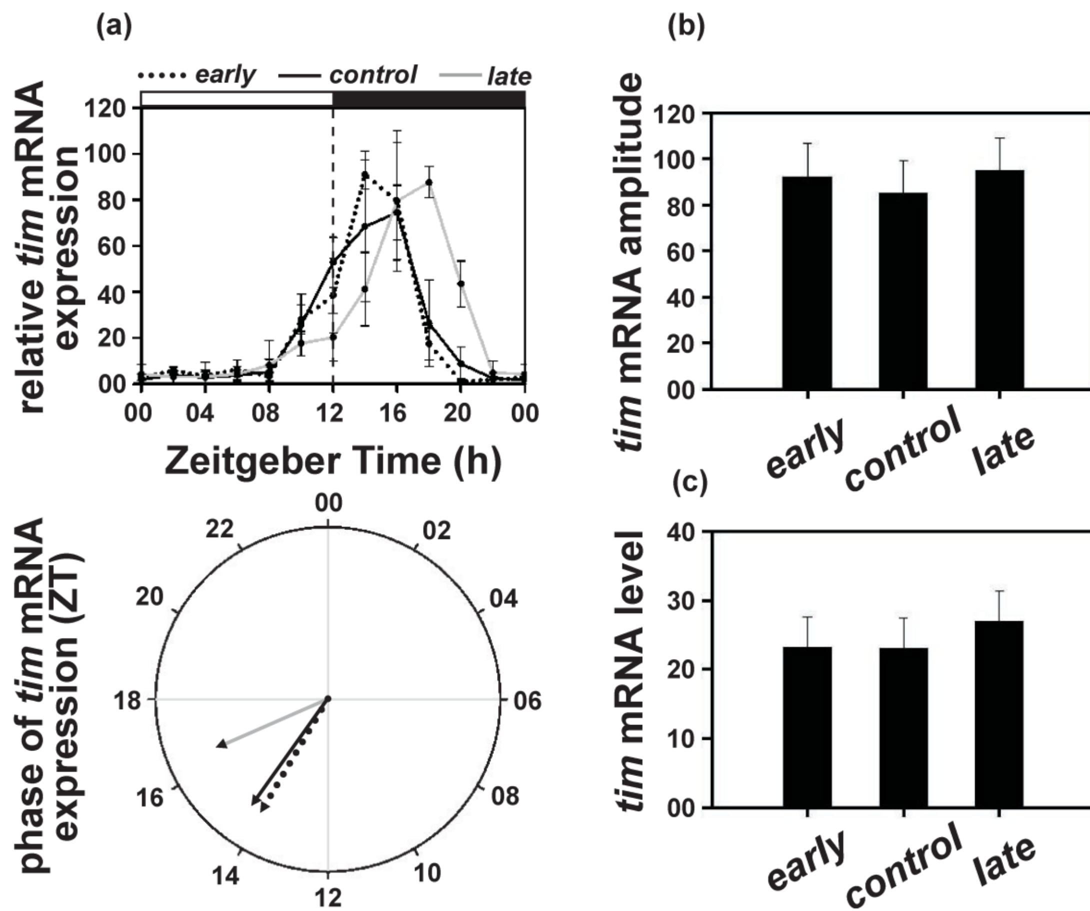


Figure 6: (a) Top panel: *timeless* (*tim*) mRNA levels (relative to *rp49*) under LD12:12 in *early*, *control,* and *late* populations across the day. Error bars depict SEM across the four replicate blocks. Bottom panel: The mean phase (in ZT) of *tim* expression (relative to *rp49*) in *early* (14.47 h), *control* (15.19 h), and *late* (16.73 h) populations. (b) Amplitude depicting difference between peak and trough, and (c) average levels depicting mean *tim* mRNA (relative to *rp49*) levels across the day for *early*, *control,* and *late* populations. All other details are same as in Figure 1.

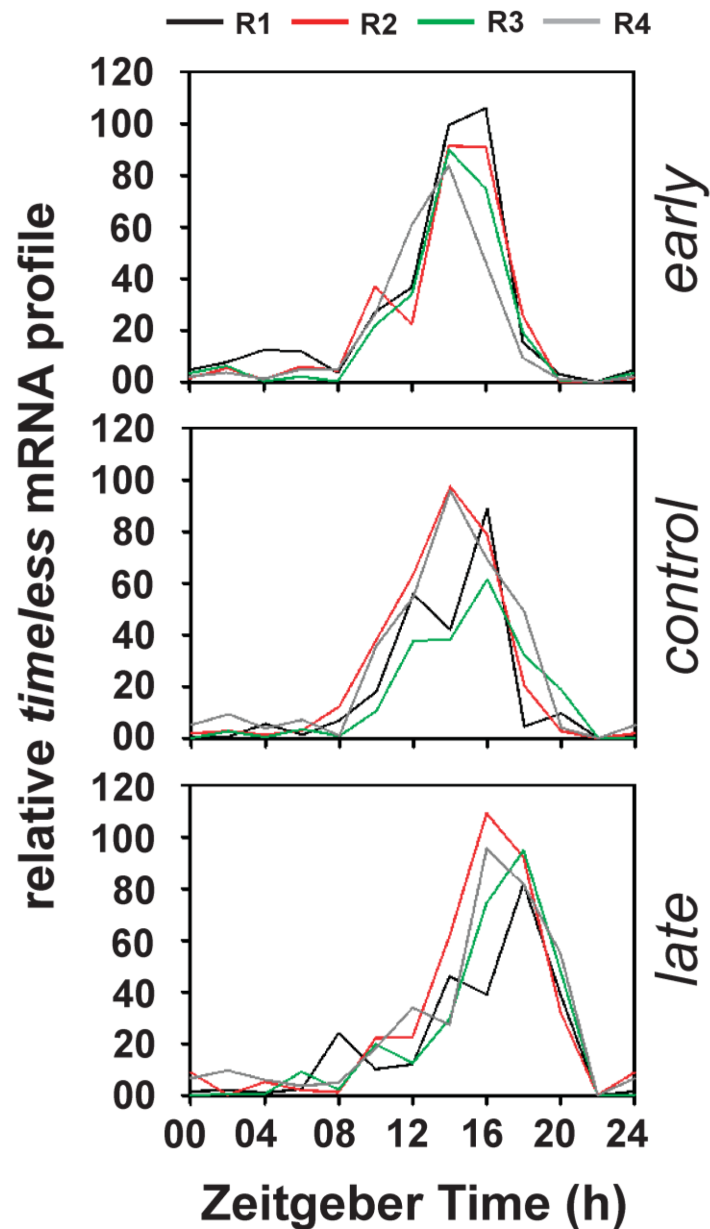


Figure 7: *timeless* mRNA expression (relative to *rp49*) profiles for each of the 4 replicate blocks (R1-R4) of *early*, *control,* and *late* populations. The values at each Zeitgeber Time represent an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

population	replicate	phase	amplitude	mRNA level
early	*1*	14.17	105.81	27.88
	2	14.34	91.22	24.11
	3	14.38	89.51	21.13
	4	13.43	82.59	20.48
control	*1*	14.14	88.18	19.67
	2	13.71	96.07	26.97
	3	15.24	61.40	17.36
	4	14.32	94.88	28.14
late	*1*	16.41	80.95	22.05
	2	16.28	108.97	30.75
	3	16.72	94.63	26.59
	4	16.58	94.74	28.56

Table 3: Mean phase (in ZT), amplitude and levels (relative to *rp49*) of *timeless* mRNA expression across 4 replicate blocks of *early*, *control,* and *late* populations. The values for each of the measures are an average of 9 reactions (3 extraction replicates with 3 reaction replicate under each extraction replicate) with 30 adult heads for each reaction.

(g) ***early and late populations differ in phase and amplitude of clk mRNA expression***

The *clk* mRNA oscillation was antiphasic to that of *per* and *tim* and showed an earlier increase in *early* followed by *control* and then *late* populations. The peak of *clk* mRNA in *late* populations occurred at ZT04, i.e. 2 h after *control* (ZT02) and 4 h after *early* (ZT00) populations (Figures 8a-top panel, 9). Accordingly, the mean phase of *clk* mRNA oscillation in *late* ($\theta = 4.23$ h) populations was significantly delayed by ~2.5 h as compared to *control* and ~3.5 h as compared to *early* populations (Figures 8a-bottom panel, 9; Table 4).

ANOVA on the amplitude values revealed a statistically significant effect of 'population' ($F_{2,6} = 5.84$, $p < 0.05$). Post hoc multiple comparisons revealed that *late* populations exhibit significantly higher amplitude *clk* mRNA oscillation (60.53) followed by *control* (55.22) and then *early* (49.50) populations (Figure 8b; Table 4).

However, average *clk* mRNA levels did not differ between the three populations (*early* = 16.78; *control* = 15.82; *late* = 15.18; $F_{2,6} = 0.24$, $p > 0.05$; Figure 8c; Table 4).

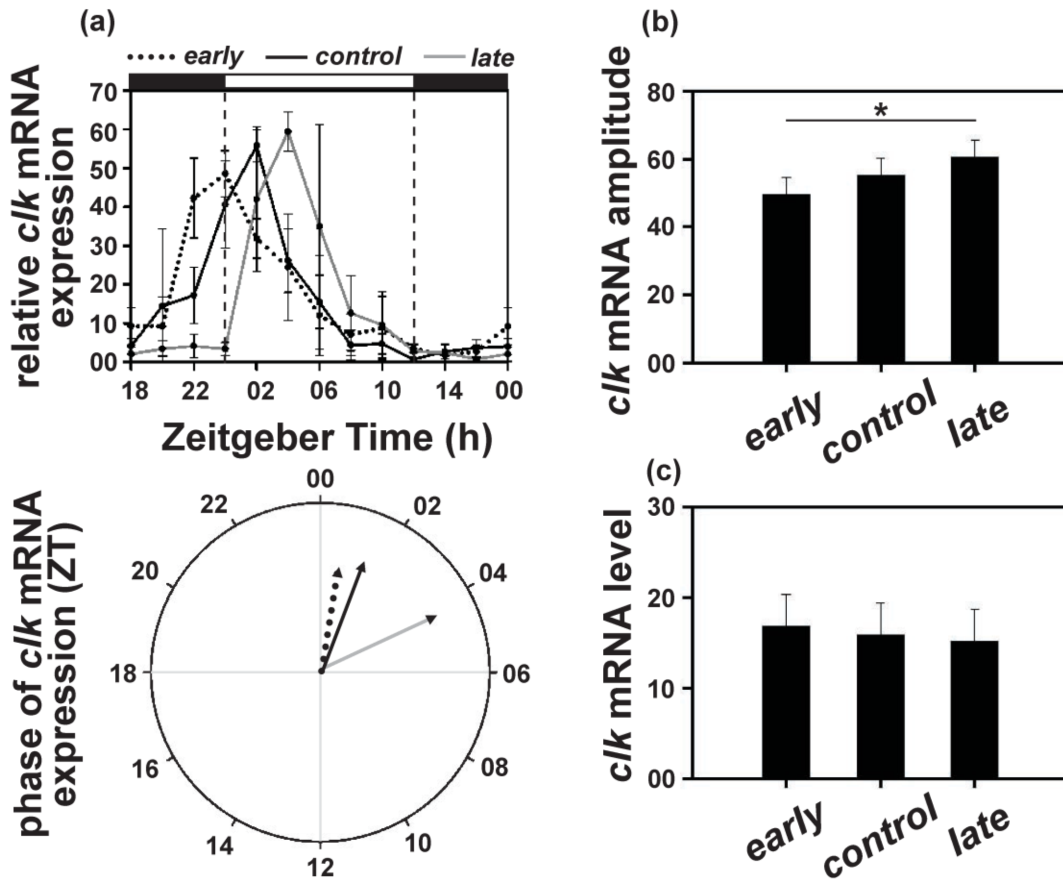


Figure 8: (a) Top panel: *clock* (*clk*) mRNA levels (relative to *rp49*) under LD12:12 in *early*, *control,* and *late* populations across the day. Error bars depict SEM across the four replicate blocks. Bottom panel: The mean phase (in ZT) of *clk* expression (relative to *rp49*) in *early* (14.47 h), *control* (15.19 h), and *late* (16.73 h) populations. (b) Amplitude depicting difference between peak and trough, and (c) average levels depicting the mean *clk* mRNA (relative to *rp49*) levels across the day in *early*, *control,* and *late* populations. All other details are same as in Figure 1.

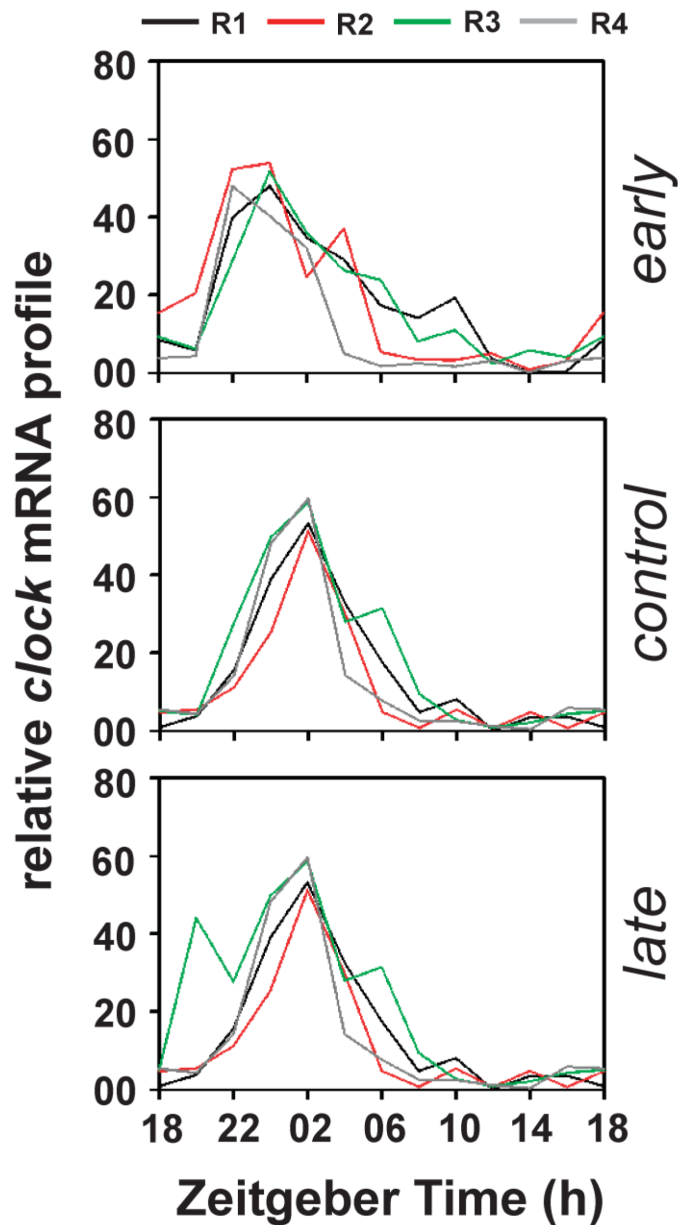


Figure 9: *clock* mRNA expression (relative to *rp49*) profiles for each of the 4 replicate blocks (R1-R4) of *early*, *control,* and *late* populations. The values at each Zeitgeber Time represent an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

population	replicate	phase	amplitude	mRNA level
early	*1*	00.66	47.74	18.43
	2	23.78	53.09	18.74
	3	01.49	49.38	17.80
	4	23.67	47.78	12.15
control	*1*	02.12	53.19	15.25
	2	01.70	50.57	12.10
	3	00.82	57.83	22.05
	4	01.04	59.30	13.86
late	*1*	03.26	66.54	11.77
	2	04.63	55.72	11.89
	3	04.97	62.97	21.03
	4	04.04	56.90	16.00

Table 4. Mean phase (in ZT), amplitude and levels (relative to *rp49*) of *clock* mRNA expression across 4 replicate blocks of *early*, *control,* and *late* populations. The values for each of the measures are an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

(h) ***early and late populations differ in level and amplitude of cry mRNA expression***

The *cry* mRNA levels remained high throughout the first half of the day followed by gradual decrease late in the evening and first half of the night. Therefore, no specific peak of expression could be detected with all the three populations exhibiting similar expression profiles (Figures 10a-top panel, 11). Consequently, the three populations (θ_e = 4.25 h; θ_c =

4.11 h; θ_l = 4.32 h) did not differ statistically in terms of the mean phase of *cry* mRNA oscillation (Figures 10a-bottom panel, 11; Table 5).

