Behavioral and Molecular Analysis of Laboratory Populations of *Drosophila melanogaster* Selected for Early and Late Adult Emergence

Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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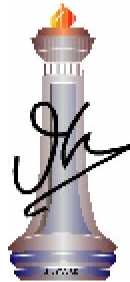

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August 2006

………to

My family and

Manish

DECLARATION

I declare that the matter presented in my thesis entitled "**Behavioral and Molecular Analysis of Laboratory Populations of *Drosophila melanogaster* Selected for Early and Late Adult Emergence"** is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Vijay Kumar Sharma and that this work has not been submitted elsewhere for any other degree.

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

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CERTIFICATE

This is to certify that the work described in the thesis entitled "**Behavioral and Molecular Analysis of Laboratory Populations of *Drosophila melanogaster* Selected for Early and Late Adult Emergence"** is the result of investigations carried out by Mr. Shailesh Kumar in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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Summary

Circadian clocks enhance the chances of survival of organisms living under periodic environment by enabling them to efficiently anticipate periodic events in their environment, because precisely and appropriately timed behavioral and metabolic processes are thought to confer greater adaptive advantage than randomly occurring activities. Among clock regulated phenomena in insects adult emergence (eclosion) rhythm is one of the most extensively studied and perhaps the best understood after activity/rest cycle. Although each individual emerges as an adult only once in its life cycle, gating of this event is under the control of an on-going oscillation present during development (Saunders, 1992). Consequently, certain intervals of time in a day constitute the "forbidden zone of eclosion", whereas a brief period of time during which adults emerge forms the "allowed zone" (also referred to as the "gate" of eclosion) (Pittendrigh, 1954). The gating is often so stringent that even if developing flies are mature enough to emerge but fail to do so during the gate they remain within the puparium until the next gate opens (Saunders, 1992). My thesis is motivated by the need for an unequivocal, systematic and rigorous approach for studying the evolution of circadian waveform of adult emergence in fruit flies *Drosophila melanogaster*. The main aim of my thesis is to study the effect of selection on timing of adult emergence on circadian clocks, clock-related rhythm and life history traits. For this purpose I derived four genetically independent, random mating, large populations each of *early* and *late* populations of *D. melanogaster* by selecting for individuals that emerge during "lights-on" (morning hours) and "lights-off" (evening hours) under 12:12 hr LD cycles. I assessed the direct response to selection by comparing the number of flies that emerged out of the morning and

evening windows of selection in the selected and control populations at regular intervals of 10-15 generations to trace the evolutionary trajectory of changes over 55 generations of selection. I observed that *D. melanogaster* populations respond to selection on timing of adult emergence, since the percentage of flies that emerge out of the morning window after 55 generations of selection is about 60% in the *early* populations while it is reduced to almost one third (~33%) in the *late* populations, and remain unchanged at ~45% in the *controls*. The percentage of flies that emerged out of the evening window in the *late* populations (~24%) is about thrice as much as in the *early* populations (~8%), while it remain unchanged at ~16% in the *controls*. To investigate the consequence of selection on circadian clocks we assayed eclosion and activity rhythms in the selected and control populations under LD and constant dark (DD) conditions. Under 12:12 hr LD cycles, the primary peaks of eclosion in the *early* populations are taller and occur earlier than the *controls*, while those in the *late* populations are relatively flatter and occur later than the *controls*. The *early* flies start and end activity earlier and are generally more active during the morning hours, while the *late* flies start and end activity later and are more active during the evening hours. In order to test the robustness of the circadian phenotypes of the selected populations we assayed the eclosion and activity rhythms under short (8:16 hr), normal (12:12 hr) and long (16:8 hr) photoperiodic regimes at the 70th generation. Although the overall eclosion and activity patterns of flies are influenced by the photoperiodic conditions, the relative phase separations between the selected and control populations are maintained; the time course and waveform of the *early* populations remain phase advanced relative to the *controls*, while those of the *late* populations are phase delayed relative to the *controls*. Consistent with the rhythmic expression of the

selected populations under LD cycles, even the circadian periodicity of eclosion and activity rhythms under DD condition is significantly longer in the *late* populations compared to the *early* populations. Such alterations in circadian phenotypes, borne out of heritable changes in genetic architecture in response to imposed selection pressure (and not because of random genetic drift or some unknown environmental or non-genetic effect) suggest that the time course and waveform of adult emergence and activity rhythms in *D. melanogaster* evolve as correlated responses to selection on the timing of adult emergence.

We further investigated the consequence of selection on life history traits such as pre-adult developmental time and adult lifespan in unmated (virgin) flies from the selected and control populations. The development time of the selected populations was altered as compared to the controls; the *early* populations develop faster than the *controls* under LD as well as DD conditions whereas the *late* flies take longer to develop under both the regimes. This suggests that selection for *early* and *late* adult emergence causes correlated change in the duration of pre-adult development. The lifespan of virgin flies from the selected populations depends upon the timing of their emergence. For example, the morning emerging *early* flies live longer than those that emerged during the evening, while the evening emerging *late* flies live longer than those that emerged during the morning. This, to the best of my knowledge is the first study of its kind demonstrating that morning emergence is adaptive for *early* populations while evening emergence is adaptive for the *late* populations.

In the last part of my thesis I have described studies on the molecular clocks of the *early*, *control* and *late* populations. I have assayed the levels of transcripts of clock

genes *period* and *clock* (*per* and *clk*) in the selected and control populations. Consistent with the circadian phenotypes, the peak of *per* mRNA in the *late* populations is phase delayed by about 4 hr compared to the *early* and *control* populations. Similarly, the peak of the *clk* mRNA in the *late* populations occurs about 4 hr later compared to the *early* and *control* populations. Besides the transcript levels even the ratio of spliced to unspliced variants in the *per* 3' UTR is significantly altered among the selected populations; the *early* populations display an overall suppressed spliced form of *per* throughout the day with a peak at ZT14 (2 hr after lights-off), whereas the *late* populations have generally higher levels of spliced form of *per* with a peak at ~ZT20. In other words, the spliced form of *per* peaks early in the *earl*y populations and later in the *late* flies. These findings are consistent with earlier studies that implicated role of *per* splicing in the determination of the phase of evening activity peak (Collins et al., 2004; Majercak et al., 2004).

Although the time course and waveform of the *early* and *control* populations appear to have diverged from each other and from the *late* populations, they do not differ from each other with regards to their free running period (τ), which raises a conundrum as to how clocks with similar τ could result in different circadian phenotypes under LD cycles. This is possible only if their clocks are differentially sensitive to light stimuli. Indeed the results of my experiment reveal that, the *early* populations undergo greater phase advance in their eclosion and activity rhythms in response to brief light stimuli administered during the late subjective night (Circadian time 20, CT20) and a modest delay during early part of the subjective night (CT14), whereas the *late* populations display exactly opposite response at these two phases. Given that circadian clocks underlying eclosion and locomotor activity in the selected and control populations

respond differently to brief light stimuli presented at CT14 and CT20, we decided to estimate the TIM levels at CT14 and CT20 to investigate the state of the molecular clock of the selected populations following a brief exposure to light. The *late* flies show drastic reduction in TIM levels at CT14 associated with large phase delays, while the *early* flies show lesser reduction in TIM levels associated with smaller delays, whereas at CT20, the light-dependent degradation is found to be greater in the *early* populations compared to the *controls*. Interestingly, the *late* populations show similar reduction to those of the *early* populations at this phase suggesting to a specific role for photoreceptor molecule (CRY) in the *late* populations. However, the role of CRY is yet to be ascertained in our selection lines.

The results of the experiments described in my thesis suggest that fruit flies *D. melanogaster* evolve morning and evening preference for adult emergence and activity as a result of periodic selection pressure imposed on the timing of adult emergence. As a consequence the *early* populations develop faster than the *late* populations and morning emergence becomes adaptive for the *early* populations and evening emergence for *late* populations. These studies also underscore the significance of timing of rhythmic phenomena for organisms living under periodic environments, and suggest a possible mechanism by which circadian rhythms may have evolved and/or fine-tuned by periodic biotic and abiotic factors of nature.

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Chapter 1

Introduction

1.1 Introduction to circadian rhythms

The phrase "as sure as night follows day" is applicable to cyclic events in all forms of life and reflects the stability of geophysical cycles in our environment as almost all organisms on our planet have evolved under a continuous exposure to the cycles of day and night caused due to the earth's rotation around its axis. Consequently, organisms adapt to appropriate and distinctive spatio-temporal niches depending upon their temporal programming of behavior and physiology (Dunlap et al., 2004). Therefore, it is not surprising that we witness a daily pattern in behavior and physiology. As a matter of fact most organisms have formed an internal representation of the external environmental cycles, which has become deeply rooted in the genome of virtually all life forms. These time-keeping mechanisms (commonly referred to as biological clocks) help organisms in predicting and preparing in advance to face the cyclic changes in their environments (Pittendrigh, 1993; Sharma 2003a; Dunlap et al., 2004). Besides anticipating cyclic events, biological clocks also provide an internal temporal order to ensure that internal changes take place in coordination with the external world (Pittendrigh, 1993). The timekeeping devices that delineate themselves from other biological clocks by having a clear functional significance are the circadian clocks. These clocks have the following characteristic features: (a) they have an endogenous periodicity of ~24 hr, (b) have the ability to synchronize to external time cues-thereby adopt a characteristic phase-relationship, a property known as "entrainment" and (c) under constant darkness (DD) their periodicities are temperature, nutrition and pH compensated, when the changes are within physiological range.

1.2 Molecular basis of circadian rhythms

The past two decades of research have provided us with great insights into the molecular mechanisms underlying circadian rhythmicity in a number of organisms. These mechanisms are primarily based on coupled transcriptional/translational feedback loops (TTLs) that appear to be conserved across a wide range of taxa (Dunlap, 1999; Glossop and Hardin, 2002; Panda et al., 2002). It is therefore believed that transcriptional and translational outputs are indispensable for the maintenance of rhythmic functions and the coordination of behavior and physiology. The TTLs consist of oscillations of gene products that regulate their own synthesis to produce a ~24 hr periodicity (Dunlap, 1999; Glossop et al., 2003; Stanewsky, 2003). In fruit flies *Drosophila melanogaster* the molecular clockwork consists of two basic helix–loop–helix (bHLH) transcription factors, CLOCK (CLK) and CYCLE (CYC), which bind to upstream E boxes and activate the transcription of the *period* (*per*) and *timeless* (*tim*) genes as well as other genes such as *vrille (vri)* and *pdp1* (Cyran et al., 2003). PERIOD and TIMELESS (PER and TIM) proteins associate with each other in the cytoplasm and the heterodimer is transported into the nucleus. The PER–TIM heterodimer then acts on the transcription factor complex CLK–CYC to inhibit the transcription of *per* and *tim* genes (for review see Hardin, 2005). A second feedback loop, which involves two transcription factors VRI and PDP1, regulate the transcription of the *clk* gene in a time dependent manner (Cyran et al., 2003; Glossop et al., 2003). Although, the molecular mechanisms through which PER-TIM represses the transcriptional activation of CLK-CYC are not yet clearly understood, some preliminary evidence point out towards posttranslational modification of clock proteins (Edery, 1999; Akten et al., 2003). Two kinases, DOUBLETIME (DBT)

and casein kinase II (CKII) have been implicated in the clock mechanisms that regulate the concentration of PER protein in the cytoplasm (Price et al., 1998; Kloss et al., 1998, 2001; Martinek et al., 2001; Lin et al., 2002). These kinases phosphorylate clock proteins in a time-dependent manner and affect their stability, a process that is believed to provide temporal gating in the nuclear localization of PER and TIM (Curtin et al., 1995; Dembinska et al., 1997; So and Rosbash, 1997; Kim et al., 2002; Shafer et al., 2002). TIM also plays a key role in the photoentrainment mechanisms of the molecular clock, mediated through the circadian photopigment CRYPTOCHROME (CRY) (for review see Helfrich-Förster, 2005). Finally, timed release of a neurotransmitter PIGMENT DISPERSING FACTOR (PDF) by the clock neurons serves as an output signal for the downstream targets that are responsible for the regulation of behaviours (for review see Stanewsky, 2002).

1.3 Phase resetting by environmental signals

Light is almost certainly one of the predominant entraining agents for circadian clocks. LD cycles align the phase of circadian clocks by evoking daily phase shifts. Although we do not have a clear understanding as to how environmental light signals synchronize circadian clocks, it is believed that light resets the *Drosophila* clock by affecting levels of the TIM protein (for review see Williams and Sehgal, 2001). Pulses of light given during the early subjective night delays the phase of the rhythm and is accompanied by a reduction in TIM level, while light stimuli administered during the late subjective night cause phase advances and a reduction in TIM level. Analysis of dose-response curves and phenotypes of genetically manipulated flies suggest that the magnitude of TIM degradation in response to light is correlated with behavioral responses (Suri et al., 1998).

An important point to note here is that both phase delays as well as phase advances in circadian rhythm are associated with TIM-degradation. This is because the direction of phase shift is likely to be determined by the amount of TIM protein present (low levels during the early part of the night and high during the second half), and/or its cytoplasmic/nuclear location (Huntor-Ensor et al., 1996; Suri et al., 1998). Following exposure to light, TIM protein undergoes phosphorylation on its tyrosine residues and ubiquitination, which eventually leads to proteasome mediated degradation (Naidoo et al., 1999). Such responses are believed to be mediated through CRY protein (Stanewsky et al., 1998; Emery et al., 1998, 2000a, 2000b), which binds TIM in a light-dependent manner (Ceriani et al., 1999). In summary, light pulses administered during the early subjective night delays the clock by postponing TIM production and therefore causing a delay in its nuclear entry, while light pulses given during the late subjective night advances the clock by prematurely removing TIM protein and by advancing the *per* and *tim* transcription (for review see Williams and Sehgal, 2001).

Although, behavioral and molecular response to light is affected, neither is completely eliminated by any visual mutation (Yang et al., 1998). Therefore, it was thought that circadian entrainment in *Drosophila* is independent of visual system, though it could aid entrainment to LD cycle when present. Furthermore, *cry*^{b} flies that are deficient in CRY function can still entrain to LD cycles, though the response to brief light pulse is severely affected (Stanewsky et al., 1998). The role of the visual system and CRY in entrainment is confirmed by the fact that the *cry*^{b}*;NorpA* double mutants have reduced ability to synchronize to LD cycles (Stanewsky et al., 1998).

1.4 Multi-oscillatory organization of circadian clocks

Organisms display a wide array of behavioral and physiological processes, which have evolved to match with environmental cycles of nature. Many of these processes have presumed a characteristic phase-relationship with the environmental cycles, so as to perform various functions at favorable time of the day or night. Circadian biologists have always been perplexed about the structure of circadian timing systems that govern such temporal organization in a wide variety of life processes. Several lines of evidence from organisms ranging from unicellular to multicellular higher organisms, accumulated over past few decades, suggest that circadian organization is multi-oscillatory nature (Pittendrigh, 1960; Sweeney, 1969; Pittendrigh, 1974; Takahashi and Menaker, 1982; Saunders, 1986; Roenneberg, 1996; Giebultowicz, 2000; Reppert and Weaver, 2002).

The circadian architecture of fruit flies *D. melanogaster* is also multi-oscillatory (for review see Howlader and Sharma, 2006). Rhythmic expression of adult emergence, locomotor activity, olfaction, and egg-laying has been shown to differ in terms of their circadian phenotypes and neurogenetic control. For example, light-induced phase response curve (PRC) of eclosion was different from that of activity/rest rhythms in *Drosophila* (Engelmann and Mack, 1978). The periodicity of egg-laying rhythm under continuous darkness (DD), unlike those of activity and adult emergence rhythms, was greater than 24 hr (Sheeba et al., 2001a). Egg-laying was found to persist rhythmically under constant light (LL) (Sheeba et al., 2001b), while activity and eclosion became arrhythmic (Paranjpe et al., 2004). This suggests that separate oscillatory systems regulate rhythmicity in behaviors such as activity, adult emergence, and egg-laying in *D. melanogaster*. The circadian pacemaker circuit of *Drosophila* consists of at least six

distinct neuronal cell groups: the small and large ventral lateral neurons (LN_vs); the dorsal lateral neurons (LN_ds) and three dorsal neurons (DN1, DN2, and DN3). These neurons are characterized by their cyclic expression of PER and TIM proteins with a near 24 hr period. In a recent study, Myers et al (2003) have shown that LN_vs are necessary for the persistence of locomotor activity rhythm, while LN_vs as well as prothoracic gland are essential for rhythmic expression of adult emergence. Therefore, though LN_v are considered to be the central pacemaker for activity and eclosion rhythms, there is also sufficient evidence to suggest that autonomous peripheral oscillators that may or may not be under the direct control of LN_v, regulate circadian rhythms in a variety of functions (for review see Howlader and Sharma, 2006). For example, in *D. melanogaster* circadian pacemakers are found in the non-innervated peripheral organs such as malpighian tubules (Giebultowicz and Hege, 1997). Autonomous pacemakers in the antennal neurons have been demonstrated to be both necessary and sufficient for the regulation of rhythmic olfaction in *D. melanogaster* (Krishnan et al., 1999; Tanoue et al., 2004). In a recent study, it was shown that egg-laying rhythm in *D. melanogaster* is controlled by non-PDF mediated non-LN_v based circadian oscillators (Howlader et al., 2006). Further, the circadian clocks governing activity rhythm in *Drosophila* are believed to comprise of separate "morning" and "evening" oscillators (Stoleru et al., 2004, 2005; Grima et al., 2004; Yoshii et al., 2004; Rieger et al., 2006). It is believed that small LN_v act as the morning oscillators and LN_d serve as the evening oscillators. These studies suggest that the circadian timing system of *Drosophila* is composed of many independent oscillators that talk to each other via neural and/or humoral signals.

1.5 Adaptive evolution and circadian clocks

The adaptive value accrued by circadian clocks is beyond any doubt, yet rigorous empirical proofs are scanty. The most appropriate way of empirically addressing this issue would be to investigate evolution of circadian clocks under the influence of periodic selection forces. Until the 1970s most evolutionary biologists believed that "if a biological phenomenon is present, it must have been selected by evolutionary forces and therefore be of adaptive significance". This view was contradicted by recent findings that suggest that existing experimental outcomes do not provide unequivocal explanations for the adaptive advantage and the driving evolutionary forces (the adaptive advantage is dealt in a subsequent section in this chapter). The evolutionary trajectory of a population driven by the forces of natural selection depends upon past selection history, ancestry, population size and chance in the form of random genetic drift (Travisano et al., 1995; Joshi, 1997). Random genetic drift takes place when allele frequencies in a population change randomly from one generation to another due to sampling error associated with finite and small number of zygotic combination from a large number of gametes. Hence, drift may pose a genetic constraint to an evolutionary process as it gradually diminishes the genetic variation present in the population, which serves as required material for evolution. Smaller populations may be at a greater risk, as the variation among the actual available genotypes to start with is very small, and this could act as a constraint on selection (Kirkpatrick, 1996). Other factor such as ancestry may in some cases act as a constraint, because certain lineages may not possess enough genetic variation in traits that would have been advantageous.

Many selection schemes to address adaptive evolution of circadian clocks have been used by previous workers, however, the approach and methodology employed were either inadequate or suffer from one or other constraints of the kind discussed earlier in this section. The observation of a consistent pattern of differentiation between a set of replicate control and selected populations, however, implicates selection as the cause, because it is unlikely that several replicate populations could undergo the same sequence of genetic changes through the operation of a random phenomenon like drift (for further discussion and empirical examples see Joshi et al., 1997). However, most previous selection studies have used only one selected population and its control (in some cases even without controls) to formulate a deterministic evolutionary outcome about the adaptive evolution of circadian clocks. Individuals live, reproduce and die, as a consequence of heritable differences in reproductive output (*i.e*. natural selection) among individuals, the genetic composition of a population changes over time in an adaptive manner. Thus, the unit of evolutionary change is the population and not the individual. The corollary to this is that the unit of replication in any study addressing evolutionary questions needs to be the population and not the individual.

Furthermore, many selection studies used isofemale lines or the so called "wild type" lines (e.g. *D. melanogaster*; Oregon R, or Canton S) wherein a study population is created by crossing several isofemale lines to eliminate problems related to paucity of genetic variation. Such populations are therefore subjected to potentially confounding large linkage disequilibrium effects unless they are first maintained for many generations (> 25) in the laboratory (Weir and Cockerham, 1979). Moreover, these lines also suffer from bottleneck effects due to small population size, which may in turn lead to

inbreeding depression. Therefore, it is difficult to rule out the fact that the results of previous studies are not masked by random genetic drift, and bad or biased genotypes (for review see Miller and Hedrick, 2001; Prasad and Joshi, 2003; David et al., 2005). Similarly, use of mutant lines, truly bred for deviant phenotypes, has been traditionally quite popular in circadian biology because of their quick and detectable effects on circadian phenotype. However, the past few decades have seen an increasing realization among biologists that the use of mutant lines (and in the case of *Drosophila*, also the traditionally popular 'chromosome extraction' lines) to study the consequence of genetic alterations is, by itself, fraught with dangers (Rose, 1984; Mueller and Joshi, 2000: chapter 1). This is because the mutant or chromosome extraction lines are typically inbred and are therefore likely to yield spurious positive genetic correlations between fitness components (for empirical evidence see Mueller and Ayala, 1981). Therefore such results cannot be used to reliably understand the course of evolution in organisms, which have random and out breeding structure in nature.

1.6 Evidence of adaptive significance of circadian clocks from studies on

(a) Circadian clocks in periodic environment

The orchestration of behavioral and metabolic processes is the hallmark of circadian clocks, and therefore has always occupied centre stage in circadian rhythm research. It has been a long held view that circadian rhythms have evolved as a result of continued interaction between biological processes and temporal structure present in the geophysical environment.

The role of circadian clocks in providing adaptive advantage has been documented in several studies under natural and semi-natural environments. For example,

Daan and Tinbergen (1980) reported that fledglings of guillemots *Uria lomvia* suffer higher mortality due to predation by gulls if their jumping activity was not restricted to certain hours in the evening. In another set of studies by DeCoursey and coworkers (1997), SCN-lesioned antelope ground squirrels *Ammospermophilus leucurus* and eastern chipmunks *Tamias striatus* were found to incur increased mortality compared to the intact controls (DeCoursey and Krulas, 1998). Such reduction in lifespan was mainly due to predation, perhaps due to increased nighttime restlessness (DeCoursey et al., 2000). Insects too exhibit a wide array of rhythmic behaviors that are directly influenced by natural environment of which emergence of adults from pupal cases (eclosion) and activity/rest cycles are the most prominent ones. Adult emergence in fruit flies *D. melanogaster* follows a circadian pattern with majority of the flies emerging during dawn when the relative humidity in the environment is high (Bünning, 1935; Kalmus, 1935; Pittendrigh, 1958). This is thought to provide the flies with enough time to dry their wings and prepare for the day. Adaptive benefits of circadian timing system in *Drosophila* is not restricted to adult emergence rhythm, adult flies exhibit bimodal activity pattern, adjusted in a way that maximum activity is scheduled during favorable time of the day (Collins et al., 2004; Majercak, et al., 2004). The evening activity bouts occur during the cooler parts of the night under summer conditions or long photoperiods, while are exhibited before dusk during ambient or extreme cold conditions or short photoperiods (Majercak et al., 1999, 2004; Collins et al., 2004).

(b) Circadian clocks and resonance

Virtually all organisms have been influenced by the temporal structure of the environment, since the origin of life on our planet. Further, it is generally believed that

only those that had circadian clocks with periodicities matching that of the geophysical world survived and others were eliminated. This led to the formulation of the "circadian resonance hypothesis" (Pittendrigh and Minis, 1972), which assumes that organisms possessing clocks with periodicities matching that of the environment, would perform "better" compared to others with deviant periodicities. Empirical evidence for the circadian resonance hypothesis came from early works of Pittendrigh and Aschöff, where they showed that fruit flies *Drosophila melanogaster* (Pittendrigh and Minis, 1972) and blowflies *Phormia terranovae* (von Saint-Paul and Aschöff, 1978) normally reared under 24 hr LD cycle live significantly longer under 24 hr LD cycles compared to any non-24 hr LD environment. The most compelling demonstration of circadian resonance came from a study on cyanobacteria *Synecococcus elongatus* (Ouyang et al., 1998). In this study, mutant strains of cyanobacteria exhibiting either short (~23 hr) or long (~ 30 hr) periodicities were competed among each other and with the wild type strain (period ~ 25 hr). The short period strain out competed other strains under 11:11 hr LD cycles and the long period strain out-competed others in 15:15 hr LD cycles. When arrhythmic strains were competed with rhythmic ones, arrhythmic strains lost to wild-type strains under LD cycle, but out-competed them under LL condition (Woelfle et al., 2004), suggesting that circadian clocks may not be beneficial under LL, in fact it might even be disadvantageous. In contrast, a study on the *per* mutants of *D. melanogaster* reported that males of *per* mutants lived significantly longer than the wild type males under various LD cycles, including those whose periods closely matched the clock periodicity of the mutants (Klarsfeld and Rouyer, 1998). Taken together, it appears that the role of circadian clocks in providing fitness advantage is restricted only to certain organisms and

environments, which warrants further systematic experimentation on a variety of organisms under a number of environmental conditions.

(c) Circadian clocks in aperiodic environments

Several lines of evidence, especially from the laboratory selection studies, suggest that any trait that does not confer adaptive advantage is eliminated from the population within 100-200 generations due to mutation accumulation and random genetic drift, and its purging will be faster if the trait has some cost associated with it (Mueller, 1987; Service et al., 1988; Rose et al., 1996; Joshi, 1997). Given this scenario, organisms inhabiting aperiodic environments such as depths of oceans, underground caves and rivers or any similar aperiodic environment may be expected to have lost the ability to exhibit circadian rhythmicity in behavior and physiology, or even if they do display rhythmic behavior, it may not be possible to synchronize them to periodic external cycles. Interestingly, results of few studies on organisms living in aperiodic environments have provided diverse results. In some cases organisms living in deep sea and subterranean caves do not exhibit overt rhythmicity at all, and if they do, its periodicities are far removed from 24 hr. For instance, eyeless crayfish *Niphargus puteanus* was found to be arrhythmic in its locomotor activity rhythm, though residual components of activity were observed which followed 10- 57 hr periodicities (Blume et al., 1962). In a separate study on Amblyopsid fishes, it was reported that the locomotor activity behavior of these fishes was arrhythmic, though oxygen consumption followed a ~24 rhythm but did not entrain to LD cycles (Poulson and White, 1969). Studies on cave dwelling millipede *Blaniulus lichensteini* (Mead and Gilhodes, 1974) and *Glyphiulus cavernicolus* (Koilraj et al., 2000) reported presence of circadian rhythmicity in activity/rest cycles. In an extensive study on

four sets of large, independent populations of *D. melanogaster* Sheeba and coworkers demonstrated that flies, which were never exposed to any periodic environment for well over 600 generation display circadian rhythmicity in adult emergence (Sheeba et al., 1999), egg-laying (Sheeba et al., 2001b) and locomotor activity (Sheeba et al., 2002). These flies were also able to synchronize their behaviors to a variety of LD cycles, suggesting that they have retained their entrainment ability even after living for several hundreds of generations under constant light (LL) conditions (Paranjpe et al., 2003, 2004). These findings led to the proposal of "intrinsic adaptive advantage" in organisms possessing free-running clocks even after rearing in aperiodic environment for more than 700 generations (Sharma, 2003a). Nevertheless, intrinsic adaptive advantage does not seem to be applicable to genetically manipulated organisms (Woelfle et al., 2004).

(d) Circadian clocks and life history traits

Circadian clocks have been implicated in the regulation of life history traits such as pre-adult development time and adult lifespan in a number of insect species including *Drosophila melanogaster* (Kyriacou et al., 1990; Sharma, 2003a; Paranjpe and Sharma, 2005). For example, in a study on the *per* mutants of *Drosophila* it was shown that the per^S mutants (τ = 19 hr) develop faster than the wild type flies (τ = 24 hr), and the wild type flies develop faster than the per^L mutants (τ = 28 hr), irrespective of the state of their circadian clocks and the environmental conditions (Kyriacou et al., 1990). In other words, whether their clock is entrained under LD cycles, or free-running under DD conditions, or rendered arrhythmic under LL conditions, the short period mutant develop faster than the wild type flies and the wild type flies develop faster than the long period mutants. In a separate study on the melon flies *Bactrocera cucurbitae,* where flies were selected for

faster and slower pre-adult development, the faster developing flies had faster (τ ~ 22.6 h) running clocks and an earlier timing of mating compared to the slower developing lines (τ ~ 30.9 h) (Miyatake et al., 1996, 1997; Shimizu et al., 1997). The authors argued that the correlation between the timing of mating behaviour and development time is mediated via pleiotropic effects of *per* gene. These studies, therefore, suggest that there may not be any causal relationship between circadian clocks and pre-adult development time.

Circadian clocks have also been implicated in regulation of lifespan. In an early study on naturally occurring *tau* mutation in hamsters, it was found that *tau* heterozygote animals (τ ~ 22 hr) lived shorter than the wild type animals (τ ~ 24 hr) under LD (14:10 hr) cycles, but the lifespan of the homozygous animals (τ ~ 20 hr) did not differ from those of the wild type controls (Hurd and Ralph, 1998). Strikingly different results were obtained in a subsequent study carried out under constant dark (DD) conditions; in this case the homozygous animals were found to live longer than the wild type controls, while average lifespan of heterozygote animals did not differ from that of the wild type controls (Okelewicz and Daan, 2002). Such differences in the outcome of studies on life history traits is not surprising, rather it suggests a complex environmentally mediated relationship between circadian clocks and lifespan (Kyriacou et al., 1990; Hurd and Ralph, 1998; Sheeba et al., 2000; Sharma and Joshi, 2002). In a separate study on *per* mutants of *D. melanogaster* it was reported that the per^T (short period mutant, τ ~ 16 hr) and per^L (long period mutant, τ ~ 29 hr) mutants lived significantly shorter than the wild type per^+ (τ ~ 24 hr) controls, even though they were kept under LD cycles of periodicity

similar to their clock period (Klarsfeld and Rouyer, 1998). This suggests the clock genes have pleiotropic role in the regulation of circadian clocks and life history traits.

