Evaluating the role of circadian clock properties and developmental processes in the evolution of accurate eclosion rhythms in *Drosophila melanogaster*

Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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DECLARATION

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

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

Vishwanath Varma

Place: Bangalore

Date: 12th April, 2018

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April 12, 2018

CERTIFICATE

This is to certify that the work described in the thesis entitled "**Evaluating the role of circadian clock properties and developmental processes in the evolution of accurate eclosion rhythms in *Drosophila melanogaster*"** is the result of investigations carried out by Mr. Vishwanath Varma in the Evolutionary and Integrative Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, largely under the supervision of late Prof Vijay Kumar Sharma. I have been closely involved in many of these studies and have taken responsibility of continuing supervision towards completion of the studies and composing of the current thesis. I certify that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Sheeba Vasu, PhD
Supervisor-in-charge

Acknowledgements

This thesis is the product of multiple contributions from different sources, including support from several people and institutions. The origin of the project that involves fly populations selected for accuracy, that forms the subject of this thesis, traces back to late Prof. V. K. Sharma who had initiated these populations along with his students. The idea for this project probably stemmed from his interest in the adaptive functions of clocks and why clocks need to be accurate in order to perform their functions, which was possibly inspired by the work of pioneers in the field of chronobiology like Colin Pittendrigh, Jurgen Aschoff and Serge Daan, and cultivated with the guidance of late Prof. Maroli K. Chandrashekaran. Hence, gratitude is owed to all of them for the streams of thought and research that they developed which I joined at a much later stage.

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eccentricities including minimal contributions to the conversations at the table and finishing my meal too soon.

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SYNOPSIS

Organisms are thought to have evolved circadian rhythms in order to coordinate the timing of their behaviour and physiological functions with the external environment. Circadian time-keeping systems produce oscillations in physiological variables and behavioural patterns which persist under constant conditions with a near-24 hour period but are also capable of synchronizing to external cycles with a fixed phase of entrainment. These circadian rhythms are known to exhibit remarkably low levels of variation in timing (or entrained phase) across days. This accuracy of circadian rhythms is thought to be a likely target of natural selection since it is essential for accurately recognizing local time and conferring extrinsic adaptive advantage to the organism by appropriately timing various behaviours.

The importance of proper phasing of circadian rhythms has been demonstrated by studies showing reduced fitness of individuals that exhibited deviant phases of behavior, possessed dysfunctional circadian clocks or were subjected to external cycle mismatches. Theoretical and empirical studies have suggested that the main factors influencing the accuracy of entrained overt rhythms are precision of the underlying internal clock, strength of the external time cue or sensitivity of the clock to the cue and clock-independent upstream or downstream processes related to the behavior. However, a comprehensive investigation of the evolution of accuracy of circadian rhythms and the roles of the probable factors involved in determining accuracy is lacking. Examining the evolved differences and possible contributions of these traits in *Drosophila melanogaster* populations which have evolved accurate circadian rhythms due to artificial selection for narrow gate of adult emergence is the subject of this thesis.

In the first chapter, a brief overview of the evolution of circadian rhythms in organisms and the organization of circadian time-keeping systems is presented. The importance of accurate phasing of circadian rhythms is outlined and analysis of accuracy through theoretical and empirical studies is discussed. Further, the factors contributing to variation, or conversely, accuracy are enumerated and various aspects of each of these factors, which are inter-connected yet partly independent, are described. The rationale for studying these traits and their biological basis has been briefly laid out in this chapter.

The second chapter introduces the selection protocol and the ancestry of the *Drosophila* populations which have been subject to artificial selection for narrow gate of adult emergence and have consequently evolved greater accuracy of circadian rhythms. While a previous study had shown that the selected populations also show a trend of greater daily precision of the internal clock period compared to controls, these differences were not statistically significant. However, the internal clock period is also subject to changes over longer durations of time and different temperatures. Therefore, this chapter examines the circadian free-running period of selected and control stocks for most of their adult life under constant darkness at three different ambient temperatures. The results showed that free-running period of both selected and control flies increased with age with no significant differences seen between the stocks. While inter-individual and intra-individual variation in period were slightly lower in the selected stocks at all temperatures compared to the controls, these differences were also not statistically significant. However, the selected stocks showed significantly better temperature compensation with lower variation in period seen across temperatures. Thus, selected stocks appear to have evolved greater stability of free-running period across temperatures even though differences in intra-individual and inter-individual variation are marginal.

Another important circadian clock property that contributes to accuracy of circadian rhythms is its sensitivity to the external time cue which in the context of our *Drosophila* populations is light. Hence, various aspects of responsiveness of the circadian system to light in selected and control stocks were compared in the third chapter. While no significant differences were observed between the stocks in the ability to entrain to light-dark cycles of different cycle lengths, phase-shifts in response to white light pulses of low intensities and long durations presented during the early subjective night were significantly larger in the selected stocks. This enhancement in magnitude of phase-shifts in response to white light could possibly be attributed to cryptochrome since light pulses of wavelengths detected by cryptochrome (blue light) resulted in greater phase-shifts in selected stocks compared to controls though no such differences were seen in response to orange light pulses. Conversely, orange light-dark cycles elicited higher levels of locomotor activity in selected stocks compared to controls while no difference was seen between the stocks under blue light-dark cycles. However, there were no observable differences between the stocks in terms of period lengthening or induction of arrhythmicity under constant light. Hence, both compound eyes, which are involved in modulation of activity levels and detection of longer wavelengths of light, as well as cryptochrome may contribute to the enhanced photosensitivity of the selected stocks.

Aside from clock properties, developmental processes and direct responses to external signals can also influence the timing of emergence rhythms. Therefore, inter-individual variation in transitions between early developmental stages, effects of manipulations of developmental rates, development time under constant conditions and masking responses of adult emergence to light were examined in the fourth chapter. There were no consistent differences seen in the time taken to pupate and exhibit wing pigmentation between selected and control stocks suggesting that

reduced variation in emergence time in the selected stocks cannot be explained by reduction in variation in early developmental stages. Manipulating developmental rates by studying emergence profiles at different larval densities resulted in greater night-time emergence in the control stocks while selected stocks continued to restrict most of their emergence to the light phase. Assays under constant light did not reveal differences in mean or variance of development time while selected stocks showed longer development time and greater variance in development time under constant darkness. These results suggest that developmental processes do not contribute to reduction in variance in emergence time seen under light-dark cycles in the selected stocks. Furthermore, masking effects of light were also less pronounced in the selected stocks compared to controls when exposed to advanced lights-ON on the day of emergence, suggesting that gating of emergence restricts light-induced emergence in these stocks.

Finally, correlated responses of fitness traits to evolution of accurate emergence rhythms were examined in the fifth chapter since these may reveal evidence of genetic correlations between emergence time and life-history traits such as pre-adult survivorship, fecundity and life span. Development time and pre-adult survivorship was not significantly different between selected and control stocks. Adult lifespan of mated males and females emerging within the selection window and an evening window was compared between selected and control stocks. While males did not show any differences in lifespan, females from the selected stocks emerging within the selection window showed reduced lifespan compared to their corresponding females from the control stocks. However, morning emerging females from the selected stocks also showed greater mid-life fecundity compared to evening emerging females from the selected stocks suggesting that the fitness costs due to lower lifespan may be compensated by higher egg-laying during the egg collection window of the regular stock maintenance regime. Hence, the morning

emerging females from the selected stocks have evolved changes in life history suggesting evidence for sex-specific genetic variation in such traits that are associated with emergence time.

In summary, the results demonstrate the evolution of clock properties, developmental and life-history traits of *Drosophila* populations that exhibit higher accuracy of circadian rhythms due to selection for adult emergence within a narrow window of time. While there appears to be little reduction in variation in free-running period both within and across individuals in the selected stocks, temperature compensation of period is significantly better in these stocks suggesting an overall increased stability of internal clock period. Moreover, various aspects of light sensitivity are enhanced in the selected stocks compared to controls. These include increased delay phase-shifts to light pulses, possibly through cryptochrome, and higher levels of activity under orange light-dark cycles which may act via the compound eyes. However, there does not appear to any evolved differences in developmental rates between selected and control stocks that may contribute to greater accuracy of emergence rhythms since variation in pupation and pigmentation times under light-dark cycles and variation in development time under constant conditions is not lower in the selected stocks. Instead, circadian gating of emergence is stronger in the selected stocks as evidenced by the reduced ability of larval density manipulation or immediate responses to light to cause emergence in the subjective night. Furthermore, evolution of accurate emergence rhythms is associated with correlated sex-specific changes in life history traits. Thus, evolution of greater accuracy of circadian rhythms in *Drosophila* populations is accompanied by increased stability of the internal clock, greater sensitivity to time cues in the environment, stronger circadian gating of behavior and changes in life history.

Introduction

Most living organisms experience predictable variation in environmental factors due to various geophysical cycles present on the earth. Cyclic variation in the environment presents opportunities and risks to organisms at various times of the day, month or year (Enright, 1970; Pittendrigh, 1981a; Daan, 1981). Internal time-keeping mechanisms are thought to have evolved in organisms to exploit the temporal order in the environment so as to increase reproductive fitness (Moore-Ede and Sulzman, 1981; Sharma, 2003; Vaze and Sharma, 2013). Among geophysical cycles present in the environment, the daily cycle (of period ~ 24 hours) due to the rotation of the earth about its axis is probably the most consistent cycle experienced by organisms (Moore-Ede, 1986). Circadian clocks (with periodicity close to 24 hours) are ubiquitously seen in most eukaryotic and some prokaryotic organisms. These internal clocks may have evolved as a consequence of daily selection pressures on preferentially allocating different physiological tasks to different parts of the day (Moore-Ede and Sulzman, 1981).

Evidence based on sequencing of circadian genes in *Drosophila*, *Neurospora*, plants and cyanobacteria suggest that the circadian clock might have independently originated in the animal and bacterial kingdoms (Rosbash, 2009). The biochemical systems involved in sustaining circadian rhythms such as post-translational regulation and transcriptional feedback found in both cyanobacteria and mammals could reflect convergent evolution or common ancestry. Although appropriate timing of various physiological and behavioural functions is thought to confer reproductive fitness to organisms (Daan and Tinbergen, 1979; Johnson and Golden, 1999), many circadian rhythms do not show any clear relation to risk or opportunity in the environment (Pittendrigh, 1981a). Hence, it is unlikely that all circadian rhythms seen in organisms have evolved in response to rhythmic selection pressure for their

corresponding functions. Instead, it has been proposed that temporal order forced by light and temperature variation on cellular metabolism in early unicellular organisms could have set a premium on the evolution of coordination of such metabolic activities by an endogenous timer (Pittendrigh, 1981a). The association of light reception and DNA repair with circadian rhythms also suggests the role of selection against light-induced DNA damage in the evolution of circadian clocks (Pittendrigh, 1993; Kay, 1997; Gehring and Rosbash, 2003). Early selection pressures on circadian clocks in primitive unicellular organisms such as cyanobacteria may have been related to coordination of cellular metabolism such as temporal partitioning of nitrogen fixation and photosynthesis (Johnson and Golden, 1999). Such temporal programs may have been progenitors of time-keeping systems capable of synchronizing with the external environment (Rosbash, 2009) with later diversification of pacemaker functions (Pittendrigh, 1981a).

While the structure and organization of the circadian system varies greatly across taxa, certain common features of the functional organization may be discerned (Menaker et al., 1997; Bell-Pedersen et al., 2005). The components of the circadian system may be heuristically characterized as input pathways, the central oscillator and output pathways (Helfrich-Förster et al., 1998; Golombek and Rosenstein, 2010; Silver and Rainbow, 2013). The input pathways include various sensory inputs from environmental variables such as light, temperature and social interactions and their signaling pathways to the central clock (Devlin, 2002; Foster and Helfrich-Förster, 2001; Mistlberger and Skene, 2004; Glaser and Stanewsky, 2005; Buhr et al., 2010). These environmental inputs feed into the central oscillator which is constituted of transcriptional and post-translational feedback loops at the cellular level and coupled cellular oscillator subgroups at the tissue level (Darlington et al.,

1998; Miller, 1998; Reppert and Weaver, 2002; Mohawk and Takahashi, 2011). The central oscillator conveys output signals to various tissues and peripheral clocks which produce overt rhythms of different behaviours and physiological processes (Helfrich-Förster et al., 1998; Giebultowicz, 2001; Reppert and Weaver, 2002; Dibner et al., 2010). Thus, the central pacemaker receives input from environmental time-cues and transmits temporal information to the peripheral tissues via various output pathways. Additionally, direct effects of environment on overt rhythms, influence of the clock on light sensitivity and feedback of overt rhythms to the central clock have also been reported, suggesting that circadian organization is more complicated than the linear hierarchical model that was initially proposed (Golombek and Rosenstein, 2010).

The organization of the circadian timing system is thought to be intimately connected to its function as an adaptation for the organism (Pittendrigh, 1961). The adaptive value of circadian rhythms may be broadly classified into intrinsic and extrinsic advantages (reviewed in Sharma, 2003; Vaze and Sharma, 2013). The intrinsic advantage hypothesis was advanced based on the fact that several different physiological variables such as body temperature, blood sugar, adrenal activity, etc., in multicellular organisms showed robust oscillations phase-locked to one another (Halberg, 1960; Moore-Ede and Sulzman, 1981). Subsequent discoveries of peripheral clocks in various tissues and their coupling to the central circadian pacemaker suggested the utility of a time-keeping system in synchronizing and appropriately timing various functions of the organism with respect to each other (Rosenwasser and Adler, 1987; Golombek and Rosenstein, 2010). In addition to such benefits, several biotic and abiotic factors exhibit cycling in the environment which results in segregation of the day into favourable and unfavourable phases for various behaviours or physiological events (Enright,

1970; Daan, 1981). These daily variations may impose selection pressures on the timing of behaviours resulting in the evolution of rhythmic patterns of behaviour better adapted to the cyclic environment (Pittendrigh, 1958; Pittendrigh, 1961; Daan, 1981), thus conferring extrinsic advantage to the organism.

Circadian time-keeping systems capable of adjusting to the daily external cycle must perform two vital functions viz. recognizing local time and measuring the lapse of time. The utility of clocks in recognizing local time is contingent on proper phasing of the internal clock with respect to the external cycle (Pittendrigh, 1981b). The synchronization of the internal clock to the external environment with a stable phase relationship is referred to as entrainment (Johnson et al., 2003). The phase of entrainment and its accuracy across days are mainly determined by the period and precision of the internal clock, and its ability to reset in response to time cues (or zeitgebers) in the environment (Pittendrigh and Daan, 1976a; Aschoff, 1981). Thus, variability in clock period and sensitivity to zeitgebers affect the accuracy of daily phase and therefore, the ability to recognize local time. Additionally, homeostasis of pacemaker period while maintaining a stable angular velocity of phase over a wide range of temperatures and other environmental variables is necessary for measuring fractions of the cycle with reliability (Pittendrigh, 1981a). Thus, circadian clocks may have evolved temperature compensation of period in order to perform the function of measuring the lapse of time (Pittendrigh, 1981a). However, measuring the lapse of time in a cyclic external environment would be useful in adjusting to the external environment only if the internal clock is accurately phased with respect to the external cycle. Hence, both aspects of the extrinsic adaptive advantage conferred by circadian clocks would benefit from greater

accuracy of phase of circadian rhythms, making accuracy a likely target of natural selection (Beersma et al., 1999).

1.1 Accuracy of circadian rhythms

The accuracy of circadian rhythms is defined as the reciprocal of the standard deviation of daily phase of a rhythm when entrained to the external environment (Daan and Beersma, 2002). The accuracy of the rhythm is a measure of the stability of the phase relationship of the circadian rhythm with its environment (Pittendrigh, 1981a). The importance of proper phasing of circadian rhythms with the environment has been demonstrated in a few empirical studies (Daan and Tinbergen, 1979; DeCoursey et al., 1997; Ouyang et al., 1998). For instance, it was seen that young guillemots which jump off their breeding cliffs around the peak of jumping behaviour during the late evening suffered lower predation than those that jumped at other times of the day (Daan and Tinbergen, 1979). However, it is difficult to separate the benefits of accurately timing the jumping behaviour accruing from swamping of the predators by synchrony among individuals as against potentially differential selection across the day. Similarly, the adaptive significance of insects emerging at times of the day with low temperature and high relative humidity is thought to be related to the vulnerability of young adults to desiccation (Pittendrigh, 1954; Daan, 1981) with a few studies substantiating this claim (Lankinen, 1986; Tanaka and Watari, 2009). Studies on circadian resonance in cyanobacteria, involving pairwise competition between strains with different periods under LD cycles of 22, 24 and 30 hours periodicities, showed that strains with period matching the period of the environmental cycle out-competed other strains (Ouyang et al., 1998). Such differences in growth rate (a measure of reproductive fitness) were speculated to be due to optimal phasing of circadian clocks of strains with periods matching the

environmental cycle (Ouyang et al., 1998). Further evidence of the importance of restricting behaviour to appropriate phases of the day comes from lesioning studies of SCN (central clock in mammals) in ground squirrels. The SCN-lesioned individuals exhibited greater night-time activity and concomitantly, experienced greater night-time predation compared to SCN-intact controls (DeCoursey et al., 1997). Additionally, accuracy of circadian clocks is necessary for measuring passage of time in sun-compass mediated navigation in birds and bees (Frisch, 1950; Kramer, 1950). Hence, multiple lines of evidence suggest that appropriate phasing of circadian rhythms with the external environment is essential for the extrinsic advantage that circadian clocks confer to their owners (Vaze and Sharma, 2013).

Accuracy of entrained circadian rhythms is thought to be associated with various circadian clock properties (Daan and Aschoff, 1975; Pittendrigh and Daan, 1976a). Since the phase of the entrained rhythm is dependent on the period of the internal clock, variation in entrained phase may also be dependent on variation in period. Variation in period may occur due to intrinsic daily variability of biochemical and physiological processes of the circadian clock or long-term changes due to age or external environments (Pittendrigh and Daan, 1976b). These aspects of variation in period will be discussed in greater detail in the next section. Additionally, the process of entrainment via resetting of the circadian clock by environmental time cues may also be variable across days, yielding variation in phase of entrainment. The stability of entrainment is also thought to be dependent on the strength of the zeitgeber and/or the sensitivity of the circadian clock to such cues. Such resetting of the circadian clock is thought to occur via phase-shifts and period changes in order to adjust to the external cycle and various aspects of such sensitivity to zeitgebers will be discussed in a later section. Alternatively, direct responses to environmental variables can also modulate the phase of

overt rhythms independent of the circadian clock and thus, affect the daily variation in phase of the rhythm (Daan and Aschoff, 1975). Such masking responses to environmental factors such as light will be dealt with in a later section discussing the clock-independent processes controlling emergence rhythms.

Hence, the main factors that may affect the accuracy of circadian rhythms can be outlined as:

1. The internal clock period (represented by τ), and its innate daily variability or precision.
2. Strength of the zeitgeber and the nature of its effects on the clock, including phase-shifts and period changes.
3. Possible effects of masking on the overt rhythms.

Aside from framing hypotheses regarding selection pressures on the evolution of accuracy and some empirical support for predictions from theoretical models of entrainment on properties of circadian clocks (Pittendrigh and Daan, 1976b; Daan and Aschoff, 1975; Daan and Aschoff, 2001), there have been few studies examining the evolution of accuracy in organisms and contributions of different clock properties to the same (Soong et al., 2006; Kannan et al., 2012a, b). Soong and coworkers examined the high accuracy of timing of diel emergence of intertidal midges *Pontomyia oceana* and sought to determine whether the selection pressure is on accuracy of phase with respect to the external cycle or on synchrony of emergence of conspecifics at a certain time (Soong et al., 2006). Based on lower variation in timing of emergence observed in summers, the authors speculated that there might be selection pressure on mating success for greater synchrony in summer, as males spend less time searching for mates under hot conditions, rather than selection on accuracy with respect

to external time (Soong et al., 2006). Another study on evolution of accuracy of circadian rhythms involves artificial selection on phase of adult emergence rhythms of *Drosophila melanogaster* populations (Kannan et al., 2012a). In this study, adult flies emerging within a narrow window of 1 hour, close to the peak emergence time, were selected for several generations, and their clock properties were examined. These studies revealed that selected populations had evolved greater accuracy of adult emergence as well as activity-rest rhythms compared to controls (Kannan et al., 2012a). The selected populations also exhibited a shorter free-running period as well as a trend of greater precision of period for both rhythms compared to control populations (Kannan et al., 2012a). Additionally, they exhibited greater synchrony of timing across individuals (lower inter-individual variation in phase) and robustness of circadian rhythms across different external environments (Kannan et al., 2012a, b). The selected populations also entrained to low intensity light-dark (LD) cycles with greater amplitude than controls suggesting increased light sensitivity in these flies (Kannan et al., 2012b). In summary, the results of studies on fly populations selected for greater accuracy of eclosion rhythms indicate changes in both the internal clock as well as sensitivity to the external environment. In the studies described in this thesis, I wished to examine these changes in clock properties of these selected stocks and their contributions to the evolved phenotype of enhanced accuracy in greater detail. Hence, I assayed various aspects of variation in period of free-running rhythms and different modes of light sensitivity of selected and control populations. I also examined the contributions of developmental processes and effects of masking on eclosion towards the evolution of the phenotype of enhanced accuracy of eclosion rhythms. Further, I was interested in whether there were correlated responses to selection for enhanced accuracy of eclosion rhythms on fitness traits.

The rationale for exploring these aspects of circadian clocks and life histories of populations selected for greater accuracy will be described in the following sections.

1.2 Stability of internal clock period

The stability of internal clock period, τ may be broadly characterized as the lack of variability of τ across days, age, temperature and a variety of other internal and external factors (Pittendrigh and Daan, 1976b; Aschoff, 1979). The precision of circadian rhythms is defined as the reciprocal of the standard deviation of daily period of a free-running rhythm under constant external conditions (Daan and Beersma, 2002). Early reports of circadian rhythms demonstrated remarkable precision in free-running rhythms with standard deviation values of the order of a few minutes (Pittendrigh, 1960; DeCoursey, 1961). Jurgen Aschoff and coworkers studied the precision of chaffinches and found differences between the precision of onset and end of activity for individual birds and that such differences were dependent on τ (Aschoff et al., 1971). They proposed a model to explain these findings where underlying oscillations may have different waveforms according to their periodicity, and locomotor activity occurs when the levels of the oscillation exceed a threshold (Aschoff et al., 1971). Comprehensive studies on the variability of τ were undertaken by Colin Pittendrigh and Serge Daan including precision of circadian rhythms of multiple species of rodents (Pittendrigh and Daan, 1976b). These studies also reported a relationship between precision and τ with highest precision seen around $\tau = 24$ hours at the individual as well as species level similar to that observed by Aschoff and colleagues in chaffinches (Pittendrigh and Daan, 1976b; Aschoff et al., 1971). They interpreted these correlations as evidence of selection for greater precision for such individuals or species due to the instability of entrained phase when $\tau \sim T$ (where T is the period of the external cycle; Pittendrigh and

Daan, 1976a), in contrast to Aschoff's interpretation of τ being related to the amplitude and waveform of the oscillation. An alternate explanation for the correlation between period and precision comes from predictions of theoretical models involving multiple oscillators where maximum precision is observed when the frequency of the system is close to that of its constituent oscillators (Daan and Beersma, 2002). Since the average period of the several neural oscillators that are coupled to one another may be around 24 hours, maximum precision would also be seen around this value (Daan and Beersma, 2002).

Few empirical studies have attempted to study correlations between τ and precision and address the hypotheses explaining the occurrence of such correlations (Sharma and Chandrashekaran, 1999; Bittman, 2012; Kannan et al., 2012a). In field mice, precision of onsets of activity were reported to be highest when τ ~ 24 hours and the authors interpreted these results as supporting the argument for evolution of greater precision when τ ~ 24 hours as suggested by Pittendrigh and Daan, 1976b (Sharma and Chandrashekaran, 1999). While Eric Bittman did not find any correlations between τ and precision in mutant hamsters with different periods, Kannan et al. reported evolution of shorter τ (further away from 24 hours) and marginally greater precision in *Drosophila melanogaster* populations selected for greater accuracy (Bittman, 2012; Kannan et al., 2012a). Although the evolution of shorter τ in populations selected for accuracy may be taken as support for the notion that greater deviation from the period of external cycle leads to greater stability of entrainment, these results may not necessarily be consistent across species and cannot conclusively validate one theory over the other. Indeed, precision itself may have multiple components and may be related to other circadian clock properties that may indirectly affect the accuracy of the entrained rhythm.

Variability of free-running periods is observed at several levels including between different species or between individuals of the same species (Pittendrigh and Daan, 1976b; Aschoff, 1979). While variation across species may represent different evolutionary strategies (Pittendrigh, 1981b), inter-individual variation within the species is more likely to represent standing genetic variation in the population. Alternatively, inter-individual and intra-individual variation may indicate the extent of canalization (evolution of phenotypic constancy) of the trait (Clarke, 1998). Studies on rodents showed that both inter and intra-individual variations in period were lowest for the species with mean period closest to 24 hours (Pittendrigh and Daan, 1976b). Intra-individual variation across days or precision may be further partitioned into variation of the underlying pacemaker and variation of output processes that control the overt rhythm (Pittendrigh and Daan, 1976b). Analyses of serial correlations, based on the notion that high output variation with low pacemaker variation would result in negative serial correlations in τ values across successive days, yielded evidence of the pacemaker being much more precise than the overt rhythm (Pittendrigh and Daan, 1976b). These results suggest that a substantial proportion of variation in overt rhythms is due to variation in processes involved in the conveyance of temporal signals from the pacemaker to downstream tissues responsible for the particular physiological or behavioural event that is rhythmic (Pittendrigh and Daan, 1976b). This inference was substantiated by studies suggesting that different phase markers and recording methods yield different precision levels mainly due to variation in the output processes and not the pacemaker (Daan and Oklejewicz, 2003). This variation in output processes is not considered as representative of variation in the core clock and will be discussed in greater detail in a later section in relation to the mechanism of insect eclosion.

Aside from output processes, core clock variation itself may be attributed to different sources. Since the circadian clock machinery in multicellular organisms is thought to be composed of multiple autonomous cellular oscillators (Welsh et al., 1995; Plautz et al., 1997; Dunlap, 1999), there may be variation due to intracellular as well as intercellular mechanisms. Internal variation in biochemical networks within the cell arises due to the stochastic nature of chemical reactions and gene expression, especially when there are only few molecules in the system (McAdams and Arkin, 1997; McAdams and Arkin, 1999; Barkai and Leibler, 2000). There are thought to be regulatory mechanisms involving feedback and redundancy which serve to reduce the variability and increase the robustness of such cellular oscillations (McAdams and Arkin, 1999; Barkai and Leibler, 2000; Mihalcescu et al., 2004). However, recording of firing rhythms of individual dissociated clock neurons revealed substantial variation in period and phase across as well as within neuronal cells (Welsh et al., 1995). The daily variation in period was found to be much greater in single cells than that seen in the SCN and behavioural rhythms (Welsh et al., 1995; Liu et al., 1997) suggesting that there may be mechanisms to reduce variations at the tissue level. Coupling between multiple oscillators has been shown to be an important process in reducing the variability of single cells both theoretically and experimentally (Winfree, 1967; Enright, 1980; Herzog et al., 2004). Hence, the variability of individual oscillators as well as the nature of interactions between them determines the variability of the core clock in multicellular organisms.

The daily variability of the circadian clock period is superimposed on more long-term changes in period due to age and the influence of external factors (Pittendrigh and Daan, 1976b). Long-term stability was found to be lower in individuals with mean periods

deviating from 24 hours than those with periods close to 24 hours, similar to the trend observed for precision (Daan and Beersma, 2002). Additionally, the correlation between short-term (precision) and long-term (stability) variability in recordings of individual animals was found to be greater than that predicted by measurement error (Daan and Beersma, 2002). Long-term stability may thus be related to precision if both measures are dependent on the strength of the underlying oscillation (Daan and Beersma, 2002). Nonetheless, long-term stability of period has received more attention in the context of aging of the circadian clock (Turek et al., 1995; Weinert, 2000). Several studies have reported period lengthening or shortening with age in various organisms (Pittendrigh and Daan, 1976b; Aschoff, 1979; Morin, 1988; Luo et al., 2012; Yadav and Sharma, 2014a). While these represent consistent changes in period, diminishing amplitude and increased fragmentation of rhythms are also seen during late life which leads to unstable overt rhythms (Weitzman et al., 1983; Nakamura et al., 2011; Rakshit et al., 2012; Penev et al., 1997; Valentinuzzi et al., 1997). These consequences of aging have been variously attributed to cellular damage or reduced coupling among oscillators or between the central and peripheral clocks (Weinert, 2000).

Apart from internal factors, various environmental factors are capable of inducing changes in the circadian period (Pittendrigh and Daan, 1976b; Aschoff, 1979). These include different temperatures, constant light (LL) of different intensities, and history of exposure to various environmental regimes such as photoperiods (different proportions of light and dark phases in a day) and *T*-cycles (different periods of external zeitgeber cycles; Pittendrigh and Daan, 1976b; Aschoff, 1979). While period changes in response to constant light of different intensities are thought to be representative of parametric effects of continuous light which may facilitate entrainment (Pittendrigh and Daan, 1976a) and will be dealt with in a later

section, the lack of period changes under different ambient temperatures (temperature compensation) is considered an important property of the circadian clock, vital to its function in measuring the lapse of time (Pittendrigh, 1954; Pittendrigh, 1981a). Although molecular and cellular mechanisms of temperature compensation are not clearly understood, models based on positive and negative components of the loop or those based on changes in limit cycle amplitudes have been proposed (Hastings and Sweeney, 1957; Lakin-Thomas et al., 1991). Since the variability of environmental temperature is different across latitudes and altitudes, variation in temperature compensation ability is also seen across populations of *Drosophila* and *Arabidopsis* inhabiting different habitats (Sawyer et al., 1997; Edwards et al., 2005). Such genetic variation in temperature compensation in *Drosophila* populations may be amenable to the forces of natural selection and thus, may also contribute to accuracy.

