Correlated response to selection for faster pre-adult development on the circadian clocks of fruit flies *Drosophila melanogaster*

Thesis

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By

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Dedicated

To
My parents,
My wife Anju
And my Baby Pulkit

For their consistent support and countless encouragement

DECLARATION

I declare that the matter presented in my thesis entitled **"Correlated response to selection for faster pre-adult development on the circadian clocks of fruit flies *Drosophila melanogaster*"** is the result of studies carried out by me at the Chronobiology Laboratory in Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Vijay Kumar Sharma and that this work has not been submitted elsewhere for any other degree.

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

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CERTIFICATE

This is to certify that the work described in the thesis entitiled "Correlated responses to selection for faster pre-adult development on the circadian clocks of fruit flies *Drosophila melanogaster*" is the result of investigations carried out by Mr. Pankaj Yadav in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

Vijay Kumar Sharma

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CONTENTS

SUMMARY

Circadian clocks are believed to confer adaptive advantages to living beings by scheduling their biological functions at appropriate times of the day. The fruit fly *Drosophila melanogaster* has been a suitable model system to study circadian rhythms and consequently, temporal regulation of behaviours such as adult emergence, locomotor activity and egg-laying have been studied extensively in them. Apart from the daily regulation of behaviours, circadian clocks are known to influence life history traits such as pre-adult development time, fecundity and adult lifespan in insects. Of these, pre-adult development time is probably the most extensively studied life history trait in relation to circadian clocks. The role of circadian clocks in the regulation of pre-adult development time has been examined by studying effects of clock manipulation on the rate of pre-adult development, or *vice-versa.* Consequently, the clock's role in timing development has been inferred from (1) correlation between circadian period and pre-adult development time in the *period* mutants of *D. melanogaster*, (2) effect of light regime manipulation on the pre-adult development time of wild-type fly strains, and (3) effect of selection for faster rate of pre-adult development on circadian rhythms in the melon fly *Bactrocera cucurbitae*.

Due to the advantages of studying circadian rhythms and life history traits using laboratory selection approach, I decided to examine the relationship between circadian clocks and pre-adult development time in populations of fruit flies *D. melanogaster*. Studies described in my thesis are motivated by the need for an unequivocal evidence for the role of circadian clocks in the regulation of pre-adult development in fruit flies *D. melanogaster*. The main aim of my thesis was to study the effects of selection for faster pre-adult development on circadian clocks and life history traits, for which I derived four populations of *D. melanogaster* by imposing selection for faster pre-adult development under constant darkness (DD) (Chapter 2).

I assessed the direct response to selection in terms of changes in pre-adult duration, at regular intervals of 5-10 generations, to trace the evolutionary trajectory of changes by comparing the egg-to-adult duration in the selected (FD) stocks with that of the controls (BD). The results revealed that FD stocks respond to selection and their pre-adult development time was reduced by ~29 h (~12.5%) compared to BD controls. The FD flies underwent concurrent shortening in their timing of hatching, pupation and wing-pigmentation stages by ~2, ~16 and ~25.2 h respectively. Interestingly, FD flies also showed a concomitant reduction in the period of their activity/rest rhythm by ~0.5 h, implying that circadian clocks and development time are positively correlated. Thus, my study provides the first ever unequivocal evidence for the evolution of circadian clocks in response to selection for faster pre-adult development (Chapter 3).

The pre-adult duration of fruit flies *D. melanogaster* is affected by a wide variety of factors including temperature, light, larval density and nutrition. Although, it is known that development time in *Drosophila* is modulated by the rearing light conditions, there is no consensus about which of the pre-adult stages are affected the most. I therefore decided to study this in FD and BD stocks by estimating the durations of various pre-adult developmental stages under different light regimes. Since under LD developmental clocks of FD and BD flies are likely to be driven at the same pace due to circadian entrainment, which would be expected to free-run with their intrinsic period under DD, the relative differences in egg-to-adult durations between the two stocks is expected to be smaller under light/dark (LD) cycles than in DD. The results revealed that, while egg-to-adult developmental duration varied in a light regime-dependent manner, the relative difference in the timing of pupation and wing-pigmentation between the FD and BD stocks was smaller under LD than in DD. Thus, my studies provide

evidence for differential timing of pre-adult events between faster developing and control flies, being mediated by light regimes, circadian clocks and other clock-independent factors (Chapter 4).

In *Drosophila*, change in ambient temperature is known to modify the rate of pre-adult development but not the clock speed, but cycles with a combination of light and temperature can alter both pre-adult development time and circadian period. To study the effect of such speeding-up or slowing-down of pre-adult development and/or circadian clocks on selection-mediated differences in pre-adult development time of FD and BD stocks, I assayed egg-to-adult duration in these flies by subjecting them (i) to DD at different ambient temperatures (18, 25, 29 ^{o}C), or (ii) to 12:12 h LD or warm/cold (WC) cycles (WC1-25:18 ^{o}C, or WC2-29:25 ^{o}C). The results revealed that FD stocks develop faster than BD controls by ~52, 28 and 21 h, at 18, 25 and 29 ^{o}C respectively, and by 28 and 26 h under WC1 and WC2 respectively. The period of activity/rest rhythm of flies from both stocks decreased at 18 ^{o}C, but unlike at 25 ^{o}C did not differ between FD and BD flies when the ambient temperature was 18 or 29 ^{o}C, which suggests a break-down in correlation between circadian period and development time under non-native conditions. While the difference in development time between FD and BD stocks increased or decreased under cooler or warmer conditions, relative difference (BD-FD/BD) in development time remained unaltered. These results thus suggest that while manipulations of ambient conditions independently change development time and clock period, selection-mediated difference in development time remains largely unaffected (Chapter 5).

Since, both BD and FD flies were never exposed to daily cyclic conditions throughout their maintenance and selection protocol for over 55 generations, it is likely that selection-mediated difference in clock period (~0.5 h) will be affected when these flies are exposed to cyclic conditions. I therefore decided to assay the activity/rest rhythm of flies from FD and BD stocks

under DD following a week-long exposure to (a) various LD cycles - LD10:10, or LD12:12, or LD14:14, or (b) two WC cycles - 12:12 h of WC1, or WC2, or (c) to LD12:12 with three different light intensities (10, 100 and 1000 lux), or (d) WC1, or WC2, in-phase or out-of-phase with LD12:12 cycles. The results revealed a strong after-effect of LD and WC exposure on the clock period of both FD and BD flies, which reduced the selection-mediated difference between the two stocks. These results thus suggest that the circadian organization of fruit flies *D. melanogaster* is plastic as it displays history-dependent effect of exposure to cyclic environmental conditions (Chapter 6).

I further investigated the effect of selection for faster pre-adult development on the pre-adult and adult fitness of flies from selected and control stocks. I found significant reduction in lifespan, fecundity and stress resistance in the faster developing flies. Thus, selection for faster pre-adult development causes reduction in adult fitness in *D. melanogaster* (Chapter 7).

In the last part of my thesis I have described studies involving life-long activity/rest recording of flies from FD and BD stocks under DD conditions. The results demonstrate various age-associated changes in several features of circadian rhythms such as lengthening of circadian period, reduction in power of rhythm, activity level and onset of arrhythmicity to happen earlier in FD flies. These results thus can be taken to suggest that age-associated changes in circadian rhythm occur earlier in faster developing flies compared to the controls (Chapter 8).

In summary, studies described in my thesis demonstrate that circadian clocks and development time are positively correlated in fruit flies *D. melanogaster*. Light regimes differentially modulate durations of various pre-adult developmental stages, but selection-mediated differences in pre-adult development time remains largely unaffected. Selection-mediated differences in circadian period between the two stocks was greatly affected by prior

exposure to various environmental cycles. Selection for faster development alters circadian rhythms and causes faster ageing of the clock with reduced adult fitness. The results describing the connection between circadian clocks and pre-adult development in my thesis is the first ever empirical demonstration of its kind, and is therefore a significant step forward towards understanding the role of circadian clocks in the regulation of life history traits. These results can also be taken to underscore the significance of faster biological processes for organisms living under periodic and aperiodic environments, and suggest a possible mechanism by which circadian rhythms may have evolved to avoid inter-specific competition and to adapt to several cyclic environmental factors.

LIST OF PUBLICATIONS

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2. Mukherjee N, Kannan NN, **Yadav P**, Sharma VK (2012) A model based on oscillatory threshold and build-up of a developmental substance can explain gating of adult emergence in fruit flies *Drosophila melanogaster.* J Exp Biol 215:2960–2968.

3. Sivaperumal R, Subramanian P, **Yadav P**, Sharma VK (2011) Analysis of circadian locomotor rhythms in *vg* and *cryb* mutants of *Drosophila melanogaster* under different light-dark regimes. Biol Rhythm Res 42:321–335.

4. Sivaperumal R, Subramanian P, **Yadav P**, Sharma VK (2010) Analysis of light pulse induced phase shift in locomotor activity rhythm of *vg* and *cryb* mutants of *Drosophila melanogaster.* J Pharm Sci Res 2:433–444.

5. **Yadav P**, Sharma VK (2013) Environmentally-mediated modulations of developmental rate does not affect selection-mediated changes in pre-adult development time of fruit flies *Drosophila melanogaster*. J Insect Physiol (In press).

6. **Yadav P**, Madhumohan T and Sharma VK: Role of circadian clocks in timing pre-adult developmental events in fruit flies *Drosophila melanogaster* (In preparation).

7. **Yadav P** and Sharma VK: Lability of circadian clocks: Evidence from studies on after-effects on exposure to cyclic environmental conditions in fruit fly populations selected for faster pre-adult development (In preparation).

8. **Yadav P** and Sharma VK: Faster pre-adult development and faster running circadian clocks are associated with lower adult life history traits in fruit flies *Drosophila melanogaster* (In preparation).

9. **Yadav P** and Sharma VK: Circadian clocks in faster developing fruit flies *Drosophila melanogaster* age faster than controls (In preparation).

10. **Yadav P** and Sharma VK: Temperature cycles are stronger Zeitgeber than light dark cycles for adult emergence of *Drosophila melanogaster* selected for faster development (In preparation).

Chapter 1

Introduction

1.1 Introduction to circadian rhythms

The Earth rotates around its axis creating alternate day/night cycles to which almost all organisms have adapted to create for themselves a distinct spatio-temporal niche (Pittendrigh, 1993; Sharma, 2003; Dunlap et al., 2004; Paranjpe and Sharma, 2005; Vaze and Sharma, 2013). It is widely believed that fluctuations in geophysical factors have aided the evolution of rhythms of a wide range of periodicities - tidal (~12 hours), semilunar (~14 days), lunar (~29 days) and annual (~a year) time scales. Among the variety of biological oscillations present in the living world, the most ubiquitous and hence the most widely studied ones are those with circadian periodicities (*circadian rhythms*: Latin: *circa* - approximately, *dies* - a day).

Circadian clocks time the day-to-day behavioral repertoire of organisms and coordinate cellular and molecular cycles to ensure that they occur at suitable times of the day (Sharma, 2003; Paranjpe and Sharma, 2005; Vaze and Sharma, 2013). These clocks are known to synchronize metabolic processes with the environmental cycles to enable organisms to anticipate cyclic changes in their environment (Pittendrigh, 1993; Sharma, 2003; Dunlap et al., 2004; Vaze and Sharma, 2013).

Circadian rhythms are found in organisms ranging from prokaryotes to mammals (Johnson and Golden, 1999; Sharma, 2003). In *Cyanobacteria*, photosynthesis and nitrogen fixation, two antagonistic processes are governed by circadian clocks such that photosynthetic genes are expressed during the day and those responsible for nitrogen fixation during the night (Kondo and Ishiura, 2000). Similarly, several metabolic programs in non-heterocystous, filamentous cyanobacterial strains such as *Oscillatoria sp.* (Stal and Krumbein, 1985; Golden et al., 1997), *Synechococcus sp*. (Ouyang et al., 1998) and ATCC51142 strain of *Cyanothece sp.*

(Colon-Lopez et al., 1997; Schneegurt et al., 1997), are temporally regulated by circadian clocks. Similarly, in plants, flowering and leaf formation occurs only in a particular season, while opening and closing of flowers and stomata and leaf movements follow daily cycles (Chandrashekaran, 2005). In insects, biological phenomena ranging from inter-pulse intervals in courtship songs to adult emergence are under clock-control (Kyriacou and Hall, 1980; Qiu and Hardin, 1996). For example, many insects including fruit flies *D. melanogaster* emerge from pupal cases close to "dawn", when relative humidity in the environment is high, possibly to avoid being desiccated due to harsh afternoon conditions (Brett, 1955; De et al., 2012). Similarly, their activity/rest rhythm is also timed probably to help them avoid predation and undue competition among sympatric species while they search for food and mates (Dunlap et al., 2004; Paranjpe and Sharma, 2005).

Circadian clocks help organisms to time seasonal events such as migration and hibernation (Dunlap et al., 2004). For example, monarch butterflies *Danaus plexippus*, use circadian clocks for their migratory seasonal flights from north-eastern America to central Mexico (Brower, 1996). Several migratory birds also use circadian clocks to organize their photoperiodic calendar in order to migrate from extreme conditions to more favorable ones at a specific time of the year (Dunlap et al., 2004). Organisms use circadian clocks as a compass to compensate for the changing position of the sun during long-distance journey (Hoffmann, 1960; Pittendrigh, 1993). Many animals use circadian clocks to time the onset and termination of hibernation and aestivation at appropriate times of the year (Dunlap et al., 2004).

Circadian timing systems can be thought of as comprising three basic components namely input pathways, central oscillators and output pathways (Eskin, 1979). Input pathways include sensory receptors such as photoreceptors, thermoreceptors, olfactory and gustatory

receptors, which receive environmental signals such as light, temperature, humidity and odours and transmit them to the central circadian oscillators. The central circadian oscillators generate rhythmic signals and activate the output pathways, which in turn control rhythmicity in various metabolic, physiological and behavioral processes. Circadian rhythms have certain general characteristic features across all levels of complexity and organization:

(1) They free-run under constant environmental conditions of light, temperature and humidity with a period close to 24 h.

(2) They can be entrained to daily cycles of light and temperature.

(3) They are innate and endogenous in origin.

(4) The period of these rhythms is protected from changes in ambient temperature, nutrition and *p*H, within physiologically permissible range.

1.2 Adaptive significance of circadian clocks

Studies spanning over the past five decades, on organisms ranging from bacteria, fungi, plants, insects, amphibians, fishes, reptiles and mammals including humans, have revealed that circadian clocks enhance the likelihood of survival of organisms under periodically varying environmental cycles by helping them anticipate predictable changes in their environments (Pittendrigh, 1993; Dunlap et al., 2004; Paranjpe and Sharma, 2005; Sharma and Chandrashekaran, 2005). The ubiquity of its occurrence at various levels of complexity and organization implies that circadian clocks must have some adaptive value (Aschoff, 1964; Hasting et al., 1991; Pittendrigh 1993; Sharma, 2003; Vaze and Sharma, 2013). Although there

exists some empirical evidence to suggest adaptive value of circadian clocks, many evolutionary aspects of this phenomenon remain unexplored (Vaze and Sharma, 2013).