(e) Circadian Clocks and photoperiodism

Circadian clocks are believed to play a non-trivial role in distinguishing long days of summers and short days of winter (for review see Saunders, 1982). These clocks time an array of developmental, physiological, and behavioral mechanisms that respond to seasonal changes in their external conditions (Edery, 2000). For example, a number of insect species enter diapause, a period of growth arrestment that is induced by short photoperiods or cold temperatures (for review see Saunders, 1982; Vaz Nunes and Saunders, 1999). Siberian hamsters typically breed only during spring and summer months, an indication of seasonal adaptation by gonadal regression, which is mediated by extended nocturnal release of melatonin (Yellon and Goldman, 1984). Photoperiodism has also been extensively studied in plants, where floral initiation can be experimentally controlled by changes in day lengths.

The adaptive significance of circadian rhythms has been much speculated about but has never been subjected to systematic and rigorous empirical investigation. The majority of the few empirical studies of the possible adaptive significance of circadian organization suffer from numerous shortcomings that we can now identify based on hindsight gained through three decades of experimental work in evolutionary genetics (Rose et al., 1996). There is also growing realization among biologists that a careful and systematic long-term selection study on laboratory populations is a worthwhile approach to understand

issues related to adaptive evolution. An unequivocal demonstration of adaptive evolution would be to show that the trait under selection evolves by enhancing fitness of organisms.

The present study was initiated on four evolutionarily independent populations of fruit flies *D. melanogaster*, in order to understand the effect of selection on timing of adult emergence on the circadian waveform and on circadian clock as well as clock related life history traits. For this purpose we have employed a selection scheme that is based on selection of individuals emerging in the morning (early) and evening (late) hours under laboratory LD cycles from four ancestral founder populations that have been maintained as separate entities without any gene flow between them and hence served as independent replicates for my study (Chapter 2). Four replicates of *early* and *late* populations were thus initiated along with four unselected controls. In order to ascertain the direct response of selection, we compared the number of flies that emerged out of the morning and evening windows of selection at regular intervals of 10-15 generations to trace the evolutionary trajectory of changes over 55 generations of selection. To investigate the consequence of selection on circadian clocks we assayed eclosion and activity rhythms in the selected and control populations under LD and DD conditions (Chapter 3). We also tested the robustness of the circadian phenotypes of the selected populations by assaying the eclosion and activity rhythms under variety of LD cycles at the 70th generation (Chapter 4). Further, I examined the consequence of selection on life history traits such as pre-adult developmental time and adult lifespan in unmated (virgin) flies from the selected and control populations (Chapter 5, 6). In the concluding part of my study, I have described my study on the molecular clocks of the *early*, *control* and *late* populations (Chapter 7).

Chapter 2

Introduction to experimental populations

2.1 Background of experimental populations

In this section the ancestry and the maintenance schedule of four baseline populations of *Drosophila melanogaster* used to derive our selection populations have been described. These populations served initially to derive three sets of populations and subjected to selection for adaptation to different light regimes. These baseline populations were derived from populations, which were initiated from a wild caught population (IV) from South Amherst, Massachusetts, USA, about 35 years ago (Ives, 1970). These populations were reared in the laboratory for about 110 generations under constant light (LL), constant temperature (25 ± 1°C) and constant humidity (75 ± 5%) on a 14-day discrete generation cycle. Five populations (B1…B5) were derived from the IV populations, and then reared under laboratory conditions similar to the ones in which the IV populations were maintained (Rose and Charlesworth, 1981; Service and Rose, 1985). After about 360 generations, a set of 5 populations was derived from the five B (B1…B5) populations (UU1…UU5; Joshi and Mueller, 1996). The UU populations were maintained under constant light, temperature and humidity on a 21-day discrete, and reared in the laboratory for about 170 generations after which four populations (JB1..JB4) were derived from UU populations (Fig. 2.1).

Populations starting from IV up to the UU were maintained on a banana-molasses food medium. The four populations (JB1..4) have been described in detail by Sheeba et al (1998). Briefly, they were maintained under alternating LD (12:12 hr) cycles (light intensity 15 ± 5 μW/cm^2/sec), temperature (25 ± 1°C), and humidity (75 ± 5%), with banana-jaggery food and water available *ad libitum*. A total of ~1200 breeding adults per population were maintained in Plexiglass cages (25 cm × 20 cm × 15 cm) with abundant

food on a 21 day discrete generation cycle. Eggs were collected by placing petri dishes with food into these cages for 2 hr (between 0900-1100 hr) and then dispensed at a density of about 300 eggs into vials (18 cm height × 2.4 cm diameter) with 10 ml of food. On the 9th to 13th days after egg collection, freshly emerged flies were collected into Plexiglass cages containing a petri dish of food. On the 18th day, a generous smear of yeast-acetic acid paste was applied on the food plates and kept in the cages. Three days later, eggs were collected to initiate the next generation. These four populations (referred as the $LD_{1..4}$ populations) which were maintained for over 100 generations as four independent evolutionary entities, served as the parental stocks for the initiation of selected populations (Fig. 2.1).

2.2 Selection protocol

From these four LD populations, four populations of early ($early_{1..4}$), and four populations of late ($late_{1..4}$) populations were initiated by imposing selection for early and late adult emergence, along with four control populations ($control_{1..4}$), where no selection pressure was applied (Fig. 2.2). Each *early* and *late* replicate population was derived from one control population, thus forming the matched selected and control pair ($early_i$, $control_i$ and $late_i$ are more closely related than $early_j$, $control_j$ and $late_j$, i,j =1-4). The four replicate populations with identical subscripts were treated as blocks (or random factor) in the statistical analysis. The selected populations were maintained in the same environment as the *control* populations with "lights-on" at 0800 hr and "lights-off" at 2000 hr, except that at each generation the adult flies were collected between 0500-0900 hr (morning window) for the *early* populations, and between 1700-2100 hr (evening window) for the *late* populations (Fig. 2.2).

2.3 Standardization of the selected populations

Imposition of different maintenance regimes may induce nongenetic parental effects (Prasad et al., 2001), and therefore all selected and control populations were subjected to one generation of common rearing conditions prior to the assays, during which no obvious selection pressure was imposed. Eggs were collected from the running cultures and dispensed into vials with about 10 ml of food, at a density of about 300 eggs per vial. On the 12th day after egg collection, adult flies were collected into Plexiglass cages with abundant food. For the assays, flies were supplied with yeast-acetic acid paste for two days prior to the egg collection. The progeny of these flies hereafter were referred as standardized flies, and were employed for all the experiments described in my thesis.

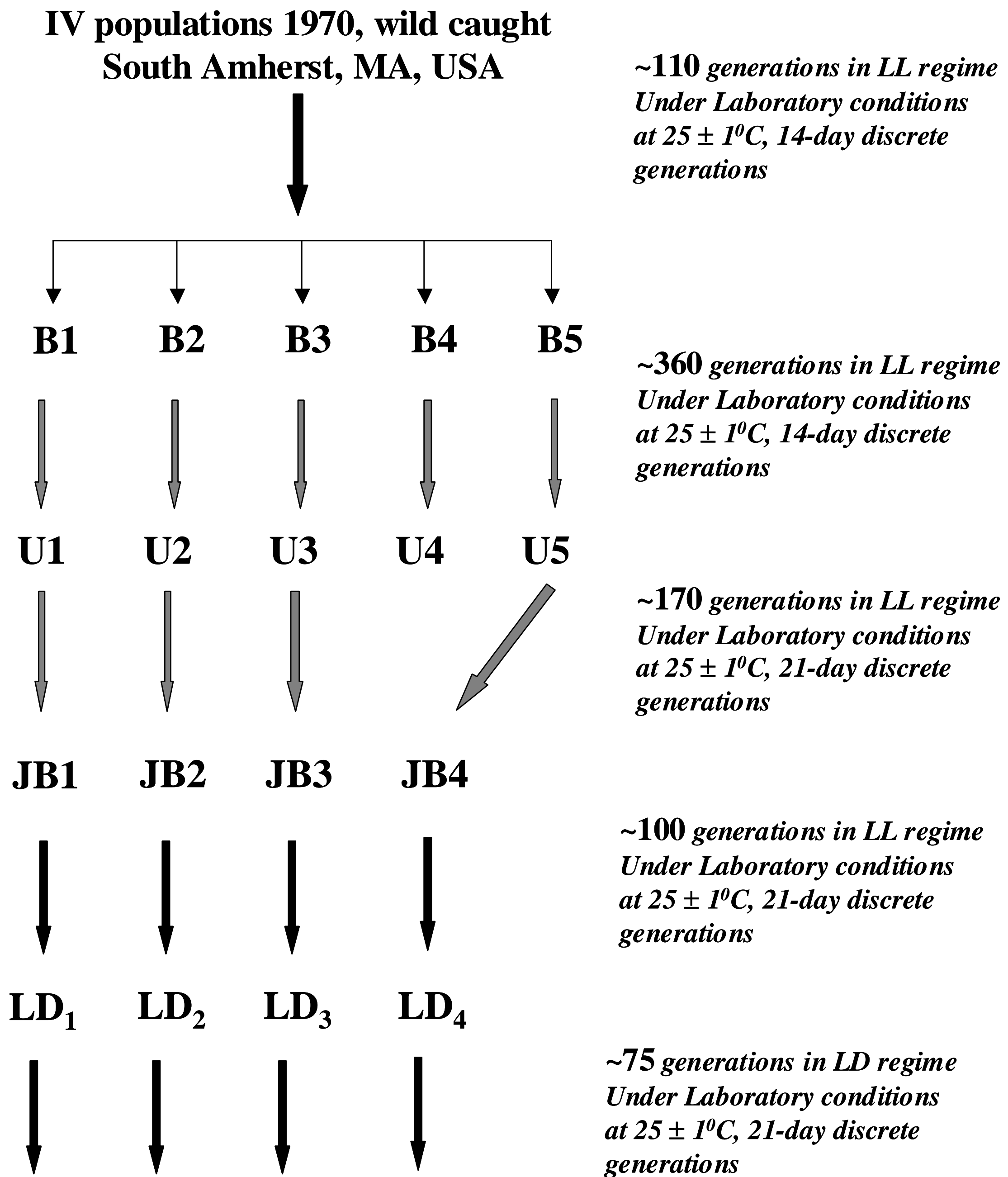

Fig. 2.1 Schematic representation of the maintenance of the ancestral populations.

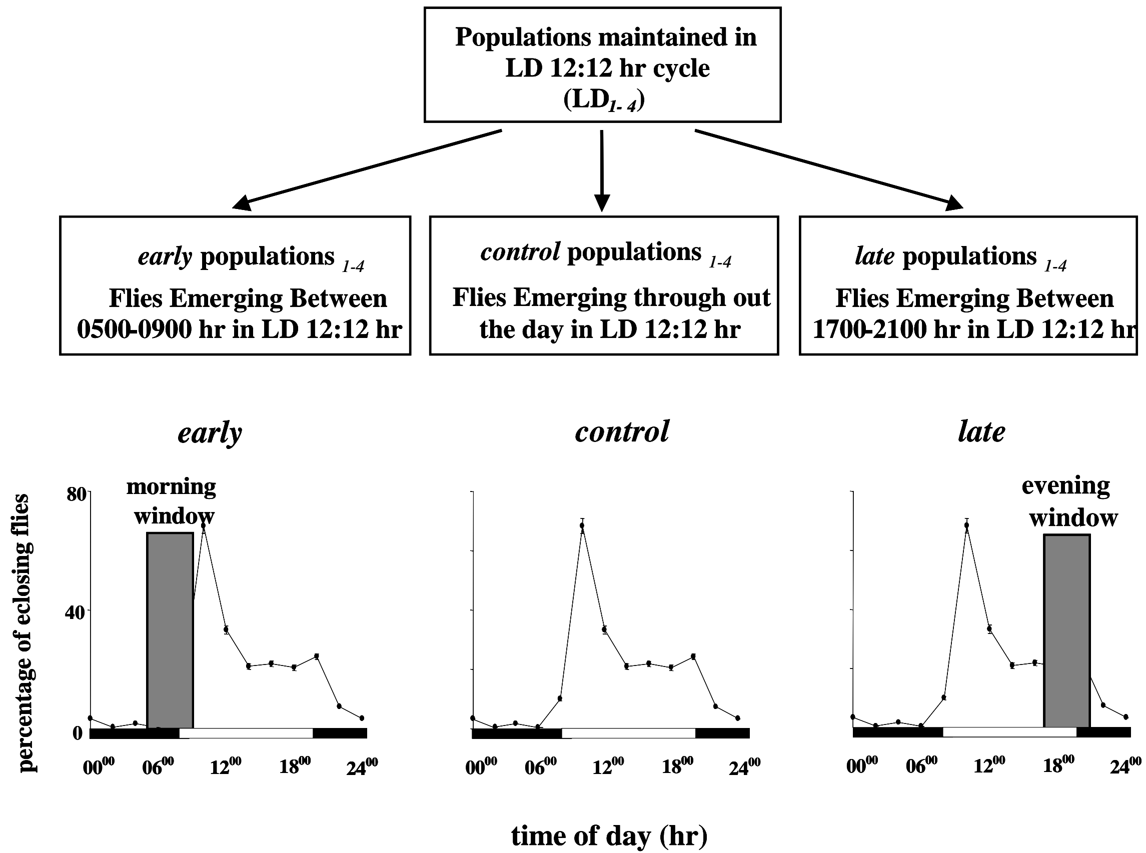

Fig. 2.2 Schematic representation of the selection protocol to derive four replicate *early, control* and *late* populations.

Chapter 3

Direct and Correlated responses to selection on timing of adult emergence

3.1 Background

In insects, adult emergence (eclosion) rhythm is one of the most extensively studied and perhaps the best understood circadian process after activity/rest cycle. Although each individual emerges as an adult only once in its life cycle, gating of this event is believed to be under the control of an on-going oscillation present during development (Saunders, 1992). Consequently, certain intervals of time in a day constitute the "forbidden zone of eclosion", whereas a brief period of time during which adults emerge forms the "allowed zone" (also referred to as the "gate" of eclosion) (Pittendrigh, 1954). The gating is often so stringent that even if developing flies are mature enough to emerge but fail to do so during the gate, they would remain within the puparium until the next gate opens (Saunders, 1992).

The emergence patterns of most insects under laboratory light/dark (LD) cycles is bimodal, where majority of the flies emerge close to "lights-on" (the primary peak), and a small fraction of them come out throughout the day with a small peak close to "lights-off" (the secondary peak) (Jackson, 1983; Sheeba et al., 2001c). Such patterns of adult emergence have facilitated efforts to derive "early" and "late" populations of *Drosophila pseudoobscura* (Pittendrigh, 1966), laboratory (Oregon R) and wild caught (W2) populations of *D. melanogaster* (Clayton and Paietta, 1972), and *Pectinophora gossypiella* (Pittendrigh and Minis, 1971). The early and late populations were raised by selecting for flies that emerge during "lights-on" (morning) and "lights-off" (evening) under 12:12 hr LD cycles. The eclosion peaks of the early and late populations diverged by about 4-5 h and the free-running period (τ) of their endogenous clock under constant darkness (DD) differed by about 2.5 hr, after fifty generations in *D. pseudoobscura*,

sixteen generations in *D. melanogaster* and nine generations in *P. gossypiella*. The early-late population difference in phase relationship of eclosion rhythm with respect to LD cycles was maintained across a variety of photoperiods in both *D. pseudoobscura* and *P. gossypiella*. Both species exhibited similar correlated response in τ of the eclosion rhythm: the early population had longer τ than the parent population, whereas the late population had shorter τ than the parent population. The light induced phase response curve (PRC, a plot of phase shift in the rhythm as a function of time of light pulse administration) of eclosion rhythm in the early, late and parental populations, however, did not differ, suggesting that the circadian pacemakers in the selected and control populations were not different. Pittendrigh (1981) interpreted these results in the context of a "master-slave oscillator model". He argued that the differences in the phase and period of the eclosion rhythm among the selected populations were due to altered coupling between master and the slave oscillators underlying eclosion rhythm. In *Pectinophora*, correlated responses to selection were observed for the egg hatching rhythm: eggs from the early population hatched earlier, and eggs from the late population hatched later than the parent population. There was however no correlated change in the parameters of the female oviposition rhythm. Though it is possible that altered coupling between oscillators may cause phase separation, it is hard to imagine how similar master oscillators can generate oscillations with widely different free-running period. In a separate study, bimodal pattern in activity/rest was used to derive early and late populations under LD cycles in an Indian population of fruit flies *D. rajashekari,* initiated from a single isofemale line (Joshi, 1999). Such a selection scheme guarantees highest degree of inbreeding and linkage disequilibrium, which can lead to inbreeding depression

and elimination of variation in the population (Sharma and Joshi, 2002). However, in spite of the high degree of inbreeding (and therefore homozygosity), not only did the flies survive fifty-nine generations of inbreeding depression and continue to respond to selection, the selected lines showed greater variation than the control (ancestral) line in their clock properties (Joshi, 1999).

Given the fact that temporal organization of behavior and physiology holds the key for understanding the adaptive significance of circadian time keeping mechanisms the most appropriate way of empirically addressing this issue would be to carry out long-term selection studies on replicate sets of populations which is likely to be more fruitful. In our opinion, this approach will provide meaningful insights into the evolutionary processes that may have been instrumental in the evolutionary fine-tuning of circadian clocks. However, of the few empirical studies on the possible adaptive significance of circadian organization, the majority suffer from numerous shortcomings that we can now identify based on the hindsight gained through three decades of empirical studies in evolutionary genetics. In all previous studies on selection for timing of adult emergence there were no replicates used at the level of population within selection regime, and there is very little information provided about the handling and rearing of the experimental populations, making it difficult to assess the degree of genetic drift or inbreeding that these populations may have undergone (Miller and Hedrick, 2001; reviewed in Prasad and Joshi, 2003; David et al., 2005).

In this chapter, I report the results from the first 55 generations of our ongoing laboratory selection study. In order to ascertain the direct and correlated response of selection we assayed eclosion and activity/rest rhythms of four replicate populations each

of *early*, *control*, and *late* populations, under LD and DD conditions. These assays were repeated in a similar manner at regular intervals of 10-15 generations of selections to trace the evolutionary trajectory of the selection pressure. The results provide interesting insights into for the evolutionary fine-tuning of circadian clocks by periodic selection forces.

3.2 Materials and methods

3.2 (a) Eclosion rhythm assay

The number of flies emerging during the morning and evening windows of selection, phase relationship (ψ) between the eclosion peak and LD cycle, the waveform of eclosion rhythm under LD cycles, as well as the waveform and the τ of the eclosion rhythm under DD were assessed at the 5th, 10th, 25th, 40th and 55th generations. For these assays, eggs of approximately same age were collected from the standardized flies (method of standardization has been discussed in details in section 2.3) and dispensed into vials with 10 ml of food, approximately at 300 eggs per vial and kept under LD and DD conditions. Ten such vials were set up per population for assays under each light condition. These vials were monitored for the first eclosion and thereafter checked regularly at every 2 hr interval for ten consecutive days and the number of eclosing flies was recorded. From the raw data, we obtained the percentage of flies eclosing from the morning and evening windows of selection, the mean ψ of the eclosion peak, and the eclosion waveform under LD cycles. The percentage of flies emerging from the morning and the evening windows was estimated at the 5th, 10th, 25th, 40th, and 55th generations by normalizing the total number of flies emerging during the morning and evening windows by the total number of flies eclosing during one complete cycle. The ψ of the eclosion rhythm under LD

cycles was estimated as the average time interval between the peak of eclosion and lights-on in the LD cycle. The ψ values were considered to be negative if the peaks followed lights-on, and were taken to be positive when the peaks preceded lights-on. Under DD condition the eclosion was monitored under dim red light (λ > 640 nm) at every 2 hr interval for ten consecutive days.

3.2 (b) Activity/rest rhythm assay

The ψ between the activity peaks and LD cycle, activity levels during the morning and evening windows of selection, waveform of activity rhythm under 12:12 hr LD, τ, and the waveform of activity/rest rhythm under DD were estimated at the 55th generation. For the assays, eggs laid between 0900-1100 hr were collected from the standardized populations and dispensed into vials with 10 ml of food, at a density of about 300 eggs per vial, and kept under LD conditions. Freshly emerged adult flies were transferred into an activity-monitoring set-up within 24 hr of their emergence, and their activity was monitored using the activity monitors, infra-red beam crossing system recording total crosses in every 15 minute bins (Sharma, 2003b). Normalized activity counts from all individuals of each population were averaged to create a single average activity profile over a period of at least 8 days. The activity/rest rhythm of the flies was monitored for the first 8-9 days under 12:12 hr LD cycles and for the next 10 days under DD. The percentage of activity during the morning and evening windows of selection was estimated by normalizing the amount of activity exhibited during the morning and evening windows by the total amount of activity during one complete cycle. The ϕ of the activity onset (ϕ_{on}) was estimated as the average time of activity onset close to lights-on

and the ϕ of the activity offset (ϕ_{off}) was estimated as the average time of activity offset close to lights-off over 10 cycles, using CLOCKLAB (Actimetrics, Evanston, IL).

Estimation of the difference waveforms of eclosion and activity/rest rhythms

The waveform of eclosion rhythm under LD and DD conditions was estimated by averaging the total number of flies eclosing at 2 hr intervals. Similarly, the waveform of activity/rest rhythm was derived by dividing hourly activity data by the total amount of activity during one complete cycle. The mean waveforms of eclosion and activity/rest rhythms were estimated from time series data obtained for 10 consecutive cycles. In order to compare eclosion and activity/rest waveforms of the selected populations, "difference waveforms" for each population were estimated by calculating the difference between the eclosion or activity waveform of a given selected population and its respective control population. For example, the difference waveform of the block 1 of the selected populations was estimated as [$(early_1\text{-}control_1)/control_1$] and [$(late_1\text{-}control_1)/control_1$].

3.2 (c) Statistical analyses

The τ of eclosion and activity/rest rhythms under DD was estimated by subjecting the time series data collected over 10 consecutive cycles to Fourier spectral analysis using Statistica^{TM} (rel.5.0B, Statistica, 1995). Statistical significance of rhythmic contributions from different frequencies in the periodogram was tested using Siegel's modification of the Fischer test (Siegel, 1980). This method delineated the frequency components present in the time series by defining a threshold value (Rao and Sharma, 2002).

The percentage of flies emerging during the morning and evening windows or the amount of activity during the morning or evening windows were used as data in a mixed model analysis of variance (ANOVA) where population replicates (blocks) were treated

as random factor, and the population and the window (morning or evening) of selection were treated as fixed factors. In all the statistical analyses population means were taken as the units of analyses, and therefore only fixed factor effects and their interactions could be tested for statistical significance. Post-hoc comparisons were done using 95% confidence interval (95%CI) around the mean.

The ψ and τ values of eclosion and activity rhythms were used as data in a separate mixed model ANOVA with replicate populations as random factor and population as fixed factor. The difference waveforms for eclosion and activity/rest rhythms of the selected and the control populations were analyzed using Kalmogorov-Smirnov 2-sample test. All the analyses were implemented using Statistica for Windows (rel.5.0B, StatSoft, 1995).

3.3 Results

3.3 (a) Direct response to selection

After 55 generations of selection the overall percentage of flies emerging from the morning window was about 60%, 45% and 33% in the *early*, *control*, and *late* populations, whereas those from the evening window were about 8%, 16% and 24% respectively (Fig. 3.1 a and b). ANOVA on the percentage of flies emerging from the morning window during the 5th-55th generations revealed a significant main effect of population ($F_{2,6}$ = 124.85; $p < 0.001$) and interaction between generation and population ($F_{8,24}$ = 10.80; $p < 0.001$), however, the main effect of generation did not reach statistically significant levels (Table 3.1 a). Multiple comparisons using 95%CI around the mean revealed that as early as 10th generation the percentage of flies emerging during the morning window was significantly greater in the *early* populations than the *late*

populations (Fig. 3.1 a). Initially the *early* and *control* populations did not differ significantly in terms of the percentage of flies emerging from the morning window, but after the 40th generation the percentage of flies in the morning window became significantly higher in the *early* populations than the *controls*. Similarly, ANOVA on the percentage of flies emerging in the evening window during 5th-55th generations revealed a significant main effect of population ($F_{2,6} = 144.562$; $p < 0.001$) and interaction between generation and population ($F_{8,24} = 6.981$; $p < 0.001$), however, the generation effect was not significant (Table 3.1 b). Multiple comparisons using 95%CI showed that, flies emerging during the evening window of selection gradually increased over a period of 55 generations in the *late* populations compared to the *control* and *early* populations. Though the difference between the *late* and *early* populations became apparent by the 10th generation, the differences between *control* and *early* populations were not noticeable until 40th generation. Over number of generations, the number of flies emerging from the morning window increased gradually while this number steadily decreased in the *early* populations. There was no significant change in the number of flies emerging from either window in the control populations. In parallel, the number of flies emerging from the evening window increased steadily in the *late* populations, while the number decreased gradually in the *early* populations. The differences between the selected and control populations reached statistical levels of significance as early as in the 10th generation, and with increasing number of generations the differences continued to increase further (Fig. 3.1 a and b).

3.3 (b) Correlated responses to selection

Eclosion waveform under LD 12:12 hr

The difference waveforms of eclosion rhythm between *early* and *control* [(*early*-*control*)/*control*], and *late* and *control* [(*late*-*control*)/*control*] populations (obtained by calculating the difference of *early* and *late* waveforms from *control* waveform and dividing by *control* waveform), shown in the Fig. 3.2 illustrate that the time course and waveforms of the selected populations diverged gradually from the control population. As revealed by the Kalmogorov-Smirnov test for 2 samples, the average difference waveforms of the *early* and *late* populations became evident in the 40th ($p < 0.05$) and 55th ($p < 0.01$) generation assays (Fig. 3.2). The results suggest that under 12:12 hr LD cycles, *early* flies emerge in greater numbers during the morning hours, whereas more *late* flies emerge during the evening hours. The eclosion waveform of the *early* populations gradually phase advanced relative to the *control* populations, while those of the *late* populations phase delayed relative to the *control* populations (Fig. 3.3). In summary, with increasing generations the primary peaks of eclosion in the *early* populations grew taller gradually and occurred earlier relative to the *control* populations, while those of the *late* populations became shorter gradually and occurred later compared to the *control* populations (Fig. 3.3).

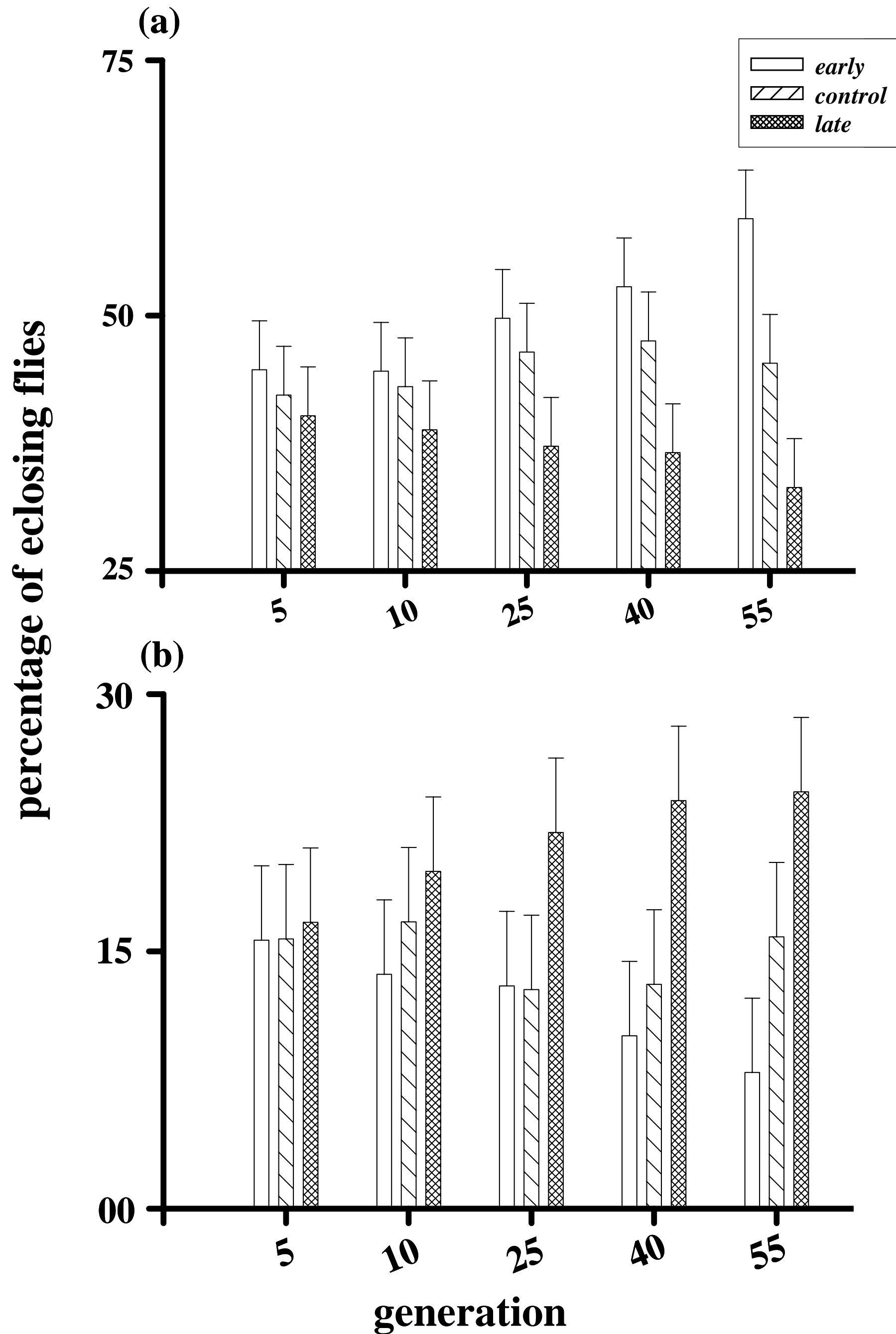

Fig. 3.1: Percentage of flies emerging from (a) the morning window (0500-0900 hr) and (b) the evening window (1700-2100 hr) of selection in the selected and the control populations during the assays done at 5th, 10th, 25th, 40th and 55th generations. The error bars represent 95% confidence interval (95%CI) around the mean across four replicate populations (10 vials per populations) for visual hypothesis testing.