Changes in circadian period due to exposure to different environmental regimes such as photoperiods and *T*-cycles are known as after-effects (Pittendrigh and Daan, 1976b; Aschoff, 1979). The magnitude of such after-effects was found to be lower in species with mean period close to 24 hours (Pittendrigh and Daan, 1976b; Aschoff, 1979). Since these organisms also showed greater precision and stability, the authors proposed that the variability of period could be determined by an overall homeostasis which protects the circadian period from changes due to internal or external variables (Pittendrigh and Daan, 1976b; Aschoff, 1979). However, other studies have suggested that after-effects may be the result of different coupling behaviours of oscillator subgroups within the central clock (Daan and Berde, 1978; Beersma et al., 2017; Azzi et al., 2017). Hence, different aspects of variability of clock period have been thought to be related to one another and also partly determined by independent processes. Thus, it would be useful to measure various sources

of variability of period in populations selected for greater accuracy in order to understand which aspects of period stability are important for the evolution of accurate circadian clocks.

1.3 Light sensitivity

Light is thought to be the predominant zeitgeber or time cue for resetting the circadian clock. Light has several effects on the circadian rhythms of organisms including phase-shifts and period changes of the circadian clocks as well as modulation of amplitude and waveform of the rhythm via direct effects on the overt behaviour (called masking). While phase-shifts and period changes are thought to be critical to the mechanism of entrainment of the internal clock to the external cycle and show persistent effects on the rhythm even upon removal of the light cue (Pittendrigh and Daan, 1976a; Aschoff, 1979), masking effects of light typically are seen only in the presence of the stimulus and do not affect the underlying clock (Aschoff, 1960). Nonetheless, both entraining and masking effects of light may influence the daily phase of the overt rhythm and thus, accuracy (Daan and Aschoff, 1975). Martin C. Moore-Ede compared the true phase of the entrained oscillation and the masked behaviour to homeostatic processes comprising predictive and reactive components, each beneficial in different contexts depending on predictability of the environment and advantages of anticipation (Moore-Ede, 1986). Indeed, it is likely that both responses may be essential to the organism's strategy and may interact with each other to give rise to patterns of variability in phase. For instance, the lower variability in phase of activity rhythms in different birds and mammals around the equinoxes was speculated to be due to the entrained phase occurring around twilight when light intensity drops steeply (Daan and Aschoff, 1975). Since activity would be cut-off by the sharp changes in light intensity due to masking, the phase of the rhythm would appear more accurate (Daan and Aschoff, 1975). Hence, light can

affect the accuracy of the circadian rhythm by stabilizing entrainment or by directly modulating the behaviour through masking.

As mentioned previously, the extent of phase-control or stability of entrained phase is thought to be dependent on the zeitgeber strength (Aschoff, 1960; Wever, 1960). The strength of the zeitgeber may be described as its ability to entrain the circadian clock and may depend on the period of the zeitgeber cycle, the ratio of duration of light and dark phase and the contrast between intensities during the two phases (Aschoff, 1960). The stability of entrained phase of oscillator models was reduced under external cycles with periods deviating from that of the oscillator or under extreme ratios of light:dark phase durations (Aschoff, 1960; Wever, 1960; Wever, 1972). Hence, these models could explain lower accuracy observed under extreme photoperiods in nature as well as under laboratory conditions in different birds and mammals (Aschoff et al., 1970; Daan and Aschoff, 1975; DeCoursey, 1972). However, these models do not explicitly describe the mechanisms of entrainment by light and the manner of its effects on the circadian clock.

The most extensively studied aspect of circadian clock sensitivity to light in relation to entrainment is the shift in phase of the clock in response to instantaneous light pulses. The plot of the phase-shifts incurred due to a light pulse as a function of the time at which the pulse is presented is known as a phase response curve (PRC; Hastings and Sweeney, 1958; Daan and Pittendrigh, 1976a). In most organisms, the phase response curve comprises delay and advance zones where delay and advance phase-shifts are seen in response to light pulses in the early and late subjective night respectively, and a dead zone in the middle of the subjective day where no significant phase-shifts are seen (Pittendrigh and Daan, 1976a). Although these features of the PRC are remarkably consistent across most nocturnal and

diurnal species, there is variation among individuals and species in the extent and magnitude of different zones and the overall slope of the PRC (Daan and Pittendrigh, 1976a). The stability of entrained phase across different photoperiods is predicted to be higher when the slope (or the amplitude) of the PRC is steeper (larger; Pittendrigh and Daan, 1976a). This is consistent with higher accuracy being correlated with greater zeitgeber strength since higher intensity light pulses and greater light sensitivity would result in large amplitude PRCs (Aschoff, 1960; Pittendrigh and Daan, 1976a). Thus, light sensitivity in terms of magnitude of phase-shift responses may contribute to accuracy of entrainment.

Additionally, period changes due to continuous effects of light are also thought to be important for stabilizing entrainment, as indicated by studies of rodents under long skeleton photoperiods (Pittendrigh and Daan, 1976b). Simulations of entrainment to a fluctuating, naturalistic light environment suggested that a combination of phase and period responses were likely to yield maximum accuracy under such variable environments (Beersma et al., 1999). However, period responses or velocity responses and their relationship with accuracy under rectangular, laboratory LD cycles have not been expounded. Additionally, accurate measurements of such velocity changes are difficult. Nevertheless, several effects of light on period such as the changes in period under constant light of different intensities and history-dependence of period on prior entraining conditions such as different zeitgeber periods (*T*-cycles) or photoperiods are thought to represent such parametric effects of light (Aschoff, 1979; Pittendrigh, 1981b). Hence, these effects may be used as a proxy to measure parametric effects of light on circadian rhythms and their contribution to evolution of accuracy.

The non-parametric and parametric effects of light are known to be associated with different photoreception circuits of the *Drosophila* circadian clock. There are several pathways of light input to the fruit fly circadian clock including the deep-brain, intra-cellular cryptochrome and various rhodopsins present in the compound eyes, ocelli and Hofbauer-Buchner eyelets (Emery et al., 1998; Helfrich-Förster et al., 2001; Rieger et al., 2003). Wheeler and co-workers had initially reported that flies could synchronize to light-dark cycles despite mutation of external photoreceptors (Wheeler et al., 1993). Thereafter, the *cryptochrome* (*cry*) gene, which was known to be a plant blue light sensor, was identified as important for circadian phase resetting in response to light in flies (Emery et al., 1998; Stanewsky et al., 1998; Ishikawa et al., 1999). However, circadian rhythms in *cry* mutant flies are still capable of entrainment via rhodopsins in the compound eyes and HB eyelets which contribute to sensitivity to light of higher wavelengths (Helfrich-Förster et al., 2001; Hanai et al., 2008; Hanai and Ishida, 2009). The compound eyes are also thought to be responsible for changes in period under constant light (Klarsfeld et al., 2004; Rieger et al., 2006) and important for entrainment to long photoperiods (Rieger et al., 2003). Hence, they may be involved in the continuous effects of light on the circadian clock and its period responses. Moreover, the compound eyes have also been implicated in masking effects of light and modulation of activity patterns under light-moonlight cycles (Rieger et al., 2003; Bachleitner et al., 2007). I wished to examine various measures of light sensitivity in terms of phase-shifts, period changes and activity patterns and their contributions to accuracy of entrainment by comparing these measures in our selected populations with their controls. Since different photoreceptors are preferentially receptive to different wavelengths of light

and are involved in different aspects of light sensitivity, I also examined responsiveness to different wavelengths of light based on the absorption spectra of these photoreceptors.

1.4 Developmental processes

The characteristics of overt rhythms of organisms are determined not only by the circadian clock and its entrained phase but also downstream components of output pathways as well as direct effects of environmental cues on the behaviour. The adult emergence of fruit flies from their pupae is the culmination of a sequence of concerted physiological events including larval development, assessment of developmental state and gating by the circadian clock and a subsequent neuro-endocrinal cascade which can also be directly induced by light (Pittendrigh and Skopik, 1970; Qiu and Hardin, 1996; Myers, 2003). In this section, the developmental processes independent of the circadian clock that are involved in determining the timing of eclosion will be discussed. These include possible developmental rate differences at different stages, inter-individual variation in development time under constant conditions, effects of manipulation of developmental rate and the role of direct effects of light on timing of eclosion.

Although transition to early developmental stages is known to be gated by the circadian clock in many species of insects (Truman, 1972; Jones and Reiter, 1975; Fujishita and Ishizaki, 1982), pupation and pigmentation in *Drosophila melanogaster* do not show circadian rhythmicity (Pittendrigh and Skopik, 1970; Qiu and Hardin, 1996). However, the timing of pupation and pigmentation is an important determinant of the timing of adult eclosion (Pittendrigh and Skopik, 1970; Qiu and Hardin, 1996). For instance, most flies showing wing pigmentation before the end of the light phase emerged between ZT2 and ZT12 (where

ZT0 or Zeitgeber Time 0 is the time of lights-on and ZT12 is the time of lights-off under a 12:12 hour light-dark cycle or LD 12:12) on the next day while those showing pigmentation during the dark phase emerged before ZT2 on the day following the subsequent light phase (Qiu and Hardin, 1996). Hence, timing of eclosion and its accuracy may be modulated in *Drosophila* populations by altering the timing of preceding developmental stages such as wing pigmentation.

Moreover, there is known to be significant inter-individual variation in developmental rates even in developmentally synchronous populations (derived from eggs laid in a 5 hour window) at constant temperature (Pittendrigh and Skopik, 1970). Additionally, the behavioural process of emergence may also show high levels of variation (Pittendrigh and Skopik, 1970). Examining the overall development time (from egg collection to eclosion) under constant conditions in populations selected for greater accuracy of eclosion under LD cycles may be useful to distinguish between variation in developmental rates independent of the circadian clock and effects of gating by the circadian clock. Further dissection of the circadian clock's effect on developmental rate while excluding the effects of gating by the entrained clock may be possible by assaying the development time in constant light as well as constant dark conditions. This may be important since the circadian clock is also known to be associated with developmental rates, with short period flies showing reduced development time even under constant conditions (Kyriacou et al., 1990) and selection for early development resulting in evolution of shorter period free-running rhythms (Shimizu et al., 1997; Yadav and Sharma, 2013). Since constant light of high intensity is known to abolish the functioning of the circadian clock in *Drosophila* (Konopka et al., 1989; Yoshii et al., 2005), the developmental rate seen under such conditions would be independent of

circadian period under constant light in contrast to constant dark conditions where effects of clocks may persist.

An alternate approach to determining the influence of developmental rates on the observed phenotype of enhanced accuracy in timing of emergence in the selected populations could be to study the effects of manipulating developmental rates. Developmental rates are known to vary across temperatures and larval densities with faster development seen at high temperatures and low densities (Bonnier, 1926; Peters and Barbosa, 1977). The eclosion rhythms of the populations selected for greater accuracy have already been observed to be robust to changes in temperature (Kannan et al., 2012b). However, temperature may be a proximate cue for eclosion behaviour apart from its effects on developmental rate. Larval density is known to affect variation in development time in *Aedes aegypti* (Couret et al., 2014). Additionally, larval crowding is known to delay the production of juvenile hormone and the release of ecdysteroids which are important hormones responsible for determining the time of eclosion (Hirashima et al., 1995). Thus, it would be useful to examine the differential influences of development time variation due to different larval densities on the eclosion profile of selected and control fly populations.

While developmental rate is determined by several hormonal processes, the mechanisms of eclosion involve input from the circadian clock as well as different peptidergic neurons in addition to hormonal signals of developmental state. The neuroendocrine control of insect eclosion has been the subject of some study, especially in moths (Truman, 1972; Truman, 1981; Truman, 1983; Mesce and Fahrbach, 2002). Juvenile hormone and ecdysteroid hormones are thought to be responsible for preparing the insect for ecdysis and the fall of ecdysteroid levels is critical for the neurohormonal cascade involving eclosion hormone and

other peptides (Truman, 1983; Rountree and Bollenbacher, 1986; Zitnan et al., 1999). These ecdysteroids are synthesized rhythmically by the prothoracic gland which is regulated by circadian clock neurons directly or indirectly via Prothoracicotrophic Hormone (PTTH) secretions from PTTH neurons (Zitnan et al., 1993; Pelc and Steel, 1997; Siegmund and Korge, 2001). Since the prothoracic gland is also known to be involved in assessment of growth and determining the critical weight (Mirth et al., 2005), this gland could be an important regulatory center for developmental readiness and circadian gating of eclosion. The decline in ecdysteroid levels in the haemolymph triggers the release of Pre-Ecdysis Triggering Hormone (PETH) and Ecdysis Triggering Hormone (ETH), both of which are responsible for initiating pre-ecdysis behaviours as well as the release of Eclosion Hormone (EH; Zitnan et al., 1996; Zitnan et al., 1999; Ewer et al., 1997; Gammie and Truman, 1997). EH causes further release of ETH as well as Crustacean Cardioactive Peptide (CCAP) which in turn, triggers the ecdysis programs in motor neurons, resulting in eclosion (Ewer et al., 1997; Gammie and Truman, 1997). While this sequence of events is mainly dependent on developmental state and circadian gating, light can also induce eclosion by suppressing the inhibition of eclosion or stimulating EH release (McNabb and Truman, 2008). Thus, examining the direct effects of light presented within or outside the normal circadian gate may reveal the relative strengths of light induced eclosion versus circadian gating of eclosion in selected and control populations, possibly indicating the strategy adopted by selected populations to evolve higher accuracy of eclosion.

1.5 Life history traits

While the proper phasing of circadian rhythms is thought to be associated with fitness (Daan and Tinbergen, 1979; DeCoursey et al., 1997; Ouyang et al., 1998), the evolution of accurate phasing in populations and corresponding changes in life history traits such as development time, pre-adult survivorship, fecundity and lifespan has not been studied. Correlated responses to selection for accuracy on life history traits may reveal evidence for trade-offs between circadian clock accuracy and fitness components since correlated responses are dependent on genetic and phenotypic covariances between characters (Lande, 1979; Reznick, 1985; Falconer and Mackay, 1996). While genetic covariances can arise due to linkage disequilibrium or pleiotropy (Lande, 1980; Lande, 1984), phenotypic covariances could arise due to environmental correlations and developmental or functional constraints in addition to genetic correlations (Searle, 1961; Cheverud, 1984; Wagner, 1988). Thus, correlated responses to selection can provide clues on the relationships between circadian clocks and fitness and consequent constraints on their evolution due to trade-offs with life history traits.

Evidence of associations of circadian clocks with fitness are mainly based on studies with mutations of clock genes or abolition of circadian rhythmicity (DeCoursey et al., 1997; Kyriacou et al., 1990; Sheeba et al., 2000; Beaver et al., 2002; Beaver et al., 2003). As mentioned previously, abolition of circadian clocks by lesioning of the SCN resulted in greater mortality due to predation in rodents (DeCoursey et al., 1997). Similarly, loss of function mutation in the core clock gene *period* resulted in lower reproductive success due to lower male sperm release and lower female fecundity (Beaver et al., 2002; Beaver et al., 2003). However, such reductions in fitness could not be rescued by expressing PERIOD protein in the clock neurons suggesting that the *period* gene may have pleiotropic effects on

fitness independent of the circadian clock. The circadian clock in fruit flies can also be rendered dysfunctional by the use of high intensity constant light. Studies on wild-type *Drosophila melanogaster* and *Phormia terraenovae* under constant light and light-dark cycles of different cycle lengths (ranging from 20 to 28 hours) showed significant reduction in longevity of flies under constant light and non-24 hour LD cycles relative to those under 24 hour LD cycles (Pittendrigh and Minis, 1972; von Saint Paul and Aschoff, 1978). Conversely, mutant flies with short and long periods also exhibited reduced longevity under 16 hour and 24 hour LD cycles compared to wild-type flies, suggesting that an aberrant periodicity can also reduce fitness (Klarsfeld and Rouyer, 1998). Similarly, among flies from an outbred population, rhythmic individuals were found to live longer than arrhythmic ones (Kumar et al., 2005). However, the effects of constant light on reducing lifespan appear to be compensated by higher fecundity seen under these conditions, suggesting that overall fitness may not be reduced due to arrhythmicity induced by constant light (Sheeba et al., 2000). Hence, we should be cautious in interpreting the correlations between circadian clocks and fitness despite evidence of circadian dysfunction with fitness deficits. The confounding factors from these studies include the use of mutations which may have pleiotropic effects, levels of inbreeding, restriction of the study to one or two fitness components and the effects of environmental regimes independent of the circadian clock. Moreover, it has been argued that only genetic correlations measured by quantitative genetic methods and correlated responses to selection are indicative of the constraints on phenotypic evolution of the population (Reznick, 1985). However, reliable estimates of genetic correlations from resemblance of relatives require large sample sizes (Klein et al., 1973) and these correlations may also be subject to change over longer time scales of evolution (Turelli, 1988; Pigliucci,

2006). Therefore, correlated responses to selection may be the most parsimonious approach to studying the trajectories of phenotypic evolution.

Several laboratory evolution studies have examined genetic correlations between circadian clocks and life history traits in insects (Shimizu et al., 1997; Miyatake, 1998; Miyatake, 2002; Takahashi et al., 2013; Yadav and Sharma, 2013; Yadav and Sharma, 2014b; Nikhil et al., 2016). Multiple studies have established the correlation between development time and circadian period, with shorter periods seen in melon flies and fruit flies selected for faster development (Shimizu et al., 1997; Miyatake, 1998; Miyatake, 2002; Takahashi et al., 2013; Yadav and Sharma, 2013). There is also some evidence for effects of selection for age at reproduction on mating time and longevity in melon flies and that these interact with rearing conditions (Miyatake, 1998; Miyatake, 2002). Additionally, fruit fly populations selected for faster development also appear to show faster aging of the circadian clock, although this may be related to reduced longevity in these flies (Yadav and Sharma, 2014a, b). Correlated changes in life history traits due to selection on circadian clock properties have been little studied (Nikhil et al., 2016). Selection on early and late phase of adult emergence rhythms resulted in correlated responses in development time, fecundity and lifespan with late populations showing longer development time, greater fecundity and reduced lifespan (Nikhil et al., 2016). Thus, these studies reveal evidence of genetic correlations and trade-offs between circadian clocks and fitness components. However, the evolution of greater accuracy of clocks and concomitant changes in life history traits has not been studied.

The restriction of adult emergence in *Drosophila* to the early part of the day by the circadian clock is believed to be adaptive (Pittendrigh, 1954; Daan, 1981). This ‘gating’ of eclosion to times of the day with high relative humidity is thought to be important for protecting newly

emerged flies which are vulnerable to desiccation from hot and dry environments (Pittendrigh, 1954; Daan, 1981). Experiments on onion flies *Delia antiqua* showed that high temperatures had a negative effect on the wing expansion of newly emerged flies suggesting that there might be selection for eclosion in the early morning (Tanaka and Watari, 2009). Latitudinal variation in eclosion timing observed in *Drosophila pseudoobscura* and *Drosophila littoralis* populations could reflect such selection pressures on genetic variation controlling eclosion time (Lankinen, 1986; Lankinen, 1993; Lankinen and Forsman, 2006). Thus, the timing of emergence in *Drosophila* populations is likely to have been subject to adaptive evolution due to such selection pressures.

Since adaptive evolution proceeds towards the optimum phenotype (Fisher, 1930), trait means of most characters in equilibrium populations are thought to be close to the optimum (Wright, 1935). However, phenotypic variation may be maintained in the population due to segregating genetic variation, balancing of opposing selection pressures, heterozygote superiority, new mutations, or variable environments (Haldane, 1937; Mather, 1943; Bulmer, 1973; Schluter et al., 1991; Turelli and Barton, 2004; Zhang and Hill, 2005; Barrett and Schluter, 2008). It is not clear which of these mechanisms may be responsible for maintenance of phenotypic variation in timing of emergence despite the vast majority of flies emerging in the early part of the day. Since we employ a protocol resembling stabilizing selection on timing of emergence (by selecting only flies emerging close to the mean), the evolutionary response of the population may present clues on the nature of phenotypic variation that has been eliminated by our selection protocol. For instance, if the extreme eclosion phenotypes represent deleterious, recessive, pleiotropic mutations (Lerner, 1954; Robertson, 1956), the overall fitness of the population may be expected to increase as a

consequence of elimination of such mutations from the populations. In contrast, if variation in eclosion is mainly due to antagonistically pleiotropic alleles maintained at intermediate frequencies, the population might reach a new equilibrium with slight differences in fitness components but no major changes in overall fitness. Hence, correlated responses to selection for accurate timing of emergence may provide hints on the nature of genetic variation underlying variation in eclosion timing.

I undertook a comprehensive study of the evolution of accuracy of circadian rhythms by examining internal clock stability, entrainability by light, developmental processes and life history traits of populations selected for narrow gate of adult emergence. The aim of this study was to explore various physiological processes that contribute to phenotypic variation in eclosion timing and how they evolve in response to selection pressure so as to confer greater accuracy to the selected populations. These would be expected to reveal not only functional relationships between various circadian clock properties and accuracy but also reflect genetic variation present in natural populations which can serve as the substrate for phenotypic evolution of accuracy. The studies on life history traits may also reflect constraints on the evolution of accuracy which may trade-off with components of fitness. Thus, this study attempts to understand the developmental and circadian clock-mediated changes that may enable the evolution of accuracy in laboratory-bred populations of flies in addition to documenting accompanying changes in life history traits.

Long-term stability of endogenous clock period under different ambient temperatures

2.1 Introduction

Circadian rhythms of organisms display remarkable accuracy in their phase relationship with the external environment across days (DeCoursey, 1961; Daan and Aschoff, 1975). Accuracy of circadian rhythms is affected by both variation in internal clock processes as well as in the mechanisms of synchronization to the external cycle (Daan and Beersma, 2002). Variation in endogenous rhythm may be measured under constant conditions as the standard deviation of free-running period values (Pittendrigh and Daan, 1976b). Variation in circadian period among individuals (inter-individual) as well as within individuals across days (intra-individual) can contribute to variation seen in population rhythms such as eclosion.

Day-to-day precision and long-term stability of circadian period are both measures of the robustness of the individual pacemaker's period that may be correlated with each other (Daan and Beersma, 2002). Analysis of intra-individual variability of period in mice as well as comparisons across different rodent species found positive associations between precision and stability (Pittendrigh and Daan, 1976b; Daan and Beersma, 2002). However, it is not clear if these measures of variation represent some property of the underlying clock or whether precision and long-term stability can evolve independently.

Long-term stability of the circadian rhythm has been studied in greater detail with regard to aging. Circadian clocks are known to exhibit changes in clock properties with age (Turek et al., 1995; Weinert, 2000) with several studies showing period lengthening or shortening with age (Pittendrigh and Daan, 1976b; Aschoff, 1979; Morin, 1988; Luo et al., 2012; Yadav and Sharma, 2013). While studies on rodents have largely yielded shortening of period in aging

individuals (Pittendrigh and Daan, 1976b; Morin, 1988), free-running period in fruit flies usually showed lengthening with age (Luo et al., 2012; Yadav and Sharma, 2014a). Additionally, amplitude of circadian rhythms as well as their output has been shown to decline in certain cases (Weitzman et al., 198; Nakamura et al., 2011; Rakshit et al., 2012). Fragmentation of activity bouts as well as reduction in overall activity levels has also been observed with aging (Penev et al., 1997; Valentinuzzi et al., 1997) suggesting a decline in the stability of the rhythm with age. However, there has been no systematic analysis of intra and inter-individual variation in period across age and how it may be correlated with precision and accuracy of circadian rhythms of populations.

Although circadian period is known to be compensated for changes in ambient temperature (Pittendrigh, 1954) in order to accurately measure passage of time (Pittendrigh, 1981a), the circadian period does show some variability with changes in temperature. Temperature compensation of period may also be an important target of selection for adaptation to different climates (Sawyer et al., 1997) since they may help conserve phase of entrainment despite variability of temperature. Moreover, aging is known to be accelerated at higher temperatures (Miquel et al., 1976) which may affect rates of period changes with age at different temperatures. Hence, minor differences in period between populations variability with age may be detectable only at certain temperatures. It is also not clear whether variation in period due to temperature changes (coefficients of temperature compensation) may be correlated with other aspects of period variation and can evolve in response to selection for accuracy.

In this study, we examined various aspects of variation in circadian period in *Drosophila* populations selected for narrow gate of adult emergence rhythms. Since these *Drosophila*

stocks had evolved greater accuracy of entrained phase of emergence and activity-rest rhythms due to selection for over 100 generations (Kannan et al., 2012a), we asked if these stocks have evolved differences in intra-individual and inter-individual variation in period as well. Previous studies had shown that selected stocks show marginally greater precision of period compared to controls though these differences were not statistically significant (Kannan et al., 2012a). In this study, we asked whether long-term stability of period and variation of period across temperatures have evolved in response to selection since these properties may also reflect period instability and contribute to variation in entrained circadian rhythms. We recorded the circadian activity-rest rhythms of individual flies and estimated circadian period across non-overlapping 8-day age-windows under three different temperatures till around the median lifespan of these flies at each temperature. We then calculated and compared intra-individual and inter-individual variation in period across the different age-windows for selected and control stocks. Our results show that period lengthens with age at all three temperatures and that inter-individual variation in period declines with age at 25 °C. However, we did not observe significant differences in any of these traits between selected and control stocks. We also observed that fly activity rhythms show temperature compensation in their period across these temperatures although selected stocks showed temperature coefficients closer to 1 than control stocks. These results indicate that intrinsic variation in internal clock period does not evolve in response to selection for greater accuracy of adult emergence rhythms in *Drosophila* populations. However, temperature compensation of period appears to have evolved as a correlated response to selection for greater accuracy in the selected stocks.

2.2 Materials and Methods

2.2.1 Selection Protocol

All assays were conducted on selected and control populations that have been maintained in the laboratory under similar environmental conditions for over 150 generations. The selected populations (also referred to as Precise Populations, or PP_{1-4}) and control populations (CP_{1-4}) were initiated from four ancestral baseline populations (LD_{1-4}) of *Drosophila melanogaster* that had been maintained for more than 100 generations in our laboratory under 12:12 hour light-dark (LD 12:12) cycles with constant temperature (25 °C) and constant humidity (~ 80%) on banana-jaggery food medium (Shindey et al., 2017). These baseline populations, in turn, were derived from four laboratory populations (JB_{1-4}) which had been maintained under constant light (LL) for over 150 generations on a 21-day generation cycle. The JB populations were derived from UU populations which had been reared on a 14-day generation cycle for about 170 generations (Sheeba et al., 1998). These four UU populations (UU_{1-4}) were derived from five B populations (B_{1-5}) which had been derived from a wild caught population (IV) from South Amherst, Massachusetts, U.S.A. (Ives, 1970) and maintained under similar laboratory conditions for ~ 400 generations (Joshi and Mueller, 1996).

From the baseline (LD_{1-4}) populations, four selected populations (PP_{1-4}) were initiated by selecting for flies emerging between ZT1 - ZT2 under LD 12:12, where time of lights-on is defined as ZT0 (Zeitgeber Time 0) and lights-off occurs at ZT12. Four control populations (CP_{1-4}) were also initiated along with the selected populations, which were maintained under similar conditions with the exception that no conscious selection pressure was applied on

these populations. Each PP and CP population was derived from one baseline population, thus forming matched selected and control pairs. The sets of four selected (PP_{1-4}) and four control (CP_{1-4}) populations will be collectively referred to as selected (PP) and control (CP) stocks respectively. A total of about 1200 breeding adults per population, with roughly equal number of males and females, were maintained as large outbred populations in Plexiglas cages (25 cm x 20 cm x 15 cm) with banana-jaggery medium as the food source. Three days prior to egg collection, yeast paste was applied on food plates and provided as the food source. After feeding flies with yeast for three days, the yeast plate was withdrawn. A cut was made on a fresh plate of banana-jaggery food such that one half-plate of food was obtained and the semicircle of food was placed such that the vertical surfaces were available for oviposition (henceforth referred to as cut-plate). Two such cut-plates were introduced into each cage, and flies were allowed to lay eggs for 2-3 hours. Thereafter, this cut-plate was withdrawn and the eggs laid on the food medium were collected and transferred into glass vials (18 cm height × 2.4 cm diameter) containing ~ 10 ml of food, at a density of about 300 eggs per vial. Flies emerging between the 9^{th} and 12^{th} days after egg collection were collected into fresh Plexiglas cages containing a petri-dish of banana-jaggery medium, thus forming the new generation of breeding adults. Fresh petri-dishes with food medium were provided every alternate day until the 18^{th} day since the previous egg collection, when yeast plate was provided before the initiation of the new generation. Three days later, egg collection was done for the next generation such that both selected and control stocks were maintained on a 21-day discrete generation cycle. The selection on the timing of adult emergence has been performed for over 180 generations till the time of writing. The assays

described in this thesis were conducted on selected stocks which had been subject to selection for 90-160 generations along with their corresponding controls.

