Behavioral and Genetic Analyses of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence

Thesis

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By

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THESIS DECLARATION

I declare that the work presented here in my thesis titled "Behavioral and Genetic Analyses of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence" is to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university. The results presented here are due to investigations carried out by me in Evolutionary and Organismal Biology Unit under the supervision of Professor Vijay Kumar Sharma.

References made to work of other researchers have been duly acknowledged. I understand that though the list of references is long, it is not exhaustive. Any omission made is not deliberate but as a consequence of misestimation.

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CERTIFICATE

This is to certify that the work described in the thesis entitiled "Behavioral and Genetic Analyses of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence" is the result of investigations carried out by Mr. Koustubh M. Vaze in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

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Synopsis

Circadian clocks are believed to confer advantage to living beings by scheduling their biological functions at most appropriate time of the day and/or through precise coordination of various interdependent metabolic processes. Although, the advantages of circadian rhythms under rhythmic environment on earth are quite apparent, available evidence is not strong enough to conclude unequivocally that circadian clocks are adaptive. Much of the evidence is primarily of circumstantial nature and merely suggests that circadian clocks may have evolved as an adaptation to natural periodic selection pressure on the earth. None of the studies conducted so far have provided any clear evidence for the evolution of circadian clocks in response to rhythmic selection pressure. Available evidence is also not very useful in deciphering mechanisms by which circadian clocks may confer adaptive advantage. Experimental evolution is a powerful tool on both these counts. By using experimental evolution one can follow adaptive evolution in action and the product of evolution (evolved populations) are available for testing the functional significance and molecular-genetic analyses of rhythmic traits. We therefore chose to investigate evolution of circadian clocks in fruit fly *Drosophila melanogaster* by using the strategy of long-term experimental evolution under laboratory conditions.

Many insects including fruit flies *D. melanogaster* exhibit diurnal rhythm in adult emergence. These flies exhibit rhythm in emergence under laboratory 12:12 hr light/dark (LD) cycles wherein adults primarily emerge during the light phase with a peak immediately after dark-to-light transition, followed by a gradual reduction leading to a zone of no emergence by lights-off. It is now well established that circadian rhythm of emergence in *D. melanogaster* is under the control of self-sustained, endogenous circadian oscillators. In a long-term selection study we created *early* and *late* stocks of fruit flies *D. melanogaster* showing preference for different timing of emergence by imposing selection for morning and

evening emergence under 12:12 hr LD cycles. Some of the salient findings of my studies discussed in my thesis are briefly described below.

***Evolution of emergence waveforms*:** With increasing generations of selection, *early* and *late* stocks showed gradual increase in the incidence of morning and evening emergence, respectively. By generation 150, morning emergence in *early* populations was more than twice (~62%) of *control* populations (~30%), while evening emergence of *late* populations was one and half times (~35%) greater than *control* populations (~20%). Increased emergence during selection windows was found to be a result of change in the phase and the time course of emergence waveform. *Early* populations start and end their emergence earlier than *control* populations every day, whereas *late* populations start emergence and stop long after *controls*. Such divergently evolved emergence waveforms (EEW) explain the enhanced morning and evening emergence in *early* and *late* populations.

***Circadian clocks (τ and PRC)*:** Evolution of divergent emergence waveforms in *early* and *late* stocks was also accompanied by divergence of their circadian clocks. Studies aimed at assessing the role of circadian clocks in determining the preference for morning and evening emergence in *early* and *late* stocks revealed that *early* stocks evolved emergence rhythms with shorter circadian period (τ) and photic phase response curve (PRC) with larger phase-advances and smaller delays than *control* stocks. On the other hand, *late* stocks evolved emergence rhythm with τ longer than *controls* and PRC with large phase-delays and smaller advances. Correlated changes in circadian clocks of *early* and *late* flies suggest that divergent clocks may underlie divergent adult emergence behavior.

***Skeleton photoperiod*:** Correlation between emergence phenotypes and clock properties in *early* and *late* stocks suggest that differences in entrainment of their circadian oscillators could be the source of their distinct adult emergence waveforms. Diverged τ partly explains their divergent emergence waveforms as the entrained phase (ψ) of emergence waveforms

and hence the entrained phases of the underlying oscillators are consistent with the known relationship between ψ and τ. Light pulse PRC provides a map of phase dependent effects of brief light pulses on the circadian oscillators but the effect of light of longer durations such as those used during entrainment is not readily deducible from such predictions made based on effects of brief light pulses. This suggests that divergent photic PRC may not be able to readily explain the differential entrainment of *early* and *late* emergence rhythms. Therefore, to probe the functional significance of divergent PRC, we decided to test if there is a correlation between PRC shape and photosensitivity schedule of the circadian oscillators under LD cycles. However, no standard procedure is available to study the photosensitivity of circadian oscillators under entrained state. We took a simple approach to assess the photosensitivity schedules of *early* and *late* flies, in which emergence of each population was studied under three types of skeleton light regimes. Skeleton photoperiod regime(s) (SPP) in which emergence waveform of a population mimicked its EEW was considered as light requirement schedule (LRS) sufficient for optimal entrainment. If emergence waveform of a population mimics its EEW under a given SPP regime, it suggests that only those portions of complete photoperiod are essential for optimal entrainment during LD and that particular light regime can be considered as LRS for that population. Therefore, populations differing in their LRS imply differences in the photosensitivity schedules of the underlying circadian oscillators. Two diametrically opposite SPP represented LRS for *early* and *late* populations, which indicate that photosensitivity schedules of *early* and *late* flies differ from each other, suggesting the functional significance of PRC to their characteristic emergence phenotypes.

***Adult emergence under natural conditions*:** The *early* and *late* flies evolved divergent emergence waveforms in response to selection for morning and evening emergence under laboratory conditions where the only time-cue present was in the form of presence and absence of light of moderate intensity. It is not known however, if such differences in

emergence would persist under natural conditions where multiple zeitgebers are present in the strongest form. Under natural conditions, the phase of emergence waveform relative to lights-on transition, in all three stocks, was advanced compared to that under laboratory conditions. Furthermore, *early* and *late* flies not only continued to exhibit divergence in their emergence waveforms but their differences became even more clear, which indicates robustness of circadian architecture underlying circadian phenotypes of *early* and *late* stocks. Change in the phase of rhythm in response to altered zeitgeber properties (strength) is typical of circadian rhythms and thus these results strongly suggest that divergent emergence waveforms of *early* and *late* flies stems from difference in the circadian regulation of emergence.

***Genetic analysis of early and late phenotypes*:** To understand the genetic architecture underlying divergence in the circadian phenotypes of *early* and *late* flies, we set sixteen types of crosses the stocks and their F1 offspring and examined emergence under LD and τ of activity rhythms in the progeny. The genetic analysis revealed that genetic basis of divergence in the circadian phenotypes of *early* and *late* stocks is primarily autosomal. Line cross analysis revealed that complex genetic architecture comprising dominance and epistasis underlie the divergent circadian phenotypes of *early* and *late* flies. While difference in τ between *early* and *late* flies could be explainable by additive effects alone, dominance and epistatic effects were necessary for explaining differences in their emergence phenotype. We found that genetic architecture underlying divergence of circadian phenotypes differed among replicate selected populations, which suggest that separate genetic mechanisms may have given rise to similar phenotypic divergence.

Evolution of circadian clocks in *early* and *late* populations of fruit fly *D. melanogaster* in response to selection for morning and evening emergence provide a strong evidence for the notion that circadian clocks evolve under the influence of rhythmic

environmental challenges. Evidence pointing towards causal involvement of circadian period and photic phase response curves in preference for morning and evening adult emergence in *early* and *late* populations provide a glimpse of the functional significance of these clock properties during evolutionary fine-tuning of circadian timing systems by periodic natural selection pressures.

Chapter 1

Background

Circadian rhythms

Almost all living organisms on the earth perceive robust 24-hr cycles of abiotic variables such as light, humidity and temperature, which occur as an inevitable consequence of unceasing rotation of the earth around its axis. Unicellular organisms such as bacteria to complex living systems such as human beings exhibit 24-hr rhythms in various behavioral and physiological processes. These rhythms persist under constant laboratory conditions with near 24-hr periodicity (hence *circadian*; *circa* - about, *dies* - day), which indicates that daily rhythms are not simply passive responses to 24-hr environmental cycles, but are the expression of some endogenous rhythm-generating systems (Dunlap et al., 2004). Scheduling of biological functions at specific time of the day is believed to be the primary function of these endogenous oscillators (Roenneberg et al., 2003a), which they achieve by using various environmental time cues such as light, temperature, social cues through a process known as entrainment (Johnson et al., 2003). The system comprising of core endogenous oscillators, mechanisms to sense environmental time cues (zeitgebers) and transduction mechanisms by which the oscillators regulate circadian rhythms are collectively known as "circadian clocks".

Theories of adaptive advantages of circadian clocks

Circadian clocks are believed to be an evolutionary adaptation to the rhythmic challenge posed by 24-hr environmental cycles on the earth. It is generally believed that circadian clocks confer advantage to living beings by (1) scheduling their biological functions to most favourable time of the day (extrinsic advantage) and/or (2) coordinating interdependent metabolic processes in the internal milieu, thereby ensuring physiological well being (intrinsic advantage). Thus, the extrinsic advantage expected to be gained by adopting

favourable temporal niche and the intrinsic advantage obtained by precise scheduling of physiological processes are probably the principle ways by which living organisms gain adaptive advantage by having circadian clocks (Sharma, 2003). Theory of adaptive significance of circadian clocks has been in vogue since early days of circadian rhythm research and is a popular belief primarily because of perceived clear functional advantage of near 24-hr endogenous rhythms in periodic environmental challenges on the earth (Sharma and Joshi, 2002). Motivated by such perception, till date several studies have reported evidence supporting the notion of adaptive significance of circadian clocks.

Evidence for adaptive significance of circadian rhythms

***Circadian clocks under non-24-hr environments*:** One of the most commonly used approaches to test the adaptive advantages of circadian clocks is based on the observation that the period (τ) of endogenous circadian rhythms is close to the geophysical periodicity (24-hr). This leads to a hypothesis that organisms would perform better when subjected to cyclic environments whose periodicity matches closely to its own, a phenomenon which is commonly known as "circadian resonance" (Pittendrigh and Minis, 1972). As a corollary to this, it is believed that to start with, there were rhythms with a range of endogenous periodicities and over the course of evolution, the ones that matched periodicity of geophysical cycles were selected and hence are observed more prominently in natural populations today (Pittendrigh, 1993; Sharma, 2003; Paranjpe and Sharma, 2005).

Pittendrigh (Pittendrigh and Minis, 1972) and others (von Saint Paul and Aschoff, 1978) tested the idea of circadian resonance for the first time in insects. In a study on fruit fly *Drosophila melanogaster*, lifespan of adult flies maintained under light/dark (LD) cycles of different periodicities showed that longevity was higher under LD 12:12 compared to non 24-hr LD cycles (LD 10.5:10.5 and LD 13.5:13.5) and constant light (LL) (Pittendrigh and

Minis, 1972). In a study on blow flies *Phornia terraenovae,* longevity of flies maintained under LL and LD cycles of periodicities ranging from 20 to 28-hr was assayed (von Saint Paul and Aschoff, 1978). Percentage longevity of flies maintained under 25 and 26-hr LD cycles was found to be equal to that seen under 24-hr LD, but reduced to 70%, 90% and 85%, under LD cycles with periodicities of 20-hr, 23-hr and 28-hr, respectively. Flies maintained under LL had the lowest longevity (between 52 and 75%), implying LL as being deleterious for physiological well being. Pittendrigh and Minis (1972) attributed the reduction in longevity under non-24-hr LD cycles to altered phase-relationship (ψ) between the endogenous rhythms and LD cycles, while von Saint Paul and Aschoff (1978) attributed it to internal desynchronization between constituent oscillatory processes. Irrespective of the cause, the above studies suggest that organisms with near 24-hr rhythms accrue fitness advantage only under a 24-hr (12:12 hr) environmental cycles. However, reduced lifespan under non 24-hr LD cycles cannot be solely attributed to altered phase-relationship or to lack of entrainment to short or long LD cycles, because deleterious effects of light responsive processes in unusual LD cycles cannot be ruled out.

While adaptive advantages of circadian clocks have been usually studied by comparing the lifespan of wild type organisms (with period of 24-hr) under 24-hr and non-24-hr LD cycles, ideally one should study this by estimating lifespan of wild type and clock mutants (with non-24-hr periods) under 24-hr and non-24-hr LD cycles. In one such study in fruit fly *D. melanogaster*, lifespan of wild type (~24-hr), short (~19-hr) and long (~28-hr) *period* (*per*) mutants, was assayed under LD 8:8 and LD 12:12 (Klarsfeld and Rouyer, 1998).

Assessment of the effect of genotype and LD on lifespan showed a significant effect of genotype but not of the LD, suggesting a contribution of *per* mutation on the lifespan. A statistically significant interaction between genotype and light regime was expected, if

circadian resonance was valid. However, analysis revealed that the effect of genotype × LD interaction on lifespan was statistically significant. However, analysis of variance (ANOVA) on pair-wise differences of mean lifespan between the genotypes showed significant interaction for the per^{+}-per^{T} pair. Analysis of difference in percent survival of genotype pairs at different ages showed significant effect of LD regime and age for the per^{+}-per^{T} pair. Magnitude of survival difference for the per^{+}-per^{T} pair at majority of ages was lesser under LD 8:8 than LD 12:12, suggesting that LD 8:8 is less deleterious for per^{T} flies compared to LD 12:12, thus in a manner supporting the notion of circadian resonance. Though these results support the circadian resonance hypothesis, results on per^{L} flies failed to do the same. Also, longevity of female flies of all three genotypes (per^{T}, per^{+} and per^{L}) was comparable under LD 12:12, further making the case for circadian resonance weaker. These drawbacks make it difficult to draw any firm conclusion from this study.

Another study, which used a similar approach, tested circadian resonance in the plant *Arabidopsis thaliana* (Dodd et al., 2005). In this study wild type strains *col*–o (with τ of 24-hr) and period mutant strains *ztl*-l (with τ ranging from 27.1 to 32.5-hr) and *toc1*-1 (with τ of 20.7-hr) grown under LD cycles with periodicity of 20-hr (*T*20), 24-hr (*T*24) and (*T*28) were assessed for growth rate and photosynthesis. Chlorophyll content was always found to be higher when endogenous τ of plant strains matched those of the LD cycles. Wild type strain (*col*–o) showed significantly higher chlorophyll content under *T*24 than in *T*20 or *T*28. Similarly, the τ mutant strains (*ztl*-l and *toc1*-1) had higher chlorophyll content when they were grown under LD cycles that matched their endogenous periods than in environmental cycles with non-matching periods. To test whether higher chlorophyll further leads to higher rate of photosynthesis, carbon fixation and subsequently greater biomass, level of CO_2 fixation and biomass was estimated in these strains. Plant strains which were grown under LD cycles matching with their τ showed, higher CO_2 fixation and greater biomass. In the

same study, authors performed reciprocal competition between short and long τ mutants by growing their mixed populations under *T*20 and *T*28. Growth assessed by several parameters such as chlorophyll content, leaf number, aerial biomass showed that plant strains grew better under LD cycles with periodicities matching their τ. Together these results suggest that having clocks with period matching with environmental periodicity, enhanced growth in plants thus providing evidence in support of circadian resonance hypothesis.

In another study aimed at testing circadian resonance, *cyanobacterial* strains with varying circadian periodicities, wild type strains (AMC149 and AMC343) (τ = 25-hr) and mutant strains SP22 (τ = 23-hr) and P28 (τ = 30-hr) were subjected to pair-wise growth competition under LD cycles of 22-hr, 24-hr and 30-hr periodicities (Ouyang et al., 1998). In pair-wise growth competitions, strains whose τ matched closely to that of the environmental LD cycles out-competed others. To further validate whether the success of the period mutant strains is due to circadian clocks and not due to any deleterious secondary mutation under competition for growth, the long period mutation was rescued (P28) by replacing the mutant allele with wild type allele and the rescued strain P28R, when competed with a wild type strain under *T24*, performed like that of the wild type. Hence, this study provides the most convincing evidence thus far for the hypothesis of adaptive significance of circadian rhythms being mediated by circadian resonance.

A recent study tested circadian resonance hypothesis in the pitcher plant mosquito *W. smithii* using a slightly different approach (Emerson et al., 2008). In pitcher plant mosquito *Wyeomyia smithii*, short photoperiods initiate and maintain larval dormancy (diapause). In this study, natural populations of pitcher plant mosquito were subjected to diapause by maintaining them under short photoperiods ranging from LD 10:14 to LD 10:62. Thus, mosquito populations experienced LD periodicities ranging from 24-hr to 72-hr with a fixed

duration of light phase (10-hr) per cycle and with increasing duration of the dark phase (14 to 62-hr). Incidence of diapause termination was minimum under LD cycles with periods multiple of 24-hr and was higher in LD cycles with periodicities which were non-multiples of 24-hr. Short photoperiod LD regimes were expected to maintain diapause, but incidence of diapause termination was found to be dependent on the period of the LD cycles, suggesting that interaction between endogenous and environmental periodicities is a critical determinant of fitness. To test the effect of this interaction on fitness, several components of fitness were estimated in populations subjected to diapause by maintaining them under LD regimens of different periodicities. Mosquitoes exposed to LD regimens which were multiple of 24 (τ of mosquitoes) had higher fitness than those exposed to non-multiples of 24.

***Effect of loss of circadian clocks on fitness in laboratory conditions*:** Several studies have examined the effect of loss of normal circadian rhythmicity on the fitness of organisms. In studies on hamsters (Menaker and Vogelbaum, 1993) and mice (King et al., 1997), arrhythmic mutant animals did not show any reduction in lifespan, at least not under laboratory conditions. Similarly, in studies which tested longevity of animals rendered arrhythmic by suprachiamatic nucleus (SCN) ablation, SCN lesioned Siberian chipmunk, *Eutamias sibiricus* (Sato and Kawamuara, 1984) and golden mantled ground squirrels, *Spermophilus lateralis* (Ruby et al., 1996) lived equally long enough as the intact controls under laboratory conditions. A separate study on the short period *tau* mutant golden hamsters tested the effect of environmentally induced rhythm disruption on longevity (Hurd and Ralph, 1998). Heterozygous *tau* mutants (τ = 22-hr) entrained to 24-hr LD cycle but with altered ψ and highly fragmented activity compared to wild type. Under 24-hr laboratory LD cycles, heterozygous (*tau*/+: τ = 22-hr) hamsters showed reduced lifespan compared to the homozygous (*tau/tau*: τ = 20-hr) and wild type animals (WT: τ = 24-hr). Fragmentation of activity was speculated to be the primary cause of reduced longevity in the heterozygous

mutants. Longevity of the three genotypes of hamsters did not differ under LL. Consolidated activity rhythm and normal longevity was rescued in heterozygous animals by transplanting SCN from a wild type animal. Inconsistency in the results with regard to expectations based on previous studies makes it difficult to infer about the role of circadian rhythms in regulating fitness of organisms in cyclic environment.

In a relatively recent study in wild type fruit flies *D. melanogaster,* it was demonstrated that fruit flies lacking consolidated activity/rest behavior under DD live shorter than their rhythmic counterparts (Kumar et al., 2005), supporting the view that intact circadian rhythms are advantageous. Many studies tested the consequence of light regime induced arrhythmicity on the fitness of *Drosophila*. Longevity of *D. melanogaster* maintained under LL or LD 12:12 was lower than DD (Allemand, 1973). In a separate study on four large, outbred, replicate populations of *D. melanogaster* maintained under LL for several hundred generations, longevity of flies was found to be significantly reduced compared to LD 12:12 or DD (Sheeba et al., 2000). Reduction in longevity under LL, however, was found to be accompanied by enhanced reproductive output, suggesting that multiple fitness components should be taken into account to draw any conclusion on adaptive advantage of circadian rhythms.

In another study, reproductive output was measured in *D. melanogaster* loss of function mutants for core clock genes *period (per), timeless (tim), clock (clk)* and *cycle* (*cyc*) (Beaver et al., 2002). Egg-output in loss of function mutants showed 40% reduction, primarily due to less number of eggs laid and higher proportion of unfertilised eggs. Male contribution to the reduction in reproductive output was confirmed by a decrease in the number of progeny from pairs of *per* or *tim* null males mated to wild type females, primarily due to poor sperm quantity. Oscillatory expression of clock genes in male reproductive tract suggests the presence of functional circadian clocks (Beaver et al., 2002). Although a rescue

of the null mutations restored the total number of progeny from crosses between rescued males mated to wild type females, sperm count in rescued males was significantly lower than those in the wild type males, suggesting that reduction in the number of progeny fathered by null mutant males is unlikely due to lesser sperm production. Moreover, no rhythm was detectable in the sperm release from the testes. Thus, these studies at the most establish the role of core clock genes in the regulation of reproductive output, however, it appears that lower reproductive output in the null mutants is likely be a result of non-clock function of clock genes, which is also consistent with other reports, suggesting a role for clock genes in non-clock functions such as regulation of pre-adult development and oogenesis (Kyriacou et al., 1990; Beaver et al., 2002).

***Importance of clocks under natural conditions*:** Adaptive advantage of circadian rhythms is also evident in the studies on free-living animals, which tested longevity of animals with and without consolidated activity/rest rhythm under wild conditions. In a study on the ground squirrels, SCN lesioned and intact control animals live equally well in laboratory conditions (Ruby et al., 1996). But under semi-natural enclosures, SCN lesioned animals were highly vulnerable to predation by feral cats and as a consequence were found to incur greater mortality compared to intact controls (DeCoursey et al., 1997). This suggests that, while lack of circadian clocks may not incur any physiological disadvantage under the laboratory conditions, they may play an important role in reducing survival under natural conditions. In a similar study on the free living chipmunks *Tamias striatus*, SCN lesioned animals suffered significantly greater mortality compared to the intact controls (DeCoursey et al., 2000). A careful analysis of the activity/rest behavior revealed that SCN lesioned animals were more vulnerable to attacks by predators due to increased night time restlessness. While it is clear that SCN lesioned animals incur greater mortality, we do not know if this is because these animals are poor in terms of their physical ability to escape predators.

***Evidence for adaptive significance of circadian clocks from studies on clines*:** According to the proposition of the extrinsic advantage of circadian rhythms, it is believed that circadian rhythms entrain "optimally" to environmental cycles by scheduling behavioral and physiological processes to anticipate, rhythmic phenomena in the environment. The levels of environmental factors such as mean light intensity, day length, temperature and wavelength of light, which influence phases of daily environmental challenges and opportunities, vary depending on the latitude and so, optimum phase of overt rhythm may be latitude-dependent. The ability to entrain optimally evolves under the influence of rhythmic environment and it is believed that this occurs through the evolution of properties of endogenous rhythms such as τ, photosensitivity, action spectra, phase response curves (PRC) and temperature compensation. Therefore, latitudinal clines in circadian phenotypes would be suggestive of adaptive evolution of circadian clocks for optimal entrainment to a gradient of environmental factors and indicates action of natural selection on circadian rhythms.

Studies on latitudinal clines of circadian clock properties are classic examples of natural selection acting on circadian rhythms. A study conducted on the latitudinal populations of *D. auraria* from 34.2^{o} to 42.9^{o} N in Japan reported a latitudinal cline of phase, period and PRC amplitude of circadian rhythm (Pittendrigh and Takamura, 1989). Clock gene *per* is a core component of *Drosophila* circadian molecular oscillator (Allada and Chung, 2010). Repetitive sequence region in *per* codes for repeats of amino acid pair Thr-Gly (Costa et al., 1992). Natural populations of *D. melanogaster* and *D. simulans* are polymorphic for these Thr-Gly repeat number alleles. Thr-Gly dipeptide repeat number alleles which code for 17 or 20-dipeptide are predominant in the European populations (over 90%; Costa et al., 1992; Rosato et al., 1994). A survey of natural populations of *D. melanogaster* collected from different latitudes of Europe showed a correlation between latitude and the frequency of the Thr-Gly repeat alleles $(\text{Thr-Gly})_{17}$ and $(\text{Thr-Gly})_{20}$. These

studies showed that repeat allele coding for 17-dipeptide repeats is predominant in the southern parts, while 20 repeats is more common in northern Europe, suggesting latitudinal cline in *per* polymorphism. Thr-Gly repeat number is associated with the thermostability of circadian behavior (Sawyer et al., 1997). Temperature compensation of *Drosophila* lines homozygous for dipeptide repeat number $(Thr\text{-}Gly)_{20}$ exhibited greater temperature compensation than lines with $(Thr\text{-}Gly)_{17}$ (Sawyer et al., 1997), thus suggesting that the distribution of dipeptide repeat allele of *per* gene was shaped by the mean temperature differences between northern and southern Europe. Similarly, in a separate study a latitudinal cline for the length of τ was reported in *A. thaliana* (Michael et al., 2003).

In a recent study on wild type populations of *Drosophila*, polymorphism in another core clock gene *tim* was also found to exhibit latitudinal cline. The gene *tim* codes for light responsive element of molecular oscillator, and recently two allele's ls-*tim* and s-*tim* which code for long and short peptide or only short peptide, respectively were identified (Rosato et al., 1997). Estimates of ls-*tim* allele frequency in populations collected from different latitudes across Europe (from southern Italy to Sweden) showed a strong latitudinal cline with ls-*tim* frequency highest in southern Italy and gradually decreasing as one goes northwards but the cline grew weaker when the ls-*tim* allele frequencies of samples from Italy and southwards were included. Same allele frequencies when plotted against direct distance or overland distance from the putative site of origin of the allele (Novoli, southern Italy), cline stronger than latitudinal cline, which raised a possibility that observed clinal variation might be due to spread of the allele in all directions from its site of emergence and not because of natural selection. A test to tease apart these possibilities suggested strong contribution of natural selection in the observed clinal variation. In a screen to identify behavioural phenotypes governed by *tim* polymorphism which could have been under selection, ovarian diapause in *Drosophila* populations collected from southern and northern

populations showed an association of diapause incidence with timeless alleles (Tauber et al., 2007). Analysis of diapauses incidence in *tim* alleles showed that ls-*tim* which is predominant in southern Europe had higher incidence of diapause than s-*tim* genotypes irrespective of the population. Ovarian dispause is believed to be a strategy in insects to survive cold temperatures in winters. Northern Europe experience much lower temperatures than the southern locations. Therefore higher incidence of diapause in ls-*tim* genotype which is predominant in southern locations seems contradictory to the notion that ls-*tim* allele is under selection for ovarian diapause. Thus based on this data, it was proposed that the observed clinal variation in ls-*tim* frequency is an outcome of spreading of recently emerged ls-*tim* allele by directional selection. However, this study could not unequivocally pinpoint behavioral phenotype under selection.

Thus, studies on latitudinal clines in circadian phenotypes have been successful in providing some evidence which suggests adaptive significance of circadian clocks. Since, latitudinal clines are purely based on the correlation between latitudes and circadian rhythm or clock allele frequencies; they do not more than suggesting the possible action of natural selection on circadian clocks. Clock genes are known to regulate processes other than circadian phenotypes such as development, and lifespan, thus it is hard to distinguish whether the observed clinal variation in clock properties or allele frequencies reflect selection acting on circadian rhythms or they are correlated response to selection on non-clock phenotypes. In addition, in the above studies, populations were collected from distant locations across range of latitudes while there is no data available to test whether the populations have diverged from a common ancestral population or there has been a mixing of genetic variation between populations, which raises a possibility of differences in genetic background between the populations which can also possibly be attributed to different genetic background rather than known differences at clock loci. Therefore, lack of knowledge about selection pressures

acting on the populations, their intensities, and knowledge about the genetic background makes it difficult to conclude that observed clinal variation in clock properties is due to the action of natural selection on circadian phenotypes.

***Intrinsic adaptive advantage of circadian rhythms*:** Corollary to circadian rhythms being advantageous to living organisms in cyclic environments, it would be logical to expect that these rhythms would be of no obvious significance to animals inhabiting aperiodic environments such as deep sea vents and caves (Sharma, 2003). Therefore, it is believed that animals living in environments where factors such as light, temperature, and humidity remain at constant level would lose their endogenous rhythms over the course of time. Empirical evidence available from studies thus far is mixed and thus debatable. Some studies reported either no circadian rhythms or rhythms with highly variable τ far from 24-hr (Blume et al., 1962; Poulson and White, 1969), while others reported presence of circadian rhythms. For example, circadian rhythms were reported in the cave dwelling catfishes *Tricomycterus sp.* (Trajano and Menna-Barreto, 1996), and cave dwelling millipedes *Glyphiulus cavernicolus sulu* (Koilraj et al., 2000). Some other studies reported the persistence of circadian rhythms of adult emergence, egg-laying and locomotor activity in *D. melanogaster* populations maintained in environment with constant light, temperature and humidity for more than 600 generations (Sheeba et al., 1999, 2001, 2002). These flies also show remarkable ability to entrain to a wide range of LD cycles, much alike their wild type counterparts from natural LD conditions (Paranjpe et al., 2004). Unlike many other organisms such as mammals and plants, fruit fly *D. melanogaster* is arrhythmic in LL. Therefore persistence of rhythms in these fly populations is still a mystery. The above evidence was however taken to support the intrinsic advantage hypothesis of circadian rhythms. However, a relatively recent growth competition study on the arrhythmic mutant strains of *cyanobacteria* show that arrhythmic strain out-compete their opponent wild type strain under LL conditions and the wild type

strain out-competes the arrhythmic strain in LD conditions, suggesting that endogenous rhythms are advantageous only in rhythmic environment and may not confer any intrinsic advantage under arrhythmic environment (Woelfle et al., 2004).

Setting the rationale

Review of evidence which is often taken as proof of the adaptive significance of circadian clocks shows that all the evidence is primarily indirect and it appears that none of the studies till date, has provided a conclusive direct evidence for the evolution of circadian clocks in response to periodic natural selection pressures. Available evidence also does not provide any clear clue about the mechanisms through which clocks confer adaptive advantage. Therefore, direct evidence that circadian clocks evolve as an adaptation to periodic selection pressures supported by functional significance and mechanistic bases of adaptive evolution of clock could greatly enhance the confidence in popularly held belief that circadian clocks are adaptive. Laboratory experimental evolutionary approach seems to be the most appropriate empirical strategy to comprehensively address the issues of adaptive trait evolution. Although, experimental evolution has been used extensively to test the hypotheses about evolution of spectrum of biological traits covering behavior, morphology, physiology and life history in diverse model systems, there were remarkably few attempts made using approach of experimental evolution and those attempts suffer from one or more shortcomings.

Many studies demonstrated that circadian rhythms evolve in response to selection on the timing of adult emergence in fruit fly populations maintained under laboratory LD 12:12 cycles. In fruit fly *D. pseudoobscura* selected for *early* and *late* adult emergence, peak of adult emergence is diverged by 4-hr after 50 generations of selection (Pittendrigh, 1967). These flies also showed divergence in the τ of emergence rhythm. The *early* flies had longer periodicities and *late* flies had periodicities shorter than control. Moreover, *early* and *late*

flies did not show any difference in their PRC (Pittendrigh, 1967). Based on these findings it was suggested that selection on timing of emergence produces changes in the "B oscillator" (slave), while the "A oscillator" (master) remains unchanged. The problem with this argument is if A oscillator does not change then how is clock period altered in the selected lines? However, selection experiments in the moth, *Pectinophora gossypiella* also showed similar results, suggesting that selection on timing of adult emergence could yield unexpected results (Pittendrigh and Minis, 1971). In a separate study, two populations of *D. melanogaster Oregon R* and wild caught W2 flies were subjected to selection for morning and evening emergence (Clayton and Paietta, 1972). After 16 generations of selection, percentage of flies emerging in the morning and evening selection hours increased significantly. This study, however, did not estimate circadian phenotypes in the selected lines. Taken together these studies suggest that circadian rhythms evolve in response to selection on the timing of rhythmic behavior, which in a way supports the idea of extrinsic advantage conferred by circadian rhythms.

In a laboratory selection experiment, melon fly *Bacterocera cucurbitae* populations were subjected to selection for faster or slower pre-adult development under LD 14:10. While faster and slower rate of development evolved as direct response to selection, faster developing lines had shorter τ (~22.5-hr) and slower developing lines had longer τ (~28-hr)(Miyatake and Shimizu et al., 1999). In yet another selection study, when populations were selected for age at reproduction, O lines which were selected for old age at reproduction survived longer, exhibited longer τ and mated later in the day than Y lines which were selected for reproduction at young age (Miyatake et al., 2002). Correlations between fitness traits such as development time, and longevity, and circadian phenotypes such as τ, time of mating suggests a possible contribution of circadian rhythms in overall reproductive fitness.