Table 3.1: Results of ANOVA on percentage of flies eclosing in the morning and evening selection windows at different generations. Since block means were used for analysis, the effect of block and interactions involving block could not be tested for significance.

(a) morning window

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p*- level
Generation (G)	4	0.004	12	0.013	0.309	0.867
Population (P)	2	0.086	6	0.001	124.847	**< 0.001**
Block (B)	3	0.002	0	0	--	--
G X P	8	0.008	24	0.001	10.802	**< 0.000**
G X	12	0.013	0	0	--	--
P X B	6	0.001	0	0	--	--
G X P X B	24	0.001	0	0	--	--

(b) evening window

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p*- level
Generation (G)	4	0.000	12	0.002	0.093	0.983
Population (P)	2	0.045	6	0.000	144.562	**<0.000**
Block (B)	3	0.000	0	0	--	--
G X P	8	0.004	24	0.001	6.981	**<0.00**
G X	12	0.002	0	0	--	--
P X B	6	0.000	0	0	--	--
G X P X B	24	0.001	0	0	--	--

Phase relationship (ψ) of eclosion peak under LD 12:12 hr

ANOVA on the ψ values revealed a significant main effect of population ($F_{2,6} = 29.31$; $p < 0.008$). Although the interaction between population and assay generation had statistically significant effect on the ψ values ($F_{8,24} = 4.40$; $p < 0.002$), the effect of assay generation did not show any statistical significance ($F_{4,12} = 0.210$; $p = 0.928$). Multiple comparisons using 95%CI around the mean revealed that the ψ values of the *late* populations were significantly shorter (more negative) than that of the *early* populations from 10th generation onwards, while the difference in the ψ values among the *control* and *early* populations became clear only after 40th generation (Fig. 3.4). Similarly, the eclosion peaks of the *late* populations occurred significantly later than the *control* populations, but the differences became clear only after 40th generation (Fig. 3.4). In summary, the primary peak of eclosion occurred earlier in the *early* populations, followed by the *control* populations and *late* populations, in that order (Fig. 3.4). Although the ψ values of the *early* populations were consistently greater (less negative) than the *control* populations in all the assays, the differences did not reach statistical levels of significance till 25th generation, whereas the *late* populations maintained a significantly shorter ψ compared to the *early* populations right since the 10th generation (Fig. 3.4).

Eclosion rhythm under DD

The Kalmogorov-Smirnov test for 2-samples revealed that the average difference waveform of adult emergence rhythm of the *early* and *late* populations were significantly different in the 10th, 40th, and 55th generation assays ($p < 0.01$ for each block pair), but did not differ significantly in the 5th and the 25th generation assays (p = n.s.).

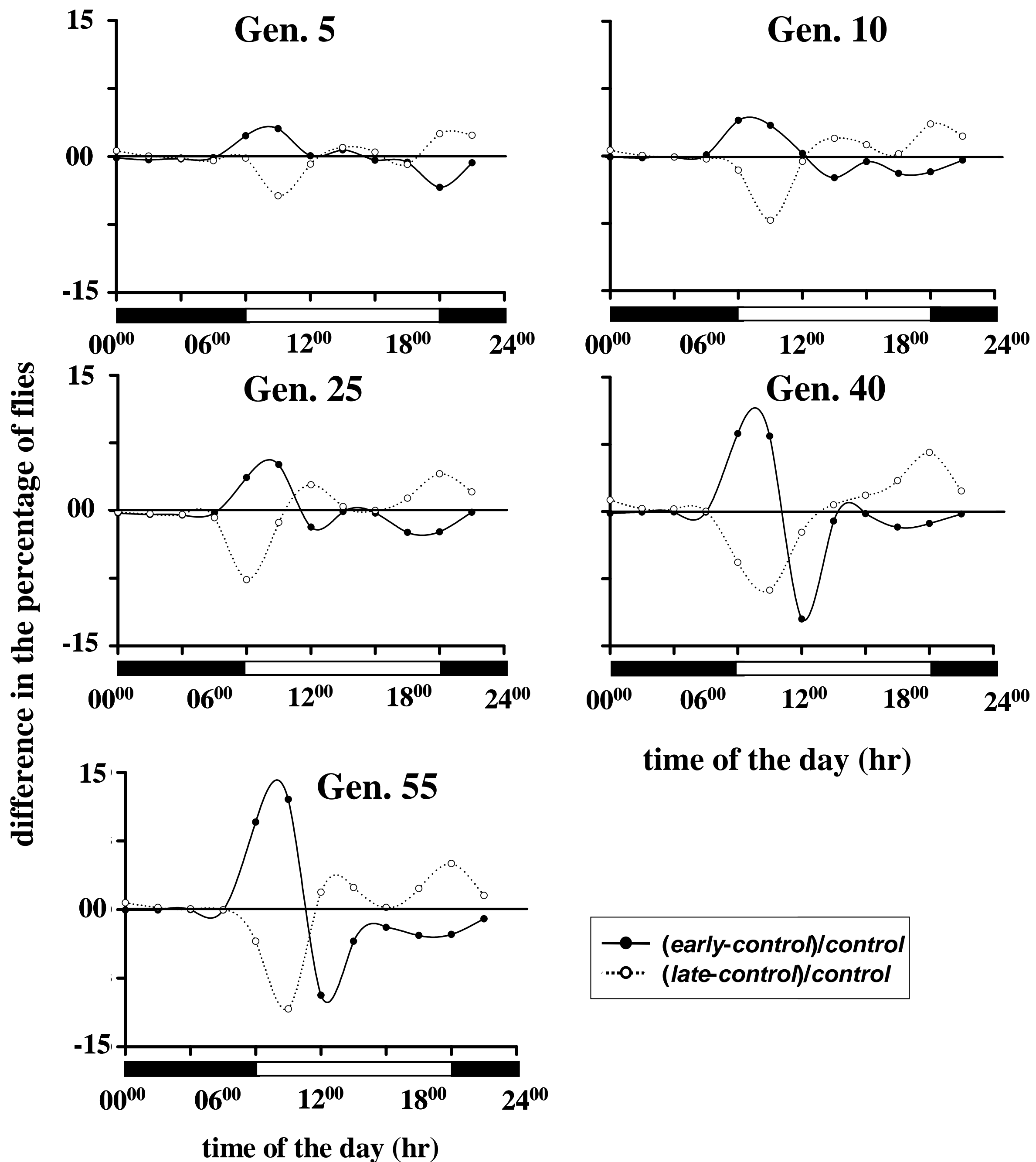

Fig. 3.2: The average "difference waveform" for the eclosion rhythm of the *early* and the *late* populations (*early-control*)/*control* and (*late-control*)/*control* under LD (12:12 hr) cycle obtained in assays done at 5th, 10th, 25th, 40th and 55th generations. Average waveforms for each strain were obtained for each replicate population from ten vials. Then the difference waveforms were generated by first subtracting the average eclosion waveforms of the four *early* and the four *late* populations from those of the *control* waveforms and then scaling it by *control* waveforms.

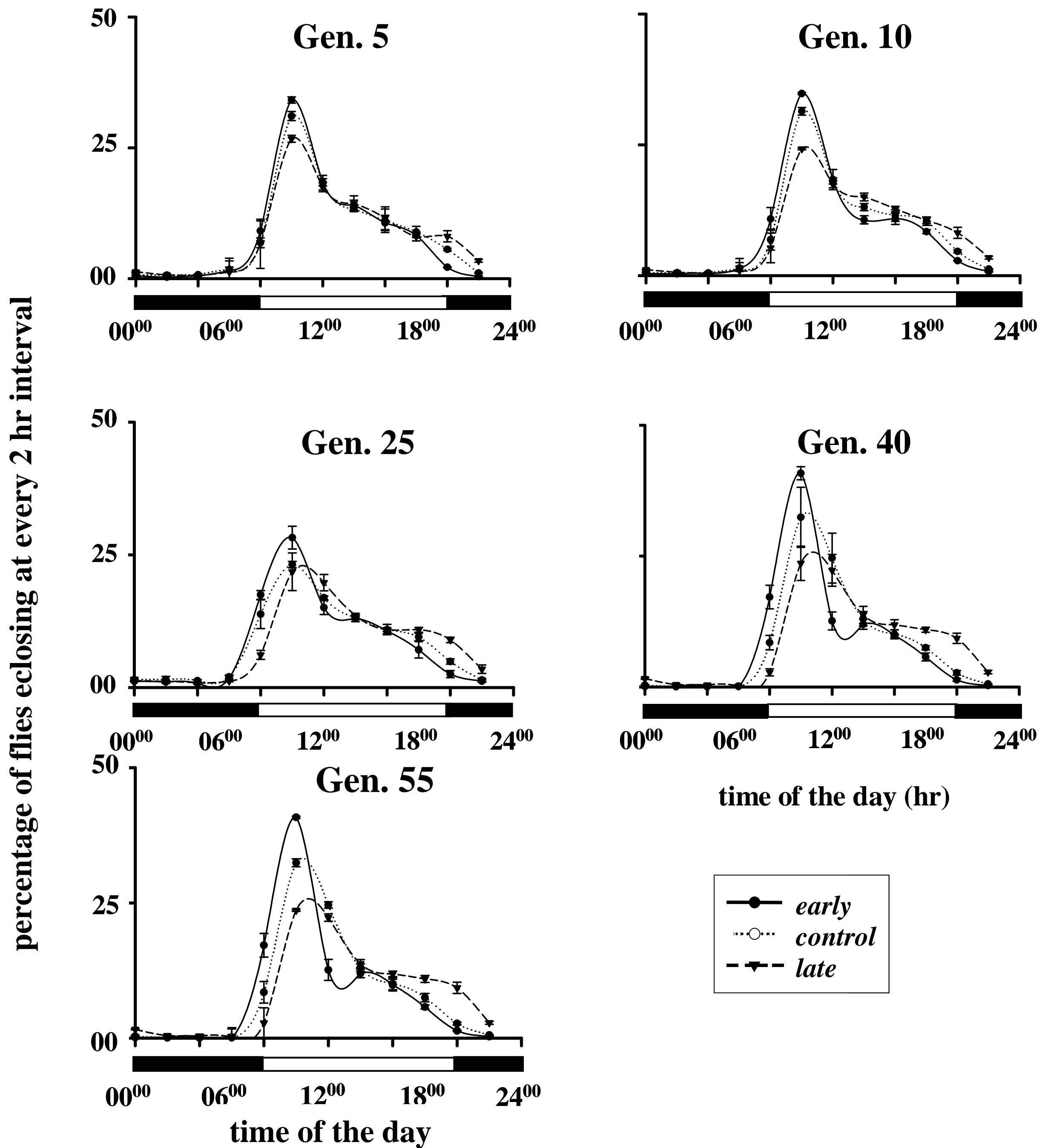

Fig 3.3: The time course and waveform of the eclosion rhythm of the selected and the control populations obtained from assays done at the 5th, 10th, 25th, 40th and 55th generations. The percentage of flies emerging at every 2 h intervals is shown along the y-axis and the time of the day along the x-axis. The filled and the empty bars denote the dark (2000 - 0800 hr) and the light (0800 -2000 hr) phases of the LD (12:12 hr) cycles, respectively. The error bars represent standard error of the mean (SEM), constructed using the variations among the replicate populations within selection regimes. A total of 40 vials were used, of which 10 were used for each of the four replicate populations in each assay. Other details as in Fig. 3.2.

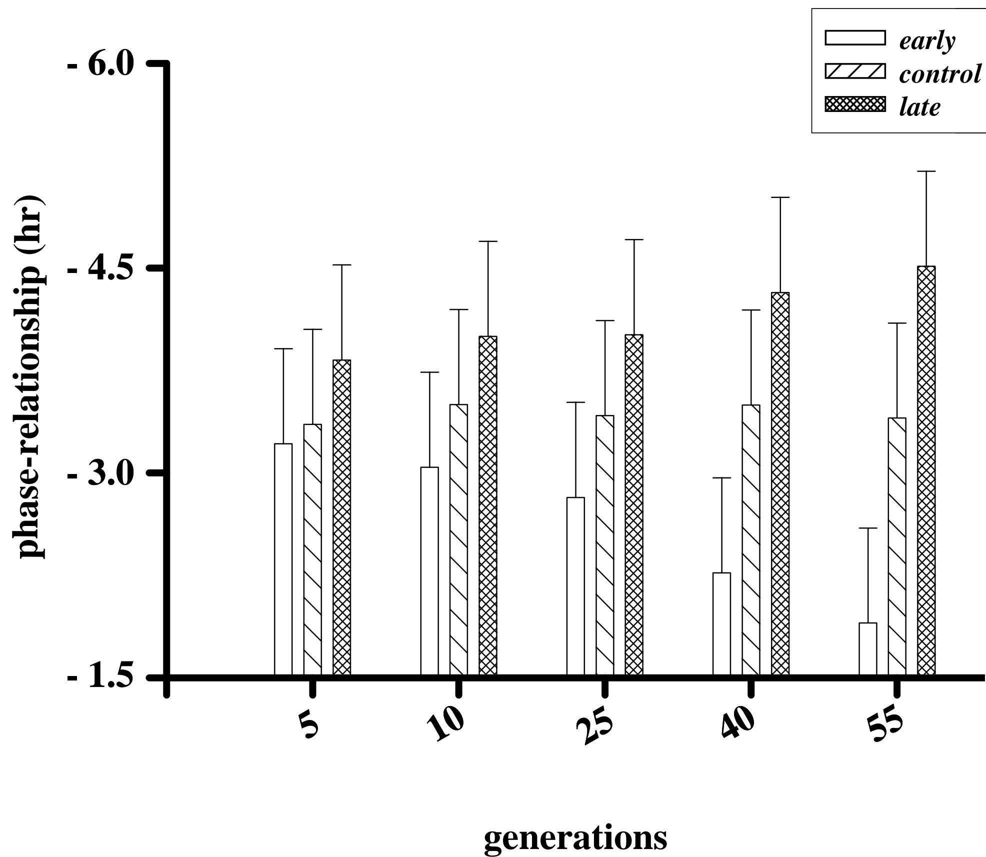

Fig. 3.4: The phase relationship (*ψ*) between the eclosion rhythm and the LD cycle obtained during the 5th, 10th, 25th, 40th and 55th generation assays. The *ψ* was estimated as the time interval between the morning peak of the eclosion and "lights-on" under LD cycles, averaged over ten consecutive cycles. The error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of 40 vials were used, 10 vials per replicate population in each assay.

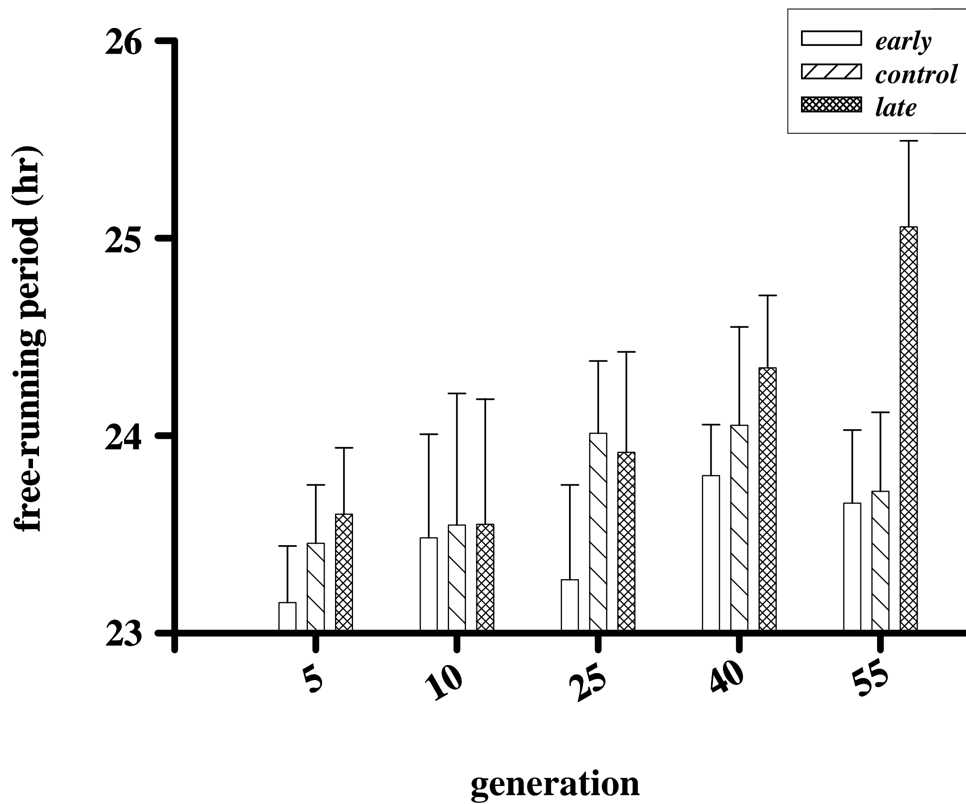

Fig. 3.5: The mean free-running period (τ) (in hr) of the eclosion rhythm of the selected and the control populations at the 5th, 10th, 25th, 40th and 55th generations under constant darkness (DD). The error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of 40 vials were used, 10 vials per replicate population in each assay.

As revealed in the ANOVA, the τ of the eclosion rhythm of the *early*, *control* and *late* populations did not differ significantly until the 55th generation (Fig. 3.5). ANOVA on the τ values of the eclosion rhythm revealed a significant main effect of population ($F_{2,6}$ = 8.149; $p < 0.019$). Multiple comparison using 95%CI showed that the τ of the *late* populations (25.06 ± 0.68 hr, mean ± 95%CI) was significantly greater than that of the *control* (23.72 ± 0.68 hr, mean ± 95%CI), and the *early* (23.66 ± 0.68 hr, mean ± 95%CI) populations, but those of the *early* and *control* populations did not significantly differ from each other (Fig. 3.5).

Activity/rest rhythm under LD

Activity levels during the morning and evening windows of selection

The activity/rest rhythm of selected and control populations were assayed after 55 generations of selection to estimate correlated responses of selection on circadian clock properties. ANOVA on the activity levels during the morning and evening windows of selection did not show any significant effect of either the population ($F_{2,6}$ = 3.92; p = 0.08) or window of selection ($F_{1,3}$ = 3.95; p = 0.14). However, the interaction of population and selection window had a significant effect on the activity levels ($F_{2,6}$ = 23.10; $p < 0.001$). Multiple comparison using 95%CI revealed that during the morning window of selection flies from the *early* populations were relatively more active than those from the *control* and *late* populations, while during the evening window flies from the *late* populations were significantly more active than those from the *control* and *early* populations (Fig. 3.6 a and b). The average activity levels during the morning window were 21.0%, 25.7% and 30.0% in the *early*, *control*, and *late* populations (Fig. 3.6 a), whereas those during the evening window were 26.2%, 21.0% and 20.1%, respectively

(Fig. 3.6 b). In summary, the overall waveform of activity/rest rhythm changed in response to selection on the timing of eclosion (Fig. 3.7 a–c); flies from the *early* populations were more active in the morning than in the evening (Fig. 3.7 a and d), flies from the *late* populations were more active in the evening than in the morning (Fig. 3.7 c and f), while flies from the *control* populations were as active in the morning as in the evening (Fig. 3.7 b and e).

The phase of activity/rest rhythm onset and offset

ANOVA on the ϕ values of the onset (ϕ_{on}) and offset (ϕ_{off}) of activity revealed a significant main effect of population ($F_{2,6}$ = 6.16; $p < 0.03$ for the morning, and $F_{2,6}$ = 8.39; $p < 0.01$ for the evening peak). Multiple comparisons using 95%CI around the mean revealed that the ϕ_{on} values of the *early* populations were significantly smaller than those of the *control* and *late* populations (Fig. 3.8 a). However, the difference between the ϕ_{on} values of the *late* and *control* populations did not reach statistical levels of significance (Fig. 3.8 a). Multiple comparisons using 95%CI revealed that the ϕ_{off} values of the *late* populations were significantly smaller compared to that of the *early* and *control* populations. The ϕ_{off} values of the *early* populations, however, did not differ significantly from those of the *control* populations (Fig. 3.8 b). In other words, the morning activity peaks in the *early* populations occurred earlier compared to the *control* and *late* populations, whereas the evening peaks occurred later in the *late* populations compared to the *control* and *early* populations (Fig. 3.8 a and b).

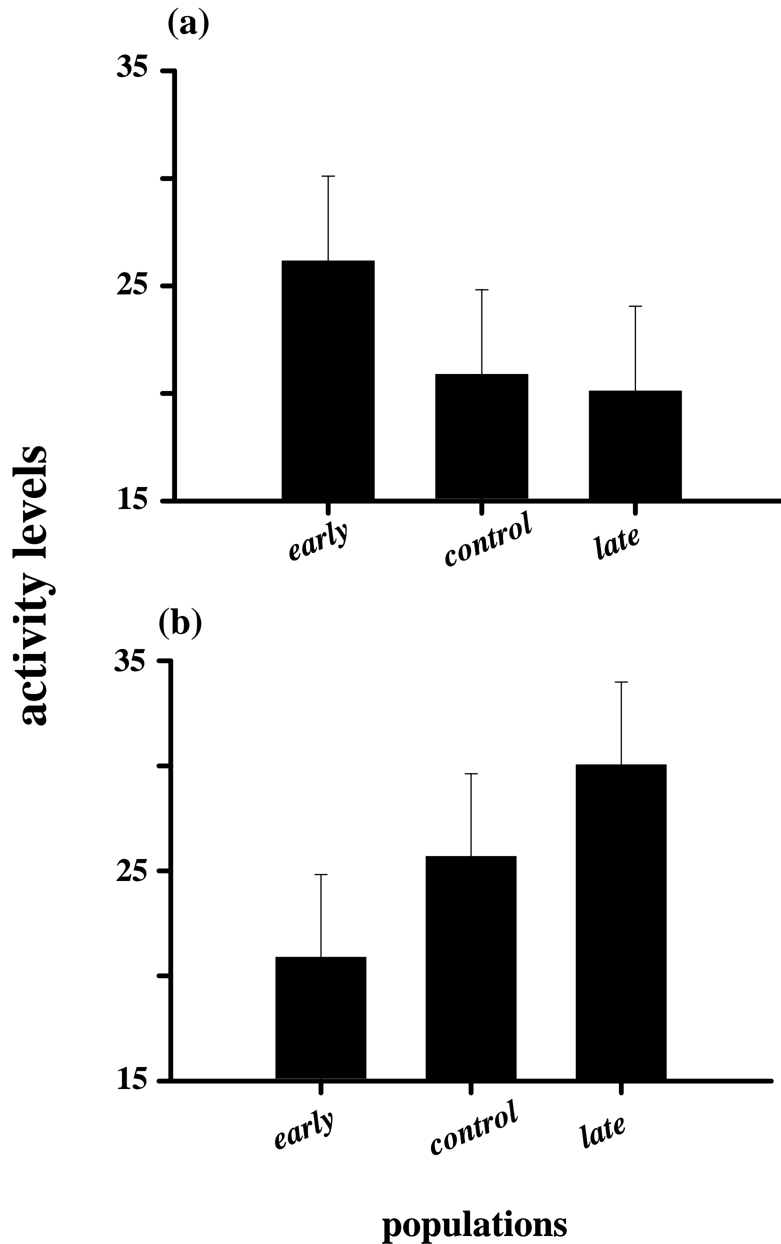

Fig. 3.6: Activity levels of the selected and the control flies during the morning and evening windows of selection under 12:12 hr LD cycle. **(a)** Percentage activity during the morning window (0500-0900 hr) in the selected and control populations. **(b)** Percentage activity during the evening window (1700-2100 hr) in the selected and control populations. The error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of (n = 161) *early* flies (n = 171) *control* flies and (n = 156) *late* flies were used in this experiment.

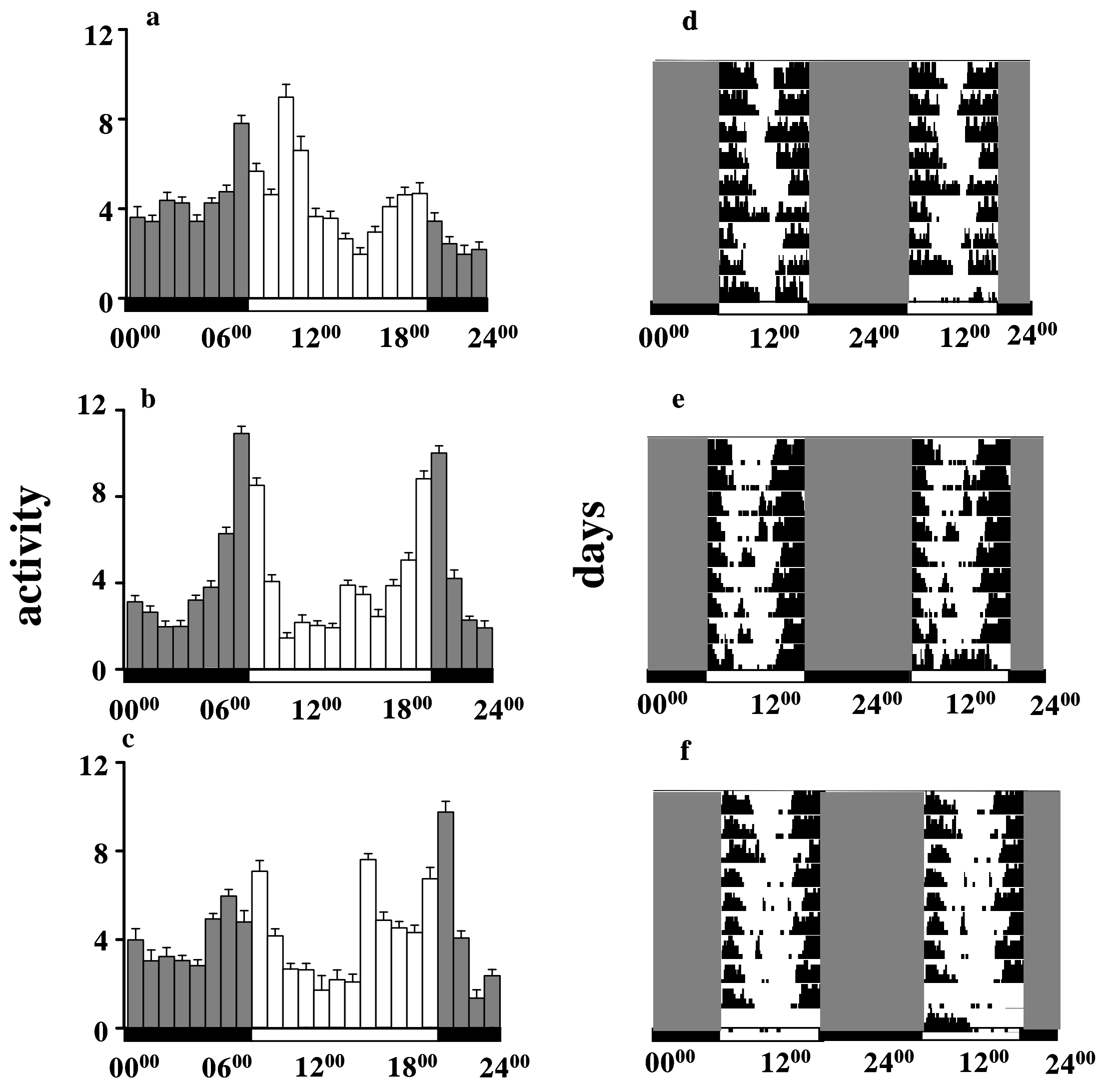

Fig 3.7: The average activity/rest plots of the *early* (n = 161) **(a)**, *control* (n = 171) **(b)**, and *late* flies (n = 156) **(c),** monitored under 12:12 hr LD cycle after 55 generations of selection. Activity/rest profiles are plotted as the mean activity/rest during one-hour bin, averaged over ten successive cycles. The percentage of activity averaged over 10 cycles successive is plotted along the ordinate and time of the day in hours along the abscissa. The error bars represent standard error around the mean (SEM), constructed using the variations among the replicate populations within selection regimes. Additionally one representative double plot activity-rest pattern of individual flies from **(d)** *early* **(e)** *control* and **(f)** *late* populations are shown under LD. The vertical filled and empty bars denote the activity levels during the night and the day, respectively. The horizontal black bars denote the dark phase (2000 – 0800 hr) and white bars represent the light phase of the 12:12 hr LD cycle.

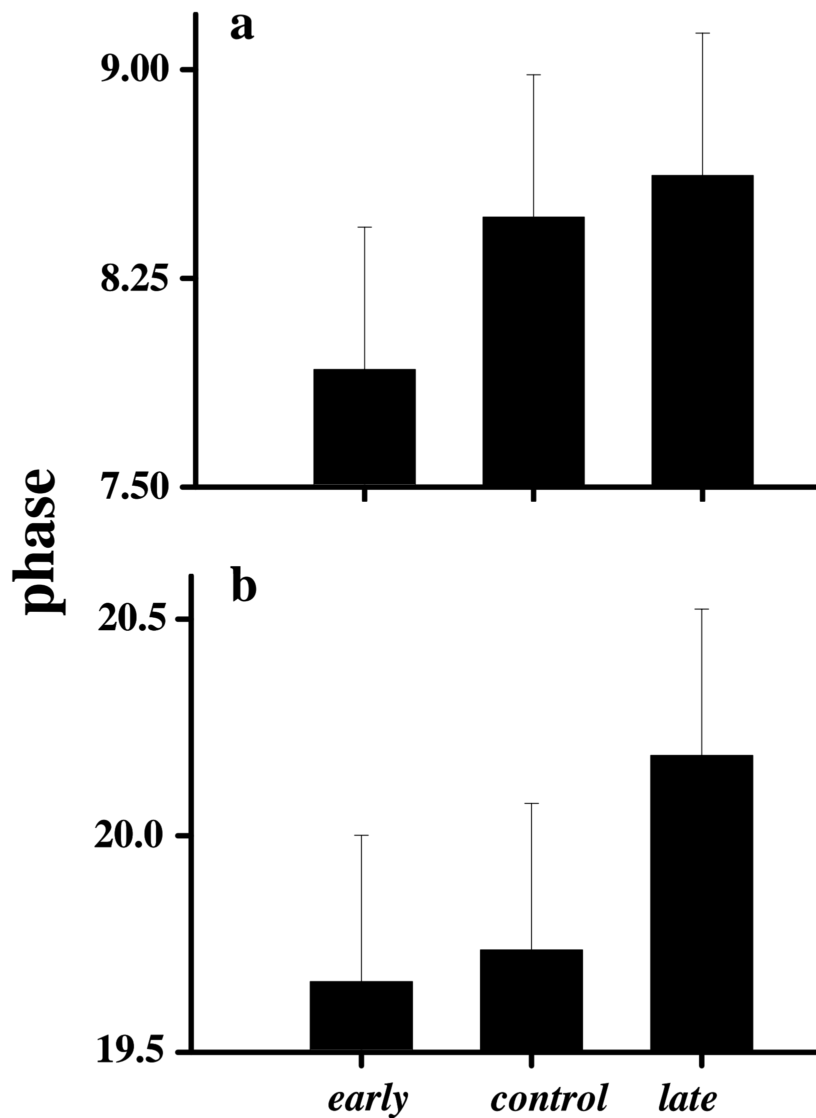

Fig 3.8: **(a)** The phase of the morning activity peak (ϕ_{on}) in an LD cycle of 12:12 hr, of the selected and the control flies. The ϕ_{on} values were estimated as the average morning activity peak over ten consecutive cycles. **(b)** The phase of the evening activity peak (ϕ_{off}) in an LD cycle of 12:12 h, of the selected and the control populations. The ϕ_{off} was estimated as the average evening activity peak over ten consecutive cycles. The error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of *early* (n = 161), *control* (n = 171), and *late* (n = 156) flies were used to estimate (ϕ_{on}) and (ϕ_{off}).