ANOVA revealed a statistically significant effect of 'population' for both amplitude ($F_{2,6}$ = 11.96, $p < 0.05$) and average *cry* mRNA levels ($F_{2,6}$ = 20.33, $p < 0.05$). The amplitude of *cry* mRNA in *late* (48.62) populations was significantly lower as compared to that of the other two populations (*early* = 74.99; *control* = 75.98; Figure 10b; Table 5) while *early* and *control* populations did not differ. Additionally, *late* (21.32) populations exhibited significantly attenuated (~35%) *cry* mRNA levels as compared to both *early* (34.14) and *control* (27.60) populations, and its levels in *control* populations were significantly lower than that in *early* populations (Figure 10c; Table 5).

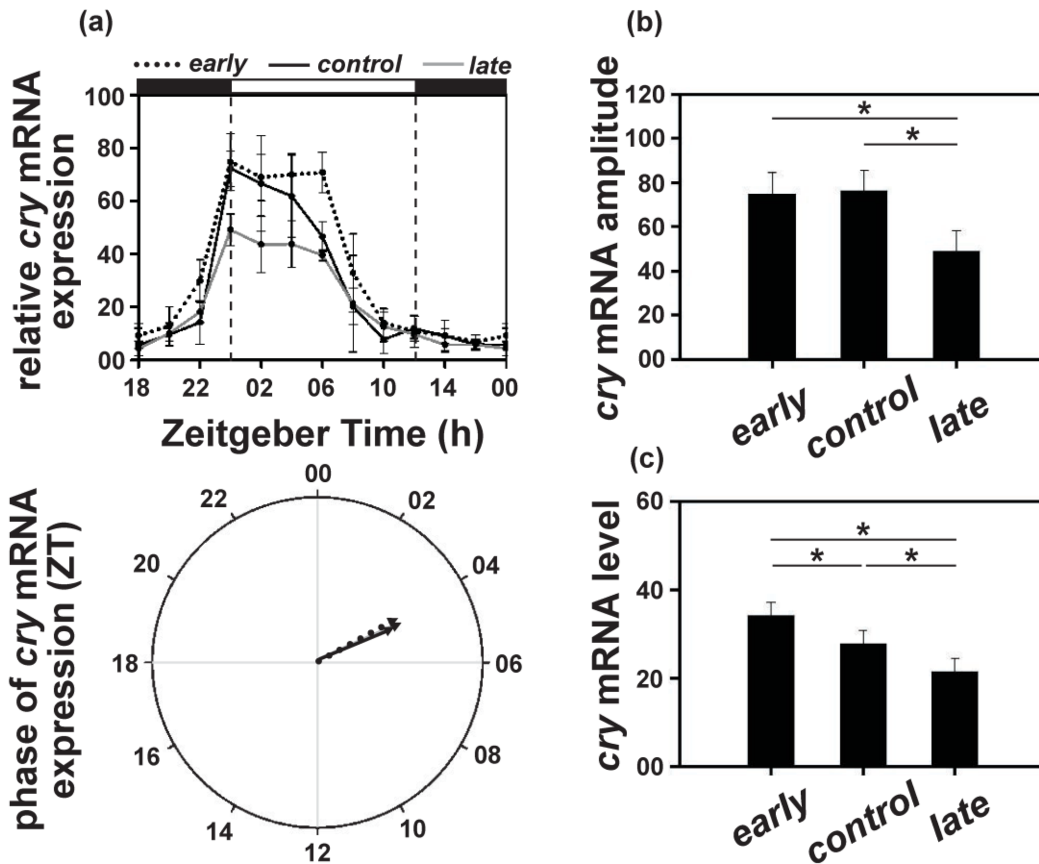


Figure 10: (a) Top panel: *cryptochrome* (*cry*) mRNA levels (relative to *rp49*) under LD12:12 in *early*, *control,* and *late* populations across the day. Error bars depict SEM across the four replicate blocks. Bottom panel: The mean phase (in ZT) of *cry* expression (relative to *rp49*) in *early* (14.47 h), *control* (15.19 h), and *late* (16.73 h) populations. (b) Amplitude depicting difference between peak and trough, and (c) average levels depicting mean *cry* mRNA (relative to *rp49*) levels across the day in *early*, *control,* and *late* populations. All other details are same as in Figure 1.

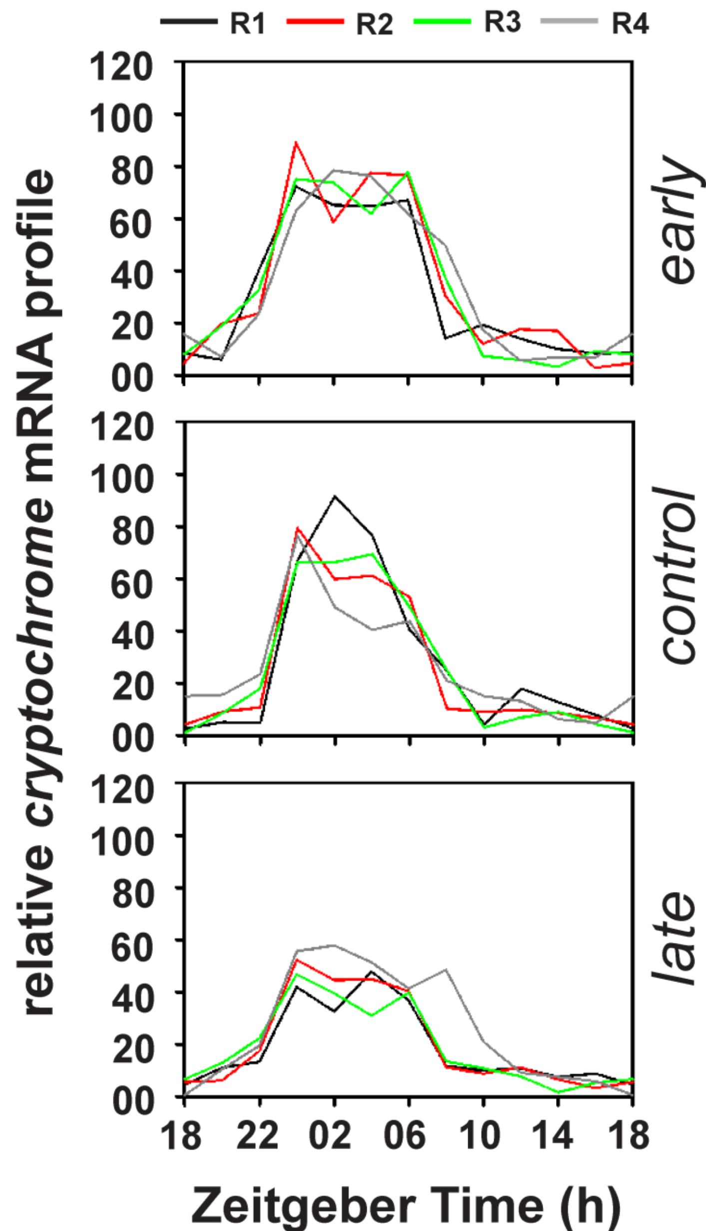


Figure 11: *cryptochrome* mRNA expression (relative to *rp49*) profiles for each of the 4 replicate blocks (R1-R4) of *early*, *control,* and *late* populations. The values at each ZT represent an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

population	replicate	phase	amplitude	mRNA level
early	*1*	04.00	66.27	32.40
	2	04.62	86.18	35.74
	3	03.90	74.54	34.16
	4	04.46	72.68	34.27
control	*1*	04.25	88.78	29.53
	2	04.18	75.17	26.75
	3	04.01	68.23	27.21
	4	04.02	71.73	26.93
late	*1*	04.45	43.17	19.66
	2	04.13	48.94	20.98
	3	03.69	45.09	17.31
	4	05.00	57.27	27.33

Table 5: Mean phase (in ZT), amplitude and levels (relative to *rp49*) of *cryptochrome* mRNA expression across 4 replicate blocks of *early*, *control,* and *late* populations. The values for each of the measures are an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

(i) ***early and late populations differ in phase and amplitude of vri mRNA expression***

The *vri* mRNA levels exhibited a trend similar to that of *per* and *tim* with the levels remaining low during the day and peaking around the first half of the night. The peak of *vri* mRNA occurred at ZT12 for *early*, at ZT14 for *control,* and at ZT16 for *late* populations (Figures 12a-top panel, 13). The phase of *vri* mRNA oscillation in *late* populations was

delayed by ~2.5 h (θ_l = 15.99 h) as compared to that for *early* (θ_e = 13.25 h) and by ~2 h as compared to *control* (θ_c = 13.83 h) populations (Figures 12a-bottom panel, 13; Table 6).

In accordance with *clk* mRNA, amplitude of *vri* mRNA oscillation was significantly higher (~12.5%) in *late* (118.40) populations as compared to *early* (104.22) and *control* (106.44) populations ($F_{2,6}$ = 16.73, $p < 0.01$), while the latter two did not differ (Figure 12b; Table 6); however, the average mRNA levels did not differ among the three populations (*early* = 35.78; *control* = 36.25; *late* = 43.38; Figure 12c; Table 6).

Thus, similar to *per, tim* and *clk*, the mean phase and amplitude of *vri* mRNA oscillation have diverged between *early* and *late* populations.

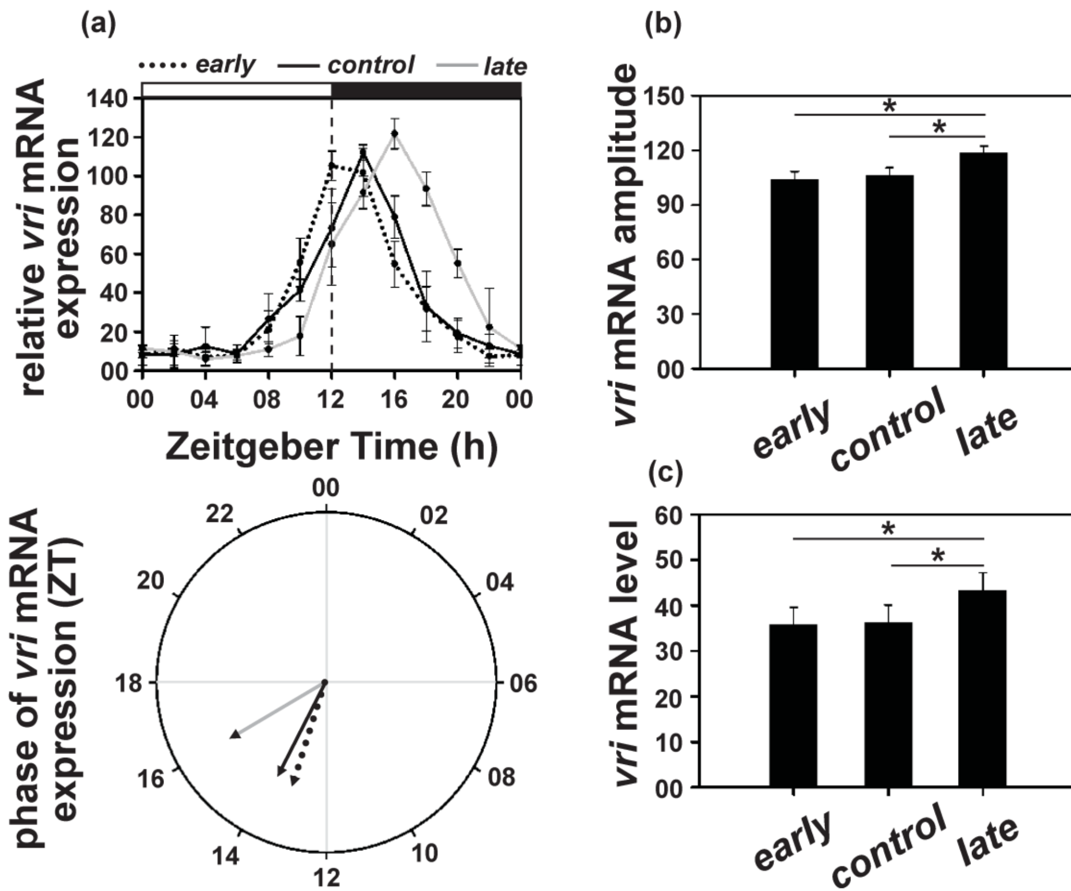


Figure 12: (a) Top panel: *vrille* (*vri*) mRNA levels (relative to *rp49*) under LD12:12 in *early*, *control,* and *late* populations across the day. Error bars depict SEM across the four replicate blocks. Bottom panel: The mean phase (in ZT) of *vri* expression (relative to *rp49*) in *early* (13.25 h), *control* (13.83 h), and *late* (15.99 h) populations. (b) Amplitude depicting difference between peak and trough, and (c) average levels depicting mean *vri* mRNA (relative to *rp49*) levels across the day in *early*, *control,* and *late* populations. All other details are same as in Figure 1.

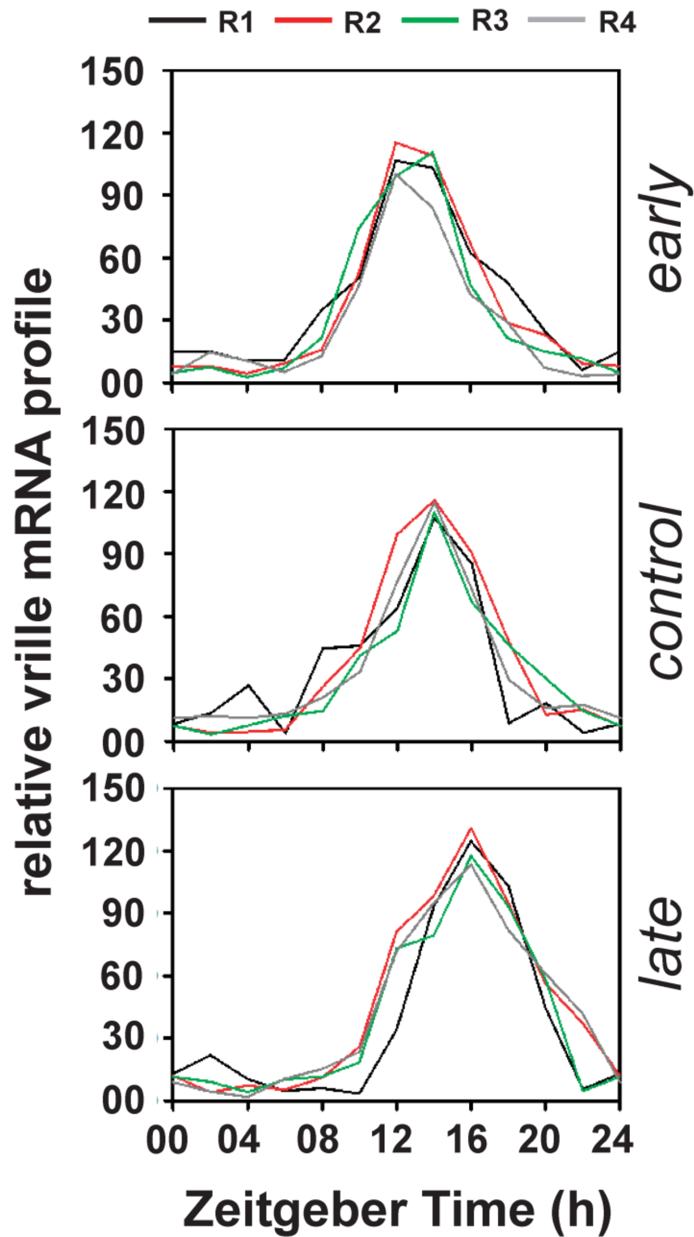


Figure 13: *vrille* mRNA expression (relative to *rp49*) profile for each of the 4 replicate blocks (R1-R4) of *early*, *control,* and *late* populations. The values at each ZT represent an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

population	replicate	phase	amplitude	mRNA level
early	*1*	13.47	100.65	40.57
	2	13.52	110.98	37.49
	3	12.97	108.22	35.14
	4	13.05	97.02	29.91
control	*1*	13.02	103.53	35.88
	2	13.93	111.63	39.47
	3	14.48	106.87	33.88
	4	13.89	103.72	35.77
late	*1*	16.40	121.39	38.75
	2	15.87	126.95	47.03
	3	15.75	113.54	43.64
	4	15.93	111.72	44.10

Table 6: Mean phase (in ZT), amplitude and levels (relative to *rp49*) of *vrille* mRNA expression across 4 replicate blocks of *early*, *control,* and *late* populations. The values for each of the measures are an average of 9 reactions (3 extraction replicates with 3 reaction replicates under each extraction replicate) with 30 adult heads for each reaction.