2.2.2 Standardization of the selected populations

Prior to the assays, all selected and control populations were subjected to a common rearing condition for one generation to minimize non-genetic parental effects which could be caused by imposition of different maintenance regimes. During this one generation, selection pressure was relaxed on the selected stocks. About 300 eggs per vial were collected from the parental populations into glass vials with ~ 10 ml of food. On the 12^{th} day after egg collection, all emerging adult flies were collected in fresh Plexiglas cages with banana-jaggery medium as the food source and the resultant populations are referred to as standardized populations. Yeast-paste was provided to standardized populations for two days prior to egg collection for the assays. Eggs were collected from the standardized populations and transferred at approximately 300 eggs per vial (unless stated otherwise) into ten vials with ~ 10 ml of food for each population. The progeny of the standardized populations will be referred to as standardized flies and were used for all the assays.

2.2.3 Long-term recording of activity rhythm in constant darkness

Freshly emerged adult flies were separated within 8 hours of their emergence and virgin males were introduced into activity tubes (5 mm x 65 mm) with corn food at one end and cotton plug at the other end when they were 2 days old and placed in Drosophila Activity Monitors (DAM 5, Trikinetics Inc, Waltham, MA, USA) which were placed in an environment of constant darkness (DD) and constant temperature. The locomotor recording was continued with regular transfer of flies into tubes with fresh food every 8^{th} day in the

presence of dim far-red light till roughly half the flies were dead, whereupon recording was terminated. This assay was conducted under three different temperatures of 18 °C, 25 °C and 29 °C and various measures of variation in period were calculated at each temperature.

2.2.4 Statistical analyses

The analysis of the activity rhythm was performed for each individual fly, dividing the recording into non-overlapping age-windows of 8 days starting from the first day of recording. Firstly, for each window of 8 days for each individual, period of the activity rhythm was estimated as the peak value in the Lomb-Scargle (LS) periodogram in the range of 18-30 hours. Those age-windows in which flies did not show a significant period value in the LS periodogram or did not show activity for at least 7 days were not considered for analysis. Individual period values for each age-window were used for repeated measures ANOVA with 'stock' as a fixed factor and 'age' as repeated measure. We used a completely randomized design instead of a randomized block design since the block effects were small, and in such cases, the randomized design may be more efficient in estimating effects of treatment (Lentner et al., 1989). For each age-window, mean period values were calculated for each population and then averaged across the four populations within a stock ($PP_{1\text{-}4}$ or $CP_{1\text{-}4}$) for plotting the figures. Next, the inter-individual variation in period within each age-window was estimated for each population by calculating the standard deviation in period across all individuals within that age-window. Repeated measures ANOVA was performed on inter-individual variation values for each population with 'stock' as fixed factor and 'age' as repeated measure. The intra-individual variation was also estimated as the standard deviation in period values for the same individual across different age-windows. The mean intra-individual variation in period was calculated for each population and subjected to one-

way ANOVA with 'stock' as fixed factor. Error bars in the figures represent SEM across the four populations.

The mean period values for each replicate population at each age-window were also used to estimate temperature compensation coefficients (Q_{10} = {period at lower temperature (T1)/period at higher temperature (T2)}$^{10°/(T2 - T1)}$; Barrett and Takahashi, 1995) for three pairs of temperatures (18-25 °C, 25-29 °C and 18-29 °C). The mean period value at each age-window for each population was used to calculate Q_{10} coefficients for each population for each pair of temperatures and these coefficients were then averaged across the four populations for each stock at each age-window. Repeated measures ANOVA was performed on temperature compensation values for each population with 'stock' as fixed factor and 'age' as repeated measure.

Post-hoc comparisons were done using Tukey's HSD test. All statistical analyses were conducted on STATISTICA^{TM} for windows release 7.0 (Statsoft Inc, 1995).

2.3 Results

2.3.1 Stability of circadian period of selected and control flies under constant darkness at 25 °C

Long-term recording of free-running rhythms of flies from selected and control stocks (starting with n = 32 for each population with 4 populations for each stock) under constant darkness at 25 °C revealed persistent rhythms till the end of recording in most flies. The free-running periods of these rhythms were estimated across 8-day age-windows and the standard deviation in period across individuals, as well as within the same individual across age-windows, were calculated and compared for both stocks. Repeated measures ANOVA

on the period values of individual flies with 'stock' as a fixed factor and 'age' as repeated measure revealed a significant main effect of 'age' ($F_{3,528} = 3.4$; $p < 0.05$) but no significant effect of 'stock' ($F_{1,176} = 2.6$; $p > 0.05$) or 'stock' × 'age' interaction ($F_{3,528} = 1.2$; $p > 0.05$). In general, flies tended to show lengthening of free-running period with age (Figure 2.1). Post-hoc multiple comparisons using Tukey's HSD revealed that flies showed shorter free-running periods during the first age-window compared to the second and fourth age-windows (Figure 2.1a, $p < 0.05$). However, there were no significant differences in the free-running period between selected and control stocks at any of the ages.

Further, the standard deviation of period values across individuals in each population was estimated for each age-window and subjected to repeated measures ANOVA with 'stock' as fixed factor and 'age' as repeated measure. ANOVA on inter-individual variation in period revealed a significant main effect of 'age' ($F_{3,18} = 4.85$; $p < 0.05$) but no significant effect of 'stock' ($F_{1,6} = 0.15$; $p > 0.05$) or 'stock' × 'age' interaction ($F_{3,18} = 0.09$; $p > 0.05$). The inter-individual variation in period within a population tended to decline with age (Figure 2.1b). Post-hoc multiple comparisons revealed that the variation in period among flies in a population was significantly lower in the fourth age-window compared to the first age-window (Figure 2.1b, $p < 0.05$). Once again, no comparisons between the selected and control stocks were significantly different though selected stocks showed a trend of lower inter-individual variation across all age-windows (Figure 2.1b). The standard deviation in period values for the same individual across the four age-windows were also calculated and compared between selected and control stocks. One-way ANOVA on the intra-individual variation in free-running period with 'stock' as fixed factor revealed no significant main

effect of 'stock' ($F_{1,6}$ = 0.009; p > 0.05; Figure 2.1c) suggesting that selected and control stocks do not differ in the stability of free-running period across age at 25 °C (Figure 2.1).

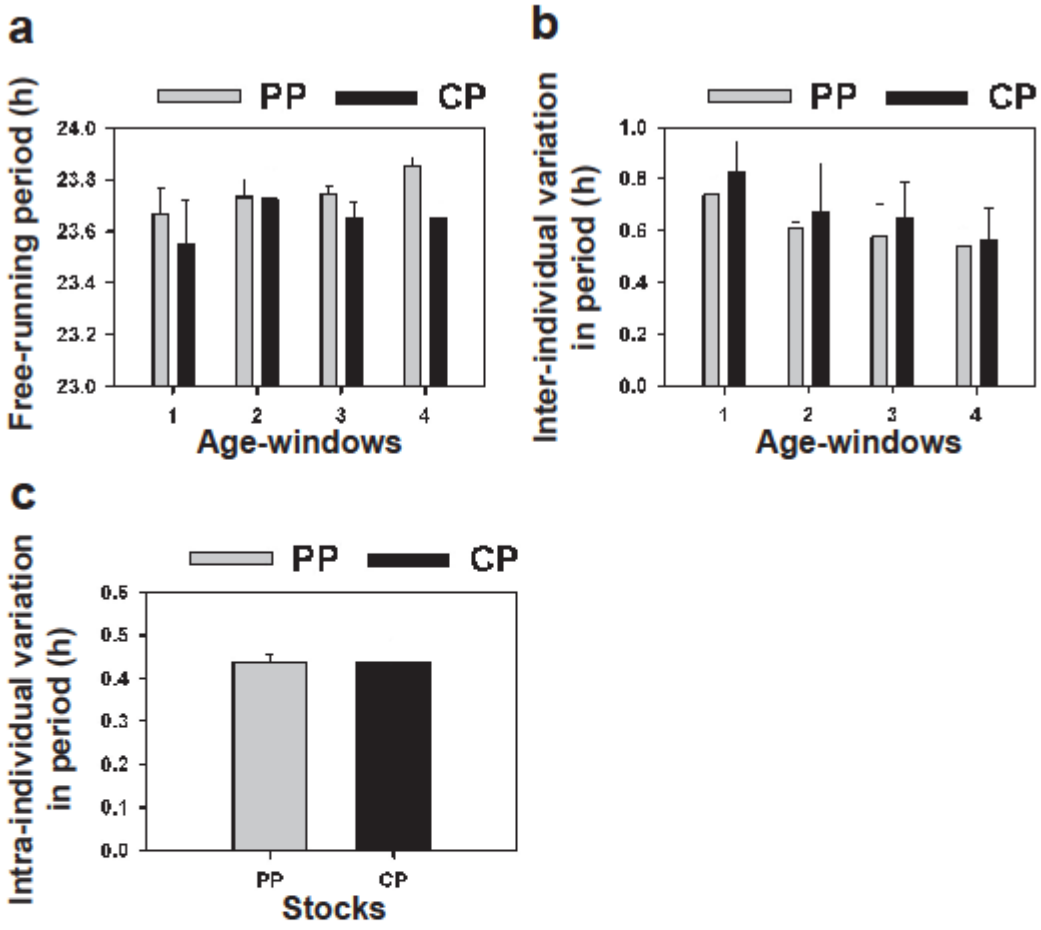

Figure 2.1. Stability of circadian free-running period at 25 °C. a) Mean free-running period of selected and control stocks over 8-day age-windows estimated by LS periodogram. b) Mean inter-individual variation in period of selected and control stocks calculated as the standard deviation of period values within a replicate population during each age-window and averaged over the four replicate populations for each stock. c) Mean intra-individual variation in period of selected and control stocks calculated as the standard deviation of period values of the same individual fly across 4 age-windows and then averaged across individuals within a population. Grey bars represent selected stocks while black bars represent control stocks in all panels. Error bars are SEM across 4 replicate populations.

2.3.2 Stability of circadian period of selected and control flies under constant darkness at 18 °C

Since flies live longer under 18 °C than 25 °C, we were able to continue recording flies for greater number of days and study the patterns of variation in their free-running periods over longer durations. We estimated periods of individual flies from eight age-windows (n ~ 15 for each replicate population at the 8^{th} age-window). Repeated measures ANOVA on the period values of individual flies with 'stock' as a fixed factor and 'age' as repeated measure revealed a significant main effect of 'age' ($F_{7,784} = 3.8$; $p < 0.05$) but no significant effect of 'stock' ($F_{1,112} = 3.2$; $p > 0.05$) or 'stock' × 'age' interaction ($F_{7,784} = 0.9$; $p > 0.05$). Although most flies showed lengthening of free-running period with age, this trend was not consistent across all age-windows (Figure 2.2a). Post-hoc comparisons revealed that flies showed significantly shorter free-running periods in the first and second age-windows compared to periods in the fourth and eighth age-windows ($p < 0.05$; Figure 2.2a). Additionally, free-running period in the second age-window was also shorter than that in the seventh age-window ($p < 0.05$; Figure 2.2a). No significant difference in period was seen between selected and control stocks though selected flies tended to show longer free-running periods compared to controls.

The variation in period among individuals in a population at particular age-windows was also computed for all the eight age-windows and subjected to repeated measures ANOVA which revealed no significant effect of 'stock' ($F_{1,6} = 0.02$; $p > 0.05$), 'age' ($F_{7,42} = 0.66$; $p > 0.05$)

or ‘stock’ × ‘age’ interaction ($F_{7,42}$ = 0.79; p > 0.05). No clear patterns of differences in inter-individual variation in period between age-windows or stocks could be discerned at this temperature (Figure 2.2b). Similarly, the intra-individual variation in period across age-windows was also not different between selected and control stocks. ANOVA with ‘stock’ as fixed factor revealed no significant effect of ‘stock’ ($F_{1,6}$ = 0.64; p > 0.05; Figure 2.2c). Thus, free-running period of selected and control stocks showed lengthening with age at 18 °C similar to that seen at 25 °C, with no significant differences seen between the stocks. Similarly, no significant differences between stocks are observed in inter-individual and intra-individual variation in period at 18 °C. However, there is no reduction in inter-individual variation in period with age at this temperature such as that seen at 25 °C.

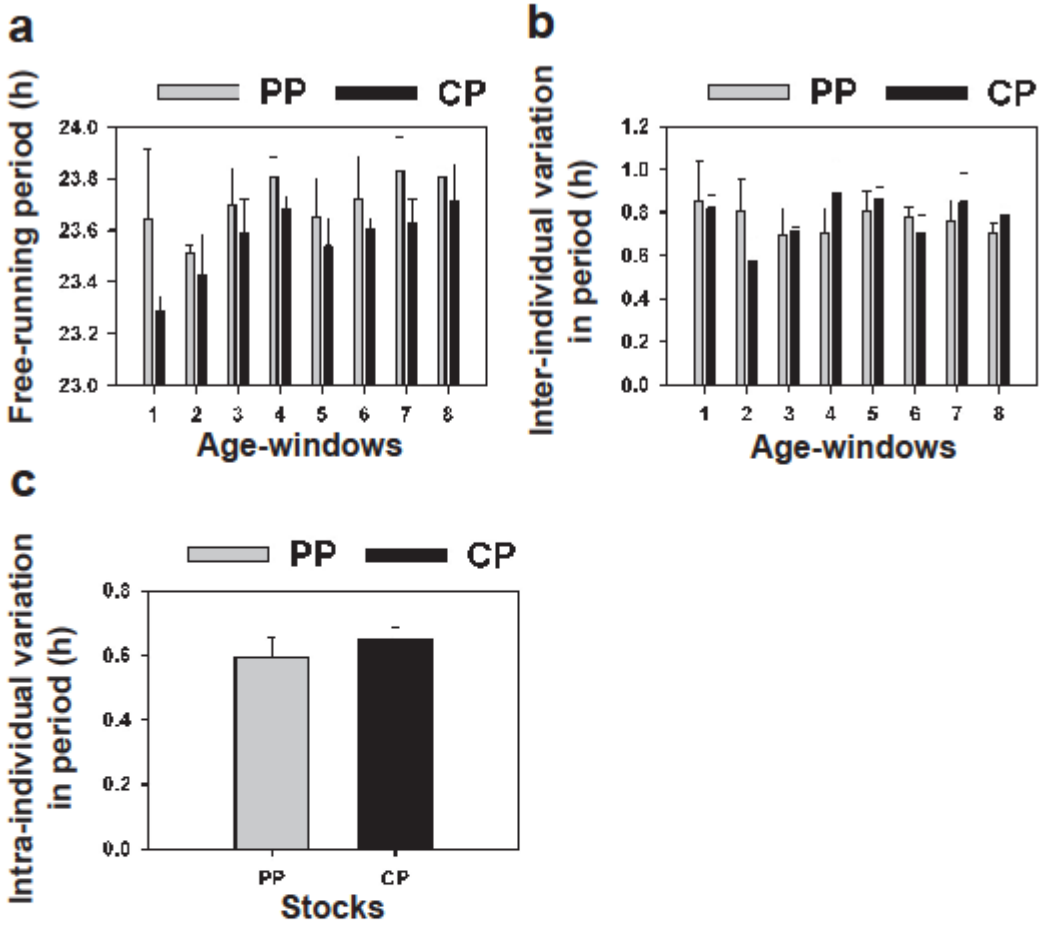

Figure 2.2. Stability of circadian free-running period at 18 °C. a) Mean free-running period of selected and control stocks over 8-day age-windows estimated by LS periodogram. b) Mean inter-individual variation in period of selected and control stocks calculated as the standard deviation of period values within a replicate population during each age-window and averaged over the four replicate populations for each stock. c) Mean intra-individual variation in period of selected and control stocks calculated as the standard deviation of period values of the same individual across 8 age-windows and then averaged across individuals within a population. Rest of the details same as figure 2.1.

2.3.3 Stability of circadian period of selected and control flies under constant darkness at 29 °C

Since flies show symptoms of accelerated aging under high temperatures (Miquel et al., 1976), we examined the clock period of selected and control stocks across different age-windows at 29 °C for detecting signatures of age-related variability of period in the two stocks. Repeated measures ANOVA on the period values of individual flies with 'stock' as a fixed factor and 'age' as repeated measure revealed a significant main effect of 'stock' ($F_{1,201}$ = 4.4; $p < 0.05$) and 'age' ($F_{2,402}$ = 4.1; $p < 0.05$) but no significant effect of 'stock' × 'age' interaction ($F_{2,402}$ = 2.5; $p > 0.05$). Post-hoc comparisons using Tukey's HSD revealed that flies showed longer free-running periods in the third age-window compared to the second age-window ($p < 0.05$; Figure 2.3a). Additionally, flies from selected stocks showed overall significantly shorter free-running period compared to control stocks ($p < 0.05$; Figure 2.3a).

However, selected and control stocks did not appear to be different in terms of the extent of change in free-running period across age.

Inter-individual variation in period within a population was estimated for three age-windows and compared between selected and control stocks. Repeated measures ANOVA on inter-individual variation revealed no significant effect of 'stock' ($F_{1,6} = 1.5$; $p > 0.05$), age' ($F_{3,18} = 2.67$; $p > 0.05$) or 'stock' × 'age' interaction ($F_{3,12} = 1.24$; $p > 0.05$) suggesting no differences in inter-individual variation between selected and control stocks or different age-windows at this temperature (Figure 2.3b). Similarly, the intra-individual variation in period across age-windows was also not different between selected and control stocks as ANOVA with 'stock' as fixed factor revealed no significant effect of 'stock' ($F_{1,6} = 3.7$; $p > 0.05$; Figure 2.3c). Hence, there do not appear to be any differences in the variation in period across age or across individual flies between selected and control stocks at 29 °C (Figure 2.3).

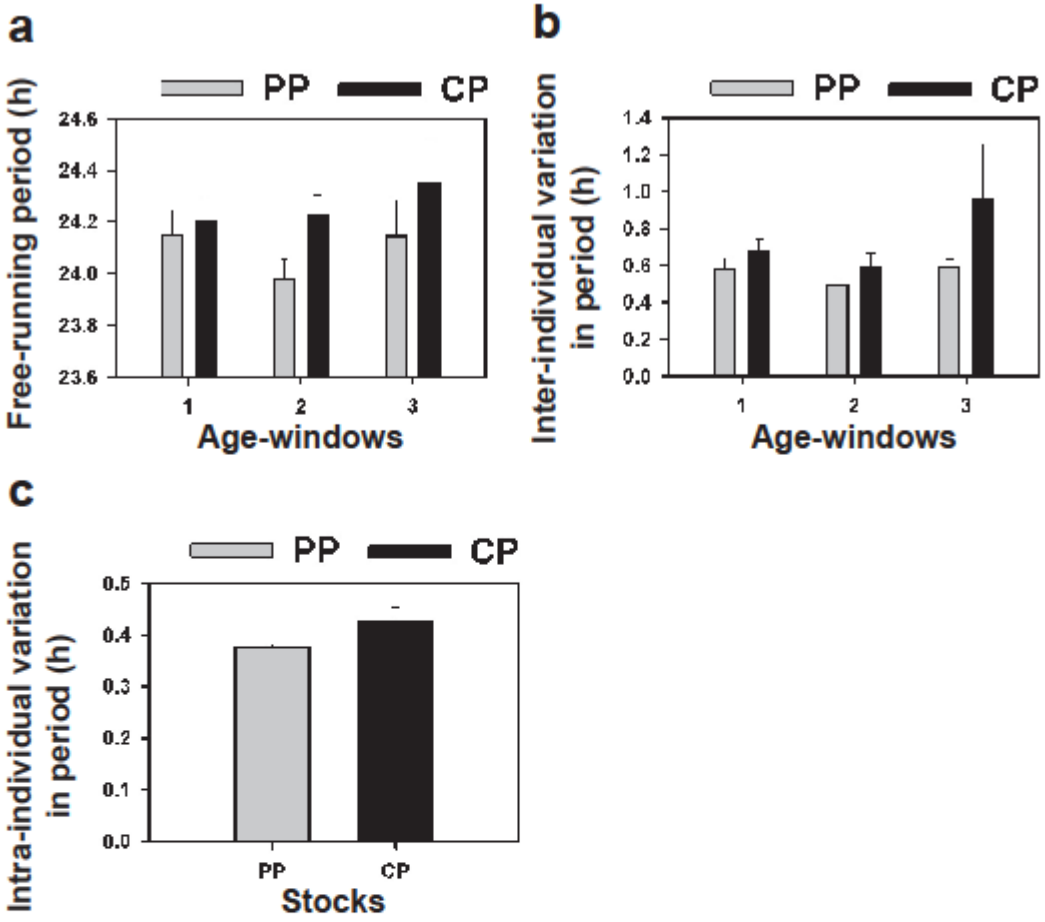

Figure 2.3. Stability of circadian free-running period at 29 °C. a) Mean free-running period of selected and control stocks over 8-day age-windows estimated by LS periodogram. b) Mean inter-individual variation in period of selected and control stocks calculated as the standard deviation of period values within a replicate population during each age-window and averaged over the four replicate populations for each stock. c) Mean intra-individual variation in period of selected and control stocks calculated as the standard deviation of period values of the same individual across 3 age-windows and then averaged across individuals within a population. Rest of the details same as figure 2.1.

2.3.4 Temperature compensation of selected and control flies across the three temperatures

While the circadian free-running period remained roughly the same across all three temperatures (Q_{10} ~ 1), we were interested in whether the magnitude of changes between the circadian period across different temperatures at multiple age-windows were significantly different between selected and control stocks. For this purpose, we calculated the coefficient for temperature compensation (Q_{10}) for each replicate population at each age-window across three pairs of temperature comparisons (18-25 °C, 25-29 °C and 18-29 °C; Figure 2.4). Repeated measures ANOVA on the Q_{10} values with 'stock' as fixed factor and 'age' as

repeated measure revealed no significant effect of 'stock' ($F_{1,6}$ = 0.26; p > 0.05 for 18-25 °C; $F_{1,6}$ = 2.16; p > 0.05 for 25-29 °C), 'age' ($F_{3,18}$ = 1.01; p > 0.05 for 18-25 °C; $F_{2,12}$ = 1.39; p > 0.05 for 25-29 °C) or 'stock' × 'age' interaction ($F_{3,18}$ = 0.37; p > 0.05 for 18-25 °C; $F_{2,12}$ = 0.13; p > 0.05 for 25-29 °C) for the 18-25 °C and 25-29 °C pairs of temperatures (Figure 2.4a, b). However, repeated measures ANOVA on Q_{10} values for periods at 18 °C and 29 °C revealed a significant main effect of 'stock' ($F_{1,6}$ = 9.7; p < 0.05) but no significant effect of 'age' ($F_{2,12}$ = 0.2; p > 0.05) or 'stock' × 'age' interaction ($F_{2,12}$ = 0.1; p > 0.05). Post-hoc comparisons using Tukey's HSD revealed that selected stocks showed greater Q_{10} values than control stocks (p < 0.05) such that selected stocks showed values closer to 1 (Figure 2.4c). This indicates that selected stocks show lower variability of period than control stocks across wide range of temperatures such as 18 and 29 °C.

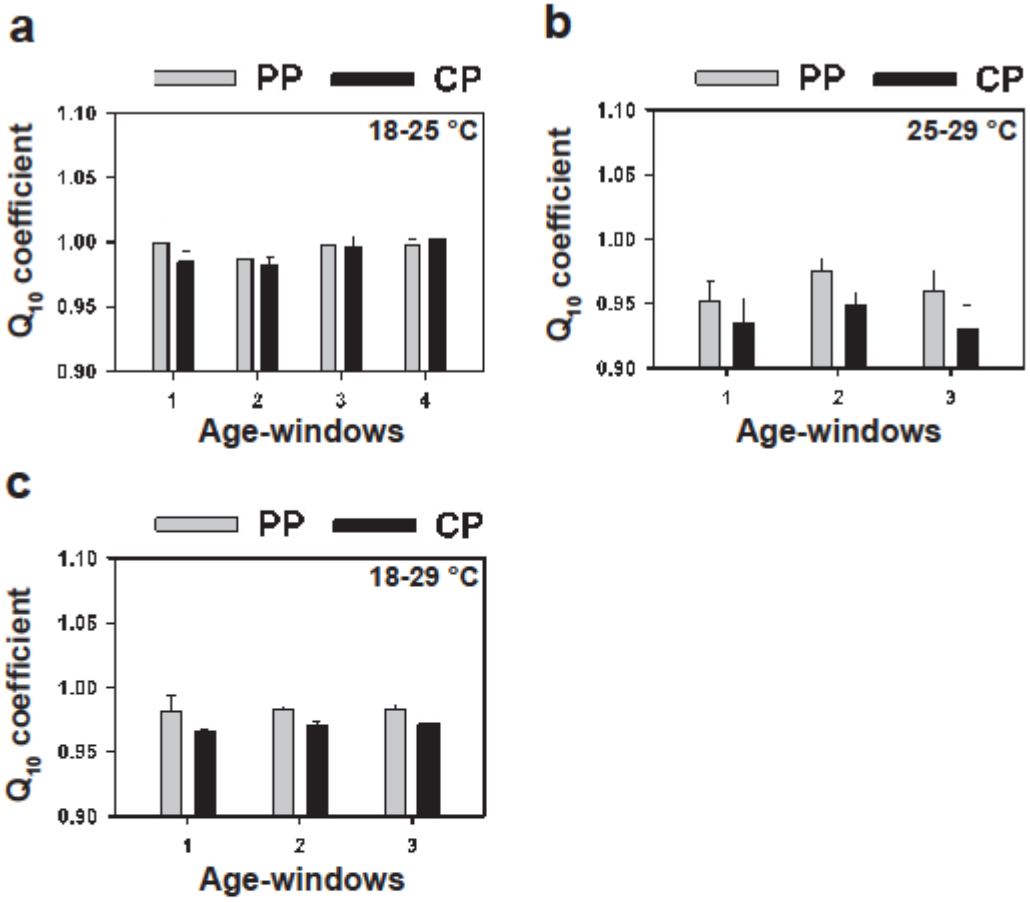

Figure 2.4. Temperature compensation of circadian free-running period across 3 pairs of temperatures. a) Temperature compensation coefficients (Q_{10} = {period at lower temperature (T1)/period at higher temperature (T2)}$^{10/(T2-T1)}$) calculated for mean circadian periods of each population at each age-window at 18 °C and 25 °C. b) Temperature compensation coefficients calculated for mean circadian periods of each population at each age-window at 25 °C and 29 °C. c) Temperature compensation coefficients calculated for mean circadian periods of each population at each age-window at 18 °C and 29 °C. Rest of the details same as figure 2.1.

2.4 Discussion

Variability in free-running period due to various factors has been an important subject of study in circadian rhythm research (Pittendrigh and Daan, 1976b; Aschoff, 1979). While it is acknowledged that such variation in period would contribute to the variation in entrained phase seen under cyclic external conditions (Daan and Aschoff, 1975; Pittendrigh and Daan, 1976a), the extent of such contributions to the evolution of greater accuracy of phase has not been explored empirically. In this study, we examined various measures of variability in circadian period in *Drosophila* populations selected for enhanced accuracy of emergence phase and compared them to controls. Circadian period is known to show day-to-day variation as well as long-term changes across age within the same individual and these measures are known to be correlated with each other (Daan and Beersma, 2002). While previous studies showed that selected stocks do not show significant increase in precision

compared to controls (Kannan et al., 2012), long-term stability was not examined. Since our selected stocks have been selected for greater accuracy in phase of adult emergence, which is a population rhythm, variation in period among individuals is also a potential contributing factor to variation in phase of the rhythm in addition to intra-individual variation in period. Hence, we estimated period across age for each individual as well as inter-individual and intra-individual variation in period for flies from selected and control stocks.

Previous studies had reported that stability and precision are reduced when the average period is further away from 24 hours (Aschoff, 1960; Pittendrigh and Daan, 1976b, Sharma and Chandrashekaran, 1999). Previous studies on our stocks also showed trend of greater precision as well as greater deviation of period from 24 hours in selected stocks compared to controls (Kannan et al., 2012a). However, the differences in precision were not statistically significant. The lack of significant differences may be due to the small magnitude of such differences which may be enlarged over longer durations or at extreme temperatures. Additionally, variation in period at a particular temperature within and across individuals may be different from such variation seen at other temperatures. Such differences in intra-individual and inter-individual variation across temperatures have been observed for morphological characters. For instance, fluctuating asymmetry (measure of developmental stability) and inter-individual phenotypic variation of morphological traits is higher at extreme temperatures though some are different at only certain temperatures (Imasheva et al., 1997). Hence, we examined long-term stability of period at different temperatures in the selected and control stocks. We found that selected stocks do not significantly differ from control stocks in terms of intra-individual variation in period across the age-windows at all three temperatures though selected stocks showed a trend of lower intra-individual variation

at 18 °C and 29 °C (Figures 2.1c, 2.2c and 2.3c). These results suggest that long-term variation in period is not reduced by selection for enhanced accuracy of phase. The absence of differences in long-term stability may be due to several reasons. Firstly, it is not necessary that long-term stability and daily precision are intimately correlated despite speculations that they might be. For instance, long-term stability of circadian period may hinge on processes related to aging of the circadian clock (Koh et al., 2006; Yadav and Sharma, 2014a) while precision is likely to be mediated by stochastic processes related to the daily functioning of the clock as well as variation due to output processes (Pittendrigh and Daan, 1976b). Thus, the selected stocks may have evolved marginally greater precision due to changes in the mechanisms associated with precision which do not affect the aging of the circadian clock. This is also consistent with the fact that selection on accuracy of phase of circadian rhythms is applied at the stage of adult emergence where factors related to aging are unlikely to contribute to variation in period and thus, phase of adult emergence.