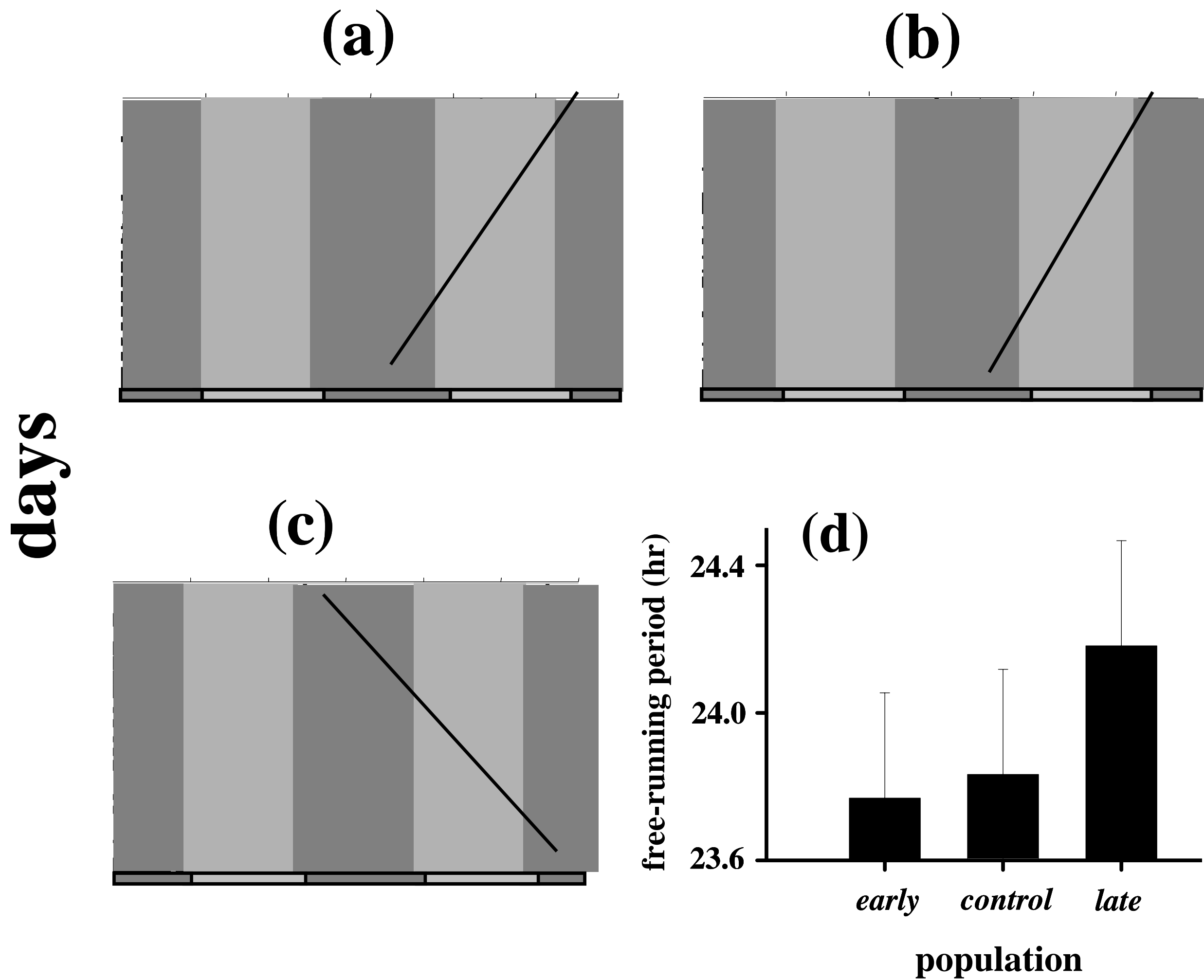

Fig 3.9: Activity/rest plots of individual (a) *early*, (b) *control* and (c) *late* flies under constant darkness (DD). The vertical dark bars denote activity and their absence as rest. Activity is plotted along the ordinate for 10 consecutive cycles and time in hours along the abscissa. (d) the mean free-running period (τ) (hr) of the activity/rest rhythm of the selected and the control populations under constant darkness (DD). The error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of *early* (n = 55), *control* (n = 59), and *late* (n = 55) flies were used to estimate τ values.

Activity/rest rhythm under DD

The τ values of activity/rest rhythm of the selected and control populations were also altered as a consequence of selection (Fig. 3.9 a-c). ANOVA on the τ values of activity rhythm under DD, obtained in the 55th generation assay revealed a significant main effect of population ($F_{2,6} = 7.27$; $p < 0.03$). Multiple comparisons using 95%CI revealed that the τ of the *late* populations (24.18 ± 0.28 hr, mean ± 95%CI) was significantly greater than that of the *early* (23.77 ± 0.28 hr, mean ± 95%CI) and the *control* populations (23.83 ± 0.28 hr, mean ± 95%CI), while those of the *early* and *control* populations did not differ significantly (Fig. 3.9 d).

3.4 Discussion

We imposed artificial selection pressure on the timing of adult emergence on four large (n > 1200) genetically independent random mating populations of *D. melanogaster*, and derived four $early_{1..4}$, four $control_{1..4}$ and four $late_{1..4}$ populations. After 55 generations of selection, the overall percentage of flies emerging from the morning window showed an approximately twofold (~60%) increase in the *early* populations compared to that of the *late* populations (~33%), whereas in the evening window, there was an approximately three fold increase in *late* populations (~24%) compared to the *early* populations (~8%), while this number remained fairly constant at about 45% in the morning window and about 16% in the evening window in the *control* populations. The percentage of flies emerging from the morning and evening windows in the *early* and *late* populations were clearly different from the *control* populations by the 10th generation, and the differences continued to increase gradually with increasing generations (Fig. 3.1 a and b). Further, the overall waveform of eclosion rhythm of the *early*, *control* and *late* populations

differed significantly; relative to the *control* populations the emergence patterns of the *early* populations were skewed to the left, while that of the *late* populations were skewed to the right (Fig. 3.3), indicating that the time course and waveform of eclosion rhythm is altered in the *early* and *late* populations due to selection on the timing of adult emergence. This suggests that *D. melanogaster* populations respond to selection on the timing of adult emergence by gradually enhancing the number of flies emerging in the respective selection windows, and by modifying the overall eclosion pattern.

Under LD cycles, the morning peak of activity rhythm of the *early* populations preceded that of the *control* populations, while those of the *late* populations occurred few hours later than the *control* populations, thus unerringly mimicking the adult emergence patterns of the selected and control populations (Fig. 3.7 a-f). The overall waveform of the activity/rest rhythm of the *early*, *control* and *late* populations also differed considerably; flies from the *early* populations started their activity earlier than flies from the *control* populations, and were generally more active in the morning than in the evening, the *control* flies showed bimodal activity patterns and were as active in the morning as in the evening, whereas the *late* flies started activity later than the *control* flies and were more active in the evening than in the morning (Fig. 3.7 a-f). This suggests that the selected populations have undergone correlated changes in their activity/rest clocks, which indicates that the adult emergence and activity/rest rhythms in *D. melanogaster* share common underlying mechanisms. These results are consistent with previous findings, which implicate common clock mechanisms for eclosion and activity/rest rhythms in *D. melanogaster*. It was shown that functional core clock genes were necessary for the rhythmic expression of eclosion as well as activity/rest rhythm

(reviewed in Williams and Sehgal, 2001). In addition, functional ventral lateral neurons (LN_vs) were necessary for the rhythmic expression of the activity/rest rhythm, whereas LN_vs plus prothoracic gland (PG) were required for the persistence of eclosion rhythm (Myers et al., 2003). It is likely that the selection on different timing of emergence may have altered temporal profiling in several clock genes in the *early* and *late* lines, hence resulting in diverged clock phenotypes.

The phase relationship (ψ) between a rhythm and LD cycles is known to depend upon the τ and light induced PRC of the underlying circadian clocks (Pittendrigh and Daan, 1976; Sharma and Chidambaram, 2002). Therefore the gradual divergence in ψ of the *early* and *late* populations seen in our study can be ascribed to gradual changes in (a) τ, or (b) PRC, or (c) both τ as well as PRC. The results of our experiments indicate that circadian clocks of the *early* populations have diverged from the *control* populations by changing the PRCs alone leaving τ unaltered, whereas the *late* populations have diverged from the *control* populations by altering both their PRCs as well as τs (discussed in subsequent chapter). These results are in some ways similar to those obtained in a separate study on the melon fly *Bactrocera cucurbitae* (Diptera: Tephritidae) selected for faster and slower pre-adult development (Miyatake, 1996; Shimizu et al., 1997). In these studies, slower developing lines had slower running clocks (τ ~ 30.9 hr), while the faster developing lines had faster clocks (τ ~ 22.6 hr). Our results are, however, in sharp contrast with Pittendrigh's early findings on the early and late populations of *D. pseudoobscura* which were raised under LD 12:12 hr, where the early population had slower and the late population had faster running clocks (Pittendrigh, 1966). In a subsequent study on *D. auraria*, when the early and late populations were raised under

short photoperiod (LD 1:23 hr) conditions (Pittendrigh and Takamura, 1987), the early population had faster clocks compared to the late population. However, it is also possible that the modes of evolutionary fine-tuning of circadian clocks depend upon a number of factors such as the genetic architecture of the founder population; especially the available genetic variance for the timing of emergence, strength of selection protocol, environmental conditions, and the population size. Moreover, lack of replicates, inadequate information about the population size and the rearing protocol in most of the previous selection make it difficult to estimate the extent of genetic drift or inbreeding that these populations may have undergone. Further, these studies were not continued for long enough to confirm whether the response to selection reached a steady state. Some studies were terminated as early as 9 generations and most of them lasted for not more than 15 generations, which may not be long enough to draw any meaningful conclusions.

Our study based on four genetically independent random mating, large populations derived from a common ancestry, suggests that the results are borne out of consistent heritable genetic alterations in response to selection pressure, and not due to random genetic drift or due to some unknown environmental or non-genetic effect. Further, the outcomes of our study are by far the most rigorous and unequivocal of all selection studies done on any rhythm or rhythm related trait. The results clearly demonstrate that the time course and waveforms of eclosion and activity rhythms evolve due to selection on the timing of adult emergence and circadian of the selected populations diverge from those of the controls. Furthermore, our study provides valuable insights into the genetic basis of behavioral traits and genetic correlations among these traits (eclosion and activity/rest). Our results can also be taken to suggest that circadian

clocks evolve through an ongoing process of adaptive evolution under the influence of periodic LD cycles of the natural environment. The cyclic factors that constituted selection forces are LD cycles, temperature, and humidity cycles, food and mate availability cycles.

Chapter 4

Selection on timing of adult emergence alters circadian phenotypes

4.1 Background

Circadian clocks track timing in the local environment by entraining to natural light/dark (LD) cycles of the environment, a phenomenon that is of paramount importance for the survival of organisms living under extreme conditions (Pittendrigh and Daan, 1976; Daan, 1981). Several studies have been carried out in past to understand how organisms adjust to daily LD cycles comprising of different proportions of light and darkness (Pittendrigh and Daan, 1976; Pittendrigh and Takamura, 1987; Sumova et al., 1995, 2003). In an early study Pittendrigh and Takamura (1987) investigated entrainment and photoperiodic induction in laboratory and wild caught populations of *Drosophila auraria* and found that systematic adaptive changes in day lengths is paralleled by changes in clock's entrained behavior and as a consequence phase relationship between the rhythm and LD cycles (ψ) changes (Pittendrigh and Takamura, 1987). These studies suggest that circadian clocks are involved in seasonal time keeping mechanism(s). However, the mechanism(s) by which such information is encoded is still unclear. As a working hypothesis it was proposed that circadian clocks comprising of morning (M) and evening (E) oscillators with different circadian periods and different responsiveness to light track day lengths for seasonal functions (Pittendrigh and Daan, 1976). This model was recently elaborated by Daan and others (2001). It was suggested that the M and E oscillators in mammalian circadian clocks comprise of *period*/*cryptochrome* (*per/cry*) gene pairs; the *per1/cry1* pair functions as the M oscillator and the *per2/cry2* pair as the E oscillator. This proposition also assumes that the morning and evening activity bouts in activity/rest cycles are the overt manifestations of these oscillators (Bünning, 1936; Aschoff, 1966; Pittendrigh and Daan, 1976). Notwithstanding the importance of this model for a wide

variety of organisms living in periodic environment, it has never been rigorously tested in wild type animals that have distinct morning and evening circadian phenotypes.

In the present chapter, I report the results of the experiments designed to study the circadian phenotypes of the *early* and *late* populations in the light of the M-E oscillator model. For this purpose four replicate population each of the *early*, *control* and *late* populations were assayed after 70 generations of selection to determine their time course and waveform of two well characterized behavioral rhythms; eclosion as well as activity/rest rhythms under periodic LD cycles. In the earlier chapter (Chapter 3) we have reported that the peak of eclosion rhythm of the *early*, *controls* and *late* populations diverged by 4-5 hr, which suggests that *D. melanogaster* populations respond to selection on phase of circadian rhythm by evolving different timing for their behavior. It is known that extreme photoperiods modulate circadian rhythms, either by magnifying or reducing circadian waveform (Sumova et al., 2003). Therefore, we decided to evaluate the circadian phenotypes of the selected and control populations under short (LD 6:18 hr), normal (LD 12:12 hr) and long (LD 16:8 hr) photoperiods. In addition, we assayed light-induced phase response of eclosion rhythm at different phases in these populations to gain further insights into the circadian architecture. The results suggest that selection on timing of adult emergence yield flies with morning and evening circadian phenotypes.

4.2 Materials and methods

This study was done on three sets of (the *early, control* and *late*) populations of *D. melanogaster* that were standardized by a method described in chapter 2. Standardized populations were used for various assays described as following.

4.2 (a) Eclosion rhythm assay

The phase relationship (ψ) between the eclosion rhythm and the LD cycle, width of the eclosion gate (g), anticipation index (AI) around lights-on and the average waveform of eclosion rhythm under three different LD cycles were estimated. For this, eggs of approximately same age were collected from the standardized populations and were dispensed into vials with 10 ml of food at an egg density of ~ 300 per vial and introduced into short (LD 8:16 hr), normal (LD 12:12 hr) and long (LD 16:8 hr) photoperiods. Ten such vials were set up for each population under each light regime. The vials were monitored for the first eclosion, and thereafter were checked regularly at every 2 hr interval and the number of emerging adults was recorded. This continued for ten consecutive days. From the primary data, we estimated the mean ψ of the primary eclosion peak, g, and the average waveform of the eclosion rhythm under short, normal and long photoperiods. The ψ for the primary eclosion peak under LD 8:16 hr, 12:12 hr and 16:8 hr was estimated as the time interval between the peak of eclosion and lights-on under a given LD cycle averaged over ten consecutive cycles. The ψ was considered to be negative if the eclosion peak occurred after lights-on, and it was taken to be positive if the peak occurred before lights-on. The g was estimated as the time interval between the first and last emergence in one complete cycle, averaged over ten consecutive cycles. From the raw data we obtained the AI of eclosion for individual vials, using the formula AI= $(b_{-1}-b_{-2}) \times (b_{+1}/b_{-1})$, where b_0 = lights-on/off bin (of 2 hr), b_{-1}= bin before lights-on/off and b_{+1}= bin after lights-on/off, $(b_{-1}-b_{-2})$ number of flies emerging in a 2 hr bin before lights-on/off, and (b_{+1}/b_{-1}) denotes the ratio of number of flies emerging in a 2 hr bin, immediately before and after lights-on/off. The τ values of eclosion rhythm were

estimated under DD (dim red light with $\lambda > 640$ nm) by monitoring eclosion for a minimum of 10 consecutive days.

4.2 (b) Light -induced phase response curve (PRC) of eclosion rhythm

For estimating phase responses of the eclosion rhythm, four replicate populations of the selected and control populations were subjected to brief light stimuli. For this assay, eggs of approximately same age were collected from the standardized populations and were dispensed into vials with 10 ml of food at an egg density of ~ 300 per vial and introduced into 12:12 hr LD cycles. Flies were transferred to DD and exposed to light stimuli of ~1000 lux intensity and 15 min duration, at four different phases (CT2, CT8, CT14 and CT20), in the first circadian cycle. Ten such vials were set up for each replicate population for each of the phase along with the controls. The control vials at each tested CT were also transported in light-tight containers (wrapped additionally with black cloth) to ensure that light pulse *per se* and not the disturbances associated with handling, transfer and human interference, caused phase shift ($\Delta\phi$). The vials were monitored for the first eclosion and thereafter were checked regularly at every 2 hr interval and the number of emerging adults was recorded. This continued for ten consecutive days. From the primary data, we estimated the mean phase of primary eclosion peaks under LD as well as DD conditions for the experimental as well as control flies. Phase shift values were obtained by subtracting control $\Delta\phi$ values (obtained for the control vials which were not subjected to a light pulse) from the experimental $\Delta\phi$ values. The $\Delta\phi$ was estimated using a regression line drawn across a minimum of eight successive peaks of eclosion, immediately following the light pulses administered under DD and a regression line

preceding in LD using a method of calculating $\Delta\phi$ has been described in Sharma and Daan (2002).

4.2 (c) Activity/rest rhythm assay

The anticipation index with respect to lights-on (AI_{on}) and lights-off (AI_{off}), duration of activity (α) and the waveform of activity/rest rhythm were estimated under LD 8:16 hr, 12:12 hr and 16:8 hr. For this experiment, freshly emerged adult flies were introduced individually into activity-monitors within 24 hr of their emergence and their activity was monitored individually using infra-red beam based activity monitoring setup (Sharma, 2003b) Approximately 60 flies were used for each population under three different light regimes. Activity of the flies was monitored for a minimum of 10 consecutive days. From the raw activity data we obtained the AI for individual flies using the formula AI= $(b_{-1}-b_{-2}) \times (b_{+1}/b_{-1})$, where b_0 = lights-on/off bin (of 1 hr), b_{-1}= bin before lights-on/off and b_{+1}= bin after lights-on/off, $(b_{-1}-b_{-2})$ amount of activity in a 1 hr bin before lights-on/off, and (b_{+1}/b_{-1}) denotes the ratio of the amount of activity in a 1 hr bin, immediately before and after lights-on/off. We also estimated the duration of activity (α) by assessing the average time interval between the onset and offset of activity in one cycle. The activity/rest rhythm of the flies was also monitored under DD conditions for about 10 days to estimate τ of activity/rest cycles of the selected and control flies. Activity/rest pattern of individual fly was plotted and analyzed using CLOCKLAB software (Actimetrics, Evanston, IL).

4.2 (d) Light-induced phase shift of activity/rest rhythm

Activity/rest rhythm of the flies was continuously monitored by placing freshly emerged adult males in glass tubes and their activity was monitored using the activity monitors, infra-red beam crossing system recording total crosses in every 15 minute bins (Sharma,

2003b). Flies were entrained to LD 12:12 hr for 9 days and released into DD at ZT12 of the 9th day and recorded for about 10 days. Flies were exposed to brief light stimuli of 15 minute duration and of ~1000 lux intensity at two phases of the clock (CT14 and CT20) at which wild type flies respond with maximum phase delay and advance under DD conditions). The offset of activity was taken as the phase reference point to calculate phase shifts in the rhythm, because generally in our flies offsets show less cycle-to-cycle variability (*Shailesh Kumar*, *personal observation*). The phase of the rhythm following light exposure was estimated and compared with those of the flies that were not subjected to a light pulse. The $\Delta\phi$ values were obtained by subtracting control $\Delta\phi$ values from the experimental $\Delta\phi$ values. The $\Delta\phi$ was estimated using two regression lines drawn across offsets of activity, one immediately following the light pulses administered under DD and another preceding in LD cycles, using the method described in Sharma and Daan (2002). The activity /rest patterns of the flies were plotted using the Clocklab software (Actimetrics, Evanston, IL).

4.3 (e) Statistical analyses

The τ of eclosion and activity/rest rhythms under DD was estimated by subjecting time series data collected over 10 consecutive cycles to Fourier spectral analysis using Statistica (Statistica^{TM}, 1995). Statistical significance in the periodogram was tested using Siegel's modification of the Fischer test (Siegel, 1980). This method delineated the frequency components present in the time series by defining a threshold value (Sheeba et al., 2001a, 2001b).

The ψ, AI, and g values of eclosion rhythm as well as AI, and α values of activity/rest rhythm, obtained for assays done under different photoperiodic regimes were

subjected to separate mixed model analysis of variance (ANOVA), by treating replicate populations as random factor and light regimes and population as fixed factor. The $\Delta\phi$ values obtained for eclosion and activity/rest rhythm for each population were subjected to ANOVA treating replicate population as random factor, populations and phase of light pulse were used as fixed factors. Also the values of τ of eclosion and activity/rest rhythms were subjected to separate one-way ANOVA treating replicate populations as random factor and population as fixed factor. In all statistical analysis, population means were used as units of analysis and therefore only fixed factor effects and the interactions could be tested for statistical significance. The error bars, 95% confidence intervals (95%CI) around the means were used as error bars to facilitate visual hypothesis testing. Overlapping error bars imply values that do not differ significantly.

4.3 Results

4.3 (a) Eclosion patterns under short, normal and long photoperiods

Phase-relationship of eclosion peak with LD cycles

The eclosion rhythm of the *early, late* and *control* populations entrained to short (LD 8:16 hr), normal (LD 12:12 hr) and long (LD 16:8 hr) photoperiods maintaining a stable ψ with the LD cycle (Figs. 4.1 a-c). The main effects of population, light regime and their interactions were tested for statistical significance using analysis of variance (ANOVA), which showed significant effects of light regime and population but not their interactions

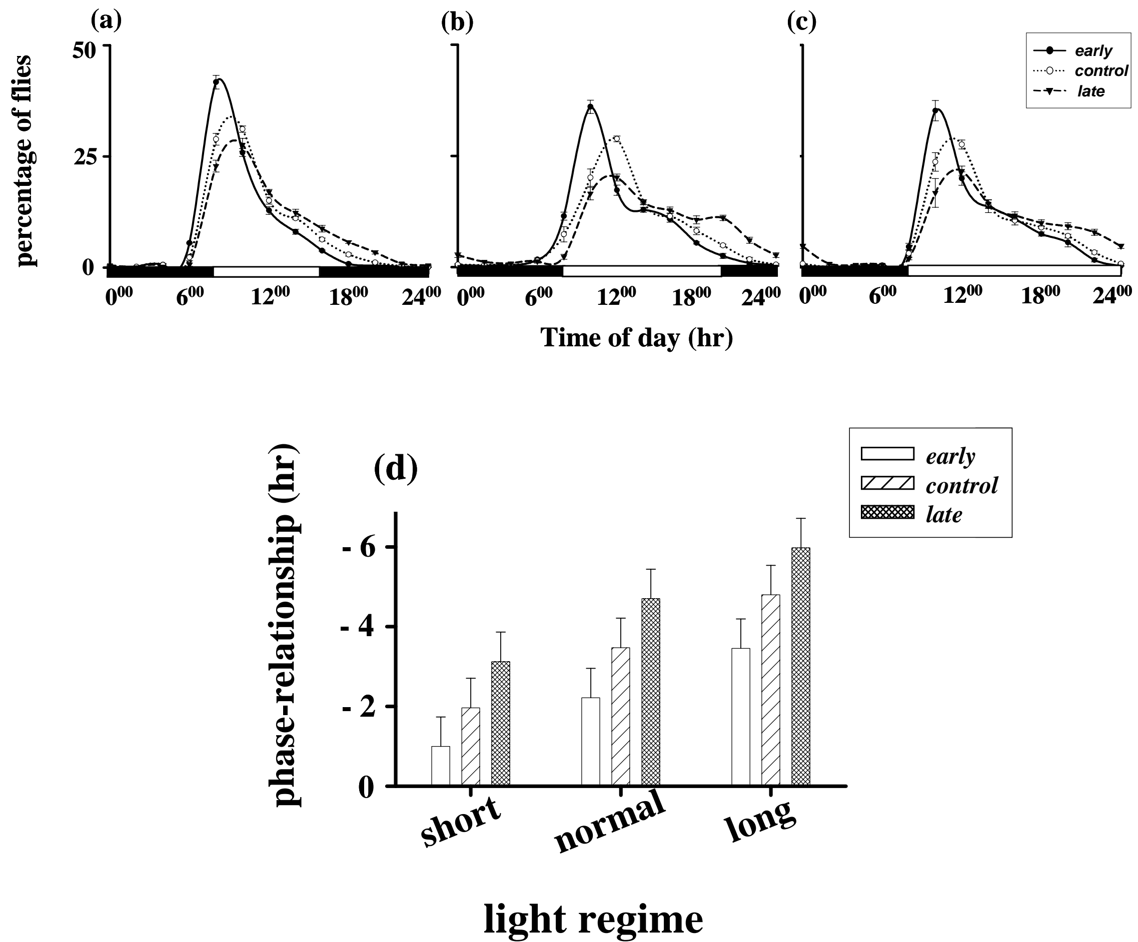

Fig. 4.1: Average eclosion waveform of the selected and the control populations under **(a)** short (LD 8:16 hr), **(b)** normal (LD 12:12 hr) and **(c)** long (LD 16:8 hr) photoperiods. Percentage of flies emerging every 2 hr, averaged across ten consecutive cycles is plotted along the y-axis. Filled and the empty bars represent the duration of dark and light in the LD cycles. **(d)** The ψ of eclosion rhythm of the selected and control populations. The ψ was estimated as the time interval between the primary peak of eclosion and lights-on, averaged over ten consecutive cycles. The error bars indicate 95%Confidence Interval (95%CI) around the mean for visual hypothesis testing. Under each photoperiodic regime, a total of 40 vials were used, of which 10 were used for each of the four replicate populations for estimation of the ψ values.

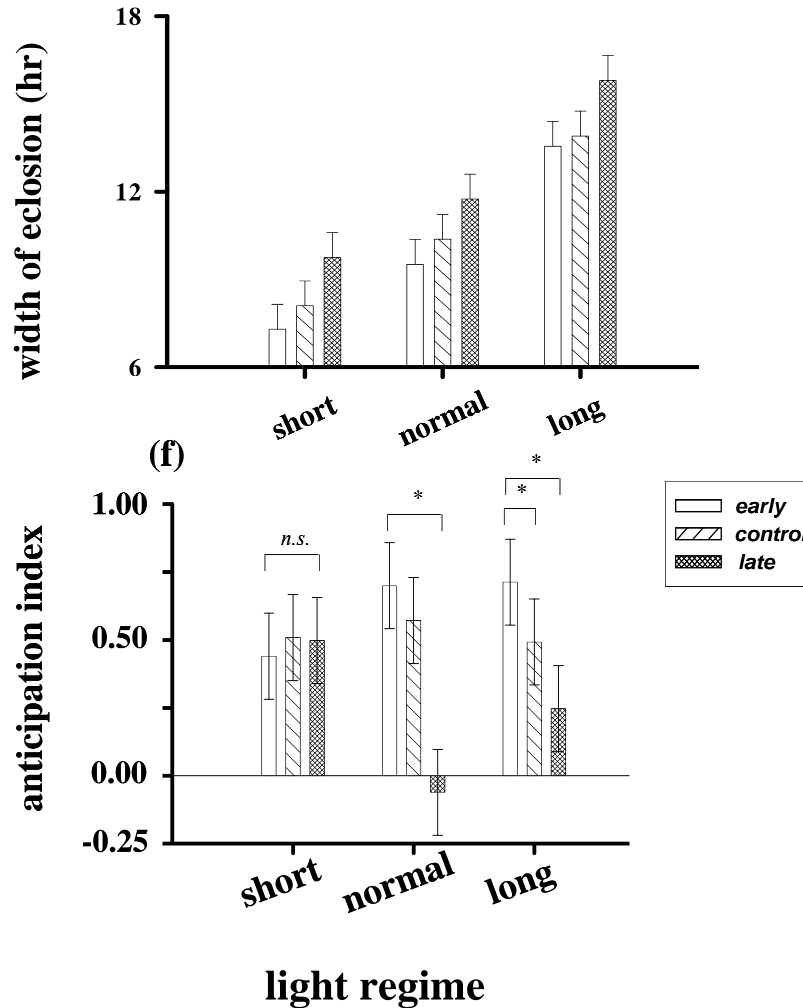

Fig. 4.1 (e) Mean width of the eclosion gate (*g*) (hr). The *g* values were estimated as the time interval between the start and the end of emergence in one complete cycle, averaged over ten consecutive cycles. **(f)** Mean eclosion anticipation index (AI) of the selected and control populations. The AI values were estimated using the formula AI= (b_{-1}-b_{-2}) x (b_{+1}/b_{-1}), where b_0 = lights-on/off bin (of 2 hr), b_{-1}= bin before lights-on/off and b_{+1}= bin after lights-on/off, (b_{-1}-b_{-2}) number of flies emerging in a 2 hr bin before lights-on/off, and (b_{+1}/b_{-1}) denotes the ratio of number of flies emerging in a 2 hr bin, immediately before and after lights-on/off. The error bars indicate 95%Confidence Interval (95%CI) around the mean for visual hypothesis testing. Under each photoperiodic regime, a total of 40 vials were used, of which 10 were used for each of the four replicate populations for estimation of the *g* and AI values. Asterisks (*) denote the significant differences, whereas differences that did not reach statistical levels of significance are denoted as *n.s.*

Table 4.1: Results of ANOVA for eclosion rhythm under three light/dark cycles. Since block means were used for analysis, the effect of block and interactions involving block could not be tested for significance.