It is generally believed that circadian clocks provide adaptive advantage to the organism - (i) by coordinating cyclic metabolic processes within the internal milieu (intrinsic advantage), and (ii) by maintaining a stable phase-relationship between behavior and cyclic external environments (extrinsic advantage).

1.2.1 Intrinsic adaptive significance

It is commonly believed that rhythmically occurring biological processes would provide greater adaptive advantage to organisms than randomly occurring ones (Roenneberg et al., 2003; Sharma, 2003). While circadian clocks are likely to provide adaptive advantage to organisms living in cyclic environments, these clocks would not be of much use to organisms living under constant environments such as deep sea, caves or polar regions. Yet, robust circadian rhythms have been reported in a number of studies involving fishes living in the deep recesses of caves (Trajano and Menno-Barreto, 1996), in cave-dwelling millipedes (Mead and Gilhodes, 1974; Koilraj et al., 2000) and in fruit fly populations maintained for extended period of time under constant light (LL) (Sheeba et al., 1999a, 2001, 2002a) or in constant dark (DD) conditions (Imafuku and Haramura, 2011), indicating that circadian clocks are advantageous even to organisms living in aperiodic environments. Robust circadian rhythms in adult emergence, activity/rest and egg-laying behaviors have been reported in laboratory-reared populations of fruit flies *D. melanogaster* maintained under LL for over 600 generations (Sheeba et al., 1999a, 2001, 2002a). These flies also exhibit stable circadian entrainment to laboratory light/dark (LD) cycles of a wide range of periodicities (Paranjpe et al., 2003). Any trait that bears some cost and does not confer adaptive advantage is expected to disappear from the population in the course of

about 25 generations (Rose et al., 1996). Therefore, persistence of circadian rhythms in organisms living for extended periods of time under constant conditions implies that circadian clocks must have some intrinsic adaptive advantage. An alternate possibility could be that unlike traits that disappear due to lack of selection pressure, complete loss of circadian rhythms might require much longer than a few hundreds of generation (Sharma, 2003). Thus, intrinsic adaptive advantage may not be sufficient to explain long-term persistence of circadian clocks under constant environments.

While there are a good number of studies that reported persistence of circadian rhythmicity under constant conditions, several studies have reported frequent and complete loss of circadian rhythms in arthropods and fishes living inside caves (Mead and Gilhodes, 1974; Lamprecht and Weber, 1978; Erckens and Martin, 1982). In a study on cave-dwelling crayfish *Orconectes pellucidus* living under constant conditions for an extended period, highly variable rhythms with periods ranging from circadian to non-circadian scale were reported (Jegla and Poulson, 1968). However, it is possible that circadian clocks exist in several steady states, and a small perturbation changes the existing state of arrhythmicity into rhythmicity or *vice-versa* (Roenneberg and Merrow, 2002).

1.2.2 Extrinsic Adaptive Significance

Several processes such as adult emergence, locomotor activity, olfaction, mating and egg-laying in fruit flies *D. melanogaster* occur rhythmically with peaks at different times of the day (Saunders, 2002a). Such segregation of timing for different behaviours is believed to have occurred to avoid deleterious effects of biotic or abiotic factors (Pittendrigh, 1993). For example, it is believed that change in the free oxygen level in the environment has acted as

selection pressure for the evolution of circadian rhythms in early eukaryotes. Under such conditions, circadian clocks schedule metabolic processes such that it minimizes the deleterious effects of diurnal photo-oxidative exposure (Paietta, 1982). This is evidenced by the findings that nuclear division in a unicellular alga, *Chlamydomonas reinhardtii* peaks at dusk when its sensitivity to UV radiations is maximum (Nikaido and Johnson, 2000).

Extrinsic adaptive advantage of circadian clocks is further evident from studies on free-living mammals. For example, in a study on guillemots (*Uria lomvia*), jumping behaviour of fledgling was observed to occur at a species-specific time of the day and deviation from the said timing was found to lead to an increased predation of young ones by gulls (Daan and Tinbergen, 1980). Hence, synchrony in the timing of jumping activity of the young fledglings with other juvenile jumpers considerably reduced predation by gulls (Daan, 1981). In another study on ground squirrels, it was shown that while intact circadian clock was not critical for the organism's survival under controlled laboratory conditions, it becomes crucial when they were in free-living conditions. Animals with their suprachiasmatic nucleus (SCN - seat for the circadian pacemakers in mammals) lesioned lived equally well under laboratory conditions as the intact controls (Ruby et al., 1998), but had significantly reduced survivorship in natural conditions probably due to predation by feral cats (DeCoursey et al., 1997). In a subsequent study aimed at identifying the cause behind frequent deaths in SCN-lesioned animals (free-living chipmunks *Tamias striatus*), DeCoursey and coworkers (2000) discovered that clock-less animals were more active at night which may have attracted their nocturnal predators. Thus, predation as a selection pressure appears to have driven the scheduling of activity at an appropriate time in free-living animals.

1.2.3 Competition studies

Lifespan alone cannot provide a true picture of the fitness of an organism when the information regarding reproductive output is absent (Sheeba et al., 2000). In an extensive study that examined the reproductive fitness of various period strains of cyanobacteria *Synechococcus elongatus*, under diadic competition assays, the strain whose clock period matched closely with that of external LD cycles wiped-out its competitor, implying higher fitness of that strain due to the attainment of circadian resonance (Ouyang et al., 1998). However, arrhythmic strains had a better competitive ability than the rhythmic competitors when they were made to compete in LL, implying that lack of circadian clocks may be advantageous for organisms living in constant conditions (Woelfle et al., 2004).

Competition among organisms belonging to the same species (intra-species) is believed to be more detrimental than that at the genus level (inter-genic), mainly because they share the same resources (Fluery et al., 2000). Circadian clocks are believed to have enabled sympatric organisms to reduce the detrimental effects of intra-species competition by enabling them to adapt to different timing based on their competitive ability. Among the best known examples of such temporal segregation of activity is that seen in three sympatric species of *Drosophila* parasitoids namely *Leptopilina heterotroma, L. boulardi* and *Ascorba tabida*. These species are found to stay out-of-phase with each other such that the inferior (*A. tabida*) parasitoid starts its activity much earlier than the superior ones, thus reducing its intrinsic competitive disadvantage in the likelihood of multi-parasitism (Fluery et al., 2000). Such temporal segregation in phase of the activity rhythm appears to be determined by the clock period, which was found to vary among these three species. The inferior species *A. tabida* had short τ and anticipated LD cycles better, while the superior species *L. boulardi* had longer τ and showed weaker anticipation to

LD. This suggests that circadian clocks regulate temporal segregation of daily rhythms in competing species favoring their coexistence, which indicates the role of natural selection in shaping up circadian clocks.

1.2.4 Links between circadian clocks and life history traits

Several studies were carried out to examine the possible correlation between circadian clocks and life history traits particularly pre-adult development time and adult lifespan. For example, in one such study selection for faster development, the melon fly *B. cucurbitae* was found to yield a faster developing strain with smaller body size (Miyatake, 1995), faster running circadian clocks (Shimizu et al., 1997) and earlier time of mating (Miyatake, 1997b), while selection for slower development produced a strain with later time of mating (Miyatake, 1997b) and lower pre-adult survivorship (Miyatake, 1995). In a relatively recent study, *B. cucurbitae* lines selected for early or late reproduction were found to diverge in their timing of mating and τ of activity rhythm with flies from the early lines had shorter τ and mated earlier than those from the late lines (Miyatake, 2002). However, this study was associated with a possible inadvertent selection for age at reproduction which is likely to have added to the selection pressure to develop faster. In addition, this study was carried out on flies whose clocks were entrained under LD14:10, and on only two replicate populations comprising of a small number of individuals, out of which only one showed significant selection response. Furthermore, selection for early reproduction resulted in lines with shorter development time and reduced adult lifespan whereas selection for reproduction at late age resulted in lines with longer development time and extended lifespan (Miyatake, 1997a). In a relatively recent study in the adzuki bean beetle, *Callosbruchus chinesis,* no evidence for correlation between development time and τ was found (Harano and Miyatake 2011), indicating that circadian clock's regulation of pre-adult development time may

be species-specific. Taken together, these studies suggest that in a number of insect species circadian clock period is genetically correlated with pre-adult development time.

1.3 Circadian clocks and physiology

Daily oscillations have also been reported at the enzymatic and hormonal levels which affect the timing of cell functions, division and growth (Gottlieb and Wallace, 2001). Most physiological functions such as digestion, body temperature and immune responses encounter cyclic perturbations that peak at a fixed time of the day (Edery, 2000). In plants, rhythmic regulation of stomatal opening, leaf movement, hypocotyl expansion and development by circadian clocks have been reported to occur through the coordination of cellular and tissue level events (Engelmann and Johnsson, 1998; Webb, 1998; Dowson-Day and Millar, 1999).

In mammals, disruption in circadian clocks alters feeding activity and induces metabolic disorders, such as obesity, hepatic steatosis, hyperglycemia, and reduces levels of circulating insulin causing reduction in lifespan (Turek et al., 2005; Maury et al., 2010), indicating that circadian clocks are critical for the regulation of metabolism which has direct consequence on human health. Naturally occurring circadian dysfunction (Kumar et al., 2005) or those caused by genetic manipulations (Hall, 1999; Sehgal, 2004) or by subjecting organisms to light/dark (LD) cycles with abnormally short or long period lengths (Pittendrigh 1965, 1966) causes significant reduction in lifespan. Studies on *Cyanobacteria* (Yan et al., 1998), *Drosophila* (Beaver et al., 2002, 2003; Hendricks et al., 2003) and *Arabidopsis* (Dodd et al., 2005) which used mutants to assess fitness consequence of circadian clocks revealed that circadian timing systems are critical for the regulation of reproduction, vegetative growth and lifespan of organisms.

Males and females of many Dipterans have a common timing for activity, which possibly increases the opportunities for reproductive encounters between the two sexes (Saunders, 2002a). Since, even closely related species of insects are found to have different distributions of daily activity (Petersen et al., 1988), circadian clocks are likely to have played some role in the speciation of insects perhaps by setting-up temporal barriers, which in turn may have restricted mating opportunities among individuals sharing the same spatial boundaries (Tychsen and Fletcher, 1971). In some insect species, the rhythm in male responsiveness to the pheromones released by females has also been found to be under clock control and it peaks only at a specific time of the day (Saunders, 2002a). For example, pheromone release and calling behavior are rhythmically regulated in the gypsy moth *Lymantria dispar* (Giebultowicz et al., 1992; Webster and Yin, 1997), tobacco hornworm *Manduca Sexta* (Itagaki and Conner, 1988), oriental tobacco budworm *Helicoverpa assulta* (Kamimura and Tatsuki, 1993), armyworm *Pseudaletia unipuncta* (Delisle and McNeil, 1987) and in bog holomelina moth *Holomelina lamae* (Schal and Carde, 1986), implying the circadian clock's role in the regulation of reproductive physiology in insects.

Egg-laying rhythm is another example of a clock-controlled event, which recurs several times in an organism's life (Howlader and Sharma, 2006; Manjunath et al., 2008). Studies on the egg-laying rhythm in pink bollworm moth *P. gossypiella* (Pittendrigh and Minis, 1964; Minis, 1965) and yellow-fever mosquito *Aedes aegypti* (Haddow and Gillett, 1957) revealed that a brief light exposure of ~5 min duration every day is enough to manifest a distinct rhythm in dark-reared populations of insects which otherwise show weak rhythmicity. However, LL exposed populations were completely arrhythmic (Gillett et al., 1959; Fleugel, 1978) and a single dark pulse exposure was unable to elicit any rhythmicity in these populations (Haddow et al., 1961). Egg-laying rhythm has also been studied in fruit flies *D. melanogaster* (Rensing and Hardeland,

1967; Allemand, 1977; McCabe and Birley, 1998; Howlader and Sharma, 2006; Manjunath et al., 2008; Sheeba et al., 2001). Under LD12:12, the peak of egg-laying occurs just after lights-off, when the daytime light intensity was greater than 60 lux, above which two peaks of egg-laying were observed (Allemand, 1977). The circadian clocks controlling this rhythm in *D. melanogaster* were shown to be temperature and nutrition compensated (Howlader et al., 2006), entrained to a wide variety of light and temperature cycles (Sheeba et al., 2001; Paranjpe et al., 2005; Kannan et al., 2012a), and comprise of novel and yet-unknown circadian pacemakers (Howlader et al., 2006).

There are population level rhythms in insects that occur only once in their lifetime. A well-known example of this is the adult emergence rhythm, which is only apparent in a group of individuals of mixed developmental stages (Saunders, 2002a). Circadian clocks gate adult emergence in many insect species such that it is allowed to occur only during the early morning hours, when relative humidity in the environment is maximum, perhaps to avoid desiccation because wings of flies do not readily expand when humidity in the environment is low (Pittendrigh, 1954, 1966, 1993; Saunders, 2002a). On the contrary, adult emergence in the mosquito *A. aegypti* does not occur rhythmically (Haddow et al., 1959). Adult emergence rhythm in *D. melanogaster* is believed to depend upon the developmental state of the fly, phase and period of its developmental clocks and upon the ambient environmental conditions (Qui and Hardin, 1996; Paranjpe et al., 2005; Mukherjee et al., 2012). It is believed that circadian clocks read the developmental states of the fly and only if the fly is mature enough to emerge it is allowed to come out of its puparium if it matures after the gate has closed it is made to wait for the next emergence gate to open (Pittendrigh, 1954; Qui and Hardin, 1996; Mukherjee et al., 2012). Preliminary studies based on rhythmicity in events during the pre-adult stages such as

egg-hatching in kissing bug *Triatoma infestans* (Lazzari, 1991), pink bollworm moth *P. gossypiella* (Minis and Pittendrigh, 1968), mosquito *Mansonia titillans* (Nayar et al., 1973) and fruit fly *Drosophila pseudoobscura* (Pittendrigh, 1966), and pupation in fruit fly *Drosophila victoria* (Rensing and Hardeland, 1967; Pittendrigh and Skopik, 1970), few other species of *Drosophila* (Bakker and Nelissen, 1963; Davidowitz et al., 2003), mosquito *Anopheles gambiae* (Jones and Reiter, 1975), *A. taeniorhynchus* (Nayar, 1967a, b) and *Aedes spp.* (Nielsen and Haeger, 1954; McClelland and Green, 1970), and wing-pigmentation in *Drosophila* (Harker, 1964, 1965), suggest that populational events in insects are 'gated' implying a role for circadian clocks in the temporal regulation of pre-adult development. On the other hand, in the flesh fly *Sarcophaga argyrostoma*, pupae are formed throughout the day (Richard et al., 1986), although larval wandering and subsequent pupal emergence occur rhythmically (Saunders, 1986). Similarly, pupation in the mosquito *A. aegypti* is not clock-controlled, although egg-laying occurs rhythmically (Haddow and Gillett, 1957). These studies suggest that circadian clocks rhythmically regulate various metabolic and physiological events in a wide range of organisms at individual as well as population levels.