We also found lengthening of circadian period with age at all three temperatures with longer periods being seen especially at the last age-windows which were considered for analysis at the respective temperatures (Figures 2.1a, 2.2a and 2.3a). However, there did not appear to be any differences in the extent of such changes with age between selected and control stocks at any of the temperatures. These results indicate again that processes related to aging do not seem to be altered between selected and control stocks. Previous studies on *Drosophila* populations selected for faster development have revealed significant acceleration in the extent of period lengthening of such flies with age compared to controls (Yadavand Sharma, 2014a). These flies also showed reduced lifespan and other signatures of faster aging suggesting that period lengthening with age is indeed a reliable marker for the aging of the

circadian clock (Luo et al., 2012; Yadav et al., 2013). Hence, the absence of differences between selected and control stocks in period lengthening can be taken as evidence to suggest lack of differences in the rate of aging of their circadian clocks and lend further credence to the proposition that mechanisms related to aging do not evolve in fly populations subjected to selection for greater accuracy of adult emergence rhythms.

We also examined inter-individual variation in period across age-windows at all three temperatures. Although we observed a general trend of lower inter-individual variation in selected stocks compared to controls at all temperatures, such differences were not statistically significant (Figures 2.1b, 2.2b and 2.3b). While inter-individual variation in period reduced with age at 25 °C (Figure 2.1b), such differences were not observed at other temperatures (Figures 2.2b and 2.3b). Reduction in inter-individual variation with age is seen at 25 °C probably due to individuals with short periods at early age showing period lengthening to match those of their long period counterparts which do not show as much period lengthening over age. The notion that variation among individuals in period can contribute to variation in phase in population rhythms stems from the principle of correlation between period and phase for individuals within populations which is derived from models of circadian entrainment (Pittendrigh and Daan, 1976a; Duffy et al., 2001). However, inter-individual variation in period may not be a major contributor to evolution of enhanced accuracy in selected stocks since there is no significant difference between selected and control stocks in this measure. Recent studies have shown that period and phase of individuals are not necessarily correlated in several cases (Duffy and Czeisler, 2002; Michael et al., 2003). Hence, inter-individual variation in period may not necessarily translate into

variation in entrained phase and thus, would not be a potential target of selection for reduced variation in phase of a population rhythm.

Although temperature compensation is thought to be a vital feature of the circadian clock system (Pittendrigh, 1981a), there is no empirical evidence of its contribution to accuracy of entrained phase. However, variation in period across different environmental conditions may be a function of general instability of period (Pittendrigh and Daan, 1976b; Pittendrigh and Caldarola, 1973). Therefore, we calculated temperature coefficients of period across the three pairs of temperatures and compared between selected and control stocks. We did observe differences in temperature compensation between selected and control stocks with selected stocks showing temperature compensation coefficients closer to 1 (Figure 2.4). Since both selected and control stocks are maintained under roughly constant temperature of 25 °C, there is unlikely to be any direct selection pressure for better temperature compensation in the selected stocks compared to controls. Hence, increased temperature compensation is probably a correlated response to selection for increased accuracy of adult emergence rhythms. Abundant genetic variation for temperature compensation abilities of the clock has been found in natural populations (Sawyer et al., 1997; Edwards et al., 2005). Furthermore, such allelic variation in temperature compensation exhibits latitudinal clines, such that certain variants that are correlated with better ability to compensate for temperature differences are more abundant at high latitudes where temperature is more variable (Sawyer et al., 1997). Thus, temperature compensation mechanisms can evolve due to selection pressures in the natural environment. Nonetheless, it is not clear why selection for greater accuracy of adult emergence rhythms results in evolution of temperature compensation. One possible explanation could be the sensitivity of adult emergence to small temperature

changes in the environment. Development time is known to show large changes when subject to alterations in ambient temperature (Bonnier, 1926). Additionally, the adult emergence profile under LD cycles is also significantly altered at different ambient temperatures (Kannan et al., 2012b). However, it had also been observed that adult emergence rhythms of selected stocks were significantly more robust than control stocks at extreme temperatures of 18 °C and 29 °C (Kannan et al., 2012b). Hence, we speculate that selected stocks may have evolved better temperature compensation in order to reduce variation in emergence phase due to small variations in temperature during development. Alternatively, reduced effects of temperature on period may simply be a measure of the general homeostasis of circadian period against changes in several variables (Pittendrigh and Caldarola, 1973) which are identifiable in the selected stocks only across the widest temperature range.

Overall, intrinsic variation in internal clock period of locomotor activity rhythms does not appear to have reduced greatly in the selected stocks compared to controls as a consequence of selection for greater accuracy of adult emergence rhythms. One possible reason for the absence of such differences is that circadian period of locomotor activity rhythms is determined by factors that are exclusive to mechanisms of locomotor function in addition to core circadian clock processes. Hence, even if there are differences in variation in core clock circadian period, these may be obscured by output processes related to locomotor activity, which contribute disproportionately to the variation seen in the overt locomotor activity rhythm. However, selected stocks show lower variability in period across temperatures suggesting that temperature compensation mechanisms can evolve in response to selection for accuracy of circadian rhythms. Thus, selection for accuracy of emergence rhythms

results in evolution of some aspects of variability of endogenous period of the circadian clock. Nevertheless, it may be worthwhile to note that since selection pressure is applied on the phase of entrained circadian rhythms, variation in the mechanism of entrainment may be a more important target of selection than variation in the free-running period. Hence, the mechanism of entrainment by resetting of the circadian clock in response to light cues is the subject of the next chapter.

Enhanced sensitivity of circadian clocks to light in the selected stocks

3.1 Introduction

The synchronization of an organism's rhythm and maintenance of a stable phase angle with an external cue is called entrainment (Johnson et al., 2003). The phase of entrainment as well as the stability of that phase across days (accuracy), are mainly determined by the organism's internal clock period (or free-running period; FRP) and its resetting ability in response to time cues (Pittendrigh and Daan, 1976a). As the characteristics of variability of free-running period in fly populations that have evolved greater accuracy have been addressed in the previous chapter, this chapter will deal with the resetting ability of the clock. The ability of the circadian clock to reset is characterized by the responsiveness of clock phase, period or other properties to changes in zeitgeber signals.

Previous studies have suggested that accuracy of circadian rhythms is dependent on strength of the zeitgeber based on theoretical and empirical studies that showed lower phase control near the limits of entrainment such as extreme photoperiods or *T*-cycles (Aschoff et al., 1970; DeCoursey, 1972; Wever, 1972; Daan and Aschoff, 1975; Daan and Aschoff, 2001). Similarly, non-parametric models of entrainment also suggest that stability of entrained phase or accuracy is greater when the slope of the Phase Response Curves (PRCs are plotted as phase-shifts in response to zeitgeber pulses as a function of time of the day) is steeper or the amplitude of the PRC is greater (Pittendrigh and Daan, 1976a). As light is the most reliable zeitgeber in the daily environmental cycle for many organisms, accuracy of entrainment to external cycles may be dependent on the sensitivity of the circadian clock to light.

Light has several effects on the behavioural rhythms of activity which may act via the circadian clock or directly on the activity of organisms (Zeng et al., 1996; Lu et al., 2008).

Phase of the activity-rest rhythm may advance or delay in response to pulses of light and the 'phase-shifts' thus obtained are consistent measures of the sensitivity of the clock to light (Pittendrigh and Daan, 1976a; Johnson, 1999). The magnitude of phase-shifts is not only determined by the time of day at which the light pulse is presented but is also affected by the duration and intensity of light (which can be measured by a Dose Response Curve; Suri et al., 1998; Comas et al., 2006). In addition to its effects on phase, light is also thought to modify the period of the oscillator to match the external cycle in order to facilitate entrainment (Swade, 1969; Aschoff, 1979). The period changes seen under low intensity constant light (Daan and Pittendrigh, 1976b) are thought to be a consequence of this effect of light on the period. However, higher intensities of constant light induce splitting of the activity-rest rhythm into two or more components of different periodicities and arrhythmicity can occur above certain intensities (Yoshii et al., 2004; Rieger et al., 2006). Besides these effects via the circadian clock, light also directly affects activity levels by masking (Lu et al., 2008).

Light input to the *Drosophila* circadian clock has been shown to occur through three pathways, namely, the compound eyes and ocelli, intracellular cryptochrome in the circadian neurons and through the HB eyelets (Emery et al., 2000; Helfrich-Förster et al., 2001; Rieger et al., 2003). Cryptochrome (CRY) is a blue-light sensor present within circadian clock neurons that can induce the degradation of the core clock protein Timeless (TIM) upon activation by light (Stanewsky et al., 1998; Suri et al., 1998; Ceriani et al., 1999). Such CRY-mediated TIM degradation has been seen to be critical for phase-shifts in response to short-duration light pulses, especially in the delay zone (Emery et al., 1998; Suri et al., 1998; Busza et al., 2004). Furthermore, there are known to be dose-dependent effects of these light pulses which are also affected by CRY levels. Flies in which CRY was overexpressed

showed greater sensitivity to low light intensities (Emery et al., 1998; Emery et al., 2000) while those without CRY showed an inability to integrate duration of light pulses (Kistenpfennig et al., 2012). These results indicate that CRY is important for the phase-shifting effects of light in the early night, especially for low intensities of light and longer durations of light pulses. In contrast, the compound eyes are thought to be mainly involved in masking and entrainment to long photoperiods (Rieger et al., 2003), changes in period under constant light (Klarsfeld et al., 2004; Rieger et al., 2006), and modulation of activity patterns (Bachleitner et al., 2007). Although compound eyes also play a role in phase-shifts in response to light pulses and sensitivity to blue light, they are thought to be more important for entrainment to longer wavelength light cues (Helfrich-Förster et al., 2001; Hanai et al., 2008; Hanai and Ishida, 2009). Hence, these different light input pathways involving photoreceptors with different absorption spectra are involved in different aspects of light sensitivity of the circadian clock.

We studied the evolution of light sensitivity via different light input pathways in our populations that have evolved greater accuracy as a consequence of selection for narrow gate of adult emergence. Since light is the only daily time-cue available to these stocks which are maintained under LD 12:12 under otherwise constant conditions, we reasoned that these stocks would display enhanced light sensitivity in order to maintain a stable phase relationship with the light cue. Indeed, it had been previously shown that under low intensity light-dark cycles, amplitude of eclosion rhythms remained relatively robust in the selected stocks compared to control stocks (Kannan et al., 2012b). However, these differences may not necessarily be due to differences in light sensitivity and could be attributed to differences in amplitude of the circadian clock as well. This led us to ask if other aspects of light

sensitivity might have evolved in response to selection for accuracy. We examined entrainment of locomotor activity rhythms to *T*-cycles, dose response curves in terms of phase-shifts in response to light of different intensities and durations, and phase-shifts and entrainment to light of different wavelengths in both selected and control stocks. Entrainment to light cycles of periodicity different from the intrinsic period is largely determined by the amplitude of the PRC (Pittendrigh and Daan, 1976a). Hence, greater sensitivity to light pulses can result in entrainment to *T*-cycles further from the intrinsic period and may indicate differences in light sensitivity between selected and control stocks. In our previous studies we did not detect differences in the amplitude of the PRC (data not shown). We speculated that such lack of differences may be due to lack of detectable differences at the strength of the light pulse used (50 lux), due to saturating effects of light at higher intensities. Therefore, we examined phase-shifts in response to light pulses over a range of light intensities and durations. Moreover, the evolution of circadian photosensitivity may be achieved through different photosensory pathways which contribute to the sensitivity to light of different wavelengths. Hence, we examined responses to light pulses and entrainment under light of different spectral compositions to dissect the roles of different photoreceptor pathways which may be involved in the evolution of light sensitivity in the selected stocks. Effects of constant light on period could help determine whether continuous effects of light may have evolved in the selected stocks compared to the controls. Hence, we also examined period lengthening under constant light of different intensities on the locomotor activity rhythms of selected and control stocks. The results suggest that some aspects of light sensitivity have evolved in the selected stocks as a consequence of selection for greater accuracy of entrained eclosion rhythms. Selected stocks showed greater delay

phase-shifts in response to white light pulses in the early night at lower intensities and longer durations compared to control stocks. These stocks also showed greater phase-shifts in response to blue light pulses but not orange light pulses compared to control stocks although they showed greater overall levels of activity under orange light-dark cycles but not blue light-dark cycles. However, both stocks showed similar patterns of activity-rest behaviour under constant light, exhibiting arrhythmicity and splitting at moderately low levels of constant white and blue light, while showing similar amount of period lengthening under extremely low level of constant white light and moderately low levels of constant orange light. Thus, our study reports differences in light sensitivity in stocks selected for enhanced accuracy which may be attributed to certain photoreceptors or pathways of light input.

3.2 Materials and Methods

All assays were conducted on standardized flies from the four replicate populations each of selected (PP) and control (CP) stocks of fruit flies *Drosophila melanogaster*. The ancestry of the stocks, the protocol for maintaining the populations and standardization prior to experiments are described in detail in the second chapter. Progeny of standardized populations were collected upon emergence and virgin males were separated within 8 hours for all the assays.

3.2.1 *T*-cycle entrainment assay

Individual virgin males of age 3-4 days (n = 32 for each population for each *T*-cycle; n = 128 for each stock) were loaded into glass tubes (5 mm × 65 mm) with standard cornmeal medium at one end sealed with paraffin wax and a cotton plug at the other end and placed in Drosophila Activity Monitors. They experienced LD 12:12 in phase with rearing conditions

until ~ 24 hours after experimental setup when lights-on was either advanced or delayed for flies going into *T*20 and *T*28 regimes respectively. Subsequently, lights came-on and went-off at 10 or 14 hour intervals under *T*20 and *T*28 cycles, respectively. The flies were exposed to 14 cycles of 20-hour light-dark cycles and 10 cycles of 28-hour light-dark cycles both of which roughly constitute twelve 24-hour days. For the sake of consistency, in both cases, only 10 cycles of LD were used to calculate the period of the rhythm for assessing entrainment. White light of intensity ~ 1 lux was used during the light phase of the *T*-cycle. Lomb-Scargle periodogram analysis combined with visual inspection of actograms was used to determine whether flies that maintained a stable phase relationship as evaluated visually from actograms also exhibited a significant period within 0.5 hours of the external cycle's period (19.5-20.5 hours for *T*20 and 27.5-28.5 hours for *T*28). Such individuals were considered entrained and others were denoted as free-running. The proportion of flies showing the two phenotypes were calculated for each population (CP_{1-4} and PP_{1-4}) and compared between the selected and control stocks.

3.2.2 Dose Response Curve

Individual 3-4 day old virgin males (n = 24 for each population for each treatment) were entrained to 6 days of 12:12 hour light-dark (LD) cycles while their activity was being recorded using DAM system. The white light sources were LED strips that were locally sourced and produced cool white light consisting of wavelengths primarily around 450 nm, 550 nm and 600 nm. On day 7 they were transferred to constant dark (DD) conditions, and were given a light pulse at CT16 or CT20 (where CT0 or Circadian Time 0 is defined as the time of the start of the subjective day under constant conditions). 24 flies from each of the 8 populations (CP_{1-4} and CC_{1-4}) were used for each treatment along with disturbance controls.

The different treatments involved the administration of 15 minute light pulses of 0.01 lux, 0.1 lux, 1 lux, 10 lux or 100 lux for intensity dose response curve (DRC) or 1 lux light pulses of 15 minutes, 1 hour or 2 hours for duration DRC, where the light pulses were initiated at CT16 or CT20. Following the pulse, the flies were left undisturbed in constant darkness for about 10 days. The administration of the light pulse involved moving the Drosophila activity monitors from the location where they were experiencing LD cycles to boxes in which different light intensities for the pulse had been set. Thus, in panels a and b of figure 3.2, the protocol involved displacing the monitors from their original location, placing the monitors inside the respective boxes at CT16 or CT20 and then removing them from the boxes after 15 minutes and placing them in their original location. Meanwhile, the disturbance control monitors were lifted and replaced at their positions each time the experimental monitors were moved to the boxes and back so as to serve as unpulsed controls. The phase-shifts occurring as a response to these light pulses were then quantified to obtain a Dose Response Curve. These were estimated by determining the difference between the pre-pulse and the post-pulse phase of offsets of the rhythm of individual flies. The phase of offset of the activity rhythm was estimated by visually determining the daily offsets of the entrained rhythm under LD and those of the free-running rhythm after the pulse and extrapolating to the day of the light pulse. The difference between pre-pulse phase and post-pulse phase (phase-shift = pre-pulse phase – post-pulse phase) was calculated for individual flies and averaged across all 24 flies of each population. These population means were further averaged across all populations of control (CP) and selected stocks (PP) and compared.

3.2.3 Phase-shifts in response to blue and orange pulses

Phase-shifts were elicited from selected and control flies (n = 24 for each population for each treatment) at CT16 (early subjective night only) in a similar protocol as above with white light pulses being replaced by 15 minute blue and orange light pulses of two different energy levels (0.003 W/m^2 and 0.15 W/m^2) and compared between selected and control stocks. Lee filters (LEE filters worldwide, Hampshire, UK) which transmit light of wavelength 400-500 nm (Tokyo blue, 071) and > 530 nm (Deep Orange, 158) were used to administer blue and orange light pulses respectively. We selected these wavelengths of light based on the spectral sensitivity of cryptochrome relative to other photoreceptors in *Drosophila* such that the Deep Orange filter transmitted wavelengths that cryptochrome was not sensitive to (Buzsa et al., 2004; Van Vickle-Chavez and Van Gelder, 2007). We further adjusted the irradiance by using neutral density LEE filters of 90%, 70% and 50% strength to obtain the necessary levels for the different experiments. The analysis was carried out similar to the experiment described above and average phase-shifts for light pulse were compared between selected and control stocks.

3.2.4 Activity rhythms under blue and orange light-dark cycles

We also recorded 32 flies from each population under 12:12 hour light-dark cycles of blue and orange light. We used two intensities (0.01 W/m^2 and 0.15 W/m^2) of blue and orange light and compared the activity profiles of selected and control stocks under such light-dark cycles. The profile of activity of each individual fly over the course of 24 hours was averaged across 7 days and across 32 individuals for each population. Mean activity profiles for each population in 1 hour bins were subsequently averaged across the four populations of

selected and control stocks and compared. Total daily activity levels were also calculated and compared across stocks.

3.2.5 Activity patterns in constant light

For comparisons of period lengthening, four day old virgin males (n = 24 for each population for each light intensity) were recorded under constant dark for eight days before subjecting them to constant light of about 0.03 lux (0.00004 W/m^2), 0.05 lux (0.00007 W/m^2) or 0.1 lux (0.00015 W/m^2) intensity for about 15 days. Individual flies were identified as exhibiting rhythmic (single significant peak in the periodogram), complex (multiple significant peaks) or arrhythmic (no significant peak) patterns of activity based on analysis using Lomb-Scargle Periodogram. The proportions of such patterns of activity were averaged across the four populations for each stock and compared between the stocks. The period of each rhythmic fly was estimated using LS Periodogram during DD and LL and averaged across all individuals of a population. The population means of period under LL were averaged across the four populations of selected and control stocks and compared for each of the light intensities. Additionally, the difference between DD and LL period values for each individual was calculated and averaged across individuals within a population before population means were compared between selected and control stocks.

Similarly, activity was recorded under constant blue and orange light of 0.003 W/m^2 after allowing flies to free-run in constant dark for eight days. Under constant blue light, most individuals were arrhythmic and hence, we compared the proportion of arrhythmic individuals between selected and control stocks. Under constant orange light, we examined

the difference between DD and LL period values and analyzed the data similar to the constant white light experiment.

3.2.6 Statistical analyses

The proportion of individuals showing entrainment to *T*20 and *T*28 cycles were calculated for each population ($CP_{1\text{-}4}$ and $PP_{1\text{-}4}$) and these proportions were used as replicate values for selected and control stocks. These proportion values were subjected to one-way ANOVA with 'stock' (CP or PP) as fixed factor. For the dose response curve, phase-shifts for individual flies were averaged within a population for each light intensity or duration and these population means served as replicate values in a 2-way ANOVA with 'stock' and 'intensity' or 'duration' as fixed factors. ANOVA was performed separately for intensity and duration DRCs of advances and delays. For phase-shifts in response to blue and orange light pulses, one-way ANOVA was performed with population mean phase-shifts as replicate values and 'stock' as fixed factor. ANOVA was conducted separately for each of the energy levels and wavelengths of light on phase-shifts in response to blue and orange light pulses. For comparing activity profiles under blue and orange light-dark cycles, population mean activity profiles were used as replicate values for 2-way ANOVA with 'stock' and 'time-point' as fixed factors. One-way ANOVA was performed on total activity levels with 'stock' as a fixed factor. ANOVA was carried out separately for each of the treatments. For period lengthening under constant light, population mean period values or population mean differences in period (between DD and LL) were compared using one-way ANOVA with 'stock' as fixed factor. Post-hoc comparisons were carried out using Tukey's Honest Significant Difference (HSD) test. All analyses were carried out on STATISTICA for Windows Release 7.0.

3.3 Results

3.3.1 Entrainment of activity rhythms of selected and control stocks to *T*-cycles

Visual examination of actograms of individual flies for a stable phase relationship with the external cycle, along with periodogram analysis for period values within 0.5 hours of the external cycle period, revealed that more than half the flies showed entrainment to both *T*20 and *T*28 cycles in both the selected and control stocks (Figure 3.1a, b). ANOVA on proportion of flies entraining to *T*20 cycles revealed no significant effect of 'stock' ($F_{1,6}$ = 0.15; $p > 0.05$). Similarly, ANOVA on proportion of flies entraining to *T*28 cycles also did not reveal any statistically significant effect of 'stock' ($F_{1,6}$ = 0.08; $p > 0.05$) suggesting that selected and control stocks do not show differences in entrainability to *T*20 or *T*28 light-dark cycles (Figure 3.1).

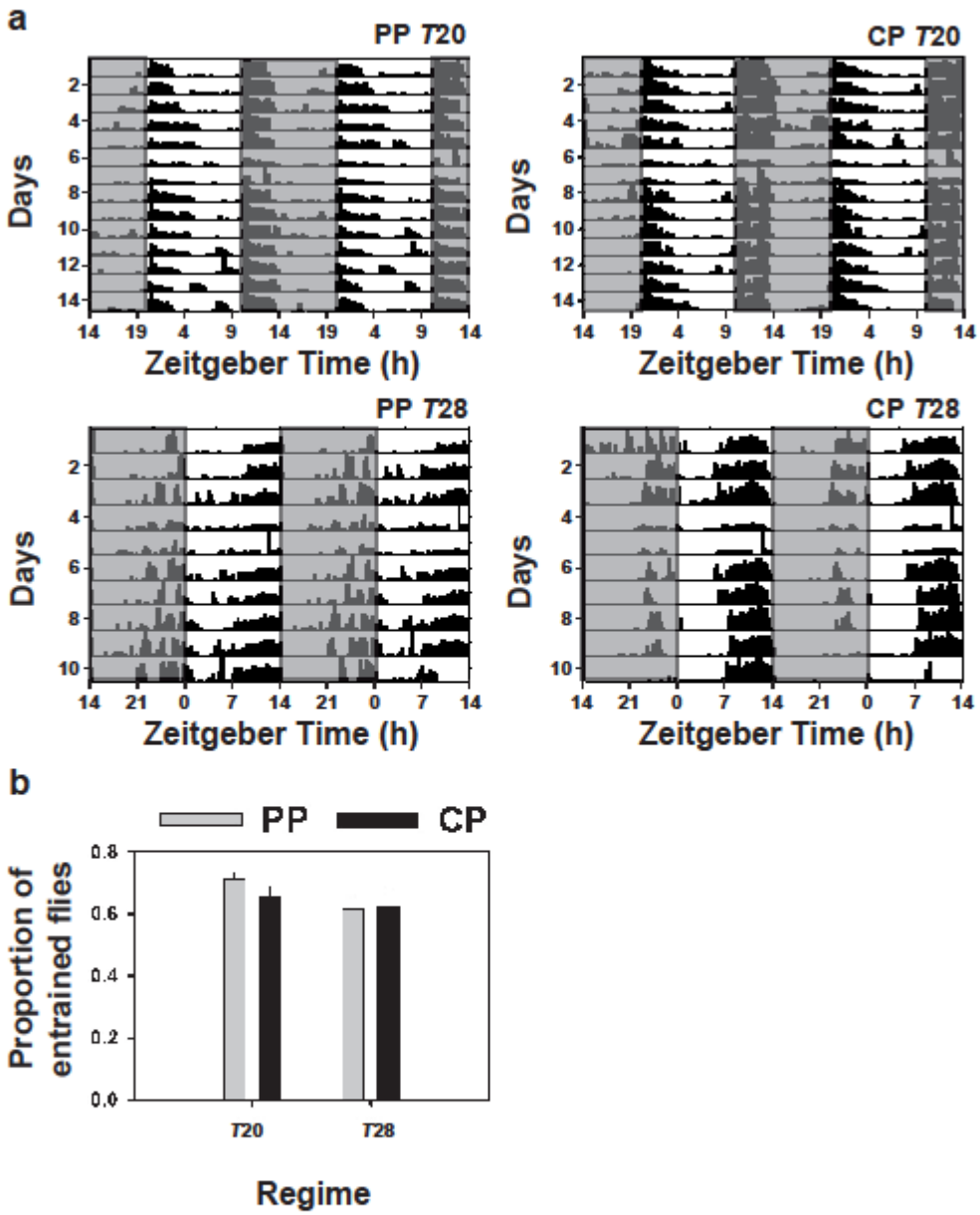

Figure 3.1. a) Entrainment of flies from selected and control stocks to non-24 hour light-dark cycles (*T*-cycles). Representative double-plotted actograms showing entrainment of selected (left panels) and control stocks (right) to *T*20 (top panels) and *T*28 cycles (bottom) respectively. Grey shading represents the dark phase of the *T* cycle. Black bars represent activity bouts of flies. b) Proportion of flies showing entrainment (period within 0.5 hours of the entraining cycle) under *T*20 and *T*28 cycles. Error bars are standard error of mean (SEM) across the four populations.

3.3.2 Dose-dependent phase-shift responses in selected and control stocks

Since previous studies on these selected stocks have suggested greater accuracy of entrainment (Kannan et al., 2012a) and higher accuracy may be associated with greater light-induced resetting of the clock (Pittendrigh and Daan, 1976a), we constructed a curve of the phase-shift responses to light of various intensities. We entrained the flies for 6 days to LD 12:12 before perturbing their rhythms with a light pulse at CT16 (or 4 hours after lights-off) where maximum phase delays are seen (Suri et al., 1998). We used 5 light intensities namely, 0.01, 0.1, 1, 10 and 100 lux along with a disturbance control which was kept in dark but subjected to the same disturbances that we subjected experimental flies to. ANOVA on phase delays with 'intensity' and 'stock' as fixed factors revealed significant main effect of both 'intensity' ($F_{5,36} = 74.15$; $p < 0.05$) and 'stock' ($F_{1,36} = 9.09$; $p < 0.05$) but no significant

interaction of 'intensity' × 'stock' ($F_{5,36} = 0.59$; $p > 0.05$). The selected stocks incurred higher mean phase-shifts at all intensities (Figure 3.2a). However, post-hoc comparisons using Tukey's HSD did not reveal any significant differences between selected and control stocks at any specific intensity. However, when phase-shifts at each light intensity were analyzed separately, a significant main effect of 'stock' was observed at 0.01 ($F_{1,6} = 7.49$; $p < 0.05$) and 100 lux ($F_{1,6} = 12.73$; $p < 0.05$) with post-hoc comparisons revealing that selected stocks show a significantly higher delay at both intensities ($p < 0.05$; Figure 3.2a). In contrast, when these flies were subjected to light pulses at CT20 (or 8 hours after lights-off) where maximum phase advances are seen, the selected stocks did not show greater phase-shifts compared to controls (Figure 3.2b). ANOVA on phase advances with 'intensity' and 'stock' as fixed factors revealed significant main effect of 'intensity' ($F_{4,30} = 98.37$; $p < 0.05$) but not 'stock' ($F_{1,30} = 3.17$; $p > 0.05$) or interaction of 'intensity' × 'stock' ($F_{4,30} = 1.56$; $p > 0.05$). This suggests that the selection for accurate phase of entrainment for emergence has resulted in evolution of higher circadian light sensitivity in the delay region of the PRC while the advance region appears to be unaffected.

Since circadian phase sensitivity to light may be assessed by differential zeitgeber strength in terms of both intensity and duration, we also assayed the phase-shifts incurred by flies in response to light pulses (1 lux) of different durations (15 minutes, 1 or 2 hours) at CT16 and CT20. ANOVA on phase delays with 'duration' and 'stock' as fixed factors revealed significant main effect of 'duration' ($F_{3,24} = 53.39$; $p < 0.05$), 'stock' ($F_{1,24} = 5.20$; $p < 0.05$) as well as interaction of 'duration' × 'stock' ($F_{3,24} = 10.64$; $p < 0.05$). Post-hoc comparisons using Tukey's HSD revealed significantly higher phase-shift in selected stocks subject to light pulses at CT16 only when the duration of the pulse was 2 hours ($p < 0.05$) with no

significant differences seen under pulses of shorter durations (Figure 3.2c). However, light pulses at CT20 of different durations failed to reveal differences between selected and control stocks (Figure 3.2d). ANOVA on phase advances with ‘duration’ and ‘stock’ as fixed factors revealed significant main effect of ‘duration’ ($F_{3,24} = 45.27$; $p < 0.05$), but not ‘stock’ ($F_{1,24} = 0.01$; $p > 0.05$) or interaction of ‘duration’ × ‘stock’ ($F_{3,24} = 0.53$; $p > 0.05$). Taken together, the results of dose response studies for both light intensity and duration indicate that selected stocks have evolved greater sensitivity to light during the early subjective night and this sensitivity results in phase delays of greater magnitude than controls.