(a): phase-relationship (ψ)

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Light regime (L)	2	22.13	6	0.29	75.92	**0.001**
Population (P)	2	16.96	6	0.13	126.06	**0.001**
Block (B)	3	1.05	0	0	--	--
L x P	4	0.06	12	0.18	0.32	0.858
L x B	6	0.29	0	0	--	--
P x B	6	0.13	0	0	--	--
L x P x B	12	0.18	0	0	--	--

(b): gate of eclosion (g)

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Light regime (L)	2	112.12	6	3.11	36.07	**0.001**
Population (P)	2	16.98	6	0.4	42.08	**0.001**
Block (B)	3	2.01	0	0	--	--
L x P	4	0.11	12	0.24	0.45	0.773
L x B	6	3.11	0	0	--	--
P x B	6	0.4	0	0	--	--
L x P x B	12	0.24	0	0	--	--

(c): anticipation Index (AI)

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Light regime (L)	2	1.93	6	2.99	0.645	0.558
Population (P)	2	23.29	6	1.38	16.84	**0.003**
Lights-on/off (on/off)	1	428.1	3	3.9	109.7	**0.002**
Block (B)	3	2.25	0	0		
L x **P**	4	7.56	12	2.81	2.69	0.083
L x **on/off**	2	1.02	6	4.63	0.22	0.809
P x **on/off**	2	26.48	6	1.22	21.66	**0.002**
L x **B**	6	2.99	0	0		
P x **B**	6	1.38	0	0		
on/off x **B**	3	3.9	0	0		
L x **P** x **on/off**	4	12.3	12	2.52	4.89	**0.014**
L x **P** x **B**	12	2.81	0	0		
L x **on/off** x **B**	6	4.63	0	0		
P x **on/off** x **B**	6	1.22	0	0		
L x **P** x **on/off** x **B**	12	2.52	0	0		

(Table 4.1 a). Multiple comparisons using 95%CI suggest that under all three light regimes the primary peak of eclosion occurs significantly earlier in the *early* populations followed by the *control* and *late* populations, in that order (Fig. 4.1 d).

Width of eclosion gate

The gate of eclosion (*g*) of the selected and control populations differed significantly from each other in all three photoperiodic regimes (Figs. 4.1 a-c and e). ANOVA on *g* values revealed a significant main effect of light regime and population, while light regime × population interaction did not show statistically significant effect, which suggests that the relative differences in the *g* values among the populations remain unchanged across the three light regimes (Fig. 4.1 e; Table 4.1 b). Multiple comparisons using 95%CI reveled that under most light regimes the mean *g* is the shortest in the *early* populations, followed by the *control* and *late* populations, in that order (Fig. 4.1 b).

Anticipatory Eclosion

The selected and control populations also differed in terms of their AI values reflecting anticipatory emergence relative to lights-on and lights-off (Fig. 4.1 a-c and f; Table 4.1 c). The selected as well as control populations clearly anticipated lights-on under all three photoperiodic regimes with the sole exception of *late* populations under normal photoperiod (Figs. 4.1 a-c and f). None of the populations showed anticipatory emergence relative to lights-off under any light regimes, except the *late* populations under normal photoperiod (Figs. 4.1 a-c). Multiple comparisons using 95%CI revealed that there is no measurable difference among the populations under short photoperiod, while under normal and long photoperiods the *early* and *control* populations showed significantly higher anticipation than the *late* populations (Fig. 4.1 f). The differences

between *early* and *control* populations becomes apparent only under long photoperiod, where *early* populations show greater AI values than the *controls*. On the other hand, the differences in the lights-off anticipation among the selected populations could be clearly seen under normal photoperiod where the *late* populations had greater AI values than the *early* and *controls* (Data not shown). Of note, the differences between the *early* and *late* populations not only remained consistent across three light regimes but also showed opposite anticipatory behavior under normal photoperiods (Fig. 4.1 f; Table 4.1 c).

4.3 (b) Activity patterns under short, normal and long photoperiods

Anticipatory activity

The time course and waveform of activity rhythm of the selected and control populations mimicked those of the eclosion rhythm (Figs. 4.2 a-c). The onset and offset of activity under all three light regimes occur significantly earlier in the *early* populations, followed by the *control* and *late* populations, in that order (Figs. 4.2 a-c; Table 2 a). The *early* and *control* flies anticipated the lights-on (AI_{on}) significantly more than the *late* flies under short and long photoperiods, while under normal photoperiods the differences did not reach statistical levels of significance (Fig. 4.2 d). In contrast, AI_{off} values of the *late* flies were greater than the *early* populations, across all three photoperiods (Fig. 4.2 e).

Duration of activity

The activity duration (α) of the selected and control populations were altered depending upon the length of light phase of the LD cycles; short photoperiod causes compression of activity whereas long photoperiod causes decompression (Fig. 4.2 f). The α values of the *early* and *controls* were significantly shorter than that of the *late* populations, while those of the *early* and *control* populations did not differ (Fig. 4.2 f, Table 4.2 b). ANOVA

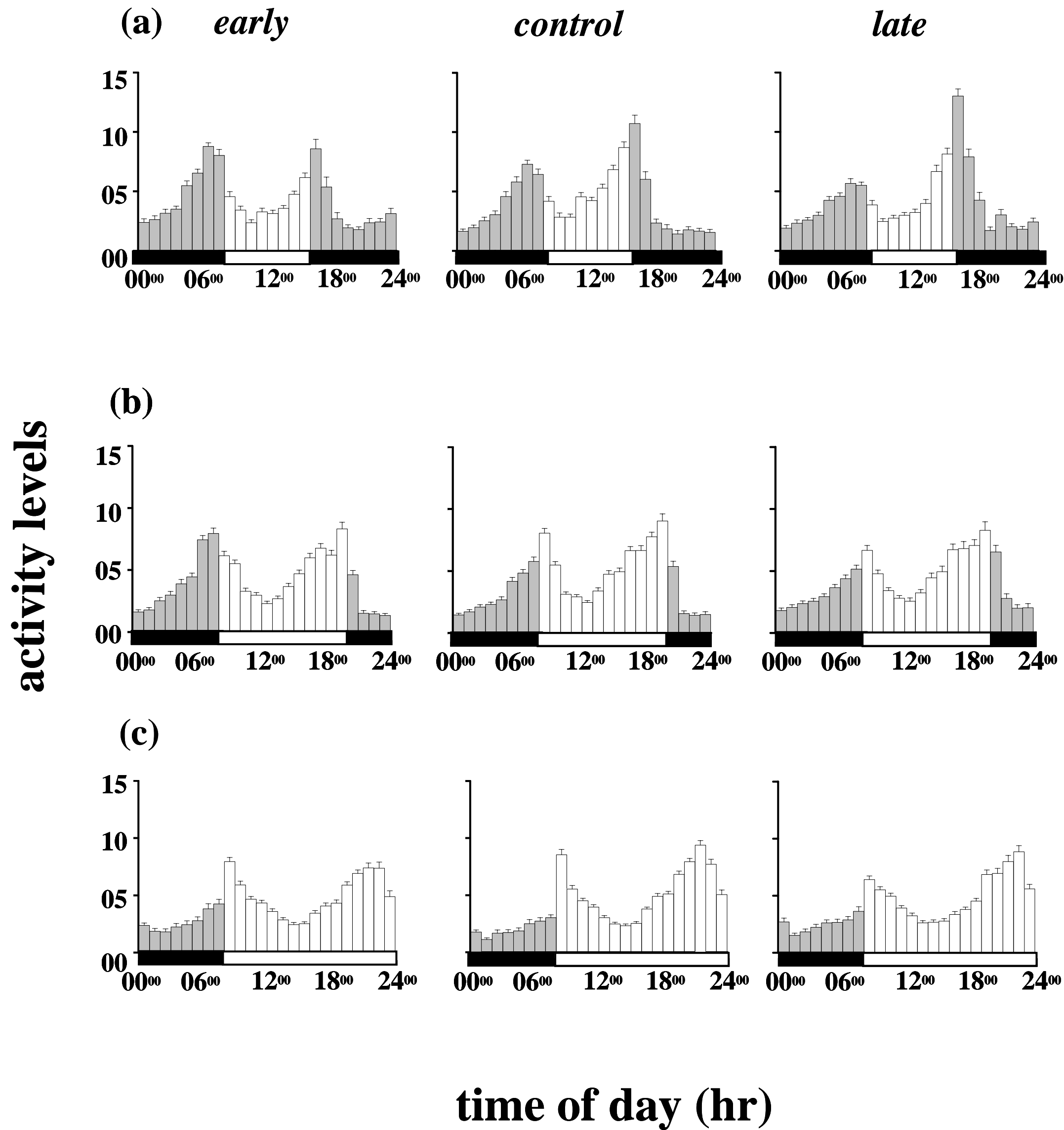

Fig. 4.2: Activity/rest plots of the *early*, *control* and *late* flies under **(a)** short (LD 8:16 hr), **(b)** normal (LD 12:12 hr) and **(c)** long (LD 16:8 hr) photoperiods**.** The vertical grey and white bars denote activity during the dark and the light phases of the LD cycles. Activity is plotted along the ordinate for 10 consecutive cycles and time in hours along the abscissa. The length of black and white boxes represents the duration of dark and light phases during different photoperiodic regimes.

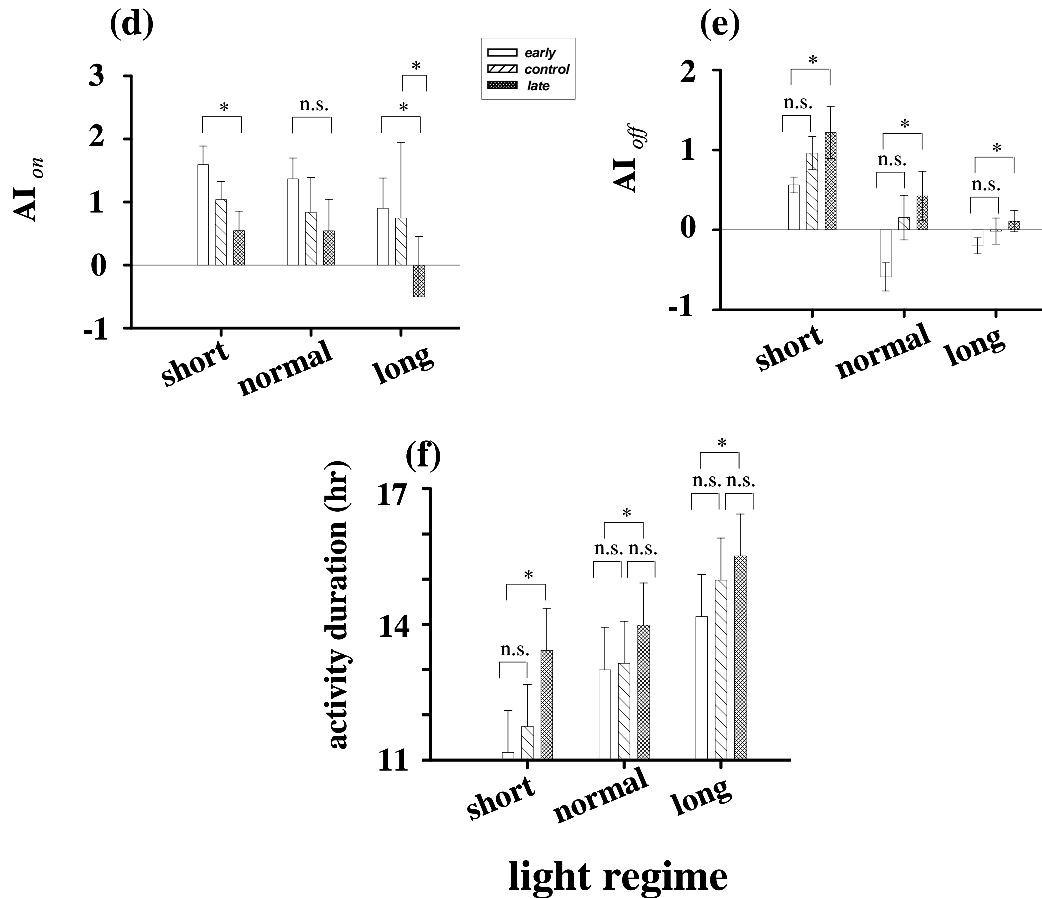

Fig. 4.2 (d) Mean activity anticipation index of the selected and control populations for the lights-on (AI_{on}). **(e)** Mean activity anticipation index of the selected and control populations for the lights-off (AI_{off}). The AI values were estimated using the formula AI= $(b_{-1}-b_{-2}) \times (b_{+1}/b_{-1})$, where b_0 = lights-on/off bin (of 2 hr), b_{-1}= bin before lights-on/off and b_{+1}= bin after lights-on/off, $(b_{-1}-b_{-2})$ amount of activity in a 1 hr bin before lights-on/off, and (b_{+1}/b_{-1}) denotes the ratio of the amount of activity in a 1 hr bin, immediately before and after lights-on/off. **(f)** Average duration of activity (α) in the selected and control populations. The α values were estimated as time interval between the onset and offset of activity in one cycle averaged over ten cycles. The error bars in panels **(d)** and **(e)** indicate standard error of mean (SEM) whereas the error bars in panel **(f)** indicate 95%Confidence Interval (95%CI) around the mean for visual hypothesis testing. For estimation of the AI and α values, 67 *early*, 70 *control* and 68 *late* flies were used under short photoperiod, 172 *early*, 162 *control* and 157 *late* flies were used under normal photoperiod, and 69 *early*, 71 *control* and 66 *late* flies were used under long photoperiod. Asterisks (*) in panels **(d)**, **(e)** and **(f)** denote the significant differences, whereas differences that did not reach statistical levels of significance are denoted as *n.s.*

Table 4.2 Results of ANOVA for activity/rest rhythm under three light/dark cycles. Since block means were used for analysis, the effect of block and interactions involving block could not be tested for significance.

(a): AI

Effect	***df*** **Effect**	**MS Effect**	***df*** **Error**	**MS Error**	***F***	***p*** **-level**
Light regime (L)	2	4.09	6	1.84	2.23	0.189
Population (P)	2	0.41	6	0.84	0.48	0.639
Lights-on/off (on/off)	1	4.38	3	1.14	3.85	0.145
Block (B)	3	3.02	0	0	--	--
L x **P**	4	0.26	12	0.64	0.41	0.798
L x **on/off**	2	0.92	6	1.45	0.64	0.561
P x **on/off**	2	4.61	6	0.56	8.17	**0.019**
L x **B**	6	1.84	0	0	--	--
P x **B**	6	0.84	0	0	--	--
on/off x **B**	3	1.14	0	0	--	--
L x **P** x **on/off**	4	0.14	12	0.53	0.26	0.898
L x **P** x **B**	12	0.64	0	0	--	--
L x **on/off** x **B**	6	1.45	0	0	--	--
P x **on/off** x **B**	6	0.56	0	0	--	--
L x **P** x **on/off** x **B**	12	0.53	0	0	--	--

(b): duration of activity (α)

Effect	***df*** **Effect**	**MS Effect**	***df*** **Error**	**MS Error**	***F***	***p*** **-level**
Light regime (L)	2	23.17	6	0.21	108.6	**0.001**
Population (P)	2	7.28	6	0.43	16.81	**0.003**
Block (B)	3	0.69	0	0	--	--
L x **P**	4	0.59	12	0.69	0.85	0.519
L x **B**	6	0.21	0	0	--	--
P x **B**	6	0.43	0	0	--	--
L x **P** x **B**	12	0.69	0	0	--	--

revealed that the interaction between population and light regime did not have statistically significant effect, which suggests that the relative differences between α of the selected and control populations were maintained across all photoperiods (Table 4.2 b). The α values of the selected and *controls* are shown in Figure 4.2 d for visual comparison using 95%CI.

4.3 (c) Periodicity of eclosion and activity/rest rhythm under DD

The free-running period (τ) of eclosion rhythm under DD differ significantly among the selected populations. ANOVA on τ revealed a significant main effect of population ($F_{2,6} = 9.07$; $p < 0.02$). Multiple comparisons using 95%CI showed that τ of the *late* populations was significantly greater than the *control* and *early* populations, but those of *early* and *control* populations did not differ (Fig. 4.3).

Similarly, we found a significant main effect of population ($F_{2,6} = 7.27$; $p < 0.03$) using ANOVA on τ values of activity rhythm obtained under DD conditions. Multiple pair wise comparisons show that τ of the *late* populations was significantly greater than those of the *control* and *early* populations, but those of the *control* and *early* populations did not differ (Fig. 4.4 a-d).

4.3 (d) Phase response to the light stimuli

Phase response of eclosion rhythm

Light induced phase shifts in eclosion rhythm were estimated by exposing flies to brief light pulse at different phases in their circadian cycle to construct phase response curves for the selected and control populations (Fig. 4.5). The effect of phase, populations on clock response in terms of phase shift was tested using ANOVA. We found that

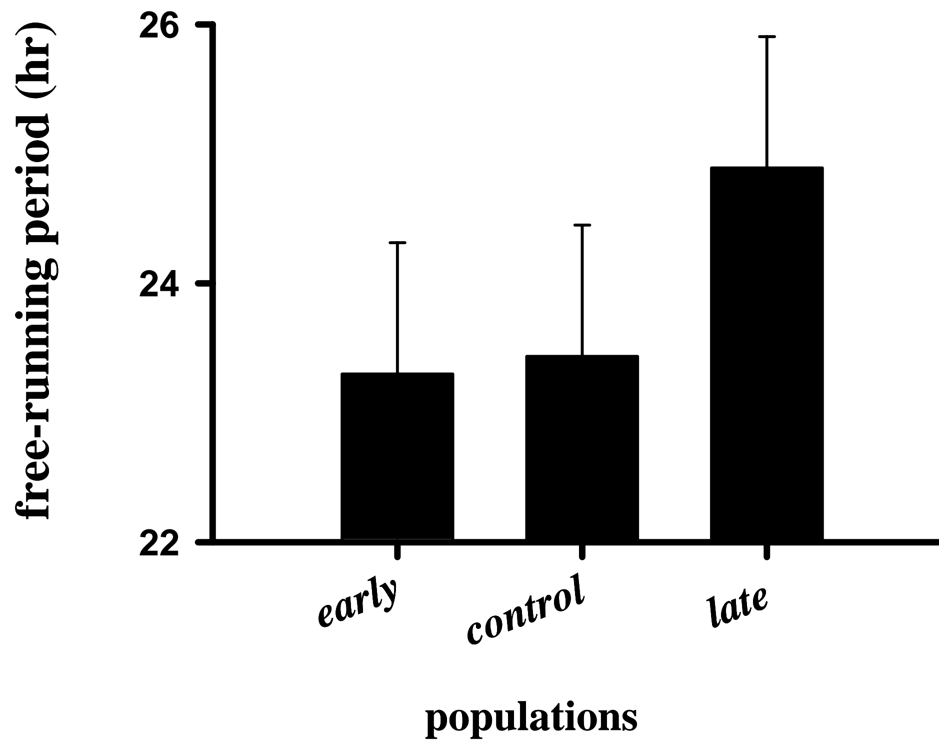

Fig. 4.3: Mean free-running period (τ) (in hours) of the eclosion rhythm of the selected and control populations, estimated under constant darkness (DD). The error bars indicate 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing. A total of 40 vials were used, of which 10 were used for each replicate population.

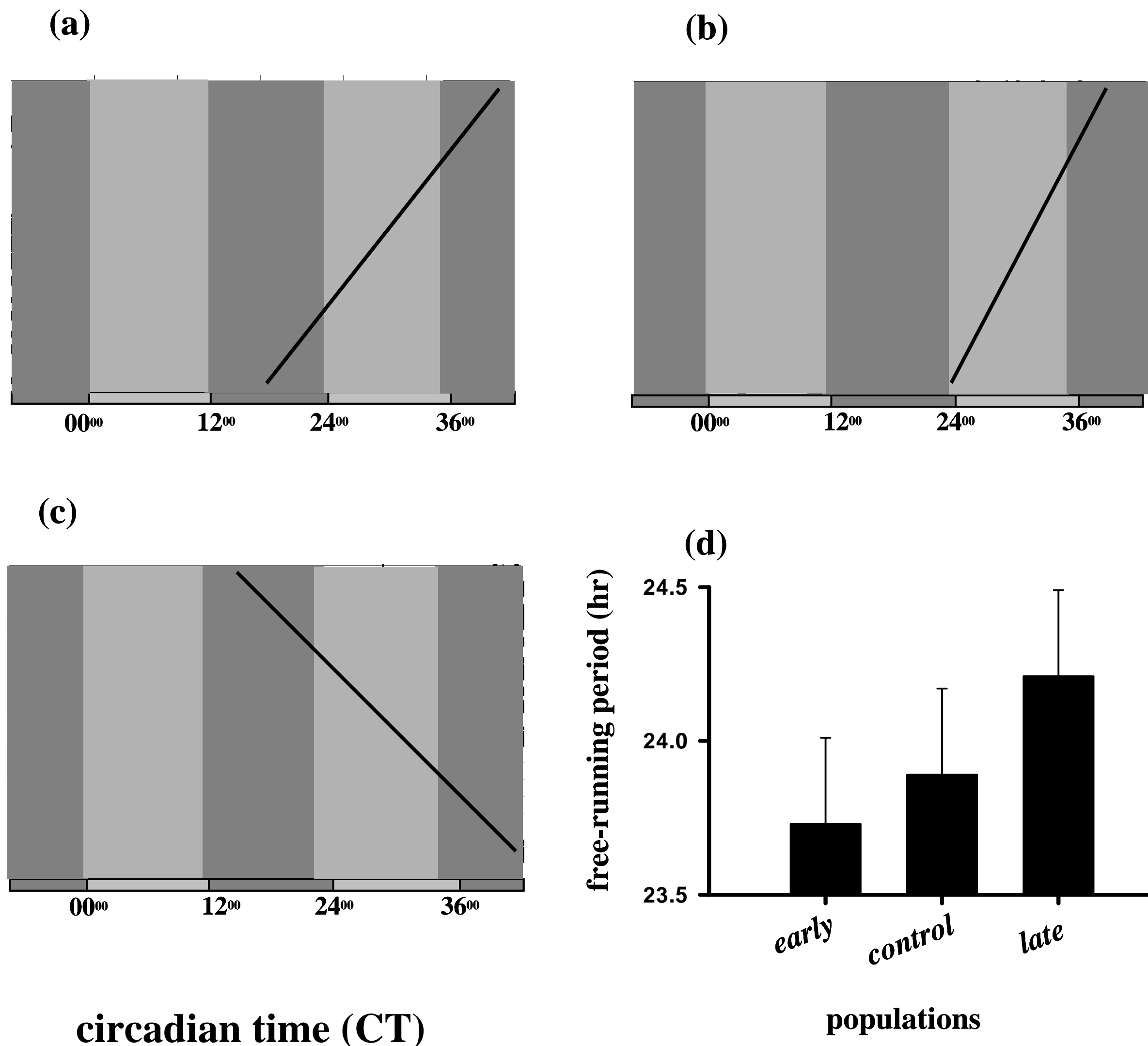

Fig. 4.4: Activity/rest plots of a representative fly from the **(a)** *early*, **(b)** *control* and **(c)** *late* populations under constant darkness (DD). The dark vertical bars and blots denote activity and their absence as rest. Activity is plotted along the ordinate and time in hours along the abscissa. **(d)** Mean free-running period (τ) (in hours) of the activity/rest rhythm of the selected and control populations, estimated under constant darkness (DD). The error bars represent 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing. A total of 55 *early*, 59 *control* and 55 *late* flies were used for the estimation of τ. Lines drawn across offset of activity indicate activity patterns in the actogram.

individual as well as interaction effects of phase and populations were statistically significant (Table 4.3 a). Multiple comparisons using 95% CI revealed that the *early* populations underwent significantly smaller phase shift at CT14 than those of the *controls*, whereas the *late* populations showed significantly larger phase delays than the other two types of populations. On the other hand, during the later part of the night at CT20, the *early* populations displayed significantly larger phase advances compared to those of the *control* and *late* populations and the *late* populations showed significantly smaller phase shifts (Fig. 4.5; Table 4.3 a). However, the selected and control populations did not differ from each other in their phase responses at CT2 and CT8 phases (Fig. 4.5).

Phase response of activity/rest rhythm

The effect of phase, populations on clock response in terms of phase shift was tested using analysis of variance (ANOVA). ANOVA on phase shift data revealed significant effect of population, and phase (Table 4.3 b). The phase responses were plotted for all three populations pooled over four replicate populations. Similar to those of the eclosion rhythm phase shifts, the relative differences among three populations for the phase response for the activity/rest rhythm too, remained consistent (Fig. 4.6), which indicates a correlated response of selection on the activity/rest rhythm. During early subjective night (CT14) the *late* flies underwent greater phase delays compared to the *control* and *early* individuals. On the other hand, during later part of the night at CT20, the *early* flies showed significantly larger phase advances than the *control* and *late* flies (Fig. 4.6). The comparison of the phase shifts of the *controls* with those of the *early* and *late* flies

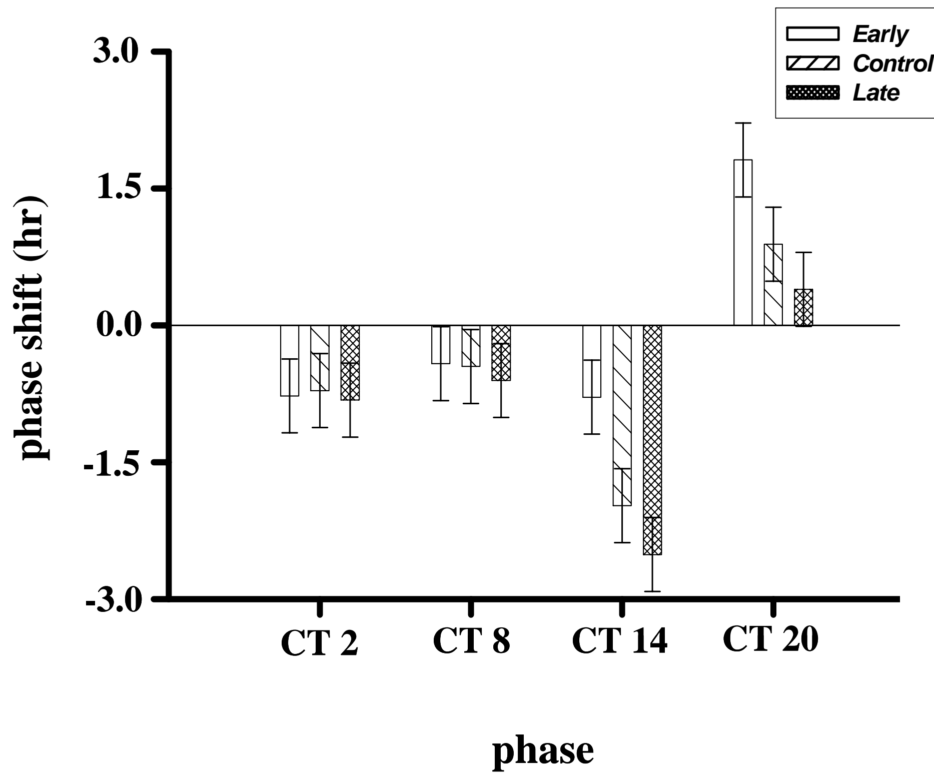

Fig. 4.5: Light pulse induced phase shift ($\Delta\phi$) (in hours) of the eclosion rhythm at four phases (CT2, CT8, CT14 and CT20), of the selected and control populations. The error bars indicate 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing. A total of 40 vials were used, of which 10 were used for each replicate population at each phase.

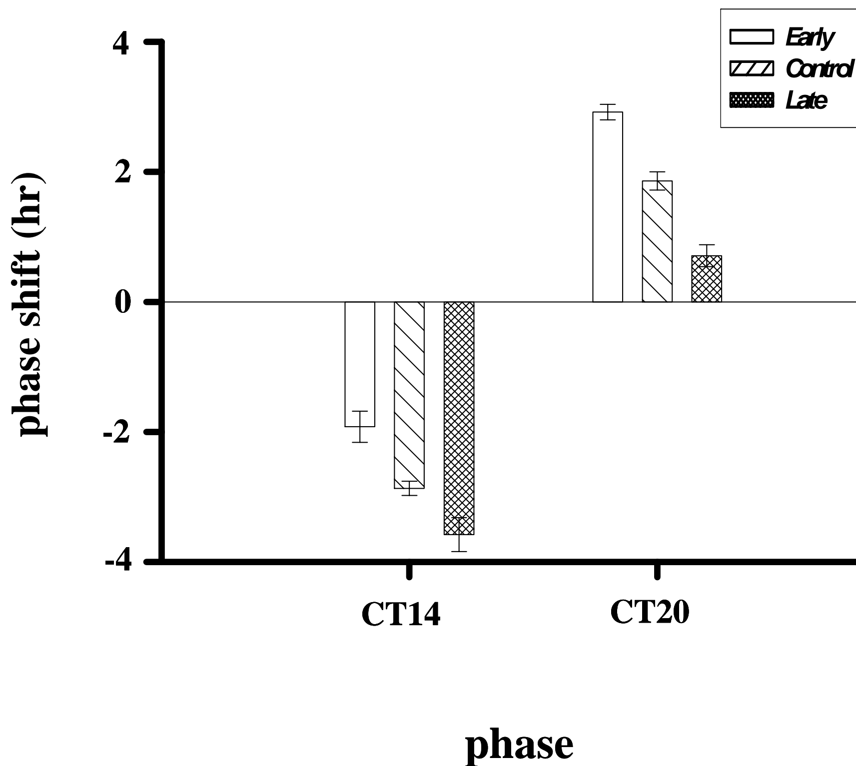

Fig. 4.6: Light pulse induced phase shift ($\Delta\phi$) (in hours) of the activity/rest rhythm at two phases (CT14 and CT20), of the selected and control populations. The error bars indicate 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing. Flies from the selected and control populations were entrained under LD 12:12 hr for 8-9 days and then given light pulse of ~1000 lux intensity and subsequently monitored in DD for at least 7-8 days. A total of at least 15-18 *early, control* and *late* flies were used for estimating phase shifts from each replicate population.