In any selection experiment, knowledge of the source of populations, population size, population replicates are very important; in order to infer that the observed changes in population traits are the result of action of natural selection forces and not due to other evolutionary forces such as mutation, migration and inbreeding or random genetic drift (Sharma and Joshi, 2002). Though all the selection experiments discussed above demonstrated effect of imposed selection pressures on circadian phenotypes most of them suffer from some problem or other. These studies did not provide adequate information about the source populations, population size, number of replicates and their maintenance regime, which makes it difficult to unambiguously conclude that the observed changes in circadian phenotypes is due to adaptive evolution under imposed selection alone.

We therefore initiated a selection experiment in which we imposed selection on the timing of adult emergence on large, out-bred replicate populations of fruit fly *Drosophila melanogaster* and followed the evolution of circadian clocks.

Chapter 2

Evolution of adult emergence waveforms in *early* and *late* stocks of fruit fly *Drosophila melanogaster*

Introduction

Circadian clocks are believed to be an adaptation to the perpetual selection imposed by 24-hr environmental cycles resulting from continual rotation of the earth about its axis (Sharma, 2003; Paranjpe and Sharma, 2005). It is believed that circadian clocks confer adaptive advantage to living beings by scheduling various biological functions to specific time of the day (Johnson et al., 2003; Roenneberg et al., 2003a; Sharma, 2003). Most studies reporting evidence supporting this hypothesis are merely suggestive of the advantage conferred by circadian clocks in terms of scheduling of biological processes at appropriate time of the day. Unequivocal evidence for the evolution of circadian clocks is still missing because previous attempts to address this issue have been associated with several loopholes. Therefore, to investigate whether and how circadian clocks evolve under periodic selection pressure of the environment, we initiated a laboratory selection experiment wherein we imposed selection on the timing of emergence in four replicate populations of fruit flies *Drosophila melanogaster*.

Fruit fly *D. melanogaster* exhibit circadian rhythms of adult emergence and under laboratory 12:12 hr light/dark (LD) cycles flies emerge during the light phase with peak immediately after dark-to-light transition, followed by a gradual reduction leading to a zone of no emergence by lights-off (Saunders, 1992). It is now well established that circadian rhythm of emergence in fruit fly *D. melanogaster* is under the control of self-sustained, endogenous circadian oscillators and steady state phase-relationship between the peak of emergence rhythm and lights-ON of LD cycles is a result of entrainment of the circadian oscillators to LD cycles (Saunders, 1992). In a long-term selection study we created four replicate stocks of *early* and *late* fruit flies *D. melanogaster* showing preference for different timing of emergence by imposing selection for morning and evening emergence under LD cycles.

Materials and methods

***Fly population maintenance and laboratory selection protocol*:** Four replicates each of *early* ($early_{i=1..4}$), *control* ($control_{j=1..4}$) and *late* ($late_{k=1..4}$) populations were initiated from four replicate large outbred ancestral populations ($JB_{1..4}$). These populations ($JB_{1..4}$; Sheeba et al., 1998) were maintained independent of each other for at least 700 generations before the initiation of *early* and *late* populations. The $early_i$, $control_j$ and $late_k$ populations sharing the same subscript ($i = j = k$) were initiated from a common ancestral JB population (Figure 1). Thus, *early*, *control* and *late* populations sharing the same subscript ($i = j = k$) indicates common ancestry. Because of genetic similarity among *early*, *control* and *late* populations sharing same subscript, replicate populations were treated as 'blocks', the significance of which will be discussed under the section on statistical analysis. Maintenance and selection protocol of *early* and *late* populations are described in detail elsewhere (Kumar et al., 2007a). Briefly, all populations were maintained as independent populations on a 21-day generation cycle with no gene flow between them. Each fly population was maintained as large population of adult individuals (sex ratio close to 1) in a plexiglass cage of dimensions 25 × 20 × 15 cm^3 with banana-jaggery (BJ) food in a petridish and water (wet ball of cotton) placed at the bottom of the cage, both of which were provided fresh every alternate day. The next generation was initiated from eggs collected from each breeding adult population whose average age was 10 days. Adult flies in the cages were fed with yeast paste for 3-days before egg collection, after which they were provided with a fresh plate of food to lay eggs for about 2-3-hr. From this plate, approximately 300 eggs were collected and dispensed into glass vials (1.5-cm diameter × 19-cm height) containing 6 ml BJ food medium. Twenty-four, sixteen, and forty-eight such vials were used at every generation for each of the *early*, *control* and *late* populations, respectively. For the *control* populations adult flies emerging between 9^{th} and

13^{th} day after egg collection from these vials were collected everyday into plexiglass cages to

Figure 1. Schematic of selection protocol

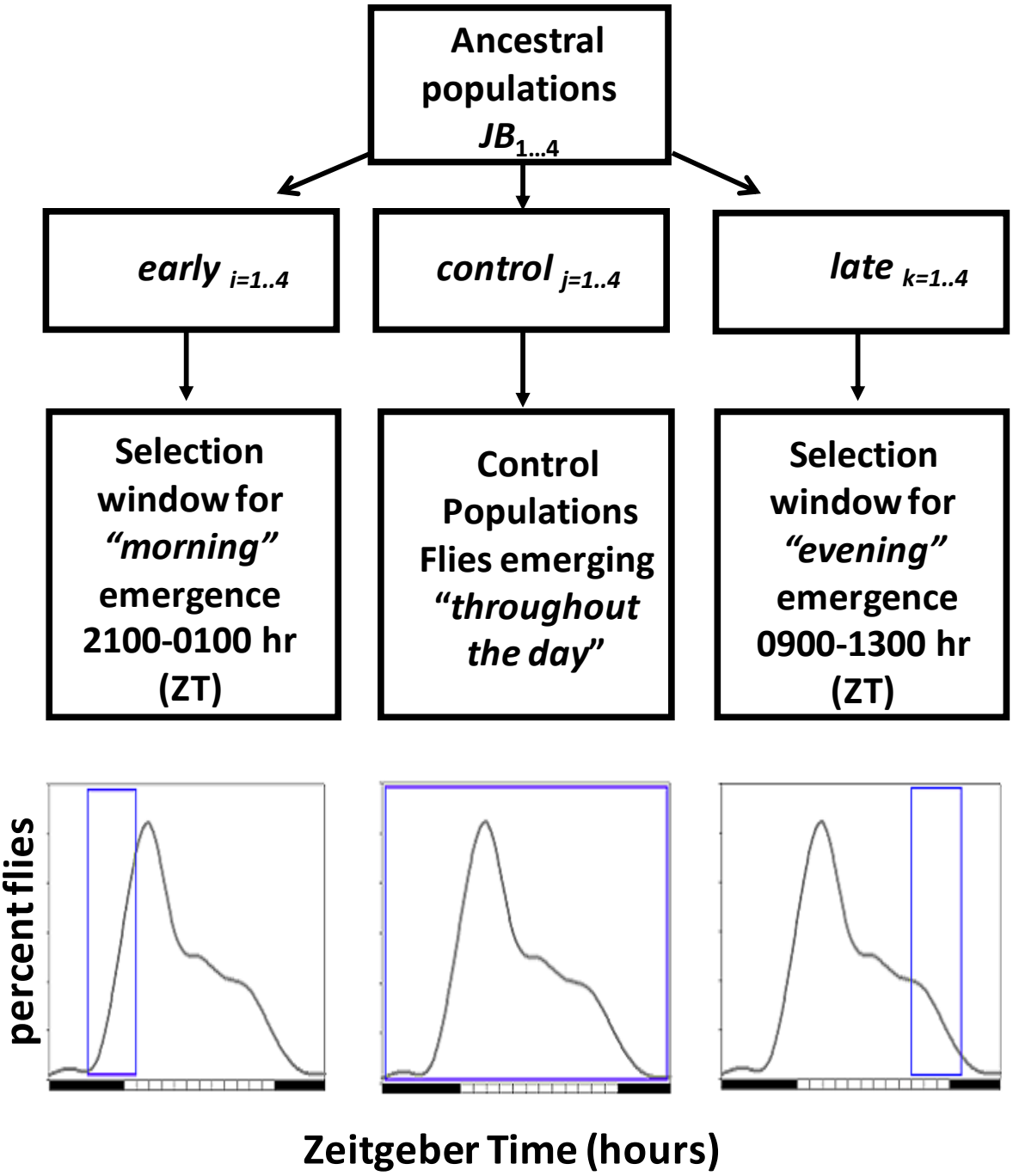
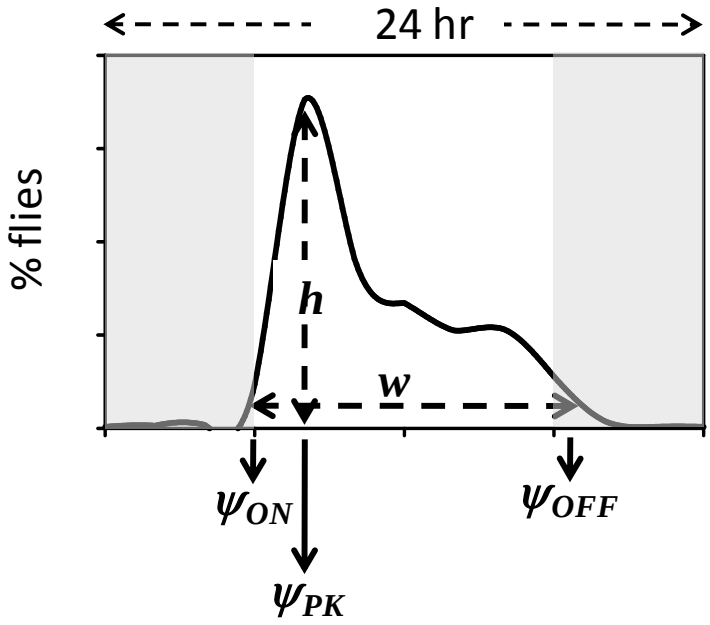


Figure 2. Quantification of waveform features



h	Height of emergence peak (%)
ψ_{PK}	Phase of emergence peak (ZT)
ψ_{ON}	Phase of emergence on-set (ZT)
ψ_{OFF}	Phase of emergence off-set (ZT)
w	Width of emergence gate (hr)

form the next generation. Therefore, *control* populations were not under any conscious selection for timing of emergence. In contrast, at every generation, breeding adults for the *early* populations were formed by collecting adult flies emerging only during a span of 4 hr in the morning (from 3-hr before lights-on to 1-hr after), for 5 successive days. Similarly, breeding adults for the *late* populations were formed out of flies that emerged during 4 hr span in the evening (from 3-hr before lights-off to 1-hr after lights-off). At every generation we ensured that each of the selected and control population consisted of approximately 1200 adult individuals with roughly equal number of males and females. Throughout their pre adult development and as adults, all populations were reared in cubicles maintained at constant temperature (25 ± 0.5 ^{o}C) and relative humidity (75 ± 5%). Alternating LD cycles were created with sharp dark to light and light to dark transitions with light intensity of about 0.15 W/m^2 during the light phase.

It is known that non-genetic factors such as altered physiology of the parents due to environmental conditions that they experience can influence the phenotypes of their progeny (Prasad and Joshi, 2003). Therefore, selection regimes (types of LD schedule) experienced by the populations could also influence the phenotype of their progeny due some unknown non-genetic effects of imposed selection. In our selection regimes, timing of emergence of parents (which differed between populations) could influence the phenotype of their progeny. Thus, experimental protocols which can rule out such unknown effects of selection regime are required to unambiguously test the genetic differences between selected and control populations. One generation of common rearing conditions for selected and control populations are known to eliminate such non-genetic parental effects (Prasad and Joshi, 2003). Therefore, for one generation we subjected selected and control populations to a common rearing condition. This was achieved by taking eggs from the running populations and collecting adults emerging throughout the day to make the required population size of

about 1200 adults with roughly equal number of males and females. Hereafter, the progeny of such flies will be referred to as "standardised populations".

***Adult emergence assay*:** Adult emergence assays were conducted at regular intervals of ≈ 15 generations to assess the generation-wise effect of selection for morning and evening emergence on the adult emergence waveform under LD cycles. For the adult emergence rhythm assay, standardised populations kept under LD cycles in plexiglass cages were provided with yeast paste (spiked with few drops of acetic acid) on petri-plate with BJ food for 3-days prior to egg collection. Eggs were collected in a manner similar to stock maintenance regime, except that here ten vials were maintained per population. After egg collection, all the assay vials were maintained under LD cycles till the assay got over. After the onset of adult emergence, all the vials were monitored every 2-hr for adult emergence and number of flies that emerged in the preceding 2-hr bin was recorded for 4-5-days. Cycles (days) in which at least 25 flies emerged were subjected to further analysis.

***Estimation of adult emergence waveforms*:** For every selected cycle of each vial, emergence in 2-hr bins were expressed as percentage of the total fly emergence in cycle by dividing fly count of each two hour bins by the total number of flies that emerged in that cycle. For each vial, percentage of flies that emerged in 2-hr bins was averaged across 4 cycles to obtain average emergence waveform of the vial. Emergence waveforms of individual replicate populations were obtained by averaging the percentage emergence over replicate vials. Individual vial emergence waveforms were analysed further to estimate morning and evening emergence and to quantify waveform characteristics as explained below.

***Estimation of adult emergence during morning and evening hours*:** Four hour morning selection window spanned from ZT21 to ZT01 (3-hr before lights-on to 1-hr) and evening selection windows from ZT09 to ZT13 (3-hr before lights-off to 1-hr after lights-off). As fly emergence was recorded at every even ZT hours (2-hourly intervals), we used pooled

emergence from ZT22, ZT00 and ZT02 (spanning morning selection window) as an estimate of emergence during morning hours and pooled emergence from ZT10, ZT12 and ZT14 (spanning evening selection window) as an estimate of emergence during evening hours. Average of percentage emergence during morning or evening hours across replicate vials was used as estimate of mean morning and evening emergence in a population.

***Quantification of emergence waveform characteristics*:** We measured some quantifiable features of the emergence waveform, such as height of emergence peak (*h*), phase of emergence peak, onset and offset (ψ_{PK}, ψ_{ON} and ψ_{OFF}), and width of the emergence gate (*w*) to characterize waveform shape (Figure 2). Adult emergence waveforms of individual vials were analysed to estimate temporal position of the peak (ψ_{PK}), onset (ψ_{ON}) and offset (ψ_{OFF}), gate-width (*w*) and height of emergence peak (*h*). The time at which maximum adult emergence was recorded under light regimes was taken as ψ_{PK}. The times at which the percentage of flies emerging in 2-hr bin increased above arbitrary cut-off of 5% near start of daily emergence or fell below 5% towards the end of the day for the first time was defined as ψ_{ON} and ψ_{OFF}, respectively. ψ_{PK}, ψ_{ON} and ψ_{OFF} were estimated as time interval between lights-on (ZT00) and peak, onset and offset of emergence rhythm. *w* was estimated as the duration between ψ_{ON} and ψ_{OFF}. Percentage emergence during the 2-hr bin during emergence peak was used as an estimate of *h*. Difference between ψ_{ON} and ψ_{OFF} was used to estimate *w*. All the waveform characteristics were first calculated for normalized waveforms from individual vials and were then averaged over all replicate vials to obtain an estimate for each replicate population.

***Statistical analyses*:** Replicate populations were considered as experimental units (blocks) and therefore for all the measures of adult emergence, block means (average across replicate vials under each replicate selected or control stocks) were used as unit of analysis. Three-way mixed model analysis of variance (ANOVA) was performed on percentage morning and

evening emergence to test the effect of generations of selection (G) in selected and control stocks (S). ANOVA enabled testing of main effects of G and S and their interaction. G and S were treated as fixed effects factors whereas replicate populations under each stock (Blocks) (B) were treated as random factor. Emergence waveforms and waveform characteristics obtained from latest generation assay was analysed to test differences among *early*, *control* and *late* stocks. Emergence waveforms were compared by performing three-way mixed model ANOVA on percentage emergence data, in which stock (S) and time of the day/phase (P) were treated as fixed effects factors and blocks (B) as random factor. Effect of stocks on all the waveform features (ψ_{PK}, ψ_{ON}, ψ_{OFF}, w and h) were tested in two-way ANOVA (stock (S) as fixed factor and block (B) as random factor) performed separately for each waveform feature. This enabled the testing of significance of main effects of fixed factors and their interactions. Post-hoc multiple comparisons were done by setting 95% comparison intervals (95%CI) around the means using minimum significant difference calculated by Tukey's HSD test at $\alpha = 0.05$ (Sokal and Rohlf, 1981). Thus, two means were considered significantly different if there was no overlap between their error bars. All statistical analyses were implemented using STATISTICA^{TM} for Windows Release 5.0 B (StatSoft, 1995).

Results

***Direct response to selection for morning and evening adult emergence*:** The *early* and *late* fly stocks were under selection for emergence during morning and evening hours, respectively, and therefore, the incidence of adult emergence during morning and evening hours was estimated every 10-15 generations during the course of long-term laboratory selection study to evaluate the direct response to selection. Here we present the data from the assays conducted till 180 generations of selection. Morning and evening emergence was estimated as percentage of the total number of flies that emerged in one cycle.

***Morning emergence*:** During the course of the selection experiment, *early* stocks showed a gradual increase in the incidence of emergence during morning hours indicating a direct response to selection, whereas *late* stocks showed gradual reduction in morning emergence (Figure 3a). By the 180th generation morning emergence in *early* stocks increased to ~65% and reduced to ~20% in *late* stocks. *Controls* showed more or less a constant level of morning emergence of ~40% throughout the selection study (Figure 3a). ANOVA on the morning emergence in *early*, *control* and *late* stocks showed a statistically significant effect of stock (S) ($F_{2,6}$ = 942.57, $p < 0.0001$) and stock × generation (G) interaction ($F_{22,66}$ = 18.76, $p < 0.001$; Table 1a). However, the effect of G did not reach statistical levels of significance. Post-hoc multiple comparisons using Tukey's test revealed that significant divergence in percentage of morning emergence in *early* and *late* stocks was apparent from generation 25 onwards, and between *early*, *control* and *late* stocks by generation 55 onwards were significantly different from control stocks. Although, *early* and *late* stocks continued to diverge during the course of experiment, after 100th generation morning emergence appeared more or less constant in all the three stocks (Figure 3a).

***Evening emergence*:** Direct response to selection for evening emergence in *late* stocks was evident from gradual increase in the incidence of evening emergence with increasing generations of selection (Figure 3b). On the other hand, emergence during evening hours reduced gradually in *early* stocks. By the 180th generation, evening emergence increased to ~35% in *late* stocks, diminished to ~3% in *early* stocks and remained constant at ~15% in *controls* (Figure 3b). ANOVA done on evening emergence revealed a statistically significant effect of S ($F_{2,6}$ = 1927.47, $p < 0.0001$) and its interaction with G ($F_{22,66}$ = 21.47, $p < 0.0001$), however, the effect of G was statistically not significant (Table 1b). Divergence between *early* and *late* stocks in terms of percentage evening emergence was apparent by the 10th generation but it became significantly different only by the 25th generation. Post-hoc

Figure 3. *Direct response to selection for morning and evening emergence in early and late stocks of fruit fly D. melanogaster*. (a) Fly emergence during morning hours (between ZT20 and ZT02) in *early*, *control* and *late* populations. (B) Fly emergence during evening hours (between ZT08 and ZT14) in *early*, *control* and *late* stocks. Morning and evening emergence is expressed as percentage of total number of flies emerged in a cycle. Error bars are 95% comparison intervals and therefore, non-overlapping error bars indicates that two means are significantly different from each other. Numbers on *x*-axis represent generations of selection and correspondence is provided .in adjacent table.

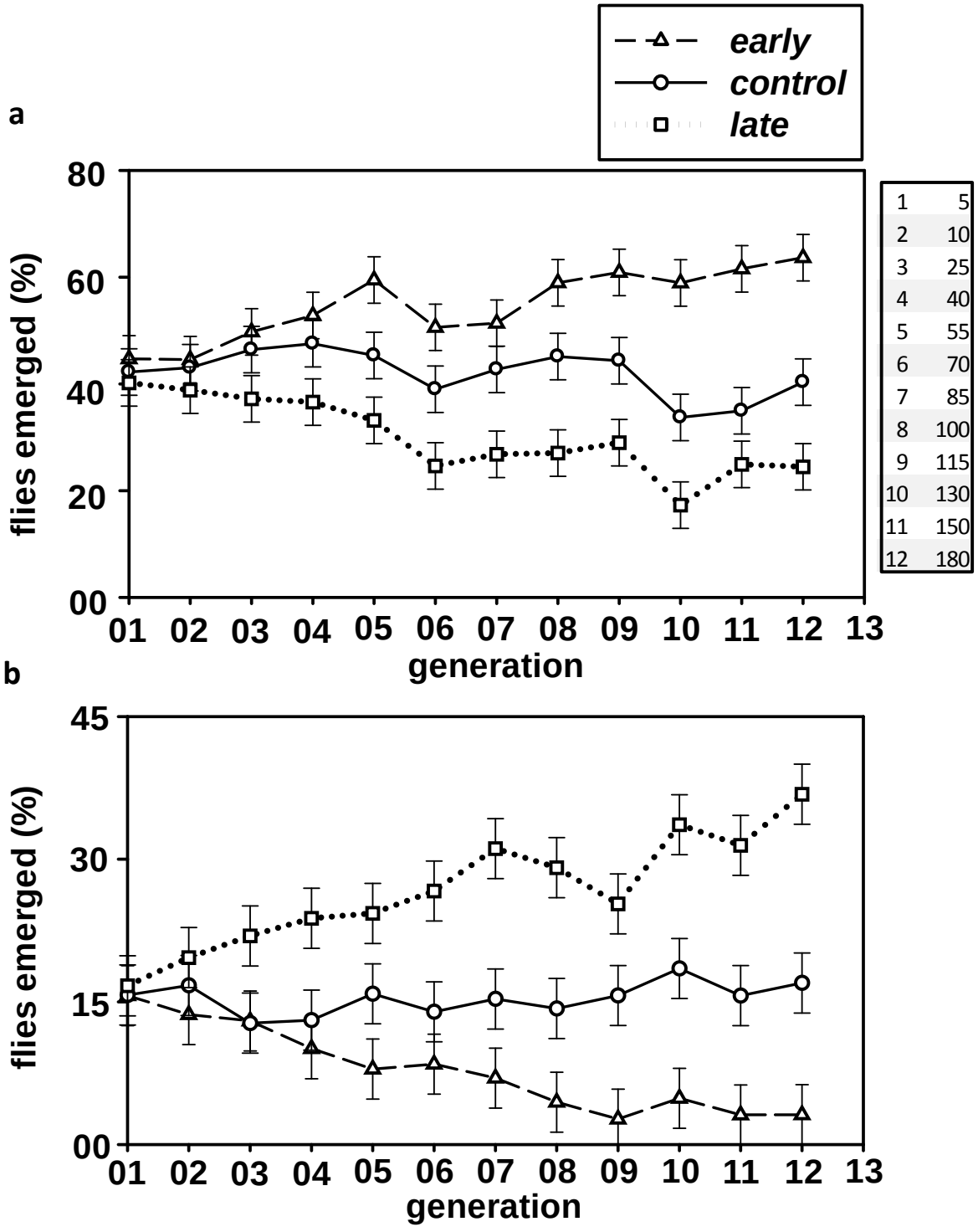


Table 1. ANOVA done on the percentage fly emergence during morning and evening hours in *early*, *control* and *late* fly stocks, assayed between generation 10 and 180 of selection. Analysis includes data from 12 such assays conducted at intervals of about 10-15 generations. (a) Morning emergence. (b) Evening emergence. Error bars are 95% comparison intervals and therefore, non-overlapping error bars indicates that two means are significantly different from each other.

a

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Generation (G)	11	103.55	33	65.43	1.58	0.15
Stock (S)	2	7355.79	6	7.80	942.57	0.0001
Block (B)	3	12.91	0	0.00	--	--
G × S	22	174.58	66	9.31	18.76	0.0001
G × B	33	65.43	0	0.00	--	--
S × B	6	7.80	0	0.00	--	--
G × S × B	66	9.31	0	0.00	--	--

b

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Generation (G)	11	21.44	33	12.31	1.74	0.11
Stock (S)	2	4332.74	6	2.25	1927.47	0.0001
Block (B)	3	8.53	0	0.00	--	--
G × S	22	104.99	66	4.89	21.47	0.0001
G × B	33	12.31	0	0.00	--	--
S × B	6	2.25	0	0.00	--	--
G × S × B	66	4.89	0	0.00	--	--

multiple comparisons showed that, although *late* stocks were significantly different from *control* 25th generation onwards, difference in evening emergence between *early* and *control* stocks reached statistical significance only by the 55th generation. Evening emergence increased gradually throughout the selection in *late* stocks, but it appeared to have reached its lower limit of evening emergence (~3%) in *early* stocks only by the 100th generation (Figure 3b).

As a direct response to selection, *early* and *late* stocks evolved increased emergence during morning and evening hours and reduced emergence in the opposite selection windows. Assays performed at intervals of about 10-15 generations showed that although the divergence between *early* and *late* stocks continued to be seen throughout selection experiment, there appears to be no measurable change in the incidence of morning and evening emergence in any of the stocks in the last 100 generations of selection.

Adult emergence waveforms of early and late stocks: Evolution of morning and evening emergence in *early* and *late* stocks implies change in the distribution of emergence and therefore we examined the emergence waveforms of *early*, *control* and *late* stocks. Adult emergence waveforms shown in Figure 4 is a representative of daily emergence distribution of approximately last 100 generations as we did not observe a remarkable change generation 85 onwards. Though there was a substantial overlap in the emergence waveforms of *early*, *control* and *late* stocks, waveforms of *early* and *late* stocks were clearly diverged. The *early* emergence waveform appeared to be advanced and *late* waveform delayed relative to that of the *control* stocks, which indicates the evolution of increased morning emergence and reduced evening emergence in *early* and reduced morning emergence and increased evening emergence in *late* stocks.

Figure 4. *Adult emergence waveforms of early, control and late fly stocks under 12:12 hr light/dark (LD) cycles, after 175 generations of selection for morning and evening emergence*. Emergence in each two hourly interval is expressed as percentage of total number of flies emerged in a cycle. Time of the day is expressed as Zeitgeber Time (hr), Lights-ON is ZT00 and lights-OFF is ZT12. Error bars are 95% comparison intervals and therefore, non-overlapping error bars indicates that two means are significantly different from each other.

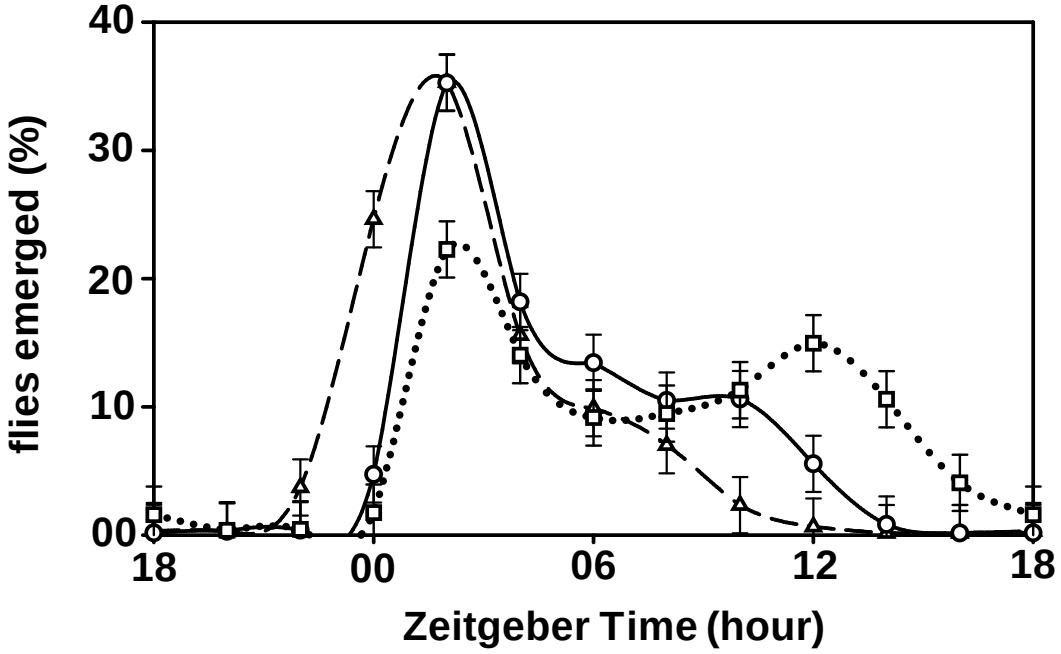


Table 2. ANOVA performed on the percentage fly emergence at twelve phases spanning complete light/dark (LD) cycles, in *early*, *control* and *late* stocks.

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	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2	0.00	6	0.00	10.61	0.01
Block (B)	3	0.00	0	0.00	--	--
Phase (P)	11	892.49	33	5.69	156.95	0.0001
S × B	6	0.00	0	0.00	--	--
S × P	22	125.47	66	2.36	53.15	0.0001
B × P	33	5.69	0	0.00	--	--
S × B × P	66	2.36	0	0.00	--	--

Figure 5. Quantification of emergence waveform features in *early*, *control* and *late* stocks. (a) Height of emergence peak (*h*). (b) Timing/phase of emergence peak (ψ_{PK}). (c) Timing/phase of daily emergence onset (ψ_{ON}). (d) Timing/phase of daily emergence offset (ψ_{OFF}). (e) Extent/width of daily emergence (*w*). *h* is expressed as percentage of total cycle count. ψ_{PK}, ψ_{ON}, ψ_{OFF} are expressed in Zeitgeber Time in hours.