1.4 Pre-adult development and circadian clock regulation

The *Drosophila* life cycle is divided into egg, larval and pupal substages and the reproductive adult stage (Ashburner et al., 2005). Usually fly eggs take 18–24 h to hatch, which is often delayed if the environmental conditions are not favorable (Prasad and Joshi, 2003; Ashburner et al., 2005). Flies often retain eggs which are eventually laid at a more advanced stage and such eggs hatch much faster than those laid under normal course (Prasad and Joshi, 2003; Ashburner et al., 2005). The larval stage in *Drosophila* is the major feeding life stage, which passes through three distinct instars in about four days. The pupal stage starts after the third instar stage, lasts

for about four days and during the late pupal stage, the wing-pigmentation stage begins. During this stage, the ocellar and bristle pigments are formed (Bainbridge and Bownes, 1981), and these events are often used as late developmental stage markers (Lorenz et al., 1989; Qiu and Hardin, 1996). While typically larval duration does not differ between the two sexes, pupal stage in females is ~6 h shorter than males (Bakker and Nelissen, 1963; Nunney, 1983). Studies have also shown that slight perturbation in the environmental factors such as nutrition (Sang, 1956; Robertson, 1960; Carpenter and Bloem, 2002; Simpson et al., 2002; Warbrick-Smith et al., 2009), larval density (Joshi, 1997; Mueller, 1997) and temperature (Ashburner et al., 2005) affects the pre-adult developmental stages and hence alters the overall development time in *D. melanogaster* (Prasad and Joshi, 2003).

Since temperature is a key environmental factor that affects the rate of development of insects, ambient temperature is believed to have played an important role in the evolution of life history traits in ectotherms (Davidowitz and Nijhout, 2004; Trotta et al., 2006). Substantial efforts have also gone into the study of temperature and/or humidity effects on the pre-adult developmental stages of fruit flies *D. melanogaster*. It is observed that *Drosophila* is able to complete its pre-adult development successfully in a wide range of temperatures (12 to 32 °C). However, temperatures below and above this range are found to be deleterious (Good, 1993; Al-Saffar et al., 1995). Absolute relative humidity (~100%) is best suited for adult emergence of *D. melanogaster* (at which pre-adult survivorship reaches the maximum ~98%), although the pupal development time gets reduced steadily as temperature increases above 12 °C (Schnebel and Grossfield, 1986; Al-Saffar et al., 1995; Ashburner et al., 2005). Al-Saffar and coworkers (1996) studied the combined effects of temperature and humidity on the survival and development time of *D. melanogaster* under simulated natural conditions in the laboratory and found that

temperature accounts for more than two-thirds of the variation in development time, while the effect of constant and varying temperature had a small but statistically significant accelerating effect on development (Al-Saffar et al., 1996). Thus, developmental stages of *Drosophila* are susceptible to changes in environmental temperature and humidity.

While developmental duration in *Drosophila* is believed to be under circadian clock control, light regimes have also been shown to have considerable effect; development is fastest under LL (a condition that renders circadian clocks dysfunctional - Konopka et al., 1989; Paranjpe et al., 2005), followed by LD and then DD (Sheeba et al., 1999b; Paranjpe et al., 2005; Lone and Sharma, 2008). Furthermore, period of LD cycles and/or emergence rhythm has been found to be correlated with pre-adult development time (Paranjpe et al., 2003, 2005), and it is believed that the phase-relationship between LD cycles and the developmental clocks is one of the key determinants of pre-adult development time (Paranjpe et al., 2005). In fruit flies *D. melanogaster*, prior to emergence, developmental clocks pass through several gating events such as those in egg-hatching, pupation and wing-pigmentation. Such gated stages and the transition time from one to another is likely to have created temporal constraints on development and thus it is believed that the duration of different pre-adult stages in *Drosophila* is determined by developmental clocks and its interaction with the LD cycles (Sehgal et al., 1992; Vallone et al., 2007).

In *Drosophila*, ecdysone, a steroid hormone released from the prothoracic gland (Gilbert et al., 2002) triggers larval molting and adult emergence (Truman et al., 1983; Riddiford et al., 2010). Premature release of ecdysone speeds-up the development of tobacco hornworm *Manduca sexta* (L.) and delayed release slows it down (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). During the larval stage, opening of the gates is considered as the

signal that triggers the release of prothoracicotropic hormone, and therefore, it is hypothesized that modulation of pre-adult development time in insects is due to altered timing of prothoracicotropic hormone release, which is primarily caused by alteration in the timing and duration of gating at different developmental stages (Rountree and Bollenbacher, 1986). In fruit flies *D. melanogaster* ecdysone is known to set the timing of its pre-adult stages by binding to the nuclear hormone receptors, thus regulating the expression of several developmental genes (Giebultowicz et al., 2008). Thus, it is possible that genes involved in various signaling pathways govern light regime-mediated developmental changes in *Drosophila* (Mensch et al., 2008).

Several studies in *Drosophila* have shown that shorter development time is associated with reduced adult body weight at emergence (Nunney, 1996), reduced adult body size (Zwaan, 1995a; Nunney, 1996; Chippindale et al., 1997; Prasad et al., 2001), reduction in life-history traits such as pre-adult survival (Chippindale et al., 1997; Prasad et al., 2001) and adult lifespan (Chippindale et al., 2004). Such changes in the timing of developmental processes may partly be due to changes caused by the genetic correlations between development time and other life history traits (Prasad and Joshi, 2003; Chippindale et al., 2004). Reduction in adult fitness traits as a result of faster pre-adult development time, implies a genetic correlation between the traits associated with pre-adult and adult stages (Chippindale et al., 2004).

However, correlation between clock period and development time was found to exist irrespective of the state of the circadian rhythms in mutants for the *period* (*per*) gene in fruit flies, i.e., while the rhythm was free-running under DD, or while it was absent under LL, or while it was entrained under light or temperature, and light plus temperature cycles, suggesting that *per* mutation has pleiotropic effects on pre-adult development time and circadian period

(Kyriacou et al., 1990). Pleiotropy is not uncommon at the *per* locus as it is known that mutation in *per* alters the inter-pulse interval (ipi) in courtship song in a similar manner as it does to circadian rhythms (Kyriacou and Hall, 1980; Konopka et al., 1996). The per^S males have ipi of ~40 sec, per^L of ~76 sec and in per^{01} males courtship song was arrhythmic. However, in a separate study aimed at examining the role of the period of LD cycles and/or adult emergence rhythm in the regulation of pre-adult development time in *Drosophila*, while bypassing possible pleiotropic effects of clock genes, it was shown that egg-to-adult development time was correlated with the period of LD cycles and/or of adult emergence rhythm (Paranjpe et al., 2005), suggesting a possible role of circadian clocks in the regulation of life history traits in *Drosophila*. Thus, in *Drosophila* the rate of development and adult lifespan are positively correlated with τ. Similar correlation between development time and clock period has also been reported in the faster/slower developing melon fly *Bactrocera cucurbitae* and in the fruit flies *D. melanogaster* selected for morning and evening emergence (Kumar et al., 2007). For example, melon flies *B. cucurbitae,* selected for faster pre-adult development evolved faster running clocks (τ ~22.6 h) and earlier timing of mating *vs.* slower clock period (τ ~30.9 h) and later time of mating in the slower developing lines (Miyatake, 1996, 1997b; Shimizu et al., 1997). Although, selection for faster and slower development resulted in positive correlation between clock period and pre-adult development time (Miyatake, 1996, 1997b; Shimizu et al., 1997), clear association was seen only in the slower developing lines since they diverged successfully from their ancestral populations. In a separate study, fruit fly populations subjected to selection for morning and evening adult emergence resulted in populations with faster and slower circadian clocks and shorter and longer pre-adult durations, respectively (Kumar et al., 2007). These two lines of

evidence further support the notion of circadian clock's involvement in the regulation of pre-adult development time in insects.

1.5 Rationale of the present study

Development time is a complex trait, which depends upon several environmental factors such as temperature (Partridge et al., 1994; de Moed et al., 1998, 1999), larval density (Joshi, 1997; Mueller, 1997), nutrition (Sang, 1956; Robertson, 1960; Carpenter and Bloem, 2002; Simpson et al., 2002; Warbrick-Smith et al., 2009) and light regime (Sheeba et al., 1999b; Paranjpe et al., 2005; Lone and Sharma, 2008). Due to living in ephemeral habitat such as rotting fruits (Robertson, 1963; Partridge and Fowler, 1992), fruit fly larvae are believed to be under directional selection to develop faster (Nunney, 1990; Prasad and Joshi, 2003). It is believed that apart from the direct benefits of developing before food starts running out, faster development may provide adults with increased opportunity to mate (Miyatake 1996, 1997b, 2002; Shimizu et al., 1997). Indeed, several studies in the past have shown that faster development in fruit flies is genetically correlated with early reproduction (Chippindale et al., 1994; Prasad et al., 2000) and therefore, insects would develop faster if they get chance to mate earlier in life (Chippindale et al., 1994, Miyatake, 1997b; Miyatake, 2002). In addition, faster pre-adult development in *Drosophila* is also correlated with life history traits such as fecundity (Chippindale et al., 2004), starvation and desiccation resistance (Rose, 1984; Partridge and Fowler, 1992; Chippindale et al., 1994) and adult lifespan (Partridge and Fowler 1992; Chippindale et al., 1994). Thus, *Drosophila* development time co-varies with various fitness and fitness-related traits.

Circadian clocks have been implicated in the regulation of life history traits including pre-adult development time in insects including fruit flies *D. melanogaster* (Kyriacou et al.,

1990; Sharma, 2003; Paranjpe and Sharma, 2005; Lone and Sharma, 2008). Since, it is believed that developmental clocks in *Drosophila* start ticking right from the first or second instar larval stage and is present throughout the development (Sehgal et al., 1992; Kaneko et al., 1997; 2000; Kowalska et al., 2010), it is likely that clocks are involved in the regulation of development right from the early pre-adult developmental stage.

While several lines of evidence suggest the notion of circadian clock's involvement in the regulation of pre-adult development time, in the literature there is no consensus on this issue. Some studies employed mutant strains to address this issue, while others used one to two replicate populations with small population sizes (N = 100), which could have led to inbreeding/ genetic drift and selection for generally poorly developing genotypes (selecting for individuals with lowered fitness being a consequence of selecting for longer development). In addition, some studies were carried out when clocks were entrained under LD cycles, given that LD cycles also have substantial effect on development time of insects, maintenance of selection lines under LD cycles might have affected the developmental rates of flies which in turn may have contributed to the response. Taken together the above factors, findings of previous studies examining the role of circadian clocks in the regulation of life history traits run into problems of misleading and spurious results.

The most logical way to study the role of circadian clocks in the regulation of pre-adult development time in fruit flies *D. melanogaster* would be to either undertake laboratory selection studies on replicate large outbred populations (to prevent inbreeding depression or genetic drift) selected for populations with (i) short or long clock period, or with (ii) faster or slower development time. The other though less attractive alternative is to explore for the possible

variability in pre-adult development time and circadian clock period in naturally occurring populations.

We chose the laboratory selection strategy to examine circadian clock's role in the regulation of life history traits in fruit flies *D. melanogaster*. We reasoned that such studies should be carried out under DD at constant temperature (25 ^{o}C) and relative humidity since light, temperature and humidity can all affect development time and circadian clocks in a number of ways. Further to rule out the effect of age of reproduction, egg collection for the stocks was done in parallel with the controls. We feel that due to these factors not being taken into consideration, several of the previous selection studies on developmental rates in *Drosophila* failed to yield any response or resulted in spurious genetic correlations. Therefore, the current study is an effort to rule-out most of the afore-mentioned shortcomings and to examine whether there exists a correlation between circadian clocks and life history traits.

Chapter 2

Experimental & Baseline populations

2.1 Background of baseline developing populations

In this section, the ancestry and maintenance of the four baseline populations of *Drosophila melanogaster* used for our study have been described. Baseline Developing populations (henceforth will be referred to as BD populations) formed the source from which we derived a set of populations that were then subjected to selection for faster development (FD_{1-4}). The baseline populations (BD_{1-4}) were in-turn derived from four separate laboratory populations (JB_{1-4}) (Details in Sheeba et al., 1998; Sheeba, 2001; Figure 2.1). These populations were maintained in the laboratory for about 100 generations under constant darkness (DD) at constant temperature (25 ± 0.5 °C; mean ± SEM) and constant humidity (~80%) under a 21 day discrete generation cycle (Figure 2.1). The physical conditions (temperature and humidity) during the course of stock maintenance were monitored regularly using Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho, Japan. Each replicate population consisted of ~1200 adults (roughly equal number of males and females), maintained in a plexiglass cage (25 × 20 × 15 cm^3) supplemented with banana-jaggery food medium (henceforth will be referred to as banana medium) available *ad libitum*. To start a new generation adult flies were provided with banana- medium supplemented with live yeast paste for two days prior to the egg collection. Flies were allowed to lay eggs on a petri plate with fresh banana medium for about 12 h (between 22:00-10:00 h) and eggs were dispensed at a density of approx. Sixty to eighty eggs into glass vials (9 cm height × 2.4 cm diameter) supplemented with ~6 ml banana medium. Twenty-four such vials were used for each population, in which eggs hatched into larvae, larvae develop into pupae and from pupae adults emerge. This developmental process is completed in about 9-10 days. On the 9^{th} to 12^{th} days after egg collection, all the freshly emerged flies were transferred from 24 glass vials into plexiglass cages containing a petri dish of banana medium. These four sets of populations in

plexiglass cages represent the next generation of the breeding population. On the18th day after previous egg collection, all four fresh generation breeding populations were supplemented with fresh banana medium covered with a smear of yeast-acetic acid paste for ~2.5 days. Twelve hours before egg collection the yeasted food plate was replaced by fresh banana medium food plate and flies were allowed to lay eggs on these plates for about 12 h (between 22:00-10:00 h) as described earlier (Figure 2.2).