Table 4.3: Results of ANOVA for light-induced phase shift in the eclosion and activity/rest rhythm. Since block means were used for analysis, the effect of block and interactions involving block could not be tested for significance.

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p*-level
(a): eclosion rhythm						
Population (P)	2	2.89	6	0.39	7.27	**0.024**
Phase (Ph)	3	16.01	9	0.72	22.29	**0.001**
Block (B)	3	0.17	0	0	--	--
P x Ph	6	0.78	18	0.25	3.16	**0.027**
P x B	6	0.39	0	0	--	--
Ph x B	9	0.72	0	0	--	--
P x Ph x B	18	0.25	0	0	--	--
(b): activity/rest rhythm						
Population (P)	2	15.315	6	0.333	46.035	< **0.001**
Phase (Ph)	1	106.65	3	0.617	172.964	< **0.001**
Block (B)	3	0.099	0	0	--	--
P x Ph	2	0.059	6	0.093	0.632	0.564
P x B	6	0.333	0	0	--	--
Ph x B	3	0.617	0	0	--	--
P x Ph x B	6	0.093	0	0	--	--

revealed interesting differences, the *control* flies exhibited larger phase shifts during early subjective nights than that of the *early* flies but less than the *late* flies. On the other hand, during later part of the night, the *control* flies response was found to be more than the *late* flies and less than the *early* flies (Fig. 4.6).

4.5 Discussion

Selection on the timing of adult emergence in fruit flies *D. melanogaster* resulted in altered time course and waveform in the *early* and *late* populations relative to the *controls*. The primary peaks of eclosion in the *early* populations occur earlier and are taller than the *controls*, whereas those of the *late* populations occur later and are shorter than the *controls* (Figs. 4.1 a-c). In an earlier study on these flies done at the 55th generation, we have shown that the number of flies that emerged during the morning selection windows in the *early* and during the evening window in the *late* populations increase significantly compared to the *controls*, which suggests that *D. melanogaster* populations respond to selection on timing of adult emergence selection by changing their circadian waveform (Chapter 3). Interestingly, the *early* and *late* populations also exhibit a correlated increase in their activity levels during their selection windows and such changes are generally accompanied by changes in circadian phenotypes. In addition the relative difference in the time course and waveform of eclosion and activity/rest rhythms among the *early* and *late* populations are maintained after 70 generations of selection. This suggests that the circadian phenotypes of the *early* and *late* populations are fairly robust and stable. Although, the time course and waveform of the *early* populations diverged from the *controls* in the course of 70 generations, their τ of eclosion and activity rhythms remain indistinguishably similar, which raises a conundrum as to how similar

circadian clocks in these populations could result in different circadian waveforms under LD cycles. This is possible only when their clocks have altered sensitivity to light pulses. Indeed, this appears to be the case in our *early* and *late* populations; the *early* populations show greater phase advances and smaller phase delays compared to the *controls*, whereas the *late* populations exhibit smaller phase advances and greater phase delays compared to the *control*s (Fig. 4.5 and 4.6). Therefore, it appears that the *early* populations adapt to emerge during the morning selection window by modifying their PRC alone because the phase adjustment required by these populations was quite small (~4 h, Fig. 4.1 a-d). On the other hand, because the phase adjustment needed by the *late* populations to adapt to emerge during the E window of selection was quite large (~8 h), and therefore these populations evolve by changing their τ as well as PRC. These results are contrary to the findings of previous studies, which reported that early and late populations of *D. pseudoobscura* had similar PRCs (Pittendrigh, 1981).

Altered light pulse induced phase response observed in the *early and late* populations provides empirical support for M-E oscillator model proposed by Pittendrigh and Daan in 1976 and elaborated more recently by Daan and others (2001). This model suggests that circadian clocks are composed of M and E oscillators with faster and slower circadian period and different light responsiveness for tracking "dawn" and "dusk", respectively. The M and E oscillator model has been implicated for *D. melanogaster* circadian rhythm in a few recent studies (Grima et al., 2004; Stoleru et al., 2004; Reiger et al., 2006) where the morning and evening activity bouts were suggested to be controlled by two separate sets of neurons residing in the fly brain; the ventro-laterally placed neurons (LN_v) is thought to be necessary for the expression of the morning

activity, while the dorso-laterally located neurons (LN_ds) are required for the evening activity.

In our study the *early* populations exhibit larger advance (A) over delay (D) ratio (A/D) compared to the *controls* and the *late* populations show smaller A/D ratio than the *controls*. These observations are analogous to findings of a study on the M (Δm*per1*) and E (Δm*per2*) knockout mice (Albrecht et al., 2001). The results of this study suggest that animals running solely on E oscillator (Δm*per1*) exhibit only phase-delays, whereas animal with functional M oscillator (Δm*per2*) display only phase advances (Albrecht et al., 2001). We have analyzed the circadian phenotypes of the selected and control populations under three different photoperiodic regimes, and found that the waveforms of eclosion and activity rhythms were left-skewed in the *early* populations and right-skewed in the *late* populations (Fig. 4.1 a-c and Fig. 4.2 a-c). This finding is similar to right and left skewed gene expression patterns seen in the M (Δm*per1*) and E (Δm*per2*) oscillator knockout mice under long and short photoperiods (Steinlechner et al., 2002). Further, in our study the *late* populations have evolved a significantly longer period compared to the *early* populations (τ_{early} < 24 h, τ_{late} >24 h), which fits very well in the M-E model, wherein the E oscillator is assumed to have longer τ compared to the M oscillator (Pittendrigh and Daan, 1976). In addition, it is intriguing to note that a characteristic anticipation of emergence and activity close to lights-on and off is also altered in the *early* and *late* populations, in a predictable manner. A strong lights-on and a weak lights-off anticipatory behavior of the *early* flies, observed under three different photoperiods suggest a dominant M oscillator in this population. On the other hand, a weak lights-on and strong lights-off anticipation of the *late* populations could be a signature of a strong

E oscillator (Fig. 4.1 a-c, 4.2 a-c). A consistent smaller width of eclosion and duration of activity in the *early* and longer *g* and α values in the *late* populations, observed across all three photoperiodic regimes, suggests that there could be some degree of asymmetry in coupling strengths of M and E oscillators in these populations, which may be critical in maintaining stable and robust morning and evening circadian phenotypes.

The results of our study based on the eclosion and activity/rest rhythms of four replicate large, random mating outbred populations of *D. melanogaster* demonstrate that the *early* populations have evolved morning circadian phenotype and the *late* populations have evolved evening phenotype in response to selection on timing of adult emergence.

Chapter 5

Selection on timing of emergence alters rate of pre-adult duration

5.1 Background

Circadian clocks enable organisms to adapt to ambient environmental conditions by coupling behavioral and physiological events to cyclic factors in their environment (Hastings et al., 1991; Dunlap et al., 2004). The timing of these rhythmic events also functions to maximize the potential of an organism to survive under fluctuating environments, suggesting a role of circadian clocks in the regulation of life history traits (Klarsfeld and Rouyer, 1998; Hurd and Ralph, 1998; Sharma, 2003a; Paranjpe and Sharma, 2005). Circadian clocks have been implicated in the regulation of pre-adult development time and adult lifespan in a few insect species including fruit flies *Drosophila melanogaster*. Faster clocks are believed to speed up pre-adult development and shorten adult lifespan while slower clocks slow down development and lengthen lifespan (Kyriacou et al., 1990; Sharma, 2003a; Paranjpe and Sharma, 2005). For example, in a study on the *period* (*per*) mutants of *Drosophila* it was shown that per^S mutants with shorter clock period (τ ~ 19 hr) develop faster than wild type (τ ~ 24 hr) flies, and wild type flies develop faster than per^L (τ ~ 28 hr) mutants (Kyriacou et al., 1990). In a separate study on the melon flies *Bactrocera cucurbitae,* where flies were selected for faster and slower pre-adult development, the τ values of eclosion rhythm of the faster developing line was found to be shorter (~ 22.6 hr) than those of the slower developing line (~ 30.9 hr) (Miyatake, 1996, 1997; Shimizu et al., 1997).

In *Drosophila,* timing of adult emergence is believed to depend upon the developmental state of the fly, phase and period of their developmental clocks, and upon the ambient environmental conditions (Qiu and Hardin, 1996; Shimizu

et al., 1997; Pittendrigh, 1974). Consequently, some time of the day acts as a "forbidden zone" for eclosion, while a narrow window of time constitutes the "allowed zone" or "gate" of eclosion (Pittendrigh, 1954, 1966; Saunders, 1992; Qiu and Hardin, 1996). It is thought that continuously consulted circadian clocks "read" the developmental state of flies, and only those that are mature enough to emerge during the gate are allowed to come out of the puparium while others are made to wait until the next gate opens.

Behavioral characterization of the circadian phenotypes of the selected populations revealed that these populations diverged from each other in their time course and waveform of eclosion and activity/rest pattern under 12:12 hr LD and DD conditions. We sought to investigate the consequence of selection on the rate of pre-adult development. In the present study, we used four populations each of *early*, *control*, and *late* populations to study the effect of selection for timing of adult emergence on the duration of pre-adult development. The pre-adult duration was assayed under 12:12 hr LD and DD conditions after about 70 generation of selections had elapsed. The results of this study provide interesting insights into genetic correlations between circadian rhythms and pre-adult developmental time of fruit flies *D. melanogaster*.

5.2 Materials and Methods

5.2 (a) Pre-adult development time assay

After 75 generations of selection, pre-adult development time of the selected and control populations was assayed. From each of the standardized replicate populations ($early_{1..4}$, $control_{1..4}$ and $late_{1..4}$), eggs laid on banana medium over a 2 hr window (between 0900-1100 hr) under LD cycles (lights-on at 0800 hr and lights-off at 2000 hr) were collected for the developmental time assays. Exactly

30 eggs were dispensed into long vials containing ~ 6 ml banana food and 20 such vials were set up from each population. Ten vials from each replicate population were introduced into DD and ten vials into LD. Thus a total of 240 vials were set up for assays (10 vials × 4 replicate × 2 light regimes × 3 populations). Fluorescent white light of 15 ± 5 μW/cm^2/sec intensity was used during the light phase of the LD cycle, and red light of $\lambda > 650$ nm was used during the dark phase and under DD conditions. Temperature and relative humidity under LD and DD regimes were monitored continuously using Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co, LTD, and were found to be constant throughout the assays. The vials were regularly monitored for emergence once pupae became dark. Eclosing adults were collected at every 2 hr intervals, and the number of males and females were counted. These continued until no flies emerged for 3 consecutive days. From these data, we obtained mean pre-adult development time for each vial. Pre-adult development time of a fly in hours was calculated as the duration between the midpoint of egg collection window and the midpoint of 2 hr period during which the fly emerged as adult.

5.2 (b) Statistical Analyses

Data from LD and DD assays were subjected to one composite mixed model analysis of variance (ANOVA), treating block as random factor and population, light regime and sex as fixed factors, crossed with block. In all cases block means (replicate means) were used as the unit of analysis and hence, only the fixed factor could be tested for significance. The eclosion waveforms of the selected and control populations were analyzed using Kolmogorov-Smirnov test.

All analyses were implemented using Statistica for Windows (StatSoft Inc. 1995).

5.3 Results

The pre-adult development time of males and females from the selected and control populations was estimated under 12:12 hr LD cycles and DD conditions. In an earlier study we found that after 70 generations of selection, primary peak of eclosion rhythm in the *early* and *late* populations diverged by about 4-5 hr (Chapter 3 and 4). Females from all three populations (*early*, *control* and *late*) develop faster than males, and flies take longer to develop under DD than LD (Table 5.1; Figs. 5.1 and 5.2). Under LD cycles, *early* populations develop faster than *control* populations, and *late* populations develop slower than *control* populations (Table 5.1; Fig. 5.1). Similarly under DD, *early* populations develop faster than *control* populations, while *late* populations develop slower than *control* populations (Table 5.1; Fig. 5.2).

A composite mixed model analysis of variance (ANOVA) on mean development time data under LD and DD conditions reveals a significant main effect of population ($F_{2,6}$ = 27.65, $p < 0.001$), light regime ($F_{1,3}$ = 11.01, $p < 0.05$), and sex ($F_{1,3}$ = 579.26, $p < 0.001$) (Table 5.2). Post-hoc comparisons using 95% confidence interval (95% CI) around mean reveal that development time of e*arly* populations is significantly shorter than *control* populations, and those of *control* populations is significantly shorter than the *late* populations (Tables 5.1 and 5.2; Figs. 5.1 and 5.2).

Table 5.1: Mean pre-adult development time of the selected and control populations under LD 12: 12 hr and DD conditions

Population	Light regime	Sex	Mean ± SEM (hr)
early	LD	M	251.581 ± 0.65
		F	244.280 ± 0.65
control	LD	M	253.982 ± 0.49
		F	247.322 ± 0.76
late	LD	M	254.621 ± 0.64
		F	249.728 ± 0.91
early	DD	M	262.158 ± 0.83
		F	257.292 ± 1.13
control	DD	M	262.591 ± 1.15
		F	258.613 ± 1.22
late	DD	M	265.960 ± 0.98
		F	261.295 ± 0.95

Table 5.2: Results of ANOVA on pre-adult development time under LD 12: 12 hr and DD conditions. Since block means were used for analysis, the effect of block and interactions involving block could not be tested for significance.

	df Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Population (P)	2	66.66	6	2.41	27.64	**0.001**
Light regime (L)	1	1469.41	3	133.52	11.01	**0.045**
Sex (S)	1	349.15	3	0.60	579.26	**0.001**
Block (B)	3	198.17	0	0	--	--
P x L	2	3.85	6	1.29	2.98	0.126
P x S	2	1.72	6	1.95	0.88	0.462
L x S	1	9.52	3	3.53	2.69	0.199
P x B	6	2.41	0	0	--	--
L x B	3	133.51	0	0	--	--
S x B	3	0.60	0	0	--	--
P x L x S	2	1.82	6	0.771	2.37	0.175
P x L x B	6	1.29	0	0	--	--
P x S x B	6	1.95	0	0	--	--
L x S x B	3	3.53	0	0	--	--
P x L x S x B	6	0.77	0	0	--	--

Post-hoc comparisons using 95%CI around mean show that development time of flies is shorter under LD than DD (Tables 5.1 and 5.2; Fig. 5.1 and 5.2). The development time of females is significantly shorter than those of males in their respective regimes (Tables 5.1 and 5.2; Figs. 5.1 and 5.2).

The interactions between population and light regime, population and sex, light regime and sex and population, light regime and sex do not have any significant effect on pre-adult development time (Table 5.2). The fact that two way interactions of population and light regime, population and sex, light regime and sex do not show any significant effect on pre-adult development time, suggests that relative differences in development time of males and females of all three populations, remain largely unaltered between LD and DD conditions.

The profiles of adult emergence suggest that the developmental rates of the selected populations have diverged from each other as well as from control populations (Figs. 5.1 and 5.2). Although, the differences between the selected and control populations appear to be consistent across both light regimes, they did not reach statistical levels of significance as revealed by Kolmogorov-Smirnov test for two samples.

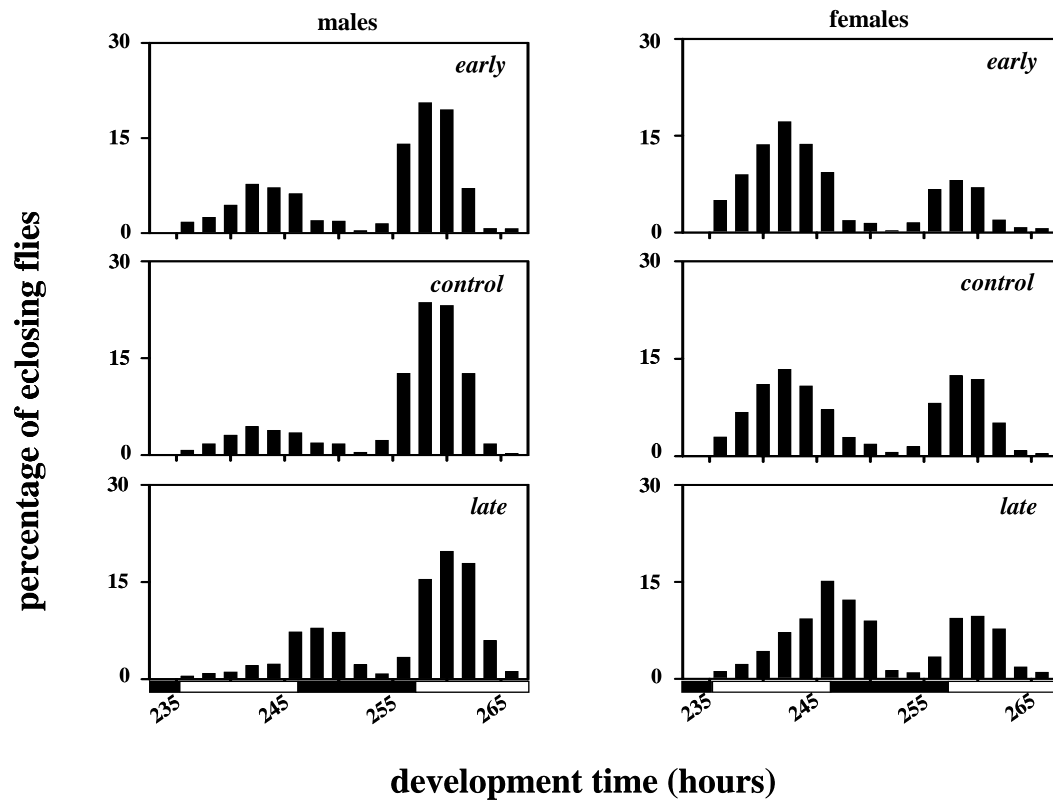

Fig. 5.1: Eclosion profiles of the *early*, *control* and *late* populations under 12:12 hr LD cycles. Pre-adult development is plotted along the x-axis, and the percentage of flies emerging in a 2 hr interval is plotted along the y-axis. The eclosion profile of males is shown in the left panel, while those of the females are shown in the right panel.

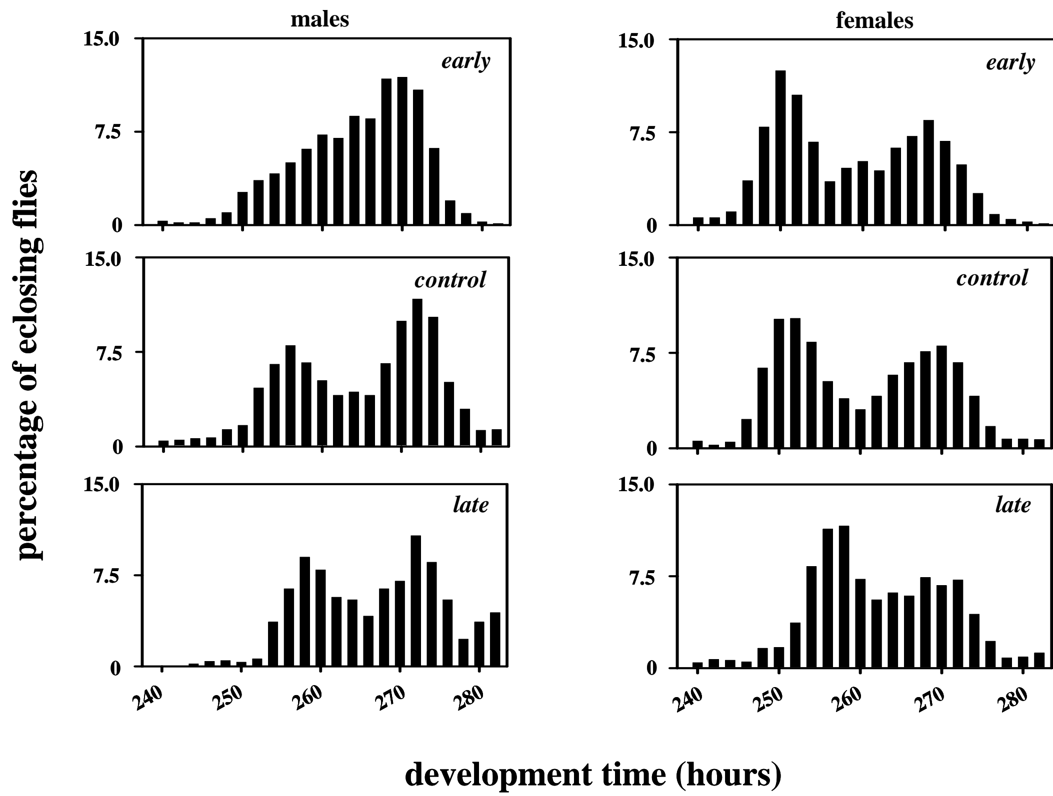

Fig. 5.2: Eclosion profiles of the *early*, *control* and *late* populations under DD conditions. Pre-adult development is plotted along the x-axis, and the percentage of flies emerging in a 2 hr interval is plotted along the y-axis. The eclosion profile of males is shown in the left panel, while those of the females are shown in the right panel.

5.4 Discussion

After 75 generations of selection, the peak of eclosion of the *early*, *controls*, and *late* populations diverged, suggesting that *D. melanogaster* populations respond to selection on the phase of circadian rhythm by evolving appropriate timing for their behavior (Chapter 3). In the present study we show that under 12:12 hr LD cycles males and females from the *early* populations develop significantly faster than those from the *control* populations, whereas males and females from the *late* populations develop slower than those from the *control* populations, suggesting that pre-adult development time in *D. melanogaster* is altered as a correlated response to selection on the timing of adult emergence. Consistent divergence between four sets of replicate control and selected populations that were treated through one generation of common rearing conditions clearly implies selection as the cause, as it is unlikely that four replicate populations would undergo similar sequence of genetic changes through random genetic drift.

The magnitude of the differences in mean development time among the *early* and *late* populations closely match those between peaks of their eclosion rhythm under 12:12 LD cycles (~ 4-5 hr), thus suggesting that "correct phase" of eclosion matches "correct development state" of the flies to cause rhythmic eclosion in *Drosophila*. Our results are in good agreement with the results from an early study by Qiu and Hardin (1996), which suggested that circadian clocks regulate pre-adult development by assessing the development state of flies and the phase of their development clocks. In this study, short period mutants (per^S) were found to develop faster than the wild type flies. The authors concluded that

per^S mutants encounter a favorable gate of emergence under LD cycles much earlier than wild type flies because eclosion peaks of *per*^{S} flies precedes lights-on whereas those of wild type flies usually follows lights-on (Konopka, 1972). This implies that under LD cycles, duration of pre-adult development in *Drosophila* would depend upon the developmental state of flies, timing of lights-on and lights-off, periodicity of LD cycles, and phase of circadian clocks (Qiu and Hardin, 1996). However, under DD conditions, development time would be expected to depend upon the developmental state and periodicity of circadian clocks. Circadian clocks in *Drosophila* are believed to be set-in early during the larval stage (Sehgal et al., 1992). Based on the assumption that circadian clocks alone determine pre-adult development time, and that the periodicity of eclosion clocks in the *early, control* and *late* populations are 23.66 ± 0.37 hr (mean ± SEM), 23.72 ± 0.40 hr and 25.06 ± 0.44 hr, respectively (Chapter 3), we would expect to see a difference of about 20 hr between the development time of the *early* and *late* populations (assuming that it takes about 9-10 days for these flies to develop). Contrary to our expectations even under DD conditions the *early* populations develop only ~3-4 h earlier than the *late* populations.

In a separate study we also tested the effect of timing of egg collection on the rate of development by collecting eggs at different time of the day (close to lights-on and lights-off) and assaying under 12:12 hr LD, DD and LL conditions (*Kaustubh Vaze, Shailesh Kumar* and *Vijay Kumar Sharma, unpublished data*). Although the magnitude of the differences between the development time of the selected and control populations changed marginally, their relative differences remained statistically significant, which circumvents

any influence of the phase of LD cycle and/or circadian clocks on the rate of pre-adult development. It is especially interesting to note that the relative differences between the rate of pre-adult development of the selected and control populations are maintained even under bright constant light (LL) conditions, wherein the circadian rhythms are abolished.

The fact that there is no influence of the phase of LD cycle and/or circadian clocks on the rate of pre-adult development results of the *early*, *control* and *late* populations suggest that the connection between circadian clocks and development time is mediated through pleiotropic effects of genes on circadian clocks and pre-adult development time. The pleiotropic effects of clocks genes have been reported in an early study on the *per* mutants of *D. melanogaster* (Kyriacou et al., 1990). In this study, development time and τ were positively correlated. The per^S flies develop faster than wild type flies, and per^L flies develop slower than wild type flies. Changing environmental conditions (DD, very bright continuous light (VLL), LD 12:12 hr and LD 12:12 hr with imposed temperature cycles) did not alter the nature of correlation between clock period and development time (Kyriacou et al., 1990). The short and long period mutants continued to develop faster and slower than wild type flies, in spite of being synchronized to LD 12:12 hr cycle, or while free-running under DD and dim light LL, or while being arrhythmic in VLL. Pleiotropic effects of clock genes were also reported in a few relatively recent studies, which involved selection for faster and slower pre-adult development in melon fly *Bactrocera cucurbitae* (Miyatake, 1996, 1997; Shimizu et al., 1997). In these studies development time was found to be correlated with the timing of mating and with the clock period. The circadian period of faster developing line was

shorter (τ ~ 22.6 hr) than slower developing lines (τ ~ 30.9 hr), and mating occurred earlier in faster developing lines compared slower developing line. The above studies suggest that the relationship between circadian clocks and life history traits are rather complex in nature, possibly due to the fact that both traits are regulated by a large number of genes, many of them interlocked through pleiotropic interactions (Kyriacou et al., 1990).

In a separate study designed to bypass pleiotropic effects of clock genes, eclosion rhythm of four populations of *Drosophila* was studied in conjunction with pre-adult development time (Paranjpe et al., 2005). In this study, eclosion rhythm of flies was speeded up or slowed down by using LD cycles of short (20 hr) and long (28 hr) periodicity. As a consequence, pre-adult duration was either shortened or lengthened, suggesting a connection between periodicities of LD cycles and/or circadian rhythm and development time. Our study suggests that selection for *early* and *late* adult emergence alters the timing of peak of adult emergence, and a correlated change in the duration of pre-adult development in *D. melanogaster*, suggesting a possible role of circadian clocks in the regulation of pre-adult development time. The effects appear to be mediated primarily through pleiotropic effects of clock genes on circadian clocks and development time. However, it is also possible that a complex and yet less understood interactions of a number of factors such as available gate of eclosion, speed of circadian clock, assessment of developmental state, regulate key life history traits such as pre-adult development time.

Chapter 6

Adaptive significance of timing of adult emergence

6.1 Background

There is a general belief among circadian biologists that lack of coordination between cyclic biological processes and environmental cycles is deleterious (Pittendrigh and Minis, 1972; von Saint-Paul and Aschöff, 1978; Klarsfeld and Rouyer, 1998; Ouyang et al., 1998). Empirical evidence for such deleterious effects are, however, sketchy and far from conclusive. A few studies have shown that organisms normally reared under light/dark (LD) cycles of certain periodicity tend to perform the best under LD cycles of similar periodicities. For example, fruit flies *Drosophila melanogaster* (Pittendrigh and Minis, 1972) and blowflies *Phormia terranovae* (von Saint-Paul and Aschöff, 1978) reared under 24 hr LD cycle were found to live significantly longer under 24 hr LD cycles compared to non-24 hr LD cycles. In cyanobacteria *Synechococcus elongatus*, when two strains with different clock periodicities were mixed in equal proportions and cultured under LD cycles of different periodicities, strains whose clock periodicity closely matched that of the prevailing LD cycle always out-competed the other (Ouyang et al., 1998). When arrhythmic strains were competed with rhythmic ones, arrhythmic strains lost to wild-type strains under LD cycle, but out-competed them under LL condition (Woelfle et al., 2004), suggesting that circadian clocks may not be beneficial under LL, in fact it might even be disadvantageous.

Circadian dysfunctions arising due to malfunctioning of circadian clocks, or disturbances in the input or output mechanisms have been shown to have adverse effects on lifespan (Hendricks et al., 2003; Cirelli et al., 2005; Kumar et al., 2005; Kume et al., 2005). For example, in a recent study in *D. melanogaster*, flies which could not consolidate rest or sleep were found to live significantly shorter than their wild type

counterparts (Hendricks et al., 2003). In a separate study, Cirelli and coworkers (2005) showed that *minisleep* (*mns*) flies, which sleep for only one-third the duration of wild-type CS flies, lived for significantly lesser number of days than the wild type flies.

Several lines of evidence suggest that the lack of the ability to schedule behavior and physiology in accordance with the local environment may prove to be fatal especially to organisms that normally live under natural conditions. For example, in an early study on the jumping of guillemot fledglings from their nests Daan and Tinbergen (1980) found that predation of flightless young ones was less severe during the daily peak of jumping activity of the fledglings, suggesting that the timing of this activity is correlated with survivorship. Adult emergence in the midge *Chironomus thummi* follows a circadian pattern with temperature-dependent phasing of emergence (Kureck, 1979), and provides an example of reproduction-mediated fitness advantage of timing of behaviour. At low temperatures (14^0 C), eclosion peak occurs during daytime, whereas at normal temperatures (above 16^0 C) it occurs during dusk. It appears that at higher temperatures night swarming is more effective for reproduction, but individuals eclosing during daytime have better reproductive success at low temperatures (Kureck, 1979). Similarly studies on SCN-lesioned antelope ground squirrels, *Ammospermophilus leucurus* (DeCoursey et al., 1997) and eastern chipmunks, *Tamias striatus* (DeCoursey and Krulas, 1998) kept under field conditions demonstrated that, relative to controls, SCN-lesioned animals suffer greater mortality, mainly through increased predation, suggesting the importance of timing of behaviour for organisms living in the wild.