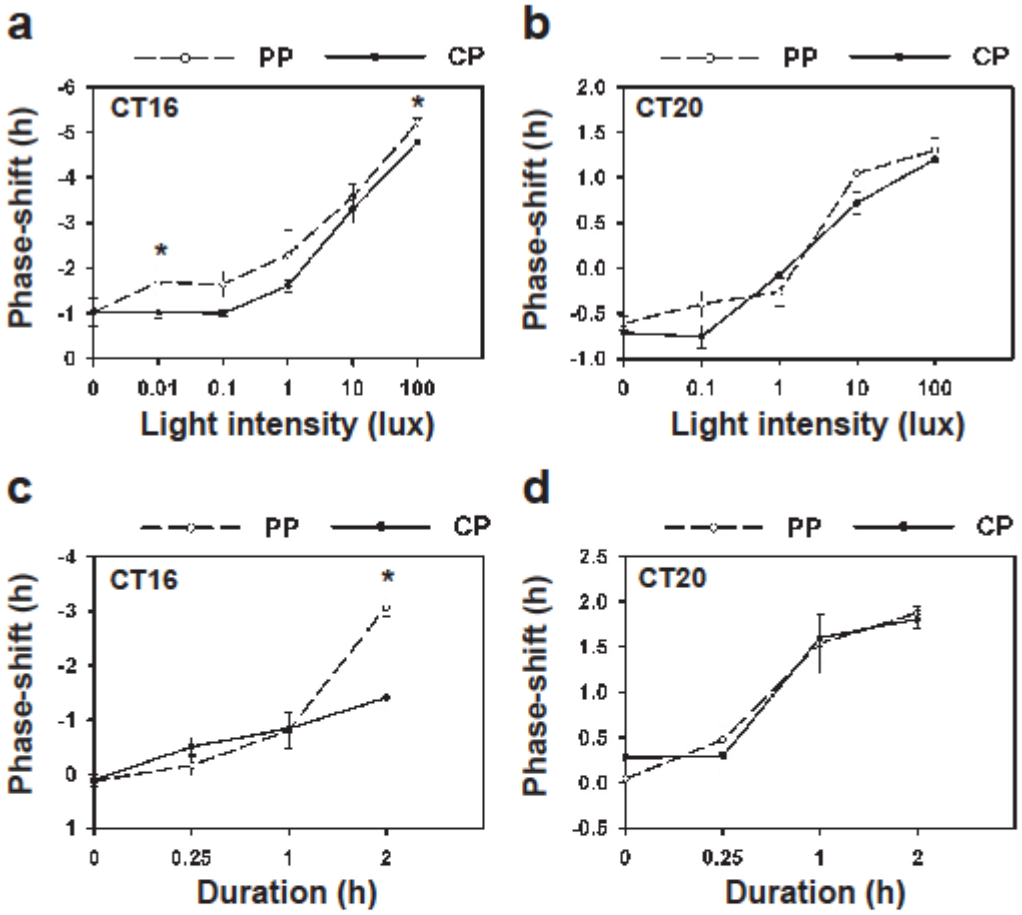

Figure 3.2. Mean phase-shifts of selected (dashed lines) and control (solid lines) stocks when subjected to light pulses at CT16 (delays; a and c) and CT20 (advances; b and d) of various intensities (a and b) or durations (c and d). Phase-shifts were calculated as the difference between pre-pulse and post-pulse phases of offsets of activity rhythm for individual flies and averaged across all individuals in a population. Population means are further averaged and plotted as mean phase-shifts for selected and control stocks. Error bars are SEM across the four populations. Asterisks indicate statistically significant differences.

3.3.3 Differential sensitivity of selected and control stocks to blue and orange light pulses

Since we found differences in magnitude of phase-shift only in the early subjective night which may be attributed to greater sensitivity to light in the selected stocks, we asked if there may be differences in photoreceptor properties that may have led to this difference. Since photoreceptors differ in their spectral sensitivity, we subjected selected and control stocks to blue and orange light pulses of two different energy levels (0.003 and 0.15 W/m^2) at CT16. ANOVA on phase-shifts in response to blue light pulses with 'stock' as fixed factor revealed significant effect of 'stock' at low energy ($F_{1,6} = 6.00$; $p < 0.05$) as well as at high energy ($F_{1,6} = 13.19$; $p < 0.05$). However, ANOVA on phase-shifts in response to orange light pulses with 'stock' as fixed factor revealed no significant effect of 'stock' at low energy ($F_{1,6} = 0.91$; $p > 0.05$) or high energy ($F_{1,6} = 1.45$; $p > 0.05$). Thus, selected stocks showed greater phase-shifts than controls when perturbed with blue light pulses of high and low intensity whereas orange pulses did not yield such differences (Figure 3.3). Hence, greater accuracy

of emergence rhythm may be achieved at least in part, by increase in blue light photosensitivity in the selected stocks, while responses to orange light pulses do not appear to have changed.

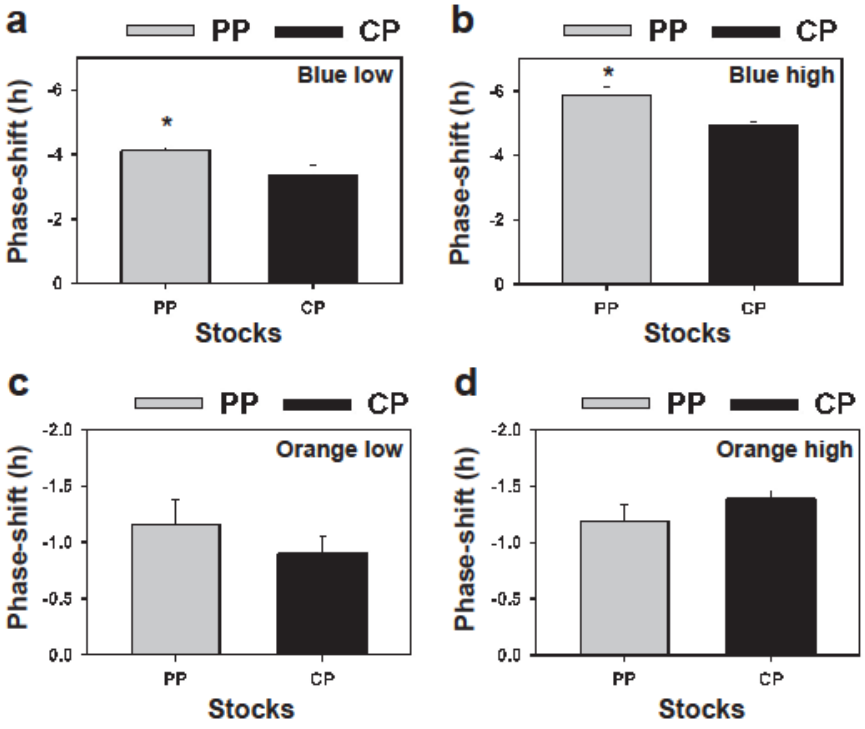

Figure 3.3. Mean phase-shifts exhibited by selected (grey bars) and control (black bars) stocks in response to 15 minute light pulses of a) blue light of 0.003 W/m², b) blue light of 0.15 W/m², c) orange light of 0.003 W/m², and d) orange light of 0.15 W/m² when presented at CT16. Rest of the details same as figure 3.2

3.3.4 Activity profiles of selected and control stocks under blue and orange light-dark cycles

Since the above results suggested differential spectral sensitivities of the underlying circadian clocks of the selected stocks to light pulses, we asked whether this may also be reflected as differences in their activity-rest rhythms when subjected to light-dark cycles where either light of blue or orange wavelength alone is provided during the light phase. For each wavelength of light, we examined rhythms under low energy as well as high energy light-dark cycles. Under low and high energy blue light-dark cycles, the activity profiles of selected and control flies did not show any difference with respect to each other (Figure 3.4a). ANOVA on the activity profile under low energy (0.01 W/m^2) blue LD cycle with 'stock' and 'time-point' as fixed factors revealed a significant effect of 'stock' ($F_{1,144} = 6.32$; $p < 0.05$) and 'time-point' ($F_{23,144} = 121.80$; $p < 0.05$) but no significant effect of 'stock' × 'time-point' interaction ($F_{23,144} = 0.76$; $p > 0.05$). However, ANOVA on the activity profile under high energy (0.15 W/m^2) blue LD cycle revealed a significant effect of 'time-point' ($F_{23,144}$= 130.58; $p < 0.05$) but no significant effect of 'stock' ($F_{1,144} = 2.33$; $p > 0.05$) or 'stock' × 'time-point' interaction ($F_{23,144} = 1.01$; $p > 0.05$). We also examined overall activity levels of selected and control stocks under these light-dark cycles. ANOVA on total daily activity with 'stock' as fixed factor revealed no significant effect of 'stock' at low energy ($F_{1,6} = 0.90$; $p > 0.05$) or high energy ($F_{1,6} = 0.25$; $p > 0.05$) blue light-dark cycles.

In contrast, activity levels of selected flies appeared to be greater than that of control flies under orange light-dark cycles. ANOVA on the activity profiles under low energy (0.01 W/m^2) orange LD cycle with 'stock' and 'time-point' as fixed factors revealed a significant effect of 'stock' ($F_{1,144} = 78.51$; $p < 0.05$) and 'time-point' ($F_{23,144} = 62.62$; $p < 0.05$) but no significant effect of 'stock' × 'time-point' interaction ($F_{23,144} = 0.56$; $p > 0.05$). ANOVA on the activity profile under high energy (0.15 W/m^2) orange LD cycle revealed a significant effect of 'stock' ($F_{1,144} = 162.57$; $p < 0.05$), 'time-point' ($F_{23,144} = 42.70$; $p < 0.05$) as well as 'stock' × 'time-point' interaction ($F_{23,144} = 1.85$; $p < 0.05$). ANOVA on total daily activity with 'stock' as fixed factor revealed a significant effect of 'stock' at low energy ($F_{1,6} = 9.71$; $p < 0.05$) and high energy ($F_{1,6} = 10.52$; $p < 0.05$) orange light-dark cycles with selected stocks showing greater activity compared to controls (Figure 3.4b). These results suggest that selected stocks show overall higher activity levels compared to control stocks under orange light-dark cycles but not under blue light-dark cycles (Figure 3.4).

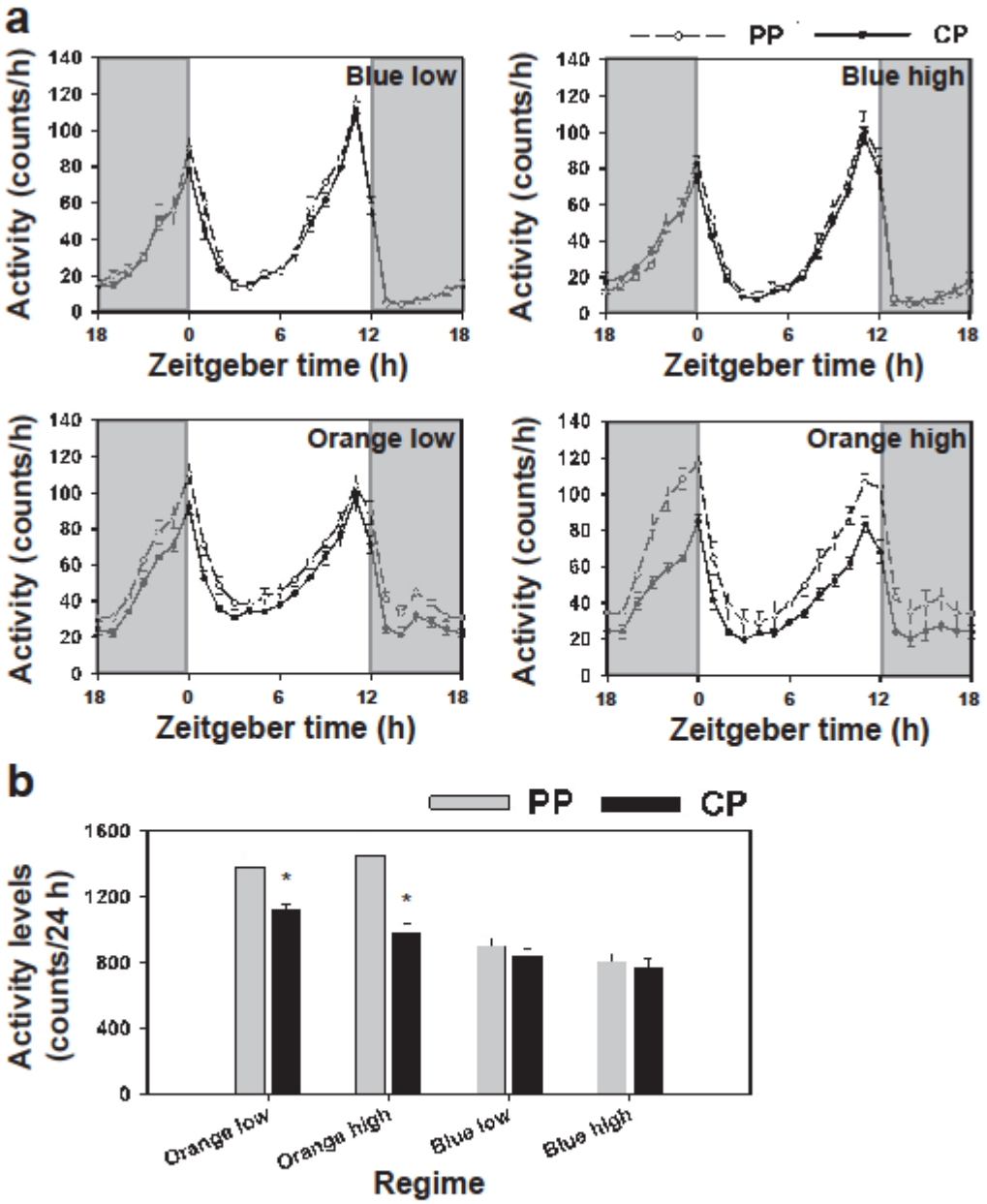

Figure 3.4. Average activity profiles in 1 hour bins of selected (dashed lines) and control stocks (solid lines) under light-dark cycles of blue light of 0.01 W/m² (top left panel), blue light of 0.15 W/m² (top right), orange light of 0.01 W/m² (bottom left), and orange light of 0.15 W/m² (bottom right). x-axis shows Zeitgeber Time (ZT) where ZT0 corresponds to time of lights-on and y-axis shows activity counts in 1 hour intervals. Grey shading represents dark phase of LD cycle. e) Average daily activity levels of flies from selected (grey bars) and control (black) stocks. x-axis denotes the LD cycle regime flies were maintained in. Error bars are SEM. Asterisks indicate statistically significant differences.

3.3.5 Period lengthening under very low intensity constant light

Since period responses are known to be important components of light sensitivity which may contribute to the accuracy of entrained rhythms (Beersma et al., 1999), we wished to determine whether selected stocks may show differences in such period responses. Since circadian rhythms are known to show lengthening of period under low intensity constant light which may reflect period responses to light, we examined the activity patterns of flies from selected and control stocks under constant white light of different intensities and estimated the proportion of flies showing rhythmic, complex or arrhythmic patterns of activity. Under white light of 0.1 lux intensity, most flies (> 50%) showed either complex rhythms or were rendered arrhythmic, hence, we did not estimate mean period values for this

intensity (Figure 3.5a). At 0.03 lux and 0.05 lux, most flies (> 70%) showed long period rhythms of around 25-26 hours periodicity in contrast to 23-24 hours periodicity seen under constant dark conditions (Figure 3.5a). ANOVA on period values with 'stock' and 'intensity' as fixed factors showed no effect of 'stock' ($F_{1,12} = 0.01$; $p > 0.05$) or 'intensity'($F_{1,12} = 0.001$; $p > 0.05$) or interactions between 'stock' × 'intensity' ($F_{1,12} = 0.43$; $p > 0.05$). Hence, the periods of the rhythms were not different between the two light intensities nor were they different between selected and control stocks.

We also subtracted the period of the respective flies under DD conditions from their period under constant light and compared the differences in period shown by the selected stocks compared to those seen in the controls (Figure 3.5b, c). ANOVA on differences in period values revealed no significant main effect of 'stock' at 0.03 lux ($F_{1,128} = 0.09$; $p > 0.05$) and 0.05 lux intensities ($F_{1,137} = 0.11$; $p > 0.05$; Figure 3.5b, c). Therefore, our results suggest that these stocks do not differ in terms of the continuous effects of light which lengthen the period of the rhythms.

Further, we also studied the effects of constant blue and orange light of 0.003 W/m^2. Under constant blue light, most flies from both stocks were arrhythmic. ANOVA on proportions of flies showing arrhythmicity revealed no significant effect of 'stock' ($F_{1,6} = 0.10$; $p > 0.05$; Figure 3.5d). Under constant orange light, most flies from both stocks continued to show rhythmicity but appeared to show a small change in period compared to constant dark conditions. The period value for each individual in constant dark was subtracted from the corresponding value for the same individual in constant orange light and this difference in period was averaged across all individuals within a population. This average difference in period was a positive value suggesting that flies show an increase in period under constant

orange light. ANOVA on mean difference in period did not reveal a significant effect of 'stock' ($F_{1,6} = 0.70$; $p > 0.05$) suggesting that the effect of constant orange light on period is not different between selected and control stocks (Figure 3.5e).

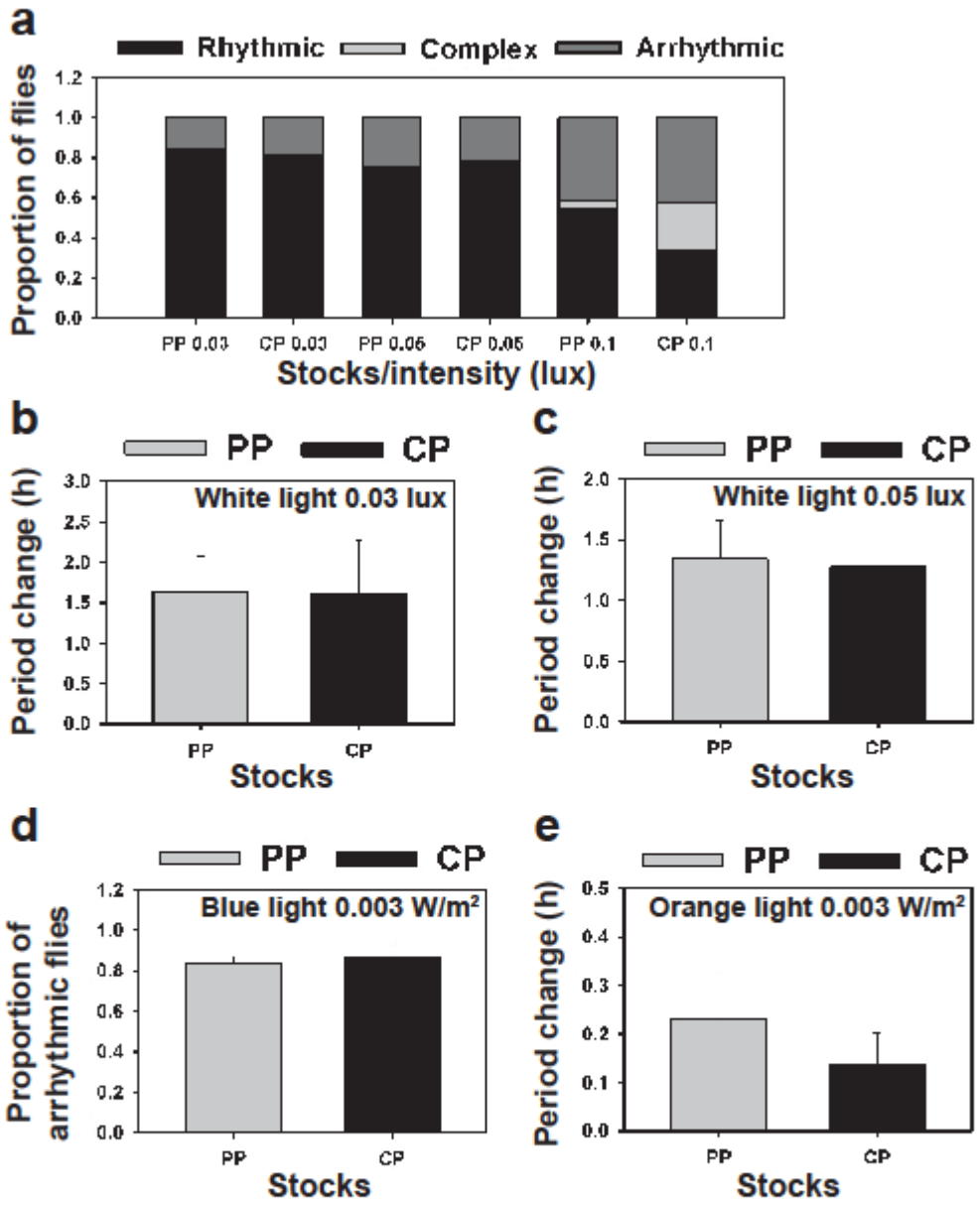

Figure 3.5. a) Average proportion of flies showing single periods (rhythmic), multiple periods (complex) and no significant periods (arrhythmic) under constant light of 0.03, 0.05 and 0.1 lux intensities. Mean difference between period under constant light of flies and constant dark when exposed to constant white light of b) 0.03 lux and c) 0.05 lux intensities. d) Average proportion of arrhythmic flies of selected and control stocks under constant blue light of 0.003 W/m² intensity e) Mean difference between period under constant orange light of 0.003 W/m² intensity and constant dark. Grey bars represent selected (PP) stocks and black bars represent control (CP) stocks in panels b-e. Error bars are SEM.

3.4 Discussion

The sensitivity of circadian clocks to light is an important determinant of the stability with which organisms are able to synchronize to external light cycles (Pittendrigh and Daan, 1976a). However, it is not clear what aspects of light sensitivity are critical for the accuracy of entrainment. In this study, we examined various features of light responsiveness of fly populations which have evolved greater accuracy of eclosion rhythms under light-dark cycles and compared them with controls. We observed that flies from the selected stocks showed no differences in ability to entrain to *T*20 or *T*28 regimes compared to controls (Figure 3.1). Entrainment of circadian rhythms to zeitgeber cycles is a function of intrinsic period of the organism, the period of the zeitgeber cycle and the strength of the zeitgeber (Pittendrigh and Daan, 1976a; Johnson et al., 2003). Since the intrinsic period of the selected and control stocks do not differ by more than half an hour (Kannan et al., 2012a), the ability to entrain to identical light-dark cycles of different lengths would be mainly determined by the sensitivity of the flies to light. Based on these results alone, the lack of differences between the two populations would suggest that selected and control stocks do not differ in terms of their light sensitivity. However, theoretical and experimental studies have postulated that entrainment ranges may also be determined by such factors as oscillator amplitude, inter-oscillator coupling and rigidity of the oscillatory system (Abraham et al., 2010). Therefore, the similarity in entrainment to light cycles of different periods does not rule out differences in light sensitivity that may have evolved in the selected stocks.

Since we had previously failed to detect significant differences in the shape of the PRC between selected and control populations and we had noted significant differences in eclosion profile under LD 12:12 mainly at low light intensities (Kannan et al., 2012b), we suspected that light sensitivity differences may be discernible only at specific intensities or durations of light pulses. The results of the dose response curve performed to elucidate such differences revealed significantly greater phase delays in the selected stocks in response to light pulses at CT16, at the lowest and the highest intensities tested, but no such differences were observed in phase advances to light pulses at CT20 (Figure 3.2a, b). Furthermore, in the selected stocks, light pulses of 2 hours evoked greater phase delays compared to controls but no such difference was seen for phase advances (Figure 3.2c, d). Previous studies on the role of cryptochrome in fly clock neurons have shown that overexpression of CRY can produce enhanced phase delays to low intensity light pulses (Emery et al., 1998; Emery et al., 2000). Cryptochrome has also been seen to be involved in the integration of duration of light pulses especially in the delay zone such that cryptochrome deficient flies do not show increases in magnitude of phase-shifts with greater durations of light pulses as much as wild-type flies (Kistenpfennig et al., 2012). Since the selected stocks in our study show both greater phase-shifts at lower intensities as well as longer durations, cryptochrome could be a possible candidate for the evolved differences in light sensitivity due to selection for accuracy of circadian rhythms. Hence, we examined the phase-shifts of selected and control stocks in response to pulses of blue light, which overlaps considerably with the spectral sensitivity of cryptochrome, and orange light which does not. Although blue light is also detected by rhodopsins present in compound eyes and ocelli, their contributions to the sensitivity of the circadian system to blue light are minimal (Fogle et al., 2011). Since

selection for accurate emergence rhythms appears to have given rise to evolution of differential spectral sensitivity such that clocks of the flies in the selected stocks are more sensitive to blue light pulses but not orange light (Figure 3.3), we propose that the observed differences in magnitude of phase-shifts in response to light pulses are mediated via changes in the light input pathway involving cryptochrome.

It may also be noted that the 0 intensity light pulse or the disturbance control does not produce phase-shifts of equal intensity. Similarly, one would expect the 15 minute light pulse in figure 3.2c to elicit similar phase-shift as the 1 lux pulse in figure 3.2a though this does not appear to be the case. The explanation for such differences may lie in the differences in protocol which are described in the materials and methods for the different dose response curve experiments. In case of figures 3.2c and 3.2d, the protocol involved moving the monitors into boxes with 1 lux intensity but with pulses set for different durations at CT16 or CT20. However, all the monitors (including those subjected to 15 minutes or 1 hour light pulses) were moved back from the boxes to their original location only after the completion of 2 hours. Disturbance controls were also subject to lifting and placing back at the same times as the experimentals with a gap of 2 hours in between. Hence, these disturbance controls (disturbed twice in an interval of 2 hours) show phase-shifts close to 0 which are different from the phase-shifts seen in disturbance controls of a and b panels (disturbed twice in an interval of 15 minutes). Interaction between phase-shifts due to light and mechanical disturbance have been shown in rodents and may explain the differences in the 15-minute 1-lux pulse phase-shifts seen in a and c or b and d, though such effects have not been characterized in flies since they are not as large or consistent.

However, phase-shifts in response to light pulses are not the only effects of light on the circadian system. Since parametric or continuous effects of light on the period may also be an important component of light sensitivity of circadian clocks and may significantly affect the accuracy of entrainment, we examined the effects of constant light of different intensities on the circadian rhythms of selected and control stocks. At lower intensities of constant light, the free-running period of both selected and control stocks showed lengthening though there was no significant difference in the magnitude of such effects between the stocks (Figure 3.5). Although these results suggest that such continuous effects of light may not evolve as a consequence of selection for greater accuracy in entrainment, we do find greater consolidation of phase-shifts in selected stocks in response to long duration light pulses (Figure 3.2c). Additionally, we would need to examine velocity response curves of these stocks to conclusively demonstrate lack of differences in parametric effects of light between these stocks. At higher intensities of constant light, a greater proportion of flies showed complex rhythms or arrhythmicity (Figure 3.5). While constant blue light also resulted in arrhythmicity, similar energy levels of orange light did not (Figure 3.5). These results suggest that the blue light component may be responsible for arrhythmicity under constant light, consistent with previous studies showing that cryptochrome mutant flies are rhythmic under high intensity constant light (Emery et al., 2000). Thus, sensitivity to blue and orange light may represent the functions of distinct photoreceptors.

Our studies to ascertain whether selected and control stocks show differences in entrainment to full photoperiods of blue or orange light-dark cycles indicated that flies from the selected populations show overall higher levels of activity under orange light-dark cycles but not blue light-dark cycles (Figure 3.4). Such direct effects of light on overall activity levels may be

mediated by the compound eyes (Tomioka et al., 1998; Bachleitner et al., 2007). Additionally, sensitivity to longer wavelengths of light such as orange light is known to be due to rhodopsins in the compound eyes, ocelli and HB eyelets (Hanai et al., 2008; Hanai and Ishida, 2009; Yamaguchi et al., 2010). Hence, it appears that the selected stocks have evolved altered sensitivity to light via such photoreceptors as well. However, there were no differences in entrainability to light of either wavelength between selected and control stocks or any differences in the period lengthening under constant light suggesting that these effects of light may be independent of the circadian clock. Although it is known that light can directly modulate activity levels of fruit flies (Rieger et al., 2007), it is not clear how such effects may contribute to the accuracy of entrainment. While period changes in response to light are thought to be important for higher accuracy under natural-like light profiles (Beersma et al., 1999), it is possible that masking or direct effects of light may also be effective in regulating phase of activity under square-wave light-dark cycles. For instance, it has been shown that negative masking effects can interact with the circadian pacemaker and determine chronotype in degus (*Octodon degus*; Vivanco et al., 2009). It has also been suggested that predictive (endogenous) and reactive (masking) mechanisms may be effective under different circumstances (Moore-Ede, 1986; Rietveld et al., 1993). While predictive mechanisms can anticipate environmental challenges and help the organism to prepare in advance, reactive mechanisms may be important in order to respond appropriately to change in environmental conditions (Moore-Ede, 1986). Since the selected stocks have been selected for emergence one hour after lights-on under square-wave light-dark cycles, a combination of circadian regulation via proper entrainment and direct effects of light may be effective in accurately determining the phase of entrainment.