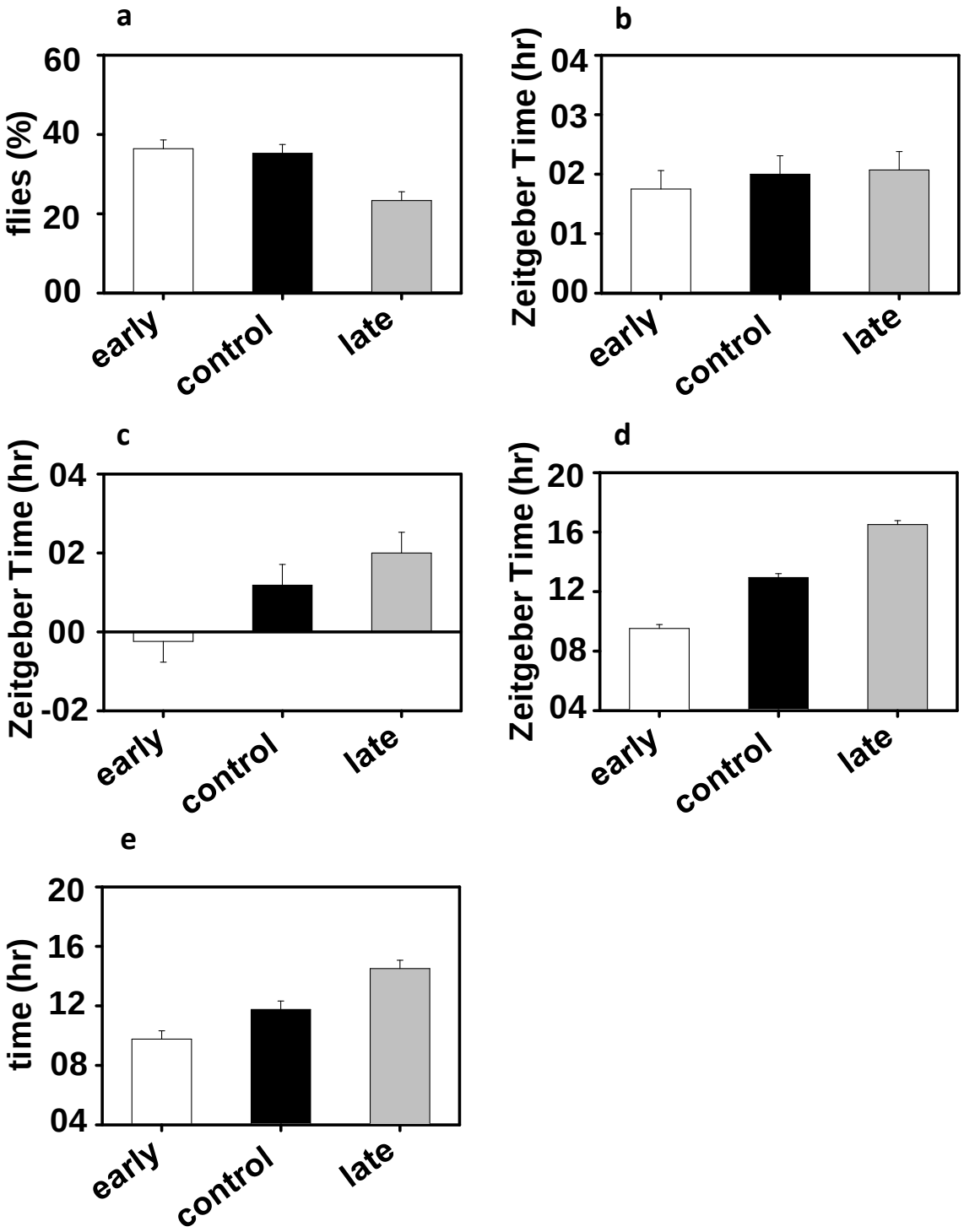


Table 3. ANOVA done on five emergence waveform features of *early*, *control* and *late* fly stocks. (a) h (b) ψ_{PK} (c) ψ_{ON} (d) ψ_{OFF} and (e) w.

		df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
a	**Stock (S)**	2	209.86	6	4.23	49.58	0.0001
	Block (B)	3	28.52	0	0.00	--	--
	S × B	6	4.23	0	0.00	--	--
b	**Stock (S)**	2	0.11	6	0.08	1.39	0.32
	Block (B)	3	0.11	0	0.00	--	--
	S × B	6	0.08	0	0.00	--	--
c	**Stock (S)**	2	5.16	6	0.24	21.87	0.002
	Block (B)	3	0.07	0	0.00	--	--
	S × B	6	0.24	0	0.00	--	--
d	**Stock (S)**	2	48.83	6	0.06	839.38	0.0001
	Block (B)	3	0.06	0	0.00	--	--
	S × B	6	0.06	0	0.00	--	--
e	**Stock (S)**	2	22.70	6	0.27	84.19	0.0001
	Block (B)	3	0.01	0	0.00	--	--
	S × B	6	0.27	0	0.00	--	--

We further characterised the shapes of emergence waveforms by measuring some quantifiable features of emergence waveforms such as *h*, ψ_{PK}, ψ_{ON}, ψ_{OFF} and *w*. Effect of S on the waveform features was tested by performing separate one-way ANOVA for each waveform feature (Table 3a-e). Except for ψ_{PK}, S showed significant effect for all other waveform features (*h*- $F_{2,6} = 49.58$, $p < 0.0001$; ψ_{ON}-$F_{2,6} = 21.87$, $p < 0.002$; ψ_{OFF}-$F_{2,6} = 839.38$, $p < 0.001$; *w*-$F_{2,6} = 84.19$, $p < 0.0001$). Post-hoc multiple comparisons using Tukey's test showed that although timing of emergence peak (ψ_{PK}) did not differ among stocks (Figure 5b), the height of emergence peak (*h*) was reduced significantly in *late* stocks compared to *early* and *control* (Figure 5a). Emergence started before lights-ON and ended almost 2-hr before lights-OFF in *early* stocks, while it started ~2-hr after lights-ON and ended more than 3-hr after lights-OFF in *late* stocks (Figure 5c, d).

Emergence gate was found to be shorter in *early* and *longer* in *late* stocks than *controls* (Figure 5e). Emergence gate of *late* stocks was ~4-hr longer than that of *early* stocks. Thus, *early* and *late* stocks evolved divergent emergence waveforms as a correlated response to selection for morning and evening emergence.

Discussion

The *early* and *late* stocks of fruit fly *D. melanogaster* were created by imposing selection for morning and evening emergence under laboratory LD cycles. Adult emergence assays conducted until 55th generation of selection showed that *early* stocks evolved increased emergence during morning hours and *late* stocks evolved greater emergence during evening hours (Kumar et al., 2007a). Analyses of adult emergence waveforms revealed that *early* and *late* stocks evolved divergent emergence waveforms. Circadian regulation of emergence in fruit fly is well established and the steady-state phase-relationship of the emergence rhythm with LD cycles is known to be a result of entrainment of the underlying circadian oscillators (Saunders, 1992). Therefore, divergence of emergence waveforms in *early* and *late* stocks

indicates that the evolution of diverged circadian clocks may be the root cause. Studies aimed at assessing the involvement of circadian clocks in the preference for morning and evening emergence in *early* and *late* stocks revealed that *early* stocks evolved emergence rhythms with shorter τ and photic PRC with larger phase-advances and smaller delays than *control* stocks. On the other hand, *late* stocks evolved emergence rhythm with τ longer than *controls* and PRC with large phase-delays and smaller phase-advances (Kumar et al., 2007a). Thus selection for adult emergence during morning and evening hours led to the evolution of divergent circadian clocks. Here we present the analysis of adult emergence rhythm under 12:12 hr LD cycles, conducted after the 55^{th} generation in the light of previous results (Kumar et al., 2007a).

We measured the proportion of flies that emerged during morning and evening selection windows in all replicate populations of *early*, *control* and *late* stocks to assess the effect of selection. The *early* and *late* stocks showed gradual increase in the proportion of flies emerged during morning and evening hours respectively with increasing generations of selection (Figure 3a, b). Selection for morning and evening emergence was carried out on large, outbred populations (harbouring substantial within-population genetic variation) of fruit fly *Drosophila melanogaster* and selection was imposed on four such independent, replicate populations under each stock. We also simultaneously maintained four replicate *control* populations in identical manner except that they were not under conscious selection for timing of emergence. The proportion of flies emerged during morning and evening hours has been more or less constant throughout the course of selection experiment in the *control* stocks. Consistent increase in the morning and evening emergence in four large, outbred independent replicate selected stocks compared to control stocks indicate that evolution of preference for morning and evening emergence in *early* and *late* stocks is a response to imposed selection (Garland and Rose, 2009).

Analysis of generation-wise morning and evening emergence data of *early* and *late* stocks collected for 180 generations shows that rate of divergence of *early* and *late* stocks from each other and from *controls* was high for the first 70 generations. We found that although divergence continued throughout the selection experiment, direct response to selection did not change much during the last 100 generations or so, which suggests that direct response to selection reached saturation somewhere between 85th and 100th generation of selection. Although at a slow rate, continued response to selection suggests the presence of substantial genetic variance for the timing of emergence in fruit fly populations.

Evolution of morning and evening emergence in *early* and *late* stocks implies change in the daily distribution of emergence and therefore we examined the emergence waveforms of *early*, *control* and *late* stocks. Emergence waveforms of *early*, *control* and *late* stocks presented here (Figure 4) represent the current status of emergence waveforms (at the 180th generation of selection). Analysis of various quantifiable features of emergence waveforms (h, ψ_{PK}, ψ_{ON}, ψ_{OFF} and w) showed that daily distribution of emergence in *early* stocks was more consolidated and phase-advanced than *controls*, while daily emergence distribution of *late* stocks was more spread out and phase-delayed relative to *controls*. Divergence of daily emergence distribution of *early* and *late* stocks explains the direct response to selection in the form of increased emergence during morning and evening selection windows (Figure 3a, b). Current emergence waveforms of *early*, *control* and *late* stocks were consistent with those reported for the same stocks at around generation 55. We found that emergence waveforms of these flies did not undergo significant change in shape and phase/timings over the last 100 generations, which corroborates our finding that direct response to selection in *early* and *late* stocks stabilized around generation 85 (Figure 3a, b).

Chapter 3

Characterization of circadian activity/rest rhythm of *early* and *late* stocks of fruit flies *Drosophila melanogaster*

Introduction

Adult emergence and activity/rest behaviors have been used extensively as read-outs to study the circadian clocks in fruit fly *Drosophila melanogaster*. As each developing individual emerges as adult only once in its lifetime, rhythmicity in adult emergence is observed only in asynchronous population of developing individuals (Saunders, 1992). Emergence of adult flies from pupal case was the first behavior used to study circadian rhythms in *Drosophila* (Allada and Chung, 2010) and in the pre-genetic era, extensive use of emergence rhythm contributed immensely to our understanding of formal properties of circadian clocks, while our understanding of molecular mechanisms underlying *Drosophila* clocks have primarily come from circadian mutants identified using activity/rest rhythm (Saunders, 1992; Price, 2005). Short or long period mutants in *D. melanogaster* identified till-date have shown that these mutations alter both behaviours in a similar manner (Konopka and Benzer, 1971, Sehgal et al., 1994). Although the neuronal circuitry underlying activity/rest rhythm is well characterized in fruit flies, very little is known about that regulating emergence rhythm. Attempts to understand the neuronal bases of emergence rhythms using mutants revealed that molecular clocks in brain neurons and cells in prothoracic gland, a tissue required for development, are essential for this rhythm (Liu et al., 1988; Emery et al., 1998; Myers and Sehgal, 2003). The above evidence indicates that at the least, common molecular oscillators underlie circadian rhythms of emergence and activity/rest rhythms in *Drosophila*.

We created *early* and *late* populations of fruit fly *D. melanogaster* in a long-term laboratory selection experiment for emergence during morning and evening hours (Kumar et al., 2007a). With increasing generations, there was a gradual increase in emergence during morning and evening hours in the *early* and *late* stocks. Careful analyses of emergence rhythm revealed that *early* and *late* populations evolved divergent time course and waveform. Emergence starts in *early* populations well before lights come on and ends before lights go

off, while it starts after lights-on and continues well beyond lights-off in *late* stocks, implying the evolution of differential preference for emergence time in these flies. Evolution of divergent emergence waveforms in *early* and *late* stocks was also accompanied by divergence in their circadian clocks underlying emergence rhythms. The *early* stocks evolved shorter circadian period (τ~23.5-hr) compared to *control* (~24.1-hr) stocks, whereas *late* populations evolved longer τ (~24.6-hr). The *early* and *late* stocks also evolved divergent photic phase response curves (PRC) with *early* flies showing smaller phase-delays and larger phase-advances compared to *controls*, whereas *late* flies showed larger phase-delays and smaller phase-advances. The central importance of photic PRC in determining the phase of entrainment in a wide variety of organisms is also well recognised due to non-parametric model of entrainment, which exclusively uses τ and PRC (Johnson, 1999). Therefore, the correlation between emergence waveforms and clock properties (τ and PRC) of *early* and *late* flies suggest that the divergent emergence waveforms of *early* and *late* populations have stemmed from the differences in circadian oscillators regulating emergence rhythms (Kumar et al., 2007a).

In case of *early* and *late* flies, selection was primarily imposed on the timing of emergence, and flies were not under conscious selection for the timing of any adult behavior. Although, circadian clocks underlying emergence rhythms of *early* and *late* populations have been well characterised, it is not yet clear whether circadian clocks underlying activity/rest rhythms of *early* and *late* populations also evolve in the same way as those underlying emergence rhythm. Preliminary studies done in the 50th generation of selection had shown that τ of activity/rest rhythm in *early* and *late* populations had evolved in a manner similar to emergence rhythm (Kumar et al., 2007a), however, whether the photic PRC of activity/rest rhythm have also diverged in a manner observed in emergence rhythm was still unknown. In order to characterize circadian clocks underlying activity/rest rhythm of *early* and *late* flies,

we estimated the τ by monitoring their activity/rest behavior at generation 150th under constant dark (DD) conditions, and also assayed the phase-dependent photosensitivity of the underlying circadian oscillator by constructing a PRC using brief pulses of light at six phases of the circadian cycle. Photic PRC of *early* and *late* flies obtained by monitoring activity/rest rhythm was found to be similar in terms of time course and waveform to already reported PRC for emergence rhythms (Kumar et al., 2007a).

Materials and methods

***Recording of fly activity/rest behavior*:** Standardised flies which were cultured on banana-jaggery (BJ) food at a larval density of ~300 per food vial (6-ml food) under 12:12 hr light/dark (LD) cycles at 25 ^{o}C were collected over 2-3-days. Virgin adults of an average age of 3-days were loaded individually into activity recording tubes provided with sufficient amount of corn-sucrose-yeast food and were installed on Drosophila Activity Monitoring system (Trikinetics Inc., Waltham, MA, USA).

***Estimation of circadian period (τ) of activity/rest rhythm*:** For estimating the τ of activity/rest rhythm, locomotor activity behaviour of virgin males and females was recorded for 12-13-days under DD at constant temperature of 25 ± 0.5 ^{o}C and relative humidity of 75 ± 5%. The τ of activity/rest rhythm was estimated for individual flies by analyzing activity data collected in 5-min bin for a minimum of 10-days using Lomb Scargle Periodogram in CLOCKLAB (Actimetrics, IL, USA). Activity data collected for the first two days were excluded from the analyses.

***Photic phase response curve* (PRC):** PRC assay was conducted on a separate set of flies, in which flies of average age 3-days were loaded into recording tubes and were then entrained to LD at 25 ^{o}C and light intensity of 40-lux for 6-cycles. We transferred individual flies to fresh tubes gently during the light phase of fifth LD cycle to prevent vials from drying out.

***Schedule of light pulse treatment for phase response curve* (PRC):** At the end of the light phase on the 6th LD cycle, flies were transferred to DD. On the 1st day in DD, flies were given white light pulses of 5-min duration and intensity of 70-lux by physically shifting DAM systems to the light pulse box at 6 equally spaced time-points. Flies were put back into DD after the light pulse. Each set of flies was given a single light pulse at one of the following time-points – CT02, CT06, CT10, CT14, CT18 and CT22, and were then left to remain under DD. These flies were named as "pulsed" flies. Additional six sets of "handling control" flies were also maintained where each set was only physically handled exactly like the pulsed flies but were not presented with light pulse. Activity recording of all three types of flies continued for next 12-days.

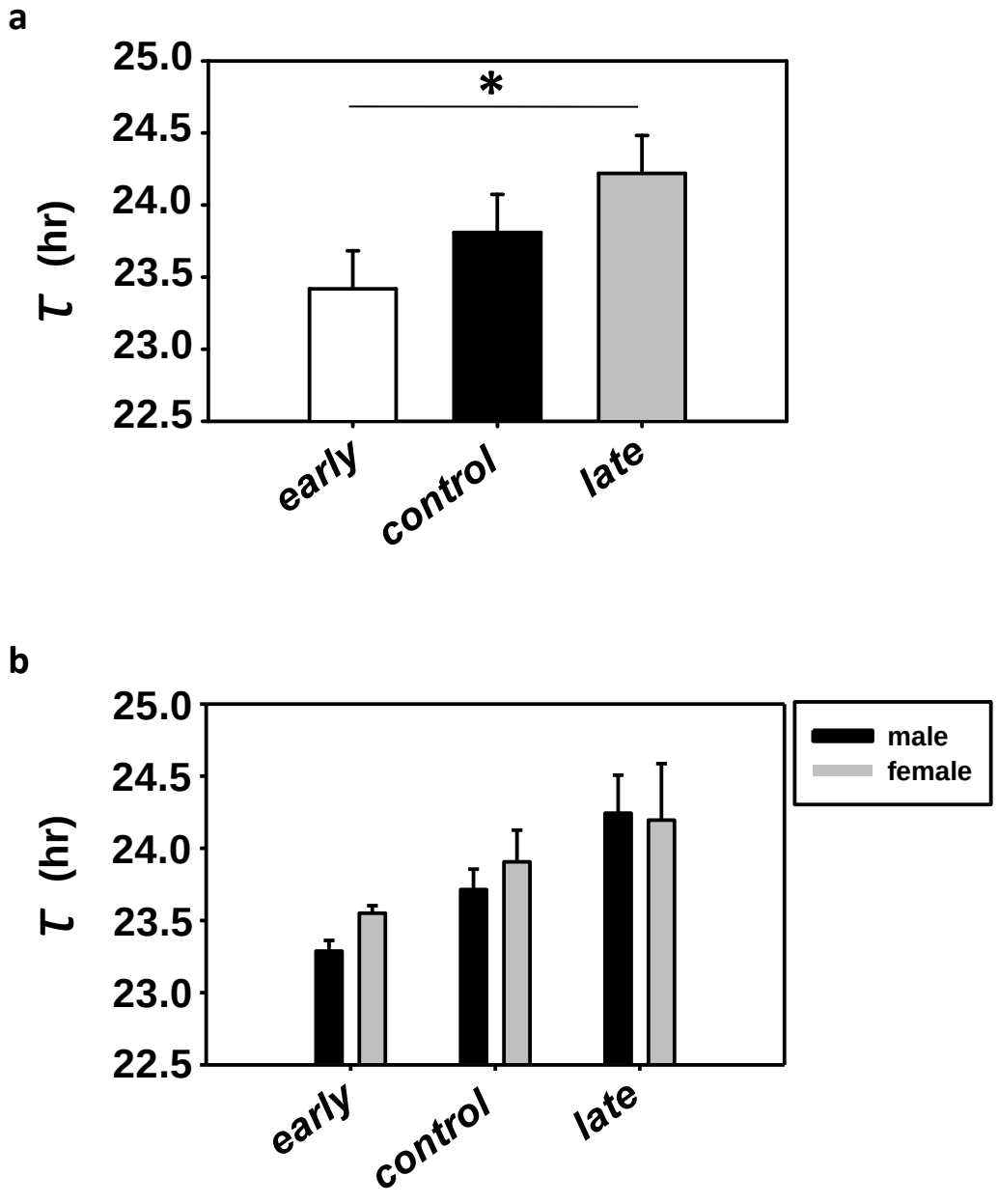
***Estimation of phase shifts*:** To estimate the magnitude and direction of phase shift in activity/rest rhythm induced by light pulse, activity offset in each cycle was identified based on actogram of individual flies. A regression line was drawn through the activity offsets during entrainment to LD and extrapolated to predict the time-point at which it would intersect the time axis of the 7th day (day of the light pulse). Another regression line was drawn through offset points identified for days 4 to 10 under DD, and was extrapolated backwards to locate the point of intersection with the time axis. Timing of this point was subtracted from that of intersection of regression line from the entrained condition. The time difference in hours between these two points represents magnitude of phase change whereas the sign indicated direction of change. Negative sign indicates delays and positive sign advances in the phase of activity/rest rhythm. Magnitude and direction of the phase shift were calculated for experimental and control flies. Differences in the mean phase shifts (in hours) between the pulsed and handling controls at a given time-point were taken as the magnitude and direction of phase shift due to light pulse alone.

***Statistical analyses*:** Mean τ of males and females was calculated for each replicate population and these population means were used as units of analysis. Effects of stock (S) and gender (G) and S × G interaction was tested by performing a mixed model analysis of variance (ANOVA), in which S and G were treated as fixed factors while replicate populations (B) were treated as random factor. Light induced phase shifts were calculated for each of the populations as described. Phase shifts elicited by light pulses at six phases were estimated for each replicate population and were subjected to a mixed model ANOVA, to test the effects of the stock (S), phase of light pulse (P) and their interaction (S × P) on phase shift. Replicate populations/blocks (B) were treated as random factor whereas S and P as fixed factors crossed with replicates (B). DRC was analysed in separate ANOVA in which light intensity was incorporated as one of the fixed factors. All the pair-wise multiple comparisons were carried out using Tukey's test. Error bars in figures are 95% comparison intervals (95%CI) calculated from minimum significant difference in Tukey's test and therefore two means were considered significantly different if there was no overlap between their error bars.

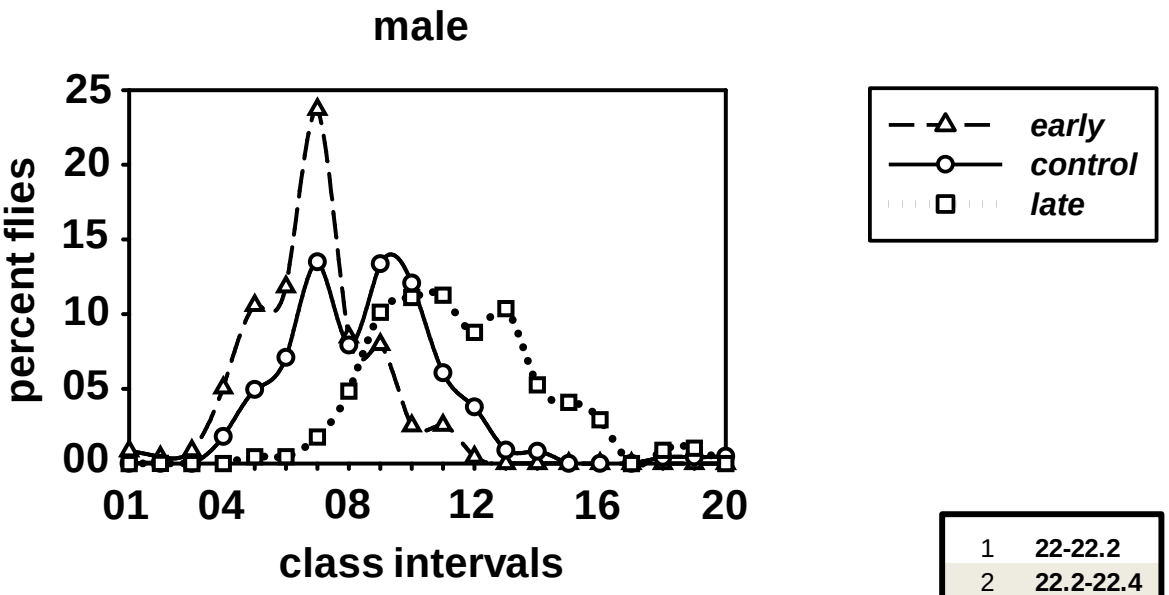
Results

***Circadian period of early and late flies*:** The τ of activity/rest rhythm in *early* flies was shorter (~23.4-hr) compared to *controls* (~23.85-hr), while that in *late* flies was longer (~24.25-hr; Figure 1a). ANOVA on the mean τ values revealed a statistically significant effect of stocks (S) ($F_{2,6}$ = 21.77, $p < 0.001$), however, the effect of gender (G) and S × G interaction did not reach statistical levels of significance (Table 1). Given that S × G interaction was not significant, post-hoc comparisons could not be carried out on different levels of S and G. Pair-wise comparisons showed that τ of *early* and *late* stocks were significantly different from each other but none of them were found to differ significantly from *controls*, which was also evident in the frequency distribution of τ in the selected and

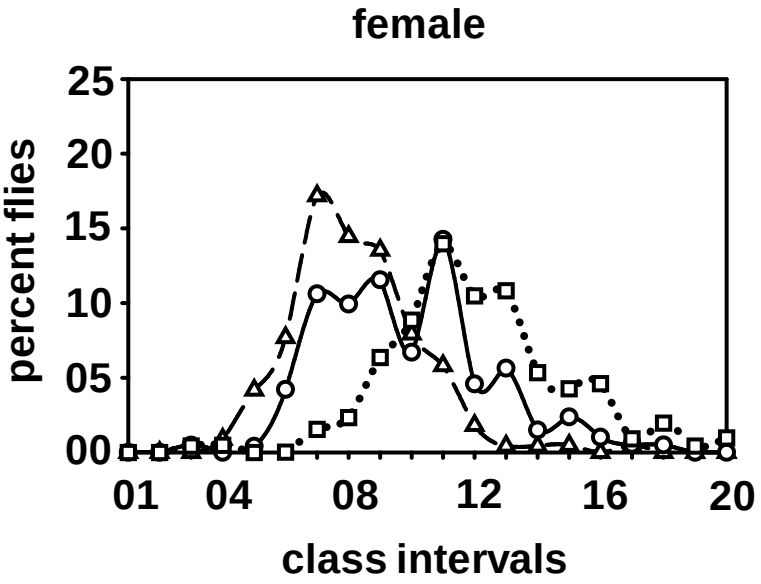
Figure 1. (a) Mean circadian period (τ) of activity/rest rhythm in *early*, *control* and *late* stocks. Mean τ of each stock is an average of four replicate population means. The τ of individual flies was calculated by analysing 10 days of activity data using Lomb Scargle periodogram. Error bars are 95% comparison intervals (calculated using minimum significant difference in Tukey's test), therefore two means are significantly different from each other if their error bars are non-overlapping. (b) Mean τ of male and female flies in *early*, *control* and *late* stocks. Error bars are standard error of means. (c) Frequency distribution of τ of activity/rest rhythm in *early*, *control* and *late* stocks. Numbers on the *x*-axis represent class intervals of τ and correspondence is given in the adjacent table. The *y*-axis is percentage of flies falling in any particular τ class interval. Left and right panels represent the frequency distribution of male and female flies, respectively.



c



d



1	22-22.2
2	22.2-22.4
3	22.4-22.6
4	22.6-22.8
5	22.8-23
6	23-23.2
7	23.2-23.4
8	23.4-23.6
9	23.6-23.8
10	23.8-24
11	24-24.2
12	24.2-24.4
13	24.4-24.6
14	24.6-24.8
15	24.8-25
16	25-25.2
17	25.2-25.4
18	25.4-25.6
19	25.6-25.8
20	25.8-26

control stocks (Figure 1a, c). Frequency distribution of τ (Figure 1c) shows that percentage of flies in short period classes was greater in *early* stocks and lesser in *late* stocks, whereas proportion of flies in long period classes was greater in *late* stocks and lesser in *early* stocks. Although, τ did not differ significantly between males and females, neither did S × G interaction reach statistical levels of significance, τ of males was shorter than females, at least in *early* and *control* stocks, whereas in *late* stocks it was comparable between males and females (Figure 1b). Together, these results suggest shortening of τ in activity/rest rhythm of *early* flies and lengthening in *late* flies.

***Photic phase response curves (PRC) of early and late flies*:** Shape of PRC in all the three stocks was consistent with a typical light pulse PRC. Flies from all three stocks showed phase-delays at CT14 and CT18 (early subjective night) and phase-advances at CT22 (late subjective night) (Figure 2). ANOVA on the mean phase shifts revealed a statistically significant effect of phase of exposure (P) ($F_{5,15} = 63.46$, $p < 0.001$), but the effect of stocks (S) or S × P interaction was statistically not significant (Table 2), and therefore post-hoc comparisons to test differences among different combinations of levels of factors S and P could not be carried out. However, *late* flies showed larger phase-delays at CT18 (early subjective night) compared to *early* and *control* flies and *early* flies exhibited larger phase-advances at CT22 (late subjective night) than *control* and *late* flies (Figure 2). Overall, PRC of *early* and *late* stocks showed a trend of divergence, with *early* flies showing larger phase-advances and smaller delays, whereas *late* flies showing larger phase-delays and smaller advances.

Figure 2. Photic phase response curves (PRC) of *early*, *control* and *late* flies. Magnitude of phase shifts in circadian hours (*y*-axis) is plotted as a function of time of day expressed as circadian time (*x*-axis). Separate sets of virgin male flies from every replicate population of each stock were subjected to 5 min white light pulses of 70 lux at six phases (CT02, CT06, CT10, CT14, CT18 and CT22) on the first day in constant dark after six days of entrainment to 12:12 hr light/dark (LD) cycles. These flies were denoted as 'pulsed' flies. Another set of flies were used for every light pulse phase which were only subjected to physical disturbance and not the light pulse and were named "handling control". Phase shift elicited by light pulse were calculated by subtracting phase shift in "handling controls" from phase shift in "pulsed" flies. Error bars are standard error of means.

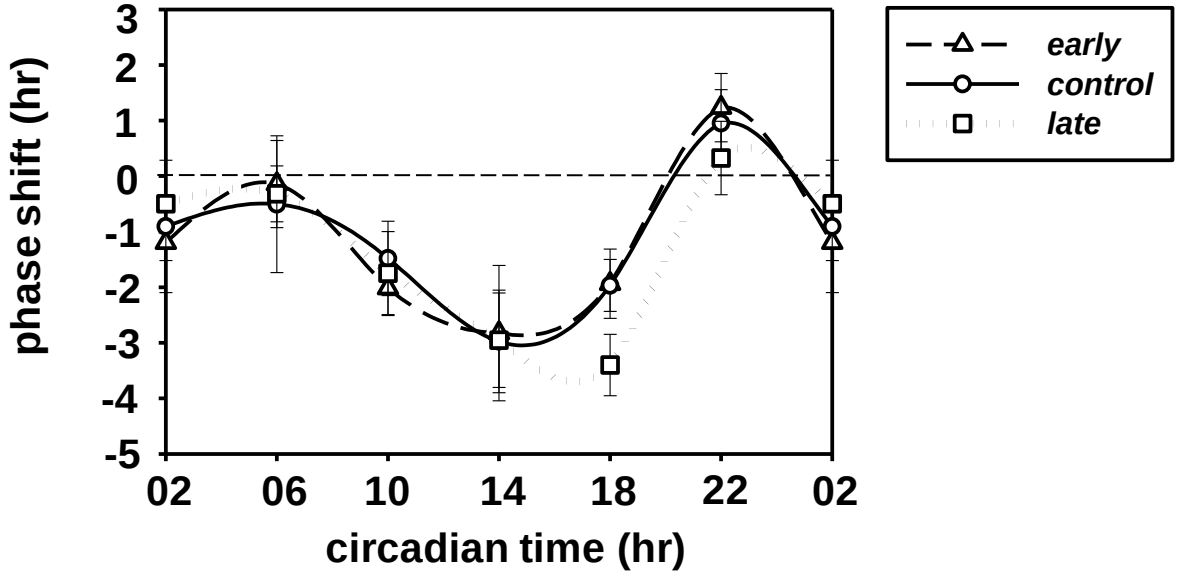


Table 1. ANOVA performed on the τ of activity/rest rhythms in male and female flies from *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2	1.28	6	0.06	21.77	0.001
Gender (G)	1	0.11	3	0.03	3.69	0.15
Block (B)	3	0.12	0	0.00	--	--
S × G	2	0.05	6	0.01	3.61	0.09
S × B	6	0.06	0	0.00	--	--
G × B	3	0.03	0	0.00	--	--
S × G × B	6	0.01	0	0.00	--	--

Table 2. ANOVA performed on the phase shifts elicited by brief pulses of light administered at 6 phases in *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2	0.64	6	1.01	0.64	0.56
Phase (P)	5	23.50	15	0.37	63.46	0.001
Block (B)	3	1.43	0	0.00	--	--
S × P	10	0.79	30	0.50	1.58	0.16
S × B	6	1.01	0	0.00	--	--
P × B	15	0.37	0	0.00	--	--
S × P × B	30	0.50	0	0.00	--	--

Discussion

The *early* and *late* stocks of fruit flies *D. melanogaster* with increased preference for emergence during morning and evening hours were created in a long-term laboratory selection experiment under laboratory LD cycles for morning and evening emergence. Increased morning and evening emergence in *early* and *late* stocks was accompanied by correlated changes in the phases of their emergence waveforms (Kumar et al., 2007a). Emergence waveform of *early* stocks was advanced relative to controls, while that of *late* stocks was delayed. Divergent phasing of emergence rhythm indicates differential entrainment of circadian clocks underlying the rhythm in *early* and *late* stocks. Indeed it was confirmed that τ and PRC of emergence rhythm of the *early* and *late* stocks were significantly different (Kumar et al., 2007a). Activity/rest rhythm assays were done on these stocks at generation 150, which revealed that τ of activity/rest rhythm was shorter (~23.45-hr) in *early* flies compared to *controls* (~23.8-hr), and longer (~24.2-hr) in *late* flies (Figure 1). These results are consistent with those from assays performed around at generation 50 (*early* ~23.6-hr, *control* ~23.75-hr and *late* ~24.2-hr; Kumar et al., 2007a). Near match of τ between assays done at 50^{th} and 150^{th} generations suggests that the response to selection for morning and evening emergence of *early* and *late* populations, at least in terms of changes in clock properties, has reached its saturation. Interestingly, the mean τ of activity/rest rhythm in the *early* and *late* stocks were very similar to those of adult emergence rhythms (*early* ~23.5-hr, *control* ~24.1-hr and *late* ~24.6-hr).