Taken together, these studies suggest a strategy in organisms towards maximizing their fitness by scheduling their day-to-day activities appropriately with the

environmental cycles. In this chapter, we report the results of our experiments aimed at studying the adaptive significance of timing of adult emergence in the *early* and *late* populations of *D. melanogaster*. We assayed adult lifespan of virgin males and females from the *early*, *control* and *late* populations that emerged during the morning (selection window for the *early* populations) and evening (selection window for the *late* populations) hours under 12:12 hr LD cycles. The results provide the first ever evidence of its kind of adaptive significance of circadian rhythm in any eukaryotic organism.

6.2 Materials and Methods

6.2 (a) Adult life span assay

From the standardized populations of each of selected and control populations (method described in details in section 2.3), eggs laid on banana medium over a 2 hr window were collected for life span assays. From each replicate population, approximately 250-300 eggs were collected into each of 24 vials. The vials were kept in LD till adults emerged. Freshly emerged male and female flies during the morning and evening selection windows from the selected and control populations, were separated and transferred in vials at a density of either 8 males or 8 females per vial. Eight such vials of virgin males and females from each selected and control populations were set up and continued in 12:12 hr LD cycle. Flies were provided with fresh banana food every alternate day and the vials were checked every day for death of flies and recorded. This procedure was carried out until all the flies died.

6.2 (b) Statistical analyses

The lifespan data of virgin males and females were used to calculate mean adult life span (in days) for each selected and control populations. The mean lifespan data was treated in

a mixed model analyses of variance (ANOVA) where replicate populations (blocks) were treated as random factor and selection regimes, eclosion windows and sex were treated as fixed factors crossed with blocks.

6.3 Results

Individuals that emerged in the morning in the *early* populations live significantly longer than their evening emerging counterparts and morning emerging *control* and *late* flies. On the other hand, individuals that emerged in the evening in the *late* populations live significantly longer than their morning emerging counterparts and evening emerging *early* and *control* flies. The trends of mean survivorship in the selected and control populations are found to be similar for males and females.

Three-way-ANOVA revealed a significant main effect of population ($F_{2,6} = 11.12$, $p < 0.019$), however, the main effect of selection window and sex on lifespan did not reach statistical levels of significance (Table 6.1). The interaction between population and selection window ($F_{2,6} = 23.35$, $p < 0.001$) has a statistically significant effect on lifespan, while population × sex, selection window × sex, and population × selection window × sex interactions do not have any statistically significant effect on lifespan (Table 6.1). Multiple comparisons using 95%CI around the mean (95%CI= 2.07) revealed that *early* males that emerge in the morning live significantly longer than their evening emerging counterparts and the morning emerging *control* and *late* males, while the lifespan of the *control* and *late* males does not differ (Fig. 6.1 a-c). The *late* males that emerge in the evening live significantly longer than their morning emerging counterparts and evening emerging *control* and *early* males, while the lifespan of evening emerging *early* and *control* males does not differ (Fig. 6.1 a-c).

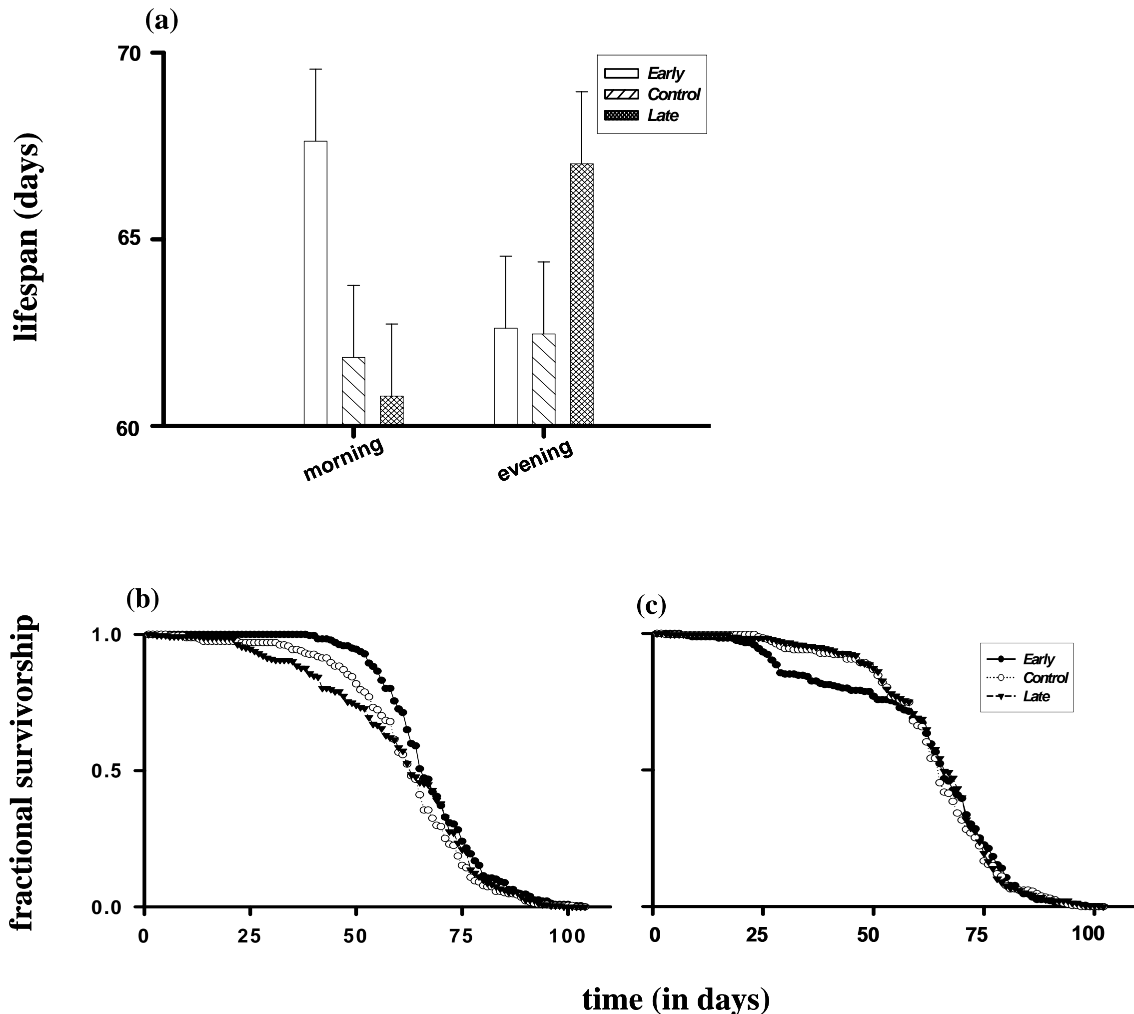

Fig. 6.1 (a) Mean lifespan of virgin males that emerged during the morning (0500-0900 h) and evening (1700 - 2100 h) windows of selection from the three laboratory selected populations (*early*, *control* and *late*) under light/dark cycles (LD 12:12 hr). The error bars indicate 95%CI around the mean for visual hypothesis testing. Adult survivorship curves of virgin males that emerged during the **(b)** morning and **(c)** evening windows of selection. The survivorship of the morning as well as evening emerging *early* (closed circles), *control* (open circles) and *late* (closed triangles) males do not differ until about 25 days. After which, they fall rapidly in the *late* males followed by the *control* males, in that order. Similarly, among the flies that emerged in the evening, survivorship of the *late* males remains higher at all times, followed by the *control* and *early* males, in that order.

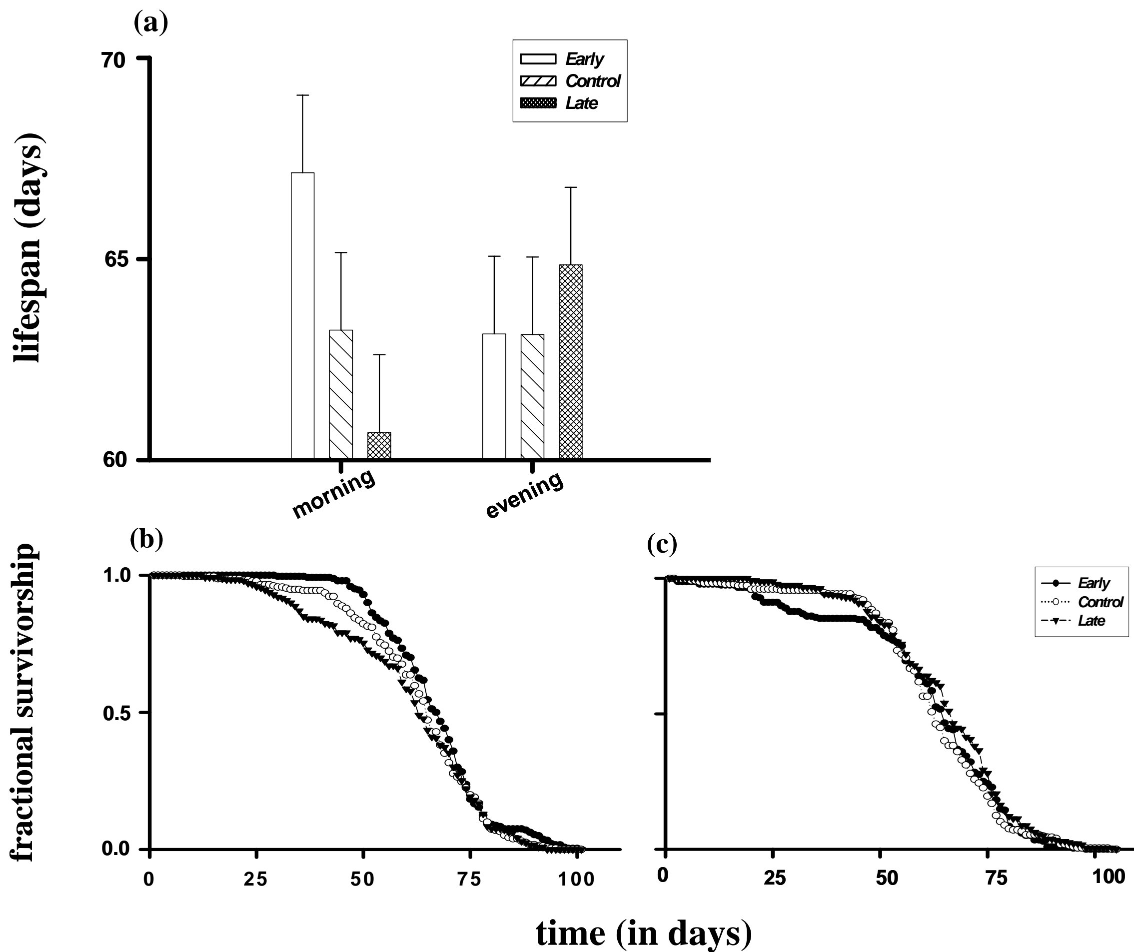

Fig. 6.2 (a) Mean lifespan of virgin females that emerged during the morning (0500-0900 h) and evening (1700 - 2100 h) windows of selection from the three laboratory selected populations (*early*, *control* and *late*) under light/dark cycles (LD 12:12 hr). The error bars indicate 95%CI around the mean for visual hypothesis testing. Adult survivorship curves of virgin females that emerged during the **(b)** morning and **(c)** evening windows of selection. The survivorship of the morning as well as evening emerging *early* (closed circles), *control* (open circles) and *late* (closed triangles) males do not differ until about 25 days. After which, they fall rapidly in the *late* females followed by the *control* females, in that order. Similarly, in the flies that emerged in the evening, survivorship of the *late* females remains higher at all times, followed by the *control* and *early* females, in that order.

Table 6.1 Results of analysis of variance (ANOVA) on adult life span of the selected and control populations emerging in the morning and evening windows. Since block means were used for data analysis, the effect of block and interactions involving blocks could not be tested in this design.

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Population (P)	2	26.014	6	2.339	11.12	**0.009**
Window (W)	1	1.206	3	3.448	0.35	0.596
Sex (S)	1	0.013	3	7.112	0.002	0.968
Block (B)	3	7.776	0	0	--	--
P x W	2	94.072	6	4.029	23.349	**0.001**
P x S	2	4.694	6	2.809	1.671	0.265
W x S	1	1.08	3	2.476	0.436	0.556
P x B	6	2.339	0	0	--	--
W x B	3	3.448	0	0	--	--
S x B	3	7.112	0	0	--	--
P x W x S	2	2.344	6	2.35	0.997	0.423
P x W x B	6	4.029	0	0	--	--
P x S x B	6	2.809	0	0	--	--
W x S x B	3	2.476	0	0	--	--
P x W x S x B	6	2.35	0	0	--	--

Multiple comparisons using 95%CI revealed that *early* females that emerged in the morning live significantly longer than their evening emerging counterparts and morning emerging *control* and *late* females, while *control* females live significantly longer than *late* females. The *late* females that emerged in the evening live significantly longer than their morning emerging counterparts and evening emerging *control* and *early* females, while the lifespan of *control* and *early* females does not differ (Fig. 6.2 a-c).

Survivorship curves for males and females are shown separately in Fig. 6.1 b, c and 6.2 b, c. The survivorship is highest in the morning emerging *early* populations followed by the morning emerging *control* and *late* populations, in that order. Similarly, survivorship is highest in the evening emerging *late* populations followed by the *control* and *early* populations, in that order. The patterns of survivorship curves are more or less similar in males and females.

6.4 Discussion

The results of our study clearly demonstrate that the lifespan of *early* and *late* populations is significantly greater when these flies emerge during morning and evening hours respectively, thus demonstrating a possible connection between circadian rhythms and life history traits. Previously done assays at a regular interval of every 10-15 generations on the selected and control populations have shown that under 12:12 hr LD cycles the emergence pattern of adults is gated in such a way that a significantly greater percentage of flies emerged during the morning window from the *early* populations than the *control* and *late* populations, whereas the percentage of flies emerging during the evening window was significantly greater in the *late* populations than the *control* and *late* populations (Chapter 3). Furthermore, under 12:12 hr LD cycles the primary peak of

adult emergence rhythm in the *early* populations was phase advanced by about 1 hr and in the *late* populations was phase delayed by about 3 hr relative to the *controls* (Chapter 3). Therefore, the *early* and *late* populations have evolved greater preference for emergence during morning and evening selection windows respectively because evening emergence for the *early* flies and morning emergence for the *late* flies would be maladaptive.

Although, adult emergence rhythm has been extensively studied in several insect species including fruit flies *D. melanogaster*, its functional significance has never been studied systematically. It is generally believed that the flies may survive better if environmental factors such as temperature and humidity during the time of emergence are favorable (Pittendrigh, 1974; Qiu and Hardin, 1996). The results of our study suggest that *early* and *late* flies gain greater fitness advantage by emerging during the morning and evening selection windows, respectively. On the other hand, lifespan of *control* flies that emerged in the morning was comparable to the lifespan of those that emerged in the evening. Although, at this point of time we do not know what could be the cause of reduction in lifespan in the flies that failed to emerge during their selection windows, it is tempting to speculate that lack of coordination between environmental, behavioral and metabolic cycles could be one of the prime factors. Desynchronization among various metabolic cycles in the internal milieu and their lack of coordination with the environmental LD cycles has been argued to be one the primary reasons behind the reduction of lifespan in previous studies on fruit flies (*D. pseudoobscura*) and blowflies (*Phormia terraenovae*) (Pittendrigh and Minis, 1972; von Saint-Paul and Aschöff, 1978). In a few recent studies lack of proper coordination in the activity/rest patterns were

shown to cause significant reduction in lifespan (Hendricks et al., 2003; Cirelli et al., 2005; Kume et al., 2005). In the *cyc*^{o} mutant of *D. melanogaster*, which exhibits arrhythmic activity/rest behavior, *cyc*^{o} males were found to live significantly shorter than the wild type males under both LD as well as DD regimes, the *cyc*^{o} females however lived as long as the wild type females (Hendricks et al., 2003). Sex specific differences in lifespan have also been reported previously in the *per* mutants of *D. melanogaster*, under LD cycles lifespan of short and long period males was significantly shorter than the wild type males, but lifespan of mutant and wild type females did not differ (Klarsfeld and Rouyer, 1998). These studies suggest that lack of coordination among cyclic processes results in reduction in fitness, but this seems to affect males more than females.

In our study, the *early* and *late* flies live longer and shorter depending upon their timing of emergence, the lifespan of *late* flies was 2-3 days shorter than those of the *early* flies when lifespan data was pooled across both sexes and selection windows. While investigating the possible reasons for such reduction in lifespan in the *late* flies, we found that under a wide range of photoperiods the *late* flies are active for longer duration of time compared to the *early* and *control* flies (Chapter 5). This suggests that increased mortality in the *late* flies compared to the *early* and *controls* is due to enhanced activity. These results are similar to the findings of a recent study on *shaker (sh)* mutants (Cirelli et al., 2005). Flies carrying *sh* mutation (a point mutation in the gene encoding for the voltage-dependent potassium channel), were more active compared to the wild type flies, however, sleep homeostasis and sleep deprivation responses remained normal. The *sh* mutant lived shorter than their wild type controls. As these mutants did not have any noticeable defect in their activity/rest rhythm, reduction in lifespan was thought to be

mainly due to lack of sleep and/or pleiotropic effects of mutation that impairs voltage-dependent potassium channels. On the other hand, in a recent study on the *fumin* (*fmn*) mutants of *D. melanogaster*, lifespan of *fmn* flies was shown to be comparable to their genetic controls, in spite of the fact that *fmn* flies were more active, had lower sleep arousal threshold and reduced rest rebound in response to sleep deprivation compared to the controls (Kume et al., 2005). These results are therefore contradictory to earlier observations on the cyc^0 and *Sh* mutants, where reduction in lifespan was attributed to increased activity levels. It was argued that since the SH protein is a voltage-activated potassium channel with widespread expression in the nervous system, and CYC is a broadly expressed basic helix-loop-helix transcription factor, mutations in these genes might have more general impact on physiology leading to shortening of lifespan, whereas, *fmn* is a mutation in *Drosophila* dopamine transporter (dDAT) gene that affects only the dopaminergic neurons, and therefore, may have a less severe impact on physiological well-being (Kume et al., 2005).

Our study clearly demonstrate that gated emergence at a favorable time of the day confers adaptive advantage to fruit flies *D. melanogaster*, implying adaptive significance of circadian clocks. This, to the best of our knowledge, is the first evidence of its kind for the adaptive significance of circadian rhythm in any higher organism. The results of our studies thus confirm a long-standing view that temporal partitioning of physiological and behavioral processes, facilitated by circadian clocks, serves to restrict activities to species-specific time of the day, and thus be of adaptive significance.

Chapter 7

Molecular characterization of the selected populations

7.1 Background

Early laboratory selection studies in insects reported that the phase of rhythmic processes could be selected for (Pittendrigh, 1967; Pittendrigh and Minis 1971; Clayton and Paietta, 1972). This suggests that the timing of these processes have a genetic basis. Subsequent studies using EMS mutagenesis and modern techniques of molecular biology established that circadian rhythms indeed have a genetic basis, and they are regulated by genes whose expression oscillates with a circadian period (Konopka and Benzer, 1971; Jackson, 1983; reviewed in chapter 5 in Young, 1993; for review see Hardin, 2005). It is generally believed that laboratory selection studies are likely to produce a near true representation of genes and their interaction with their environments (Tully, 1996). Following laboratory selection approach on large outbred populations of *D. melanogaster* we created the *early* and *late* populations that were selected for morning and evening emergence. Previous characterization of the behavioural phenotypes of these populations revealed that they have diverged from each other as well as from the *controls* in terms of their time course and waveform of adult emergence rhythms (Chapter 3 and 4). Further, the circadian phenotypes of adult emergence and activity/rest rhythms have undergone correlated changes in response to the selection. The *early* flies display robust morning circadian phenotype and the evening flies exhibit evening phenotype (Chapter 4). The behavior of the *early* populations resembles those of the *psi* mutants that were discovered in an early study while screening for unusual adult emergence patterns in chemically (EMS, ethyl methane sulfonate) mutagenenized flies (Jackson, 1983). The primary eclosion peak of adult emergence of the phase angle-2 (*psi*-2), and phase angle-3 (*psi*-3) mutants occurred ~ 2-3 hr prior to lights-on, while it occurred ~ 2 hr after lights-on in the

wild type flies (Jackson, 1983). Another striking similarity between our *early* populations and the *psi* mutants is that their circadian periodicities were comparable to the *controls*. In a separate study, it was shown that manipulation in the expression of *lark* gene produces early and late emerging flies in *D. melanogaster* (Newby and Jackson, 1996). Loss of function mutation in this gene caused early emergence, while its over-expression caused late emergence. Once again the circadian periodicity of the mutants was similar to those of the wild type flies. Having carried out detailed analyses of the behaviors of the *early* and *late* populations we decided to study the molecular correlates of morning and evening circadian phenotypes in our flies by analyzing the core clock genes in the molecular clockwork of *Drosophila*. We therefore assayed the temporal profiles of the transcripts of *period* (*per*) and *clock* (*clk*) genes under 12:12 hr LD cycles using real-time quantitative PCR (RT-qPCR).

While studying the circadian phenotypes of the selected and control populations we had seen that though the peak of the adult emergence rhythm in the *early* populations occurs 1-2 hr before those of the *controls*, they did not differ from each other with regards to their τ (Chapter 3). We had evoked differential sensitivities of their clocks to light stimuli in the selected populations to explain this discrepancy. Subsequent studies revealed that the eclosion and activity/rest clocks of the *early* and *control* flies are indeed differentially sensitive to light (Chapter 4). The TIM protein has been thought to mediate light responses in the molecular clock of *D. melanogaster* (for review see Williams and Sehgal, 2001). This led to the proposal of the molecular basis of circadian entrainment to LD cycles (Hunter-Ensor et al, 1996), which suggests that phase shifts in circadian rhythms during early and late subjective nights are brought about by light induced

degradation of the TIM protein (Suri et al., 1998; reviewed in Williams and Sehgal, 2001). Therefore, it will be interesting to determine whether the *early* and *late* populations differ from their *controls* in terms of the rate of TIM degradation in response to light stimuli, particularly during the early and late subjective nights, when their clocks are maximally responsive to light.

It is a well-known fact that the association of TIM with PER proteins is necessary for the functioning of molecular clockwork in *Drosophila*. Therefore, appropriate adjustment in the TIM levels would ensure proper timekeeping by the clocks (Suri et al., 1998; Yang et al., 1998). Furthermore, effect of light on TIM proteins is closely associated with the photic and temperature regulated *per* splicing (Chen et al., 2006). It has been shown that the 3' untranslated region (UTR) of *per* mRNA undergoes alternate splicing, which acts as a molecular switch in the phase resetting processes of circadian rhythm (Majercak et al., 1999, 2004; Collins et al., 2004). These studies have shown that an elevated level of splicing (removal of an intron, *dmpi8* from the 3'UTR region of *per*) is coupled with advanced evening activity. Given that the *early* and *late* populations have evolved a morning and evening expression in their eclosion and activity/rest rhythms, it would be interesting to investigate if these populations employ differential splicing of *per*. In order to study the molecular correlates underlying *early* and *late* circadian phenotypes, I decided to analyze the (a) temporal expression of two core clock genes (*per* and *clk*) using real-time qRT-PCR, (b) TIM-degradation at different circadian phases in response to light stimuli, and (c) levels of *per* splicing in the selected and control populations.

7.2 Material and Methods

7.2 (a) Rearing and handling of populations -The *early*, *control* and *late* populations, used in this study have been described in details in Chapter 2. The populations were maintained in vials containing standard banana-jaggery medium, containing ~ 30 young (2 to 6 day old) adult flies and were placed in controlled environmental chambers at the indicated temperature (24 ± 1°C) and exposed to at least 7-8 cycles of 12:12 hr LD cycles (where Zeitgeber time 0 (ZT0) is defined as lights-on), and in some cases subsequently maintained in constant darkness (DD). White fluorescent light (~100 lx) was used during the light phase of the LD cycle, and the temperature did not vary by more than 0.5 °C between the light and dark periods. At specific ZTs under LD and CTs under DD, flies were collected and frozen.

7.2 (b) TIM degradation assay- Adult flies from the standardized selected and control populations were entrained under 12:12 hr LD cycles for 7-8 cycles and then transferred to DD. Brief light pulses of 15 min duration and ~1000 lux intensity were administered at CT14 and CT20 after completion of the first cycle in DD. Selected and control flies were frozen immediately after exposure to light pulse, along with unpulsed controls which were not presented with any light pulse. Flies were also frozen after an interval of 30 and 60 min in order to study temporal degradation patterns in these populations. Fly head extracts were obtained as follows; approximately 70-80 heads from the frozen flies were homogenized in the head extraction buffer (100 mM KCl, 20 mM HEPES, 5% glycerol, 10 mM EDTA, 0.1% TritonX100, 1 mM DTT, pH 7.5) with a handheld homogenizer (Wheaton). Extracts were clarified by centrifugation (13,000 × *g*, 15 min at 4°C), and the protein concentration was determined by Bradford method at 595 nm wavelengths using

UV-Visible spectrophotometer (Bio-Rad, USA). A total of 50 μg of protein from the clarified supernatant was loaded onto SDS-polyacrylamide gel electrophoresis gels and transferred to a nitrocellulose membrane. For Western blots, equivalent amounts of protein from whole head extracts were loaded. After being blocked in 2% bovine serum albumin in 1 X PBS, the blot was incubated with either a 1:2,000 dilution of rat anti-TIM antibody (provided by Amita Sehgal, Univ. of Pennsylvania Medical School, USA), a 1:5,000 dilution of mouse anti- β-ACT antibody (Invitrogen) in blocking solution at room temperature for overnight. Subsequently, the blot was washed three times for 10 min each in PBS and then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; B. Genei). The signal was visualized with Super Signal Chemiluminscent kit from Pierce, USA. To ensure equal loading in each lane, the blot was stripped in stripping buffer (62.5 mM Tris-HCl pH 7.6, 100 mM 2-mercaptoethanol, 5% SDS) at 55°C for 30 min, rinsed in PBS, blocked, and processed for detecting positive control proteins, β-ACT. The Western blots were quantified by Image Gauge (V4.0) software from Fuji film, and the relative optical density (ROD) of the protein of interest was then determined by background subtraction method.

7.2 (c) RNA isolation- Total RNA from approximately 15 fly heads was isolated using Trizol (Invitrogen Life technology, Carlsbad, CA, USA) following manufacturer's protocol. The heads were homogenized in 1 ml Trizol and 200 µl of chloroform was added. After mixing, the sample was centrifuged at 13 000 x *g* for 15 min (4°C). The upper aqueous phase was transferred to a tube containing an equal volume of isopropanol. Mixture was thoroughly vortexed and centrifuged at 13,000 x *g* for 10 min (4°C). Supernatant was discarded and the precipitated RNA pellets were washed twice

using 1 ml of 75% ethanol. RNA pellet was centrifuged at 13,000 x *g* for 5 min (RT). After discarding supernatant, pellet was allowed to air-dry for 10-15 min, then resuspended in DEPC-treated water. Total RNA was quantified by UV absorbance at 260 nm using Bio-Rad UV-Visible spectrophotometer.

7.2 (d) Reverse-transcription and real-time RT-PCR- The transcript levels of *per, Clk* gene along with a reference gene *elf1-α* were estimated in the selected and control populations by quantitative real time PCR carried out on 3 parallel replicate sets. All of the molecular studies on only one of the replicate populations except real-time RT-qPCR assays. The primers used in the RT-PCR amplification for *per* specific sequences were, PER (F5'CAGTTCAACTCGCTGGTCAA3') and (R5'TCTGTCTGGGCTCGATTACC3'), and for *clk* were (F5'AAGAGCACCTTCTGCGTGAT3'), and (R5'AACTTGGGGCTCTTCTGTGA3'), whereas the first strand cDNA synthesis was performed using 1 μg of total RNA with Superscript II (Invitrogen Lifetechnology, Carlsbad, CA, USA) under the recommended conditions using oligo $(dT)_{18}$ primer. As a control for sample-to-sample differences in total RNA, we also included primers for the noncycling mRNA encoding elongation factor alpha (ELF-α). We chose *elf-α* RNA as an internal control because the levels of *per* were not affected by *elf-α* mRNAs and were in a similar range in total head extracts, as inferred by the staining intensities following RT-PCR done in the exponential phase (Fig. 7.1). The primers used in the RT-PCR for amplification of *elf-α* sequences were ELF-α F (5' ACATTGCCTGCAAGTTTTCC 3') and ELF-α R (5' AGGACTTGCGGTGACGATAC 3'). This amplified region of *elf-α* PCR products, which was visualized by electrophoresis on 2% agarose gels followed by

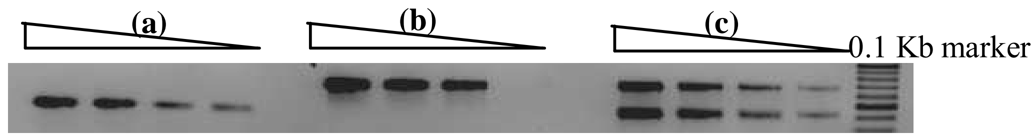

Fig. 7.1: Standardization of semi-quantitative RT-PCR method to estimate relative abundance of clock transcripts. Shown in (a) is PCR amplified product of *per* when 10-fold dilution of cDNA were taken, (b) similar dilution for *elf-*α mRNA levels were used for amplification and (c) co-amplification of the *per* and *elf-*α cDNA pools. It is quite evident that the noncycling positive control, in this case *elf-*α did not affect the amplification efficiency of gene of interest (clock genes) thus serving as suitable control.

staining with 1-D analysis (BioRad Co.), and the bands were quantified using 1-D analysis software. Amplification of cDNA was carried out in BioRad iCycler iQ Real-time detection system. Each sample consisted of: 1 µl of cDNA, 500 nM of primers, 12.5 µl of iQ SYBR Green Supermix (BioRad Laboratories), in a reaction volume of 20 µl. The qPCR results were analyzed by the $2^{-\Delta\Delta CT}$ method as described by Pierson et al (2003).