2.2 Background of experimental populations

In this section, the ancestry and maintenance of four experimental faster developing (FD_{1-4}) populations of *Drosophila melanogaster* used for our study have been described. They were derived from baseline developing populations described in the previous section. For example, the FD_1 population was derived from BD_1, and thus each FD population was derived from the corresponding BD control population. Therefore, selected and control populations bearing identical numerical subscripts are more closely related to each other than to the populations with which they share selection regime (Figure 2.1). Experimental populations (FD_{1-4}) were maintained along with control baseline (BD_{1-4}) populations under constant dark regime with constant temperature (25 ± 0.5 °C) and constant humidity (75 ± 5%) in the laboratory for about 70 generations. Each pairs of FD_{1-4} - BD_{1-4} populations will henceforth be referred to as blocks and are maintained as independent entities. All the steps of stock maintenance protocol for the FD populations are similar to that of BD controls described in previous section for baseline populations except that, (i) instead of collecting all flies from every vial, here we collect only the earliest eclosed flies (only the first 25%, i.e., ~12-15 flies were collected from each vial) (ii) instead of 24 vials/population 80 vials were used and (iii) the faster developing flies were transferred to breeding cages on the day of emergence, whereas the controls were transferred into

breeding cages only after all the flies had emerged (Figure 2.2). As with BDs, eggs were collected after 21 days from the previous egg collection date to start the next generation.

2.3 Selection protocol

From these four BD (BD_{1-4}) populations, four populations of faster developing (FD_{1-4}), were initiated by imposing selection for faster development in constant dark (DD), along with four control populations (BD_{1-4}), where no selection pressure was applied (Figure 2.3). Each FD replicate population was derived from one control population, thus forming the matched selected and control pair. The four replicate populations with identical subscripts were treated as blocks (random factor) in the statistical analysis. The selected populations were maintained in the same environment as the control populations.

2.4 Standardization of populations

Imposition of different maintenance conditions and strategies may induce non-genetic parental effects (Prasad et al., 2001); in order to eliminate such possible non-genetic effect of parental rearing condition, before every assay, flies from both stocks were subjected to a common rearing condition for one generation wherein selection for faster development was relaxed. For this, from the running cultures of FD_{1-4} and BD_{1-4} stocks, eggs laid on banana medium over a period of 12 h were collected and 60-80 eggs dispensed into 24 vials per population. Twelve days later, adult flies were transferred into plexiglass cages with fresh banana jaggery food *ad libitum*. These caged populations will be referred as the "standardized populations" and maintained under constant dark regime with constant temperature (25 ± 0.5 °C) and constant humidity (75 ± 5%) in the laboratory and were employed for all the experiments described in my thesis.

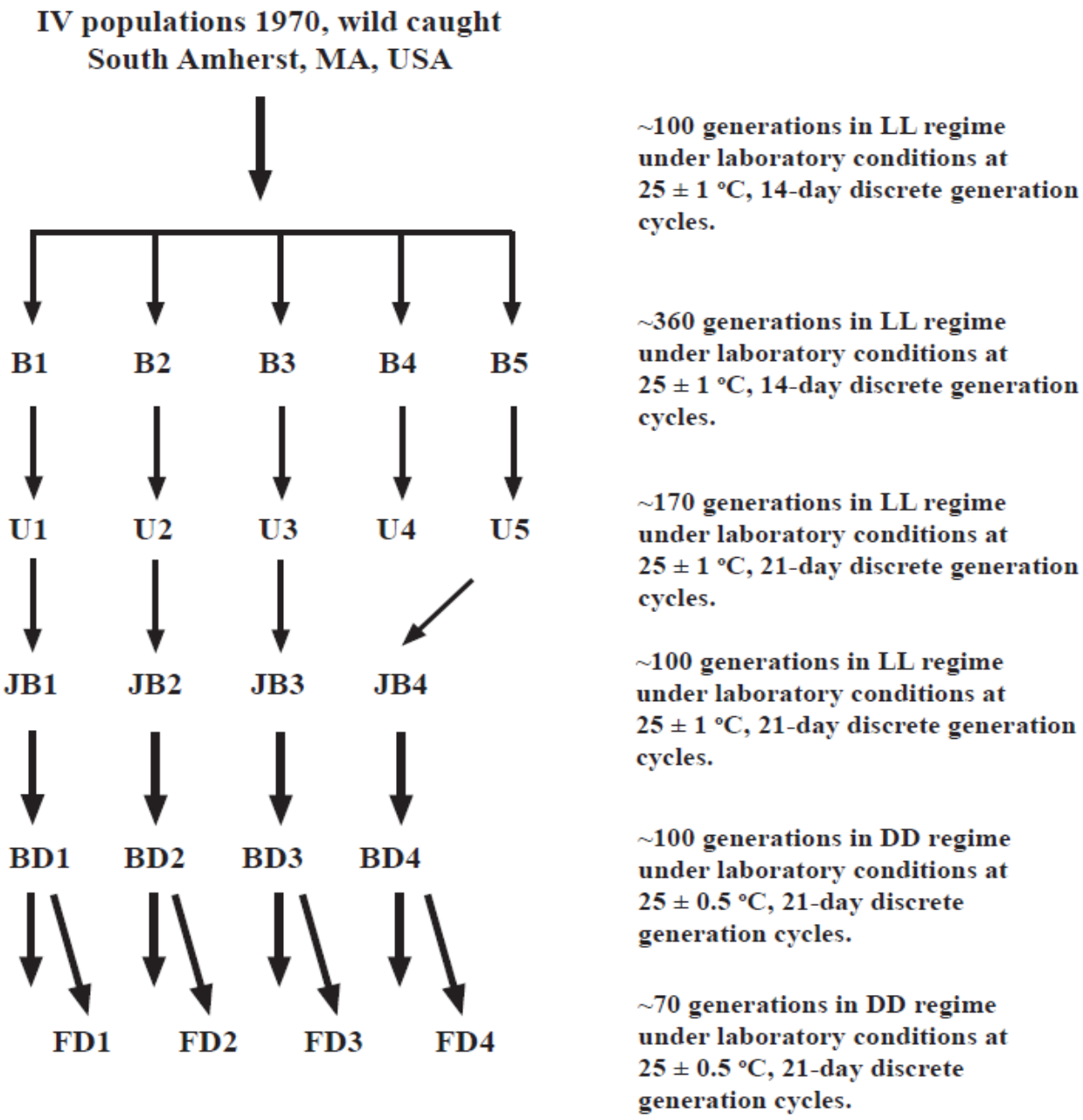
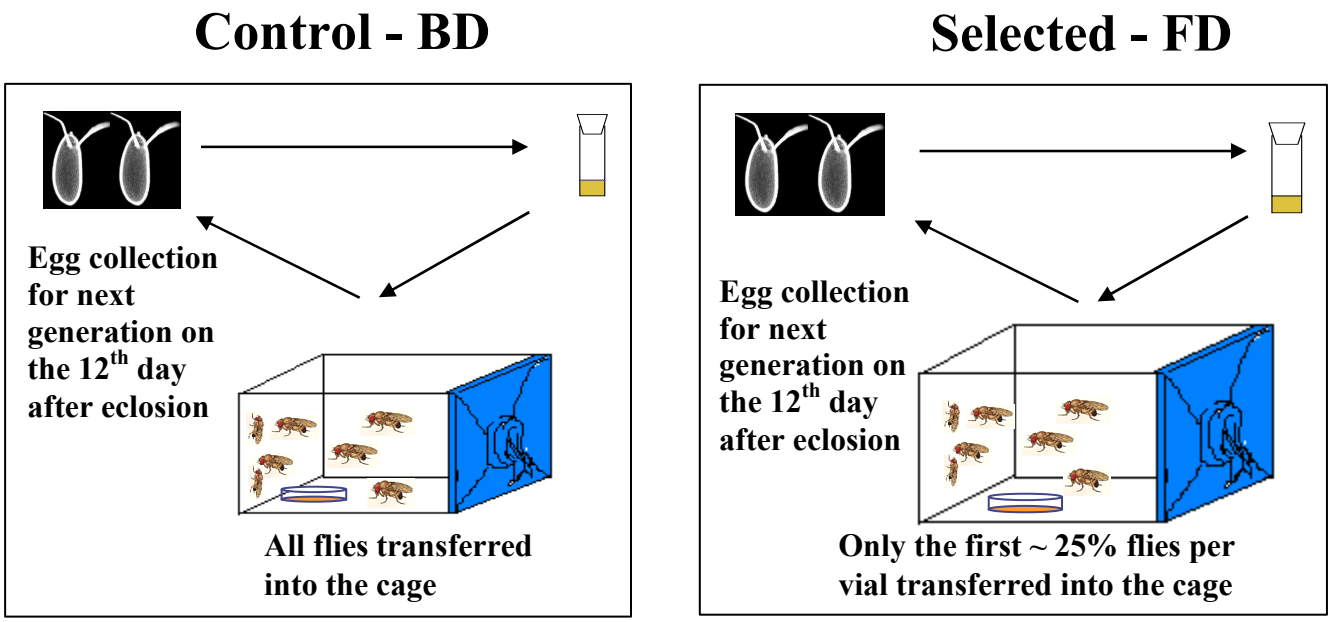


Figure 2.1. Schematic representation of stock ancestry: The maintenance and ancestry of four replicate outbred baseline and experimental populations.



(Modified from schematic by Dr. Shampa Ghosh)

Figure 2.2. Schematic representation of stocks maintenance: The steps followed during maintenance of baseline control (BD) and selected experimental (FD) populations.

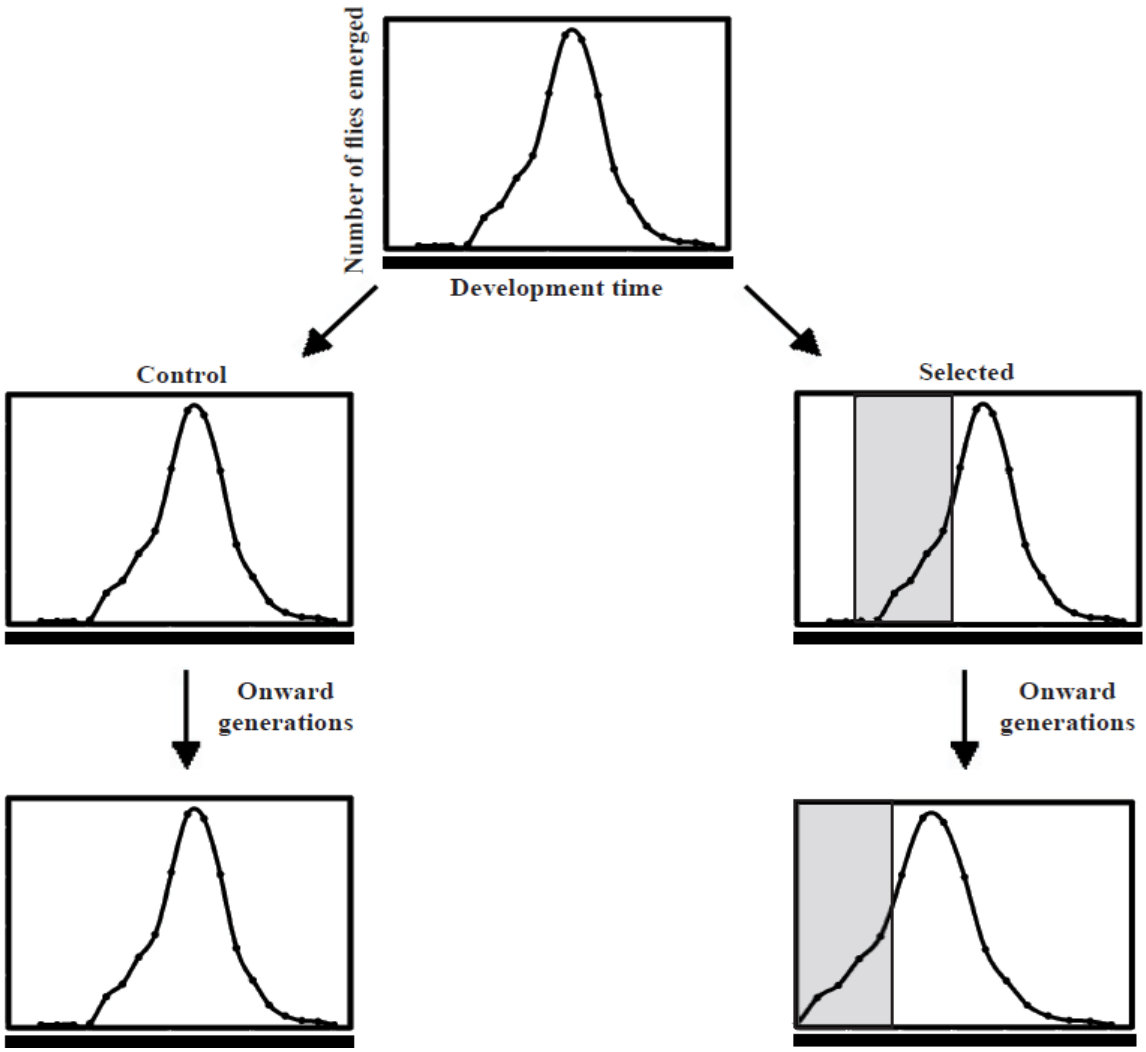


Figure 2.3. Schematic representation of the laboratory selection protocol: The number of flies emerging under constant dark condition (DD) is plotted along the *y*-axis and development time is plotted along the *x*-axis. The gray shaded area represents the portion of development profile falling under selection duration. The black horizontal bar represents constant dark (DD), which is the maintenance regime for both stocks.

Chapter 3

Correlated changes in circadian clocks in response to selection for faster pre-adult development in fruit flies *Drosophila melanogaster*

3.1. Introduction

It is a long held belief that when clocks tick faster or slower, the processes that they regulate occur sooner or later (Pittendrigh, 1981). For example, in the nematode *Caenorhabditis elegans*, a mutation in developmental genes (*clk-1*, *clk-2*, *clk-3* and *gro-1*) that slowed down embryonic cell cycles also slowed down its development, ageing and affected several adult behaviors such as swimming, pharyngeal pumping and defecation (Wong et al., 1995; Lakowski and Hekimi, 1996). In *Drosophila,* mutations in the *period* (*per*) gene that speeds-up or slows-down circadian clocks, shortened or lengthened the frequency of inter-pulse beats in courtship songs (Kyriacou and Hall, 1980) and the duration of pre-adult development (Kyriacou et al., 1990). The short period mutant (per^S) developed faster than the wild type strain which in turn developed faster than the long period mutant (per^L). However, in this study, development time differences between the short and long period mutants also persisted under constant light (LL), a regime where adult emergence is arrhythmic (Konopka et al., 1989) and under light/dark (LD) cycles, where it is entrained, suggesting that changes in the development time of *per* mutants may not be mediated via circadian clocks *per se*. In studies aimed at studying genetic correlations between traits such as development time and other components of fitness, use of mutant lines is not an ideal choice (Sharma and Joshi, 2002), because mutants are often inbred for a particular phenotype, yielding spurious genetic correlations between fitness components (Muller and Ayala, 1981). The most ideal strategy for studying the role of circadian clocks in the regulation of development would be to examine correlation between circadian period (τ) and development time in natural populations, where sufficient variation in both these traits is likely to exist. The other alternative would be to study such correlations in large replicate populations selected either

for different τ or for different pre-adult development time.