Photic PRC of activity/rest rhythm of these stocks showed characteristics typical of photic PRC reported earlier for other organisms, with phase-delays during early subjective night, phase-advances during late subjective night and a dead zone during the subjective day (Figure 2). For most parts, PRC of all three stocks looked similar except at CT18 and CT22, phases where maximum phase delays and advances were observed. Phase-delays at CT18 in

late stocks were larger than *early* and *controls* and phase-advances in *early* and *control* stocks at CT22 were larger than *late* stocks. Thus, *early* and *late* stocks also showed a trend of divergence in their photic PRC for activity/rest rhythm (Figure 2). This trend was similar to their emergence rhythm PRC as observed ~120 generations earlier. Such similarity in the τ and PRC of emergence and activity/rest rhythm hints at a strong connection between the mechanisms underlying emergence and activity/rest rhythms in *early* and *late* stocks. This is consistent with previous reports of correlation in τ of emergence and activity/rest rhythms in *Drosophila*. For example, short and long period mutations in several clock genes altered the τ of activity/rest and emergence rhythms in a similar manner (Konopka and Benzer, 1971; Sehgal et al., 1994; Rothenfluh et al., 2000). Such correlations have also been reported for egg-laying and activity/rest rhythms (McCabe and Birley, 1998). Correlation between the properties of circadian clocks underlying emergence and activity/rest rhythms observed in *early* and *late* flies observed in the present study is thus consistent with those reported previously (Kumar et al., 2007a).

Divergence of circadian phenotypes of *early* and *late* flies is a result of long-term laboratory selection and thus the differences can be attributed to the genetic architecture of the underlying traits under selection. The relationship between phase of an entrained rhythm and circadian period is now well established (Pittendrigh and Daan, 1976; Sharma et al., 1998; Roenneberg et al., 2003a; Sharma and Chidambaram, 2003). The phase of a circadian rhythm leads more or lags less relative to that of the zeitgeber for clocks with shorter τ and *vice versa* (Pittendrigh and Daan, 1976). This explains the advantages associated with short and long τ of emergence rhythm in *early* and *late* flies, respectively, which were selected for emergence during early morning (advanced phase) and late evening (delayed phase). However, adult traits in *early* and *late* stocks were not under any conscious selection for timing of any rhythmic behavior(s). Evolution of divergent circadian clocks underlying

activity/rest rhythm in the absence of any selection, suggests that it is primarily a correlated response resulting from genetic linkage/pleiotropic effects. In other words, despite the absence of selection on timing of adult behavior(s), maintenance of strong correlation between emergence and activity/rest rhythms after 150 generations of selection suggests that the clocks regulating these rhythms cannot be decoupled. It could also be possible that disadvantages associated with having τ of activity/rest rhythm deviating from optimum τ (as in controls) in adults may not be large enough to act as selection against divergence of adult clocks and to break the correlation.

Chapter 4

Early and late emerging fruit flies *Drosophila melanogaster* differ in their sensitivity to light during morning and evening

Introduction

Drosophila melanogaster fruit flies exhibit a 24-hr rhythm in adult emergence. Under 12:12 hr laboratory light/dark (LD) cycles, emergence primarily occurs during the light phase, with the peak just after the dark-to-light transition (Saunders, 1992). The incidence of emergence reduces gradually as the day progresses, leading to a zone of non-emergence by evening. The circadian clock's control of the 24-hr emergence rhythm is well known, and a stable phase-relationship of the rhythm with LD cycles results from entrainment of the underlying circadian clocks (Saunders, 1992). In one study, Kumar et al. (2007a) derived *early* and *late* populations of *D. melanogaster* fruit flies exhibiting increased adult emergence during morning and evening hours, respectively. These populations were derived in a long-term study by imposing selection for morning or evening emergence. From the analysis of their emergence waveforms, it was evident that increased preference for emergence during the morning or evening hours occurred through the evolution of characteristic emergence waveforms in *early* and *late* flies, which was different from *controls* (Kumar et al., 2007a). Evolution of such divergent emergence waveforms in large, outbred, random mating replicate populations in response to selection for morning or evening emergence indicated that the emergence waveforms of *early* and *late* populations were "adaptive". Furthermore, *early* and *late* populations also evolved correlated changes in their circadian clocks underlying adult emergence and activity/rest rhythms (Kumar et al., 2007a, b). The *early* and *late* populations had, respectively, a shorter and longer clock period (τ) than *controls*. These populations also evolved divergent photic phase response curves (PRC) for adult emergence (Kumar et al., 2007a) and activity/rest rhythms (*Koustubh Vaze, Nisha N Kannan, Nikhil KL, and Vijay Kumar Sharma, unpublished data*). Relative to *controls*, *early* populations showed smaller

delays and larger advances, while *late* populations showed larger delays and smaller advances (Kumar et al., 2007a).

The relationship between the phase of the entrained rhythm (ψ) and τ is now well established (Pittendrigh and Daan, 1976; Sharma et al., 1998; Roenneberg et al., 2003a; Sharma and Chidambaram, 2003). For a given period of the environmental LD cycles (*T*), the phase of the entrained rhythm leads more or lags less relative to that of the zeitgeber if τ is shorter than *T* or *vice versa* (Pittendrigh and Daan, 1976). Indeed, relative to wild-type controls, *period* mutations of *D. melanogaster* with shorter (~19-hr) and longer τ (~29-hr) exhibited an advanced and delayed evening peak of the activity/rest rhythm under LD cycles (Hamblen-Coyle et al., 1992). The τ and emergence waveform under LD cycles of *early* and *late* populations also exhibited a similar correlation (Kumar et al., 2007a). The functional significance of photic PRC in determining the phase of entrainment is well recognized, and the non-parametric model of entrainment exclusively uses τ and PRCs to predict phase of the entrained rhythm (Johnson, 1999). Therefore, looking at the correlation between emergence waveforms and clock properties (τ and PRC) of *early* and *late* populations, it appears likely that their "evolved emergence waveforms (EEW)" have stemmed from differences in the properties of their circadian clocks (Kumar et al., 2007a). Although, the non-parametric model of entrainment was successful in predicting entrainment to repetitive brief light pulses, it failed to satisfactorily explain entrainment under complete photoperiod regimes, such as LD 12:12 (Daan, 2000; Roenneberg et al., 2010). This limitation in predicting accurately the phase of entrainment under LD cycles was attributed to the lability of τ and PRC, which are believed to change with zeitgeber conditions (Aschoff, 1979; Daan, 2000; Roenneberg et al., 2010). Because of the labile nature of τ and PRC, the effect of light during complete photoperiods, which are typically much longer than light pulses, is not readily deducible from the predictions made on the basis of the light PRC (Sharma and Daan, 2002; Sharma, 2003;

Comas et al., 2006). This suggests that probing the photosensitivity behavior of circadian clocks under LD cycles will be more insightful in understanding the functional significance of diverged τ and PRC for the *early* and *late* flies.

We reasoned that investigating the phase-dependent photosensitivity of circadian clocks under LD cycles would explain the functional significance of PRC for *early* and *late* EEWs, although no standard procedure is available to study this. Therefore, we designed an assay based on the following premise – (i) Entrainment of circadian oscillators can be effected by modulation of the velocity of phase progression in a phase-dependent manner (Swade, 1969). (ii) Change in τ_{LL} relative to τ_{DD} is correlated with the shape of the PRC (Daan and Pittendrigh, 1976); it was found that circadian systems having PRC with large advance to delay ratio showed shortening of their τ_{LL} relative to τ_{DD}, whereas those having PRCs with small advance to delay ratio exhibited lengthening of τ_{LL}. (iii) There was good agreement between τ_{LL} predicted from the velocity response curves (VRC, constructed from PRCs) and those observed empirically (Daan and Pittendrigh, 1976), which suggests that circadian clocks entrain to LD cycles via light-induced acceleration of phase progression during morning and deceleration during evening, and also predicts the presence of a 'dead zone', when the oscillator is refractory to light (Daan and Pittendrigh, 1976; Roenneberg and Foster, 1997; Taylor et al., 2010). These predictions imply that during the entrained state, the velocity of the circadian oscillator is modulated by light, primarily during stretches contiguous with morning and evening transitions, while light in the intervening duration may be superfluous (Taylor et al., 2010). It also implies the shape of the VRC is a determinant of the length of light portions contiguous to the morning and evening hours, which may be essential for circadian entrainment. From this, it could be predicted that animals with different PRC may need light for different durations during the morning and evening hours. Given that *early* and *late* populations exhibited divergence in their PRC, it is likely that they

use light for different durations during the morning and evening hours in order to achieve their EEWs.

Entraining actions of a wide range of photoperiods can be successfully simulated by skeleton photoperiods consisting of two brief light pulses/cycle, marking the start and end of the photophase, provided the length of the dark interval encompassed by the two light pulses is shorter than $\tau/2$ (Pittendrigh, 1964). Therefore, in principle, light regimes consisting of only the essential portions of photoperiod should be sufficient to entrain circadian clocks in a manner similar to their entrainment by complete photoperiod regimes. Such light regimes will henceforth be referred to as light requirement schedules (LRS). For practical purposes, we defined LRS for any population as a light regime which can entrain the emergence rhythm to produce the EEW of that population. We used LRS of *early* and *late* populations to test our hypothesis that these populations may require light for different durations during the morning and evening hours to achieve their EEWs. Our hypothesis is based on the model which assumes that light contiguous with morning and evening transitions form the primary entraining stimuli; therefore, we approximated the LRSs of *early* and *late* populations by studying their emergence waveforms under three different skeleton photoperiod (SPP) regimes. The SPP regime in which a population exhibited the most resemblance to its EEW was considered as the LRS for that population. The results showed that two diametrically opposite SPP regimes represented LRSs of *early* and *late* populations, suggesting the temporal profile of photosensitivity of *early* and *late* circadian clocks controlling the emergence rhythms of *D. melanogaster* fruit flies differ while they are entrained to 12:12 hr laboratory LD cycles.

Materials and methods

Adult emergence assay: The adult emergence waveform was estimated for selected and control populations under four different light regimes described in Figure 1. Briefly, they

were (i) LD 12:12, (ii) two 0.25-hr of light interruptions 11.5-hr apart in an otherwise dark condition (SPP1), (iii) 0.25-hr light followed by 5.75-hr darkness and 6-hr light followed by 12-hr darkness (SPP2), and (iv) 6-hr light followed by 5.75-hr darkness and 0.25-hr light followed by 12-hr darkness (SPP3). For the adult emergence rhythm assay, standardized populations kept under LD 12:12 cycles in plexiglass cages were provided with yeast paste (spiked with few drops of acetic acid) on petridish containing banana-jaggery food for 3-days prior to egg collection. Eggs were collected in a manner similar to the stock maintenance regime, except that here 10 vials were maintained/population for every light regime. After egg collection, vials were maintained under LD cycles for the first 3-days, and then developing cultures were introduced at ZT00 of the 4th day into different photoperiod regimes (Figure 1). After the onset of emergence, these vials were monitored every 2-hr for adult emergence, and the number of flies that emerged in the preceding 2-hr bin was recorded for 4-5-days. Cycles (days) in which at least 25 flies emerged were subjected to further analysis. For every selected cycle, the number of flies that emerged per 2-hr bins was normalized by dividing the fly count of the 2-hr bins by the total number of flies that emerged in that cycle. For each vial, the percentage of flies that emerged in the 2-hr bins was averaged across four cycles. The adult emergence waveform of individual vials was analyzed to estimate ψ_{PK}, ψ_{ON}, and ψ_{OFF} of the adult emergence rhythm. Emergence data were analyzed to estimate the temporal position of the peak (ψ_{PK}), onset (ψ_{ON}), and offset (ψ_{OFF}), plus the gate-width (w) and height of the emergence peak (h). The time when maximum adult emergence was recorded under the different light regimes was taken as ψ_{PK}. The time when the percentage of flies emerging in 2-hr bins increased above or fell below an arbitrary cut-off of 5% for the first time was defined as ψ_{ON} and ψ_{OFF}, respectively. This time point, referred to as the onset of emergence, and ZT00 was used as phase reference to estimate the phase-relationship of the emergence rhythm with the light regime (Figure 1). ψ_{PK}, ψ_{ON} and ψ_{OFF} were estimated as

time interval between lights-on (ZT00) and peak, onset and offset of the emergence rhythm. *w* was estimated as the duration between ψ_{ON} and ψ_{OFF}. Percentage emergence per 2-hr bin during the emergence peak was used as an estimate of *h*. All the waveform characteristics were first calculated for normalized waveforms from individual vials and were then averaged over all replicate vials to obtain an estimate for each replicate population.

We used the sum of squares (SS) of the differences in the two waveforms over all time points to estimate the degree of match between any pair of waveforms. SS was calculated for individual blocks of each population to estimate the degree of match between emergence waveforms under LD and in each of the three skeleton photoperiod regimes.

***Statistical analysis*:** Replicate populations were considered as experimental units (blocks); therefore, for all measures of the adult emergence waveform, block means were used as the unit of analysis. Effect of selection (P) and light regime (L) was tested by subjecting block means to mixed-model analysis of variance (ANOVA), where replicate populations (blocks) were treated as a random factor, whereas population, light regime, and time point were treated as fixed factors crossed with replicates (blocks). This enabled testing the significance of main effects of fixed factors and their interactions. Post-hoc multiple comparisons were done by setting 95% confidence intervals (95%CI) around the means using the minimum significant difference calculated by Tukey's HSD test at $\alpha = 0.05$. Thus, two means were considered significantly different if there was no overlap between their error bars. All statistical analyses were implemented using STATISTICA^{TM} for Windows Release 5.0 B (StatSoft, 1995).

Figure 1. (a) Each of the four panels represents light regimes experienced by each of the four sets of developing vial cultures of any given population (*early*, *control*, and *late)*, from the time of egg collection to the end of emergence assay. For each population, eggs were collected in 40-replicate food vials during light phase of day 1 from the standardized adult population maintained in 12:12 hr light/dark (LD) cycles. For the first 3-days, all the 40 replicate developing vial cultures of any given population were kept in LD cycles as depicted in first 3 layers of each panel. At ZT00 of the 4^{th} day, 40 developing vial cultures of each population were randomly divided into four sets of 10 vial cultures each. Each set was randomly assigned to one of the four treatment light regimes and was maintained in the assigned light regime until the end of the assay, as depicted by layers 4^{th} to 14^{th} of each panel. White portion represents light phases, whereas gray portions represent dark phases of the light regimes. (b) Schedule and duration of light and dark phases in the four light regimes that were applied to replicate vial-cultures of *early*, *control*, and *late* populations from ZT00 on the 4^{th} day. LD cycles, one of the four treatment light regimes, was continued from the LD cycle of the previous 3-days, and as per convention the lights-on phase of LD cycle was considered as ZT00 and lights-off as ZT12. Three skeleton photoperiod (SPP) regimes - SPP1, SPP2, and SPP3 - were applied in such a way the SPP regimes acted as skeleton of the photoperiod from the previous LD cycle; therefore, reference phases of the SPP were extrapolated from the phases of previous LD cycles. Thus in any SPP regime, lights-on, which could be extrapolated from lights-on in the previous LD, was considered as ZT00 and lights-off, which could be extrapolated from lights-off in previous LD, was considered as ZT12.

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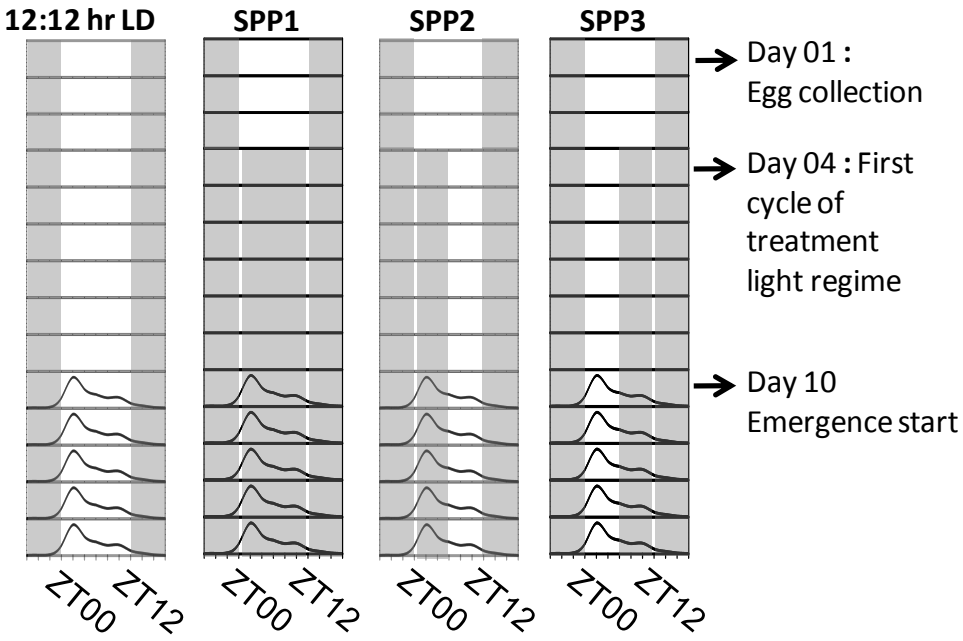
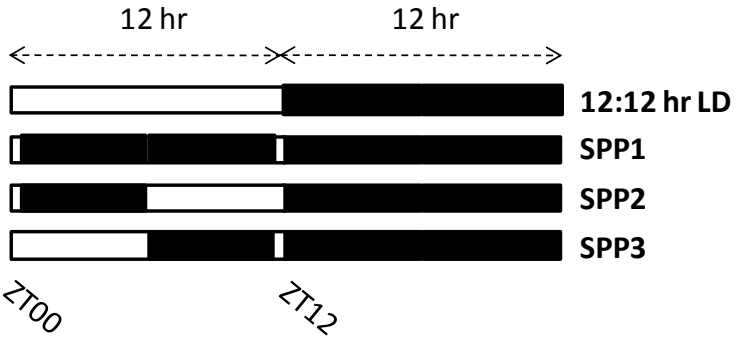
a


12:12 hr LD
SPP1
SPP2
SPP3
Day 01 :
Egg collection
Day 04 : First
cycle of
treatment
light regime
Day 10
Emergence start
ZT00
ZT12
ZT00
ZT12
ZT00
ZT12
ZT00
ZT12

b


12 hr
12 hr
12:12 hr LD
SPP1
SPP2
SPP3
ZT00
ZT12

Results

***Populations achieved their EEWs in a light regime-dependent manner*:** We assessed the effect of different light regimes on the emergence waveforms of selected and control populations by measuring some of the quantifiable features of the emergence waveform, such as height of emergence peak (*h*), phase of emergence peak, onset and offset (ψ_{PK}, ψ_{ON} and ψ_{OFF}), and width of the emergence gate (*w*). The SPP for any population, whether it did or did not mimic the LD to achieve its EEW, was assessed by comparing the *h*, ψ_{PK}, ψ_{ON}, ψ_{OFF}, and *w* values to those found under LD. The SPP regime showing all waveform characteristics matching the LD waveform was considered to mimic LD and thus was designated as the LRS for that population. Figure 2 shows the emergence waveforms of all the three populations under SPP1, SPP2 and SPP3 along with their EEWs (LD waveforms; Table 1).

***Height of emergence (h)*:** In all three populations, *h* under SPP1 was significantly reduced compared to LD. The *h* of *early* populations under SPP2 was similar to that under LD, but for *late* populations under SPP2 and SPP3, it resembled those under LD (Figure 3a; Table 1). ANOVA on *h* data showed a statistically significant effect of selection (P) ($F_{2,6}$ = 75.95; p < 0.001), light regime (L) ($F_{3,9}$ = 35.70; p < 0.001), and P × L interaction ($F_{6,18}$ = 10.95; p < 0.001; Table 2). Post-hoc multiple comparisons showed that for all three populations, *h* was significantly reduced under SPP1 compared to LD (Figure 3a). Population-specific comparisons of emergence waveforms under the different light regimes showed *h* of *early* and *control* populations under SPP3 was significantly shorter than that under LD, whereas *h* of the two populations under SPP2 was comparable to that under LD. For *late* populations, *h* under SPP2 and SPP3 did not differ under LD (Figure 3a). The *h* of *early* populations was comparable to that under LD only in SPP2, while *h* of *late* populations under SPP2 and SPP3 was similar to that in LD.

Figure 2. Adult emergence waveforms of *early*, *control*, and *late* populations under SPP1, SPP2, SPP3, and LD. Emergence waveforms of *early* and *control* populations under SPP2 resembled their LD waveforms, while the *late* waveform under SPP3 resembled its LD waveform. The emergence waveforms were estimated by recording flies that emerged in 2-hr bins over the 4-days from individual replicate vials. Percentage emergence at any given point of time represents an average over four replicate populations. Percentage of adults emerging in the 2-hr bins is plotted along the *y*-axis, and time of day in hours of Zeitgeber Time (ZT) is plotted along the *x*-axis.

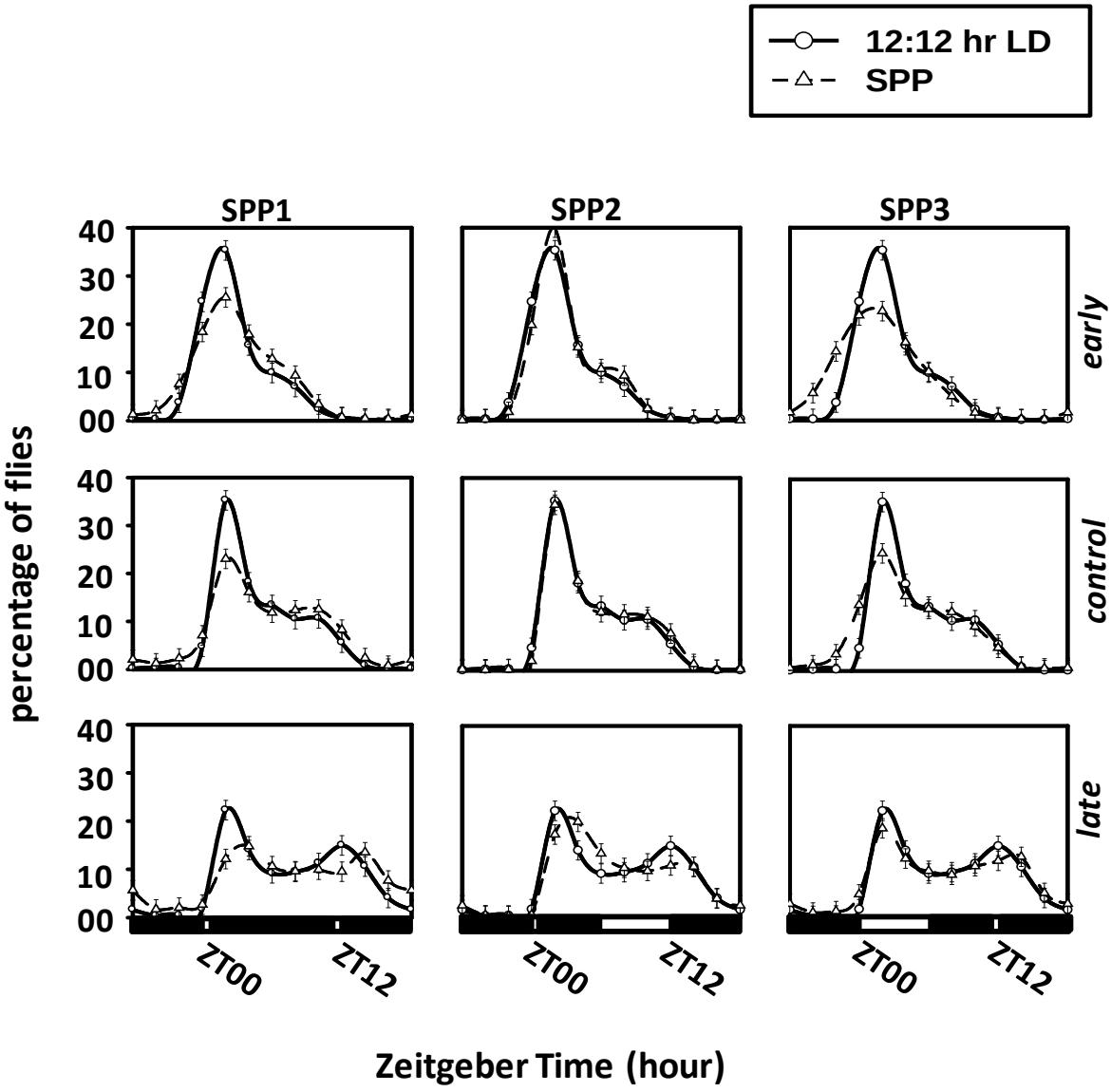
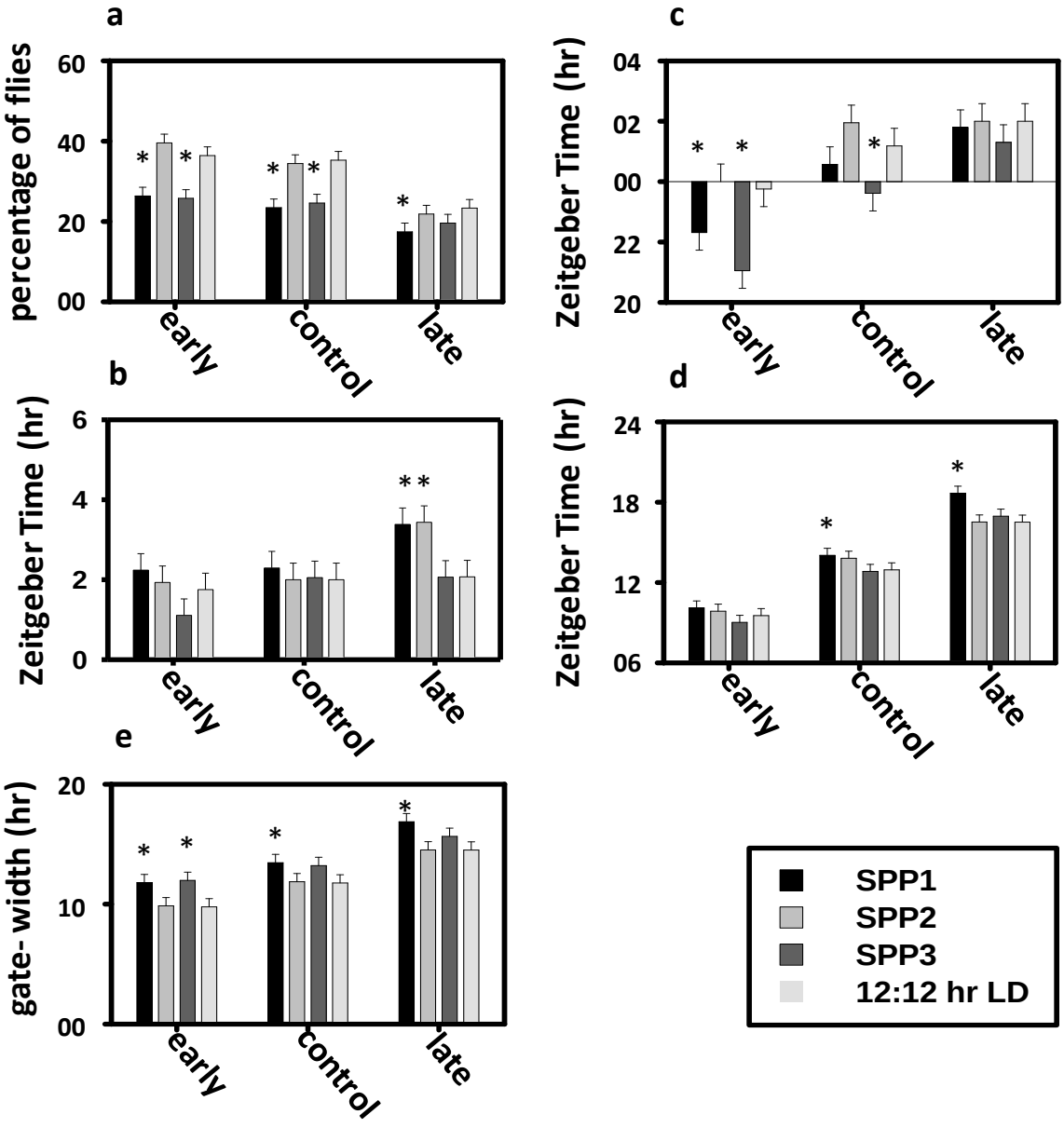


Figure 3. Many features of the emergence waveform of the selected populations are altered compared to controls. Shown are the: (a) height of the emergence peak (*h*), (b) phase of the emergence peak (ψ_{PK}), (c) onset (ψ_{ON}) and (d) offset (ψ_{OFF}) of emergence, and (e) width of emergence gate (*w*) of *early*, *control*, and *late* populations under SPP1, SPP2, SPP3, and LD regimes. Phase values were estimated in hours of Zeitgeber Time. Mean values plotted along *y*-axis represent the average of replicate populations. Asterisks (*) in the figures indicate statistically significantly difference between the test photoperiod and LD, and its absence imply lack of statistically significant differences. Error bars in all panels are 95% Confidence Interval (95%CI) for visual hypothesis testing.



Phase of emergence peak (ψ_{PK}): The ψ_{PK} of *late* populations under SPP1 was phase delayed by ~1.5-hr compared to LD, while the ψ_{PK} of *early* and *control* populations remained unaffected. The ψ_{PK} of *early* populations under SPP2 and SPP3 was similar to LD, while that of *late* populations was comparable to LD only in the SPP3 regime (Figure 3b; Table 1). ANOVA on ψ_{PK} data revealed a statistically significant effect of P ($F_{2,6}$ = 21.68; $p < 0.01$), L ($F_{3,9}$ = 9.28; $p < 0.01$), and P × L interaction ($F_{6,18}$ = 6.36; $p < 0.01$; Table 3). Post-hoc comparisons revealed the ψ_{PK} of *early* and *control* populations under SPP1 was not different from that in LD, but the ψ_{PK} of *late* populations was phase delayed by ~1.5-hr under SPP1 compared to that in LD (Figure 3b). Under SPP2, the ψ_{PK} of *early* and *control* populations was similar to that in LD, but that of *late* populations was significantly phase-delayed compared to that under LD. In SPP3, the ψ_{PK} of *early* and *control* was equal to that under LD. Under SPP3, the ψ_{PK} of *late* populations was similar to that in LD, but under SPP1 and SPP2 it was different than LD, being significantly phase-delayed (Figure 3b). The ψ_{PK} of the *early* populations under SPP2 and SPP3 was comparable to that in LD, while the ψ_{PK} of the *late* populations was similar to that under LD only in SPP3.