In summary, *Drosophila melanogaster* populations that were selected for greater accuracy in timing of adult emergence were seen to have evolved greater light responsiveness in some respects. The selected flies showed higher delay phase-shifts in response to light pulses of certain intensities and durations compared to controls and these differences were especially pronounced when they were subjected to light pulses of wavelengths overlapping with the spectral sensitivity of cryptochrome. Although the selected and control stocks did not differ in their entrainability to light-dark cycles of different wavelengths or in terms of period changes under continuous light, selected stocks showed enhanced activity levels under orange light-dark cycles, which may be attributed to the compound eyes. These results suggest that selected stocks may have evolved greater light sensitivity in terms of both phase-shifts in response to instantaneous light pulses as well as greater clock-independent induction of activity by light. Thus, our results suggest that selection for narrow gate of adult emergence results in enhancement of light sensitivity via multiple light input pathways.

Contributions of developmental processes and circadian gating to evolution of accurate emergence rhythms

4.1 Introduction

Eclosion rhythms are thought to have evolved as a consequence of adaptive benefits conferred to insects by emerging in the early part of the day (Pittendrigh, 1954; Cloudsley-Thompson, 1960; Tanaka and Watari, 2009). If there is an adaptive benefit of emerging at a particular time of the day, it would be necessary for the circadian clock to accurately restrict the timing of emergence in order to maximize these benefits.

Accuracy of circadian phase or maintaining a stable phase relationship of circadian rhythms with respect to the external environment is recognized as an important function of the circadian clock and the relationship between accuracy and internal periodicity and phase and period-resetting has been explored theoretically (Pittendrigh and Daan, 1976a; Beersma et al., 1999) and discussed in the previous chapters. However, other factors independent of the circadian clock may also affect the accuracy of circadian phase of behavioural output. For instance, Pittendrigh and Daan (1976b) noted that the core circadian clock appeared to be more precise than the overt rhythm and suggested that variability in output processes is an important component of variability in phase of the rhythm. Hence, accuracy of circadian behaviours could possibly evolve by reducing variability of such output processes. Further, studies from intertidal midges have suggested that addition of a proximate cue can improve synchronization in emergence timing (Soong et al., 2006). Therefore, increased sensitivity to proximate cues can potentially enhance accuracy of circadian rhythms as well. However, the evolution of accuracy in circadian rhythms when subject to artificial selection has not been studied with the intention of examining which of these factors may contribute to enhancement of accuracy in natural populations.

Adult emergence in fruit flies, *Drosophila melanogaster* is a widely studied rhythm, the timing of which is determined by developmental state and circadian gating (Qiu and Hardin, 1996; Myers, 2003). There are several physiological processes which interact among each other and with the environment at various stages of development. Developmental rate itself is affected by larval density and internal clock period (Peters and Barbosa, 1977; Kyriacou et al., 1990) in addition to light and temperature (Bonnier, 1926; Paranjpe et al., 2005). Unlike some other insects such as *Anopheles gambiae* and *Triatoma infestans* in which egg hatching and pupation are rhythmic (Jones and Reiter, 1975; Lazzari., 1991), early developmental stages of fruit flies do not appear to be under circadian control (Pittendrigh and Skopik, 1970; Qiu and Hardin, 1996). Although circadian clocks appear to be operating early during development (Sehgal et al., 1992) and PDF-secreting circadian clock neurons appear in early larval stages (Helfrich-Förster, 1997), they assess developmental state for competence to eclose in the next available gate only around the time of wing pigmentation (Qiu and Hardin, 1996). Since clock neurons project to neurons involved in prothoracicotrophic hormone (PTTH) secretion (Zitnan et al., 1993) as well as neurons that synapse onto the prothoracic gland (PG; Siegmund and Korge, 2001) which are involved in assessment of growth (Mirth et al., 2005), the circadian clock may gate eclosion by regulating ecdysteroid production from the PG. Ecdysteroid production follows the decline of Juvenile Hormone (JH) levels and decreasing ecdysteroid level is an important cue for the initiation of eclosion behaviours in *Manduca sexta* (Truman, 1983). These eclosion behaviours are triggered by a hormonal cascade involving pre-ecdysis triggering hormone (PETH), ecdysis triggering hormone (ETH), eclosion hormone (EH) and crustacean cardioactive peptide (CCAP; Zitnan et al., 1996; Zitnan et al., 1999; Ewer et al., 1997; Gammie and Truman, 1997). The release of

CCAP triggered by EH is thought to be the final step in the ecdysis pathway which induces the ecdysis motor program (Gammie and Truman, 1997). While expression of LARK protein which is known to regulate neuronal excitability in CCAP neurons (Huang et al., 2009) exhibits circadian control and may regulate circadian gating of adult eclosion (McNeil et al., 1998; Zhang et al., 2000), the circuit connecting these cells to clock neurons remains uncharacterized. Hence, circadian clocks interact with neuroendocrine pathways at multiple levels and stages of development. In addition to these neuropeptidergic signals and developmental factors, eclosion can be directly induced by light input via the compound eyes and ocelli (McNabb and Truman, 2008). The lights-on signal may induce eclosion by stimulating release of EH or suppressing the inhibition of eclosion following EH release (McNabb and Truman, 2008). Thus, several developmental and physiological processes are involved in determining the timing of adult emergence in flies and can possibly contribute to variation in eclosion timing and therefore, accuracy of phase of eclosion rhythms.

The selected populations in our study had evolved greater proportion of emergence in the selection window (Kannan et al., 2012a) as well as lower day-to-day variability or enhanced accuracy in the timing of the peak of emergence (Kannan et al., 2012). Although some properties of core circadian clocks have been altered as a consequence of selection (Kannan et al., 2012a), it is not clear whether developmental factors independent of the clock determining timing of emergence also contribute to the phenotype of greater accuracy seen in these stocks. Hence, in this study, we examined various aspects of developmental processes such as timing of transition between early developmental stages, development time under constant external environments, effects of manipulation of developmental rate by varying larval density and masking effects of light on eclosion timing in selected and control stocks.

Reduction in variability in the timing of early developmental stages such as pupation and pigmentation which are not known to be under circadian control may directly result in reduced variability in eclosion timing. The influence of developmental rates on the eclosion profile of the two stocks can be further examined by assaying eclosion rhythms of both stocks under lower larval densities where development is faster. Furthermore, clock independent processes leading up to the final stage of eclosion may have innately lower variability in the selected stocks. This can be tested under constant light and dark conditions where the role of clock is absent or limited. Finally, accuracy may be improved simply by enhancing the sensitivity to a proximate cue such as light, which we tested by assaying the masking response of selected and control stocks to light. Our results did not find any reduction of inter-individual variation in timing of pupation or pigmentation. Further, variation in eclosion time under constant conditions was not lower in selected stocks compared to controls suggesting the necessity of circadian gating for higher accuracy of selected stocks. Additionally, selected stocks did not show significant differences in response to manipulations of developmental rate or timing of light cue with respect to controls though they continued to restrict their emergence to the duration of the circadian gate more effectively than controls under all circumstances. These results suggest that selected stocks have evolved greater accuracy of eclosion rhythms primarily due to enhanced gating of eclosion by the circadian clock and not due to accumulated reduction in variation at previous stages of development or differences in masking responses between the stocks.

4.2 Materials and methods

The study was conducted on four replicate populations each of selected (PP) and control (CP) stocks of fruit flies *Drosophila melanogaster*. The protocol for maintaining the populations and standardization prior to experiments are described in detail in the second chapter.

4.2.1 Pupation, pigmentation and eclosion assays

Standardized selected and control populations were given yeast plates for two days prior to the day of egg-collection to increase egg-laying. On the day of egg-collection, a cut-plate was given to all populations at ZT0 (time of lights-on) which was discarded and replaced by another cut-plate after 2 hours. The second cut-plate was withdrawn two hours later and individual eggs were transferred on to agar pieces.

Agar pieces with exactly 30 eggs each were then transferred to ten long vials (25 mm × 200 mm) with ~ 10 ml of food for each replicate population and maintained under LD 12:12. The duration of pupation and wing pigmentation was estimated by counting the number of pupae formed or pigmented every two hours. Pupation was identified by the formation of two spiracles on the head and pigmentation was recorded when wings were pigmented. Finally, the number of flies emerging every 2 hours was recorded to estimate the timing of eclosion. All handling and counting in the dark phase of the light-dark cycle were performed in the presence of dim far-red light.

4.2.2 Adult emergence at different larval densities

The waveform of emergence rhythm under LD 12:12 was estimated for the selected and control stocks at different larval densities. For these assays, eggs were collected from the

standardized populations and transferred into vials with ~ 10 ml of food at approximately 75, 150, 225 and 300 eggs per vial. Five such vials per replicate population for each density were maintained under LD 12:12. These vials were monitored till the onset of emergence and thereafter the number of flies emerging was counted every 2 hours for 4 consecutive days.

4.2.3 Development time under constant conditions

The development time assay under constant light was carried out using a similar protocol as that of the pupation, pigmentation eclosion assay under LD 12:12 described above with the exception that the vials with eggs were placed under constant light and only eclosing flies were counted. Vials were examined routinely for the start of emergence after which emerging flies were counted every 2 hours.

Similarly, development time assay was carried out under constant dark conditions and flies emerging every 2 hours were counted and recorded for estimating eclosion profile and mean development time.

4.2.4 Immediate responses to light of adult emergence

Eclosion assays were conducted with 15 vials for each replicate population with eggs collected from standardized selected and control stocks at a density of ~ 300 eggs per vial under three different regimes. Vials of all three regimes were placed under LD 12:12 till the day before emergence started. While one set of vials continued to remain in LD 12:12, two other sets of vials were removed from the LD 12:12 cubicle after the lights-off on the previous day. One set of vials was exposed to a regime wherein lights-on occurred one hour prior to the time of lights-on in the regular LD 12:12 under which they were maintained until

then, while the other set of vials was exposed to lights-on occurring one hour after the time of regular lights-on. Emergence was monitored every half an hour from ZT22 - ZT2 and subsequently, the total number of flies emerged at ZT20 were counted to normalize the emergence profile by the total number of flies emerging in the day. Emergence under respective regimes was monitored over 3 days with different sets of vials used every day.

4.2.5 Statistical analyses

Pupation profiles with 2 hour resolution were estimated for each vial and averaged across ten vials for each replicate population. These population mean profiles were then averaged across four replicate populations for selected and control stocks and plotted with standard error of mean across the four populations as error bars. Mean pupation time for each replicate population was also calculated and these population means were used as replicate values for one-way ANOVA with 'stock' as fixed factor. Variation in pupation time was also estimated for each replicate population by calculating the standard deviation in pupation time across all individuals and was analyzed similar to the mean. Similarly, pigmentation profiles, mean pigmentation time and variation in pigmentation time were estimated for selected and control stocks and compared. Eclosion profiles were calculated across 2 days and plotted similar to pupation and pigmentation profiles. Mean eclosion time was calculated for all flies emerging over two days and compared using ANOVA with 'stock' as fixed factor. However, variation in eclosion time was calculated only for the second day of emergence since there were very few numbers of flies emerging on the first day. Eclosion profiles, mean eclosion time and variation in eclosion time under constant dark and constant light conditions were analyzed similar to pupation and pigmentation time.

For eclosion assays with different larval densities, eclosion profiles were calculated for each vial and averaged across 5 vials for each replicate population at each larval density. These population mean profiles were further averaged across four replicate populations to obtain average profiles for selected and control stocks and plotted with SEM as error bars. Further, % emergence in the 6 hour window prior to lights-on (ZT18 - ZT0) was calculated as this part of the profile was the most variable across densities. ANOVA was performed on % emergence before lights-on with 'density' and 'stock' as fixed factors. The phase of peak of adult emergence was also recorded as the time at which maximum emergence was observed on a given day for a particular vial. This phase of emergence peak was averaged across days and across all vials within a population and subsequently averaged across four replicate populations for each stock and plotted. ANOVA with 'density' and stock' as fixed factors was performed on phase of emergence peak.

For eclosion assays to study the direct effect of light on emergence, half hour emergence profiles were calculated from ZT22 – ZT2. The % emergence in each half hour interval was calculated as a fraction of the total emergence in a day for a particular vial and averaged across all vials within a population and subsequently, averaged across the four populations and plotted. ANOVA with 'stock' and 'time-point' as fixed factors was performed separately for each regime.

All post-hoc comparisons were done using Tukey's Honest Significant Difference. All statistical analyses were performed on STATISTICA 7.0.

4.3 Results

4.3.1 Pupation, pigmentation and emergence of selected and control stocks under LD 12:12

We assayed the timing of transitions between developmental stages such as pupation and wing pigmentation that are not known to be under clock control in *Drosophila*, to determine whether the selected stocks have evolved differences in developmental rate (Figures 4.1a-f). We found no significant change in the pupation profile between selected stocks and control stocks (Figure 4.1a). Whereas maximum pupation was seen in control stocks at ~ 106 hours after egg-collection, pupation peak was seen at ~ 112 hours for the selected stocks (Figure 4.1a). However, ANOVA on the mean pupation time of selected and control stocks revealed no main effect of 'stock' ($F_{1,6} = 5.32$; $p > 0.05$; Figure 4.1c). Additionally, ANOVA on the standard deviation in pupation time revealed no significant effect of 'stock' ($F_{1,3} = 2.73$; $p > 0.05$; Figure 4.1e). These results suggest that pupation profile of selected and control stocks are largely similar and the mean pupation time and variation between individuals are not significantly different between selected and control stocks (Figure 4.1a, c, e).

We also recorded the time that these pupae started showing wing pigmentation and found that the timing of wing pigmentation was largely similar in the selected and control stocks. The peak of wing pigmentation was at ~ 190 hours after egg-collection in the selected stocks while the peak was at ~ 192 hours in the controls (Figure 4.1b). These results suggest that wing pigmentation time is marginally advanced in the selected stocks with respect to the controls. ANOVA on the mean pigmentation time revealed no significant main effect of 'stock' ($F_{1,6} = 2.1$; $p > 0.05$; Figure 4.1d). However, ANOVA on the standard deviation in

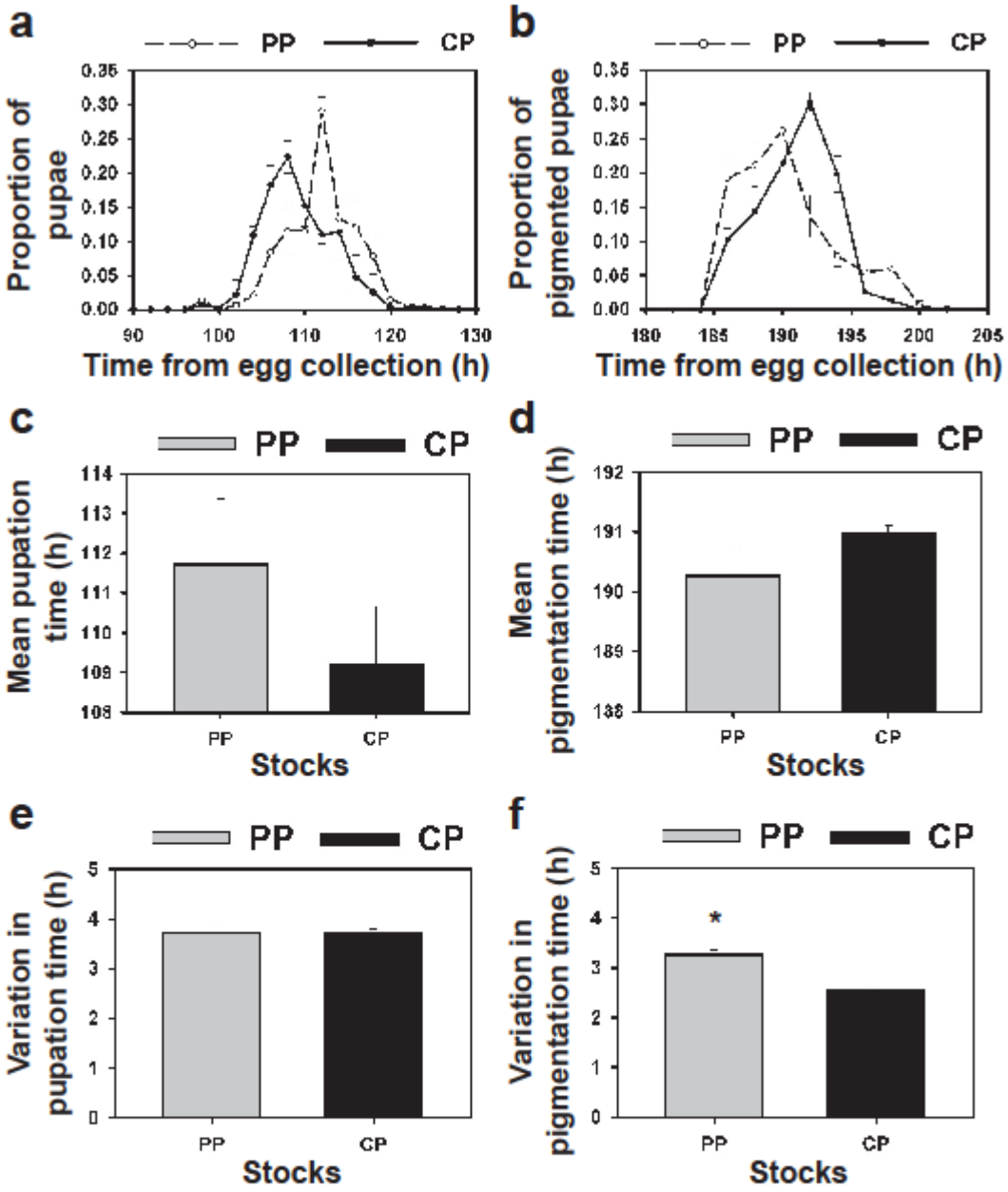

Figure 4.1. a) Proportion of larvae that pupated in every 2 hour interval measured from the time of egg collection in selected (PP) and control (CP) stocks under LD 12:12. b) Proportion of pupae that showed wing pigmentation in every 2 hour interval under LD 12:12. c) Mean pupation time of selected and control stocks under LD 12:12. d) Mean pigmentation time of selected and control stocks under LD 12:12. e) Variation in pupation time estimated by the standard deviation in pupation time across all individuals of selected and control stocks. f) Variation in pigmentation time across all individuals of selected and control stocks. Error bars are SEM. Asterisks denote statistically significant differences.

pigmentation time revealed a significant effect of 'stock' ($F_{1,6}$ = 16.16; p < 0.05). Post-hoc comparisons using Tukey's HSD revealed that selected stocks showed higher standard deviation in pigmentation time compared to control stocks (p < 0.05; Figure 4.1d). Hence, it appears that the selected stocks are not different in their mean pigmentation time but show

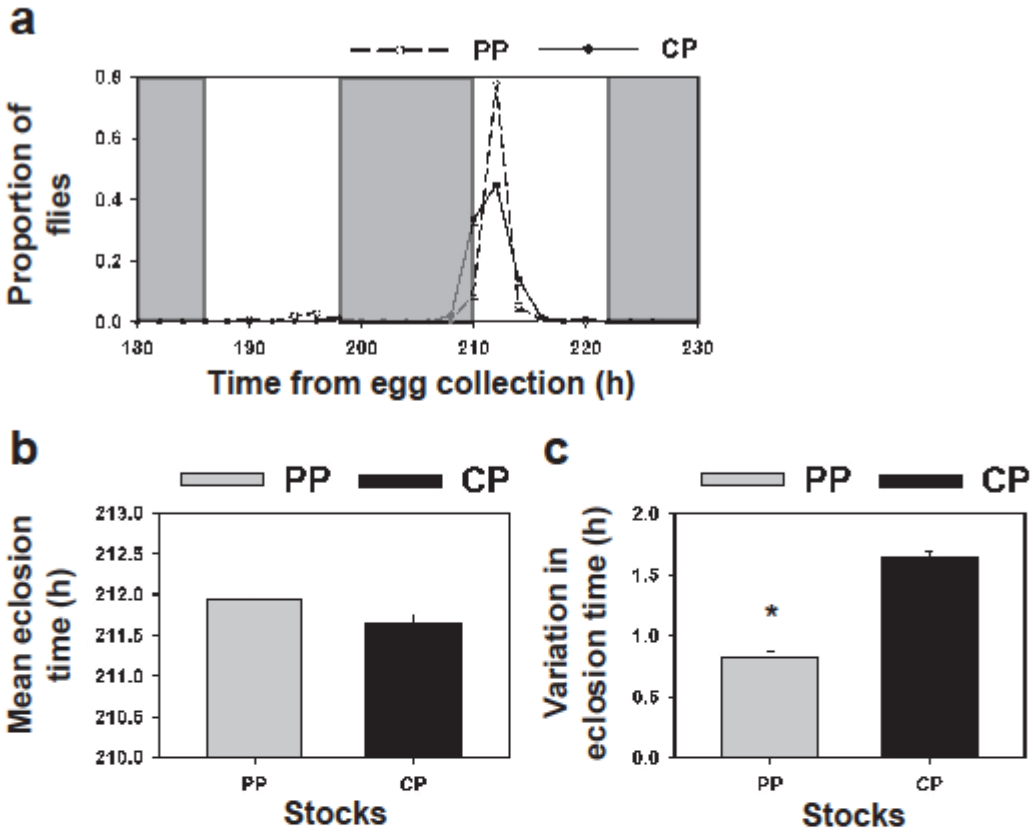

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Figure 4.2. a) Proportion of adult flies that emerged in every 2 hour interval measured from the time of egg collection in selected (PP) and control (CP) stocks under LD 12:12. Grey shading represents dark phase. b) Mean eclosion time of selected and control stocks under LD 12:12. c) Variation in eclosion time estimated by the standard deviation in eclosion time across all individuals of selected and control stocks emerging on second day of emergence. Error bars are SEM. Asterisks denote statistically significant differences.

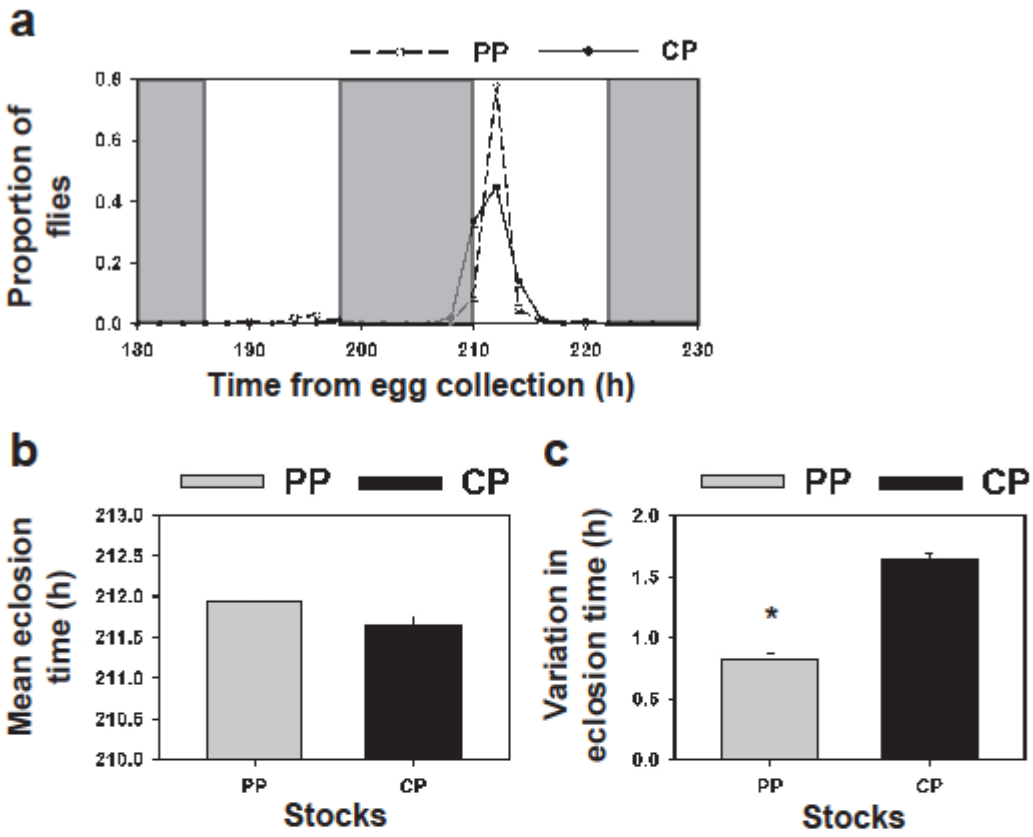

Figure 4.2. a) Proportion of adult flies that emerged in every 2 hour interval measured from the time of egg collection in selected (PP) and control (CP) stocks under LD 12:12. Grey shading represents dark phase. b) Mean eclosion time of selected and control stocks under LD 12:12. c) Variation in eclosion time estimated by the standard deviation in eclosion time across all individuals of selected and control stocks emerging on second day of emergence. Error bars are SEM. Asterisks denote statistically significant differences.

4.3.2 Adult emergence profiles of selected and control stocks at different larval densities

There was a clear effect of larval density on the adult emergence profile of both stocks (Figure 4.3a, b). The emergence prior to lights-on was greatly enhanced at lower densities in the control stocks (Figure 4.3b). Although selected stocks also had greater emergence at ZT0 at lower densities in contrast to the minimal emergence at ZT0 at higher densities, there was still much lower emergence seen before lights-on compared to the control stocks at these densities (Figure 4.3a). ANOVA on the percentage emergence in the second half of the night (6 hours before lights-on) revealed a significant main effect of 'density' ($F_{3,24} = 24.44$; $p < 0.05$), 'stock' ($F_{1,24} = 137.14$; $p < 0.05$) as well as 'stock' × 'density' interaction ($F_{3,24} = 5.53$; $p < 0.05$). Post-hoc comparisons using Tukey's HSD revealed significantly higher emergence before lights-on at 75 and 150 eggs/vial compared to 225 and 300 eggs/vial in control stocks ($p < 0.05$; Figure 4.3c). However, only emergence at 75 eggs/vial was lower than that at 225 and 300 eggs/vial in the selected stocks ($p < 0.05$; Figure 4.3c) suggesting that these stocks do not respond as much to changes in larval density as the controls. Moreover, the selected stocks showed lower emergence before lights-on at 75, 150 and 225 eggs/vial compared to control stocks ($p < 0.05$; Figure 4.3c). Additionally, ANOVA on the phase of peak of adult emergence in selected and control stocks across different densities revealed significant effect of 'density' ($F_{3,24} = 15.19$; $p < 0.05$), 'stock' ($F_{1,24} = 85.87$; $p < 0.05$) as well as 'stock' × 'density' interaction ($F_{3,24} = 15.19$; $p < 0.05$). Post-hoc comparisons of phase of emergence peak showed that the peak of emergence in selected stocks (which occurred at ZT2 at all densities) was significantly different from that of control stocks which occurred earlier at 75, 150 and 225 eggs/vial ($p < 0.05$; Figure 4.3d). Additionally, emergence peak of control stocks at 300 eggs/vial were significantly different

from the peak of control stocks at all other densities ($p < 0.05$; Figure 4.3d) while no such variation was seen in selected stocks across densities. Hence, the adult emergence profiles and timing of emergence peak of selected stocks are relatively more robust to changes in larval densities as compared to controls.

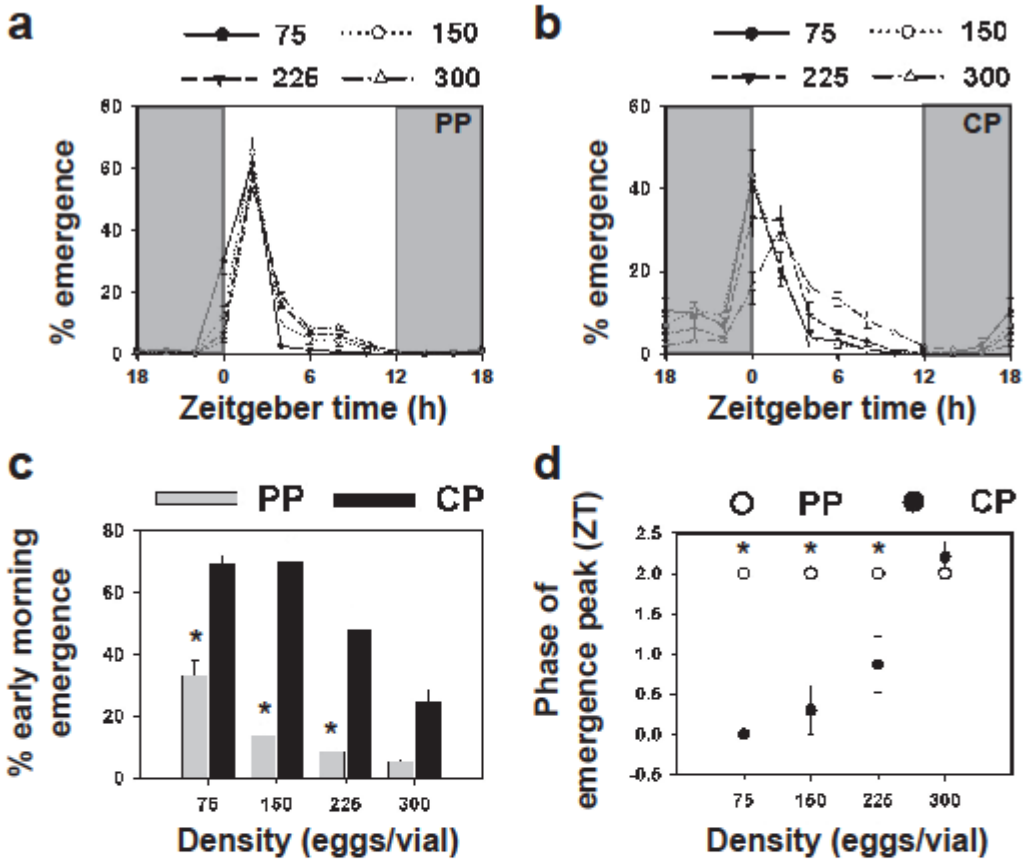

Figure 4.3. Adult emergence profiles in 2 hour intervals of a) selected (PP) and b) control (CP) stocks at different egg densities (eggs/vial) under LD 12:12. Grey shading represents the dark phase. c) Percentage emergence in the early morning (or late night; ZT18 - ZT0) as a fraction of total emergence in the day. Black bars indicate selected populations and grey bars indicate controls. d) Phase of peak of adult emergence (expressed in Zeitgeber Time) as a function of egg densities in selected (open circles) and control stocks (filled circles). Error bars are SEM. Asterisks denote statistically significant differences.