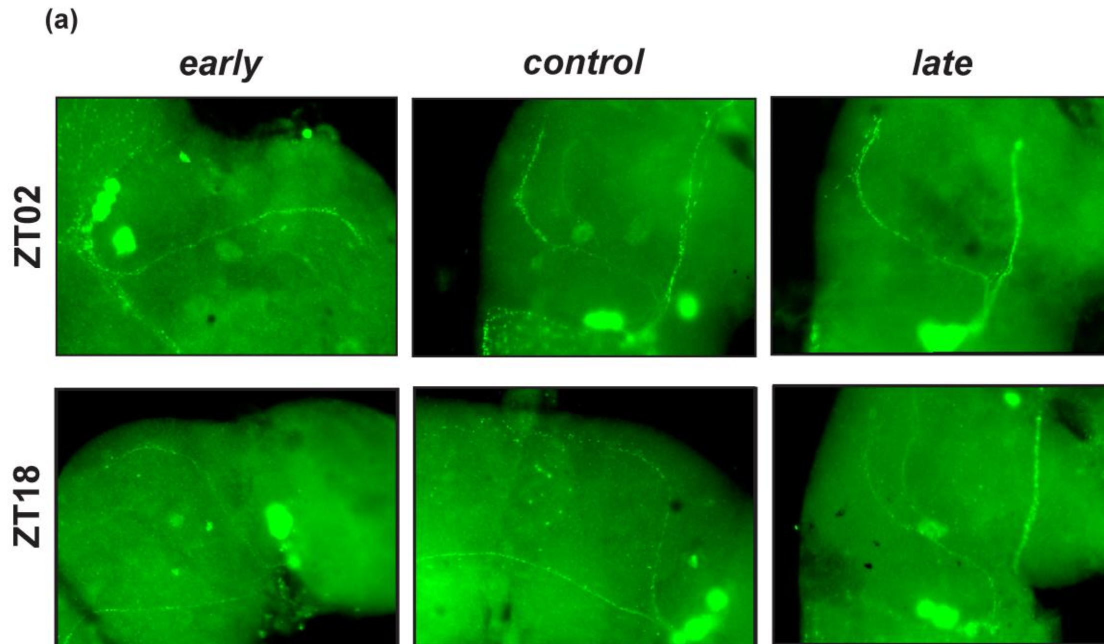
(j) ***early and late populations differ in phase, amplitude and levels of PDF oscillation***

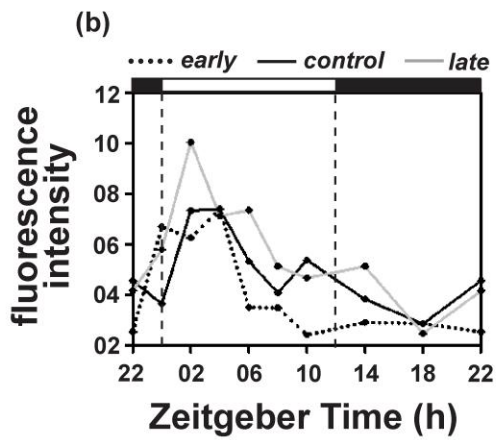
In agreement with previous reports (Park et al. 2000), PDF levels exhibited clear diurnal oscillation in all the three populations (Figures 14a, b-top panel) with peaks around morning and gradually fall through the day to low levels at night. Although, *early* and *late* populations show a similar trend, PDF levels falls more rapidly in *early* populations and more gradually in *late* populations (Figures 14b-top panel, 15).

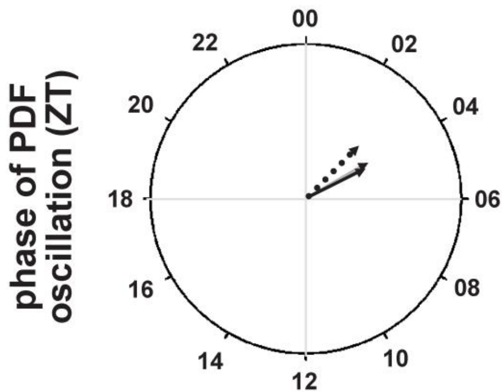
The mean phase of PDF oscillation in *late* (θ_l = 4 h) populations was significantly delayed as compared to *early* (θ_e = 3 h) populations while phase of *control* (θ_c = 4.16 h) populations was close to but slightly delayed in comparison to *late* populations ($F_{2,1497}$ = 130294.9, $p < 0.01$; Figure 14b-bottom panel).

late (7.57) populations exhibited a significantly higher amplitude PDF oscillation as compared to *early* (4.99) populations while amplitude of *control* (4.58) populations was close to but significantly lower ($F_{2,1497}$ = 76115.16, $p < 0.01$; Figure 14c) than *early* populations. Also, *late* (5.77) populations showed higher levels of PDF differing significantly from both *early* (4.23) and *control* (4.94) populations with *early* populations exhibiting the lowest PDF levels ($F_{2,1497}$ = 362521.7, $p < 0.01$; Figure 14d).

Thus, *early* and *late* populations have evolved divergent mean phase, amplitude, and average PDF levels.


(a)
early
control
late
ZT02
ZT18


(b)
early
control
late
fluorescence intensity
Zeitgeber Time (h)
12
10
08
06
04
02
22
02
06
10
14
18
22


phase of PDF oscillation (ZT)
00
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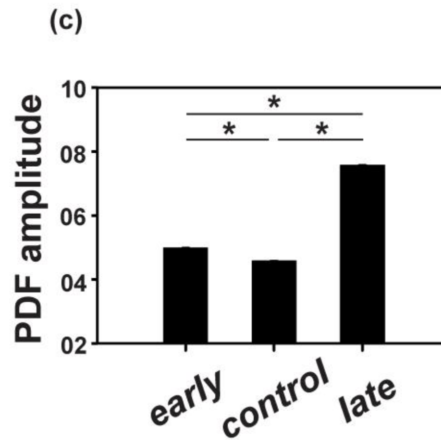
(c)
PDF amplitude
10
08
06
04
02
*
*
*
early
control
late

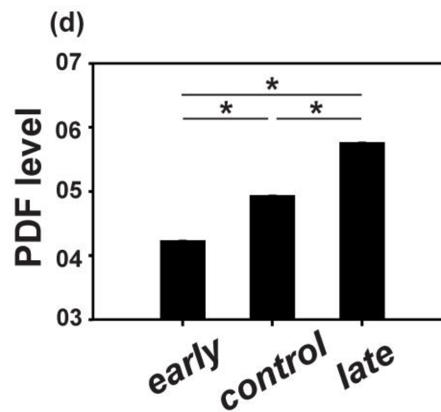
(d)
PDF level
07
06
05
04
03
*
*
*
early
control
late

Figure 14 (previous page): (a) Representative images depicting PDF levels in the sLNv terminals at ZT02 (top panel) and ZT18 (bottom panel) in individuals sampled from *early*, *control,* and *late* populations. The cell bodies appear to be blurred as they are present on a different stack/plane, as the images are focused for clarity of small ventral-lateral neurons (sLNv) terminals from which the florescence intensity was quantified. (b) PDF staining intensity in the sLNv terminals across the day (top panel) and corresponding mean phase of PDF oscillation (bottom panel) (n = 6-10 brains per time point). Note that fly brains were sampled at 2 h intervals between ZT22-10, and at 4 h intervals between ZT10-22. (c) Amplitude depicting difference between peak and trough, and (d) average levels depicting mean PDF levels across the day in the brains of individuals sampled from *early*, *control,* and *late* populations. Error bars depict 99% CI calculated by Tukey's HSD following ANOVA, and asterisks (*) indicate statistically significant differences ($p < 0.01$). Data presented in panels b-d are bootstrapped from experimental raw data (presented in Figure 15).

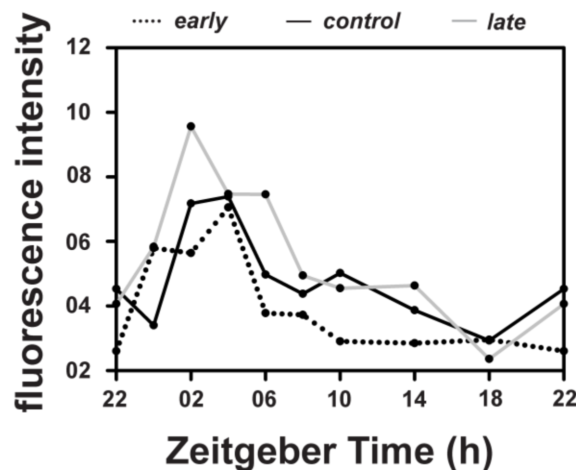

Figure 15: PDF staining intensity profiles in the brains (n = 6-10 brains per time point each) for the *early*, *control,* and *late* populations across the day. The intensity values presented here were obtained by calculating the fluorescence intensity in the small ventral-lateral (sLNv) neuron terminals separately for each hemisphere and subtracted from the background intensity using ImageJ. Note that fly brains were sampled at 2 h intervals between ZT22-10, and at 4 h intervals between ZT10-22.

6.4 Discussion

In spite of widely observed circadian clock-chronotype associations, our understanding of the underlying molecular-genetic bases remains largely elusive primarily due to (a) unavoidable shortcomings of the existing methodologies for such studies in humans (reviewed in Levandovski et al. 2013), and (b) lack of suitable model systems (see Introduction). With these shortcomings in consideration, we raised *D. melanogaster* populations that exhibit *early* and *late* emergence chronotypes, and have reported the coevolution of divergent circadian clocks by generation 55 (Kumar et al. 2007a). To examine if the circadian phenotypes of the *early* and *late* populations persisted or have further diverged over 250 generations, we assayed the emergence rhythm of the three sets of populations under LD12:12, winter and summer type photoperiods, and observed that the mean phase of emergence rhythm of the *early* and *late* populations has diverged by ~4 h under LD12:12, and the chronotype differences persist robustly in winter type and summer type photoperiods (Figure 1a-c). However, under summer type photoperiod, gating of emergence was drastically affected in *late* populations with emergence spread throughout the day unlike the other two populations, which exhibited clear gating of emergence (Figure 1c). In a previous study (Nikhil et al. 2016b), we had reported that under dim LL condition *late* populations exhibit higher incidence of arrythmicity in their activity-rest behaviour as compared to the two other sets of populations. These results collectively suggest that *late* populations have evolved weaker gating/consolidation mechanisms, rendering them more vulnerable under unfavourable environments. The τ of emergence rhythm in the *early* and *late* populations has diverged by 1.35 h while that of activity-rest rhythm by 0.7 h (~0.1 h and ~0.2 h greater than that reported in Kumar et al. 2007a). Thus, while the phase of emergence has drastically diverged between the *early* and *late* populations, the circadian

period exhibits a relatively smaller divergence. Interestingly, in spite of divergence in τ of activity-rest rhythm, the mean phase of the *early* and *late* populations did not differ under LD12:12, but were different under winter and summer type photoperiods (Figure 2a, b). Under all the three photoperiods, *late* populations consistently exhibited higher evening activity as compared to the *early* populations (Figure 2c). An interesting yet intriguing result was that of phase advancement of the evening activity peak in *late* populations under summer type photoperiod. In *Drosophila*, the activity-rest rhythm is postulated to be controlled by a Morning (M) - Evening (E) oscillator network (Stoleru et al. 2007) which is coupled by PDF and mediates the morning and evening activity components (reviewed in Yoshii et al. 2012). Such population dependent differences in activity-rest rhythm across photoperiods discussed above appear to suggest that the M-E oscillators might be differentially coupled in *early* and *late* populations and may possibly involve PDF (reviewed in Yoshii et al. 2012) as has been hypothesized in our recent study (Nikhil et al. 2016a).

We also studied the molecular correlates of *early* and *late* emerging populations, and found that the mean phase of mRNA expression of *per, tim,* and *clk* genes in *early* and *late* populations have diverged significantly (Figures 4-9). In accordance with the differences in their circadian periods, *late* populations exhibited a significantly delayed phase of *per* (~1.5 h) and *tim* (~2.5 h) mRNA expression as compared to *early* populations (Figures 4a, 5a), which is consistent with the results of a previous study which reported a delayed phase of *per* and *tim* mRNA oscillations in the long period mutant of *Drosophila* (Hardin et al. 1990; Rothenfluh et al. 2000). Accordingly, *late* populations also exhibited a delayed (by ~2.5 h) phase of *clk* mRNA expression (Figure 8a) as compared to *early* populations suggesting that

the observed differences in the phase of *per* and *tim* mRNA oscillations might be due to divergent evolution of their transcriptional regulation by CLK. While CLK in association with CYC is known to mediate transcription of *per* and *tim*, *cyc* mRNA expression has not been observed to oscillate across the day and therefore we did not assess *cyc* mRNA expression. We had previously reported that *late* populations exhibit high amplitude activity-rest rhythm under both dim LD and DD conditions, which prompted us to hypothesize that these populations might have evolved high amplitude circadian oscillators (Nikhil et al. 2016a), and this is corroborated by our findings of high amplitude oscillations of *per* and *clk* mRNA in *late* populations as compared to *early* populations.

The *early* and *late* populations show clear difference in their circadian photosensitivity. To display their characteristic LD12:12 emergence waveform *early* populations require more light in the evening while *late* populations require more light in the morning (Vaze et al. 2012a). To explore the molecular correlates of the observed differences in circadian photosensitivity we assessed *cry* mRNA expression, and interestingly, *late* populations although did not differ in the mean phase of mRNA expression, exhibited significantly lower amplitude oscillation and reduced average levels of *cry* mRNA as compared to *early* populations (Figure 5). Lower *cry* mRNA level in *late* populations is also consistent with the reduced ability of *late* populations in recovering from jetlag (Nikhil et al. 2016a).

Since the experimental protocol employed to generate *early* and *late* emerging populations involved selection for timing of an overt circadian behaviour (emergence rhythm), divergent evolution of molecular circadian clockwork in *early* and *late* populations can also arise due to differences in downstream effectors/pathways linking the circadian

clocks to emergence rhythm. Initiation of emergence in insects requires 20-hydroxyecdysone (20-HE), which acts via the ECR/HSP receptors (Truman et al. 1983; Riddiford et al. 1993; Gilbert, 2011). Furthermore, another nuclear receptor, ecdysone-induced protein 75 (Eip75/E75) has been implicated in the maintenance of circadian rhythms in *Drosophila* linking the steroid hormone pathways with circadian clocks (Kumar et al. 2014). Since *early* and *late* populations exhibit significant differences in their egg-to-adult developmental rate (Kumar et al. 2006), which is known to be mediated by ecdysteroids, these results suggest that neuroendocrine factors might also mediate chronotype divergence between *early* and *late* populations. The clock gene *vri* which is under the direct control of CLK and constitutes an additional interlocked loop of core molecular clock, has been attributed as a downstream effector of circadian clocks thereby mediating rhythmicity in overt behaviours (Cyran et al. 2003; Glossop et al. 2003; Zheng et al. 2009). *vri* is also one of the direct targets of ECR/HSP (Beckstead et al. 2005), making it a putative candidate linking circadian clocks to the timing of emergence. Therefore, we assessed *vri* mRNA profiles in *early* and *late* populations and found that *vri* expression is significantly phase delayed in *late* populations as compared to *early* populations (Figure 12). Furthermore, in corroboration with the *clk* mRNA oscillation, *late* populations also exhibited higher amplitude and levels of *vri* expression as compared to *early* populations. Overexpression of *vri* is known to lengthen the clock period and causes higher incidence of behavioural arrythmicity (Blau and Young, 1999), a phenotype akin to *late* populations (Figures 1, 2; Nikhil et al. 2016a). These results suggest that selection for altered phase of emergence leads to divergent expression of *vri* (and other possible downstream effectors),

which by virtue of its role as transcriptional activator of *clk,* might have resulted in the divergent evolution of molecular circadian clockwork.