Phase of emergence onset (ψ_{ON}): Under SPP1, ψ_{ON} was affected only in *early* populations, in which it was phase advanced by ~2-hr compared to LD (Figure 3c; Table 1). The ψ_{ON} of *early* populations under SPP2 was similar to that in LD, while that of *late* populations under SPP2 and SPP3 was similar to LD. ANOVA on ψ_{ON} values showed a statistically significant effect of P ($F_{2,6}$ = 194.29; $p < 0.001$), L ($F_{3,9}$ = 49.54; $p < 0.001$), and P × L interaction ($F_{6,18}$ = 6.23; $p < 0.001$; Table 4). Post-hoc multiple comparisons showed that under SPP1 the ψ_{ON} of *early* populations was significantly phase advanced compared to LD, whereas the ψ_{ON} of *control* and *late* populations was comparable to that in LD. The ψ_{ON} of all three populations

under SPP2 was similar to that under LD. In SPP3, the ψ_{ON} of *early* and *control* populations

Table 1. Results of ANOVA done on percentage emergence recorded in selected and control populations (P) over twelve time-points day (T) in four different light regimes (L) 12: 12 hr LD, SPP1, SPP2, SPP3.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	0.00	6	0.00	1129.31	0.001
Light regime (L)	3	0.00	9	0.00	1.37	0.31
Block (B)	3	0.00	0	0.00	--	--
Time point (T)	11	2480.01	33	6.03	411.55	0.001
P × B	6	0.00	18	0.00	15.15	0.001
P × B	6	0.00	0	0.00	--	--
L × B	9	0.00	0	0.00	--	--
P × T	22	392.37	66	5.47	71.71	0.001
L × T	33	56.21	99	2.52	22.30	0.001
T × B	33	6.03	0	0.00	--	--
P × L × B	18	0.00	0	0.00	--	--
P × L × T	66	17.17	198	1.77	9.70	0.001
P × T × B	66	5.47	0	0.00	--	--
L × T × B	99	2.52	0	0.00	--	--
P × L × T × B	198	1.77	0	0.00	--	--

Table 2. Results of ANOVA done on height of emergence peak (h) in selected and control populations (P) under four light regimes SPP1, SPP2, SPP3 and 12:12 hr LD.

height (h)	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	578.20	6	7.61	75.96	0.001
Light regime (L)	3	321.45	9	9.00	35.71	0.001
Block (B)	3	24.13	0	0.00	--	--
P × L	6	29.66	18	2.71	10.96	0.001
P × B	6	7.61	0	0.00	--	--
L × B	9	9.00	0	0.00	--	--
P × L × B	18	2.71	0	0.00	--	--

Table 3. Results of ANOVA done on phase of emergence peak (φ_{PK}) in selected and control populations (P) under four light regimes SPP1, SPP2, SPP3 and 12:12 hr LD.

peak (φ_{PK})	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	3.99	6	0.18	21.68	0.001
Light regime (L)	3	2.14	9	0.23	9.28	0.001
Block (B)	3	0.11	0	0.00	--	--
P × L	6	0.62	18	0.10	6.37	0.001
P × B	6	0.18	0	0.00	--	--
L × B	9	0.23	0	0.00	--	--
P × L × B	18	0.10	0	0.00	--	--

Table 4. Results of ANOVA done on phase of emergence onset (φ_{ON}) in selected and control populations (P) under four light regimes SPP1, SPP2, SPP3 and 12:12 hr LD

ON (φ_{ON})	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	37.48	6	0.19	194.30	0.001
Light regime (L)	3	9.43	9	0.19	49.55	0.001
Block (B)	3	0.39	0	0.00	--	--
P × L	6	1.23	18	0.20	6.24	0.001
P × B	6	0.19	0	0.00	--	--
L × B	9	0.19	0	0.00	--	--
P × L × B	18	0.20	0	0.00	--	--

Table 5. Results of ANOVA done on phase of emergence offset (φ_{OFF}) in selected and control populations (P) under four light regimes SPP1, SPP2, SPP3 and 12:12 hr LD

OFF (φ_{OFF})	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	227.05	6	0.29	790.18	0.001
Light regime (L)	3	4.52	9	0.33	13.59	0.001
Block (B)	3	1.16	0	0.00	--	--
P × L	6	1.00	18	0.16	6.29	0.001
P × B	6	0.29	0	0.00	--	--
L × B	9	0.33	0	0.00	--	--
P × L × B	18	0.16	0	0.00	--	--

Table 6. Results of ANOVA done on width of emergence gate (*w*) of selected and control populations (P) under four light regimes SPP1, SPP2, SPP3 and 12:12 hr LD (L).

Width (*w*)	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	84.01	6	0.48	176.50	0.001
Light regime (L)	3	13.04	9	0.23	57.93	0.001
Block (B)	3	0.75	0	0.00	--	--
P × L	6	0.45	18	0.27	1.64	0.19
P × B	6	0.48	0	0.00	--	--
L × B	9	0.23	0	0.00	--	--
P × L × B	18	0.27	0	0.00	--	--

Table 7. Results of ANOVA done on sum of squares (SS) in selected and control populations (P) under 3 light regimes SPP1, SPP2, SPP3 (L).

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Population (P)	2	5799.20	6	11350.09	0.51	0.62
Light regime (L)	2	78418.30	6	8596.41	9.12	0.02
Block (B)	3	17148.31	0	0.00	--	--
P × L	4	41222.25	12	5758.32	7.16	0.001
P × B	6	11350.09	0	0.00	--	--
L × B	6	8596.41	0	0.00	--	--
P × L × B	12	5758.32	0	0.00	--	--

was significantly phase advanced, whereas that of *late* populations was comparable to that under LD (Figure 3c). The ψ_{ON} of *early* populations under SPP2 was similar to that in LD, while that of *late* populations under SPP2 as well as SPP3 was comparable to that in LD.

***Phase of emergence offset (ψ_{OFF})*:** The ψ_{OFF} under SPP1 was delayed by ~1-hr in *control* and by ~2-hr in *late* populations compared to LD, while it was unaffected in *early* populations. The ψ_{OFF} of all three populations under SPP2 and SPP3 was indistinguishable from their respective ψ_{OFF} in LD (Figure 3d; Table 1). ANOVA on ψ_{OFF} data demonstrated a statistically significant effect of P ($F_{2,6}$ = 790.18; $p < 0.001$), L ($F_{3,9}$ = 13.59; $p < 0.01$), and P × L interaction ($F_{6,18}$ = 6.29; $p < 0.01$; Table 5). Post-hoc multiple comparisons showed the ψ_{OFF} of *early* populations did not differ between SPP1 and LD, while that of *control* and *late* populations was significantly delayed under SPP1 compared to LD. Under SPP2 and SPP3, ψ_{OFF} of all the three populations did not differ from that under LD.

***Width of emergence gate (w)*:** The *w* of the emergence rhythm also varied under the light regimes in a population-dependent manner (Figure 3e). ANOVA on *w* data revealed a statistically significant effect of P ($F_{2,6}$ = 176.5; $p < 0.001$) and L ($F_{3,9}$ = 57.93; $p < 0.001$), however, the P × L interaction was not statistically significant ($F_{6,18}$ =1.64; $p > 0.05$; Table 6). As the effect of P × L interaction was not statistically significant, the effect of the light regimes on *w* for individual populations could not be tested.

***Sum of squares (SS)*:** For all three populations, we also quantified the similarity of the SPP2/SPP3 waveforms with those under LD cycles by the estimating sum of squares (SS) of the differences between the LD and SPP2 or SPP3 waveforms (Figure 4). It is obvious that the smaller is the SS value, the better is the agreement between the two waveforms. Emergence waveforms of *early* and *control* populations under LD showed a better match with SPP2 than SPP3. In *late* populations, the emergence waveform under LD showed a better match with SPP3 than SPP2. To test whether the SS of a population depends on the

Figure 4. SS of differences between percentage emergence under light/dark (LD) and skeleton photoperiod (SPP) regimes. SS was used as a measure of the degree of match between the two waveforms. If two profiles are identical, SS will be zero. Type of population is plotted along *x*-axis and SS along *y*-axis. Asterisks (*) denote statistically significant differences of the emergence waveforms under SPP2/SPP3 from those in LD cycles. *Early* and *control* populations showed minimum sum of squares (SS) under SPP2, while *late* populations showed minimum SS under SPP3.

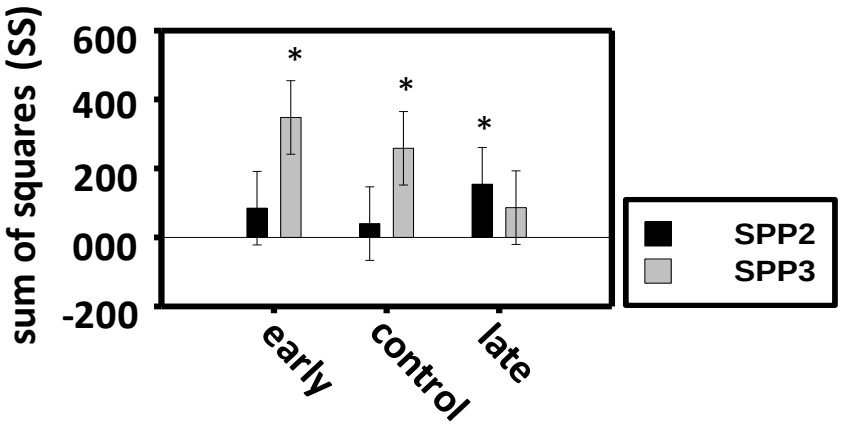


Table 8. Compilation of emergence waveform characteristics in early, control and late populations under SPP1, SPP2 and SPP3 expressed relative to those seen under 12:12 hr LD. * implies significance.

		h	ψ_{PK}	ψ_{ON}	ψ_{OFF}	w
early	**SPP1**	reduced*	same as LD	advanced*	same as LD	greater*
	SPP2	same as LD	same as LD	same as LD	same as LD	same as LD
	SPP3	reduced*	same as LD	advanced*	same as LD	greater*
control	**SPP1**	reduced*	same as LD	same as LD	delayed*	greater*
	SPP2	same as LD	same as LD	same as LD	same as LD	same as LD
	SPP3	reduced*	same as LD	advanced*	same as LD	same as LD
late	**SPP1**	reduced*	delayed*	same as LD	delayed*	greater*
	SPP2	same as LD	delayed*	same as LD	same as LD	same as LD
	SPP3	same as LD	same as LD	same as LD	same as LD	same as LD

light regime (SPP2/SPP3), ANOVA was done on SS values. The results showed a statistically significant effect of L ($F_{2,6} = 9.12$; $p < 0.05$) and P × L interaction ($F_{4,12} = 7.15$; $p < 0.01$; Table 7). Post-hoc multiple comparisons indicated the SS of *early* and *control* populations was significantly smaller under SPP2 than SPP3, suggesting the *early* and *control* emergence waveforms under LD better resembled their SPP2 than SPP3 waveform (Figure 4). In case of *late* populations, the SS value was smaller under SPP3 than SPP2, however, the differences between the SS values did not reach statistical levels of significance. Moreover, the SS of *early* and *control* populations did not differ statistically from 0 only under SPP2, which implies better matching of the emergence waveforms in flies under this regime than with those under LD. In *late* populations, SS did not differ significantly from 0 under either SPP2 or SPP3, but there was a trend of greater SS under SPP2 than SPP3, which suggests that SPP3 mimicked LD better than SPP2. Together, these results suggest that SPP2 mimicked the effects of LD for *early* and *control* populations, whereas SPP3 appeared to mimic effects of LD better than SPP2 for *late* populations.

Discussion

Symmetric skeleton photoperiod regimes can be interpreted such that either of the two dark intervals can be considered as day or night depending on the duration of the dark interval and the phase of the animal's circadian clocks when exposed to the first light pulse (Pittendrigh, 1964). In our study, the SPP1 regime was initiated on the 4th day after egg collection, before which developing populations were maintained under LD 12:12. The two brief pulses of light under SPP1 were presented in such a way that they marked the morning and evening transitions of the previous LD cycles as shown in Figure 1. The rhythm of all three populations entrained to SPP1, displaying emergence predominantly during the dark interval, corresponding to the light phase of the previous LD cycles. This suggests that all three populations interpreted the light pulses marking morning and evening transitions of previous

LD cycles as dawn and dusk while entraining to SPP1. Based on the analyses of waveform characteristics, we conclude that the emergence waveforms of all three populations under SPP1 were different from those seen under LD cycles, which suggests that SPP1 does not mimic the entraining effects of the complete photoperiod.

In a way, our results appear to contradict those of a previous study on the emergence rhythm in *D. pseudoobscura* where skeleton photoperiods were shown to successfully mimic the effects of complete photoperiod as long as the simulated photoperiod was shorter than $\tau/2$, and simulation was fairly good even for skeleton photoperiods slightly longer than $\tau/2$ (Pittendrigh, 1964). The skeleton photoperiod in our present study simulated a photoperiod of 12-hr (LD 12:12), which was within the required range for all three populations (τ of *early* populations ~23.4-hr, *control* populations ~23.8-hr and *late* populations ~24.3-hr). Despite that, our assessment showed that SPP1 did not mimic the effects of LD cycles. This apparent discrepancy seems to be primarily due to the difference in methods used by Pittendrigh (1964) and by us in the present study to estimate the phase of the emergence waveform. The previous study used the median of the emergence waveform as phase marker of the rhythm and found that emergence occurred at the same zeitgeber time under complete and their corresponding skeleton photoperiods. We measured various quantifiable features of the emergence waveform - h, ψ_{PK}, ψ_{ON}, ψ_{OFF} and w, which enabled us to detect finer differences in the emergence waveform. Our estimation of the median of emergence waveform also did not reveal difference between SPP1 and LD for any of the populations, just as that described in the earlier study by Pittendrigh (1964).

Comparison of waveform characteristics of *early*, *control*, and *late* populations under SPP1 and LD revealed that under SPP1 one or more waveform characteristics did not match with those under LD (Figure 3; Table 8). This implies that cumulative phase adjustments caused by two brief light pulses/cycle was not sufficient to mimic the overall effects of the

LD cycles. If one were to go by the view that light around the dawn and dusk transitions constitute primary entraining stimuli, then these results suggest the lack of adequate light at either one of the two or both transitions, as the length of the two light pulses was merely 15-min. So, we went on to test if asymmetric SPP regimes can simulate the effects of LD 12:12. In this case, populations were exposed to skeleton photoperiods consisting of one brief light pulse and another light exposure of longer duration (6-hr), marking the start and end of the photoperiod under LD (SPP2), or the other way round (SPP3), assuming that insufficient light at one of the two transitions in SPP1 could be the cause of lack of SPP1 in mimicking the effects of LD cycles (Figure 1). We found that all waveform characteristics (5/5) of *early* and *control* populations under SPP2 resembled those in LD, whereas only some characteristics under SPP3 (2/5 for *early* and 3/5 for *controls*) resembled those in LD (Figure 3; Table 8). All waveform characteristics (5/5) of *late* populations under SPP3 resembled those under LD, but a lesser number (4/5) of waveform characteristics in SPP2 resembled those under LD (Figure 3; Table 8). Since *early* populations achieved their EEW under SPP2, and *late* populations under SPP3, we conclude that SPP2 and SPP3 represented the LRS of *early* and *late* populations. This suggests that *early* and *late* populations use light primarily contiguous to dawn and dusk transitions to entrain their adult emergence rhythms. The LRSs of *early* and *late* populations approximated in our study are based on assessment of emergence waveforms only under three SPP regimes (SPP1, SPP2 and SPP3). Since the purpose of our study was to test whether *early* and *late* populations require light for different durations during the morning and evening transitions to achieve their EEWs, three SPP light regimes were sufficient to start with. Interestingly, two diametrically opposite SPPs (SPP2 and SPP3) represented LRSs for *early* and *late* populations, respectively, strongly indicating that the temporal profiles of light essential for circadian entrainment of the emergence rhythm in *early* and *late* populations are different. Although SPP2 and SPP3 represented LRSs of

early and *late* populations, respectively, they should not be considered as the 'minimal light requirement schedules, as the durations of light shorter than those provided in SPP2 and SPP3 (e.g., in SPP2, 3-hr of light in the second half of the day instead of 6-hr) could also produce similar effect on the emergence waveform. Circadian systems are capable of integrating light over longer durations and the magnitude of phase shift depends on the number of photons received (Dkhissi-Benyahya et al., 2000). A study on hamsters by Takahashi et al. (1984) showed a reciprocal relationship between the intensity and duration of light, on the magnitude of phase shift, which can work for durations as long as 45-min. Considering the intensity/duration reciprocity in the phase shifting effects of light, it would be interesting to study if skeleton photoperiod regimes consisting of one low and one high intensity pulses could produce similar effects as SPP2 and SPP3 regimes on the emergence waveforms of *early* and *late* populations.

Our results show that *early* populations closely resembled their EEW under SPP2 than SPP3, whereas resemblance of *late* populations to their EEW was better under SPP3 than SPP2, which suggests that entrainment of *early* circadian clocks under SPP2 and *late* clocks under SPP3 were closest to their entrained states under LD cycles. This further implies that SPP2 and SPP3 provided most components of light essential for circadian entrainment of *early* and *late* populations to achieve their EEW; therefore, SPP2 and SPP3 were designated as the LRSs of *early* and *late* populations. A segment of the photoperiod under LD cycles that forms the essential entraining stimulus implies circadian clocks entrained to LD are sensitive to light only during that particular segment. Therefore, the LRSs of *early* and *late* populations could be considered to represent differences in the photosensitivity of their circadian clocks. Thus, differences in photosensitivity of circadian clocks in *early* and *late* flies while entrained under LD cycles provides evidence corroborating the difference in their PRCs, which in turn could represent optimal entrainment mechanisms required to achieve

their characteristic EEWs. These results thus strengthen the idea that divergent clocks play a critical role in divergent emergence phenotypes of *early* and *late* populations.

Chapter 5

Genetic architecture of circadian clocks in morning and evening emerging fruit flies *Drosophila melanogaster*

Introduction

Most living organisms on earth perceive daily cycles of abiotic factors such as light and temperature that occur as an inevitable consequence of the earth's rotation around its axis. Organisms ranging from bacteria to humans exhibit daily rhythms in various behavioral and physiological processes. These rhythms are not merely the organism's passive response to environmental cycles because they are found to persist under constant laboratory conditions with near 24-hr period (hence circadian; *circa* - about, *dies* - day), which indicates that these rhythms are governed by endogenous rhythm generating systems (Dunlap et al., 2004). Scheduling of biological functions at a specific time of the day is the primary function of such circadian clocks (Roenneberg et al., 2003a). It is thought that the endogenous timekeeper attains a stable temporal relationship with the environmental cycles, using time cues such as light and temperature, and then schedules the biological functions under its control at specific time of the day (Johnson et al., 2003). The phase-relationship (ψ) of the entrained rhythm relative to a reference phase in the environmental cycle is known to be a function of two basic properties of the circadian oscillator - its period (τ) and phase response curve (PRC) (Johnson et al., 2003; Roenneberg et al., 2003a).

Like many other quantitative traits, properties of circadian rhythms exhibit continuous variation (Hofstetter et al., 2003; Sharma and Chandrashekaran, 1999). Quantitative genetic analyses of variations in clock properties suggest that multiple genetic loci contribute to such variations (Shimomura et al., 2001; Hofstetter et al., 2003; Michael et al., 2003; Edwards et al., 2005; Kim et al., 2007). According to the quantitative genetic theory, alleles segregating at multiple loci, each having small effect, produces variations in the quantitative traits through three distinct classes of genetic effects, (i) additive effects of alleles (at the same locus and across multiple loci), (ii) effects of interaction between alleles at the same locus

(dominance) and (iii) effects of interaction among alleles at two or more loci (epistasis) (Falconer and Mackay, 1996; Hamilton, 2009).

Molecular mechanisms underlying circadian clocks have been fairly well understood in a broad spectrum of organisms ranging from bacteria to mammals (Takahashi et al., 2008). Identification of genes involved in the functioning of core circadian oscillators, pathways by which environmental cues such as light and temperature are sensed and mechanisms by which rhythmic biological functions are regulated, has remained central to the molecular-genetic studies in circadian biology (Bell-Pedersen et al., 2005). Natural populations provide rich source of quantitative variation in circadian phenotypes which has been extensively studied in many systems such as mammals, birds, insects and plants (Aschoff, 1969; Lankinen, 1986, 1993; Pittendrigh and Takamura, 1989; Pittendrigh et al., 1991).

Life on earth began, evolved and flourished in the face of rhythmic challenges (Tauber and Kyriacou, 2005), and therefore, it is believed that circadian timing systems evolved as an adaptation to cyclic environmental conditions on earth (Pittendrigh, 1960, 1993). Although, every point on the earth's surface experiences robust 24-hr cycles of light, temperature and humidity, several features of such diurnal cycles are highly variable from one location to another. Daylengths, magnitudes of temperature and humidity fluctuations between day and night are some of the features which exhibit remarkable variation, and thus invoke the need for organisms to fine-tune their rhythmic behavior and physiology (Sharma and Joshi, 2002; Sharma, 2003; Johnson, 2005; Yerushalmi and Green, 2009). Intra-specific variations in circadian rhythms in populations from different geographic locations have been studied in great detail in several insect and plant species (Kyriacou et al., 2007). Correlation between circadian phenotypes in populations from different geographic locations and latitude of their origin provides one of the most convincing yet indirect evidence for the role of natural selection in shaping circadian clocks. Lankinen (1986, 1993) reported latitudinal

variation in ψ and τ of adult emergence rhythms in *Drosophila littoralis* (30^{o} to 70^{o}N) and *D. subobscura* (56^{o} to 63^{o}N) strains collected from different regions of northern Europe. In another study, Pittendrigh and Takamura (1989) reported latitudinal variation in τ and PRC in *D. auraria* strains collected from different latitudes (34.2^{o} to 42.9^{o}N) in Japan. Similarly, study of geographical variation in the circadian phenotypes of *Arabidopsis thaliana* revealed latitudinal variation in τ of leaf movement rhythm (Michael et al., 2003). Extensive surveys of intra-specific genetic variations at loci regulating circadian rhythms revealed latitudinal variation in the frequencies of alleles of two core clock genes (Kyriacou et al., 2007), which suggests that natural selection shapes genetic variation in circadian phenotypes leading to the evolution of circadian clocks. The *period* (*per*) locus of *D. melanogaster* was found to be polymorphic for Threonine-Glycine (T-G) repeat length in strains collected from north Africa and Europe (30^{o} to 55^{o}N), and T-G allele frequencies were found to be correlated with latitude (Costa et al., 1992). The T-G alleles were later found to be linked to the clock's ability to maintain a stable τ over a wide range of temperatures, which suggests that latitudinal variations in *per* allele frequencies could be a result of natural selection acting on circadian clocks (Sawyer et al., 1997). Furthermore, in a separate study the frequency of alleles at another core clock locus (*timeless*) was found to show latitudinal variation, and this variation was associated with those in light induced PRC (Tauber et al., 2007).

Laboratory selection studies provide another source of variation in circadian phenotypes. Pittendrigh (1967) and Pittendrigh and Minis (1971) raised *early* and *late* strains of fruit flies *D. pseudoobscura* and moths *Pectinophora gossypiella* respectively, having earlier and later phases of emergence, by imposing selection for timing of adult emergence. Circadian clocks evolved in both species as a correlated response; *early* strains had longer τ of emergence rhythm, while *late* strains had shorter τ, however, PRC of *early* and *late* strains did not differ. In another selection experiment, melon fly *Bacterocera cucurbitae*

populations selected for faster and slower pre-adult development evolved circadian clocks with shorter and longer τ, respectively (Miyatake and Shimizu, 1999). Evolution of rhythmic phenotypes as a correlated response to artificial laboratory selections suggests the role of clocks in adaptive response to imposed selection pressure, which implies adaptive significance of circadian clocks.

Studies on natural populations and populations selected in the laboratory revealed quantitative variation in clock properties, which suggests the significance of such variations in negotiating local environmental conditions. Contribution of additive effects, dominance, maternal effects and epistasis to the variation in clock properties have been reported in pitcher-plant mosquito *Wyeomyia smithii* (Mathias et al., 2006) and in bean beetle *Callosobruchus chinensis* (Harano and Miyatake, 2010). Despite considerable progress made in our understanding of the genetic bases and the molecular mechanisms underlying circadian rhythms, very little is known about the genetic basis of such adaptive variation and the relative contribution of additive effects, dominance, maternal effects and epistasis (Sharma and Joshi, 2002).

It is well known that circadian rhythm of adult emergence in fruit flies *D. melanogaster* is under clock control, and steady-state ψ of the rhythm with respect to light/dark (LD) cycles is a result of entrainment of these oscillators to LD cycles. Here we report the results of genetic analysis of adaptive circadian phenotypes of *early* and *late* fruit fly *D. melanogaster* populations (henceforth stocks), created in a long-term laboratory selection study by imposing selection for adult emergence during morning and evening hours under 12:12 hr LD cycles (Kumar et al., 2007a). With increasing generations, *early* stocks evolved preference for increased morning emergence, while *late* stocks evolved preference for increased evening emergence (Kumar et al., 2007a). By the 150th generation of selection,

percentage emergence during morning hours increased to ~60% in *early* and reduced to ~24% in *late* stocks, whereas emergence during evening hours decreased to ~3% in *early* and increased to ~35% in *late* stocks (*Vaze KM, Nikhil KL, Sharma VK, unpublished data*). From the analysis of daily emergence waveforms it was evident that *early* flies start emerging well before lights-on and end well before lights-off. On the other hand, flies in *late* stocks start emerging after lights-on and continue emerging well beyond lights-off. In addition, *early* and *late* stocks evolved divergence in their basic clock properties such as τ and PRC (Kumar *et al*., 2007a). Compared to *controls*, *early* stocks had shorter τ, smaller delays and larger advances in their PRC, and *late* stocks had longer τ, larger delays and smaller advances in their PRC (Kumar et al., 2007a). Evolution of specific temporal preference for emergence and circadian period suggests a causal role of circadian clocks in the regulation of morning and evening emergence in the *early* and *late* stocks.

In order to understand the genetic bases of morning and evening emergence in these phenotypically diverged populations we set crosses between *early* and *late* stocks and obtained F1, F2 and backcross progeny. We scored morning and evening emergence and τ of activity rhythm in the progeny from sixteen different types of crosses (Figure 1). This scheme of crosses allowed us to test the contribution of X, Y chromosomes and two types of cytoplasmic factors. Apart from the chromosomal genetic material, offspring receive maternal cytoplasm in the form of egg cytoplasm, which in insects is known to influence several pre-adult and adult traits (de Belle and Sokolowski, 1987). Short-lived maternal cytoplasmic factors such as hormones, mRNAs and proteins are known as transient maternal factors (TMF), whereas maternally inherited non-chromosomal genetic factors such as mitochondria, chloroplasts persist throughout the lifetime and are known as permanent cytoplasmic factors (PCF) (de Belle and Sokolowski, 1987). Our analyses revealed that genetic basis of timing of emergence and τ of activity rhythm in *early* and *late* stocks is

primarily autosomal. In addition, we performed line cross analyses to examine the relative contribution of additive, dominance, maternal and epistasis effects to the divergence in the morning or evening preference for emergence and in τ of activity rhythm. The results revealed that complex genetic architecture comprising dominance and epistasis underlies divergent circadian phenotypes in *early* and *late* flies.

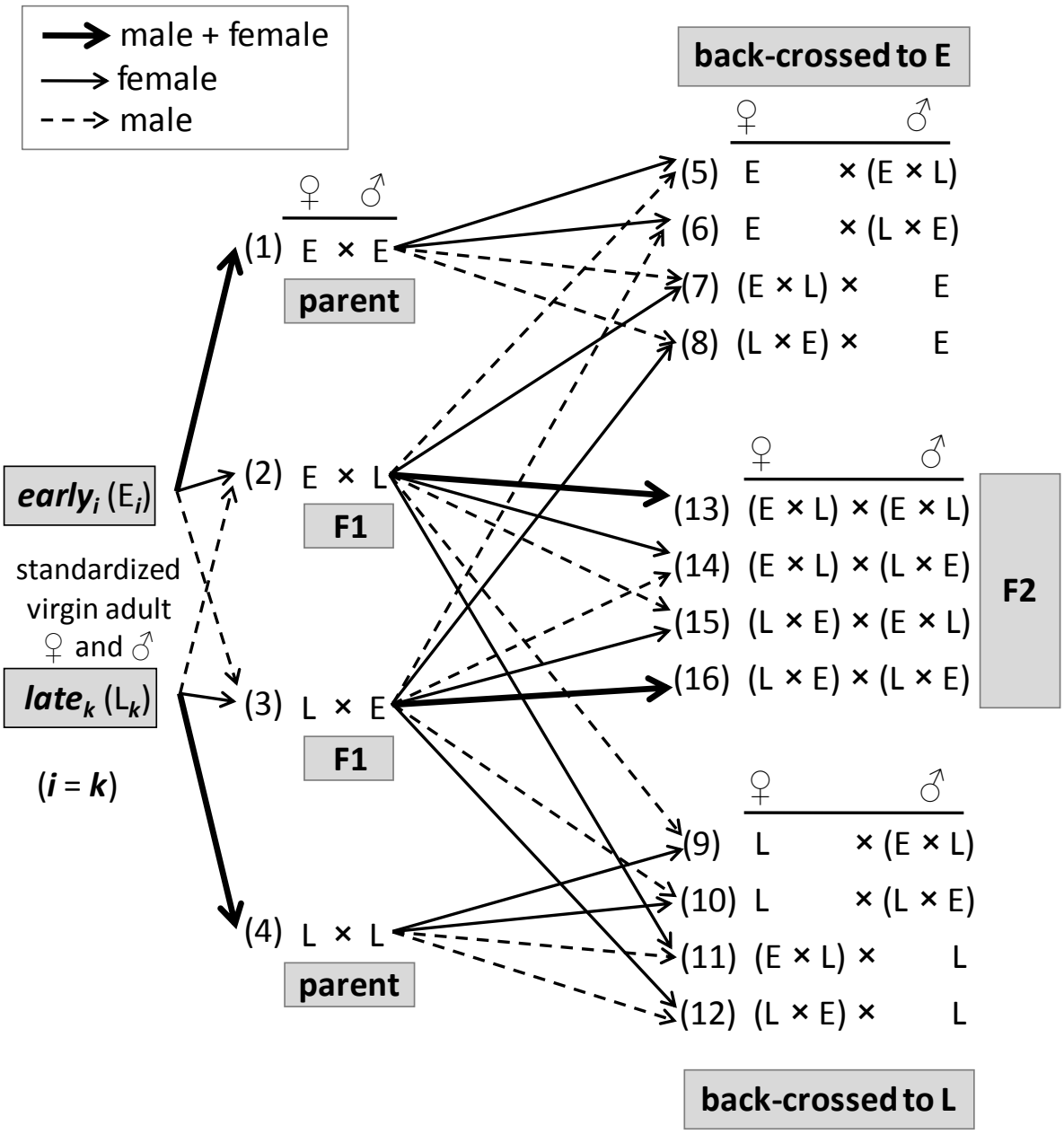
Materials and methods

***Crosses between early and late flies*:** A total of 16 crosses were set-up in a sequential manner as shown in Figure 1 for every pair of $early_i$ and $late_k$ (where $i = k$) stocks. All the crosses were set-up by mixing 200 virgin males and 200 virgin females of an average age of 6-days in Plexi-glass cages of 25 × 20 × 15 cm^3 dimension with BJ food and water *ad libitum*. On the second day of setting-up crosses, populations were provided with yeast plates and 3-days later ~300 eggs per glass vial were collected in about 8 vials per cross. The entire set of crossed populations and their progeny populations were stored in cubicles maintained at constant laboratory conditions (as described earlier). Progeny flies emerging throughout the day for 2-3 consecutive days were collected to set-up crosses for the next stage. In the first stage, virgin males and virgin females from a pair of standardized $early_i$ and $late_k$ stocks (where $i = k$) were used to set-up four crosses, which yielded parental - E (*early*, 1), parental - L (*late*, 4) and two reciprocal F1 hybrid (2) and (3) (Figure 1). Virgin males and females of (1), (2), (3) and (4) were used to set 12 crosses in the second stage. Reciprocal crosses between parental - E (1) and two types of F1 hybrids (2) and (3) produced four types of backcross to E hybrid (5-8) progeny. Similarly, reciprocal crosses between parental - L (4) and two F1 hybrids (2) and (3) yielded four backcrossed to L (9-12) hybrid progeny. Self crosses of (2) and (3) and two reciprocal crosses between (2) and (3) produced four types of F2 hybrid progeny (13-16) (Figure 1). As hybrid progeny from the crosses came in two

stages their circadian phenotypes were also studied in two stages. All crosses and scoring of circadian phenotypes of hybrid progeny for each of the four replicate *early* and *late* stocks were performed separately. All the analyses performed on progeny phenotypes are presented separately for each replicate pair of *early* and *late* stocks and four replicates are named as R1, R2, R3 and R4, respectively.

***Adult emergence rhythm assay*:** Eggs were collected from each crossed populations on the 4^{th} day of setting-up crosses, in 8 food vials, at a density (~300 eggs per vial) similar to that used for population maintenance. Eggs were counted under microscope using cool light source. After egg collection, all vials were stored in cubicles maintained at constant laboratory conditions (as described earlier). After the start of adult emergence, flies emerging from individual vials were collected 4 times a day at ZT02, ZT08, ZT14 and ZT20 as an estimate of emergence during morning, daytime, evening and night windows and therefore number of flies recorded at any of the time points represents emergence during 6-hr interval preceding the time of fly collection. For example, flies collected at ZT08 are the ones which emerged in the duration spanning ZT02 to ZT08. Flies were collected from each vial for 2-4 consecutive days. Number of males and females that emerged from each vial was counted. To calculate the percentage, males or females emerging every 6-hr from each vial were normalised respectively by the total number of males or females that emerged from the same vial in that particular LD cycle. ZT20 (4-hr before lights-on) was considered as start of each cycle and therefore number of flies that emerged during 24-hr interval following start of the day was used for normalization. Vial average for percentage emergence in every window was calculated by taking average over 2-4 consecutive days (cycles) from the same vial. Minimum of 8 males or females per cycle was set as arbitrary cut-off for inclusion of a cycle in the percentage emergence estimation. All the vial-means for percentage emergence values were arc-sine square root transformed before doing any further analysis.

Figure 1. Schematic diagram of crosses and the sequence in which they were performed while doing the genetic analysis of *early* and *late* circadian phenotypes.