4.3.3 Adult emergence of selected and control stocks under constant light and constant dark conditions

We also assayed the development time of selected and control stocks under constant conditions of light or darkness to assess clock-independent development in selected and control stocks (Figure 4.4a-f). Under constant light (LL) conditions where the *Drosophila* clock is rendered arrhythmic, the mean development time of control and selected stocks was

~ 170 hours with no apparent difference between the stocks in the mean or variation in emergence time (Figure 4.4a, c, e). ANOVA on the mean emergence time in LL revealed no significant main effect of 'stock' ($F_{1,6} = 0.4$; $p > 0.05$). Similarly, ANOVA on the standard deviation in emergence time revealed no significant effect of 'stock' ($F_{1,6} = 0.37$; $p > 0.05$). This suggests that the differences in the emergence profile of selected and control stocks seen in LD are due to differences in the effects of gating by the circadian clock, rather than due to differences in the developmental rate.

We also assayed the development time of selected and control stocks under constant dark (DD) conditions where the *Drosophila* clock is rhythmic, though individual flies are not in synchrony with one another due to lack of a synchronizing time cue from the egg stage. The mean development time of control and selected stocks was ~ 220 hours with some differences in the development time profiles (Figure 4.4b, d). ANOVA on the mean

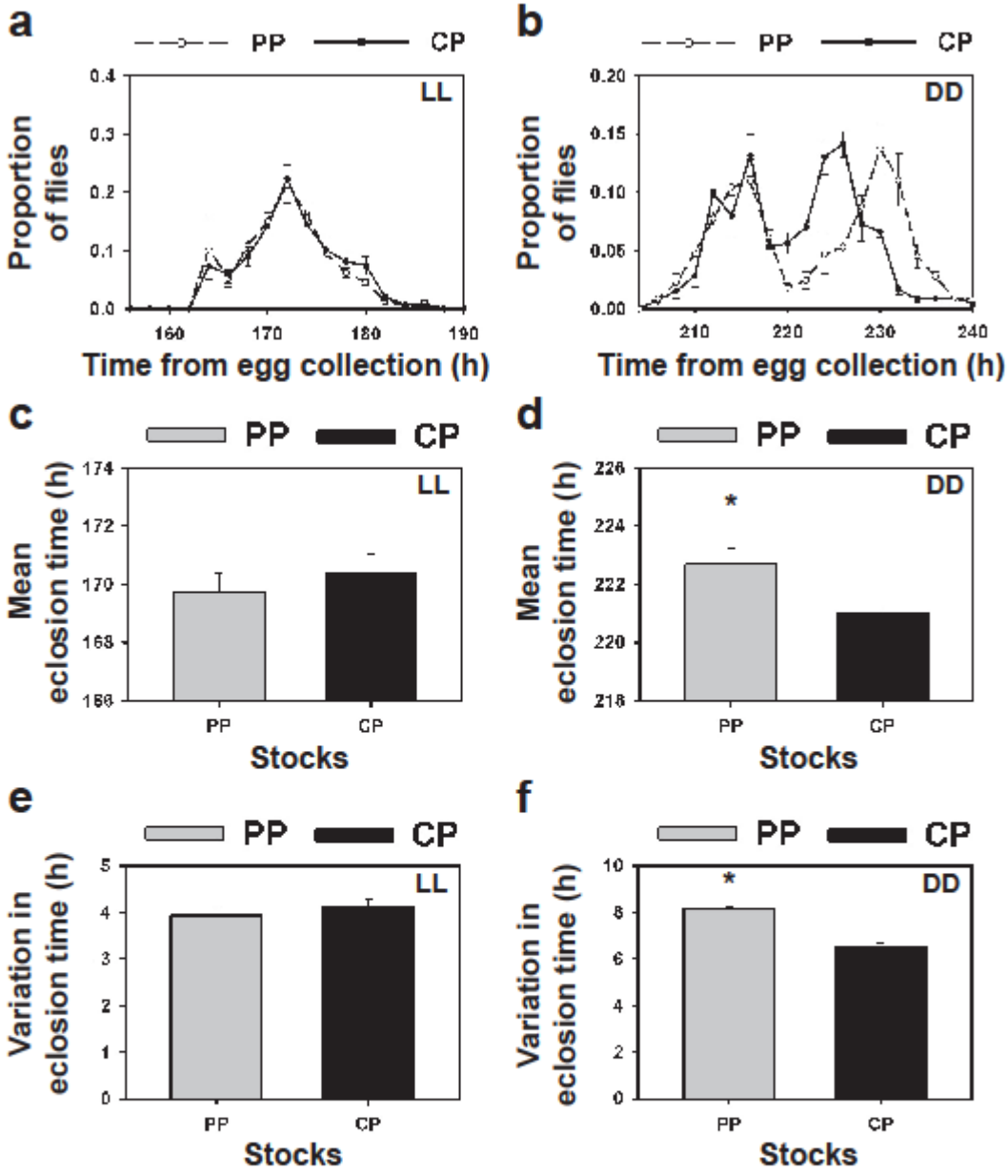

Figure 4.4. a) Proportion of flies emerging in every 2 hour interval measured from the time of egg collection in selected (PP) and control (CP) stocks under constant light (LL). b) Proportion of flies emerging in every 2 hour interval under constant darkness (DD). c) Mean eclosion time of selected and control stocks under LL. d) Mean eclosion time of selected and control stocks under DD. e) Variation in eclosion time estimated by the standard deviation in eclosion time across all individuals of selected and control stocks under LL. f) Variation in eclosion time across all individuals of selected and control stocks under DD. Grey bars represent selected stocks and black bars represent controls. Error bars are SEM. Asterisks denote statistically significant differences.

emergence time in DD revealed a significant main effect of 'stock' ($F_{1,6}$ = 83.68; p < 0.05). Post-hoc comparisons revealed that selected stocks showed significantly greater mean emergence time compared to controls (p < 0.05; Figure 4.4b). Similarly, ANOVA on the standard deviation in emergence time also revealed a significant effect of 'stock' ($F_{1,6}$ = 33.67; p < 0.05). Post-hoc comparisons revealed that selected stocks showed significantly greater standard deviation in emergence time compared to controls (p < 0.05; Figure 4.4f). Hence, selected stocks show significantly greater variation in emergence time compared to controls under constant dark conditions in contrast to reduced variation seen under LD cycles. Therefore, the differences in the emergence profile and variation in emergence time of selected and control stocks seen in LD are probably due to differences in the effects of gating under LD cycles, rather than due to differences in the clock-independent developmental rate or developmental differences with free-running clocks.

4.3.4 Immediate response of adult emergence to advance or delay of lights-on in selected and control stocks

In order to study the immediate responses to light, we subjected the pupae to advance or delay of lights-on by 1 hour on the day of emergence with respect to the time of regular lights-on experienced during development. We assayed the % emergence of flies from ZT22 - ZT2 in bins of half hour each for selected and control stocks under the advance, delay and regular LD regimes. Under normal timing of lights-on (Figure 4.5, middle panel), we see that the maximum emergence in the control stocks is seen at the time-points of ZT0.5 and ZT1 whereas the peak of adult emergence in the selected stocks lies at ZT2. ANOVA on % emergence of selected and control stocks revealed a significant main effect of 'time-point' ($F_{11,72}$ = 212.45; p < 0.05), and significant effect of 'stock' × 'time-point' interaction ($F_{11,72}$ =

4.46; $p < 0.05$). Post-hoc comparisons using Tukey's HSD revealed significantly greater emergence at ZT0.5 and ZT1 and significantly lower emergence at ZT2 in control stocks compared to selected stocks ($p < 0.05$; Figure 4.5, middle panel).

When the lights-on is advanced by 1 hour (Figure 4.5, top panel), the control stocks respond by increasing their emergence at ZT23.5 and ZT0 (where ZT0 is taken as the time of regular lights-on to which the flies are entrained during development and not the time of advanced lights-on on the day of emergence). The peak emergence of selected stocks is also advanced due to advance in the timing of lights-on but not to the extent seen in the control stocks and the % emergence before the regular lights-on remains negligible (Figure 4.5, top panel). ANOVA on % emergence of selected and control stocks revealed a significant main effect of 'time-point' ($F_{11,72} = 253.34$; $p < 0.05$), and significant effect of 'stock' × 'time-point' interaction ($F_{11,72} = 17.5$; $p < 0.05$). Post-hoc comparisons using Tukey's HSD revealed significantly greater emergence at ZT23.5 and ZT0 and significantly lower emergence at ZT0.5 and ZT1 in control stocks compared to selected stocks ($p < 0.05$; Figure 4.5, top panel). This indicates that flies from the selected stocks do not respond to advance in lights-on as much as the control stocks and do not emerge outside the gate of their circadian rhythm which is entrained to the regular light-dark cycle experienced during development.

In contrast, when lights-on was delayed, both control and selected stocks peaked immediately after the delayed lights-on occurred. However, the peak of emergence was greater in the selected stocks compared to the controls (Figure 4.5, bottom panel). ANOVA on % emergence of selected and control stocks revealed a significant main effect of 'time-point' ($F_{11,72} = 224.16$; $p < 0.05$), and significant effect of 'stock' × 'time-point' interaction ($F_{11,72} = 11.24$; $p < 0.05$). Post-hoc comparisons using Tukey's HSD revealed significantly greater

emergence at ZT0 and ZT0.5 and significantly lower emergence at ZT1.5 in control stocks compared to selected stocks ($p < 0.05$; Figure 4.5, bottom panel). These results suggest that the selected stocks do not differ in their immediate response to light; rather they are more tightly gated by the circadian clock as they do not emerge outside their gate despite the masking effects of light when lights-on is advanced.

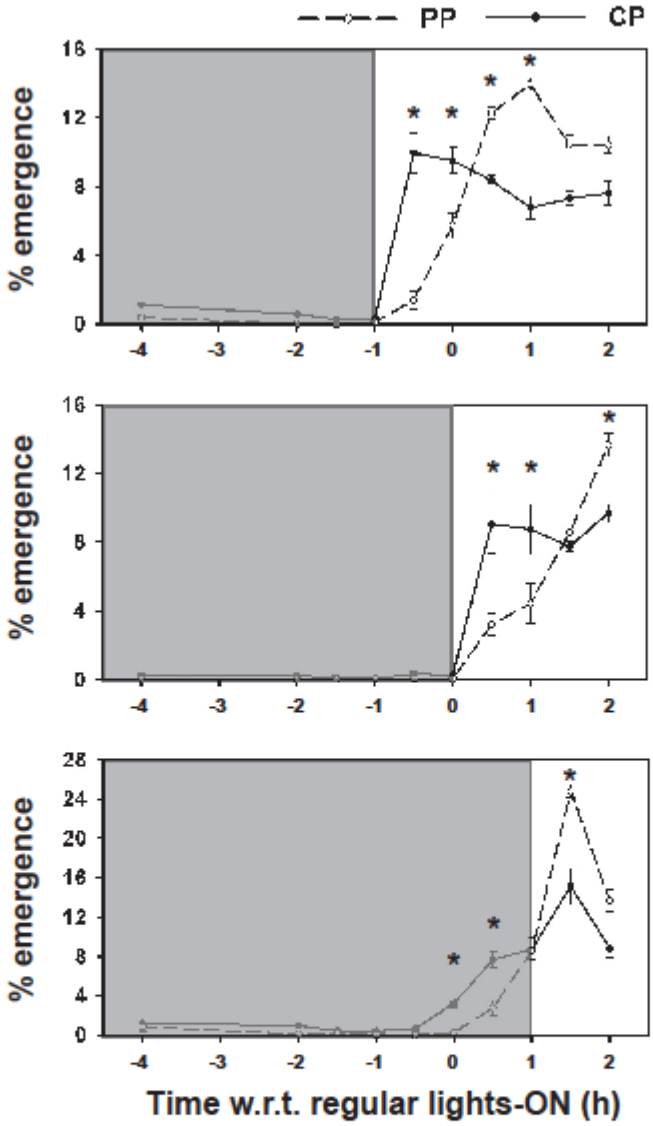

Figure 4.5. Percentage of emergence in half-hour intervals from 2 hours before regular lights-on in selected (dashed lines) and control stocks (solid lines) when timing of lights-on is advanced (top panel) by 1 hour relative to time of regular lights-on (taken as ZT0 for all regimes), occurs at the regular time of lights-on (middle panel), or is delayed by 1 hour relative to time of regular lights-on (lower panel). Lights-on occurred at the regular time during the entire development of the flies and was advanced or delayed only on the day of emergence. Grey shading represents the time when the flies experience darkness on the day of emergence. Error bars are SEM. Asterisks denote statistically significant differences.

4.4 Discussion

The evolution of accuracy of timing of circadian behaviours has been the subject of considerable interest (Pittendrigh and Daan, 1976a; Pittendrigh, 1981b; Beersma et al., 1999; Sharma, 2003). While there is some evidence for evolution of clock properties as a consequence of selection for accuracy of circadian rhythms (Kannan et al., 2012a; and see chapters 2 and 3), it is not clear how circadian clocks interact with clock-independent physiological processes to enhance accuracy of behavioural output. Since it had been previously suggested that downstream output processes may contribute substantially to variation in overt rhythms (Pittendrigh and Daan, 1976b), we examined the evolution of developmental processes independent of the circadian clock that may affect eclosion timing in *Drosophila* populations selected for enhanced accuracy of emergence rhythm.

While it is known that fruit flies *Drosophila melanogaster* do not show circadian rhythms of pupation and pigmentation (Pittendrigh and Skopik, 1970), reduced variation between individuals in timing of transitions between these developmental stages could potentially reduce the variation in timing of emergence independent of circadian clock control. However, the selected stocks which show reduced variation in eclosion timing under LD 12:12 do not show any changes in the mean duration to pupation and pigmentation nor is the

variation in timing among individuals reduced (Figures 4.1 and 4.2) compared to controls. Hence, the mechanisms underlying enhanced accuracy in emergence time seen in selected stocks are likely to be restricted to advanced stages of development post pigmentation.

Although early development does not appear different between selected and control stocks, eclosion processes independent of circadian clock control could still potentially result in reduced variation in eclosion time under LD 12:12. To test this possibility, we examined overall development time from egg collection to eclosion in selected and control stocks when reared under constant conditions. While the circadian clock is rendered dysfunctional under constant light (Marrus et al., 1996), individual flies still possess functional circadian clocks under constant darkness though they are out of synchrony with one another due to the absence of an external synchronizing cue (Sehgal et al., 1992). Under constant light conditions, selected stocks did not show differences in mean eclosion time or variation in eclosion time relative to controls (Figure 4.4). These results suggest that functional circadian clocks are necessary for the lower variability in eclosion time seen in the selected stocks. Under constant dark conditions, selected stocks exhibited significantly greater mean eclosion time compared to controls as well as greater variation in eclosion time among individuals (Figure 4.4). These results indicate that presence of functional circadian clock is insufficient to result in reduced variation in eclosion time in the selected stocks compared to controls and entrainment to external cycle is necessary for the gating effects of circadian clocks to result in reduced inter-individual variation. Although previous results had indicated that synchrony (inverse of inter-individual variation of circadian period) of circadian clocks was greater in selected stocks due to narrower distribution of free-running periods across individuals (Kannan et al., 2012a), this synchrony does not appear to translate to lower variability in

eclosion time under constant darkness. Thus, entrainment of circadian clocks to an external cycle is necessary for reduction in variation in eclosion time in selected stocks.

In order to further substantiate evidence from these experiments that the differences in eclosion profiles between selected and control stocks are not due to developmental rate differences, we examined the eclosion profiles of both stocks while manipulating developmental rates by varying larval densities. Under greater larval densities, development of flies is known to be significantly delayed (Peters and Barbosa, 1977) with variance in development time also possibly being a function of larval crowding (Mukherjee et al., 2012). Hence, we assayed and compared eclosion profiles of selected and control stocks at different larval densities. While control stocks showed greater early morning emergence prior to lights-on, at lower larval densities, selected stocks continued to restrict their emergence to the light phase even at low larval densities (Figure 4.3). Furthermore, the phase of emergence peak was remarkably consistent in the selected stocks across all densities compared to control stocks, which showed earlier phases at lower densities (Figure 4.3). These results indicate that the emergence profiles of selected stocks are robust across manipulations of larval density. Larval crowding results in delayed production of juvenile hormone esterase (JHE) and delayed release of ecdysteroids, both of which are important for timing of metamorphosis in *Tribolium freeman* (Hirashima et al., 1995). If such effects of crowding are similar in *Drosophila*, then we may conclude that the timing of release of ecdysteroids and the degradation of JH by JHE may not be critical to the enhanced accuracy of eclosion time in the selected stocks. Thus, the greater accuracy of eclosion timing in the selected stocks is largely independent of such developmental processes unconnected to the circadian clock.

Aside from developmental processes leading up to eclosion, eclosion timing can be affected by masking responses due to the direct induction of eclosion hormone release and subsequent ecdysis by light (McNabb and Truman, 2008). Such masking effects of light involve a photoreception pathway independent of circadian entrainment to light-dark cycles and act in addition to the gating of eclosion by the circadian clock (McNabb and Truman, 2008). Since the selected stocks had been subject to selection for a narrow window of emergence that starts 1 hour after lights-on (Kannan et al., 2012a), these stocks could potentially enhance accuracy of eclosion by evolving greater masking response to light in addition to stronger circadian gating of eclosion. However, our experiments did not reveal any enhancement of the effects of lights-on in the selected stocks compared to controls (Figure 4.5). While the control stocks showed masking response to light when lights-on was advanced, such effects were minimal on the selected stocks (Figure 4.5). Since the advanced lights-on was 1 hour earlier than the time at which circadian gate would be opened in the selected stocks, the relative absence of masking due to light at this time suggests that circadian gating in these stocks is stronger than the direct effects of light. In contrast, both selected and control stocks showed masking response to light when the lights-on was delayed by an hour (Figure 4.5). Thus, the lack of masking response to advanced lights-on in the selected stocks is probably not due to reduction in the direct effects of light, rather it may be due to the greater restriction of eclosion by circadian gating. Therefore, these experiments also support our previous results by demonstrating the dominant role of circadian gating in enhancing the accuracy of eclosion in the selected stocks relative to developmental processes independent of the circadian clock.

Overall, our results demonstrate that various factors independent of the circadian clock that possibly influence timing of adult emergence, such as developmental rate in pupal stages and under constant conditions, differences in larval densities and masking responses to light do not significantly influence or contribute to the phenotype of enhanced accuracy in timing of emergence observed in the selected stocks. Hence, we may conclude that the increase in accuracy of eclosion of selected stocks is primarily the consequence of more stringent gating of eclosion by the circadian clock.

Correlated responses to selection for accuracy of circadian rhythms on life history traits

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

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5.1 Introduction

The stability of circadian time-keeping in the face of fluctuating internal as well as external environments is likely to confer organisms with adaptive advantages (Daan, 2000). Therefore, accuracy of circadian clocks, which is key to their functioning as reliable time-keepers, is believed to be under selection pressures in the environment (Sharma and Chandrashekaran, 1999; Clodong et al., 2007). While accuracy or precision of circadian clocks in assessing time in the local environment is thought to be closely related to fitness, no studies have examined the adaptive significance of such accuracy in natural populations. Laboratory selection for accurate timing of circadian rhythms in large, outbred populations may be a suitable strategy to study the adaptive value of accuracy (Abhilash and Sharma, 2016).

In large populations at equilibrium, traits directly related to fitness such as growth rates, adult lifespan and fecundity, often bear negative genetic correlations (trade-offs) with one another (Roff, 1996). Such trade-offs are interpreted to be due to pleiotropic alleles that influence two or more components of fitness (Rose and Charlesworth, 1981a). While positively pleiotropic alleles either get quickly fixed (if they increase fitness of both traits) or lost (if they are deleterious), antagonistically pleiotropic alleles persist at intermediate frequencies in a population under the influence of balancing selection (Connallon and Clark, 2013; Barton and Keightley, 2002). Therefore, correlated responses in fitness traits to selection for clock precision is likely to suggest the existence of pleiotropic effects of genes influencing both traits (Reznick, 1985), and negative correlations would indicate the cost of possessing stable clocks on fitness (Roff, 1996).

The duration of pre-adult development and circadian periodicity are reported to be positively correlated in clock mutant flies (Kyriacou et al., 1990) and in wild-type fruit flies (Kumar et al., 2007; Yadav and Sharma, 2013; Takahashi et al., 2013), suggesting the association of circadian clocks with developmental processes (Saunders, 2002). Circadian clocks have also been implicated in the regulation of reproductive fitness in *D. melanogaster*; males carrying a loss of function mutation in the core clock gene *period* (*per*) release fewer sperms causing reduction in the fecundity of females (Beaver et al., 2002; Beaver et al., 2003). Nevertheless, we should be cautious in drawing inferences from studies on life history traits in inbred populations, as they often yield spurious correlations between traits (Rose and Charlesworth, 1981a). Other studies on the relationship between circadian clocks and fitness have manipulated environmental regimes and examined the effects on fitness traits. These studies have reported a tendency for greater longevity in resonating environments (regimes with periods matching that of internal clock periods; Pittendrigh and Minis, 1972; von Saint Paul and Aschoff, 1978) though *period* mutants showed reduced adult lifespan compared to the wild-type flies even under LD cycles of period close to their intrinsic period (Klarsfeld and Rouyer, 1998). Under LL, mean lifespan of flies was shorter than that in LD or DD, although flies laid more eggs in LL than the other two regimes (Sheeba et al., 2000). Hence, there seems to be some fitness advantage for being rhythmic in terms of extended lifespan (Kumar et al., 2005), although this cannot be generalized for overall fitness. Although previous studies suggest the role of circadian clocks in the regulation of fitness of organisms, the genetic basis for such phenotypic correlations is still unclear.

In the present study, we compared fitness traits of selected and control stocks to assess correlated responses to selection for accuracy of circadian rhythms on life history traits. We

assayed pre-adult (development time and pre-adult survivorship) and adult (lifespan and lifetime fecundity) fitness traits of fly populations subjected to selection for narrow gate of emergence to examine their fitness, and asked if a correlation exists between the timing of emergence and these life history traits. We chose flies emerging within the selection window in the morning (henceforth the morning window) to represent the adaptive mean phenotype, and flies emerging in an evening window (henceforth the evening window) to represent the extreme non-adaptive phenotype, to examine correlations in adult life history traits that may reveal the consequences of stabilizing selection for the mean timing of emergence. We found that inter-individual variation in pre-adult development time was reduced in the selected stocks without any cost to its pre-adult fitness. Mated males from the selected stocks live as long as the controls, while mated females have significantly shorter lifespan compared to the controls. Morning emerging mated males from the selected stocks live as long as their evening emerging counterparts. On the other hand, morning emerging females have reduced adult lifespan and higher mid-life fecundity than those emerging in the evening, suggesting higher reproductive fitness in the morning emerging flies, and a trade-off between reproduction and lifespan. Interestingly, such correlations between life history traits and timing of adult emergence are not seen in flies from the control stocks. These results suggest that stabilizing selection for narrow gate of emergence in the morning results in reduced adult lifespan and enhanced reproductive output in morning emerging females revealing sex-specific changes in life history in response to selection.

5.2 Materials and methods

The protocol for maintenance and standardization of populations used in the present study has been described in the second chapter.

5.2.1 Development time and survivorship assays

After ~ 90 generations of selection, the pre-adult development time and survivorship of flies from the selected and control stocks were assessed. From each standardized population, eggs laid during a 2 hour window (ZT1 - ZT3) were collected and exactly 30 eggs were dispensed into each long vial (18 cm height × 2.4 cm diameter) containing ~ 6 ml food medium. For the assays, ten such vials from each replicate population were maintained under LD 12:12. To estimate pre-adult development time and survivorship, adults were collected every 2 hours and counted similar to the protocol described in the previous chapter. The development time of a fly, in hours, was calculated as the time interval between the mid-point of the 2 hour egg collection window and the mid-point of the 2 hour interval during which the fly emerged as adult. For the analysis on time of emergence, percentage of flies emerging every 2 hours was calculated. Pre-adult survivorship was estimated as the fraction of eggs, in each vial, that successfully developed and emerged as adults. The gate-width of emergence was taken as duration between the onset of emergence (first 2 hour window in the day showing greater than 5% emergence) and the offset of emergence (last 2 hour window showing greater than 5% emergence). The threshold of 5% has been used as a standard cut-off for measuring gate-width in previous studies on these stocks as well as other flies (Kannan et al., 2012a; Prabhakaran et al., 2013).

5.2.2 Adult lifespan assay

Adult lifespan of flies from the selected and control stocks was assessed after ~ 100 generations of selection. From the standardized populations of selected and control stocks, eggs were collected and ~300 eggs were transferred into glass vials (18 cm height × 2.4 cm diameter) containing ~ 10 ml of food medium. From among the progeny of the standardized populations, we collected flies which emerged during the selection (ZT1 - ZT2: morning or M) and evening windows (ZT10 - ZT11: evening or E) to represent the mean and extreme emergence phenotypes, respectively. For the adult lifespan assay of mated flies, 4 males and 4 females were introduced in each vial and adult lifespan of twenty such vials for each replicate population were monitored until all flies in all the vials died. Flies were provided with fresh food medium every alternate day and vials were checked every day for dead flies. The overall sample size of the study was n = 1739 flies including roughly equal numbers of males and females from the selected and control stocks.

5.2.3 Fecundity assay

After ~ 100 generations of selection, fecundity of flies from the selected and control stocks was assayed to examine if there was any effect of selection on the reproductive fitness. From the progeny of standardized populations we collected flies, which emerged during the morning (ZT1 - ZT2) and evening windows (ZT10 - ZT11), similar to the lifespan assay. From these two sets of flies (morning and evening emerging), males and females were introduced in pairs into glass vials containing ~ 3 ml of food medium. Twenty such vials from each population were maintained under LD 12:12. The number of eggs laid every day post-emergence until the day of death of the female was counted to assess lifetime fecundity

of the fly. During the fecundity assay, flies were transferred into fresh food vials every day and the number of eggs laid on the previous day was recorded. The duration of average lifespan was divided into equal windows of 12 days based on the intervals of age with distinct patterns of egg-laying to compare across selected and control stocks and the two emergence windows. Thus the age of females was divided into early (1–12 days), mid (13–24 days) and late-life stages (25–36 days). Since very few flies lived until the last life stage (37–48 days) and number of eggs laid at this stage was very low, we did not include this age-window in the analyses. Overall, the sample size of the fecundity assay was n = 187 flies with roughly equal numbers of flies from selected and control stocks.

5.2.4 Statistical analyses

Adult emergence profiles were calculated by estimating the % of flies emerging at each time-point and averaging across vials and then populations. Variation in development time was analyzed separately using analysis of variance (ANOVA) treating 'stock' as fixed factor. For the adult lifespan assay, 'stock', 'sex' and 'emergence window' were treated as fixed factors. All the analyses for adult lifespan were performed on natural log transformed values of the individual lifespan data, since the adult lifespan data have a long right-hand tail for which mean value is not an appropriate measure for comparison. Repeated measures ANOVA was used for the lifetime fecundity data with 'stock' and 'emergence window' as fixed factors and daily number of eggs as the repeated measure across 'age-windows'.

Post hoc multiple comparisons were done using Tukey's test. The error bars used in the figures are standard error of mean (SEM). All our analyses were implemented on STATISTICA 7.0.

5.3 Results

5.3.1 Pre-adult fitness traits in selected and control stocks

To determine the effect of selection for narrow gate of adult emergence on pre-adult fitness traits we assayed pre-adult development time and survivorship of the selected and control stocks. While development time under LD 12:12 has also been assayed in the previous chapter, we report the results of this assay which was conducted after similar number of generations of selection as other life history traits, since such traits may evolve across generations. Adult emergence profiles of selected and control stocks were largely similar apart from the higher peak of emergence seen in the selected stocks on the second day (Figure 5.1a). However, the pre-adult development time (Figure 5.1b) and pre-adult survivorship (Figure 5.1c) of the selected and control stocks did not differ. The gate-width of emergence of the selected and control stocks also did not differ under these conditions of low larval density (Figure 5.1d).

Although ANOVA on the pre-adult development time data showed no statistically significant effect of 'stock', ANOVA on inter-individual variance in development time showed a statistically significant effect of 'stock' ($F_{1,3} = 29.73$; $p < 0.01$; Figure 5.1b, e). Post-hoc multiple comparisons using Tukey's test revealed that although the mean development time of the selected and control stocks did not differ, selected flies showed reduced variation in development time compared to the controls (Figure 5.1e; $p < 0.05$), similar to that seen in the previous chapter. These results suggest that flies selected for narrow gate of emergence do not differ in their pre-adult development time, but have become more coherent in their emergence time compared to the controls without incurring any cost to their pre-adult fitness.