Furthermore, we have previously reported that some of the behavioural phenotypes observed in *late* populations mirror the dynamics of a weakly coupled circadian network including that of higher susceptibility to exhibiting complex and arrhythmic activity-rest behaviours (Nikhil et al. 2016a). While we hypothesized that lower PDF levels might drive reduced coupling, flies with null mutations for *pdf* (pdf^{01}) are known to exhibit shorter clock period (Renn et al. 1999), which is contrary to that observed in *late* populations. Alternatively, overexpression of PDF has been reported to increase activity levels in DD, lengthen clock period (and shorten it in certain neurons), and induce complex and arrhythmic behaviours in *Drosophila* possibly due to mutual desynchronization of the constituent oscillators (Wülbeck et al. 2008). To gain further insights into the possible molecular correlates of such hypothesized coupling differences, we assessed PDF levels in sLNvs of the three sets of populations. Interestingly, we observed that *late* populations exhibit significantly higher amplitude oscillation and levels of PDF, which is also phase delayed as compared to *early* populations (Figure 14). In light of the reported effects of enhanced PDF levels by Wülbeck et al (2008), PDF profiles of *late* populations appear to corroborate their behavioural phenotypes including complex and arrhythmic behaviours, longer clock period and high amplitude activity-rest behaviour. Furthermore, Helfrich-Förster et al (2000) reported that overexpression of PDF leads to loss of gating in emergence rhythm shortly following transfer to DD thereby resulting in apparent arrhythmic emergence profiles. Interestingly, we also observe a somewhat similar phenotype in *late* populations with the emergence gate widening by day 4 following transfer

to DD and relatively higher subjective night time emergence during days 5-6 (Figure 1d). Therefore, higher PDF levels in *late* populations appear to underlie multiple behavioural phenotypes.

We report that evolutionary divergence of *early* and *late* emergence chronotypes is associated with correlated changes in the phase, amplitude and average levels of mRNA expression of the core clock genes *per*, *tim, clk,* and *vri*. This is in striking contrast to the results of Pegoraro et al (2015) who surprisingly did not observe difference in the expression of any circadian genes in another set of *early* and *late* emerging *Drosophila* strains, possible reasons for which have been discussed earlier. In addition, we report differences in *cry* mRNA levels which might underlie differential entrainment to LD cycles thus contributing to divergent ψ_{ent} between the two populations. Thus we report that selection for early and late phase of emergence leads to divergent evolution of molecular circadian clocks and individuals with advanced and delayed phase-of-entrainment (chronotypes) are associated with a similar advanced and delayed phase of entrained molecular oscillation of core clock genes, and propose that such differences in entrained molecular oscillations might drive an advanced or delayed expression of output components such as *vri* and other downstream factors thereby driving different phases-of-entrainment. Furthermore, in accordance with our previously reported behavioural differences, we observe that divergent phase-of-entrainment is associated with the coevolution of differential photosensitivity and coupling of constituent oscillators which appear to be driven by *cry* and PDF.

To the best of our knowledge, ours is the first study of its kind to report the evolution of divergent molecular circadian clockwork in response to artificial selection for

the timing of emergence. Although it is likely that mRNAs expression may not entirely reflect their respective protein profiles as differences in circadian phenotypes can also stem from differential post transcriptional and post translational regulations, in light of considerable variation exhibited by the outbreeding populations (compare across block variation) we adopted a more stringent quantitative method to assay the expression differences and therefore decided to assay mRNA and not protein expression as the latter would involve a semi quantitative method that might reduce the chance of detecting inter population differences. Therefore, this would remain a caveat of the current study which will be addressed in future studies, since the fact that mRNA level differences are by themselves statistically significant provides the motivation for future studies. Nevertheless, our study highlights *early* and *late* populations as a potential model system for the study of chronotypes and underlying molecular genetic correlates, and with the preliminary groundwork established in the current study, future studies will help further elucidate several other molecular underpinnings of the clock-chronotype associations.

Chapter 7

Differences in life-history traits between *early* and *late* populations

The contents of this chapter has been submitted as the following research article:

KL Nikhil, K Ratna and VK Sharma (2016). Life-history traits of *Drosophila melanogaster* populations exhibiting *early* and *late* eclosion chronotypes. (*Submitted manuscript*).

7.1 Introduction

It is believed that circadian time keeping mechanisms underlying rhythmic processes provide adaptive advantage to organisms (Cloudsley-Thompson, 1960; Aschoff, 1967; Pittendrigh, 1993; Fleury et al. 2000; Emerson et al. 2008; Vaze and Sharma, 2013; West and Bechtold, 2015), and has prompted studies employing a variety of strategies to examine the adaptive benefits of possessing functional circadian clocks. Surgical ablation of the mammalian 'master circadian clock' - suprachiasmatic nucleus (Dunlap et al. 2004), and genetic manipulation of circadian clocks in fruit flies *Drosophila melanogaster* (Saunders, 2002), which are both known to cause loss of rhythmicity in several key daily behaviours, result in reduced longevity (DeCoursey et al. 1997, 1998, 2000; Daan et al. 2011). Additionally, environmentally induced, or naturally occurring circadian dysfunction has been reported to reduce longevity in *D. melanogaster* (Allemand et al. 1973; Kumar et al. 2005). In separate sets of studies, Beaver et al (2003) reported that *D. melanogaster* strains carrying loss of function mutation in two core clock genes exhibit reduced reproductive output. In addition, studies on organisms inhabiting different latitudes as well as those living in constant conditions reported large variation in circadian phenotypes in accordance to their local habitats, suggesting that the underlying clocks might have evolved as an adaptation to the presence or absence of local cyclic environmental conditions (Blume et al. 1962; Poulson and White, 1969; Lankinen, 1986, 1993; Pittendrigh and Takamura, 1989; Costa et al. 1992; Rosato et al. 1994; Sawyer et al. 1997, 2006; Michael et al. 2003; Tauber et al. 2007; Vaze and Sharma, 2013). Nevertheless, conclusions drawn from such studies are limited by the lack of adequate information about the ancestry, population size and

history of the environmental conditions pertaining to organisms' ecology (Vaze and Sharma, 2013).

The emergence waveform of *D. melanogaster* comprises a primary peak at dawn (under natural conditions) or around night-day transition (in the laboratory), which gradually reduces through the day with little or no emergence occurring at night (Skopik and Pittendrigh, 1967; Saunders, 2002). The restriction/gating of emergence primarily during dawn is hypothesized to be an adaptation to avoid desiccation of pharate adults due to high temperature and low humidity prevailing during the rest of the day (Pittendrigh, 1993), that appears to be partly supported by the results of a recent study (De et al. 2012). Laboratory selection approach has been previously adopted to study how circadian clocks evolve in response to selection for phasing of adult emergence. Selection for early and late emerging strains of *Drosophila pseudoobscura* and moth *Pectinophora gossypiella* under LD12:12 resulted in the evolution of divergent phase of emergence (4 h in *D. pseudoobscura* and 5 h in *P. gossypiella* (Pittendrigh, 1967; Pittendrigh and Minis, 1971). As a correlated response to selection, early flies in both studies evolved longer circadian clock periods while late flies evolved shorter period. However, these studies also suffered from some major shortcomings such as lack of population level replication and details of population ancestry and selection protocols employed (population maintenance methodology, population size, and sex ratio) which are known to considerably affect the evolutionary trajectories of populations under selection; and thus might have led to misinterpretation of the observed responses to selection (reviewed in Vaze and Sharma, 2013). Although the studies outlined above suggest that circadian clocks might have evolved to ensure temporal order in behaviour and physiology thus enhancing Darwinian

fitness (reviewed in Vaze and Sharma, 2013), our understanding of how selection for timing of clock controlled behaviours influences life-history traits remains nominal.

To explore the evolutionary trajectory of circadian clocks in response to selection for timing of adult emergence, we initiated a long term study on *D. melanogaster* populations by imposing selection for emergence during early morning and late evening hours, which is in contrast to the usual time of emergence in this species. From a set of 4 ancestral *control* populations we derived a set of 8 populations - 4 replicate *early* populations using flies that emerge early in the morning and 4 replicate *late* populations using flies that emerge late in the evening (see materials and methods for detailed selection protocol). Consequently, $early_{1\text{-}4}$ and $late_{1\text{-}4}$ populations evolved significantly higher morning and evening emergence respectively relative to $control_{1\text{-}4}$ populations, and exhibited several properties analogous to the well-known 'morning/early' and 'evening/late' chronotypes in humans. Similar to *early* and *late* human chronotypes (Duffy et al. 1999, 2002; Roenneberg et al. 2003), *early* and *late Drosophila* populations evolved shorter and longer clock periods respectively with *control* populations exhibiting intermediate period (Kumar et al. 2007a), and also exhibited diverged photic phase response curves (PRCs) for both adult emergence (Kumar et al. 2007a) and activity-rest rhythms (Nikhil et al. 2016a). These results indicate that the circadian clocks of the two sets of populations ‘entrain’ differently to light/dark (LD) cycles, or in other words they are differentially sensitive/interact differentially with LD cycles. This is corroborated by previous findings that *early* populations are primarily sensitive to light in the evening while *late* populations are sensitive to light primarily in the morning (Vaze et al. 2012a). Collectively, these results suggest that the divergent correlated evolution of the underlying circadian clocks in

early and *late* populations might further mediate differential interaction/entrainment to regulate time of emergence.

In the present study, we used *early* and *late* populations to examine genetic correlations between the mechanisms that underlie emergence at specific times of the day and various pre-adult (pupariation and development time, egg-to-puparium, egg-to-adult survivorship and puparial dry-weight) as well as adult life-history traits (dry-weight at emergence, fecundity, dry-weight pre- and post-fecundity assay and longevity). Pre-adult traits such as development time are known to be correlated with circadian clock period, and additionally *early* and *late* emergence chronotypes have been shown to be associated with different circadian clock periods and differential entrainment to LD cycles (Kumar et al. 2007a; Vaze et al. 2012a). Therefore, to assess the relative contribution of these two factors to possible differences in life-history traits between *early* and *late* populations we performed some of the experiments under both 12:12 h light/dark cycles (LD12:12) as well as constant darkness (DD). The rationale being that if differences in life-history traits between the populations are solely determined by circadian clock period, as can be observed under DD when the circadian clock is not under the influence of external LD cycles, such differences would either decrease or cease to exist under LD12:12 where clock period of all the populations would be held at 24 h by virtue of entrainment (Dunlap et al. 2004). Persistence of differences between populations under both light regimes would imply that the observed life-history trait differences are also under the influence of clock period independent factors.

As mentioned earlier, since *D. melanogaster* emerge predominantly during "dawn", emergence at other times of the day is considered to be maladaptive. If this is true, then the

proportion of individuals which normally emerge early in the morning in *control* populations might also differ in terms of fitness from those that emerge late in the evening. To test for such a possibility, one generation before the assays we derived 8 additional populations from *controls* - 4 populations comprising individuals emerging early in the morning, referred to as *early-control*, and similarly, 4 populations comprising individuals emerging late in the evening, referred to as *late-control*. *early-control* and *late-control* populations are likely to reveal whether the observed differences in fitness between *early* and *late* populations (if any) are indeed evolved responses to the selection imposed, or are merely environment driven.

We report that *late* populations have evolved significantly longer pupariation time leading to longer development times, are more fecund on day 11 post-emergence which is the usual day for egg collection as per the selection protocol (discussed later), and also exhibit reduced longevity compared to *early* populations, whereas no difference in the aforesaid life-history traits was observed between *early-control* and *late-control* populations, suggesting that the observed differences between the selected populations (*early* and *late*) are evolutionary responses to selection for timing of emergence. Also, *early* populations even though differed significantly from *late*; they were similar to *control* populations for most of the traits assayed, the possible reasons for which are discussed later. Our results thus highlight possible genetic correlations between emergence chronotypes and life-history traits in *D. melanogaster* populations.

7.2 Materials and Methods

(a) *Experimental populations*: Details of experimental populations and maintenance protocol are described in chapter 2. In addition to the four replicate populations each for *early*, *control* and *late*, we used four replicates each for the two other populations (*early-control* and *late-control*; see 'Introduction'). From *control* populations, flies emerging in the morning window (ZT21-01) were collected to form *early-control* populations and similarly, flies emerging in the evening window (ZT09-13) formed *late-control* populations. This procedure was implemented on all four replicates of *control* populations, for only one generation prior to the assays, and therefore, unlike *early* and *late* populations, *early-control* and *late-control* populations were not subjected to any long term selection protocol.

To minimize the effects of non-genetic inheritance (reviewed in Garland and Adolph, 1991) due to different selection regimes, all populations were subjected to one generation of standardization with the maintenance protocol same as that used for *control* populations. This was achieved by relaxing selection on timing of emergence by collecting all flies that emerged throughout the first 4 days similar to that for *control* populations, and the population size was kept constant at ~1200 flies per replicate population. Since the primary purpose of using *early-control* and *late-control* populations was to asses if the observed differences between *early* and *late* populations are evolved responses to selection as not merely environmental in origin, these populations were also subjected to standardization by deriving them from *control* populations followed by relaxation of selection for one generation as described above. All assays described in the present study were performed on the progeny of the standardized populations at the 242nd generation (~14

years) either in LD12:12 or DD, or both, with light intensity, temperature, and humidity same as that for the maintenance of populations.

(b) ***Pupariation time assay*****:** Pupariation time for all the populations was assayed under two light regimes - LD12:12 and DD. After having provided yeast paste supplemented media for three days, all populations were provided with media plates for 1 h as substrate for oviposition. These plates were then replaced by fresh media plates for the next 1 h. Eggs laid on these plates were collected and 30 eggs were dispensed into each vial. A total of 10 such vials were used per replicate population per light regime making a total of 300 eggs per population per light regime. These vials were transferred to respective light regimes and monitored for the first pupariation event. After the first puparium was observed, vials were checked every two hours to count the number of puparia formed thereafter, and the assay was terminated when no pupariation event was seen for 24 consecutive hours. It was observed that a small proportion of larvae took relatively longer to pupariate, thus rendering the pupariation time distribution right skewed (Figures 1a, b, 2). Mean pupariation time cannot be used as a reliable measure for such distributions (Sokal and Rohlf, 1995) and therefore, we used median pupariation time (calculated as the time from egg collection for 50% of total pupariation events in a vial) for the same. The median pupariation time was estimated for every replicate vial and then averaged across vials to obtain average median pupariation time for a given replicate population.

(c) ***Egg-to-adult development time assay*****:** Egg collection protocol and environmental conditions for the egg-to-adult development time assay were identical to pupariation time assay. After egg collection and transfer to LD12:12 or DD, emergence of the first adult fly was monitored following which vials were subjected to two hourly checks to count the

number of flies that emerged thereafter. The assay was terminated when no emergence event was observed for 24 h. To facilitate comparisons between pupariation and development times, we used median development time as a measure for analysis. The procedure to estimate median development time was same as that described for median pupariation time.

(d) ***Estimation of egg-to-puparium and egg-to-adult survivorship*****:** Egg collection protocol and environmental conditions for the survivorship assays were same as that for the pupariation and development time assay. Proportion of 30 eggs (total number of eggs dispensed per vial for the assay) that successfully pupariated was used as a measure for egg-to-puparium survivorship while proportion of adults that successfully emerged was used to estimate egg-to-adult survivorship. Individuals that were stuck in the pupal case and died within the pupa were considered as individuals that did not emerge successfully. Percentage survivorship was calculated for every replicate vial and then averaged across vials to obtain average survivorship per replicate population.

(e) ***Dry-weight at pupariation*****:** The protocol for egg collection and subsequent environmental conditions for development under LD12:12 and DD was the same as that described for pupariation time assay. From the initiation of the first pupariation event, freshly formed puparia (P1 stage) were collected every 2 h and frozen at -20 ^{o}C. These puparia were later sorted into 10 replicate groups with 5 puparia in each group; dried at 70 ^{o}C for 36 h after which their dry weights were assayed. Dry-weight of each group was measured at least thrice to account for instrument error and then normalized by the number of puparia (5 puparia). The dry-weight measurements from 10 such groups were then averaged to obtain mean puparium dry-weight per replicate population.

(f) ***Dry-weight at emergence*****:** The protocol for assaying dry-weight at emergence was the same as that for puparium dry-weight assay except that freshly emerged adult flies (within 2 h of emergence) in LD12:12 or DD were used.