***Activity/rest rhythm assay*:** Eggs were collected from each crossed population in a separate set of 5-ml food-vials along with egg collection for adult emergence assay. Progeny flies from each of the crossed populations, emerging throughout the day were collected separately over 2-3 consecutive days. Within 6-hr of their emergence, males and females were separated by anesthetizing them with CO_2, and maintained as virgins at a density of 50-60 flies per vial. Activity of about 48 male and 48 female flies per cross were recorded under DD for over 10 days using *Drosophila* activity monitoring system (Trikinetics, Waltham, MA). Activity of individual flies was recorded inside locomotor activity tubes (glass tube of length 5-cm and inner diameter 4-mm) provided with standard corn-sugar-yeast medium at one end and cotton plug at the other. Activity recording began when flies were 4-days old and continued for more than 12-days. The τ of activity/rest rhythm was calculated for individual flies by analyzing activity data collected in 5-min bin for a minimum of 10-days using Lomb-Scargle Periodogram in CLOCKLAB (Actimetrics, IL). Activity data collected for the first 2-days was excluded from the analysis. Values lying outside mean ± 3 standard deviations (SD) were considered as outliers, and were excluded while calculating the means.

***Statistical analyses*:** Percentage emergence during morning or evening hours and τ of activity/rest rhythm was measured to quantify the circadian phenotypes. Differences among circadian phenotypes of selected and control populations were tested by carrying out mixed model analysis of variance (ANOVA). Comparisons of adult emergence behaviour among selected and control populations was analysed by mixed model two way ANOVA, in which selection regime (*early*/*control*/*late*) (S), window of emergence (morning/evening) (W) were considered as fixed effect factors and four replicate populations (blocks - B) were treated as random factor. Comparisons of τ of activity/rest rhythm among selected and control populations were carried out by mixed model one-way ANOVA. Post-hoc multiple comparisons were carried out using Tukey's test. Error bars in figures are 95% CI

(comparison intervals calculated from minimum significant difference obtained in Tukey's test), therefore absence of overlap between error bars of two means indicates significant difference (Sokal and Rohlf, 1981).

The scheme of crosses between *early* and *late* stocks (as shown in Figure 1 and Table 1a) allowed us to test the contribution of four hereditary factors – sex chromosomes (X, Y), permanent cytoplasmic factors (PCF) and transient maternal factors (TMF) to the difference between their circadian phenotypes following the approach adopted by de Belle and Sokolowsky (1987) and Huttunen and Aspi (2003). Table 1a provides scheme of crosses and inheritance of four hereditary factors in the progeny flies. Comparisons among mean circadian phenotypes (morning and evening emergence, τ of activity/rest rhythm) of appropriately chosen progeny allowed us to test the contribution of each of the four factors and their interactions to the difference between *early* and *late* phenotypes as shown in Table 1B following de Belle and Sokolowski (1987) and Huttunen and Aspi (2003). Null hypothesis of no difference among mean circadian phenotypes of 16 types of progeny from crosses between each pair of *early* and *late* stocks was analysed by one factor ANOVA, followed by test of hypotheses concerning the contribution of various hereditary factors (Table 1B) were performed by planned contrast analysis of variance. Differences between progeny which share three of the four hereditary factors were tested for significance to assess the contribution of fourth factor. For example, in a test for contribution of PCF, female progeny of crosses 13 and 14 have same type of X chromosomes, autosomes, and TMF but only differ in the type of PCF. Therefore, comparison of (13 + 14) *vs* (15 + 16) was used to test the contribution of PCF to differences between *early* and *late* phenotypes (Table 1a and 1b). This analysis was performed on each replicate pair of *early* and *late* stocks (R1, R2, R3 and R4) separately.

Table 1. (a) Scheme of crosses between *early* (E) and *late* (L) stocks and inheritance of four hereditary factors in the progeny flies.

Cross No.		♀		♂	Autosome	Female XX	Male X	Male Y	Permanent Cytoplasmic factors	Transient Maternal factors
1	(1)	E	×	E	E	E	E	E	E	E
2	(3)	E	×	L	F1	F1	E	L	E	E
3	(4)	L	×	E	F1	F1	L	E	L	L
4	(2)	L	×	L	L	L	L	L	L	L
5	(5)	E	×	(E × L)	B_E	E	E	L	E	E
6	(6)	E	×	(L × E)	B_E	F1	E	E	E	E
7	(9)	(E × L)	×	E	B_E	B_E	E/L	E	E	F1
8	(10)	(L × E)	×	E	B_E	B_E	E/L	E	L	F1
9	(7)	L	×	(E × L)	B_L	F1	L	L	L	L
10	(8)	L	×	(L × E)	B_L	L	L	E	L	L
11	(11)	(E × L)	×	L	B_L	B_L	E/L	L	E	F1
12	(12)	(L × E)	×	L	B_L	B_L	E/L	L	L	F1
13	(13)	(E × L)	×	(E × L)	F2	F2	E/L	L	E	F1
14	(14)	(E × L)	×	(L × E)	F2	F2	E/L	E	E	F1
15	(15)	(L × E)	×	(E × L)	F2	F2	E/L	L	L	F1
16	(16)	(L × E)	×	(L × E)	F2	F2	E/L	E	L	F1

Cross number in brackets indicate equivalent cross number in de Belle and Sokolowski, 1987.

Notations used for different forms of a hereditary factor arising from crosses between parental stocks

F1 = F1 hybrid
BE= Back-cross to *early* parent
BL= Back-cross to *late* parent
F2 = F2 hybrid

Table modified from de Belle and Sokolowski, 1987

Table 1. (b) Comparisons/contrasts used to test contribution of various hereditary factors to the difference between *early* and *late* circadian phenotypes.

	Test	♂	♀	Comparison
1	Difference between parents (P)	♂	♀	1 *vs* 4
2	Dominance (D)		♀	1 + 4 *vs* 2 + 3
3	X chromosome (X) + maternal effects (M)	♂		2 *vs* 3
4	Matrenal effects (M)		♀	2 *vs* 3
5	X chromosome (X)		♀	5 + 9 *vs* 6 + 10
6	X chromosome interactions		♀	5 + 10 *vs* 6 + 9
7	Y chromosome (Y)	♂		14 + 16 *vs* 13 + 15
8	Y chromosome interactions	♂		6 + 9 *vs* 5 + 10
9	Permanent cyctoplasmic factors (PCF)	♂	♀	13 + 14 *vs* 15 + 16
10	Permanent cytoplasmic intearctions	♂	♀	7 + 12 *vs* 8 + 11
11	Transient maternal factors (TMF)	♂		6 + 12 *vs* 7 + 9
12	Transient maternal factor interactions	♂		6 + 9 *vs* 7 + 12

***Analysis of line crosses*:** Analysis of line cross was performed to examine the contribution of additive effects (A), dominance (D), maternal effects (M) and epistasis (E) to the difference between *early* and *late* circadian phenotypes. Analyses of each of the four replicate pair of $early_i$ and $late_k$ stocks (where $i = k$) were performed separately. Analysis of each trait was done separately for males and females. Mean phenotypes and variances were calculated for progeny from each of the crosses, which will be called 'generation mean' hereafter. Generation means were analysed following the protocol described in Mather and Jinks (1982). Briefly, the analysis was aimed at identifying the genetic models which best explain the variation in observed generation means. Observed generation means were fitted to genetic models comprising different combinations of A, D, M and E using weighted least square regression to estimate parameters of the genetic models, which were in turn used to calculate the generation means expected under respective models. Goodness-of-fit of the observed generation means with those expected from the model was examined using chi-square test. Significant difference between observed and expected generation means in chi-square test would indicate that the observed means were not in agreement with those expected from the model, which then prompted testing of higher models. Lack of significance indicates that observed means are explainable by the model being tested and thus suggest that genetic effects incorporated in a model are sufficient to explain the variation among generation means. The parameter coefficients used while estimating model parameters by weighted least square regression are given in Table 2. Assignment of parameter coefficients was according to Tables 11.4 and 13.2 in Kearsey and Pooni (1996) as followed by Gilchrist and Partridge (1999) and Kennington et al. (2001).

Table 2. Coefficients used to estimate the model parameters. Assignment of parameter coefficients was according to Kearsey and Pooni (1996).

	Crosses ♀		♂	*m*	*a*	*d*	a_m	d_m	*c*	*a.a*	*a.d*	*d.d*
1	**E**	×	**E**	1	1	0	1	0	1	1	0	0
2	**L**	×	**L**	1	-1	0	-1	0	-1	1	0	0
3	**E**	×	**L**	1	0	1	1	0	1	0	0	1
4	**L**	×	**E**	1	0	1	-1	0	-1	0	0	1
5	**E**	×	**(E × L)**	1	0.5	0.5	1	0	1	0.25	0.25	0.25
6	**E**	×	**(L × E)**	1	0.5	0.5	1	0	1	0.25	0.25	0.25
7	**L**	×	**(E × L)**	1	-0.5	0.5	-1	0	-1	0.25	-0.25	0.25
8	**L**	×	**(L × E)**	1	-0.5	0.5	-1	0	-1	0.25	-0.25	0.25
9	**(E × L)**	×	**E**	1	0.5	0.5	0	1	1	0.25	0.25	0.25
10	**(L × E)**	×	**E**	1	0.5	0.5	0	1	-1	0.25	0.25	0.25
11	**(E × L)**	×	**L**	1	-0.5	0.5	0	1	1	0.25	-0.25	0.25
12	**(L × E)**	×	**L**	1	-0.5	0.5	0	1	-1	0.25	-0.25	0.25
13	**(E × L)**	×	**(E × L)**	1	0	0.5	0	1	1	0	0	0.25
14	**(E × L)**	×	**(L × E)**	1	0	0.5	0	1	1	0	0	0.25
15	**(L × E)**	×	**(E × L)**	1	0	0.5	0	1	-1	0	0	0.25
16	**(L × E)**	×	**(L × E)**	1	0	0.5	0	1	-1	0	0	0.25

Please note: order of crosses is different from the one in Table 1A

E – *early* parent, **L** – *late* parent

m – mean, **a** – additive effect, **d** – dominance

$\mathbf{a_m}$ – additive maternal effect, $\mathbf{d_m}$ – dominance maternal effect

c – cytoplasmic factors, **a.a** – additive-additive interaction

a.d – additive-dominance interaction, **d.d** – dominance-dominance interaction

We tested the goodness of fit of models consisting up to eight composite genetic parameters – additive [*a*], dominance [*d*], three types of diagenic epistatic interactions –additive–additive [*a.a*], additive–dominance [*a.d*], dominance–dominance [*d.d*], maternal additive effect [a_m], maternal dominance effects [d_m] and cytoplasmic effects [*c*]. In traditional joint scaling test, model testing starts with simplest possible model such as the one containing only additive effects and if simpler models are found to be inadequate in explaining variation in the observed means, further genetic effects are added sequentially in the order – dominance, epistasis and maternal effects, until the observed means match those expected under the model. However, the order in which the parameters are added influences the detection of effects of parameters added later and thus the best-fit model obtained in this manner may not provide us with the most parsimonious model. Therefore, we followed another method adopted by Bieri and Kawecki (2003), wherein every parameter could either be added or removed from the model. Thus a total of 256 models were possible from 8 parameters (2^8). Therefore, to reduce the number of models to be tested, three types of - diagenic interactions ([*a.a*], [*a.d*] and [*d.d*]) or maternal effects ([a_m], [d_m] and [*c*]) were either added or subtracted from the analysis, which left us with four working effects – additive (A), dominance (D), maternal (M) and epistasis (E). Sixteen candidate models consisting of mono, di, tri and tetrameric combinations of four parameters were tested for each data set following Bieri and Kawecki (2003). Although, more than one model could have adequately described the observed variation in the generation means, to find the most parsimonious model we used Akaike's Information Criterion (*AIC*) following Bieri and Kawecki (2003), which detects the best compromise between the amount of variation explained and the number of parameters used. AIC was calculated for every candidate model tested for each trait and the model with minimum AIC was selected as a model which best explains the observed variation in generation means.

Results

***Divergence of emergence preference and circadian period in early and late stocks*:** We used the proportion of flies emerging during morning and evening hours as a surrogate measure for circadian emergence phenotypes of *early* and *late* stocks under LD. The *early* stocks were characterised by higher preference for emergence during morning (~63%) and lower during evening (~3%), while *late* stocks were characterised by higher preference for emergence during evening (~35%) and lower during morning (~15%). Discrepancy between the percentage emergence values provided here and those in Figure 2a is because for figures and analyses we used mean of arc-sine square root transformation of percentage emergence. In our selection study, during morning hours all four replicate *early* populations exhibited higher emergence (R_1 – 71.43, R_2 – 60.24, R_3 – 62.22 and R_4 – 58.45), while all four *late* populations showed reduced emergence (R_1 – 16.19, R_2 – 16.28, R_3 – 15.11 and R_4 – 16.26) compared to *control* populations (R_1 – 48.13, R_2 – 44.37, R_3 – 37.12 and R_4 – 34.78). On the other hand, during the evening hours, emergence in all four replicate *late* populations was greater than controls (R_1 – 30.23, R_2 – 43.08, R_3 – 45.58 and R_4 – 37.15), while in all four *early* populations it was lower (R_1 – 1.22, R_2 – 2.98, R_3 – 4.27 and R_4 – 3.34) than *control* populations (R_1 – 17.73, R_2 – 16.89, R_3 – 16.99 and R_4 – 16.15). Significant differences between *early* and *late* stocks in terms of proportion of flies emerging during morning and evening selection windows were evident from statistically significant interaction of selection regime (S) and window of emergence (W) ($F_{2,6}$ = 550.76, $p < 0.001$; Table 3a) in ANOVA done on the percentage emergence (arc-sine square root transformed) data. Post-hoc multiple comparisons using Tukey's test showed that percentage emergence during morning and evening hours in *early* and *late* stocks were significantly different from *controls* (Figure 3a). Such differences in the preference for emergence during morning and evening between *early*

and *late* stocks (at generation 165) were consistent with those observed in an assay carried out on the same populations at an earlier generation (at generation 55; Kumar et al., 2007a).

The τ of activity rhythm in *early* and *late* stocks diverged from that of *controls*, with *early* flies having shorter τ (~23.45-hr), while *late* flies having longer τ (~24.20-hr) compared to *controls* (~23.90-hr, Figure 2b). The *early* populations had shorter τ of activity rhythm (R_1 – 23.37, R_2 – 23.54, R_3 – 23.51 and R_4 – 23.51), while *late* populations had longer τ (R_1 – 24.14, R_2 – 24.38, R_3 – 24.23 and R_4 – 24.23) compared to *controls* (R_1 – 23.98, R_2 – 23.83, R_3 – 23.90 and R_4 – 23.93). ANOVA on τ data showed a statistically significant effect of S ($F_{2,6}$ = 73.65, $p < 0.001$). Post-hoc multiple comparisons using Tukey's test revealed that τ of *early* flies was significantly shorter than *controls* and *late* flies. Although, τ of *late* flies was longer than *controls* it did not reach statistically significant levels (Figure 2b; Table 3b). Divergence in τ of *early* and *late* flies was consistent with earlier reports on the same stocks (Kumar et al., 2007a).

Table3. (a) ANOVA on the percentage of adult emergence during morning and evening hours in *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p* - level
Selection (S)	2	2.52	6	7.13	0.35	0.716
Window (W)	1	1287.35	3	24.08	53.46	0.005
Block (B)	3	3.62	0	0.00	--	--
S × W	2	1718.86	6	3.12	550.76	0.0001
S × B	6	7.13	0	0.00	--	--
W × B	3	24.08	0	0.00	--	--
S × W × B	6	3.12	0	0.00	--	--

Table 3. (b) ANOVA on circadian period of activity/rest rhythms in *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p* - level
Selection (S)	2	0.59	6	0.01	73.65	0.0001
Block (B)	3	0.00	0	0.00	--	--
S × B	6	0.01	0	0.00	--	--

***Hereditary factors contributing to early and late emergence phenotypes*:** Contribution of four hereditary factors X and Y chromosomes, PCF and TMF to the difference between *early* and *late* circadian emergence phenotypes were tested by performing planned contrasts analysis of variance following de Belle and Sokolowski (1987). Each of the comparisons tested are shown in Table 1b. Separate analysis was performed for each replicate pair of *early* and *late* stocks and within each replicate data was analysed separately for males and females. ANOVA on percentage emergence during morning and evening hours (arc-sine square root transformed) in progeny flies showed a statistically significant effect of cross in all the replicates ($p < 0.001$; Table 4a, b). Planned contrasts on morning emergence showed that in males, Y chromosome (in R_1), X chromosome + maternal factors (in R_3) and TMF (in R_4) showed a statistically significant effect (Table 5a), whereas tests for none of the hereditary factors showed a statistically significant effect in females (Table 5a). In case of evening emergence, except in R_4 males (significant effect of TMF), tests for none of the hereditary factors showed statistically significant effect (Table 5b).

***Hereditary factors contributing to circadian period of activity/rest rhythm*:** ANOVA on τ of activity rhythm data in progeny flies showed a statistically significant effect of cross in all four replicates (Table 4c). Planned contrasts analysis revealed that in males TMF (in R_2) and interaction of TMF with other factors (in R_4) had statistically significant effect (Table 5c), whereas, tests for none of the hereditary factors showed statistical significance in R_1 or R_3 males (Table 5c) and any of the replicates in females (Table 5c).

Table 4. Replicate wise ANOVA on the circadian phenotypes of *early* and *late* parental stocks and in their progeny. (a) morning emergence (b) evening emergence (c) circadian period

		df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
a	R1	15	469.08	101	15.59	30.09	<0.001
♂	R2	15	286.44	103	14.95	19.16	<0.001
	R3	15	247.87	85	17.52	14.15	<0.001
	R4	15	243.54	89	20.04	12.15	<0.001
	R1	15	636.73	101	18.80	33.87	<0.001
♀	R2	15	479.92	103	13.30	36.09	<0.001
	R3	15	365.88	85	16.59	22.06	<0.001
	R4	15	383.40	89	17.59	21.80	<0.001
b	R1	15	561.27	101	17.67	31.77	<0.001
♂	R2	15	435.52	103	19.41	22.43	<0.001
	R3	15	304.12	85	17.50	17.38	<0.001
	R4	15	392.18	89	19.18	20.45	<0.001
	R1	15	490.47	101	25.15	19.50	<0.001
♀	R2	15	507.90	103	16.07	31.61	<0.001
	R3	15	572.76	85	23.10	24.79	<0.001
	R4	15	440.84	89	14.85	29.69	<0.001
C	R1	15	2.40	523	0.22	10.72	<0.001
	R2	15	3.86	603	0.29	13.26	<0.001
♂	R3	15	2.44	535	0.22	11.32	<0.001
	R4	15	1.92	582	0.26	7.43	<0.001
	R1	15	1.24	521	0.18	6.79	<0.001
	R2	15	0.80	481	0.12	6.64	<0.001
♀	R3	15	1.27	467	0.17	7.60	<0.001
	R4	15	0.79	464	0.16	5.04	<0.001

Table 5. Results of planned comparisons performed to test the contribution of various hereditary factors to differences in circadian phenotypes of *early* and *late* stocks
(a) morning emergence (b) evening emergence (c) circadian period of activity/rest rhythm

a

		R1		R2		R3		R4	
♂		df	101	df	103	df	85	df	89
		α'	0.0064	α'	0.0064	α'	0.0064	α'	0.0064
Test		F		F		F		F	
1	**P**	259.95	S	119.14	S	109.26	S	64.40	S
3	**X + M**	1.46	NS	1.06	NS	9.02	S	0.00	NS
7	**Y**	9.07	S	0.57	NS	0.02	NS	1.15	NS
8	**Y interaction**	5.67	NS	3.12	NS	0.00	NS	0.10	NS
9	**PCF**	4.21	NS	0.43	NS	2.01	NS	0.06	NS
10	**PCF intearctions**	1.05	NS	0.26	NS	1.00	NS	1.38	NS
11	**TMF**	0.05	NS	0.21	NS	1.96	NS	13.59	S
12	**TMF interaction**	0.50	NS	0.82	NS	0.12	NS	2.91	NS

♀		df	101	df	103	df	85	df	89
		α'	0.0073	α'	0.0073	α'	0.0073	α'	0.0073
Test		F		F		F		F	
1	**P**	284.17	S	280.98	S	192.29	S	136.99	S
2	**D**	0.07	NS	0.07	NS	4.70	NS	0.09	NS
4	**M**	2.62	NS	0.11	NS	0.07	NS	0.25	NS
5	**X**	2.81	NS	0.36	NS	0.89	NS	0.26	NS
6	**X interactions**	0.21	NS	0.61	NS	0.99	NS	0.23	NS
9	**PCF**	0.04	NS	0.03	NS	0.87	NS	0.00	NS
10	**PCF interactions**	0.03	NS	3.92	NS	0.05	NS	1.31	NS

b

		R1		R2		R3		R4	
♂		df	101	df	103	df	85	df	89
		α'	0.0063	α'	0.0063	α'	0.0063	α'	0.0063
Test		F		F		F		F	
1	**P**	199.01	S	174.22	S	131.28	S	109.68	S
3	**X + M**	0.01	NS	0.11	NS	0.84	NS	1.09	NS
7	**Y**	7.57	NS	0.03	NS	0.30	NS	0.76	NS
8	**Y interaction**	0.59	NS	0.46	NS	0.24	NS	0.39	NS
9	**PCF**	1.70	NS	0.09	NS	1.15	NS	0.83	NS
10	**PCF intearctions**	0.36	NS	0.03	NS	1.37	NS	0.46	NS
11	**TMF**	3.13	NS	0.13	NS	1.65	NS	17.27	S
12	**TMF interaction**	0.68	NS	0.41	NS	3.18	NS	0.08	NS

♀		df	101	df	103	df	85	df	89
		α'	0.0073	α'	0.0073	α'	0.0073	α'	0.0073
Test		F		F		F		F	
1	**P**	110.34	S	287.50	S	156.21	S	146.64	S
2	**D**	0.13	NS	0.04	NS	9.93	S	5.10	NS
4	**M**	0.73	NS	0.03	NS	0.07	NS	3.52	NS
5	**X**	1.26	NS	0.85	NS	2.14	NS	4.39	NS
6	**X interactions**	1.18	NS	1.63	NS	0.63	NS	2.06	NS
9	**PCF**	0.03	NS	0.00	NS	0.34	NS	2.68	NS
10	**PCF interactions**	0.04	NS	1.05	NS	0.41	NS	1.95	NS

C

		R1		R2		R3		R4	
♂		df	523	df	603	df	535	df	582
		α'	0.0064	α'	0.0064	α'	0.0064	α'	0.0064
Test		F		F		F		F	
1	**P**	84.14	S	80.17	S	66.62	S	42.03	S
3	**X + M**	4.64	NS	5.78	NS	1.70	NS	1.29	NS
7	**Y**	0.59	NS	0.24	NS	0.50	NS	0.36	NS
8	**Y interaction**	1.08	NS	2.84	NS	0.39	NS	5.59	NS
9	**PCF**	0.01	NS	2.22	NS	0.21	NS	0.12	NS
10	**PCF intearctions**	2.70	NS	0.45	NS	0.47	NS	5.09	NS
11	**TMF**	2.47	NS	17.01	S	6.06	NS	6.21	NS
12	**TMF interaction**	1.23	NS	4.90	NS	0.29	NS	11.26	S

♀		df	521	df	481	df	467	df	464
		α'	0.0073	α'	0.0073	α'	0.0073	α'	0.0073
Test		F		F		F		F	
1	**P**	48.43	S	33.22	S	34.80	S	18.32	S
2	**D**	2.34	NS	0.04	NS	2.39	NS	0.53	NS
4	**M**	0.00	NS	5.13	NS	0.01	NS	0.02	NS
5	**X**	0.69	NS	3.31	NS	0.08	NS	0.01	NS
6	**X interactions**	0.00	NS	0.59	NS	1.15	NS	0.20	NS
9	**PCF**	1.47	NS	3.11	NS	0.72	NS	0.01	NS
10	**PCF interactions**	0.58	NS	0.99	NS	2.28	NS	0.00	NS

P - Difference between parents

D - Dominance

X - X- chromosome

Y - Y-chromosome

PCF - Permanent cytoplasmic factors

TMF - Transient maternal factors

M - Maternal effects (PCF and TMF together)

df - Denominator degrees of freedom
(Numerator degrees of freedom is 1 for all the comparisons)

α' - Per comparison error rate to keep experiment-wise error rate at α' = 0.05, calculated according to Dunn-Sidak method (Sokal and Rohlf, 1981)

S - Difference is significant

NS - Difference is not significant

***Line cross analyses*:** Analysis of line crosses between *early* and *late* flies revealed a complex genetic architecture underlying differences in their circadian phenotypes. Genetic architecture underlying τ difference was relatively simpler than that underlying morning and evening emergence. For each trait, genetic architecture varied among the replicate pairs of *early* and *late* stocks and differed between males and females. Tables 6, 7 and 8 shows the parameter estimates for the genetic model which best explained the variation in the generation means among all the models tested (i. e., the model with smallest AIC value).

***Emergence during morning hours*:** Crosses between *early* and *late* flies revealed that additive, dominance and epistatic effects contribute to the differences in emergence during morning hours (Figure 3; Table 6). In males, difference in morning emergence in only one out of four replicates (R2) was explainable by simple additive effects, but incorporation of dominance and epistasis improved the fit (Tables 6, S1-Supplementary data is provided at the end of this chapter). In the remaining three replicates, none of the genetic effect models including dominance, maternal effects and diagenic epistasis were able to explain the differences between *early* and *late* stocks (Tables 6, S1). In females, differences in morning emergence were not explainable by simple additive model in the four replicates (Tables 6, S2). In two replicates (R_1 and R_2) additive and epistatic effect model was sufficient to explain the differences, but in R1 addition of dominance effects improved the fit (Tables 6, S2). In other two replicates (R_3 and R_4) none of the models could explain the differences (Tables 6, S2). Together these results indicate a complex genetic architecture involving additive, diagenic epistatic interactions and even higher order interactions.

Figure 3. Morning emergence in *early-late* parental stocks and in their F1, F2 and backcross progeny.

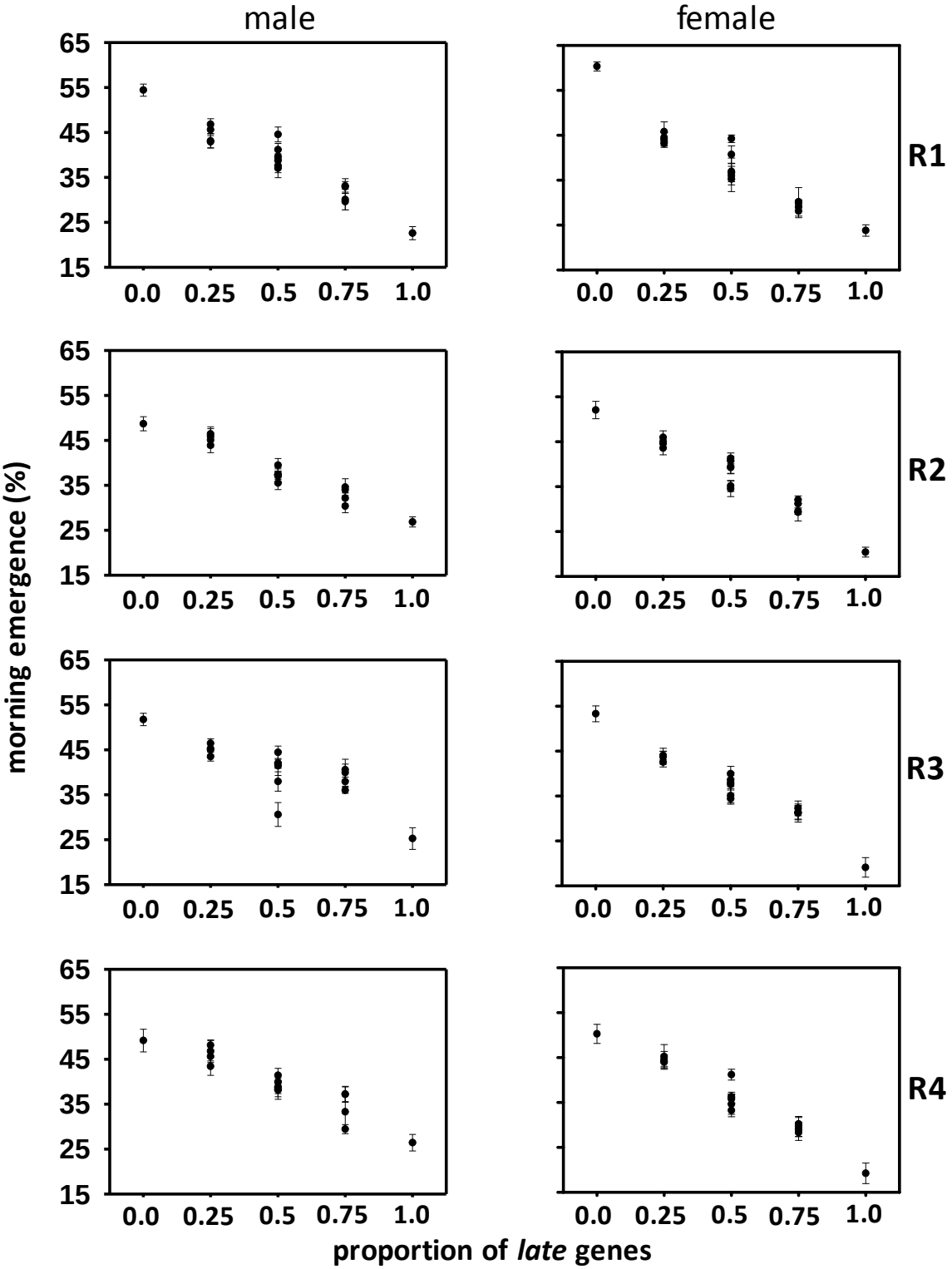


Figure 4. Evening emergence in *early-late* parental stocks and in their F1, F2 and backcross progeny.