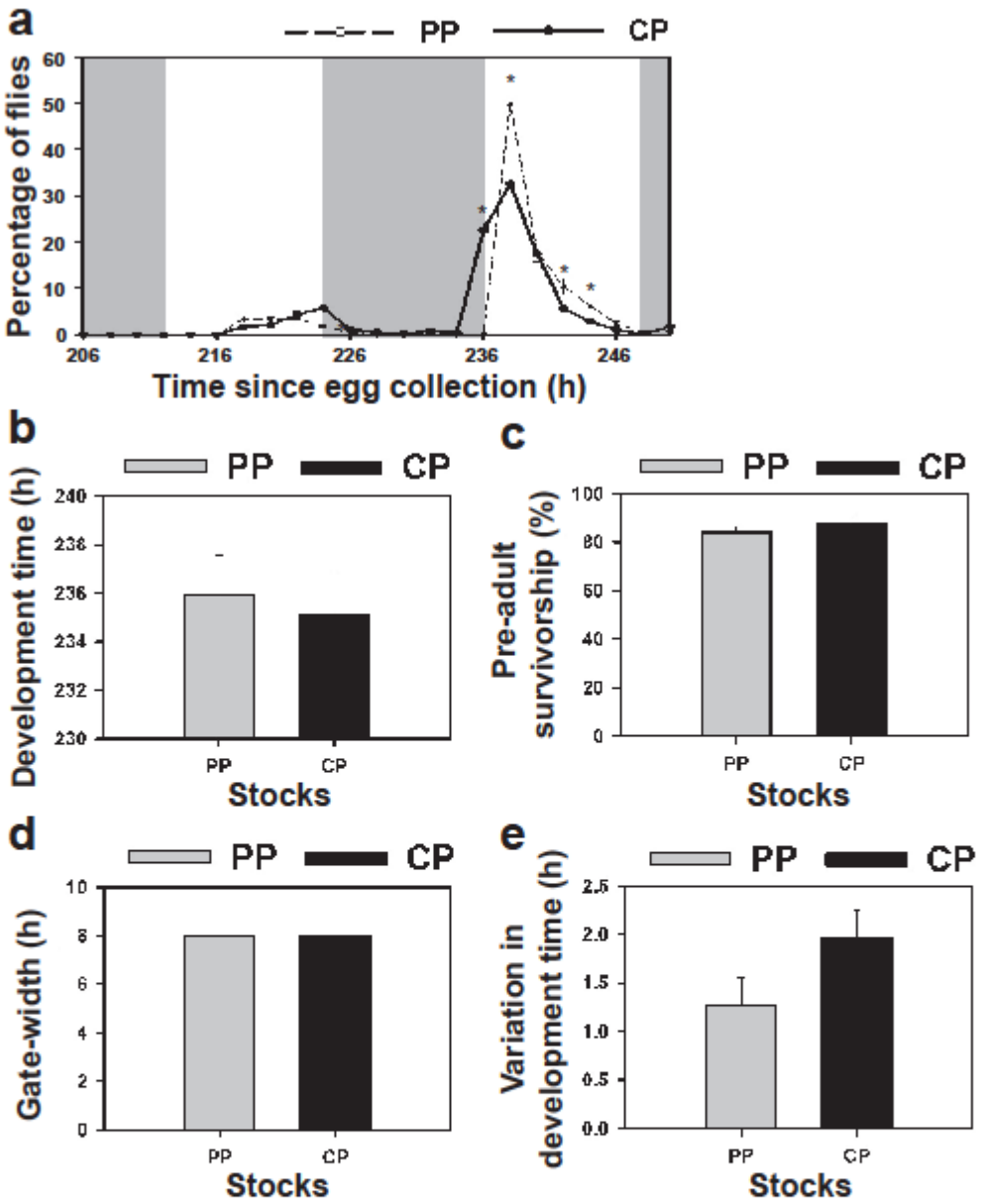

Figure 5.1. Development time and survivorship of the selected and control stocks (a) Percentage of flies of selected (dashed lines) and control stocks (solid lines) emerging in 2 hour windows under LD 12:12. Grey shading represents the dark phase of the LD cycle. Time of emergence on the x-axis is measured from the time of egg-collection. (b) Mean development time of the selected and control stocks. (c) Percentage of flies surviving from the egg to adult stage (pre-adult survivorship) in selected and control stocks. (d) Gate-width measured as duration between the onset of emergence (first 2 hour window in the day showing greater than 5% emergence) and the offset of emergence (last 2 hour window showing greater than 5% emergence) in selected and control stocks. (e) Variation in development time measured as standard deviation across individual flies in selected and control stocks. Error bars are standard errors of mean (SEM). Grey bars indicate selected stocks and black bars indicate control stocks in panels b-e. Asterisks denote significant differences.

5.3.2 Lifespan of morning and evening emerging males and females from selected and control stocks

We next compared mean lifespan, which is an important adult fitness trait, in mated flies from both selected and control stocks. We chose flies emerging within a narrow window of 1 hour each in the morning and evening as representatives of adaptive (mean) and non-adaptive (extreme) emergence phenotypes, respectively, since our selection regime is such that only flies emerging in the morning window contribute to the next generation. We found that males, irrespective of the time of emergence, lived longer than females both in the selected and control stocks. ANOVA revealed statistically significant effects of 'sex' ($F_{1,1707}$ = 23.72; $p < 0.05$) and 'stock' ($F_{1,1707}$ = 10.78; $p < 0.05$). Post-hoc multiple comparisons using Tukey's test revealed that morning emerging mated females from the selected stocks lived significantly shorter than morning emerging controls (Figure 5.2; $p < 0.05$). Post-hoc multiple comparisons also revealed that in the selected stocks, adult lifespan of morning and evening emerging males did not differ. Morning emerging females from the selected stocks lived shorter than those emerging in the evening, although this difference was not statistically significant (Figure 5.2a, b; $p > 0.05$). Such a time of emergence dependent difference in adult lifespan was not seen in the control flies. These results suggest reduced lifespan in mated females from the selected stocks and a link between timing of emergence and adult lifespan resulting in a sex-specific correlated response to selection for narrow gate of emergence.

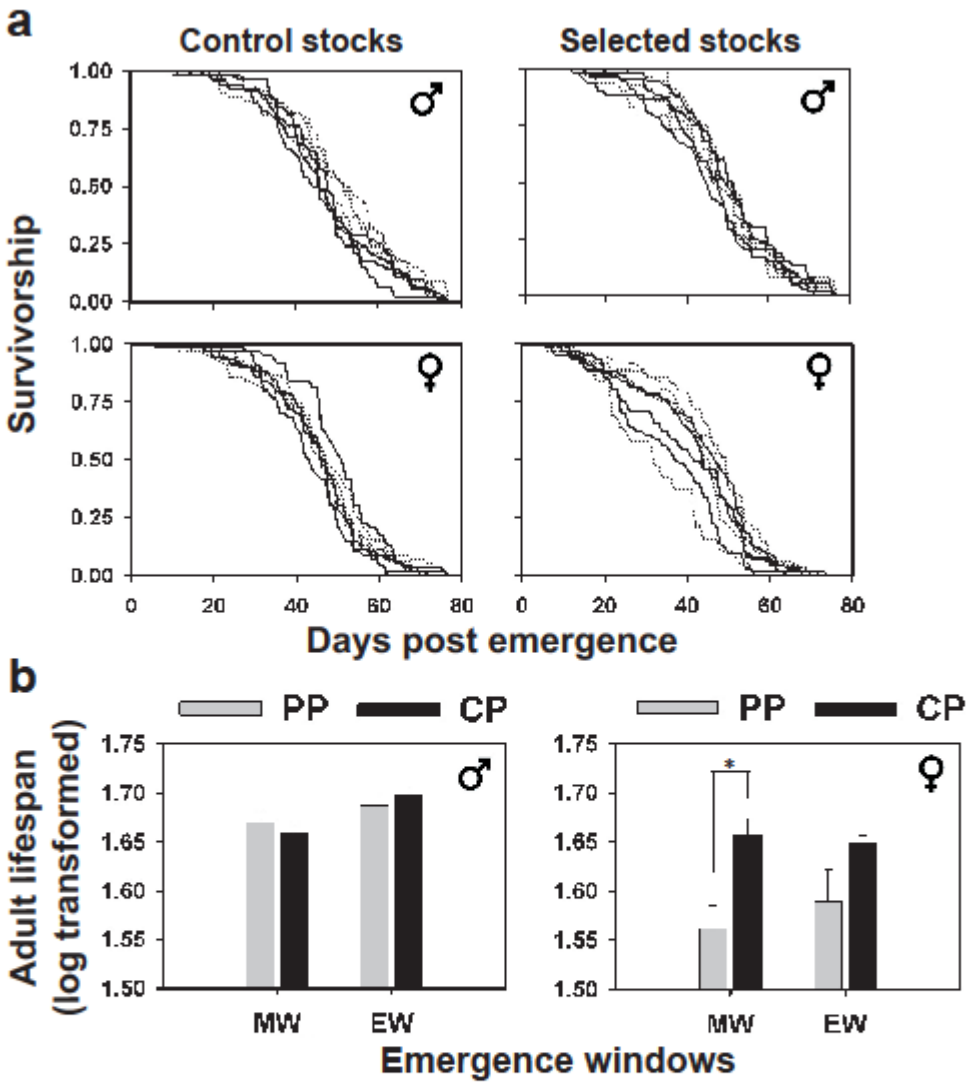

Figure 5.2. Survivorship curves and adult lifespan of mated males and females from the selected and control stocks. (a) Proportion of flies survived (y-axis) plotted against the days post emergence (x-axis). Left and right panels show survivorship curves of the control and selected stocks, respectively, while top and bottom rows represent male and female flies, respectively. Each panel compares the survivorship of flies emerging in the morning with those emerging in the evening window. Continuous lines represent morning emerging (ZT1 - ZT2, M window) flies whereas dashed lines represent evening emerging (ZT10 - ZT11, E window) flies. (b) Comparisons of the mean log transformed adult lifespan data between the selected and control stocks of the morning and evening emerging flies. Left and right panels show adult lifespan data of male and female flies, respectively. Emergence window is indicated on the x-axis as morning (MW) and evening (EW) windows. Grey bars indicate the selected stocks and black bars indicate the control stocks. Error bars are SEM. Asterisks denote statistically significant differences.

5.3.3 Fecundity of morning and evening emerging females from selected and control stocks

We also assayed daily fecundity across the adult lifespan of flies by counting the number of eggs laid by individual females every day, from the day of emergence until death (Figure 5.3a, b). The daily fecundity of these flies is around 10 eggs per day, which is on the lower side for outbred populations of *D. melanogaster* (Figure 5.3a). One of the reasons for this low fecundity could be the smaller body size of these females owing to their rearing under crowded larval conditions. The fly populations used in our study have been maintained on a 21-day generation cycle, which requires them to lay eggs on the 12th day after emergence to contribute to the next generation. Therefore, we divided the stages of adult life of flies into age-windows of 12 days each, with early (days 1–12), mid (days 13–24) and late life stages or age-windows (days 25–36). Only flies that survived and laid eggs until the age of 36 days were considered for the analysis. Repeated measures ANOVA on the lifetime fecundity data with 'window of emergence' and 'stock' as fixed factors and fecundity across age-windows as repeated measure, revealed statistically significant effects of 'window of emergence' ($F_{1,183} = 6.07$; $p < 0.05$), 'age-window' ($F_{2,366} = 121.35$; $p < 0.05$) and 'stock' × 'age-window' ($F_{2,366} = 4.72$; $p < 0.05$) and 'window of emergence' × 'stock' × 'age-window' interactions ($F_{2,366} = 10.87$; $p < 0.05$). Post-hoc multiple comparisons using Tukey's HSD revealed that the total egg output of the selected and control flies did not differ statistically; however, there was a trend of lower early-life fecundity and greater mid-life fecundity in the selected stocks compared to the controls. Post-hoc multiple comparisons on the early-life fecundity (days 1–12) revealed that flies from the control stocks emerging in the morning had

greater fecundity than those emerging in the evening (Figure 5.3b; $p < 0.05$). This trend was reversed in the selected stocks, although the difference was not statistically significant.

Post-hoc comparisons also showed that mid-life fecundity (days 13–24) of the morning emerging flies from the selected stocks was significantly greater than that of those emerging in the evening ($p < 0.05$), whereas mid-life fecundity of the morning and evening emerging flies from the control stocks did not differ (Figure 5.3a, b). Mid-life fecundity of the selected stocks was also greater than that of the controls, although this difference was not statistically significant. Hence, the correlation of time of emergence with early-life fecundity, seen in the controls, is broken in the selected stocks as a result of selection. Instead, the lower early-life fecundity in the morning emerging flies from the selected stocks was compensated by an increase in their mid-life fecundity. The late-life fecundity was reduced in the evening emerging flies (compared to morning emerging flies) in both selected and control stocks (Figure 5.3b). Similar trends were consistently seen even when the age of the flies was divided into 7 or 10 day age-windows. However, since we excluded those flies that did not lay eggs even though they were alive for 36 days, there was negligible increase in sample size using these smaller age-windows; hence, we persisted with the analysis on 12 day age-windows. Thus, the results of our assays revealed that although selected flies did not show differences in their total egg output, they displayed an age dependent enhancement in reproductive output during their mid-life stage. These results also demonstrate the effects of selection for narrow window of emergence on egg output across different life stages of flies, which provide evidence of a trade-off between early and mid-life fecundity in the morning emerging flies from the selected stocks.

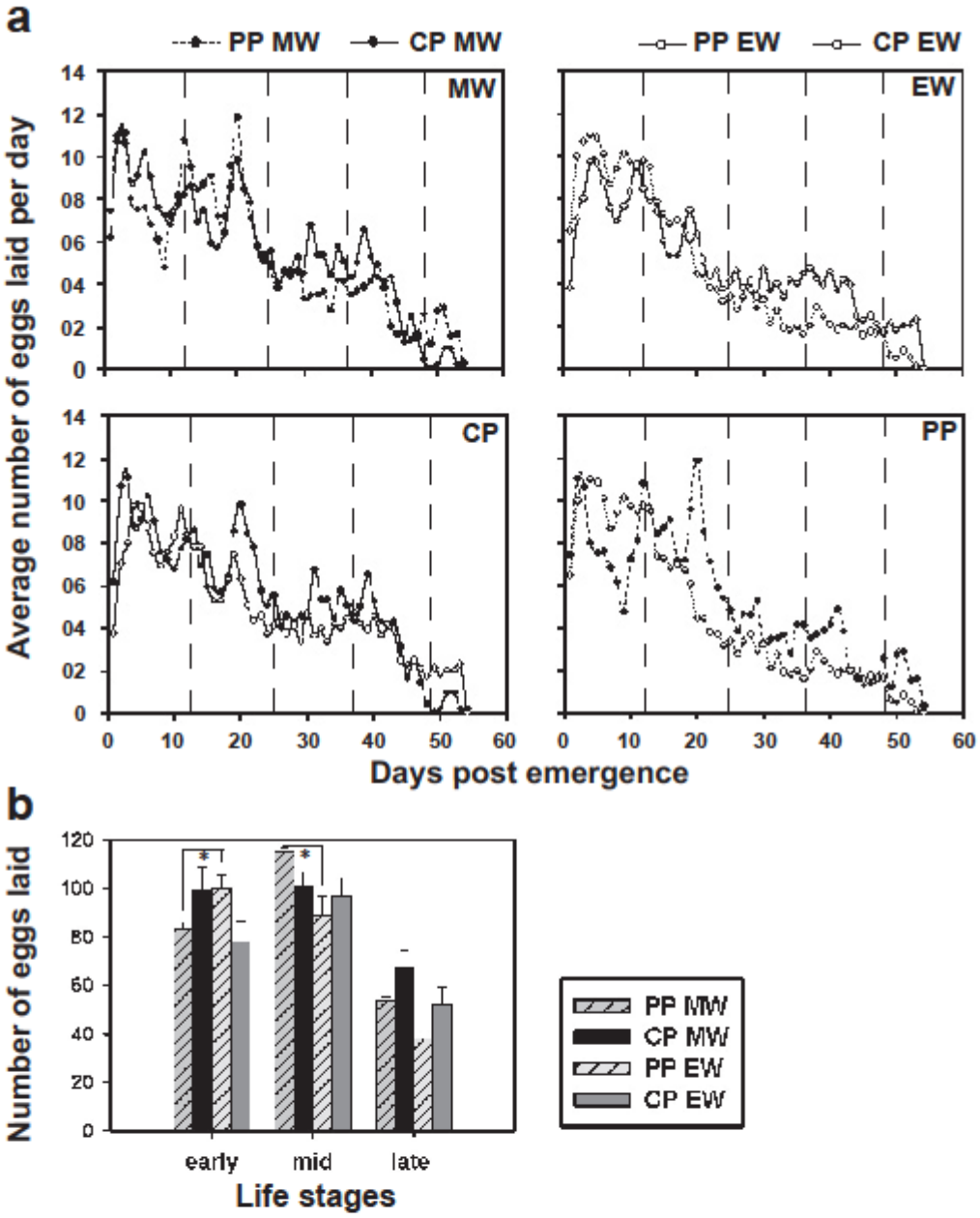

Figure 5.3. Lifetime fecundity and fecundity across age windows in flies from the selected and control stocks emerging in the morning and evening windows. (a) Top panels show comparisons of lifetime fecundity between the selected and control flies emerging in the same window whereas bottom panels show comparisons across the emergence windows. The number of eggs laid on a particular day averaged across individuals is plotted against the age of the fly measured as days after emergence. The dotted lines represent the divisions of the lifetime into relevant age windows, which are then pooled and analysed in panel b. (b) Comparison of fecundity of the selected and control flies across early (1–12 days), mid (13–24 days) and late (25–36 days) life stages. Error bars are SEM. Asterisks denote statistically significant differences.

5.4 Discussion

Correlations between life history traits suggest a common genetic architecture or somatic cost of certain traits on others. Correlated responses to selection further affirm that genetic variation of a particular trait upon which selection acts, also includes pleiotropic effects on other traits. The occurrence of such pleiotropic effects may be due to functional relatedness among the traits. Although there is evidence of the role of circadian clocks in the regulation of development time (Kyriacou et al., 1990; Miyatake, 1998; Miyatake, 2002; Kumar et al., 2007) and adult lifespan (Pittendrigh and Minis, 1972; Klarsfeld and Rouyer, 1998; Hendricks et al., 2003), it is still unclear whether such effects are solely based on phenotypes or if genetic causation can be attributed.

The results of our studies suggest that stabilizing selection for narrow gate of emergence in the morning does not affect pre-adult development time and survivorship (Figure 5.1). Although we observed no difference in gate-width between the selected and control stocks, it must be noted that the development time assay was done under uncrowded conditions (30 eggs per vial) in contrast to the normal maintenance conditions for the populations where the larval density is about 300 eggs per vial. In the crowded maintenance conditions, the gate-width of emergence of the selected stocks has evolved to be significantly shorter than the controls as a direct response to selection (Kannan et al., 2012a). Such differences in the expression of response to selection in assay environments different from the maintenance environment have also been reported previously (Ackermann et al., 2001). However, despite the absence of differences in their mean development time, flies from the selected stocks showed lower inter-individual variance in development time. This results in greater coherence in emergence time in selected stocks.

Circadian period and development time are usually positively correlated, with short period individuals developing faster than those with long period (Kyriacou et al., 1990; Yadav and Sharma, 2013; Miyatake, 1998), therefore, the lack of change in the mean development time of the selected stocks (Figure 5.1b) despite shortening of period (Kannan et al., 2012a) is counterintuitive. This observation can be partly explained by the fact that flies from the selected stocks show enhanced synchrony and reduced gate-width of emergence, due to strengthened effect of emergence gate, which may prevent flies from emerging outside the morning selection window even if they are developmentally mature (Mukherjee et al., 2012). This is consistent with an overall increase in accuracy of emergence in the selected stocks (Kannan et al., 2012a).

Mated males from the selected stocks live as long as those from the controls, whereas mated females from the selected stocks have significantly reduced adult lifespan compared to the controls (Figure 5.2). Thus, there appears to be a sex-specific evolution of reduced lifespan in the females of the selected stocks. Sexual dimorphism in aging and mortality has been reported in several species with males usually being shorter lived than females (Owens, 2002; Clutton-Brock et al., 1985; Promislow and Harvey, 1990) due to factors such as competition, physiological costs of sex hormones and high-risk, high-return reproductive strategies (Trivers, 1972; Vinogradov, 1998). However, empirical studies confirm that such male biased mortality is not universal across species due to factors such as selection pressure, increased male mating success with age and variable sex roles (Bonduriansky et al., 2008; Promislow, 2003). Since our assay conditions presumably do not result in intense male competition, we do not see a reduction in male lifespan (Figure 5.2). Additionally, it is known that artificial selection can result in sex-specific responses (Winkler et al., 2012;

Hoffmann et al., 2005). Our observations are consistent with the fact that quantitative trait loci for longevity in *D. melanogaster* show sex-specific effects on lifespan (Nuzhdin et al., 1997).

Although adult lifespan of morning and evening emerging males from the selected stocks does not differ, morning emerging females live shorter than their evening emerging counterparts (Figure 5.2). Interestingly, flies from the control stocks do not show such time of emergence dependent difference in adult lifespan. This reduction in lifespan of the morning emerging females from the selected stocks can be attributed to mating and reproductive costs on survival since this lower lifespan is compensated by greater mid-life fecundity in these flies (Figure 5.3). These results suggest that morning emergence is correlated with greater mid-life fecundity around the day of egg collection in the maintenance regime of the selected stocks, and has a fitness advantage for flies from the selected stocks in terms of becoming a part of the breeding pool for the next generation. The enhancement of egg output around the day of egg collection has been reported in studies where early or late fecundity has been selected for in fly populations (Rose and Charlesworth, 1981b). This may be due to age-specific genetic variance in fecundity (Tatar et al., 1996; Leips et al., 2006). Lower adult lifespan and higher mid-life fecundity in females from the selected stocks emerging in the selection window as a correlated response to selection for narrow gate of emergence can be taken as evidence of antagonistically pleiotropic effects or trade-offs between these traits (Reznick, 1985). This evolution of enhanced egg production at the cost of adult lifespan in females is understandable, given the importance of fecundity in female reproductive fitness. Such sex-specific effects on reproductive trade-offs have been previously reported in natural populations of crickets

(Zajitschek et al., 2009). Thus, stabilizing selection on timing of emergence enhances mid-life fecundity at the cost of reduced adult lifespan in female flies.

Morning emerging flies from both the stocks show greater late-life fecundity relative to those emerging in the evening. Late-life fecundity plateaus in females that lay fewer eggs early in their life (Rose and Charlesworth, 1981a). However, in control stocks, the morning emerging flies not only have higher early-life fecundity, they also lay more eggs during their late-life stage compared to the evening emerging flies. On the other hand, morning emerging flies from the selected stocks compromised their early-life fecundity for greater mid-life fecundity. These results are consistent with the notion of trade-off in reproductive efforts between successive life stages (Williams, 1966; Gadgil and Bossert, 1970), which predicts that females that lay fewer eggs early in their life, live longer and lay more eggs later in their life (Rauser et al., 2003). Such negative correlations between fecundity at different stages of life, and between high fecundity and adult lifespan have been reported earlier in *Drosophila* (Rose and Charlesworth, 1981a; Rose and Charlesworth, 1981b). Thus, selection for narrow window of emergence yields morning emerging females with greater mid-life fecundity at the cost of reduced early-life fecundity and adult lifespan, consistent with the expectation of trade-offs between life history traits.

Morning emerging mated females have higher mid-life fecundity than those emerging in the evening, which is consistent with the fact that evening emergence is maladaptive in flies from the selected stocks. Since in these fly stocks, emergence in the morning is strictly selected for, the proportion of flies emerging in the morning is much greater than those emerging in the evening (Kannan et al., 2012a). Thus, although fecundity of selected stocks is reduced compared to the controls at late-life stage, under the given protocol of a 21-day

generation cycle, flies from the selected stocks would have an adaptive advantage in terms of survival and egg output on the day when it matters the most. Hence, we may conclude that female flies selected for narrow gate of emergence have greater reproductive fitness than the controls under the maintenance regime. However, we have only considered flies with mean and extreme phenotypes of emergence timing and compared their life history traits, while conclusions regarding overall fitness of a population should be based on a more random sampling of flies from the population. Since a great majority of the flies emerge around the morning window of selection, our conclusions are likely to be robust for flies emerging across the day.

In summary, the results of our study revealed reduction in the variation in development time at no cost to pre-adult fitness. Adult lifespan of females from the selected stocks is shorter compared to the controls. However, this reduction of lifespan in morning emerging females is compensated by a concurrent increase in their mid-life fecundity. Thus, flies from the selected stocks show time of emergence dependent difference in adult fitness, albeit in a sex-specific manner. Interestingly, such time of emergence based differences in adult fitness traits are not seen in the controls. Thus, we find evidence of enhanced age-specific reproductive output in females from the selected stocks for emergence in the morning compared to evening. We interpret these results as evidence of genetic correlations between timing of emergence and life history traits, which may provide clues to the adaptive significance of enhanced clock accuracy in fly populations.

Future Directions

The results in this thesis are indicative of certain trends in the evolution of accurate circadian rhythms which may be confirmed and further probed to describe the detailed mechanisms by following different approaches.

For instance, the results of the first chapter are suggestive of a non-specific increase in robustness of period of selected populations (due to marginal but non-significant increases in inter-individual and intra-individual variation) that may be detected at significance levels only between large temperature differences since the magnitude of differences in period are greater by this manipulation. This hypothesis could be tested by assaying these flies under conditions where daily period variation is larger, such as mild starvation conditions, low humidity conditions or with external (non-light or temperature based) disturbances. Moreover, it may be interesting to see if such differences in robustness may be detectable at the cellular and molecular level by examining levels of PERIOD protein in clock neurons over the course of few days. These experiments could lend insight into the nature of variation in period due to different factors and whether or not they are dependent or independent of one another and whether they would also be reflected in the molecular rhythms or they are the consequence of cellular and tissue interactions appearing only at the behavioural level.

Similarly, the second chapter suggests that blue light sensitivity especially is enhanced in the selected populations compared to controls. The obvious candidate, as pointed out in the discussion, is Cryptochrome. There may be several approaches to validate this assertion. One approach may involve sequencing the Cry gene and its upstream sites to identify whether any sequence variants that may increase levels or activity of Cry gene are more frequently seen in the selected populations. Alternatively, one could examine CRY protein

levels by western blotting, especially during the early night, where phase-shift differences are seen. We could also perform TIM (Timeless protein) degradation assays to verify if the increase in phase-shifts occurs via this pathway while expecting to see faster degradation in the selected populations in the early subjective night. These experiments could verify whether the differences in phase-shifts seen in the selected populations are due to changes in the canonical pathways responsible for blue-light induced phase-shifts in fruit flies and provide clues regarding the genetic variation in light sensitivity present in natural populations of *Drosophila melanogaster*.

The third chapter essentially points towards gating of emergence by the circadian clock as the primary cause of the emergence phenotype seen in the selected populations by ruling out the role of developmental and other non-clock based processes involved in emergence in fruit flies. Since we know that several neurohormonal signals may be involved in gating of emergence by the circadian clocks, including ecdysone and Eclosion Hormone, the levels of these could be measured around the time that maximum differences are seen between selected and control populations (i.e. the 2 hours immediately preceding lights-ON). Additionally, these levels may be measured under constant light conditions with the expectations that no differences between the populations would be seen under such conditions. These experiments could confirm the role of the clock in mediating the neurohormonal processes responsible for restricting the timing of adult emergence to the selection window in the selected populations. Further experiments on tracing the differences in signalling from the circadian clock may be conducted by examining the timing of PDF signals to the prothoracic gland by immunocytochemical staining of these axons at different times of the day.

Finally, the chapter on life-history traits suggests the evolution of lifespan and fecundity in the females of the selected populations such that reduced lifespan is compensated by increased mid-life fecundity. Such patterns are usually indicative of antagonistically pleiotropic, moderate frequency alleles which govern both life history traits, rather than rare deleterious recessive alleles, which upon elimination by normalizing or stabilizing selection would result in overall increase in fitness. Preliminary experiments were carried out to address the nature of genetic changes in the selected populations by conducting a new regimen of artificial selection for emergence in an evening window where the number of flies emerging in the selected populations had substantially reduced. This selection was performed on both selected and control populations for 12 generations and the responses to selection were compared. Although the initial response (first 2 generations) in the selected populations was slower than control populations, within 3-4 generations, the number of flies emerging in the evening window gradually started increasing in the selected populations and the proportion of flies emerging in this window reached parity with the control populations by the 10^{th} generation. This suggests that genetic variation controlling emergence has not been eliminated from the population, but is merely not represented at the phenotypic level (or hidden from selection) and was slowly released as the selection on morning emergence was relaxed and selection on evening emergence continued. This pattern of response to selection is also consistent with moderate frequency alleles contributing to standing genetic variation rather than phenotypic variation in timing of emergence being caused primarily by rare, deleterious recessive alleles. This may also be verified by creating inbred lines from selected and control populations to examine whether inbreeding results in lower variation in emergence time in inbred lines derived from selected populations which may have purged

recessive alleles during the course of selection on emergence time. Hence, these are possible future directions that may be undertaken to follow up on this thesis.

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Varma, V., Kannan, N. N., & Sharma, V. K. (2014). Selection for narrow gate of emergence results in correlated sex-specific changes in life history of *Drosophila melanogaster*. *Biology Open, 3*, 606-613.