(g) ***Fecundity assay*****:** Fecundity was assayed only under LD12:12 since populations used in the present study are maintained on a 21 day discrete generation cycle where eggs for the next generation are collected on day 21 post egg collection (average adult age of 11 days). Since only eggs laid around this day would determine an individual's contribution to the gene pool for the next generation and consequently to its fitness we estimated fecundity only under LD12:12 around day 11 (post-emergence) in the progeny of standardized populations, which were collected in plexi glass cages and maintained under LD12:12 in mixed sex groups similar to that used for regular maintenance of populations. On day 8 (average adult age), flies from plexi glass cages were collected, separated using mild carbon di oxide anaesthesia and transferred into vials containing ~4 ml BJ medium for conditioning at a density of 10 flies/vial (5 of each sex). In parallel, additional sets of conditioning vials were set aside from which flies for pre-fecundity dry-weight assay were to be collected later (described in the following section). On day 10, flies from the conditioning vials were sorted into single male female pairs and transferred into 20 vials/population containing 1 ml BJ medium. After 24 h (day 11), flies were transferred to fresh set of vials and the same was repeated on day 12. Average number of eggs laid per female across days 10-12 was used as a measure of mean fecundity/female around day 11. Only vials from which data could be collected for all three days were used and those in which either male or female died within the three days were not used for data analysis.

(h) ***Estimation of pre- and post-fecundity dry-weights*****:** To assess pre-fecundity dry-weight of females, 20 females (for every replicate population) from separate sets of conditioning vials (which were not used for fecundity assay) as described in the preceding section were frozen at -20 ^{o}C at the beginning of day 10. Additionally, at the end of the fecundity assay (end of day 12), females used for the assay were collected and frozen. All flies were then dried at 70 ^{o}C for 36 h, sorted into groups of 5 individuals each and weighed at least thrice to estimate dry-weight/female. Dry-weight measurements were then averaged across groups to calculate mean pre- and post-fecundity dry-weight/female/replicate population. Further, dry-weight loss during fecundity assay was estimated by calculating the difference in pre- and post-fecundity dry-weights and was used to normalize the fecundity/female values to calculate fecundity per unit dry-weight lost as an estimate for biomass to egg conversion ratio. However, this is under the assumption that the biomass lost is entirely converted to eggs laid which may not necessarily be the case but nevertheless can be used as a proxy for assessment of for biomass to egg conversion ratio.

(i) ***Longevity assay*****:** Longevity of flies was assayed only in LD12:12 with environmental conditions same as described previously. Freshly emerged virgin males and females were collected from the progeny of standardized populations every 6 h over three consecutive days. On the fourth day, all flies of a given sex and population were mixed and randomly distributed in groups of 10 flies/vial/sex into 10 replicate vials containing ~4 ml BJ medium. Therefore, every replicate population comprised 20 vials in total with 10 vials for each sex and each vial housing 10 flies (average age of 2 days). Thereafter, flies were transferred to fresh BJ medium every 3rd day and longevity was estimated by counting the number of dead flies in each vial every 24 h. The assay was continued until all flies were

dead. While care was taken to ensure no flies escaped when they were being transferred to fresh vials, a few of them either escaped or were crushed between the cotton plug and the vial, and hence were not considered for calculating percentage survivorship for that vial. Similar to pupariation time, longevity distribution was also right skewed and therefore, we used median longevity (time taken for the death of 50% of individuals in a given vial) as the measure of longevity.

(j) ***Statistical analyses*****:** All measures of survivorship, pupariation time, development time, fecundity, dry-weight and longevity were estimated for every replicate vial and then averaged across replicate vials to obtain mean values for all replicate populations. These replicate means served as data for statistical analyses by a randomized block design mixed model analysis of variance (ANOVA) with 'population', 'light regime', 'stage (at which fecundity was assayed)' or 'sex' (whichever was appropriate) as fixed factors and 'replicate population' as random factor. All percentage and ratio values were arcsine square root and log transformed respectively before subjecting them to ANOVA. Post hoc multiple comparisons were performed at a significance level (α) of 0.05 by method of Tukey's HSD. All statistical analyses were implemented on STATISTICA for Windows, Release 5.0B (Statsoft, 1995).

7.3 Results

(a) ***late populations exhibit longer pupariation time under both LD and DD***

ANOVA on median pupariation time showed statistically significant effect of population, light regime and population × light regime interaction (Table 1a). Across light regime comparisons revealed that pupariation time for all the populations was significantly longer

(8.4 h or 7%) in LD12:12 as compared to DD suggesting that LD cycles have a delaying effect on egg-to-pupariation duration (Figures 1a, b, c, 2; Table 2).

In LD12:12, *late* populations had a significantly longer (6.5 h or 5.4%) pupariation time (129.11 h) compared to all other populations (*early* = 122.43 h, *early-control* = 123.01 h, *control* = 121.84 h, and *late-control* = 122.89 h) while pupariation times of the remaining four sets of populations did not differ among each other (Figures 1a, c, 2; Table 2).

In DD, *late* populations took significantly longer (118.14 h) by 5 h or 3.6% to pupariate compared to *early* (113.57 h) and *control* (114.47 h) populations but did not differ from *early-control* (114.83 h) and *late-control* (116.32 h), whereas *early, early-control*, *control* and *late-control* populations did not differ among each other (Figures 1b, c, 2b; Table 3).

trait	effect	df	MS	*F*	*p*
(a) median pupation time	population	4	42.60	48.80	<0.0001
	regime	1	704.4	2261.7	<0.0001
	population × light-regime	4	5.70	3.70	0.0346
(b) egg-to-pupa survivorship	population	4	14.70	1.47	0.2711
	regime	1	0.03	0.004	0.9540
	population × light-regime	4	10.6	1.05	0.4218

Table 1: Summary of results of ANOVA on (a) median pupariation time and (b) arcsine square root transformed egg-to-puparium survivorship values of all populations in LD12:12 and DD light regimes.

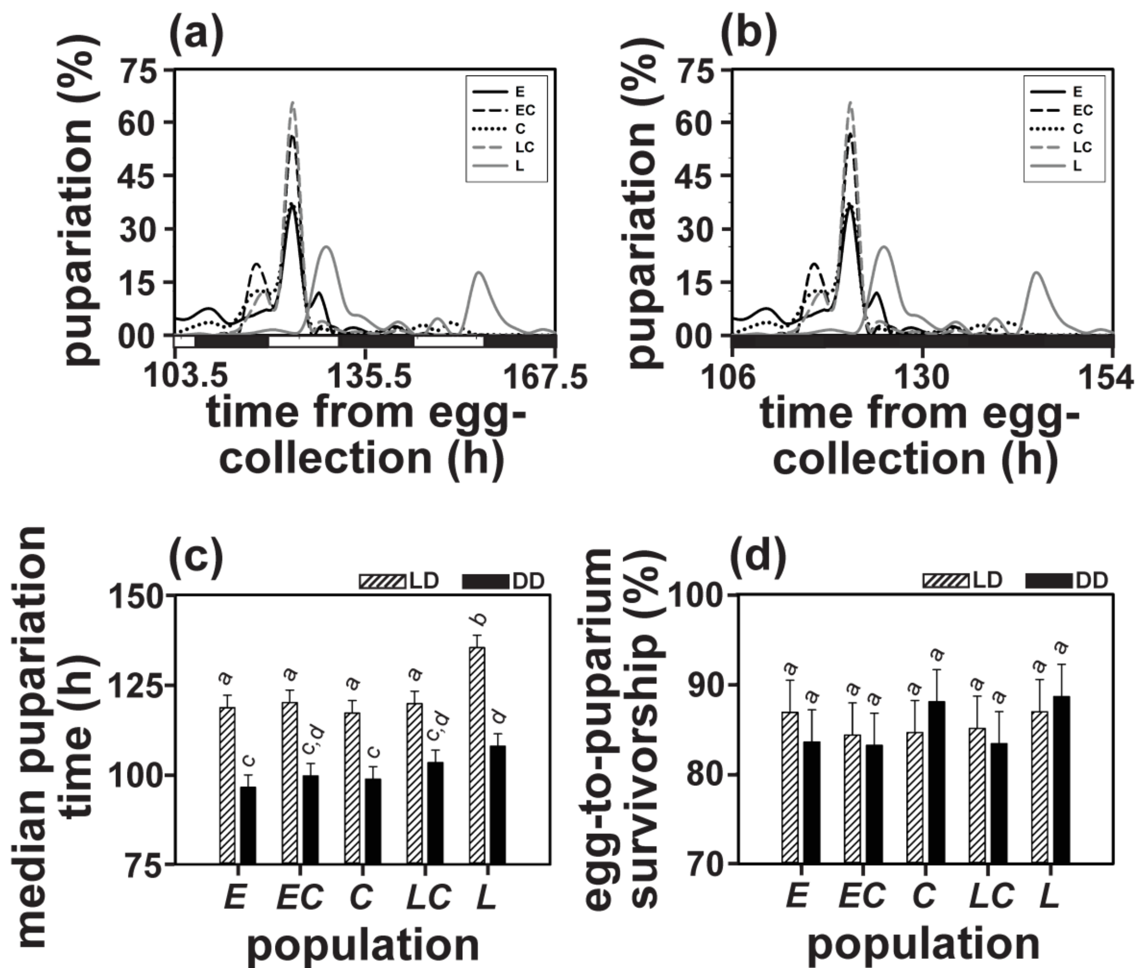

Figure 1: Percentage pupariation as a function of time (in hours) from egg collection in (a) LD12:12 and (b) DD for *early* (*E*), *early-control* (*EC*), *control* (*C*), *late-control* (*LC*), and *late* (*L*) populations. The black and white bars at the bottom represent night and day respectively. (c) Median pupariation time (time taken for 50% of total pupariation events) of all populations in LD12:12 and DD, and (d) Percentage egg-to-puparium survivorship values in LD12:12 and DD. Error bars for panels c, d indicate 95% CI calculated by method of Tukey's HSD. Bars sharing same letters do not differ statistically while those with different letters are significantly different from each other.

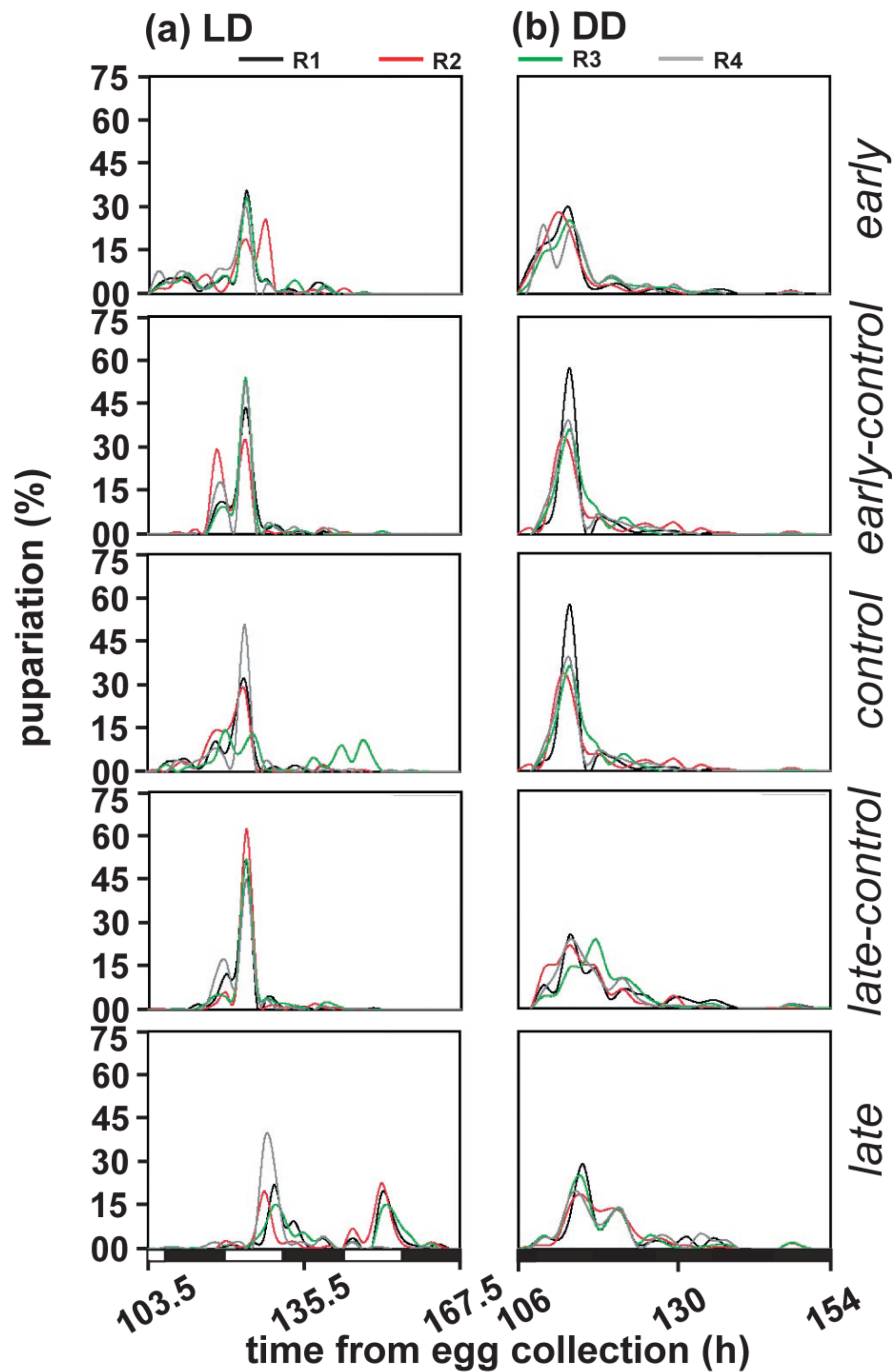

Figure 2: Proportion of individuals pupariated as a function of time from egg collection for *early* (panel 1), *early-control* (panel 2), *control* (panel 3), *late-control* (panel 4), and *late* (panel 5) populations in (a) LD12:12 and (b) DD. R1-R4 represents the four replicates of the respective populations used for the study. The black and white bars at the bottom represent night and day respectively.

population	replicate	median pupariation time	
		LD12:12	DD
early (E)	*1*	122.75 (1.48)	112.80 (1.39)
	2	123.50 (1.63)	113.00 (1.51)
	3	123.50 (1.15)	114.20 (0.66)
	4	120.00 (1.41)	114.25 (1.67)
early-control (EC)	*1*	123.50 (0.00)	116.57 (2.76)
	2	121.90 (1.67)	114.50 (2.33)
	3	123.16 (0.81)	114.28 (0.75)
	4	123.50 (0.00)	114.00 (0.00)
control (C)	*1*	121.72 (2.10)	115.00 (1.06)
	2	121.16 (1.50)	114.66 (1.63)
	3	121.25 (2.25)	113.75 (1.67)
	4	123.25 (0.70)	114.50 (1.41)
late-control (LC)	*1*	122.83 (1.48)	116.89 (3.17)
	2	123.78 (0.75)	114.40 (1.57)
	3	123.05 (1.33)	118.00 (1.06)
	4	121.90 (2.27)	116.00 (1.41)
late (L)	*1*	129.90 (1.57)	119.20 (3.55)
	2	127.72 (0.66)	118.22 (1.20)
	3	130.10 (0.96)	117.14 (1.57)
	4	128.75 (1.03)	118.00 (1.85)

Table 2: Median pupariation time in hours (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(b) ***The populations do not differ in egg-to-puparium survivorship under LD or DD***

ANOVA on egg-to-puparium survivorship revealed that the effect of population, light regime and population × light regime interaction was not statistically significant (Table 1b), indicating that the populations did not differ in their egg-to-puparium survivorship both within and across light regimes.