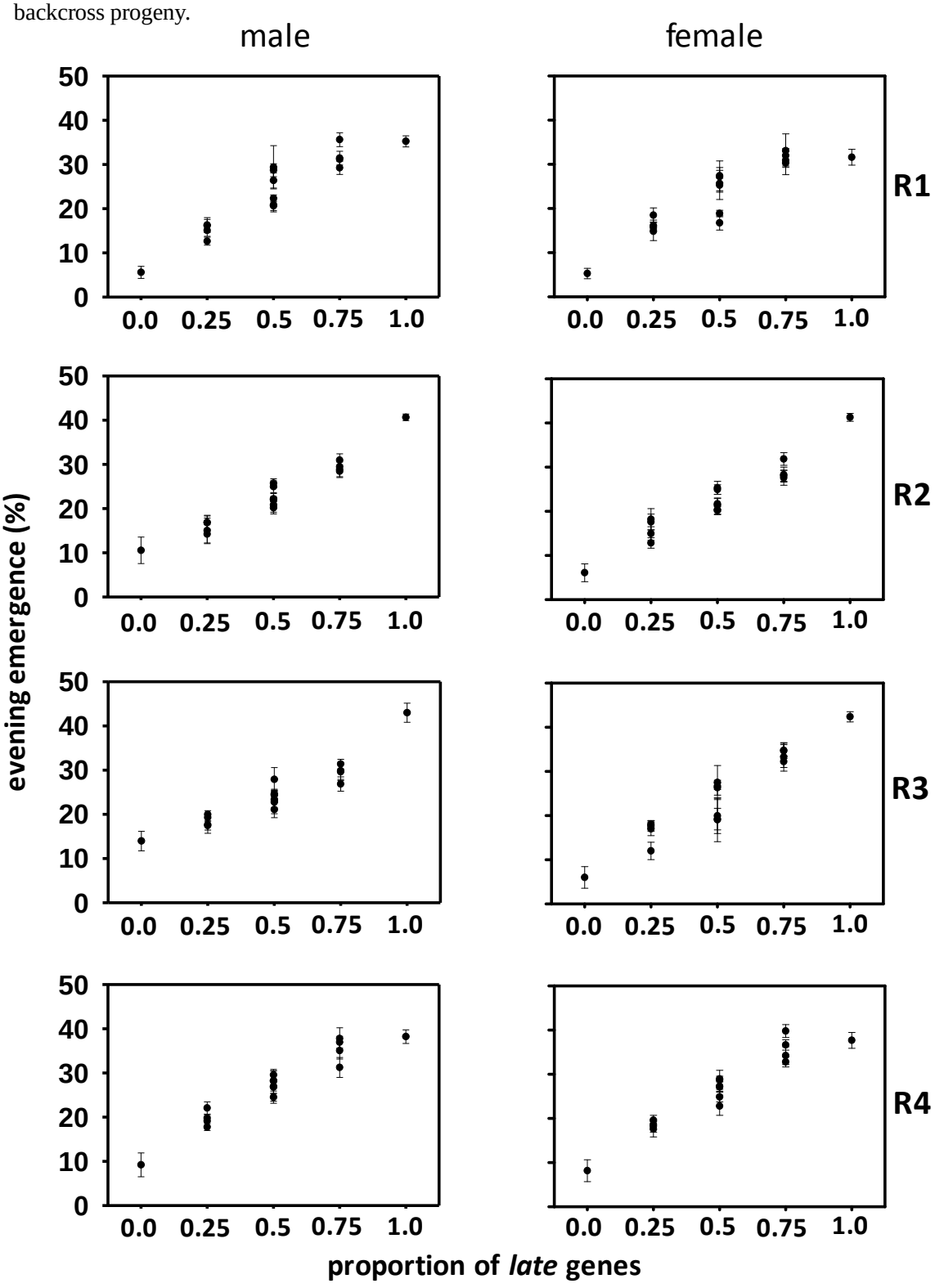
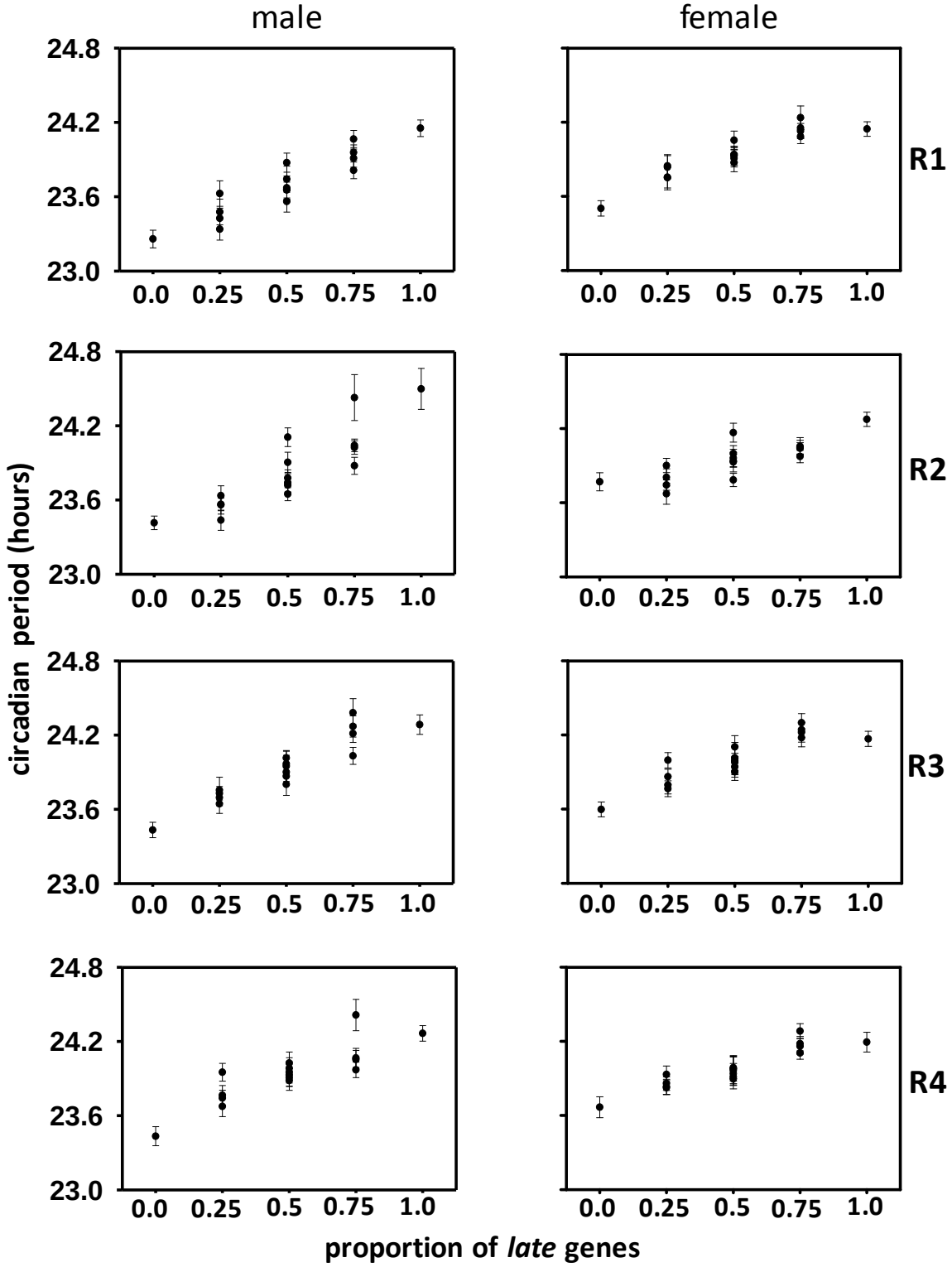


Figure 5. Circadian period of activity/rest rhythm in *early-late* parental stocks and in their F1, F2, and backcross progeny.



Emergence during evening hours: Like the genetic architecture of morning emergence, additive and epistatic effects were required to adequately explain differences in evening emergence between *early* and *late* stocks (Table 7; Figure 4). In males, additive–dominance model was sufficient to explain the differences in evening emergence in one of the replicates (R_3) whereas in (R_2) differences were explainable by additive and diagenic epistatic effect model. In case of one replicate (R_1 and R_4), none of the models were sufficient to explain the differences between *early* and *late* flies (Tables 7, S3). In females, additive and diagenic epistasis model adequately explained differences in evening emergence in two of the four replicates (R_1 and R_3) whereas in other two replicates (R_2 and R_4) none of the models were sufficient to explain differences in evening emergence (Tables 7, S4). These results suggest the contribution of additive, dominance and epistatic effects to the differences in preference for evening emergence.

***Circadian period (τ) of activity/rest rhythm*:** Crosses between *early* and *late* flies showed that genetic architecture of difference in the τ of activity rhythm was relatively simpler than that underlying emergence preference (Table 8; Figure 5). Difference in the τ of two replicates each in male (R_1 and R_3) and female (R_1 and R_4) was explainable by simple additive model (Tables 8, S5, S6). In case of males (R_1 and R3) fit of the observed generation means was better after the addition of maternal effects (Tables 8, S5), whereas in R1 female addition of epistatic effects improved the fit (Tables 8, S6). Remaining replicates in both males and females could not be explained by any of the higher order models (Table 8).

Table 6. Estimates of model parameters, chi-square values for model which best explained variation in morning emergence in progeny from crosses between *early* and *late* stocks. Model with smallest AIC was chosen as best fitting model.

		R1	R2	R3	R4
male	**m**	38.95 ± 0.04	29.01 ± 2.99	44.21 ± 1.1	39.34 ± 0.53
	a	14.22 ± 1.04	10.92 ± 0.96	12.84 ± 1.86	13.2 ± 1.32
	d		22.64 ± 7.8		
	aa		8.73 ± 2.83	-4.95 ± 2.06	
	ad			-9.85 ± 4.34	
	dd		-13.1 ± 5.27	-8.81 ± 3.22	
	χ^2	32.37	11.58	21.75	36.05
	df	14	11	11	14
	p	0.003	0.396	0.026	0.001
		S	NS	S	S
	AIC	36.37	21.58	31.76	40.06
female	**m**	42.39 ± 2.48	41.09 ± 0.84	38.69 ± 0.48	36.44 ± 0.42
	a	18.74 ± 0.8	15.25 ± 1.27	12.91 ± 1.54	15.13 ± 1.2
	d	-23.8 5.58			
	aa		-5.99 ± 1.65		
	ad	-7.35 ± 1.79			
	dd	24.77 ± 3.61	-6.47 ± 1.79		
	χ^2	11.17	16.16	54.8	24.66
	df	11	12	14	14
	p	0.429	0.180	0.000	0.038
		NS	NS	S	S
	AIC	21.17	24.17	58.8	28.66

s - significant χ^2 test, ns - not significant
significant χ^2 test means model is inadequate to explain variation

Table 7. Estimates of model parameters, chi-square values for model which best explained variation in evening emergence in progeny from crosses between *early* and *late* stocks. Model with smallest AIC was chosen as best fitting model.

		R1	R2	R3	R4
male	**m**	22.75 ± 0.55	20.36 ± 1.1	27.75 ± 1.23	30.05 ± 0.97
	a	16.25 ± 1.12	14.78 ± 1.11	11.86 ± 0.86	14.28 ± 1.89
	d			-6.09 ± 2.29	
	aa		5.04 ± 1.61		-5.92 ± 2.19
	dd		4.91 ± 1.34		-5.4 ± 1.71
	χ^2	56.53	7.82	17.07	22.85
	df	14	12	13	12
	p	0.001	0.80	0.19	0.03
		S	NS	NS	S
	AIC	60.54	15.83	23.08	30.85
female	**m**	28.79 ± 0.65	19.25 ± 0.8	28.89 ± 1.15	27.09 ± 0.57
	a	13.14 ± 0.78	17.52 ± 1.2	18.11 ± 1.4	15.94 ± 1.4
	aa	-10.4 ± 1.16	4.49 ± 1.52	-4.62 ± 1.88	
	ad	4.72 ± 1.98			
	dd	-10.3 ± 0.93	5.8 ± 1.52	-9.42 ± 3.15	
	χ^2	6.04	23.69	15.68	45.45
	df	11	12	12	14
	p	0.87	0.02	0.21	0.001
		NS	S	NS	S
	AIC	16.05	31.7	23.68	49.45

s - significant χ^2 test, ns - not significant
significant χ^2 test means model is inadequate to explain variation.

Table 8. Estimates of model parameters, chi-square values for model which best explained variation in circadian period of activity/rest rhythm in progeny from crosses between *early* and *late* stocks. Model with smallest AIC was chosen as best fitting model.

		R1	R2	R3	R4
male	**m**	23.68 ± 0.03	23.79 ± 0.03	23.92 ± 0.02	23.92 ± 0.02
	a	0.33 ± 0.05	0.43 ± 0.06	0.37 ± 0.04	0.34 ± 0.05
	a_m	0.11 ± 0.04		0.1 ± 0.05	
	χ^2	9.60	45.40	13.19	24.96
	df	13	14	13	14
	p	0.72	0.001	0.43	0.034
		NS	S	NS	S
	AIC	15.61	49.40	19.19	28.96
female	**m**	23.97 ± 0.04	23.92 ± 0.02	23.99 ± 0.02	23.99 ± 0.02
	a	0.32 ± 0.04	0.25 ± 0.04	0.322 ± 0.04	0.3 ± 0.03
	aa	-0.13 ± 0.06			
	χ^2	9.35	32.49	25.91	13.36
	df	13	14	14	14
	p	0.75	0.003	0.03	0.50
		NS	S	S	NS
	AIC	15.36	36.50	29.91	17.37

s - significant χ^2 test, **ns** - not significant
significant χ^2 test means model is inadequate to explain variation

Discussion

Among the spectrum of biological processes, circadian rhythms are probably one of the best understood quantitative behaviors at the molecular-genetic level (Takahashi et al., 2008). Forward genetics tools employed to understand the genetic bases of circadian rhythms in a wide variety of organisms identified a dozen genes involved in the regulation of rhythmic functions (Takahashi et al., 2008). Although, identification of such genes greatly facilitated our efforts to decipher the molecular-genetic nature of circadian clocks, it is still an open question whether such canonical clock genes portray the true genetic basis underlying the natural variation in circadian rhythms. Questioning the validity of the contribution of canonical clock genes to the natural variation in circadian rhythms is not an unwarranted scepticism; it is based on some valid concerns. The roots of this scepticism can be traced back to the popular methods used in gene identification by forward genetic screens (Kim et al., 2007).

The current picture of the genetic bases of circadian clocks painted with the canonical clock genes is in striking contrast with the notion that variation in quantitative traits is due to alleles segregating at multiple loci, each having small effect (Mackay, 2001; Sharma and Joshi, 2002). This is likely to be due to the fact that forward genetic approaches based on mutational screens are biased towards the identification of mutations with large effects (Mackay, 2001; Kim et al., 2007) and therefore the search for clock genes may have missed out those genetic loci that when mutated will have small effect on clock properties.

Another issue of prime concern, which has its roots in quantitative genetics, is the use of inbred genetic model systems. In the forward genetics approach, phenotypic effects of single gene mutations are typically tested against highly inbred genetic background (with little or no genetic variation). According to quantitative genetic model, each trait is

influenced by alleles segregating at multiple genetic loci of small effects, and the phenotype of individual genotypes is determined by homozygous, heterozygous effects of alleles at individual loci or pair-wise or higher order interactions among the alleles at multiple loci (Mackay, 2001). Therefore, phenotypic effects attributed to the mutation which was tested against a particular, highly inbred genetic background of the laboratory population may not be reproducible in the context of varying genetic backgrounds (which is usually the case with natural populations) and therefore phenotypic effect of mutations identified by such an approach cannot be taken to represent phenotypic variation in nature (Sharma and Joshi, 2002; Garland and Rose, 2009).

Circadian clocks are believed to confer adaptive advantage to organisms by scheduling rhythmic functions to a specific time of the day (Johnson et al., 2003; Roenneberg et al., 2003a; Sharma, 2003). Therefore, understanding entrainment is central to the understanding of functioning of circadian timing systems (Johnson et al., 2003; Roenneberg et al., 2003a). While the study of genetic bases of circadian rhythms has helped immensely to our understanding of its physical nature and general organization, its contribution to the understanding of circadian entrainment has been very little (Roenneberg et al., 2003a). The underlying reason could be the choice of circadian phenotype used in the genetic screens. Most studies screened for period variants of activity rhythm under constant laboratory conditions (Kim et al., 2007). Although, such variants facilitated subsequent genetic and molecular analyses of circadian molecular clockwork, this approach predominantly identified the components essential for oscillator function but not the components necessary for circadian entrainment. The result is, apart from the identification of the specialized circadian photoreceptor (Emery et al., 1998; Stanewsky et al., 1998) and demonstration of regulation of core clock components by light (Crosthwaite et al., 1995; Hunter-Ensor et al., 1996), its contribution in understanding the process of entrainment has been limited, and how

entrainment occurs at the molecular-genetic level is still an open question (Roenneberg et al., 2003a). Therefore, it is still reasonable to ask whether our understanding of the genetic bases of circadian clocks is representative of those underlying adaptive natural variation.

We created *early* and *late* stocks of *D. melanogaster* in a long-term laboratory selection study by imposing selection for emergence during morning and evening hours. The *early* and *late* populations evolved preferences for morning and evening emergence, and shorter and longer circadian period respectively (Kumar et al., 2007a). Considering the relationship between phase of the rhythm and circadian period (Pittendrigh and Daan, 1976; Sharma et al., 1998; Roenneberg et al., 2003a; Sharma and Chidambaram, 2003), co-evolution of period and preference for emergence (morning/evening) in our large, outbred, independent replicate populations suggests that circadian clocks in *early* and *late* stocks are adaptation to selection for timing of emergence. Though we are aware of the fact that artificial laboratory selection does not mimic natural selection, studying the genetic bases of divergent circadian phenotypes evolved as an adaptation to laboratory selection could possibly be the closest approximation to studying the genetic variation of circadian rhythms in nature.

***Circadian phenotypes of early and late flies after 165 generations of selection*:** After 165 generations of selection, all four *early* stocks emerged in greater numbers in the morning and had shorter τ of activity rhythm, while all four *late* stocks emerged in higher numbers in the evening and had a longer τ compared to *control* stocks. Evolution of divergent circadian phenotypes in four independent replicate *early* and *late* stocks selected for emergence during morning and evening hours, otherwise maintained under identical conditions, indicates that such changes are due to imposed selection for timing of emergence.

***Contribution from sex chromosomes*:** In fruit flies *D. melanogaster*, male flies get their single X chromosome from their mothers and therefore difference in the phenotypes of male progeny from the reciprocal crosses would indicate contribution of X chromosome and/or maternal cytoplasmic factors. Comparison of circadian phenotypes of male progeny from reciprocal crosses allowed us to test the contribution of the X chromosome and the effects of maternal cytoplasm (de Belle and Sokolowski, 1987; Huttunen and Aspi, 2003). The results revealed that circadian phenotypes of males did not differ (except in R_3 males) between the reciprocal crosses (Table 4a), which suggests that X chromosome does not contribute to the differences between the circadian phenotypes of *early* and *late* stocks. Progeny from reciprocal F2 and backcrosses allowed us to test the main/independent effects of X and Y chromosomes. The analyses revealed that none of the sex chromosomes had any significant role in the divergence of circadian phenotypes in *early* and *late* stocks.

***Maternal effects*:** Typically, characterization of genetic architecture of continuously varying traits involves identification of genetic loci influencing the trait variation (QTLs), relative magnitude of their effects, identification of segregating allelic variation at those loci, their homozygous-heterozygous effects, epistatic interactions among loci, and plieotropy (Lynch and Walsh, 1998; Mackay, 2001). Traditional approaches of QTL identification are based on correlation between allelic state at the loci and the associated phenotype, where phenotypic variation among individuals is mapped on to the genetic variation among individuals (Wolf et al., 2002). Thus this approach is based on the implicit assumption that phenotype of the individual is a result of only the direct effect of the underlying genes and its interaction with the environment. However, this assumption may not be true for all traits, particularly those where phenotypes are influenced by the genotypes of the interacting individuals. These approaches are based on the assumption that observed phenotype of a given individual is solely determined by the genotype of the individual, which is also not true for all traits.

Therefore, the undue focus on the study of direct-effect genes, in cases where this assumption is not valid, may result in false characterization of underlying genetic architecture of the trait (Wolf et al., 2002). One of the most common examples of this is the maternal effects in which mother's genotype influences the phenotype of its offspring's through some mechanisms other than transmission of genes (Mousseau and Fox, 1998). Design of crosses between *early* and *late* stocks allowed us to test the contribution of main effects of TMF and PCF and their interaction with remaining factors (sex chromosomes and TMF) to the differences between *early* and *late* circadian phenotypes. Analyses revealed that there is no role of TMF or PCF in the difference between the circadian phenotypes of emergence and activity rhythms in *early* and *late* stocks. Only in R_4 males, TMF showed a significant effect on morning and evening emergence, and TMF interactions showed a significant effect on circadian period (Table 4a-c). Absence of any direct influence of TMF in other three replicate populations indicates that the differences in circadian phenotypes of *early* and *late* stocks are directly regulated by the underlying genes. Absence of any effect of PCF suggests that non-chromosomal genetic factors do not contribute to the differences in the circadian phenotypes of *early* and *late* stocks.

***Genetic architecture of flies with preference for morning and evening emergence*:** We studied the genetic architecture underlying divergence of emergence rhythm and τ of activity/rest rhythm between *early* and *late* stocks, separately for each replicate pair of $early_i$ and $late_k$ ($i = k$) stocks. Our analyses revealed that complex genetic interactions underlie the differences in morning and evening emergence preference between the *early* and *late* stocks. Divergence in morning emergence in R2 males alone was explainable by simple additive effects, however, addition of other interactions improved the fit (Table S1). Dominance or epistatic interactions were additionally necessary to explain the differences in morning or evening emergence in the other three replicates (Figures 3, 4; Tables 6, 7). In case of

morning emergence, R_1, R_3, R_4 – males; R_3, R_4 – females, and in case of evening emergence, R1, R4 – males, R2, R4 - females, none of the tested models could explain the differences in morning and evening emergence preference (Figures 3, 4; Tables 6, 7, S1-S4), which suggests that the differences in morning and evening emergence in the these replicates are due to linkage or higher order interactions.

Correlated changes in the circadian period of the *early* and *late* stocks allowed us to study the underlying genetic architecture. Our analyses revealed that the genetic architecture underlying the divergence in circadian period was relatively simpler than that of emergence rhythm. Difference in period, at least in two out of four replicates (R_1, R_3 – males; R_1, R_4 – females) was explainable by simple additive model, but addition of dominance and epistatic interactions improved the fit (Tables S5, S6). In the remaining two, none of the models could adequately explain the differences (Figure 5; Table 8), suggesting the contribution of linkage or higher order interactions.

***Epistasis and its implications*:** While our analyses revealed that epistasis contributed to the divergence of emergence preference in *early* and *late* stocks, it also had some role in the divergence of the period of activity rhythm. Contribution of epistasis to such divergence implies that genes do not merely influence the trait through additive effects and the phenotypic effect of allele at one particular locus also depends on the allele(s) present at other loci.

Forward genetic screens have been the workhorse in identification of clock genes. In forward genetics approach, clock mutations are identified by screening for abnormal circadian phenotypes of large magnitudes (phase and period variants). Phenotypic effects of such mutations are then typically studied on single, inbred genetic background (lacking genetic variation) and thus phenotypic effects of possible interactions of mutation with

variation at other loci are rarely tested. Evidence for the involvement of epistasis to the divergence of emergence and activity rhythms in the *early* and *late* stocks indicates the contribution of inter-locus interactions to the occurrence of variation in circadian phenotypes. Thus our study suggests the need for a cautious approach towards defining the roles of genes in circadian clockwork without knowing the phenotypic effects of mutations on different genetic backgrounds.

In summary, the results of our study suggest that directly acting autosomal loci primarily contribute to the adaptive divergence of *early* and *late* circadian phenotypes. Line cross analyses revealed that complex genetic architecture underlies circadian phenotypes of morning and evening emergence in *early* and *late* stocks. While epistasis is likely to be involved in the divergence of emergence preference, differences in circadian period of activity rhythm are possibly due to additive effects. Differences in the genetic architecture of divergence among replicate selected populations suggest that the divergent circadian phenotypes of *early* and *late* stocks are achievable through the evolution of distinct genetic mechanisms. More such line cross studies on the naturally occurring genetic variation with some genetic models describing higher order interactions might throw some light on the genetics underlying circadian clocks.

Legends for supplementary Tables S1-S6

Upper case alphabet(s) (A, D, M, E) in upper most row of each tables represent type of genetic models tested. Each of those alphabets represent presence of genetic effect(s) in the model being tested.

A - m, a D - m, d M - m, a_m, d_m, C E - m, a.a, a.d, d.d

Small letter alphabets in the middle rows of each table show model parameters which were significant and their estimates were used in the calculation of expected generation means .

m – mean, **a** – additive effect, **d** – dominance
$\mathbf{a_m}$ – additive maternal effect, $\mathbf{d_m}$ – dominance maternal effect
c – cytoplasmic factors, **a.a** – additive-additive interaction
a.d – additive-dominance interaction, **d.d** – dominance-dominance interaction

K = number of significant parameters
df= degrees of freedom used for testing goodness of fit (df= 16-k)
AIC = Akaike information criteria value (calculated as described in Bieri and Kawecki, 2003).

Non significant χ^2 values are shown in bold letters.

Table S1. Results of all the models tested on generation means of males for morning emergence in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m	m a	m a	m a	m	m a
k	2	1	2	2	2	2	2	2	2	1	2	2	2	1	2
df	14	15	14	14	14	14	14	14	14	15	14	14	14	15	14
χ^2	32.37	464.80	255.32	286.50	36.23	38.39	39.78	273.14	567.68	752.83	51.42	226.10	54.22	466.02	105.61
AIC	36.37	466.80	259.32	290.50	40.23	42.39	43.78	277.14	571.68	754.83	55.42	230.10	58.22	468.02	109.61

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m a_m a.d	m a	m a d a.a d.d	m a	m a.d	m a d a.a
k	2	1	2	2	2	2	2	2	2	3	2	5	2	2	4
df	14	15	14	14	14	14	14	14	14	13	14	11	14	14	12
χ^2	**19.55**	344.06	140.55	162.40	**20.63**	24.46	24.74	133.43	1740.9	86.16	**22.99**	**11.58**	27.82	679.96	191.43
AIC	23.55	346.06	144.55	166.40	24.63	28.46	28.74	137.43	1744.9	92.16	26.99	21.58	31.82	683.96	199.43

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a a.a a.d d.d	m a_m	m a.d	m	m a	m a a.d	m a a.d d.d	m	m a a.d
k	2	1	2	2	2	2	5	2	2	1	2	3	4	1	3
df	14	15	14	14	14	14	11	14	14	15	14	13	12	15	13
χ^2	47.92	525.52	136.19	213.80	87.55	66.15	21.76	198.66	768.42	258.49	61.21	45.66	37.62	321.54	59.82
AIC	51.92	527.52	140.19	217.80	91.55	70.15	31.76	202.66	772.42	260.49	65.21	51.66	45.62	323.54	65.82

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a a_m	m a.d	m a	m a	m a	m a_m	m a.d	m	m a	m a	m a	m	m a
k	2	1	2	2	2	2	2	2	2	1	2	2	2	1	2
df	14	15	14	14	14	14	14	14	14	15	14	14	14	15	14
χ^2	36.06	311.28	79.19	110.17	36.56	66.79	43.49	75.04	359.72	298.36	60.14	83.58	82.05	1093.7	738.02
AIC	40.06	313.28	83.19	114.17	40.56	70.79	47.49	79.04	363.72	300.36	64.14	87.58	86.05	1095.7	742.02

Table S2. Results of all the models tested on generation means of females for morning emergence in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a d_m	m a a.a a.d d.d	m a_m	m a.d	m	m a d_m	m a d a.d d.d	m a a.a a.d d.d		m a d a.d d.d
k	2	1	2	2	2	3	5	2	1	1	3	5	5	0	5
df	14	15	14	14	14	13	11	14	15	15	13	11	11	16	11
χ^2	139.21	1137.9	1079.8	1522.6	146.09	69.24	**15.25**	932.66	21838.	1494.50	72.51	**14.89**	**19.23**	2011.7	**11.17**
AIC	143.21	1139.9	1083.8	1526.6	150.09	75.24	25.25	936.66	21840.	1496.50	78.51	24.89	29.23	2011.7	21.17

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.a a.d	m a	m a d_m	m a a.a d.d	m a_m	m a.d	m a.d	m a d_m	m a a.a	m a d.d	m a.d	m a
k	2	1	2	3	2	3	4	2	2	2	3	3	3	2	2
df	14	15	14	13	14	13	12	14	14	14	13	13	13	14	14
χ^2	42.58	924.25	408.60	314.03	44.66	28.65	**16.17**	456.85	395.74	1100.73	29.05	710.89	49.20	424.08	935.15
AIC	46.58	926.25	412.60	320.03	48.66	34.65	24.17	460.85	399.74	1104.73	35.05	716.89	55.20	428.08	939.15

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m	m a	m a	m a	m	m a
k	2	1	2	2	2	2	2	2	2	1	2	2	2	1	2
df	14	15	14	14	14	14	14	14	14	15	14	14	14	15	14
χ^2	54.80	335.19	209.76	197.67	63.87	72.22	105.87	383.97	1285.6	620.90	118.68	476.64	90.76	329.60	145.50
AIC	58.80	337.19	213.76	201.67	67.87	76.22	109.87	387.97	1289.6	622.90	122.68	480.64	94.76	331.60	149.50

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m	m a.d	m a	m a	m a	m a_m	m a.d	m a.d	m a	m a	m a	m	m a
k	2	1	1	2	2	2	2	2	2	2	2	2	2	1	2
df	14	15	15	14	14	14	14	14	14	14	14	14	14	15	14
χ^2	24.66	306.18	350.00	126.47	25.45	25.38	30.04	259.21	140.06	166.12	25.88	76.30	49.05	426.35	37.35
AIC	28.66	308.18	352.00	130.47	29.45	29.38	34.04	263.21	144.06	170.12	29.88	80.30	53.05	428.35	41.35

Table S3. Results of all the models tested on generation means of males for evening emergence in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m c	m a.d	m a	m a	m a	m a_m	a.d	m a_m a.d	m a	m a	m a	a_m a.d	m a
k	2	1	3	2	2	2	2	2	1	3	2	2	2	2	2
df	14	15	13	14	14	14	14	14	15	13	14	14	14	14	14
χ^2	56.54	925.65	380.17	371.69	56.78	110.21	137.31	917.19	7904.3	235.45	135.11	197.91	182.44	9009.5	478.05
AIC	60.54	927.65	386.17	375.69	60.78	114.21	141.31	921.19	7906.3	241.45	139.11	201.91	186.44	9013.5	482.05

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m d	m	m a.a a.d	m a	m a d_m	m a a.a d.d	m	m a.d	m a.a	m a d_m	m a	m a a.a d.d	m a.d	m a
k	2	2	1	3	2	3	4	1	2	2	3	2	4	2	2
df	14	14	15	13	14	13	12	15	14	14	13	14	12	14	14
χ^2	33.40	449.09	646.23	193.50	34.34	**17.91**	**8.78**	1076.4	1508.8	460.17	**17.73**	66.68	**7.83**	845.65	86.38
AIC	37.40	453.09	648.23	199.50	38.34	23.91	16.78	1078.4	1512.8	464.17	23.73	70.68	15.83	849.65	90.38

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m	m a.d	m a d	m a	m a	m	m a.d	m a.d	m a d	m a	m a	a.d	m a
k	2	1	1	2	3	2	2	1	2	2	3	2	2	1	2
df	14	15	15	14	13	14	14	15	14	14	13	14	14	15	14
χ^2	26.39	352.15	286.10	111.83	**17.08**	28.38	39.76	450.30	327.37	117.58	**18.34**	185.26	77.71	5394.3	81.68
AIC	30.39	354.15	288.10	115.83	23.08	32.38	43.76	452.30	331.37	121.58	24.34	189.26	81.71	5396.3	85.68

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a a.a d.d	m a_m	m a.d	m a_m a.d	m a	m a	m a a_m a.a	m a_m a.d	m a a_m
k	2	1	2	2	2	2	4	2	2	3	2	2	4	3	3
df	14	15	14	14	14	14	12	14	14	13	14	14	12	13	13
χ^2	39.19	488.92	197.65	142.59	43.43	46.27	22.85	324.50	743.23	77.83	62.31	56.62	32.62	1188.8	82.34
AIC	43.19	490.92	201.65	146.59	47.43	50.27	30.85	328.50	747.23	83.83	66.31	60.62	40.62	1194.8	88.34

Table S4. Results of all the models tested on generation means of females for evening emergence in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m	m a.a a.d d.d	m a	m a d_m	m a a.a a.d d.d	m	a.d	m a.d	m a d_m	m a a.a	m a a.a d.d	a.d	m a a.a d.d
k	2	1	1	4	2	3	5	1	1	2	3	3	4	1	4
df	14	15	15	12	14	13	11	15	15	14	13	13	12	15	12
χ^2	77.18	564.97	567.62	159.81	118.13	40.03	**6.05**	549.45	6933.9	1857.2	57.11	462.01	**17.33**	6735.8	**15.98**
AIC	81.18	566.97	569.62	167.81	122.13	46.03	16.05	551.45	6935.9	1861.2	63.11	468.01	25.33	6737.8	23.98

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.a a.d	m a	m a d_m	m a a.a d.d	m a_m	a.d		m a	m a	m a d.d		m a
k	2	1	2	3	2	3	4	2	1	0	2	2	3	0	2
df	14	15	14	13	14	13	12	14	15	16	14	14	13	16	14
χ^2	45.23	968.90	491.57	370.10	46.65	32.86	23.70	639.42	6021.5	969.25	63.94	167.52	52.57	1753.7	179.36
AIC	49.23	970.90	495.57	376.10	50.65	38.86	31.70	643.42	6023.5	969.25	67.94	171.52	58.57	1753.7	183.36

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.a	m a	m a	m a a.a d.d	m a_m	m a.d	m	m a	m a	m a a.a d.d		m a a.a
k	2	1	2	2	2	2	4	2	2	1	2	2	4	0	3
df	14	15	14	14	14	14	12	14	14	15	14	14	12	16	13
χ^2	24.72	1240.7	266.09	290.28	30.96	28.99	**15.68**	486.44	1616.4	975.28	29.18	86.02	26.22	764.35	397.92
AIC	28.72	1242.7	270.09	294.28	34.96	32.99	23.68	490.44	1620.4	977.28	33.18	90.02	34.22	764.35	403.92

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m a.d	m a	m a	m a	a.d	m a
k	2	1	2												
df	14	15	14	14	14	14	14	14	14	14	14	14	14	15	14
χ^2	45.45	479.36	262.17	140.83	47.88	52.47	88.33	330.29	260.98	137.98	88.25	52.98	89.07	7516.8	85.47
AIC	49.45	481.36	266.17	144.83	51.88	56.47	92.33	334.29	264.98	141.98	92.25	56.98	93.07	7518.8	89.47

Table S5. Results of all the models tested on generation means of males for circadian period in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a a.a	m a	m a_m	m a.d	m a_m	m a a_m	m a	m a a_m	m a_m	m a a_m
k	2	1	2	2	2	3	2	2	2	2	3	2	3	2	3
df	14	15	14	14	14	13	14	14	14	14	13	14	13	14	13
χ^2	**20.83**	179.35	44.15	121.94	**22.64**	**10.16**	34.40	44.78	160.39	86.77	**9.61**	101.45	**19.32**	319.89	124.86
AIC	24.83	181.35	48.15	125.94	26.64	16.16	38.40	48.78	164.39	90.77	15.61	105.45	25.32	323.89	130.86

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m	m a	m a	m a	m	m a
k	2	1	2	2	2	2	2	2	2	1	2	2	2	1	2
df	14	15	14	14	14	14	14	14	14	15	14	14	14	15	14
χ^2	45.40	341.19	79.28	105.39	49.92	67.67	129.57	105.66	873.26	199.73	58.45	73.70	59.41	676.36	66.64
AIC	49.40	343.19	83.28	109.39	53.92	71.67	133.57	109.66	877.26	201.73	62.45	77.70	63.41	678.36	70.64

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a a_m	m a	m a_m	m a.d	m a_m	m a	m a	m a	m a_m	m a
k	2	1	2	2	2	3	2	2	2	2	2	2	2	2	2
df	14	15	14	14	14	13	14	14	14	14	14	14	14	14	14
χ^2	**17.65**	204.15	73.28	104.34	**23.21**	**13.19**	**18.30**	78.81	403.69	120.93	25.32	181.96	41.62	223.84	146.50
AIC	21.65	206.15	77.28	108.34	27.21	19.19	22.30	82.81	407.69	124.93	29.32	185.96	45.62	227.84	150.50

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m	m a	m a	m a	m a_m	m	m a_m	m a	m a	m a	m a_m	m a
k	2	1	2	1	2	2	2	2	1	2	2	2	2	2	2
df	14	15	14	15	14	14	14	14	15	14	14	14	14	14	14
χ^2	24.96	115.51	42.44	115.50	29.89	31.53	30.47	46.19	128.87	55.41	35.50	29.31	38.69	43.29	26.01
AIC	28.96	117.51	46.44	117.50	33.89	35.53	34.47	50.19	130.87	59.41	39.50	33.31	42.69	47.29	30.01

Table S6. Results of all the models tested on generation means of females for circadian period in four replicates.