Correlation between τ and development time has also been implied in laboratory selection studies where fly populations were subjected to selection for faster or slower pre-adult development (Miyatake 1996, 1997a, 2002; Shimizu et al., 1997) or for *early* and *late* adult emergence (Kumar et al., 2007). In the melon fly *Bactrocera cucurbitae*, pre-adult development time and phase of mating rhythm was positively correlated with τ of the activity rhythm (Miyatake, 1996, 1997a; Shimizu et al., 1997). In this study, τ of lines selected for longer developmental time differed from that of the ancestral populations. However, there were large differences in the mean phenotype of the two replicate slower developing lines (Miyatake, 1997a). Moreover, population sizes in this study were also quite small (N = 100), which taken along with other facts discussed above make it difficult to rule out inbreeding/ genetic drift and selection for generally poorly developing genotypes (selecting for individuals with lowered fitness being a consequence of selecting for longer development) as an alternative explanation for the results. In a relatively recent study, lines of *B. cucurbitae* selected for early or late reproduction were found to diverge in terms of time of mating and τ of activity rhythm, with flies from the early reproducing lines with shorter τ mating earlier in the day than those from the late lines (Miyatake, 2002). The results of this study, too, were equivocal because the early reproducing lines also developed faster as pre-adults, possibly due to inadvertent selection for faster development through advantage gained from having longer maturation time between emergence and reproduction (Miyatake, 2002). In a separate study morning and evening emerging flies respectively evolved faster and slower circadian clocks and shorter and longer development time (Kumar et al., 2007). However, difference in

the mean development time between the two selected populations was of the order of the difference in their mean timing of emergence. This is not surprising because fly populations in this study were subjected to selection for different timing of adult emergence and not for different rates of pre-adult development. No evidence for genetic correlation between τ and development time was observed in a recent study in the adzuki bean beetle, *Callosbruchus chinesis*, suggesting that circadian clocks do not regulate pre-adult development (Harano and Miyatake, 2011). Thus, results of previous studies only provide suggestive evidence for involvement of circadian clocks in the temporal regulation of development.

Fruit flies inhabit ephemeral habitats and therefore they are exposed to directional selection for faster development (Nunney, 1990; Prasad and Joshi, 2003). Moreover, faster development may provide flies with the opportunity for early mating and reproduction. Timing of adult emergence depends upon the developmental state of the fly, the phase, and period of developmental clocks and upon ambient environmental conditions (Qui and Hardin, 1996; Paranjpe et al., 2005; Mukherjee et al., 2012). Further, it is believed that a continuously consulted clock reads the developmental state of the fly and only those flies that are mature to emerge during the gate are allowed to emerge while others are forced to remain within their puparium until the next gate opens. Therefore, links between circadian clocks and pre-adult development time can also be taken to suggest that circadian clocks have adaptive significance and it is likely that some insect species accrue greater fitness advantage by speeding up pre-adult development. Studies on selection for faster pre-adult development showed pronounced reduction in development time (Chippindale et al., 1994, 1997; Zwaan et al., 1995a; Nunney, 1996; Prasad et al., 2000, 2001) and trade-offs between development

time and adult weight (Chippindale et al., 1994; Nunney, 1996), viability (Chippindale et al., 1997; Prasad et al., 2000) and adult lifespan (Partridge and Fowler, 1992; Chippindale et al., 1994). These trade-offs are inevitable as time is a fundamental aspect in insect development and each stage of development right from cell division to the formation of phenotypes needs to be timed appropriately (Moss, 2007).

Here we report the results of our long-term study on four large, outbred, populations of *D. melanogaster* where flies were selected to develop faster as pre-adults in order to examine if circadian clocks are linked to pre-adult development. Since we were interested in flies with functional circadian clocks we decided to carry out our selection study under constant dark (DD) conditions because under LL, circadian clocks of *Drosophila* are rendered dysfunctional (Konopka et al., 1989), while under LD, variation in τ is likely to be very low (Paranjpe et al., 2005). At intervals of 5-10 generations, we assayed time to hatching, pupation, wing pigmentation and emergence, and also τ of locomotor activity rhythm, to study the effect of selection, on the durations of various pre-adult stages and on the τ of circadian clocks. The results provide evidence for a role of circadian clocks in the regulation of pre-adult development time in *Drosophila*.

3.2. Materials and Methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by a method described in detail in chapter 2. Standardized populations were used for various assays described below.

3.2.1. Assay of pre-adult (egg-to-adult) development time: Temperature (25 ± 0.5 °C) and relative humidity (75 ± 10%) under DD were monitored continuously using Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co, Japan and were found to be constant throughout the assays. For the development time assays, standardized flies from $FD_{1\text{-}4}$ and $BD_{1\text{-}4}$ stocks were allowed to lay eggs on banana medium. In order to increase the egg laying capacity of females, flies were provided with banana medium supplemented with live yeast paste, two days prior to egg collection. Eggs laid under DD regime over a period of 2 h were collected with the help of a moistened fine brush under dim red light ($\lambda > 650$ nm). Exactly 30 eggs were collected and dispensed into glass vials containing 10 ml banana food. Ten such vials from each replicate population were introduced into DD thus, making a total of 80 vials for each assay (10 vials × 4 blocks × 2 selection stocks). After the pupae became dark, vials were monitored regularly for adult emergence and the number of males and females emerging in 2 h intervals was estimated. From this data, we obtained pre-adult development time of each fly, which was then used to calculate the mean development time of all flies in each vial. Pre-adult development time of a fly was defined as the duration between the midpoint of 2 h egg collection window and the midpoint of 2 h period during which the fly emerged as adult.

3.2.2. Assay of life-stage specific developmental durations: To assess the contributions of different pre-adult stages to the overall egg-to-adult emergence duration, we estimated time taken for eggs to hatch, for larvae to pupate and for the formation of wing-pigmentation. The egg-hatching time of FD and BD stocks was assayed by collecting eggs from the standardized populations and dispensing on ~0.5 sq cm agar pieces of 0.1 to 0.2 mm thickness, with

exactly 30 eggs arranged in 5 rows and 6 columns. During the collection, eggs were moistened every 4-5 min with few drops of water to prevent them from desiccating. Hatching time assay was carried out every 1 h under DD starting 12 h after egg collection. Similarly, pupation time was recorded every 2 h as time taken for flies to complete the larval instars and enter into the pupal stage (identified by two prominent spiracles on the head). The newly formed pupae were scored and their position was marked using circles. The pupae darkened in ~3 days and thereafter we continued to monitor the pupae every 2 h for the formation of wing-pigmentation. It is generally considered that no further changes in pupae occurs after the wing pigments are formed (Harker 1965; Lorenz et al., 1989; Qiu and Hardin 1996) and therefore for all practical purposes, time till pigmentation can be considered as the maturation time of flies. The pigmentation time was recorded by examining the vials every 2 h and by marking a cross on the previously encircled pupae when pigmentation occurred.

3.2.3. Locomotor activity assay: Starting at the 5th generation, locomotor activity rhythm of flies from $FD_{1\text{-}4}$, and $BD_{1\text{-}4}$ stocks was monitored every 5-10 generation for a minimum of 10 days. The locomotor activity rhythm of freshly emerged virgin males was individually recorded under DD using Drosophila Activity Monitoring (DAM) system from Trikinetics, USA. The DAM system is a simple computer-aided device that uses a pair of infrared emitter and sensor to detect the movement of individual flies in a narrow glass tube (0.6 cm inner diameter × 4 cm long). The τ of the locomotor activity rhythm was estimated using Lomb Scargle Periodogram in CLOCKLAB from Actimetrics, USA.

3.2.4. Statistical analyses: Data from development time assays were subjected to composite mixed model analysis of variance (ANOVA), treating replicate population (block - B) as a

random factor and stock (S) and generation (G) as fixed factors crossed with block. In all cases block means were used as the unit of analysis and hence, only the fixed factor could be tested for statistical significance. Also the τ values were subjected to separate two-way ANOVA treating replicate population as random factor and stock and generation as fixed factors. Post hoc multiple comparisons were done using Tukey's HSD test. All statistical analyses were implemented using STATISTICA^{TM} for Windows Release 5.0 B (StatSoft Inc. 1995).

3.3. Results

3.3.1. Shortening of egg-to-adult duration in flies selected for faster pre-adult development: Although the mean development time of flies varied considerably from one assay generation to another (Figure 3.1a), the difference in development time between selected and control stocks increased gradually with increasing generations (Figure 3.1b). The rate of shortening of development time in FD slowed down twice, first between 5th and 15th generations and then between 25th and 40th generations (Figure 3.1b). After 25 generations of selection, FD began developing faster to an extent that BD began to emerge only after all FD flies had emerged (Figure 3.2). Thereafter, FD continued to develop faster than BD and after 50 generations of selection, its mean development time became shorter than BD by ~29 h (12.5%; Figure 3.1a, b). Reduction in male development time (~30 h) was marginally greater than females (~28 h; Figure 3.1c). ANOVA on the development time data showed a statistically significant effect of generation (G) ($F_{9,27}$ = 170.96; $p < 0.0001$), stock (S) ($F_{1,3}$ = 23.51; $p < 0.0001$) and G × S interaction ($F_{9,27}$ = 45.37; $p < 0.0001$; Table 3.1). Post hoc multiple comparisons using Tukey's test revealed that in all the assay generations,

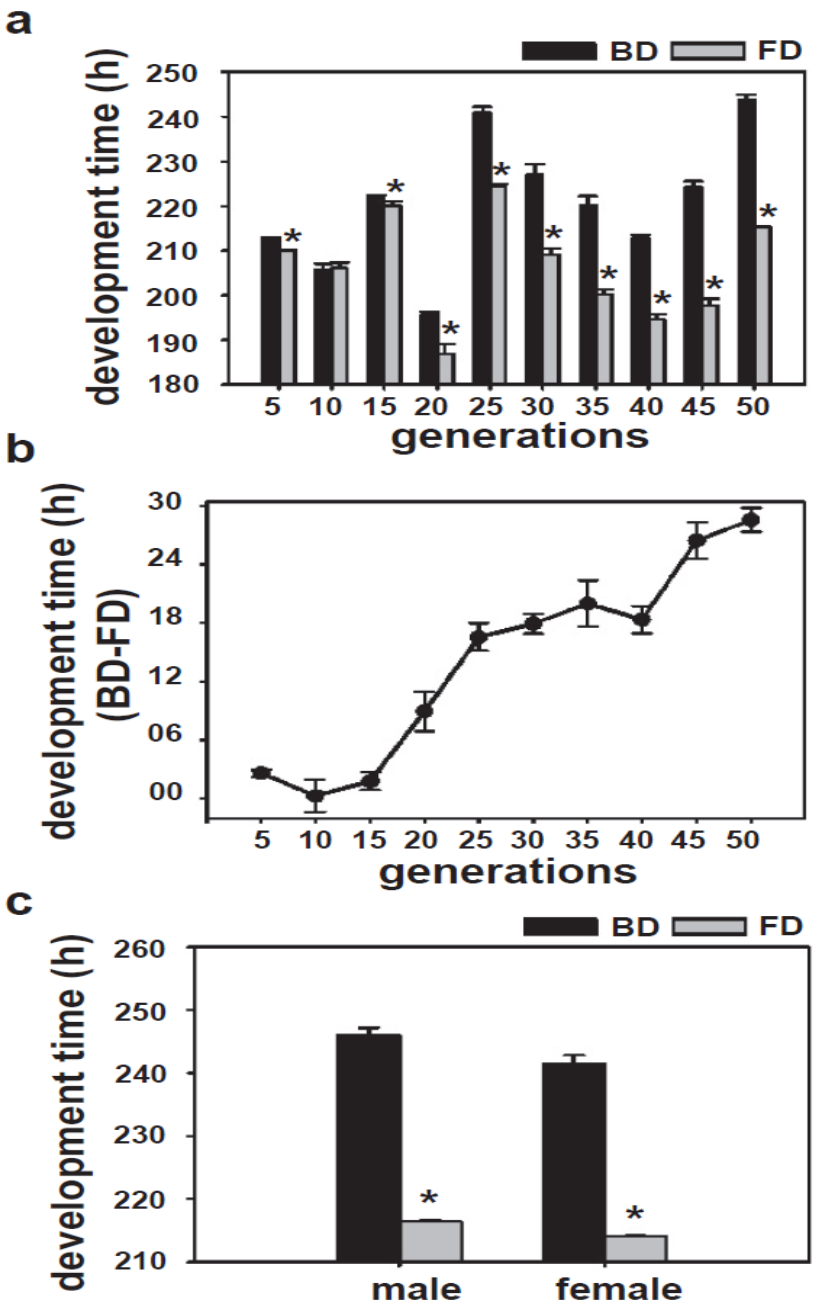


Figure 3.1. Shortening of pre-adult development time in the selected (FD) compared to control (BD) stocks: Average pre-adult development time of FD and BD stocks (under DD) at various assay generations (a). The differences in pre-adult development time between FD and BD stocks at regular intervals of 5 generations shows the effect of selection on pre-adult development time (b). By the 50th generations of selection, males and females from FD stocks developed significantly faster than those of BD stocks (c). The error bars are standard error around the mean (SEM). Statistically significant differences are indicated by asterisks.

Table 3.1 Results of ANOVA on development time data across generations.

Effect	*df*	MS Effect	df Error	MS Error	*F*	*p*-level
Generations (G)	9	11833.25	27	69.22	170.96	0.0001
Stocks (S)	1	38084.86	3	119.21	319.51	0.0001
Blocks (B)	3	119.66	704	5.09	23.51	0.0001
G × S	9	2010.13	27	44.30	45.37	0.0001
G × B	27	69.22	704	5.09	13.59	0.0001
S × B	3	119.21	704	5.09	23.41	0.0001
G × S × B	27	44.30	704	5.09	8.71	0.0001

Table 3.2a. Results of ANOVA on time until pupation (gen 50).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	7129.42	3	14.11	505.53	0.0002
Blocks (B)	3	10.11	72	0.47	21.68	0.0001
S × B	3	14.11	72	0.47	30.28	0.0001

Table 3.2b. Results of ANOVA on time until pigmentation (gen 50).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	13232.51	3	57.53	229.99	0.0006
Blocks (B)	3	77.25	72	1.88	41.13	0.0001
S × B	3	57.53	72	1.88	30.63	0.0001

Table 3.2c. Results of ANOVA on time until hatching (gens 40 and 50).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Generation (G)	1	77.99	3	2.49	31.34	0.0113
Stocks (S)	1	31.87	3	0.17	184.52	0.0008
Blocks (B)	3	2.07	74	0.47	4.39	0.0067
G × S	1	2.80	3	1.04	2.70	0.1991
G × B	3	2.49	74	0.47	5.29	0.0023
S × B	3	0.17	74	0.47	0.37	0.7768
G × S × B	3	1.04	74	0.47	2.21	0.0943

development time of FD was significantly shorter than BD. This suggests that flies selected for faster development evolve shorter pre-adult development time.