The average egg-to-puparium survivorship across populations was 85.56 ± 1.24% (mean ± SD) in LD12:12 (*early* = 86.86%, *early-control* = 84.33%, *control* = 84.60%, *late-control* = 85.08%, and *late* = 86.93%) and 85.35 ± 1.72% (mean ± SD) in DD (*early* = 83.56%, *early-control* = 83.18%, *control* = 88.03%, *late-control* = 83.36%, and *late* = 88.61%; Figure 1d; Table 3).

population	replicate	egg-to-puparium survivorship	
		LD12:12	DD
early (E)	*1*	84.16 (08.86)	81.00 (13.15)
	2	89.52 (07.31)	78.33 (09.59)
	3	88.33 (09.92)	87.40 (05.95)
	4	85.41 (17.45)	87.50 (07.50)
early-control (EC)	*1*	82.22 (09.00)	76.19 (18.70)
	2	87.33 (08.62)	86.66 (09.42)
	3	80.00 (08.94)	89.04 (07.62)
	4	84.66 (11.92)	80.83 (10.80)
control (C)	*1*	87.03 (03.88)	92.91 (09.16)
	2	87.22 (18.57)	90.95 (09.56)
	3	83.75 (06.77)	82.85 (09.70)
	4	85.41 (09.58)	85.41 (10.97)
late-control (LC)	*1*	84.07 (06.82)	77.77 (15.89)
	2	86.66 (08.35)	88.00 (08.91)
	3	85.92 (13.09)	82.50 (14.45)
	4	83.66 (15.10)	85.18 (14.04)
late (L)	*1*	87.66 (06.85)	82.66 (10.16)
	2	84.44 (11.66)	87.40 (08.46)
	3	88.00 (09.83)	91.90 (07.66)
	4	87.62 (06.58)	92.50 (10.94)

Table 3: Average percentage egg-to-puparium survivorship (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(c) ***late populations exhibit longer development time under both LD and DD***

ANOVA on median development time revealed statistically significant effect of population, light regime and population × light regime interaction (Table 4a). As observed for pupariation time, development time in LD12:12 was also significantly longer (16 h or 7.5%) for all populations as compared to that in DD (Figures 3a-c, 4).

In LD12:12, development time of *late* population (239.15 h) was significantly longer by 4 h or 1.9% than all populations (*early* = 234.69 h, *early-control* = 235.70 h, and *late-control* = 234.68 h) except for *control* (236.44 h) populations whereas development time of all other populations (*early, early-control*, *control,* and *late-control*) did not differ significantly among each other (Figures 3a, c, 4; Table 5).

The trend of *late* populations exhibiting longer (by 8 h or 3.5%) development time (226.41 h) as compared to all other populations was also observed in DD (*early* = 218.90 h, *early-control* = 217.96 h, *control* = 219.18 h, and *late-control* = 217.79 h; Figures 3b, c, 4; Table 5).

trait	effect	df	MS	*F*	*p*
(a) median development time	population	4	58.00	20.00	<0.0001
	regime	1	2588	2189	<0.0002
	population × light-regime	4	8.00	4.00	0.0207
(b) egg-to-adult survivorship	population	4	3.20	0.38	0.8179
	regime	1	22.7	40.52	0.0078
	population × light-regime	4	8.30	3.78	0.0326

Table 4: Summary of results of ANOVA on (a) median development time and (b) arcsine square root transformed egg-to-adult survivorship values of all populations in LD12:12 and DD light regimes.

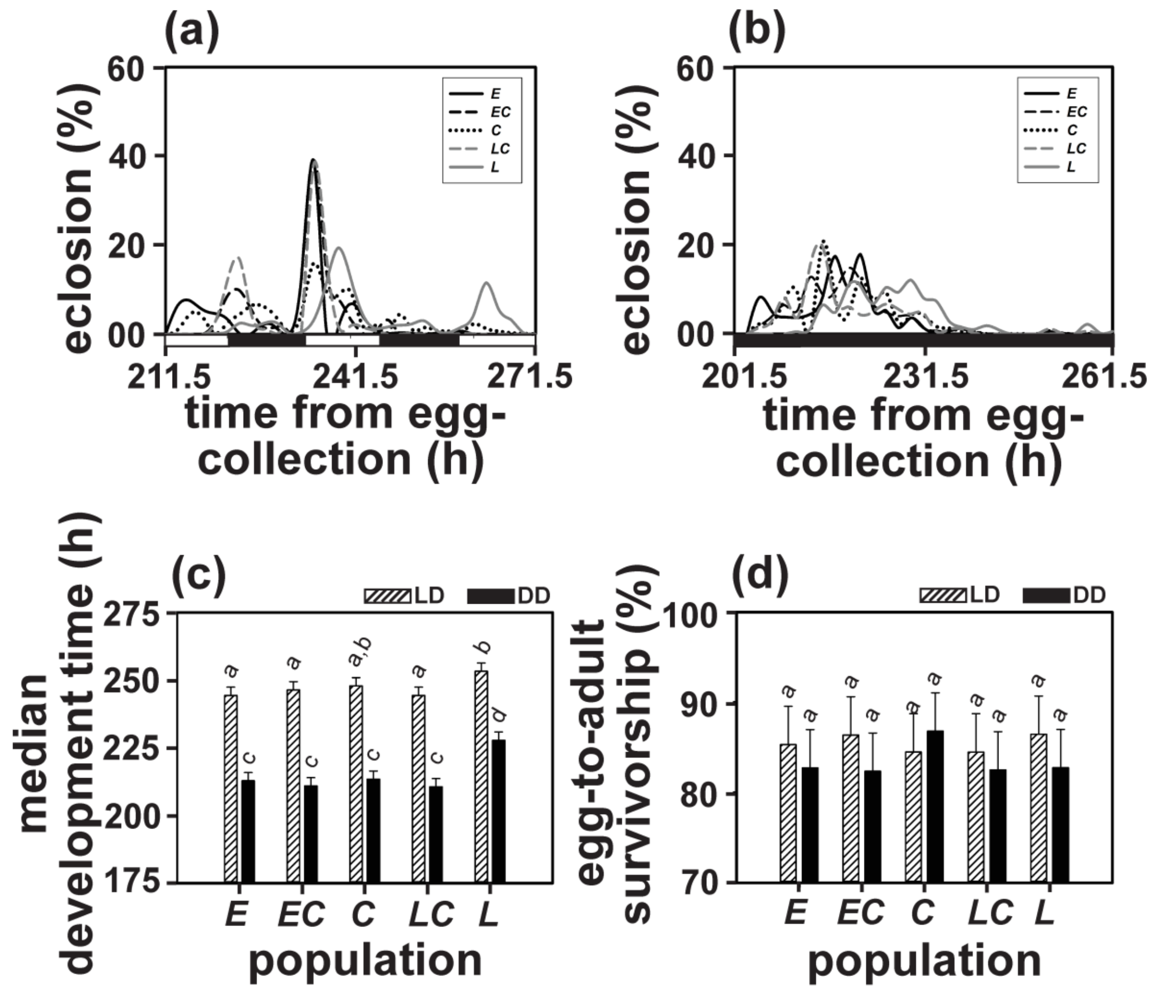

Figure 3: Percentage of individuals emerging as a function of time (in hours) from egg collection in (a) LD12:12 and (b) DD for *early* (*E*), *early-control* (*EC*), *control* (*C*), *late-control* (*LC*), and *late* (*L*) populations. The black and white bars at the bottom represent night and day respectively. (c) Median egg-to-adult development time of all populations in LD12:12 and DD, and (d) Percentage egg-to-adult survivorship values of all populations in LD12:12 and DD. Error bars for panels c, d indicate 95% CI calculated by method of Tukey's HSD. Bars sharing same letters do not differ statistically while those with different letters are significantly different from each other.

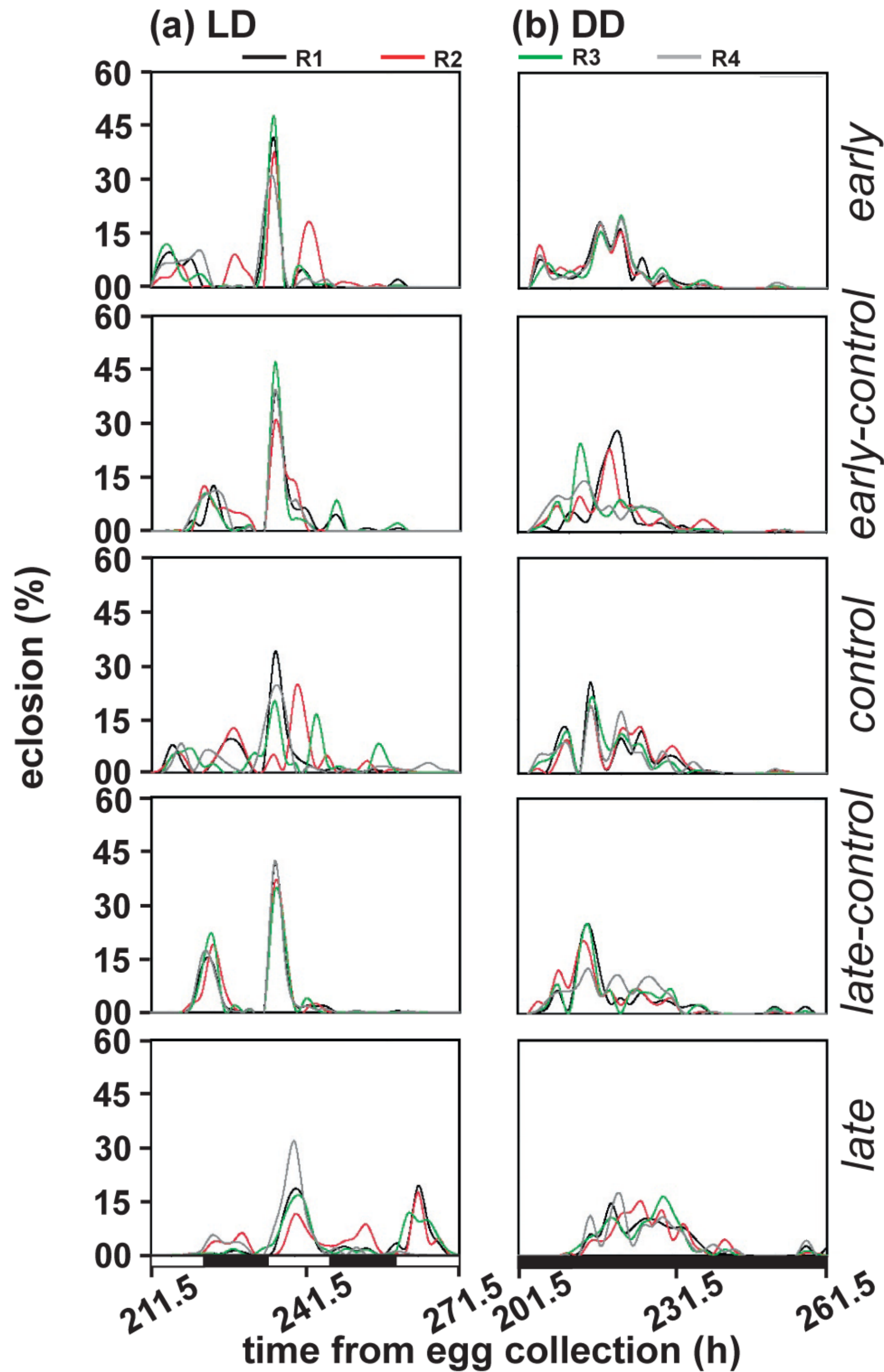

Figure 4: Proportion of individuals emerged as a function of time from egg collection for *early* (panel 1), *early-control* (panel 2), *control* (panel 3), *late-control* (panel 4), and *late* (panel 5) populations in (a) LD12:12 and (b) DD. R1-R4 represents the four replicates of the respective populations used for the study. The black and white bars at the bottom represent night and day respectively.

population	replicate	median development time	
		LD12:12	DD
early (E)	*1*	234.50 (1.06)	218.66 (1.73)
	2	235.50 (0.00)	217.50 (1.41)
	3	235.00 (0.92)	220.44 (1.33)
	4	233.78 (0.75)	219.00 (2.39)
early-control (EC)	*1*	235.90 (0.89)	220.25 (1.66)
	2	235.90 (0.89)	219.55 (1.33)
	3	235.50 (0.00)	216.28 (1.38)
	4	235.50 (0.00)	215.75 (2.49)
control (C)	*1*	235.21 (0.75)	218.50 (2.97)
	2	238.17 (1.63)	222.57 (2.76)
	3	237.75 (2.49)	217.00 (2.61)
	4	234.64 (1.06)	218.66 (2.64)
late-control (LC)	*1*	234.70 (2.53)	217.71 (2.13)
	2	234.25 (3.53)	215.11 (1.45)
	3	235.50 (0.00)	218.33 (3.20)
	4	234.30 (3.79)	220.00 (3.38)
late (L)	*1*	240.10 (0.96)	226.66 (3.60)
	2	238.30 (2.52)	226.75 (2.12)
	3	239.70 (1.13)	226.22 (3.23)
	4	238.50 (1.07)	226.00 (2.82)

Table 5: Median development time in hours (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(d) ***The populations do not differ in egg-to-adult survivorship under LD or DD***

ANOVA on egg-to-adult survivorship revealed statistically significant effect of light regime and population × light regime interaction but not of population (Table 4b). However, post hoc multiple comparisons using Tukey's HSD did not reveal any statistically significant difference in egg-to-adult survivorships across LD12:12 (*early* = 85.33%, *early-control* = 86.38%, *control* = 84.51%, *late-control* = 84.50%, and *late* = 86.48%) and DD (*early* = 82.74%, *early-control* = 82.37%, control = 86.82%, *late-control* = 82.52%, and *late* = 82.76%) light regimes or across populations within a light regime (Figure 3d; Table 6).

population	replicate	egg-to-adult survivorship	
		LD12:12	DD
early (E)	*1*	82.50 (09.21)	80.00 (12.13)
	2	85.55 (09.81)	83.75 (06.77)
	3	87.08 (10.14)	85.55 (09.57)
	4	86.19 (09.89)	81.66 (09.08)
early-control (EC)	*1*	89.33 (07.95)	85.41 (09.74)
	2	86.66 (10.54)	84.07 (14.69)
	3	85.55 (09.35)	80.00 (07.93)
	4	84.00 (10.64)	80.00 (10.54)
control (C)	*1*	85.23 (09.97)	89.58 (14.52)
	2	84.44 (10.88)	87.14 (09.11)
	3	84.58 (12.46)	85.00 (11.12)
	4	83.80 (08.62)	85.55 (09.42)
late-control (LC)	*1*	84.66 (06.70)	79.52 (06.78)
	2	86.25 (04.86)	86.66 (09.72)
	3	84.44 (07.81)	78.33 (12.78)
	4	82.66 (18.13)	85.55 (14.90)
late (L)	*1*	84.66 (14.33)	80.37 (09.19)
	2	87.00 (15.59)	82.96 (10.19)
	3	89.66 (10.82)	89.63 (08.06)
	4	84.58 (10.94)	78.09 (12.74)

Table 6: Average percentage egg-to-adult survivorship (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(e) *The populations do not differ in dry-weights at pupariation or emergence*

Since *late* populations exhibited significantly longer pupariation and development times, we further tested if this delay in developmental rate manifested in higher dry-weight at pupariation and adult emergence.

ANOVA on pupal dry-weight revealed statistically significant effect of population and light regime but not of population × light regime interaction (Table 7a). In accordance with their pupariation time difference between light regimes, the pupal dry-weights were found to be significantly higher (on an average 6.3%) in LD (*early* = 576.16 µg, *early-control* = 570.53 µg, *control* = 572.17 µg, *late-control* = 575.11 µg, and *late* = 580.16 µg) as compared to that of DD (*early* = 533.52 µg, *early-control* = 536.46 µg, *control* = 533.33 µg, *late-control* = 544.07 µg, *and late* = 544.83 µg; Figure 5a; Table 8) whereas no differences were observed between populations within either of the light regimes.

ANOVA on dry-weight at emergence reported statistically significant effect of light regime but not of population or population × light regime interaction (Table 7b). In accordance with development time differences across light regimes, the dry-weight at emergence was also found to be significantly higher (on an average by 4.35%) in LD (*early* = 359.39 µg, *early-control* = 358.30 µg, *control* = 361.71 µg, *late-control* = 362.64 µg, and *late* = 369.94 µg) as compared to that in DD (*early* = 342.63 µg, *early-control* = 347.85 µg, *control* = 346.19 µg, *late-control* = 348.12 µg, and *late* = 348.06 µg; Figure 5b; Table 9) whereas no differences were observed between populations within either of the light regimes.

trait	effect	df	MS	*F*	*p*
(a) dry-weight at pupation	population	4	142	4.7	0.0167
	regime	1	13231	136.3	0.0013
	population × light-regime	4	40	00.60	0.6407
(b) dry-weight at emergence	population	4	71	2.4	0.1107
	regime	1	2505	150.9	0.0011
	population × light-regime	4	34	2.0	0.1612

Table 7: Summary of results of ANOVA on (a) dry-weight at pupariation and (b) dry-weight at emergence values of all populations in LD12:12 and DD light regimes.