R1	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a a.a	m a_m	m a.d	m	m a	m a	m a a.a	m	m a
k	2	1	2	2	2	2	3	2	2	1	2	2	3	1	2
df	14	15	14	14	14	14	13	14	14	15	14	14	13	15	14
χ^2	**14.29**	116.06	62.90	74.67	24.63	**15.12**	**9.36**	89.38	187.52	120.03	31.18	152.62	**22.21**	329.09	87.16
AIC	18.29	118.06	66.90	78.67	28.63	19.12	15.36	93.38	191.52	122.03	35.18	156.62	28.21	331.09	91.16

R2	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m a_m	m a.d	m a	m a	m a	m a_m	m a.d	m	m a	m a	m a	m	m a
k	2	1	2	2	2	2	2	2	2	1	2	2	2	1	2
df	14	15	14	14	14	14	14	14	14	15	14	14	14	15	14
χ^2	32.50	108.65	62.85	141.32	32.51	35.65	85.43	63.30	152.65	215.21	34.43	63.93	113.10	105.17	46.70
AIC	36.50	110.65	66.85	145.32	36.51	39.65	89.43	67.30	156.65	217.21	38.43	67.93	117.10	107.17	50.70

R3	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m	m a.d	m a	m a	m a	m	m a.d	m	m a	m a d.d	m a a.a	m	m a
k	2	1	1	2	2	2	2	1	2	1	2	3	3	1	2
df	14	15	15	14	14	14	14	15	14	15	14	13	13	15	14
χ^2	25.91	142.17	128.07	83.65	40.04	29.15	41.10	132.47	636.25	284.72	46.05	1381.58	48.76	283.69	237.85
AIC	29.91	144.17	130.07	87.65	44.04	33.15	45.10	134.47	640.25	286.72	50.05	1387.58	54.76	285.69	241.85

R4	A	D	M	E	AD	AM	AE	DM	DE	ME	ADM	ADE	AME	DME	ADME
Parameters	m a	m	m	m a.d	m a	m a	m a	m	m a.d	m a.d	m a	m a d.d	m a	m a.d	m a d a_m d_m c a.a d.d
k	2	1	1	2	2	2	2	1	2	2	2	3	2	2	8
df	14	15	15	14	14	14	14	15	14	14	14	13	14	14	8
χ^2	**13.37**	95.62	97.36	46.77	**14.84**	**16.40**	31.07	92.65	242.27	34.32	**14.29**	754.22	**14.00**	725.80	**3.92**
AIC	17.37	97.62	99.36	50.77	18.84	20.40	35.07	94.65	246.27	38.32	18.29	760.22	18.00	729.80	19.92

Chapter 6

Chronotypes in emergence rhythms of *early* and *late Drosophila* populations are determined by circadian clocks in consultation with environmental cycles

Introduction

Although humans are predominantly diurnal, there is a considerable inter-individual variation in our daily sleep/wake timings. Larks tend to be morning-active with advanced phase of sleep and wake-up timings relative to owls (Roenneberg et al., 2003b). Several studies have reported association between clock period (τ) and morningness/eveningness chronotypes, which suggest that differences in circadian clocks underlie variation in human sleep/wake schedules (Baehr et al., 2000; Duffy et al., 2001; Roenneberg et al., 2003b). Duration of daylight exposure is also reported to be correlated with morningness/eveningness chronotypes suggesting that the strength of environmental time-cues influences sleep/wake timings (Roenneberg et al., 2003b; Goulet et al., 2007). Although, the terms 'lark' and 'owl' are often metaphorically used to refer to extreme human chronotypes, in principle, they can also be taken to represent two extremes of distribution in temporal organization of any diurnal behaviour. Fruit flies *Drosophila melanogaster* exhibit diurnal rhythm in adult emergence which is known to be under the control of circadian clocks. Emergence under laboratory light/dark (LD) cycles starts by lights-ON followed by a peak shortly after lights-ON and end of emergence by lights-OFF. In a continuing long-term selection study, we derived '*early*' and '*late*' stocks of *Drosophila*, exhibiting preference for emergence during morning and evening, respectively (Kumar et al., 2007a). Divergence of emergence timings in *early* and *late* stocks, resembling larks and owls, is associated with divergence of their circadian clocks (Kumar et al., 2007a). The *early* flies have shorter τ (~23.4-hr) and phase response curve (PRC) with smaller phase-delays and larger advances, while *late* flies have longer τ (~24.5-hr) and PRC with larger phase-delays and smaller advances compared to controls. Considering the circadian regulation of rhythmic emergence, evolution of clocks implies that diverged clocks may underlie morning-evening chronotypes in the emergence behaviour of *early* and *late* flies. Studying such lark and owl-like pattern in the timing of

emergence in a model system such as *Drosophila* may therefore help better understand the circadian regulation of lark and owl sleep/wake chronotypes in humans.

Although *early* and *late* flies exhibited clearly divergent emergence waveforms under laboratory conditions where the only time-cue present was light of low intensity, their emergence waveforms overlapped substantially and it was not clear whether such differences in emergence preference would persist under natural conditions where a vast repertoire of zeitgebers is present in the strongest form. Therefore, to assess the utility of *early* and *late* flies as a model for human chronotype, we characterised their emergence behaviour under semi-natural conditions.

Material and Methods

Maintenance and selection protocol of *early* and *late* stocks are described in detail in Kumar et al. (2007a). Briefly, each stock is maintained as four replicate populations and each replicate population as a large group of adult individuals (~1200; sex ratio close to 1) provided with banana-jaggary (BJ) food *ad libitum* under 12:12 hr LD cycles. Every generation, ~300 eggs/vial are collected for each of the four *early* (24-vials), *control* (16-vials) and *late* (48-vials) populations, respectively. In *early* and *late* stocks, breeding populations are formed at each generation from flies emerging on 5 successive days during 4-hr span in the morning and 4-hr in the evening respectively. In *controls*, flies that emerged throughout the day were collected for the same 5-days.

Adult emergence was assayed for all stocks under Laboratory (LAB) and semi-natural conditions (SN). For emergence assay, eggs were collected in ten replicate vials/ population/ light regime, and vials were shifted to respective light regime. Emergence under SN was assayed by keeping vials in an outdoor-enclosure at JNCASR, Bangalore (12°59'N 77°35'E). Developing flies were exposed to natural illumination inside the enclosure but were not

exposed to direct sunlight. Light intensity, temperature, humidity were recorded every 5-min throughout experiment using DEnM (Trikinetics, USA). Moon phases during the course of the experiment (29th December 2010 to 16th January 2011) were as follows: 4th January–new moon, 20th January–full moon.

Number of flies that emerged every 2-hourly time-point was recorded for 4-5-cycles and emergence waveforms plotted in the form of percentage emergence as function of time of the day expressed as time-difference in hours relative to mid-point of dark phase (external time, ExT; Daan et al. 2002). Individual vial waveforms were analysed to estimate timings of emergence rhythm phases such as, peak (ψ_{PK} = time at which frequency of emergence reached maximum), onset and offset (ψ_{ON}/ψ_{OFF} = time at which emergence increased above an arbitrary cut-off of 5%, for the first time in a cycle or dropped below an arbitrary cut-off of 5% towards the end of the day). Under SN, the time-point when light intensity increased above or dropped to 0-lux was considered as lights-ON and lights-OFF, respectively. Timings of lights-ON and lights-OFF under SN were about 06:25 am and 06:35 pm, respectively.

Population means were used as units for mixed-model analysis of variance (ANOVA) while testing among stock differences in emergence rhythm waveforms and its phases (ψ). Stocks (S), time–point (T) and environment (ER) were treated as fixed effect factors, whereas replicate populations as random factor. Post-hoc multiple comparisons were done by Tukey's test. Error-bars in figures are 95%CI (comparison intervals), therefore absence of overlap between error-bars of two means indicates significant difference.

Results

Under SN, emergence waveforms of *early* and *late* flies became distinctly separated from *controls* (Figure 1a); this divergence was more prominent than that seen in LAB (Figure 1b). Separate ANOVA on emergence data revealed a statistically significant effect of S (SN-$F_{2,6}$ = 16.18, $p < 0.001$; *LAB*-$F_{2,6}$ = 34.93, $p < 0.001$), T (SN-$F_{11,33}$ = 192.76, $p < 0.001$; LAB-$F_{11,33}$ = 253.85, $p < 0.001$), and S × T interaction (SN-$F_{22,66}$ = 96.71, $p < 0.001$; LAB-$F_{22,66}$ = 65.40, $p < 0.001$; Table 1a, b). Post-hoc multiple comparisons using Tukey's test revealed that emergence of *early* flies was higher than *controls* shortly before dawn (ExT5.5 in SN and ExT6 in LAB) and *late* flies emerged in greater numbers towards the evening (ExT11.5 in SN and ExT18 in LAB). Emergence of *early* and *late* flies overlapped substantially between ExT5.5 and 11.5 in SN and between ExT6 and ExT18 in LAB (Figure 1).

For all three phase measurements (ψ), ANOVA showed a statistically significant effect of S (ψ_{ON}-$F_{2,6}$ = 198.33, $p < 0.001$; ψ_{OFF}-$F_{2,6}$ = 435.83, $p < 0.001$; ψ_{PK}-$F_{2,6}$ = 27.93, $p < 0.001$), ER (ψ_{ON}-$F_{1,3}$ = 75.07, $p < 0.001$; ψ_{OFF}-$F_{1,3}$ = 2798.45, $p < 0.001$) and S × ER interaction (ψ_{ON}-$F_{2,6}$ = 32.92, $p < 0.001$; ψ_{PK}-$F_{2,6}$ = 40.84, $p < 0.001$; Table 2a-c). Post-hoc multiple comparisons using Tukey's test revealed that under SN, ψ_{ON} and ψ_{OFF} of *early* flies were phase-advanced compared to *controls* by ~3-hr and of *late* flies was phase-delayed by ~3-hr (Figure 2b). ψ_{ON} was advanced by ~4-hr in *early* and ~0.5-hr in *late* flies, but compared to LAB ψ_{OFF} of all the stocks were phase-advanced under SN by ~4-hr (Figure 2b). The ψ_{PK} of *early*, *control* and *late* stocks did not differ under LAB, but in SN it was phase-advanced by ~3.5-hr in *early* and phase-delayed by ~2-hr in *late*, compared to *controls* (Figure 2b).

Figure 1a. Adult emergence waveforms of *early*, *control* and *late* flies under semi-natural (SN) conditions. Upper panel show average profiles of light intensity (lux), temperature (ºC) and humidity (%RH) for the entire duration of the experiment.

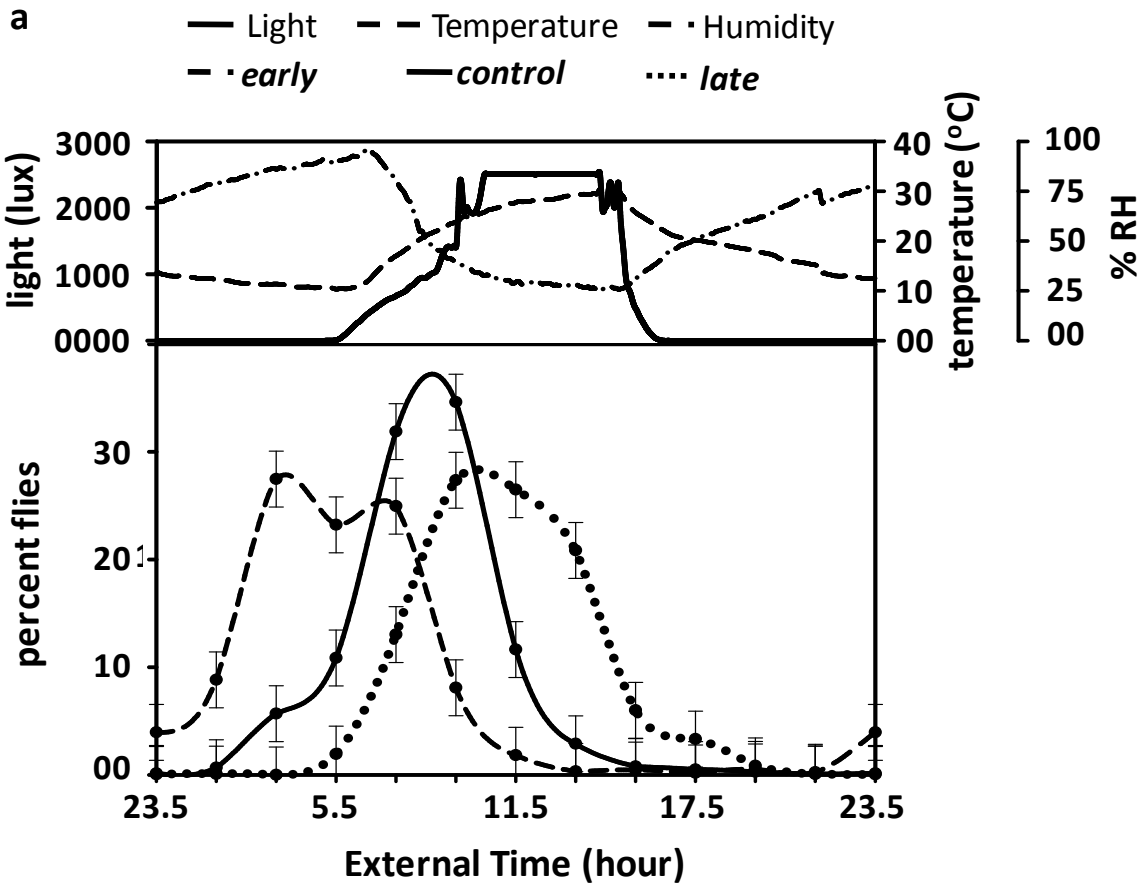


Figure 1b. Adult emergence waveforms of *early*, *control* and *late* flies under laboratory conditions (LAB). Upper panel show average profiles of light intensity (lux), temperature (°C) and humidity (%RH) for the entire duration of the experiment.

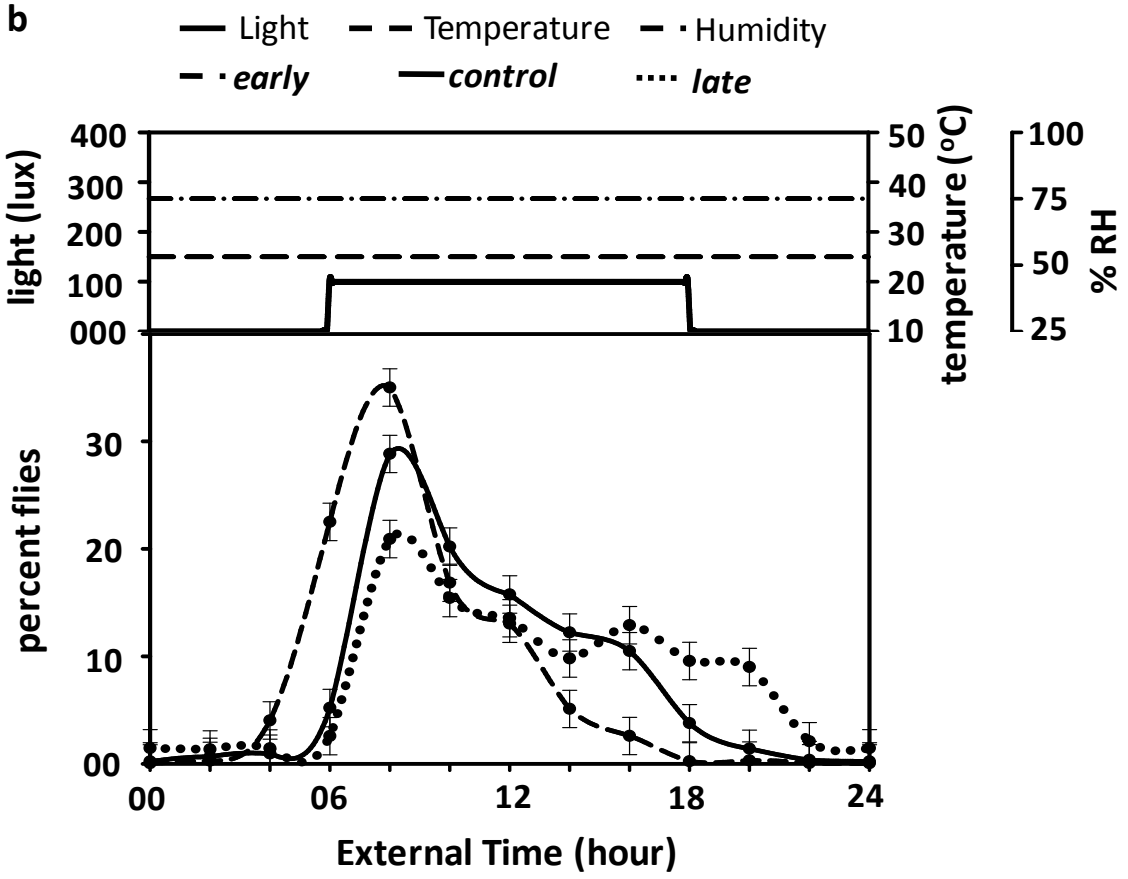


Figure 2. The phase of onset (ψ_{ON}), peak (ψ_{PK}) and offset (ψ_{OFF}) of adult emergence rhythm in *early*, *control* and *late* flies under semi-natural (SN) and laboratory (LAB) conditions. Panels (a) and (b) are the plotted using the same data in two different ways. Panel (a) compares ψ_{ON}, ψ_{PK} and ψ_{OFF} for a given stock of flies under SN/LAB. Panel (b) compares ψ_{ON}, ψ_{PK} and ψ_{OFF} among *early*, *control* and *late* flies under SN/LAB. Error-bars are 95% comparison intervals. Meaningful comparisons can be done only for the same type of phase measurements.

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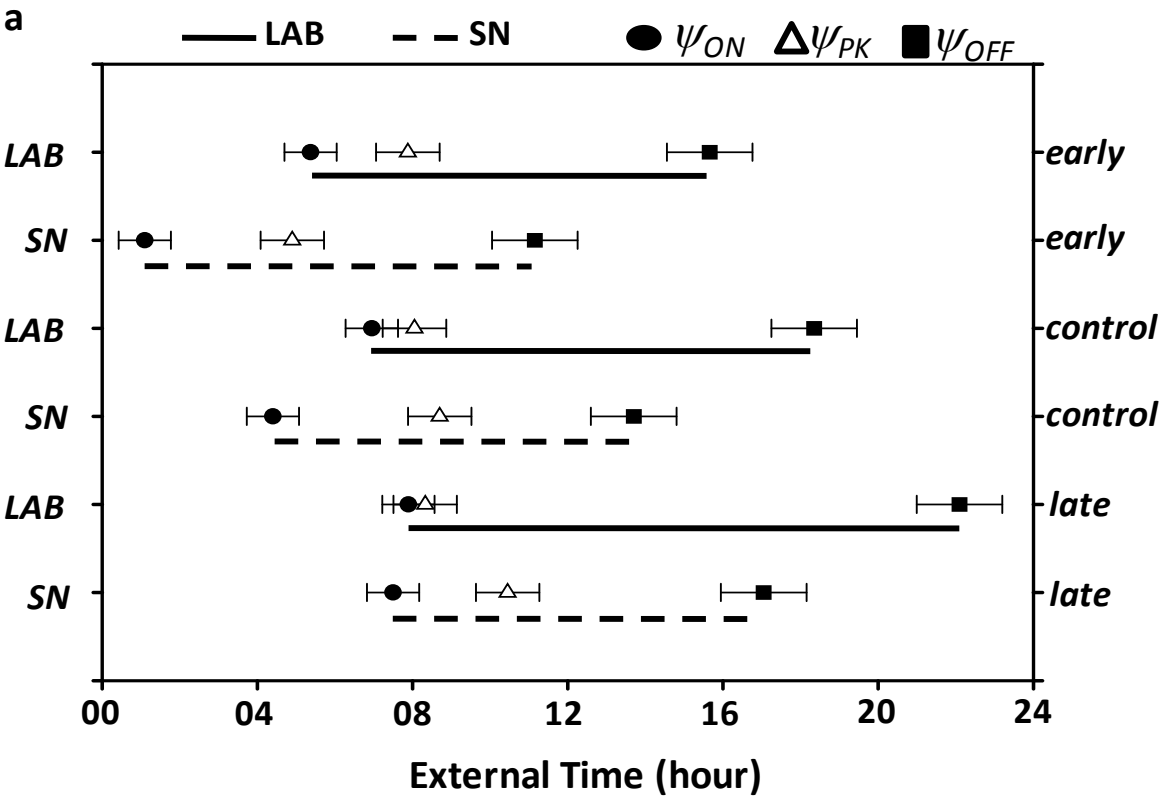

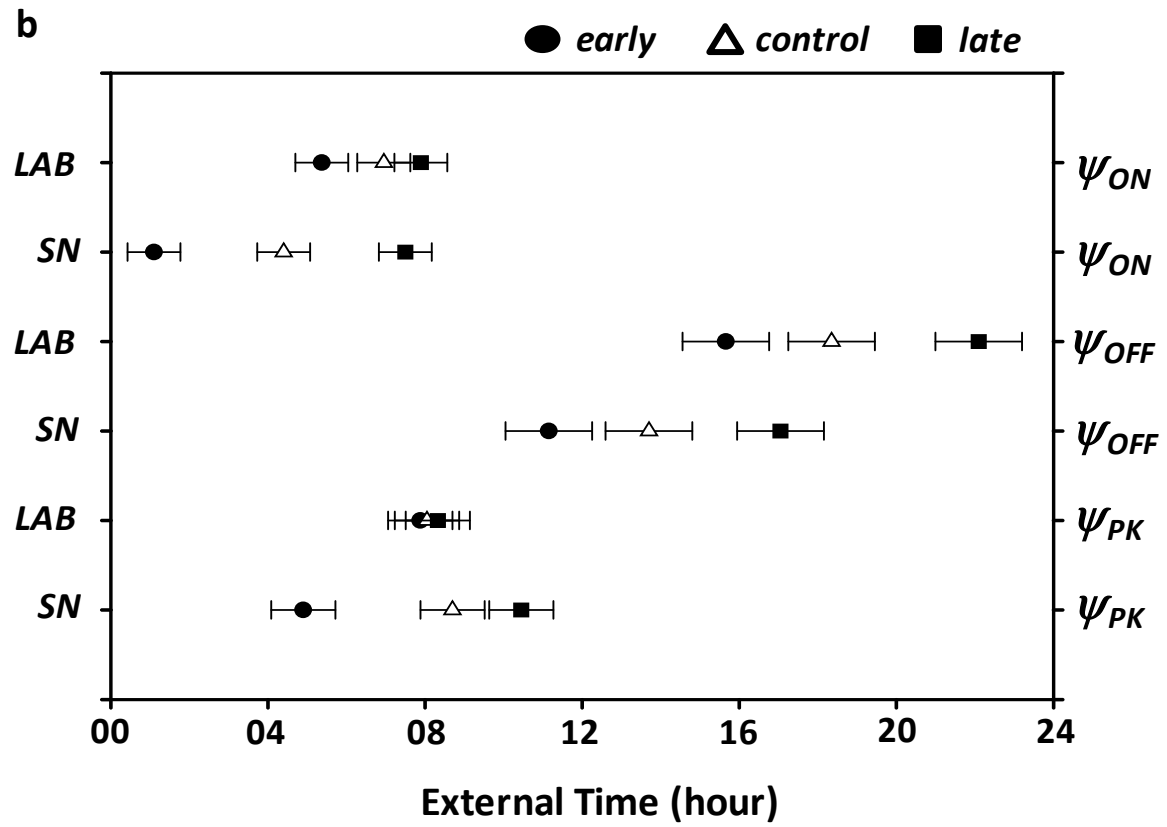

Discussion

We found that emergence waveforms were more phase-advanced but consolidated under SN compared to LAB (Figures 1, 2a). SN is characterised by the presence of multiple zeitgebers in the strongest form and twilight zones with gradually changing environmental factors. Aschoff and Weaver (1965) predicted advancement of the phase of entrained rhythm (ψ) with increasing zeitgeber strength and found supporting evidence in two species of finches. Studies on human chronotypes reported that long exposure to daylight is associated with morningness (Roenneberg et al., 2003b; Goulet et al., 2007), implying that under strong zeitgeber conditions circadian rhythms of humans are phase-advanced. Aschoff and Weaver, also predicted advanced ψ with increasing duration of twilight transitions which was also empirically validated (Wever, 1967; Daan and Aschoff, 1975). Overall advancement of emergence waveforms in the three stocks of *Drosophila* under SN (compared to LAB) could therefore be a result of combined effect of strong zeitgeber with twilight zones.

Consolidation of emergence waveform under SN in all our stocks is consistent with earlier observations of tighter gating of emergence under SN compared to LAB (De et al., 2012).

The phase of entrained rhythm leads-more or lags-less relative to zeitgeber, if τ of the rhythm is shorter than 24-hr and vice-versa (Pittendrigh and Daan, 1976). Under LAB, emergence waveform of *early* flies with shorter τ was phase-advanced compared to *controls*, and that of *late* flies with longer τ was phase-delayed, which suggest that morningness-eveningness chronotypes in emergence rhythm of *Drosophila* are primarily due to the differential entrainment of their diverged circadian clocks. Empirical evidence suggests that correlation between ψ and τ under stronger, in weak zeitgeber conditions and weaker, in strong zeitgeber conditions (Pittendrigh and Daan, 1976). Circadian rhythms are believed to be "driven" by zeitgeber rather than being entrained by them under strong zeitgeber conditions. Under such conditions ψ may not vary with environmental cycles (Rémi et al., 2010). Corollary to that, oscillatory systems with small differences in τ, which show differences in ψ under weak environmental cycles, are likely to assume similar phases when subjected to strong environmental cycles. Contrary to our expectations, emergence waveforms of *early* and *late* flies showed greater divergence under SN than in LAB (Figures 1, 2b), indicating that ψ–τ relationship in *early-late* emergence rhythm seen under LAB persists under SN. Our observation is thus consistent with recent observation on the activity rhythms of short (per^S) and long (per^L) period mutants of *Drosophila* under natural conditions (Vanin et al., 2012), wherein it was reported that the phase of evening activity peak of per^S and per^L flies was advanced and delayed relative to wild-type. Stronger correlation between ψ and τ, was also reported under LD cycles with twilight transitions compared to square wave LD cycles in field mouse *Mus booduga* (Sharma et al., 1998). Consistently, greater separation of *early* and *late* emergence waveforms under SN could therefore be due to gradual changes in environmental factors.

Table 1a. ANOVA performed on the percentage fly emergence at twelve phases spanning entire natural light/dark (LD) cycle under semi-natural (SN) condition, in *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2	0.00	6	0.00	16.18	0.004
Block (B)	3	0.00	0	0.00	--	--
Phase (P)	11	853.30	33	4.43	192.77	0.0001
S × B	6	0.00	0	0.00	--	--
S × P	22	336.09	66	3.48	96.71	0.0001
B × P	33	4.43	0	0.00	--	--
S × B × P	66	3.48	0	0.00	--	--

Table 1b. ANOVA performed on the percentage fly emergence at twelve phases spanning complete light/dark (LD) cycles under LAB conditions, in *early*, *control* and *late* stocks.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2	0.00	6	0.00	34.94	0.0001
Block (B)	11	837.25	33	3.30	253.85	0.0001
Phase (P)	3	0.00	0	0.00	--	--
S × B	22	97.14	66	1.49	65.41	0.0001
S × P	6	0.00	0	0.00	--	--
B × P	33	3.30	0	0.00	--	--
S × B × P	66	1.49	0	0.00	--	--

Table 2. ANOVA done on five emergence waveform features of *early*, *control* and *late* fly stocks under LAB and SN conditions. (a) ψ_{PK} (b) ψ_{ON} (c) ψ_{OFF}

		df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
a	**Stock (S)**	2	18.61	6	0.67	27.93	0.001
	Environment Regime(ER)	1	0.03	3	0.37	0.08	0.802
	Block (B)	3	0.44	0	0.00	--	--
	S × ER	2	13.79	6	0.34	40.84	0.0001
	S × B	6	0.67	0	0.00	--	--
	ER × B	3	0.37	0	0.00	--	--
	S × ER × B	6	0.34	0	0.00	--	--
b	**Stock (S)**	2	39.93	6	0.20	198.34	0.0001
	Environment Regime (ER)	1	34.71	3	0.46	75.08	0.003
	Block (B)	3	0.87	0	0.00	--	--
	S × ER	2	7.55	6	0.23	32.93	0.001
	S × B	6	0.20	0	0.00	--	--
	ER × B	3	0.46	0	0.00	--	--
	S × ER × B	6	0.23	0	0.00	--	--
c	**Stock (S)**	2	76.70	6	0.18	435.84	0.0001
	Environment Regime (ER)	1	134.46	3	0.05	2798.45	0.0001
	Block (B)	3	0.16	0	0.00	--	--
	S × ER	2	0.16	6	0.61	0.25	0.784
	S × B	6	0.18	0	0.00	--	--
	ER × B	3	0.05	0	0.00	--	--
	S × ER × B	6	0.61	0	0.00	--	--

The *early* and *late* populations evolved lark and owl-like emergence chronotypes as a consequence of selection for morning and evening emergence in LAB. Under SN, *early* and *late* flies continued to show difference in emergence timings, greater separation of emergence waveforms, with *early* and *late* flies closely following humidity and light profiles, respectively. This suggests that circadian architecture of *early* and *late* flies is fine-tuned by natural conditions to display strong preference for different timing of emergence specifically. Our results thus provide strong evidence for the notion that human chronotypes are products of interaction between circadian clocks and natural environmental cycles.

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