Amplification conditions

Cycle 1: (1X)

Cycle 1: (1X)			
	Step 1:	95.0ºC	for 02:00
Cycle 2: (40X)			
	Step 1:	94.0ºC	for 01:00
	Step 2:	55.0ºC	for 00:40
	Data collection and real-time analysis enabled.		
	Step 3:	72.0ºC	for 00:40
Cycle 3: (40X)			
	Step 1:	55.0ºC	for 00:20

7.2 (e) RT-PCR splicing assay- The relative levels of the spliced and unspliced *per* RNA variants were quantified using a reverse transcriptase PCR (RT-PCR) assay based method previously described in Majercak et al (1999). At each time point, total RNA was extracted from 15-20 fly heads using TriZol based extraction method (Invitrogen). Approximately 1 µg of total RNA was incubated in a final volume of 20 µl, and reverse transcription was performed using ThermoScript RT-PCR kit from Ambion following manufacturer recommended procedure with oligo $(dT)_{18}$ as a primer. A 2-µl aliquot of the reaction mixture was further processed by PCR in a final volume of 50 µl using the *per*-

specific primers P7197 (5' TCTACATTATCCTCGGCTTGC 3') and P6869 (5' TAGTAGCCACACCCGCAGT 3'). The amplification conditions were as following:

Amplification conditions

Cycle 1: (1X)

Step 1:	94.0ºC	for 03:00

Cycle 2: (3X)

Step 1:	94.0ºC	for 00:45
Step 2:	50.0ºC	for 00:50
Step 3:	72.0ºC	for 00:50

Cycle 3: (30X)

Step 1:	94.0ºC	for 00:40
Step 2:	53.0ºC	for 00:50
Step 3:	72.0ºC	for 00:50

Cycle 3: (1X)

Step 1:	72.0ºC	for 10:00

This amplified a region of the 3' UTR of *per* from bp 6869 to 7197 (numbered according to Citri et al., 1987). The ratio of two *per* band intensities for each population was analyzed by 1-D analysis software (BioRad Co. USA). Two *per*-specific bands of expected size for PCR products were detected in total RNA that contained spliced (240 bp) and unspliced (329 bp) forms of *per* transcripts (Fig. 7.5 a).

We performed several control experiments to ensure the accuracy of our results. To verify that our assays were restricted to the exponential phase of PCR, we collected flies at different times during a daily LD cycle and after mRNA isolation and cDNA synthesis, the aliquots were used for PCR ranging in cycle lengths between 20 and 30. Under the conditions used, all cycle lengths resulted in curves for *per* RNA levels that

had indistinguishable amplitudes, peak times, and overall shapes as a function of time in a daily cycle (data not shown). In addition, at least 2-3 replicates of cDNA synthesis reactions were performed and were subsequently used for PCRs as additional controls. No *per*-specific amplicons was detected when the RT-PCR was performed in the absence of RTase. We also optimized our experimental conditions by using several primers, incubation temperatures, RTases and amounts of total RNA and cDNA.

7.2 (f) Statistical analyses

The relative mRNA levels (normalized by *elf*-α mRNA levels) of the *per* and *clk* genes obtained from real-time qRT-PCR method were subjected to ANOVA treating replicate population as random factor, and population and phase of mRNA estimation as fixed factors (Statsoft Inc., 1995). Multiple comparisons based on 95% confidence intervals (95%CI) around the mean were used as error bars to facilitate visual hypothesis testing. Overlapping error bars imply values that do not differ significantly. The block means were used for data analysis, hence their interaction with other factors cannot be tested for significance and are not shown in the ANOVA table. The relative levels of TIM proteins and splice/unspliced ratio of *per* 3'UTR from separate assays were subjected to separate mixed model analysis of variance (ANOVA), by treating population and phase as fixed factors. Since TIM degradation and splicing assays were done on only one replicate population, the variations obtained were mainly from the replicate sets of experiments within and among the selected and control populations. In such cases the post-hoc multiple comparisons were performed using Tukey's test (Statsoft Inc., 1995).

7.3 Results

7.3 (a) Transcriptional profiles of *per* and *clk* genes

As a starting point for identifying molecular expressions underlying the morning and evening circadian phenotypes of the *early* and *late* populations (Chapter 3 and 4), we estimated the levels of *per* and *clk* transcripts from fly heads at different times of the day in the selected and control populations using real time quantitative PCR (RT-qPCR).

The levels of *per* transcript obtained from flies harvested at every 4 hr intervals, over a period of 24 hr, show a distinct rhythmic pattern for each stock (Table 7.1 a; p = 0.002 for phase). Maximum level of *per* occurs at ZT14 in the *early* and *control* flies, and at ~ZT20 in the *late* flies (Fig. 7.2). The timing of peak in *per* transcript differs significantly between the *early* and *late* populations (Table 7.1 a; Fig. 7.2). However, those between *early* and *control* populations did not differ significantly (Table 7.1a; Fig. 7.2). On the other hand, although the *clk* mRNA display rhythmic expression patterns, the oscillation did not reach statistical levels of significance (Table 7.1 b; $p>$ 0.056 for phase). The *clk* mRNA peaks at ZT22 and ZT18 in the *early* and *control* flies respectively, while it peaks in the *late* flies at ZT2 (Fig. 7.3). Lack of significant interaction between phase and population suggests that selected and control populations do not differ from each other in terms of *clk* mRNA levels, though the phase difference (~ 4 hr) in the peak of *clk* mRNA levels between the *early* and *late* populations does follow a similar pattern like that of *per* expression (Table 7.1 b).

7.3 (b) TIM degradation pattern in response to light pulse

TIM protein levels from head lysates were estimated by western blotting method (Huntor-Ensor et al., 1996). The effects of population, phase, and their interactions on TIM degradation were tested using ANOVA (Table 7.1 c). Light treatment has an acute effect on the TIM levels in all three populations; TIM levels were reduced invariably

Table 7.1: Results of analysis of variance (ANOVA) obtained from different sets of experiments on the selected and control populations.

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p*-level
(a) *per* mRNA						
Population (P)	2	427.15	6	151.267	2.824	0.137
Phase (Ph)	5	1582.602	15	244.654	6.469	**0.002**
Block (B)	3	1531.379	0	0	--	--
P x Ph	10	1159.604	30	235.279	4.929	**> 0.001**
P x B	6	151.267	0	0	--	--
Ph x B	15	244.654	0	0	--	--
P x Ph x B	30	235.279	0	0	--	--
(b) *clk* mRNA						
Population (P)	2	47.076	6	29.41	1.601	0.277
Phase (Ph)	5	200.877	15	71.91	2.794	0.056
Block (B)	3	150.477	0	0	--	--
P x Ph	10	53.467	30	36.696	1.457	0.204
P x B	6	29.41	0	0	--	--
Ph x B	15	71.91	0	0	--	--
P x Ph x B	30	36.696	0	0	--	--
(c) TIM levels						
Population (P)	2	0.126	50	0.051	2.455	0.096
Phase (Ph)	1	0.001	50	0.051	0.011	0.916
P x Ph	2	0.268	50	0.051	5.231	**0.009**
(d) *per* splicing levels						
Population (P)	2	0.063	80	0.025	2.532	0.086
Phase (Ph)	5	0.07	80	0.025	2.809	**0.022**
P x Ph	10	0.057	80	0.025	2.269	**0.021**

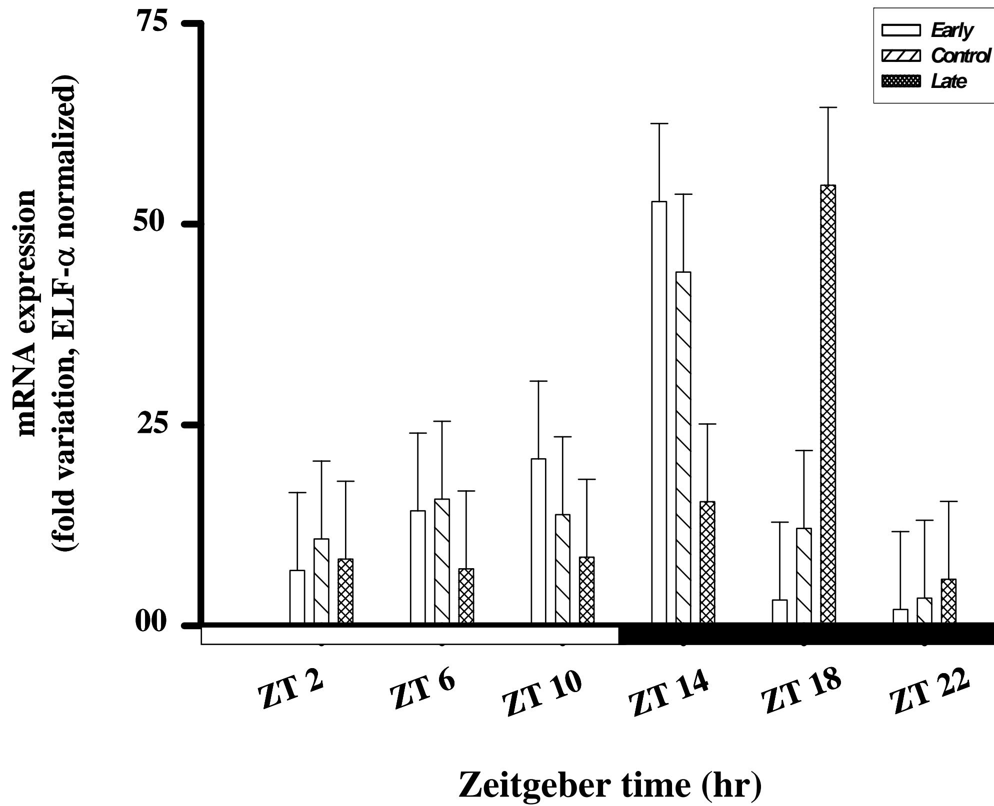

Fig. 7.2: Relative *per* mRNA levels under light/dark cycles of 12:12 hr at different phases for the selected and control populations. The relative abundance of *per* transcripts were estimated by $2^{-\Delta\Delta CT}$ method. The error bars denote the 95% confidence interval (95% CI) around the mean for visual hypothesis testing. At least 3-4 independent parallel sets of qPCR runs were performed for all three populations.

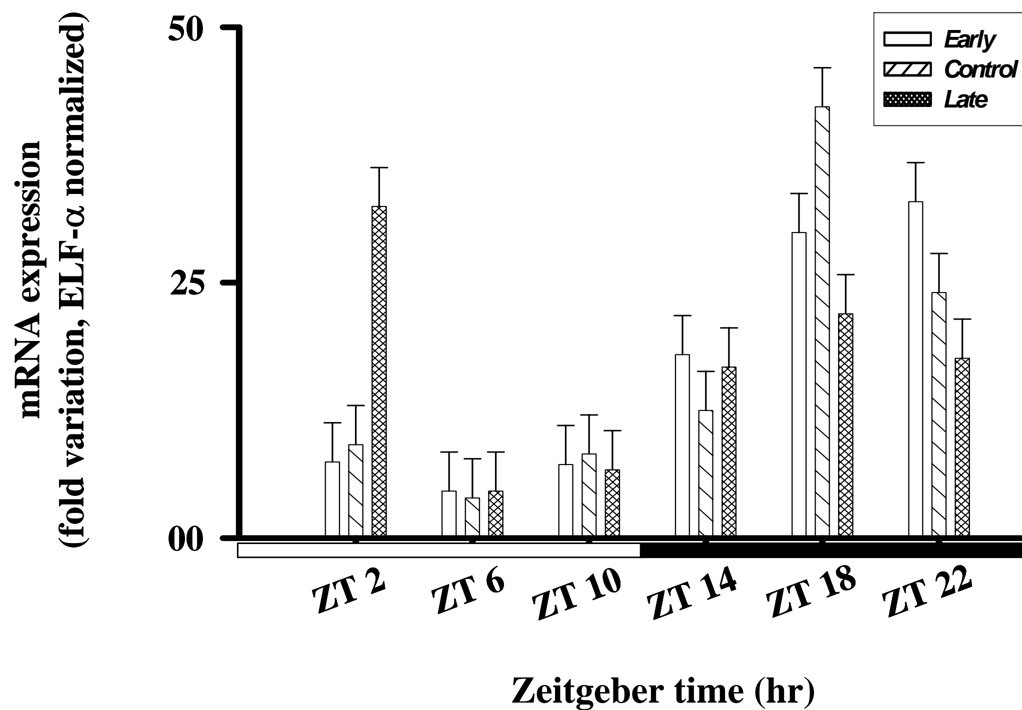

Fig. 7.3: Relative *clk* mRNA levels under light/dark cycles of 12:12 hr at different phases for the selected and control populations. The relative abundance of *per* transcripts were estimated by $2^{-\Delta\Delta CT}$ method. The error bars denote the 95% confidence interval (95% CI) around the mean for visual hypothesis testing. At least 3-4 independent parallel sets of qPCR runs were performed for all three populations.

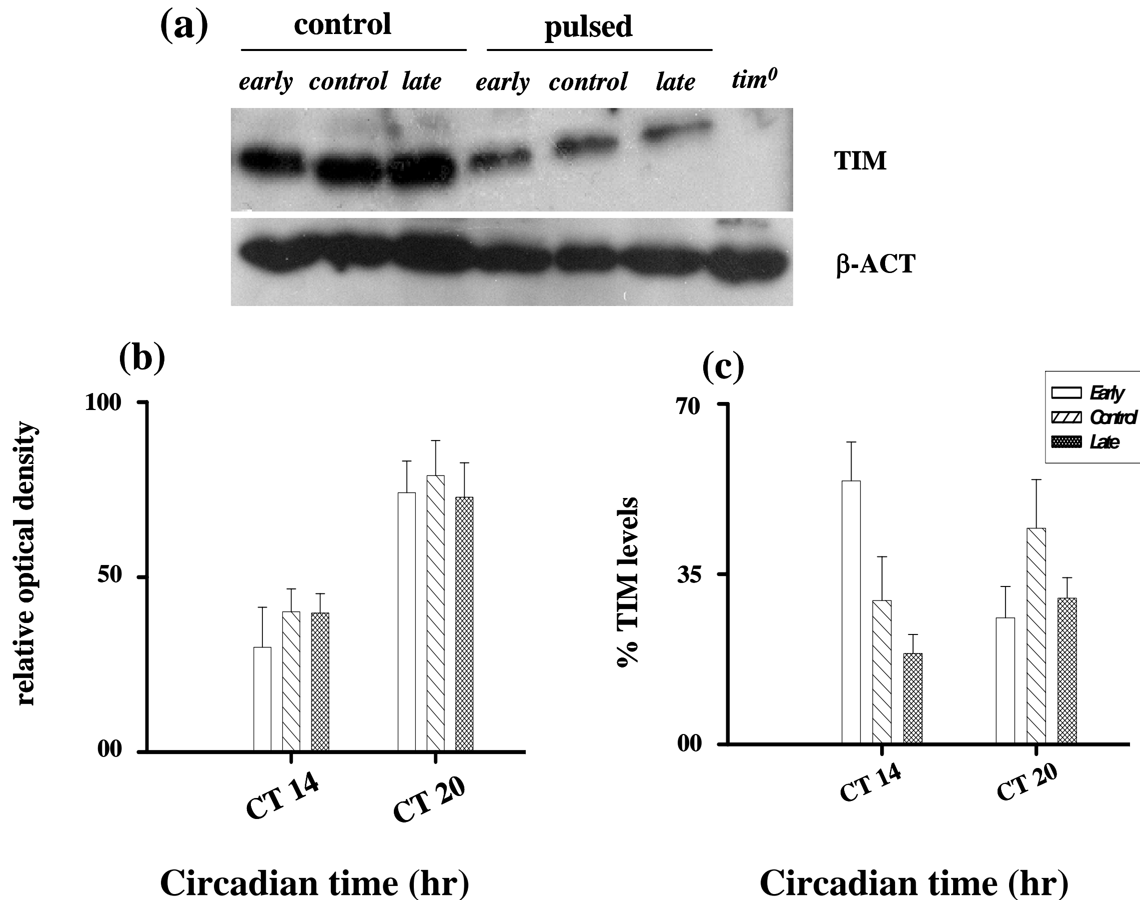

Fig. 7.4: Western analysis of selected and control populations. (**a**) Total head extracts were prepared from selected and control populations collected at CT20 and probed using anti-TIM to reveal TIM levels. Lower panel shows β- ACT levels used as loading control in the experiment. (**b**) Comparisons of TIM levels in the unpulsed controls from the selected and control populations, though the levels of TIM showed circadian fluctuations, they did not differ significantly among three populations (**c**) comparisons of remaining TIM present in the selected and control populations. Flies were pulsed for 15 min. with ~1000 lux light intensity and kept in dark over 60 min to see temporal degradation patterns. Extract were run on western blot, and TIM levels were quantitated using densitometry (see section 7.2 (B) for details. To generate the data, the amount of TIM in nonpulsed lane was set as 100%; and this sample was taken as 0% disappearance for each of the population separately. tim^0 flies used to test the specificity of anti-TIM antiserum. The *early* and *late* populations differed from each other significantly as well as from *control* populations (see ANOVA Table 1 d) as revealed by multiple comparisons using Tukey's test.

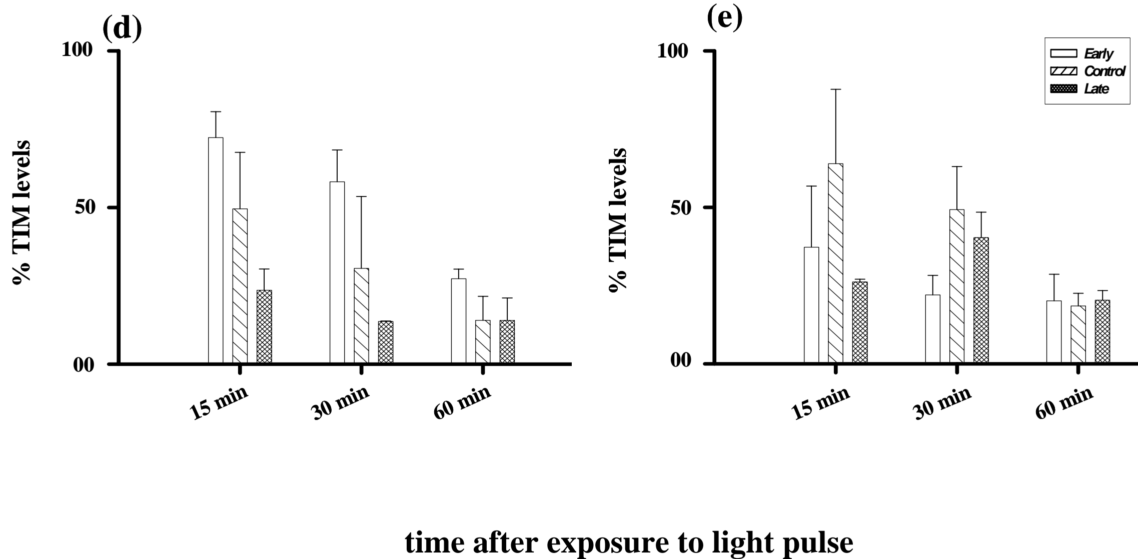

Fig. 7.4 (d) Temporal pattern of TIM degradation in the selected and control populations at CT14. Flies were pulsed for 15 min. with ~1000 lux light intensity and allowed to recover TIM levels at indicated times. (e) Comparisons of remaining TIM present in the selected and control populations CT20 after indicated time intervals. The error bars denote standard error of means (SEM) around the mean. Each experiment was replicated at least 5-6 times to generate data.

following light pulse exposure (Fig. 7.4 a). Under unpulsed control conditions, the levels of TIM were lower at CT14 and higher at CT20, indicating circadian fluctuations in the protein level (Fig. 7.4 b). Although the levels of TIM in the three populations under unpulsed conditions are similar, it significantly differs among the selected and control populations following light exposures during the early and late subjective nights (Fig. 7.4 c). Following light exposure at CT14 the *late* flies suffer greater reduction in TIM levels compared to the *control* and *early* flies, while the reduction in the *early* flies was smaller compared to the *control* flies. On the other hand, following light pulse treatment at CT20, a significantly higher reduction in TIM levels is seen in the *early* flies compared to the *controls* (Fig. 7.4 c). However, at this phase the *late* flies also have reduced levels of TIM protein compared to the *controls* (Fig. 7.4 c). In the TIM degradation assay in response to light, we estimated time dependent degradation of TIM at CT14 and CT20, after 15, 30 and 60 min following light pulse administration. Levels of TIM were significantly reduced after 15 min and continued to decline in the course of next one hour in all three populations (Fig. 7.5 d, e).

7.4 (c) *per* splicing under LD cycles

We assayed the ratio of spliced to unspliced forms of *per* mRNA in the selected and the control flies at various phases under 12:12 hr LD cycles. The level of spliced form of *per* transcript is lower during the light phase of LD cycles (Figure 7.5 a-c), whereas the levels are higher throughout the dark phase (Fig. 7.5 b). This suggests that the spliced form of *per* transcript is suppressed by light and induced by darkness. Interestingly, a significant phase dependent difference is seen among the *early* and *late* populations in terms of the

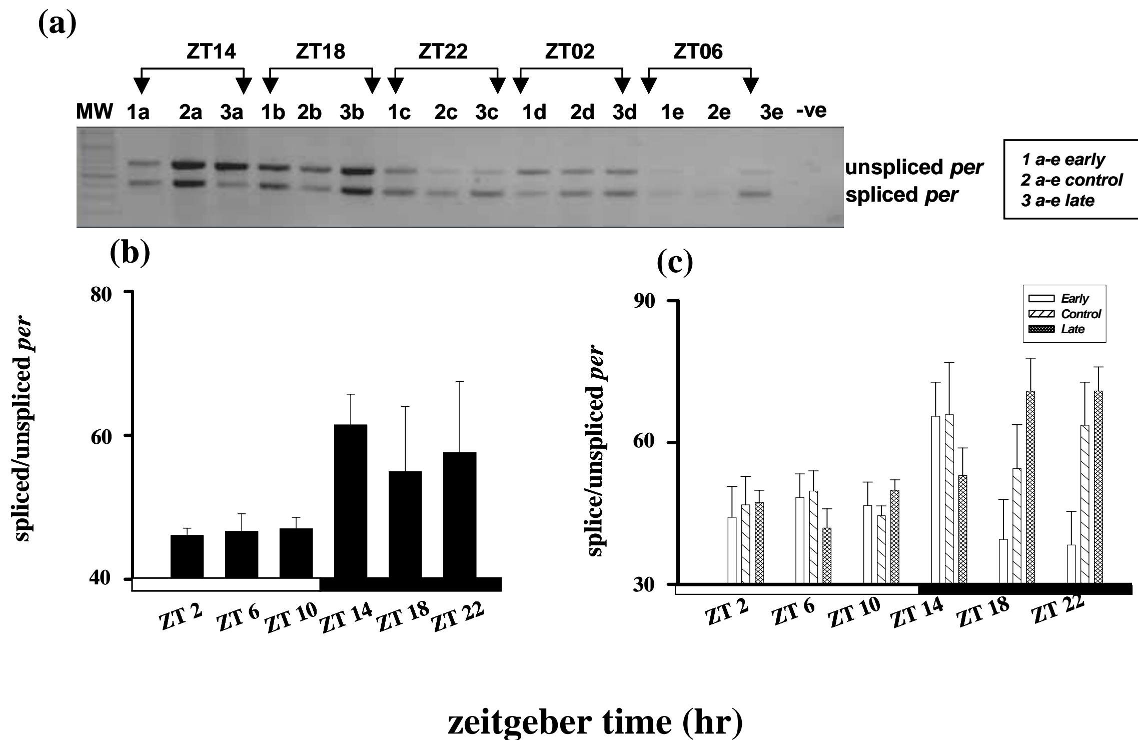

Fig. 7.5: **(a)** Representative agarose gel showing the *per* (spliced and unspliced forms) RT-PCR products from the selected and control populations at different times during LD 12:12 hr conditions. Different phases of LD are indicated at the top of the panel, -ve denotes the absence of RT; and MW denotes 0.1 Kb marker **(b)** overall levels of spliced to unspliced forms (pooled across all three populations) under LD cycles at six different phases. During light phase the levels were found to be suppressed but elevated after lights-off. **(c)** comparisons of spliced/unspliced ratio of *per* mRNA among the selected and control populations. Throughout the light phase the levels remain low and there is no significant differences between three populations, however during darkness the levels of spliced forms are elevated. The e*arly* and *control* flies displayed a peak of splicing at ZT14 whereas this peak occurred between ZT18-22 in case of the *late* flies. The error bars denote standard error of means (SEM) around the mean. Each experiment was replicated at least 5-6 times to generate data. The error bars denote standard error of means (SEM) around the mean. White horizontal bars denote light phase whereas the dark bars, dark phase of LD cycle.

peak in splicing (Fig. 7.5 c; Table 7.1 d). The peak of splicing events occurred at ZT14 in the *early* and *control* flies, which is 4-8 hr earlier than *late* flies (Fig. 7.5 c). However, *early* and *control* flies do not differ significantly from each other at any of the phase of LD cycles (Fig. 7.5 c).

7.4 Discussion

We have shown that selection on timing of adult emergence yields *early* and *late* populations, whose time course and waveform of adult emergence diverge from the *controls* (Chapter 3). As a consequence clocks governing eclosion and activity/rest rhythms also diverge from those of the *controls*, and the *early* populations exhibit morning circadian phenotype and *late* populations display evening phenotype. The results of studies on the transcripts suggest that the peaks of *per* and *clk* mRNA have also diverged in concordance with adult emergence and activity rhythms. Compared to the *controls* and *early* populations, the peaks of both the transcripts are delayed by about 4-8 hr in the *late* populations. This suggests that molecular clocks underlying circadian behaviour undergo correlated changes in response to selection on timing of emergence. These results could serve as a starting point for studies aimed at investigating the putative mechanisms and candidate clock genes that regulate morning and evening behaviours in *D. melanogaster*.

Consistent with earlier reports (Myers et al., 1996; Zeng et al., 1996), the overall TIM levels in the unpulsed experimental controls, followed a circadian pattern, with higher levels at CT20 and lower at CT14 (Fig. 7.4 b). However, the TIM levels did not differ among the *early*, *control* and *late* populations (Fig. 7.4 b). In contrast, the light dependent TIM degradation studies on these populations yielded quite intriguing results.

Following light pulse exposure at CT14, TIM level in the *early* populations is reduced to ~ 60% compared to their unpulsed experimental controls, and is about twice as much as the *control* populations. At CT20, TIM level is reduced to ~ 40% compared to unpulsed experimental controls, and is about half as much as the *control* populations (Fig. 7.4 c). On the other hand, following light pulse exposure at CT14, TIM level in the *late* flies is reduced to ~ 20% compared to unpulsed experimental controls, and is about one third of the *control* populations. At CT20 TIM is found to be reduced to ~ 30% of the unpulsed experimental controls, and is substantially lesser than the *control* populations (Fig. 7.4 c). The results of time dependent TIM degradation assay done after 15, 30 and 60 min of the exposure to light pulse demonstrates that the rates of TIM degradation in the selected and control populations are different (Fig. 7.4 d and e). As can be seen, the results of TIM degradation assay does not entirely explain the behavioural phase shift data of the selected and control populations (discussed in Chapter 4). We speculate that this may be due to the fact that CRY may be playing a greater role in light responses in the *late* populations compared to *early* and *control* populations. However, the role of CRY is yet to be ascertained in the *early*, *control* and *late* populations.

Studies on the differential splicing in the 3' UTR of *per* gene demonstrate that the selected and control populations have differential splicing mechanisms. Similar to earlier reports (Collins et al., 2004; Majercak et al., 2004), we found that *per* splicing levels are reduced during the light phase of the LD cycle and starts rising gradually during the dusk. The levels remain high throughout the night (Fig. 7.5 a-c), suggesting that light represses splicing in *per* transcription. Previous studies on *per* splicing were based on *glass* (gl^{60j}) and $norpA^{p41}$mutants of *Drosophila*, which show higher levels of *per* splicing under LD

as well as DD conditions, implicating defects in light input pathways (Collins et al., 2004; Majercak et al., 2004). In our study, splicing profile of the *early* and *late* flies were strikingly different particularly during the dark phase of the LD cycle, *early* flies exhibit reduced levels of splicing forms compared to the *controls*, whereas *late* flies display higher levels of spliced forms than the *controls* (Fig. 7.5 c). This confirms our view that different pathways in the molecular clockwork might be the direct/indirect targets of selection on timing of adult emergence in *D. melanogaster*.

Further, in our opinion advanced peak of emergence and activity rhythm in the *early* flies reflect a preference for light, while the delayed peak of emergence and activity rhythms in the *late* flies reflect a preference for darkness. This is also corroborated by the fact that the *early* flies were more active in the morning and less in the evening compared to the *controls*, while the *late* flies were more active in the evening and less in the morning compared to the *controls* (Chapter 3). This is also consistent with the temporal pattern of spliced to unspliced ratios in the *early* and *late* populations (Fig. 7.5 c). Although, the selected and control populations do not differ from each other during the light phase of LD cycle, during darkness *early* and *control* flies show an earlier peak in splicing events (ZT14), while in the *late* flies it occurs at much later phase (between ZT18-22) (Fig. 7.5 c). Given that tightly orchestrated molecular pathways underlie *Drosophila* circadian clock, it is quite possible that the difference in *per* splicing is also affected by altered oscillatory mechanism ranging from transcription to protein phosphorylation to rhythmic behaviors among the selected and control populations. We propose that expression of core clock genes, light dependent TIM degradation, and

differential *per* splicing during the dark phase of the LD cycles play crucial roles in the regulation of morning and evening behaviors in *D. melanogaster*.

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List of publications

1. Shailesh Kumar, Ambika Mohan and V.K.Sharma. Circadian dysfunction reduces life span in *Drosophila melanogaster*. Chronobiol Int 2005; 22:641-653.

2. Shailesh Kumar and V. K. Sharma. Entrainment properties of the locomotor activity rhythm of *Drosophila melanogaster* under different photoperiodic regimes. Biol Rhythm Res 2004; 35:377-388.

3. Shailesh Kumar, Dhanya Kumar, Dhanashree A. Paranjpe, C. R. Akarsh and Vijay Kumar Sharma. Selection on the timing of adult emergence results in altered circadian clocks in fruit flies *Drosophila melanogaster.* (unpublished manuscript).

4. Shailesh Kumar, Dhanya Kumar, Harish V S, Divya S, and Vijay Kumar Sharma. Evaluating the morning and evening oscillator model using *Drosophila melanogaster* populations selected for early and late adult emergence. (unpublished manuscript).

5. Shailesh Kumar, Koustubh M Vaze, Dhanya Kumar, and Vijay Kumar Sharma. Selection for early and late adult emergence alters the duration of pre-adult development in *Drosophila melanogaster.* (manuscript submitted).