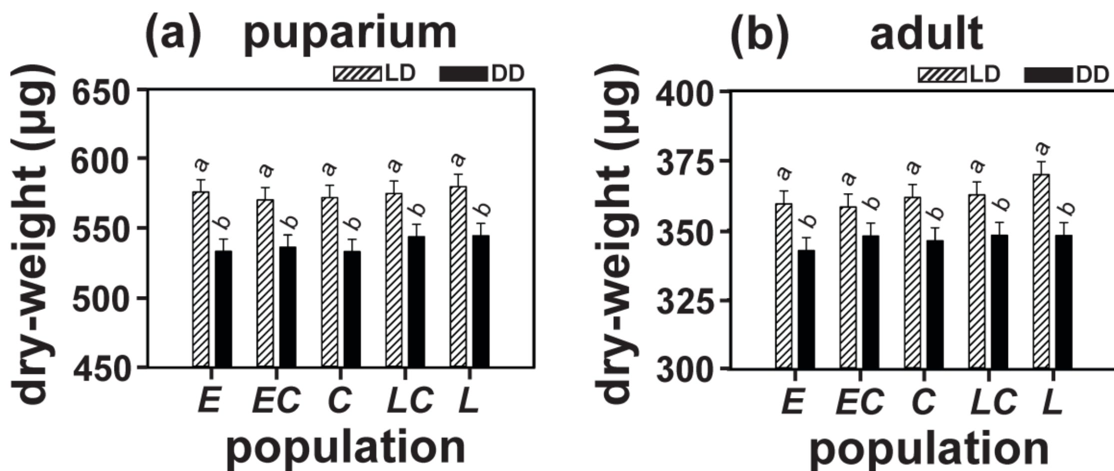

Figure 5: (a) Dry-weight per individual at pupariation, and (b) dry-weight per individual at emergence for *early* (*E*), *early-control* (*EC*), *control* (*C*), *late-control* (*LC*), and *late* (*L*) populations in LD12:12 and DD regimes. Error bars indicate 95% CI calculated by method of Tukey's HSD. Bars sharing same letters do not differ statistically while those with different letters are significantly different from each other.

population	replicate	dry-weight at pupariation	
		LD12:12	DD
early (E)	*1*	564.26 (11.56)	538.65 (13.21)
	2	588.96 (16.25)	536.35 (15.41)
	3	585.92 (16.16)	519.33 (12.03)
	4	565.45 (15.42)	539.74 (10.50)
early-control (EC)	*1*	564.13 (19.09)	535.71 (12.29)
	2	573.60 (18.05)	542.43 (19.62)
	3	576.16 (19.68)	536.47 (15.94)
	4	568.21 (20.39)	531.28 (19.13)
control (C)	*1*	571.64 (18.55)	539.77 (14.53)
	2	565.35 (16.66)	534.71 (08.61)
	3	571.38 (18.82)	536.93 (18.54)
	4	580.10 (15.51)	521.89 (19.46)
late-control (LC)	*1*	565.93 (20.90)	546.58 (15.21)
	2	581.53 (17.81)	542.20 (18.74)
	3	572.73 (11.76)	540.00 (17.01)
	4	580.50 (07.98)	547.50 (09.62)
late (L)	*1*	580.50 (14.46)	584.40 (11.10)
	2	583.14 (15.30)	549.00 (10.36)
	3	575.64 (19.31)	539.55 (09.04)
	4	581.12 (16.41)	542.37 (14.03)

Table 8: Average dry-weight at pupariation in µg (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

population	replicate	dry-weight at eclosion	
		LD12:12	DD
early (E)	*1*	360.85 (08.49)	345.33 (10.65)
	2	364.41 (15.16)	347.18 (12.39)
	3	361.98 (10.55)	338.26 (11.04)
	4	350.33 (11.96)	339.75 (09.53)
early-control (EC)	*1*	364.46 (15.19)	344.14 (09.18)
	2	352.95 (12.64)	345.18 (13.30)
	3	353.26 (11.47)	345.98 (10.92)
	4	362.51 (07.97)	356.08 (12.99)
control (C)	*1*	358.01 (12.40)	348.40 (16.00)
	2	358.60 (07.96)	351.93 (10.27)
	3	360.31 (10.57)	339.48 (09.99)
	4	369.43 (08.65)	344.95 (10.43)
late-control (LC)	*1*	362.00 (11.88)	348.30 (10.72)
	2	359.46 (10.95)	343.68 (14.34)
	3	366.03 (11.71)	348.60 (12.41)
	4	363.06(12.64)	351.93 (06.10)
late (L)	*1*	371.86 (16.61)	347.80 (09.09)
	2	367.44 (11.08)	350.02 (08.96)
	3	366.86 (11.50)	341.20 (06.24)
	4	373.41 (13.67)	353.23 (06.31)

Table 9: Average dry-weight at emergence in µg (mean ± SD) of all populations in LD12:12 and DD light regimes. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(f) Females of *late populations exhibit higher fecundity*

ANOVA on average fecundity on day 11 (post-emergence) revealed a statistically significant effect of population (Table 10a). Fecundity of *late* populations (10.80 eggs/fly) was significantly higher (by 32%) as compared to that of the other populations (*early* = 7.32 eggs/fly, *early-control* = 7.74 eggs/fly, *control* = 7.01 eggs/fly, and *late-control* = 7.68 eggs/fly), whereas none of the other populations differed significantly among each other (Figure 6a; Table 11).

(g) *Pre-fecundity dry-weight does not differ between populations whereas late populations exhibit lower post-fecundity dry-weight*

ANOVA on female dry-weight measurements at pre- and post-fecundity assay stages showed statistically significant effect of 'stage' (pre/post-fecundity assay) and population × stage interaction but not of population (Table 10b). Post-hoc multiple comparisons revealed that post-fecundity dry-weight of all the populations was reduced by about 52.40 µg (22%; Figure 6b) as compared to the pre-fecundity dry-weight. Pre-fecundity dry-weight did not differ statistically between populations (*early* = 231.95 µg, *early-control* = 233.62 µg, *control* = 229.95 µg, *late-control* = 232.87 µg, and *late* = 238.45 µg) but post-fecundity dry-weight of *late* populations was significantly lower (167.16 µg) by 17 µg or ~10% as compared to all other populations (*early* = 182.79 µg, *early-control* = 184.30 µg, *control* = 188.41 µg, and *late-control* =182.16 µg; Figure 6b; Table 12).

(h) *The populations do not differ in fecundity per unit loss in dry-weight*

When normalized by the dry-weight lost (difference in pre- and post-fecundity dry-weight), fecundity per unit dry-weight lost did not differ statistically (Table 10c) across populations (*early* = 0.15 eggs/µg, *early-control* = 0.16 eggs/µg, *control* = 0.17 eggs/µg, *late-control* =

0.15 eggs/μg, and *late* = 0.15 eggs/μg; Figure 6c), suggesting that *late* populations even though have higher fecundity, lose more dry-weight post-fecundity probably due to higher number of eggs laid. To further confirm this, we performed a linear correlation between egg output and dry-weight loss by pooling data from all the populations and found that the two variables were significantly positively correlated ($r = +0.75$, $p < 0.0001$; Figure 6d).

trait	effect	df	MS	*F*	*p*
(a) eggs laid/female	population	4	9.31	24.315	<0.0001
(b) pre- and post-fecundity dry-weight	population	4	53	2.00	0.1567
	stage	1	27465	6835.6	<0.0001
	population × stage	4	249	18.10	<0.0001
(c) fecundity/unit dry-weight loss	population	4	0.002	0.43	<0.7782

Table 10: Summary of results of ANOVA on (a) average eggs laid/female, (b) dry-weight at pre- and post-fecundity assay stages, and (c) log transformed fecundity per unit dry-weight loss of all populations in LD12:12.

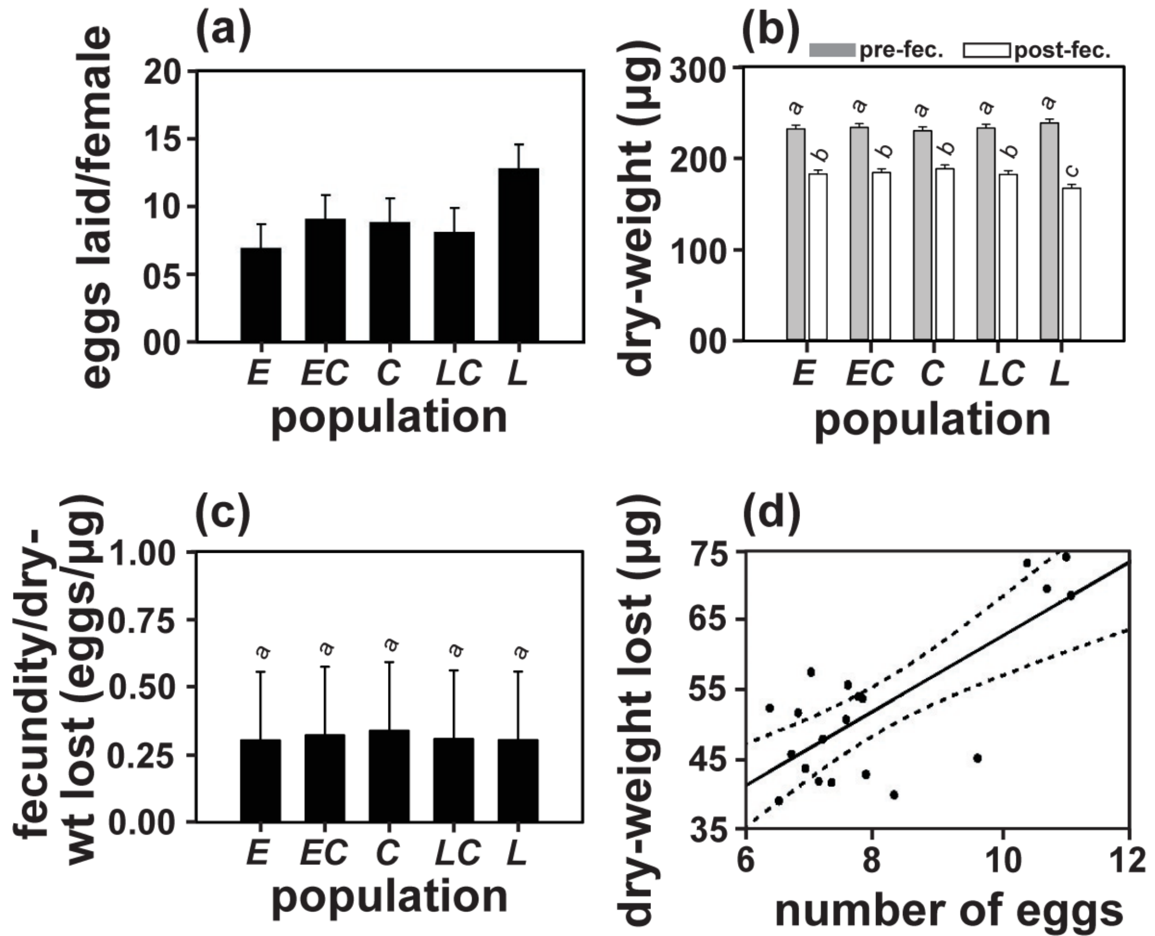

Figure 6: (a) Average fecundity per female on day 11 (post-emergence), (b) dry-weight per female at pre- and post-fecundity stages, and (c) fecundity per unit dry-weight loss (difference in pre- and post-fecundity dry-weight) for *early* (*E)*, *early-control* (*EC*), *control* (*C*), *late-control* (*LC*), and *late* (*L*) populations in LD12:12. Error bars indicate 95% CI calculated by method of Tukey's HSD. (d) Pearson correlation of average fecundity/female with that of dry-weight lost during the fecundity assay ($r = +0.75$, $p < 0.0001$). The data points for correlation were obtained by pooling values across all 20 populations. The dotted line indicates 95% CI. Fecundity and dry-weight measurements assays were performed only in LD12:12. Bars sharing same letters do not differ statistically while those with different letters are significantly different from each other.

population	replicate	eggs laid
early (E)	*1*	7.05 (2.47)
	2	6.74 (1.97)
	3	7.59 (2.42)
	4	7.90 (2.21)
early-control (EC)	*1*	8.33 (3.06)
	2	7.23 (2.71)
	3	7.79 (2.78)
	4	7.62 (1.71)
control (C)	*1*	7.17 (1.97)
	2	6.96 (2.79)
	3	6.56 (2.11)
	4	7.37 (1.80)
late-control (LC)	*1*	7.85 (2.32)
	2	6.41 (3.64)
	3	6.85 (2.46)
	4	9.62 (3.72)
late (L)	*1*	10.7 (2.84)
	2	10.3 (3.47)
	3	11.0 (1.90)
	4	11.0 (2.24)

Table 11: Average eggs laid /female (mean ± SD) on day 11 post-emergence of all populations in LD12:12. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

population	replicate	dry-weight	
		pre-fecundity	post-fecundity
early (E)	*1*	237.50 (7.03)	180.00 (2.83)
	2	227.66 (5.47)	182.00 (2.29)
	3	229.83 (2.03)	179.16 (4.45)
	4	232.83 (3.18)	190.00 (6.30)
early-control (EC)	*1*	225.00 (3.45)	185.16 (6.18)
	2	234.83 (2.79)	187.00 (4.86)
	3	237.16 (4.27)	183.20 (1.48)
	4	237.50 (6.37)	181.83 (1.05)
control (C)	*1*	232.16 (4.00)	190.33 (7.48)
	2	232.50 (5.58)	188.83 (4.65)
	3	228.66 (1.38)	189.66 (9.74)
	4	226.50 (8.50)	184.83 (4.70)
late-control (LC)	*1*	238.50 (1.02)	184.83 (1.32)
	2	237.83 (6.76)	185.50 (3.44)
	3	228.66 (1.38)	177.00 (1.50)
	4	226.50 (8.50)	181.33 (3.98)
late (L)	*1*	233.16 (5.65)	163.66 (1.09)
	2	240.50 (2.82)	167.33 (7.00)
	3	241.16 (2.67)	168.16 (4.79)
	4	238.00 (3.72)	169.50 (1.60)

Table 12: Average dry-weight in µg (mean ± SD) at pre- and post-fecundity stages for all populations in LD12:12. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

(i) *Females of late populations exhibit reduced median longevity*

ANOVA on median longevity reported statistically significant effect of population, sex, and population × sex interaction (Table 13). With the exception of *late* populations where individuals of both the sexes had an average median longevity of 41.89 ± 0.004 (mean ± SD) days, the average female longevity of all the other populations (*early* = 47.5 days, *early-control* = 46.38 days, *control* = 47.81 days, and *late-control* = 45.22 days) was ~7% higher than the males (*early* = 41.41 days, *early-control* = 42.67 days, *control* = 44.25 days, and *late-control* = 44.13 days; Figure 7; Table 14).

Within sex comparisons revealed that the females of *late* populations exhibited significantly shorter (~12%) median longevity as compared to females of all the other populations with the exception of *late-control* which did not differ statistically from *late* populations. No significant difference in median longevity was observed for males across populations (Figure 7; Table 14).

effect	df	MS	*F*	*p*
population	4	17.99	17.22	<0.0001
sex	1	83.45	70.88	0.0035
population × sex	4	11.44	5.27	0.0011

Table 13: Summary of results of ANOVA on median longevity of virgin males and females of all populations in LD12:12.