3.3.2. Shortening of time to pupation and pigmentation in flies selected for faster pre-adult development: To study the effect of selection for faster pre-adult development on the pupation and pigmentation time, we assayed these durations in FD and BD stocks in the 50th generation. The average reduction in pupation and pigmentation time of FD stocks compared to BD stocks was ~16.9 h and ~25.2 h, respectively (Figure 3.3a, b). ANOVA followed by post hoc multiple comparisons using Tukey's test revealed that pupation and pigmentation time of FD was significantly shorter than BD (pupation – $F_{1,3}$ = 505.53; p < 0.0002; pigmentation – $F_{1,3}$ = 229.99; p < 0.0006; Figure 3.3a, b; Table 3.2a, b). These results suggest that selection for faster pre-adult development shortens time to pupation and pigmentation.

3.3.3. Shortening of egg hatching time in flies selected for faster pre-adult development: By the 50th generation, even the egg hatching time was reduced in FD compared to BD (Figure 3.4a-c). ANOVA on the egg-hatching time data at 40 and 50th generation, showed a statistically significant effect of stock (S) ($F_{1,3}$ = 184.52; p < 0.0008) and generation (G) ($F_{9,3}$ = 31.34; p < 0.01), however, the effect of S × G interaction did not reach statistically significant levels ($F_{1,3}$ = 2.70; p = 0.20; Table 3.2c). Post hoc comparisons using Tukey's test revealed that egg hatching time in FD was significantly shorter than BD. These results suggest that selection for faster pre-adult development results in a significant reduction in egg hatching time.

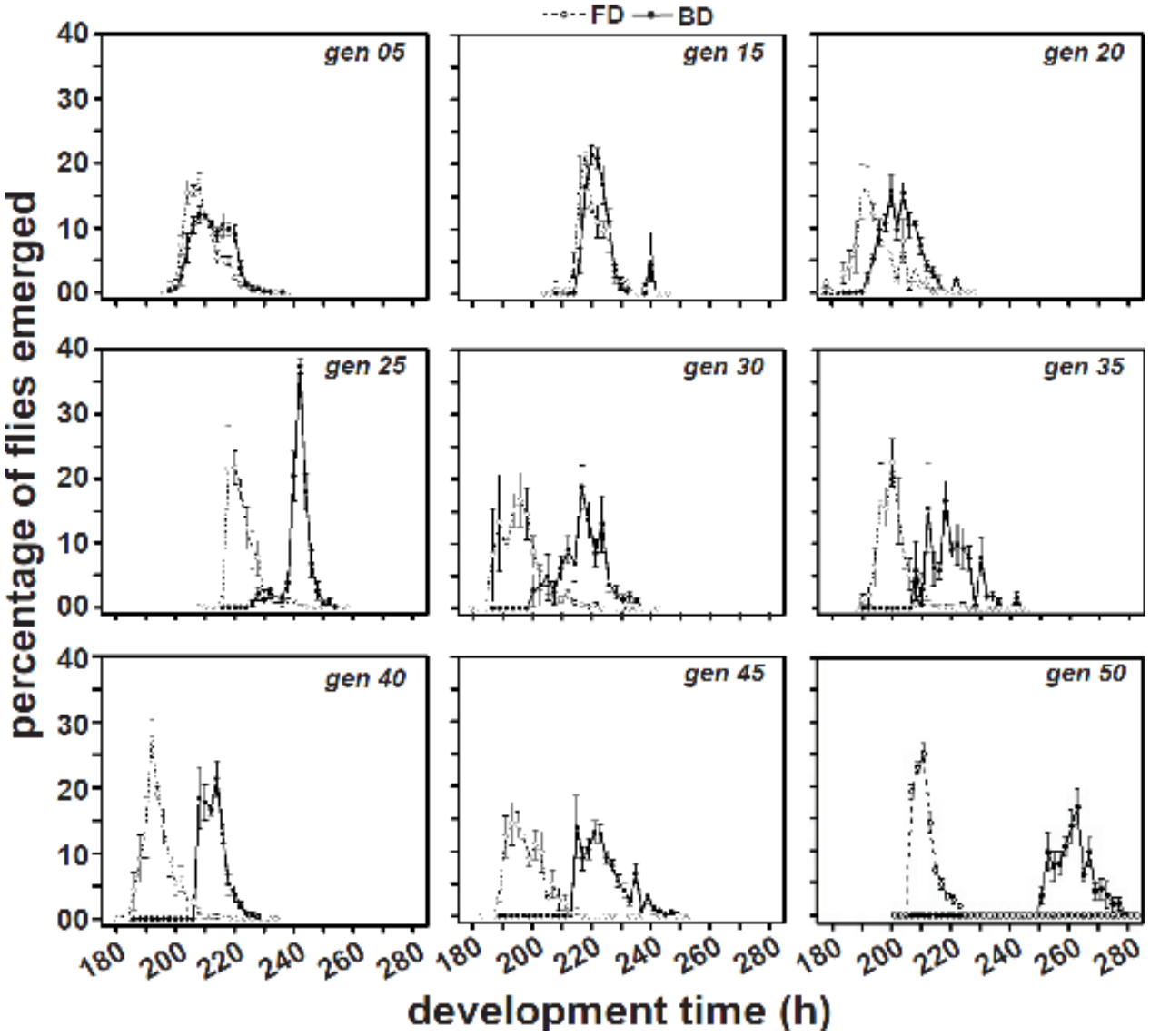


Figure 3.2. Response of selection for pre-adult development on adult emergence profile: Selection for faster preadult development under constant dark (DD) caused a gradual shift in the emergence profiles of the selected flies towards lower values. The waveforms showed a significant difference in the development time of selected (FD) and control (BD) stocks at all assay generations between 5 and 50. FD and BD stocks are indicated by broken lines with open circles and black lines with close circles, respectively. The error bars are standard error around the mean (SEM). The percentage of adults emerging in 2 h bins is plotted along the *y*-axis and pre-adult development time (from the egg stage) in h is plotted along the *x*-axis.

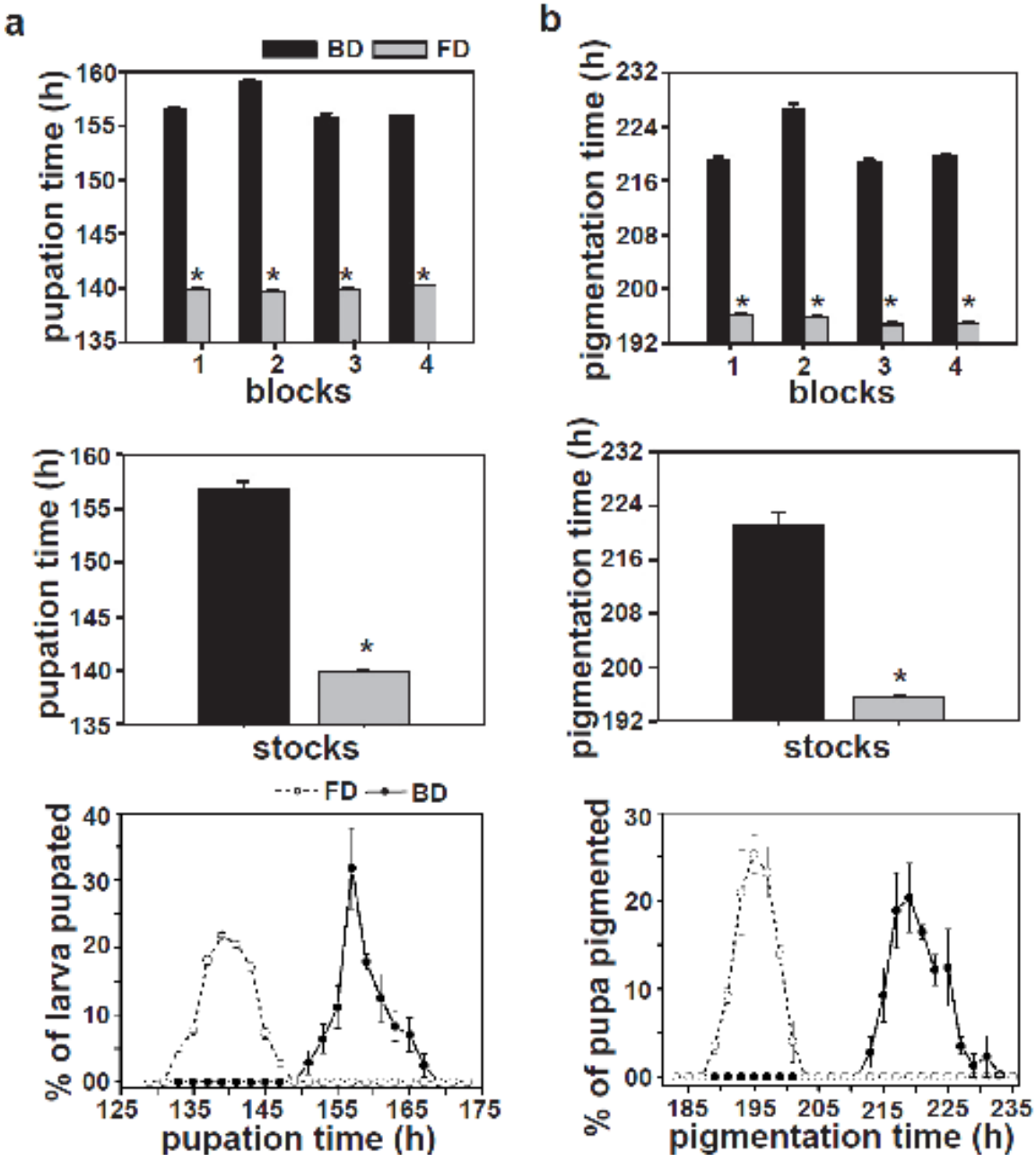


Figure 3.3. Shortening of time to pupation and pigmentation in the selected (FD) stocks: Average pupation (a) and pigmentation (b) time of four replicate populations, stock and profile (from top to bottom panels respectively) of FD and BD flies in the assay done at the 50th generation. The error bars represents standard error around the mean (SEM). Statistically significant differences are indicated by asterisks.

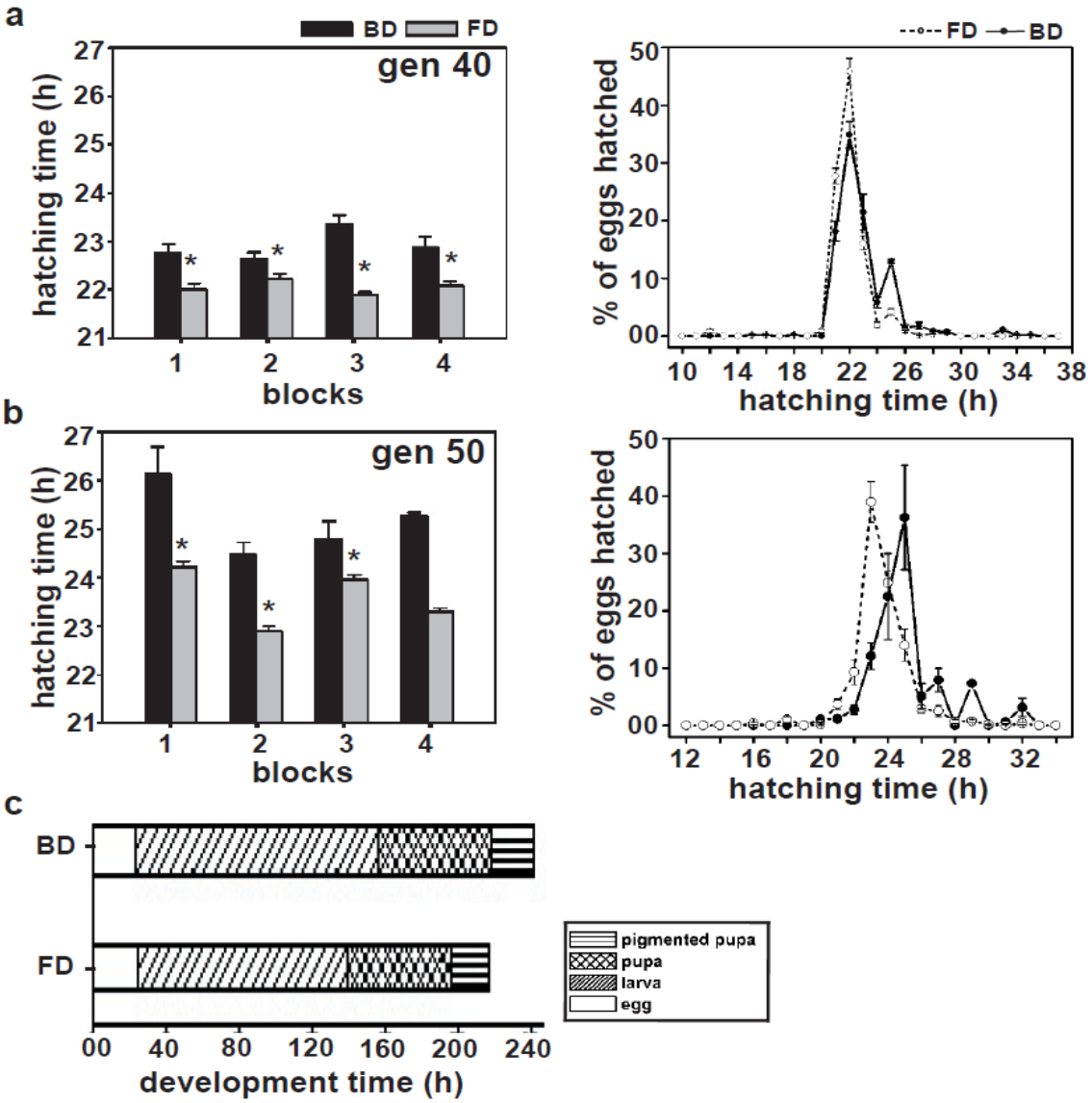


Figure 3.4. Shortening of hatching time: Egg hatching time is significantly reduced in selected (FD) stocks compared to control (BD) stocks at 40 th (a) and 50 th (b) generations. Average hatching profiles of selected (FD) and control (BD) stocks under constant darkness (DD) at 40th and 50th generations are shown in right panel of figure (a) and (b) respectively. The reduction in the durations of various pre-adult stages that contributed to the overall reduction in pre-adult development time in the faster developing flies (c). The error bars are standard error around the mean (SEM). Statistically significant differences are indicated by asterisks.