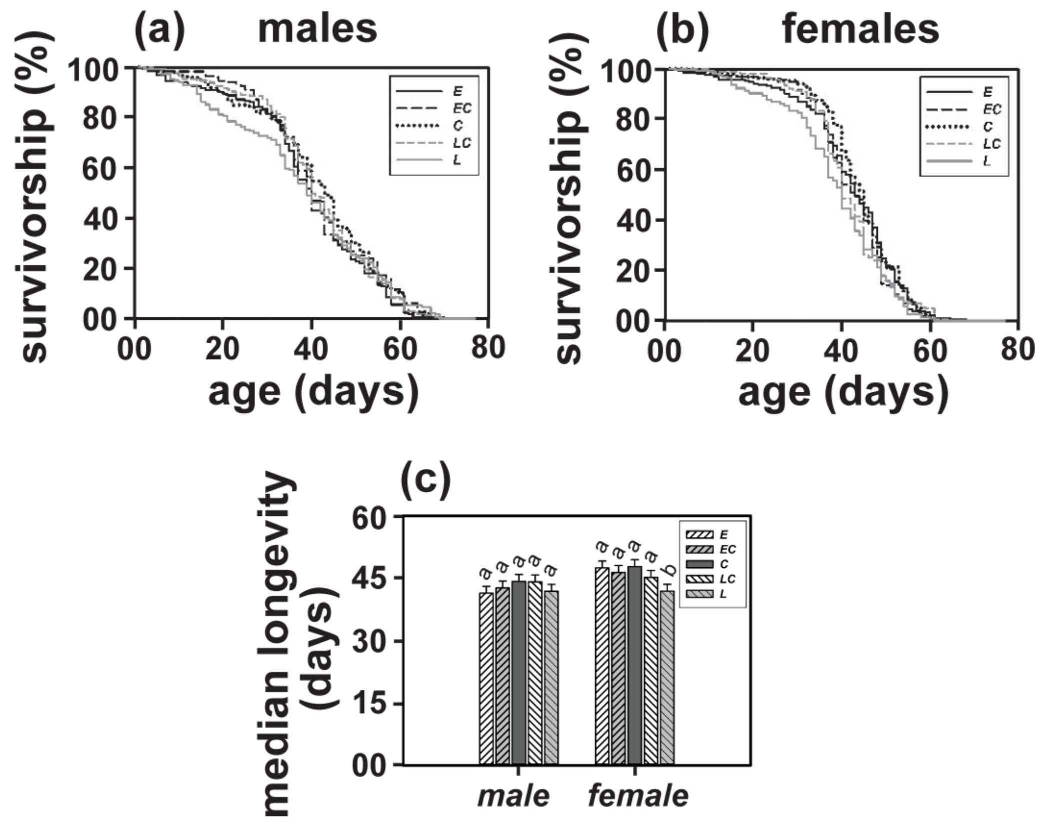

Figure 7: Survivorship curves of (a) virgin males and (b) virgin females of *early* (*E)*, *early-control* (*EC*), *control* (*C*), *late-control* (*LC*), and *late* (*L*) populations in LD12:12. (c) Median longevity (time taken for 50% of individuals to die) of virgin males and females of all populations in LD12:12. Error bars indicate 95% CI calculated by method of Tukey's HSD. Bars sharing same letters do not differ statistically while those with different letters are significantly different from each other.

population	replicate	median longevity	
		males	female
early (E)	*1*	41.00 (3.09)	47.16 (3.76)
	2	41.50 (6.41)	46.00 (5.23)
	3	42.10 (4.70)	47.83 (3.43)
	4	41.00 (5.52)	49.00 (3.76)
early-control (EC)	*1*	45.50 (1.73)	45.00 (3.77)
	2	40.83 (3.81)	46.40 (6.06)
	3	42.71 (3.59)	46.42 (5.41)
	4	41.66 (7.60)	47.71 (4.71)
control (C)	*1*	43.16 (7.13)	47.85 (5.04)
	2	44.37 (5.95)	48.88 (5.51)
	3	43.80 (1.92)	47.50 (3.11)
	4	45.66 (3.20)	47.20 (3.39)
late-control (LC)	*1*	44.50 (3.73)	44.25 (3.91)
	2	45.37 (5.20)	45.57 (3.55)
	3	41.83 (6.08)	46.57 (4.27)
	4	44.83 (8.63)	44.50 (3.93)
late (L)	*1*	42.83 (6.17)	42.42 (5.02)
	2	41.14 (6.38)	42.00 (2.89)
	3	40.60 (6.73)	40.60 (8.82)
	4	43.00 (3.28)	42.57 (3.25)

Table 14: Median longevity in days (mean ± SD) of virgin males and females of all populations in LD12:12. Figures within brackets indicate standard deviation of values across replicate vials used for each replicate population.

7.4 Discussion

early and *late* populations in our study were selected to emerge at different 'gates'/times of the day, and were not under direct selection for faster and slower egg-to-puparium or egg-to-adult development; nevertheless, *late* populations evolved delayed pre-adult pupariation and development times (Figures 1a-c, 3a-c). A closer look at the data reveals that the difference in pupariation time between populations is of the same order as that for development time (compare Figures 1a-c, 3a-c) suggesting that selection for evening emergence has primarily resulted in coevolution of delayed egg-to-puparium development rate, which consequently lead to the egg-to-adult development time differences. The egg-to-adult development time differences in these populations after 242 generations of selection are consistent with those reported in a previous study on the same populations after 55 generations of selection (Kumar et al. 2006). Furthermore, in addition to divergent phase of emergence *early* and *late* populations have evolved shorter and longer clock periods differing by 40 min (Kumar et al. 2007a; Nikhil et al. 2016a). Small magnitude correlations between clock period and development time have been reported earlier in insects melon flies *Bactrocera cucurbitae* (Miyatake, 1997; Shimizu et al. 1997), and between clock period and pupariation and development time in fruit flies *D. melanogaster* (Kyriacou et al. 1990; Takahashi et al. 2013; Yadav and Sharma, 2013), suggesting that clock period differences influence developmental rates, albeit weakly. In DD, pupariation time of *late* populations was 118.14 h (4.9 days) and development time was 226.41 h (9.4 days) as opposed to 113.56 h (4.7 days) and 218.90 h (9.1 days) respectively in *early* populations. If the differences in pupariation and development time were entirely driven by circadian clock period difference, *early* and *late* populations would drift apart by 0.66 h (40

min) every day under DD, and consequently the two populations would be expected to show 3.12 h difference in pupariation time (in 4.7 days which is equal to the time taken for *early* population to pupariate) and 6.01 h difference in development time (in 9.12 days) which is lower than that observed (Figures 1c, 3c). In addition, pupariation time of *late* populations in LD12:12 was 6.68 h and development time 4.46 h longer than *early* populations (Figures 1c, 3c), which is same as the 4.5 h difference in the mean chronotypes of the two populations (Nikhil et al. 2014). Furthermore, the magnitude of difference in pupariation and development times between populations was considerably enhanced under LD cycles as compared to that of DD (Figures 1c, 3c) suggesting that such differences might be primarily driven by differential interaction of the populations with the LD cycles (significant population × light regime interaction reported in Tables 1a, 4a). Taken together these results suggest that difference in pre-adult developmental rates is not entirely circadian clock driven and may involve both clock dependent and independent mechanisms. Since eggs for the pupariation and development time assays were collected from all the twenty populations at the same time of the day (thus were age matched), the observed differences in pupariation and development time between *early* and *late* populations are unlikely to be due to the differences in the age of eggs. Moreover, the time of egg collection or the age of eggs does not affect difference in developmental time between *early* and *late* populations (Kumar et al. 2006). Light mediated reduction in the developmental rates is apparent as both pupariation and development times of all the populations were 7%-7.5% longer in LD12:12 as compared to DD (Figures 1a-c, 3a-c). While effects of light on pupariation and development time have been documented earlier (Paranjpe et al. 2005; Yadav et al. 2014), precise mechanisms underlying the action of LD12:12 on

developmental rate are still not explored. The timing of adult emergence in *Drosophila* depends upon the developmental state of the fly, the phase and period of circadian rhythm, hormonal cascade, and environmental conditions (Qui and Hardin, 1996; Mukherjee et al. 2012). It is believed that LD cycles interact with the circadian clock controlled gate of emergence such that even if adult flies have completed development, they are allowed to emerge only during certain time of the day, and not merely in accordance with their developmental state and circadian clocks (as would be the case in DD), and consequently the developmental time would be delayed in LD12:12 compared to DD by virtue of gating (Pittendrigh and Skopik, 1970; Qui and Hardin, 1996; Mukherjee et al. 2012; Yadav et al. 2014). Additionally, the time of emergence in a given day is also a function of the circadian clock period such that individuals with shorter period emerge earlier than those with longer period (Konopka and Benzer, 1971). This further supports the idea that pre-adult development is probably mediated by the interaction of circadian clock with the LD cycles and that such an interaction is a function of clock period as well.

Pupariation and developmental rates in *early* and *late* populations did not seem to influence egg-to-puparium and egg-to-adult survivorship between populations; nor did the light regime appear to differentially influence survivorships (Figures 1d, 3d). It is plausible that the magnitude of difference in pupariation and development times between populations within a light regime might not be large enough to influence egg-to-puparium and egg-to-adult survivorships. However, not surprisingly LD cycles significantly influenced both pupariation and development times but not that of egg-to adult and egg-to-puparium survivorship (Figures 1, 3). These results are in accordance with earlier reports suggesting that the developmental rates as has been reported earlier are relatively labile and thus

sensitive to LD cycles (Markow, 1981; Qui and Hardin, 1996; Paranjpe et al. 2004, 2005) while egg-to-puparium and egg-to-adult survivorships do not seem to be influenced strongly by LD cycles.

Although *late* populations have evolved significantly longer pupariation and development times, their body-weight at pupariation and adult emergence did not differ from all the other populations (Figure 3). However, dry-weight of puparia and adults were found to be significantly higher for all populations in LD compared to DD (Figure 5) which is not surprising as pupariation and development time is significantly reduced in DD (Figures 1, 3).

Coevolution of pre-adult life-history traits in response to selection for timing of emergence is intuitive, as changes in pre-adult stages can directly affect the time course and waveform of adult emergence. It would be important to know whether selection for emergence at different times of the day also led to correlated changes in adult life-history traits that may not necessarily influence emergence time but would highlight the underlying genetic correlations. In this regard we observed that *late* populations exhibited significantly higher fecundity compared to all other populations (Figure 6a). Pre-adult traits such as development time are known to be highly correlated with fecundity as delayed development is associated with higher dry-weight, which consequently enhances fecundity in *D. melanogaster* (Robertson, 1960, 1963; Hillesheim and Stearns, 1992). This did not appear to be the case in *late* populations since neither their dry-weight at emergence nor pre-fecundity dry-weight differed from that of the other populations (Figures 5b, 6b). The post-fecundity assay dry-weight of *late* populations was significantly lower as compared to that of the other populations (Figure 6b), and when normalized by the loss in dry-weight

(difference in pre- and post-fecundity dry-weights), the fecundity per unit dry-weight lost was similar for all populations (Figure 6c). Therefore, significant reduction in post-fecundity dry-weight in *late* populations appears to be a consequence of higher number of eggs laid which is also substantiated by a significant correlation observed between number of eggs laid and dry-weight lost (Figure 6d). Therefore, contrary to the well-known positive correlations between development time, dry-weight and fecundity, our results suggest that the observed higher fecundity in *late* populations is not due to higher dry-weight attained by delay in development time, but different mechanisms might underlie such phenomena. However, we also acknowledge the possibility that higher fecundity in *late* populations might have evolved as an artefact of the nature of selection protocol employed. For instance, to ensure that the number of adults in all populations is ~1200 every generation, we collect a larger number of eggs for *late* followed by relatively smaller number of eggs for *early* as compared to *control* populations (see materials and methods), which is 24 vials per replicate population for *early* populations, 48 vials for *late* populations as opposed to 16 vials for *control* populations with each vial housing approximately 300 eggs. Therefore, the number of eggs collected from *late* populations (~14400 eggs) is approximately twice that of *early* (~7200 eggs) and thrice that of *control* (~4800 eggs) populations. This would lead to an inadvertent selection for higher fecundity in *late* populations, and also possibly as a consequence of higher effective population sizes (N_e) because of which *late* populations might experience relatively lower extent of inbreeding depression followed by *early* populations with *control* populations experiencing highest degree of inbreeding depression. If this were to be true, then *early* populations would be expected to exhibit higher fecundity compared to *control,* but does not seem to be the case. Therefore, it is unlikely that this

might be the reason for evolution of higher fecundity in *late* populations even though its effects cannot be entirely disregarded. Additionally, given that fecundity in *drosophila* is not constant across lifespan, the evolved changes in fecundity on day 11 ± 1 post emergence might also have influenced early life and late life fecundity. However, as discussed earlier the nature of selection protocol imposes in a direct selection for fecundity on day 11 which determines the fitness in this regime and therefore fecundity during other life-stages were not assayed but nevertheless will be interesting to study.

Further, we found that females of *late* populations live significantly shorter as compared to those from *early* and *control* populations, while no differences was observed for males (Figure 7). In the light of fecundity and dry-weight results, the observed reduction in longevity might represent a classic trade-off between fecundity and lifespan due to the antagonistic pleiotropic effects of underlying genes (Rose, 1984; Chippindale et al. 1993; Partridge and Barton, 1993). However, since the results presented here are of virgin males and females, the observed reduction in longevity might entirely be a trade-off for higher fecundity. Therefore, even though higher reproductive output might have evolved as an artefact of selection protocol, reduced longevity in *late* populations compared to *early* and *control* populations may have evolved as a correlated response to selection for late evening emergence and not directly as a consequence of higher fecundity.

Thus, we report that selection for late evening emergence in fruit flies *D. melanogaster*, in addition to driving the evolutionary divergence of circadian clocks (Kumar et al. 2007a), is also associated with in the coevolution of several life-history traits while no differences were observed in *early*, *control, early-control,* and *late-control* populations. Most of the differences observed correspond to *late* populations relative to *control* populations, while

very little difference was observed between *early* and *control* populations. Also, most of the life-history traits assayed in *late* populations differ by a small magnitude varying from 2-10% compared to *early* and *control* populations. This is not surprising considering the larger time difference between the selection windows of *late* populations from the emergence peak of *control* populations, and proximity of the selection window of *early* populations from the emergence peak of *control* populations. Since evening emergence is not predominantly seen in *control* populations, *late* populations would experience much stronger selection pressure compared to *early* populations, which in turn might drive stronger coevolution of life-history traits.

In summary, selection for late evening emergence leads to delay in pre-adult development time without any increase in body-weight at emergence, increased fecundity associated with greater post-fecundity dry-weight loss and reduced virgin female lifespan. The observed life-history traits in *late* populations being evolved responses to selection are further supported by *early-control* and *late-control* populations. Life-history traits of *early-control* and *late-control* populations did not differ significantly from each other and between *early* and *late* populations in most cases, the results suggest that the observed life-history trait differences between *early* populations and *late* populations are indeed evolutionary responses to the imposed selection pressure and are not merely environmentally driven. Further, since the pre-adult and adult life-history traits studied here are known to be highly correlated, enhanced fecundity in *late* populations does not seem to be a consequence of higher biomass attained by delayed development time. Thus the differences in adult traits do not seem to be associated with pre-adult trait differences and

the two appear to be driven by independent mechanisms evolved as a consequence of selection.

Thus, in contrast with studies which demonstrated the effect of direct manipulation of circadian clock on fitness aspects, we report evolution of life-history traits differences in independently evolving replicate populations of *D. melanogaster* exhibiting *early* and *late* emergence chronotypes suggesting that the genetic architecture underlying emergence gate/emergence at specific times of the day (emergence chronotypes) appears to be correlated with several life-history traits; and these correlations are mediated by both clock dependent and independent mechanisms. While the difference in the assayed life-history traits of these populations appear to be correlated with altered sensitivity/differential interaction with the LD cycles, the extent of circadian clocks' influence (if any) in mediating such interactions, and the underlying genetic architecture remains to be explored.

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List of Publications

KL Nikhil, L Abhilash and VK Sharma (2016). Molecular correlates of circadian clocks in fruit flies *Drosophila melanogaster* populations exhibiting *early* and *late* emergence chronotypes. *Journal of Biological Rhythms*. (*In press*).

KL Nikhil, KM Vaze and VK Sharma (2016) Late emergence chronotypes of fruit flies *Drosophila melanogaster* exhibit higher accuracy of entrainment. *Chronobiology International.* (*In press*).

KL Nikhil, KM Vaze, K Ratna and VK Sharma (2016) Circadian clock properties of fruit flies *Drosophila melanogaster* exhibiting early and late emergence chronotypes. *Chronobiology International.* (*In press*).

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

KL Nikhil, K Ratna and VK Sharma Life-history traits of *Drosophila melanogaster* populations exhibiting *early* and *late* eclosion chronotypes. (*Manuscript submitted*).

KM Vaze, **KL Nikhil** and VK Sharma (2013) Genetic architecture underlying morning and evening circadian phenotypes in fruit flies *Drosophila melanogaster*. *Heredity.* 111:265-274.

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