3.3.4. Shortening of clock period in stocks selected for faster pre-adult development: To study the consequence of selection for faster pre-adult development on circadian clocks, we estimated various characteristics (percent rhythmicity, period length, power and total activity) of locomotor activity rhythm of FD and BD stocks, at intervals of 5-10 generations (Table 3.3). While all the rhythm characteristics differed from one assay generation to another, FD and BD stocks did not differ with regards to any of them except period length (Table 3.3). Starting at 10^{th} generation, and in all subsequent generations, τ of activity rhythm of FD was significantly shorter than BD (Figure 3.5c), and by the 55^{th} generation it became 24.08 ± 0.04 h (mean ± SEM) in FD and 24.61 ± 0.08 h in BD (Figure 3.5c; Table 3.3). ANOVA on the τ data showed a statistically significant effect of stock (S) ($F_{1,3}$ = 89.51; $p < 0.003$), generation (G) ($F_{7,21}$ = 30.95; $p < 0.000$) and S × G interaction ($F_{7,21}$ = 4.38; $p < 0.004$; Table 3.4). Post hoc multiple comparison using Tukey's test revealed that in all except 5^{th} generation, τ of FD was significantly shorter than that of BD. These results suggest that selection for faster pre-adult development results in a correlated shortening of τ of activity rhythm.

3.4. Discussion

Selection for faster pre-adult development resulted in a significant reduction of pre-adult development time. After 40 generations of selection, the overall egg-to-adult duration in selected stocks became ~18 h shorter than controls (Figure 3.1), with a concurrent reduction in pupation time by ~12.5 h. This suggests that almost 70% of the total reduction in pre-adult development time occurs during the egg and larval stages (Figure 3.4c), while the remaining stages account for ~30% reduction. While these results are in a way consistent with those of Prasad et al. (2001), it is in sharp contrast to those of Chippindale et al. (1997) study, where

pupal duration was found to remain unchanged in populations selected for faster pre-adult development. We also observed a significant reduction in egg hatching time in the selected stocks compared to controls (Figure 3.4a), which once again is in contrast to the findings of Chippindale et al. (1997) study. Such differences in outcome are likely to be due to the way selection was carried out in these studies. For example, in our study we collected eggs on the 11^{th} day after emergence, whereas in the Chippindale et al. (1997) study, eggs were collected as soon as there were enough eggs available (i.e., within 24 h of emergence). This suggests that by collecting eggs when flies were matured allowed flies to compensate for the postponement of some of their reproduction related processes such as maturation of ovary, ovariole and sperm (Prasad and Joshi, 2003). Therefore, relatively relaxed selection pressure on the timing of reproduction in our study as opposed to very early reproduction in Chippindale et al. (1997) or moderately early reproduction in Prasad et al. (2001) study may have allowed the evolution of reduced egg and pupal durations in the faster developing stocks. Furthermore, this suggests that, in our study, reduction in development time in the faster developing populations was entirely due to selection for faster development and not due to selection for reproductively immature flies. In our generation-wise assays, development time was found to vary considerably from one assay generation to another (Figure 3.1a), however, the selected stocks invariably developed significantly faster than controls which is evident from the plot where difference in development time between the selected and control populations is plotted as a function of assay generation (Figure 3.1b).

Table 3.3 Generation-wise details of circadian rhythms of selected and control stocks

Generations	Stocks	N (flies used)	% rhythmicity	Period (h mean ± SEM)	Power (mean ± SEM)	Total activity (mean ± SEM)
5	FD	102	85.3	24.55 ± 0.05	146.94 ± 9.13	2481.5 ± 39.7
	BD	115	84.3	24.53 ± 0.06	188.59 ± 9.07	2205.3 ± 36.9
10	FD	113	86.3	24.36 ± 0.06	155.11 ± 7.62	2278.9 ± 60.2
	BD	114	88.6	24.57 ± 0.04	179.03 ± 8.75	1947.1 ± 15.1
15	FD	58	91.4	24.45 ± 0.08	199.21 ± 16.4	481.5 ± 39.7
	BD	61	78.7	24.77 ± 0.07	153.98 ± 9.7	1565.2 ± 14.1
20	FD	75	76.4	23.92 ± 0.06	80.51 ± 4.62	1975.4 ± 17.7
	BD	60	81.7	24.69 ± 0.10	58.46 ± 2.41	1936.6 ± 30.7
25	FD	98	71.4	23.09 ± 0.05	125.41 ± 6.15	1801.6 ± 37.8
	BD	95	75.8	24.31 ± 0.07	115.96 ± 5.71	1750.8 ± 31.6
35	FD	80	81.2	24.01 ± 0.05	145.63 ± 8.52	2186.0 ± 19.0
	BD	100	80.0	24.26 ± 0.07	57.91 ± 9.71	2530.1 ± 25.1
45	FD	103	82.5	23.81 ± 0.04	255.74 ± 16.5	3015.3 ± 17.4
	BD	106	81.1	24.19 ± 0.06	249.65 ± 13.6	2924.1 ± 14.3
55	FD	100	95.0	24.08 ± 0.05	305.02 ± 14.6	2983.1 ± 56.0
	BD	119	85.7	24.61 ± 0.06	251.57 ± 14.1	2936.8 ± 30.2

Table 3.4 Results of ANOVA on circadian period data across generations.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Generations (G)	7	11.11	21	0.36	30.95	0.000
Stocks (S)	1	34.55	3	0.39	89.51	0.003
Blocks (B)	3	1.04	1495	0.28	3.69	0.012
G × S	7	1.38	21	0.32	4.39	0.004
G × B	21	0.36	1495	0.28	1.27	0.187
S × B	3	0.39	1495	0.28	1.36	0.252
G × S × B	21	0.32	1495	0.28	1.12	0.323

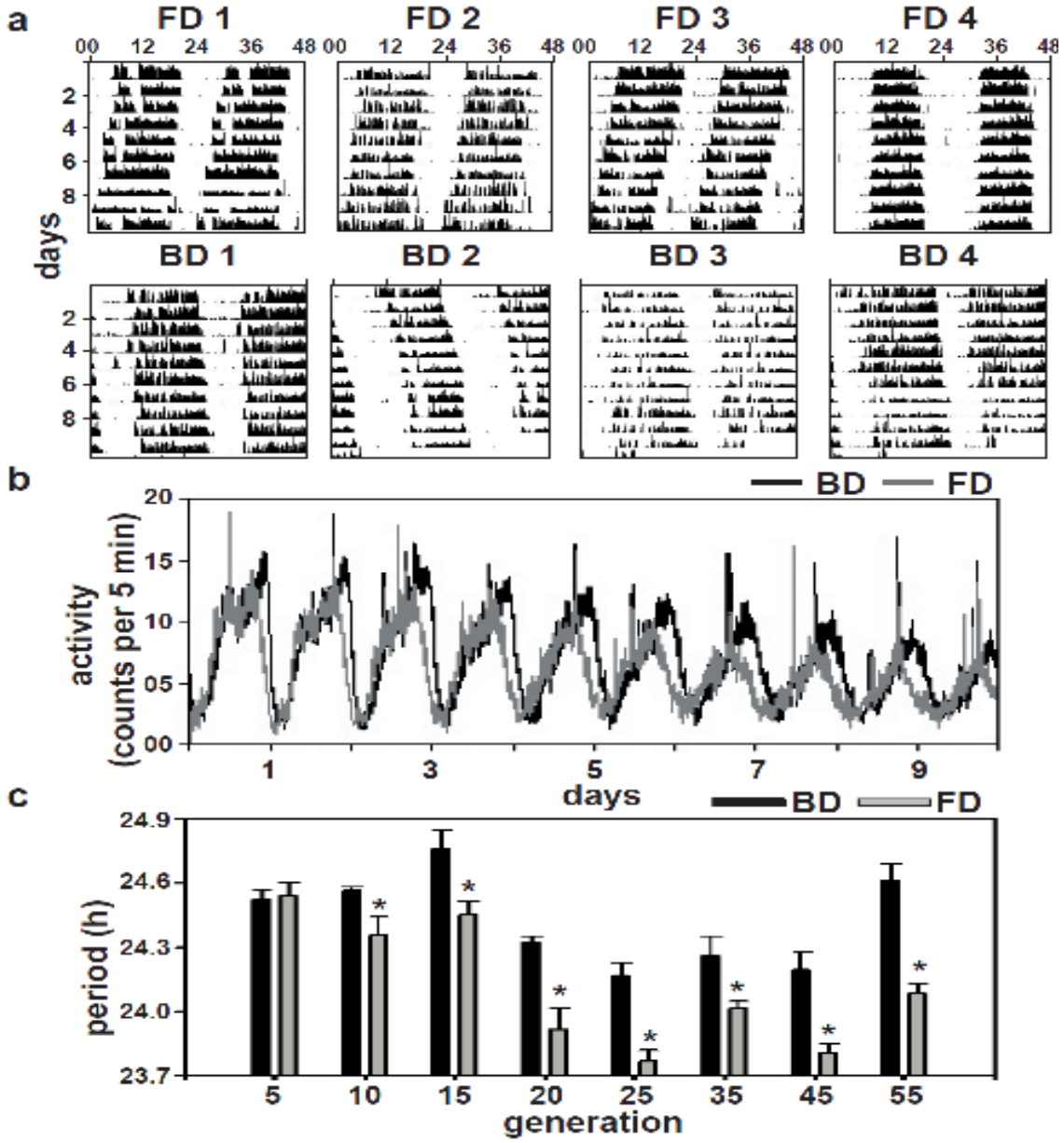


Figure 3.5. Shorter mean circadian period in selected flies: One representative actogram each of male flies from each replicate populations of selected (FD) and control (BD) stocks. Time (in h) is plotted along the *x*-axis and days in chronological manner are plotted along the *y*-axis (a). The double plotted actograms show activity data from the locomotor activity assays done under constant darkness (DD) at the 50th generation. The average activity profiles (b) of FD (grey curve) and BD (black curve) stocks clearly shows a gradual shift in the daily activity profile of selected population compared to controls. Shorter mean circadian period of locomotor activity rhythm of FD stocks compare to controls (BD) in generation-wise assays (c). The error bars are standard error around the mean (SEM). Statistically significant differences are indicated by asterisks.

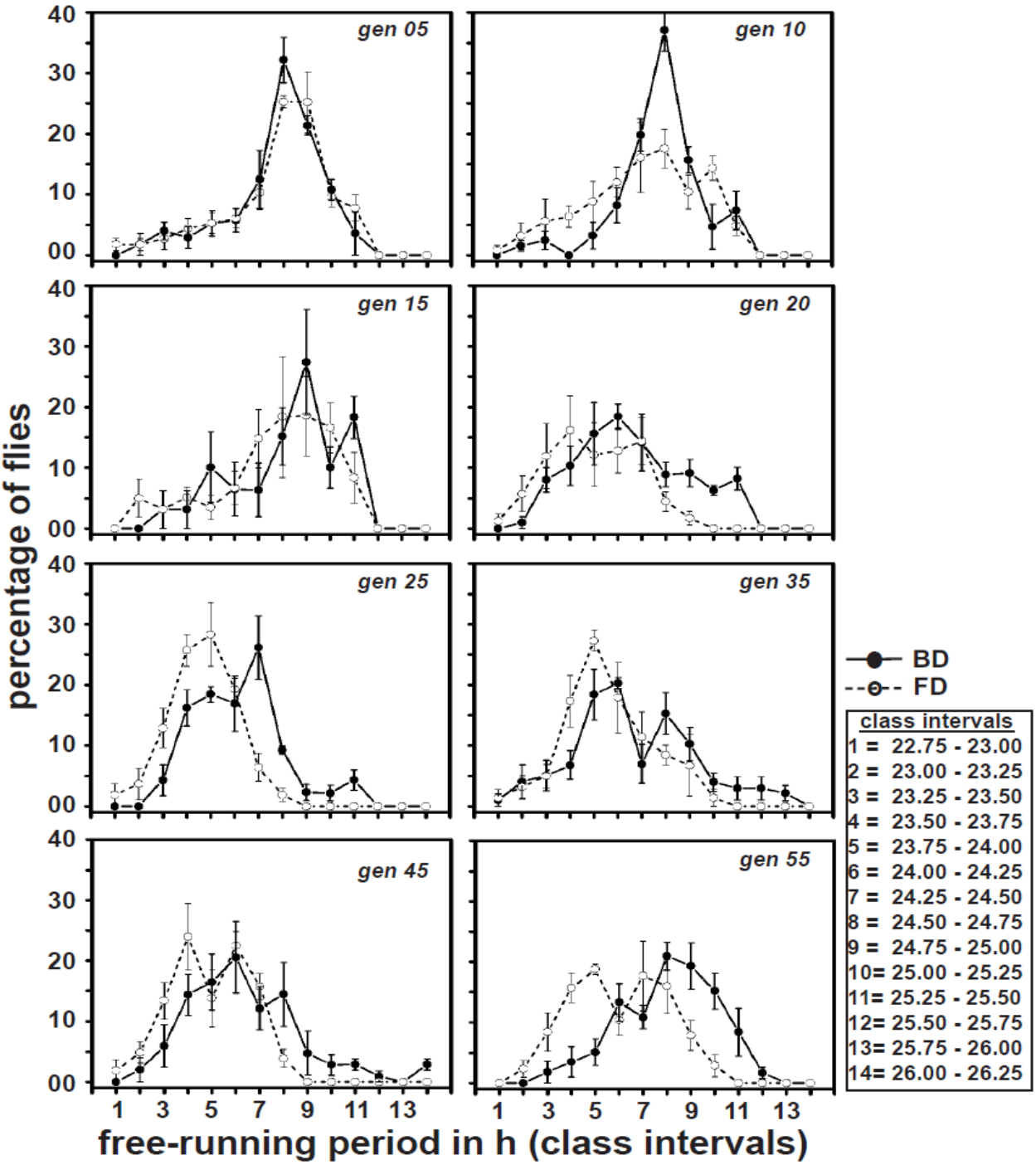


Figure 3.6. Frequency distribution of mean circadian period of FD and BD stocks: The distribution of circadian period of FD (broken line with open circle) diverged gradually from that of the BD (black line with close circle) stocks. The error bars are standard error around the mean (SEM). Percent of flies is plotted along *y*-axis, whereas the class intervals of circadian period are plotted along *x*-axis.

Such generation-wise variation was also noticed in pupation, pigmentation and hatching time when we compared data collected in the 40th, 50th (Figures 3.2, 3.4) and 70th generation assays (data not shown). This is not unexpected as it is very well established that slight perturbation in environmental factors such as temperature (Ashburner et al., 2005), larval density (Mueller, 1997) and nutrition (Sang, 1956) affects the pre-adult development time of *D. melanogaster* by a large magnitude. Such environment-dependent differences in pre-adult development time between separate assays are not unusual even in the most stringently controlled experimental protocol (Chippindale et al., 1997; Prasad et al., 2001). Therefore, comparisons for genetic differences in development time should always be made between the selected and control stocks and not across generations.

Larval stages are most critical in the life-history of *Drosophila* because final size of the third instar larvae eventually determines the body size of the adult (Bakker, 1959). Furthermore, selection for faster pre-adult development is largely mediated via the reduction of larval duration, which is often constrained by the necessity for third instar larvae to attain a minimum critical size in order to successfully pupate and emerge. The minimum critical size is greatly determined by the environment (de Moed et al., 1999) and genetic background (Robertson, 1963). Although, we did not collect data on minimum critical size, given the extent of reduction in pupation time of the selected populations (Figure 3.3a), it is likely that selected stocks have evolved the ability to attain minimum critical size much faster than controls.

The end of pupal stage is marked by wing pigmentation, which comprises of ocellar and bristle pigmentation (Qiu and Hardin, 1996). Interestingly we found that there was a further ~3.4 h shortening (data not shown) in the pupal duration post wing-pigmentation

(Figure 3.3a, b), which suggests the possibility of some yet unknown mechanisms involved in the shortening of pre-adult duration in the selected stocks.

Drosophila females mature faster than males and thus the sex ratio of flies that were selected in every generation is likely to be skewed (Prasad and Joshi, 2003). Therefore, it is possible that selection for faster pre-adult development would lead to either an altered primary sex ratio in favour of females, or a reduction in development time difference between the two sexes, because males are likely to experience stronger selection pressure for faster development than females (Prasad and Joshi, 2003). However, in our study neither did we find any sex-specific difference in development time, nor any change in primary sex ratio in the selected stocks (data not shown).

The distribution of pre-adult development time and τ of activity rhythms in the selected stocks gradually diverged from those of controls (Figures 3.2, 3.6). Such changes in circadian period of selected stocks was due to heritable changes in its genetic architecture, as a result of selection for faster development, which suggests that circadian clocks evolve as a correlated response to selection for faster pre-adult development. Until the 5^{th} generation, τ of selected stocks did not differ from controls (Figure 3.5c). By the 20^{th} generation, a shortening of ~0.38 h was noticed in the selected stocks, which subsequently increased to ~0.65 h (2.5% of τ shortening) by the 55^{th} generation. Such gradual shortening of τ in the selected populations in conjunction with similar reduction in development time (~29 h corresponds to shortening of 12% of fruit flies development) suggests that circadian clocks and pre-adult development time in *D. melanogaster* are linked. However, the connection is unlikely to be causal, because magnitude of the differences in circadian period of faster

developing and control stocks (~0.65 h) is unlikely to create a difference of ~29 h in the mean development time, implying that connections between circadian clocks and pre-adult development in *Drosophila* may not be linear.

Under DD, circadian clocks are expected to free-run and therefore pre-adult duration is likely to be determined by an interaction between circadian gating and developmental states of the fly. Considering that circadian gate would set-in early in faster developing stocks, these flies should develop faster than controls only by ~7 h, assuming that it takes about 10 days for control flies to complete development under DD at 25 ^{o}C (Paranjpe et al., 2005). Contrary to this expectation, the selected stocks emerged ~29 h faster than controls. This suggests the following possibilities: (i) only a part of the pre-adult developmental processes is under the control of circadian clocks, and/or (ii) period of circadian clocks that operate during pre-adult developmental stages are correlated to but not the same as those in adults, or (iii) circadian clocks may not have any causal role in the temporal regulation of development, and inadvertently we may have independently selected for flies with faster development and shorter circadian period.

3.5. Conclusions

Our study on selection for faster pre-adult development on four large outbred populations of fruit flies *D. melanogaster* performed under DD for more than 50 generations provides the first ever rigorous, systematic and unequivocal evidence for the connection between circadian clocks and development in fruit flies *D. melanogaster*.

Chapter 4

Role of circadian clocks in timing pre-adult developmental events in fruit flies *Drosophila melanogaster*

4.1. Introduction

In a number of holometabolous insects, circadian clocks have been implicated in the regulation of life history traits such as pre-adult development time and lifespan (Kyriacou et al., 1990; Miyatake, 1996, 1997a; Shimizu et al., 1997; Sharma, 2003; Paranjpe and Sharma, 2005; Kumar et al., 2006, 2007; Lone and Sharma, 2008; Yadav and Sharma, 2013; Takahashi et al., 2013). Studies in insects including some species of *Drosophila* reported rhythmicity in events such as egg-hatching (Pittendrigh, 1966; Minis and Pittendrigh, 1968; Nayar et al., 1973; Lazzari, 1991), pupation (Bakker and Nelissen, 1963; Pittendrigh and Skopik, 1970) and wing-pigmentation (Harker, 1964, 1965), which suggests a role of circadian clocks in the temporal regulation of pre-adult development. In fruit flies *D. melanogaster,* strains with faster-running clocks were found to complete their pre-adult development earlier than strains with slower clocks (Kyriacou et al., 1990). Such correlation between clock period (τ) and development time was also reported in studies on populations selected for faster or slower pre-adult development (Miyatake, 1996, 1997a; Shimizu et al., 1997; Yadav and Sharma, 2013), or for morning and evening adult emergence (Kumar et al., 2007). Furthermore in fruit flies *D. melanogaster*, pre-adult developmental duration and period of adult emergence rhythm were reported to be positively correlated, suggesting pre-adult development time to be a function of the period of circadian rhythm and/or of LD cycles (Paranjpe et al., 2005). Together, this evidence has led to the notion that circadian clocks play a key role in the regulation of pre-adult development time in *D. melanogaster* (Prasad and Joshi, 2003; Sharma, 2003; Paranjpe and Sharma, 2005).

Like other holometabolous insects, pre-adult development in fruit flies *D. melanogaster* includes three discrete developmental stages – eggs, larvae and pupae. At an ambient temperature of 25 ^{o}C, pre-adult development spans ~9 days. Eggs typically take 18–24 h to

hatch into larvae. The larval stage spans for about 4 days, during which developing larva passes through 3 instars (substages) (Ashburner et al., 2005). The pupal stage starts after the third instar larval stage, lasts for another 4 days, subsequently leading to wing-pigmentation followed by adult emergence.

Previously we reported that (Yadav and Sharma, 2013; Chapter 3), populations of fruit fly *D. melanogaster* subjected to selection for faster pre-adult development under constant dark (DD) conditions evolved faster rate of pre-adult development (~29 h shorter than control at 50th generation) and circadian clocks with shorter free-running period (~0.5 h faster than controls). Evolution of circadian clocks in the faster developing (FD) populations suggests links between circadian clocks and developmental processes, similar to what has been inferred from many other previous studies (Kyriacou et al., 1990; Miyatake, 1996, 1997a; Shimizu et al., 1997; Paranjape et al., 2005, Kumar et al., 2007; Takahashi et al., 2013). Additionally, we found that speeding-up of development time in the FD populations was achieved by a concurrent reduction in the duration of almost all pre-adult stages.

The event of adult emergence in many insects including *Drosophila* is known to be under the control of circadian clocks (Pittendrigh, 1954; Brett, 1955; Konopka and Benzer, 1971; Saunders, 2002b) and evidence suggests that these clocks begin ticking as early as third instar larval stage, and are functional during most part of the pre-adult development (Sehgal et al., 1992; Kaneko et al., 1997; 2000; Kowalska et al., 2010). Additionally, *Drosophila* larvae are known to show rhythmicity in light avoidance behaviour (Mazzoni et al., 2005), which is probably the first and only clock-driven behavioural rhythm in *Drosophila* during pre-adult development reported thus far. Light is known to be a potent zeitgeber for the adult emergence rhythm of fruit flies as it plays a key role in entraining developing circadian oscillators present

during early larval stages (Saunders, 1992; Vallone et al., 2007). Furthermore, adult emergence rhythm in *Drosophila* is known to be influenced by environmental light/dark (LD) conditions (Paranjpe et al., 2005; Mukherjee et al., 2012) and hence has been used as a means of modulating the underlying circadian oscillators (Saunders, 2002b). Moreover, LD regimes have been shown to have significant impact on pre-adult development time; being fastest under constant light (LL), followed by LD12:12 and slowest under DD (Sheeba et al., 1999b; Paranjpe et al., 2005; Lone and Sharma, 2008). Further, pre-adult developmental duration in *Drosophila* is also found to be positively correlated with the period of LD cycles (*T*-cycles), suggesting a role of the rhythm period and/or of LD cycles in the regulation of pre-adult development time (Paranjpe et al., 2005). Hence, entrainment of *Drosophila* circadian clocks under LD cycles would result change in development time according to the period of *T*-cycles. While it is expected that pre-adult development time would be affected by light regimes, which of the developmental stages are affected the most is largely unknown. In this study, considering circadian clock's ability to entrain to LD cycles of a range of periodicities, we assayed the duration of various pre-adult stages (egg-to-hatching, larva-to-pupation, wing-pigmentation and adult emergence) of selected and control flies under three different LD cycles (*T24*, *T20* and *T28*) and in constant light/dark conditions (LL and DD), to test the causal role of differences in circadian clock period between FD and BD stocks to the differences in their pre-adult development time. If difference in development time between FD and BD (i.e., under DD) is clock-mediated, then difference in development time between FD and BD (for a given developmental stage) would be expected to reduce (a) under LD cycles, owing to the same pace of their circadian clocks due to their entrainment to LD cycles and (b) also under LL, as their circadian clocks would become dysfunctional. If a difference in development duration persisted in these regimes, it can be

attributed to clock-independent developmental factors. Analysis of durations of various pre-adult developmental stages such as egg, larva, pre and post–wing-pigmentation pupal stages relative to the egg stage revealed that results are consistent with our hypothesis, i.e., durations of most of these stages were shorter in LD cycles (and LL for egg-hatching) compared to those in DD. These results therefore imply involvement of circadian clocks to the difference in durations of pre-adult developmental events between FD and BD stocks. However, entraining LD cycles or rhythm abolishing condition LL could not eliminate the difference in durations of developmental stages between FD and BD completely, thus suggesting the role of clock-independent developmental factors also, to the difference in development time between FD and BD.

4.2. Materials and Methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by a method described in detail in chapter 2.

Standardized populations were used for various assays described below.

4.2.1. Egg-hatching time assay: Eggs of approximately identical age were collected from the standardized populations by placing a fresh food plate in the population cage for 1 h. The plate was then replaced by a fresh food plate for 1 h. Therefore, antecedent eggs retained in female body were avoided in all assays. Eggs were collected from the food plate and dispensed on 0.5 cm^2 agar piece with exactly 30 eggs arranged in 5 rows and 6 columns and placed in petri-dishes for ease of observation of the hatching process. During the egg collection, eggs were moistened every 4-5 min with few drops of water to prevent them from drying. Since *D. melanogaster* eggs start hatching ~18-24 h after being laid and all eggs hatch in few hours, we assayed egg-hatching

time every 1 h, starting 12 h after egg collection. The egg-hatching time assay was done under LL, *T24* and DD conditions. From this data, egg-hatching time was estimated as the time interval between the mid-point of 1 h egg collection window and of the 1 h assay duration during which it hatched. Since each FD population was derived from its respective BD population, egg-hatching time difference was calculated by subtracting hatching time of FD_1 from BD_1, similarly egg-hatching time of FD_2, FD_3 and FD_4 were subtracted from BD_2, BD_3 and BD_4, respectively. This method was also applied for other assays such as pupation, pigmentation and egg-to-adult development time assays.

4.2.2. Pupation and pigmentation time assays: In order to assess the contribution of two major pre-adult stages (larval and pupal) to the light regime mediated changes in the overall egg-to-adult emergence duration, we performed three separate experiments namely the pupation time (duration from egg-to-termination of third instar larval stage), wing-pigmentation time (duration of egg-to-wing pigmentation formation stage) and pre-adult development time assays (duration from egg-to-adult emergence stage). Starting the third day after egg collection, vials were continuously monitored every 2 h for pupae (when third instar larva encapsulates inside a hard and dark colored puparium). In each regime, 30 eggs were placed in each glass vial containing 6 ml of banana food and 10 such vials were used for each population. The number of larvae that had pupated in each vial was checked at every 2 h interval. Newly formed pupae were scored and marked with a circle. These 2 hourly checks were continued until no new pupae were formed for 2 consecutive days. The pupae develop pigments on its wings before emerging, so we continued the same experimental set-up for pigmentation assay (blackening of mature a pupa, which is considered as the complete maturation of pre-adult development). The wing-

pigmentation time was recorded every 2 h by marking a cross sign on the encircled mature pupae already marked during the pupation time assay.

4.2.3. Egg-to-adult development time assay: For the pre-adult development time assay, flies from standardized $FD_{1\text{-}4}$ and $BD_{1\text{-}4}$ stocks were allowed to lay eggs on banana food. In order to increase the egg-laying capacity of flies, 2 days prior to egg collection live yeast paste was supplemented on banana food. Flies were allowed to lay eggs for 2 h and exactly 30 eggs were collected and dispensed into long glass vials containing 10 ml banana food. Ten vials for each replicate population were introduced into five light regimes (LL, *T20*, *T24*, *T28* and DD). Thus, a total of 400 vials were set-up for this assay (10 vials × 8 populations × 5 light regimes). Eggs were collected under microscope with the help of a moistened '000' size brush and introduced into *T20*, *T24* and *T28* at the start of the light phase. Egg collection for the assays and observation and handling of flies under DD and dark phase of LD was done with the help of a red lamp ($\lambda > 650$ nm). The light phase of LD and LL was created with the help of a fluorescent white light of intensity ~100 lux (0.15 W/m^2). To estimate egg-to-adult development time, we monitored daily the vials with eggs for darkened pupae. Once the pupae became dark, vials were regularly monitored for freshly emerging adults. The individuals were sexed and number of males and females emerging every 2 h from each vial were counted. These 2 hourly checks were continued until no flies emerged from the vials for 3 consecutive days. Mean pre-adult development time for eggs in each vial was calculated. Pre-adult development time of a fly was calculated as the duration between the midpoint of 2 h egg collection window and the midpoint of 2 h period during which the fly emerged as adult.

4.2.4. Statistical analyses: Egg-to-adult developmental durations and the durations of various pre-adult events relative to beginning of egg stage such as egg-to-hatching, egg-to-pupation and

egg-to-wing pigmentation under different light regimes were analyzed separately using mixed model analysis of variance (ANOVA) in which replicate populations (Block-B) were treated as random factor, light regime (L) and stocks (S) as fixed factors crossed with populations. Post-hoc multiple comparisons were done using Tukey's test. All analyses were implemented on STATISTICA for Windows Release 5.0 B (StatSoft, 1995). In all cases block average, i.e., average of replicate vials in a population was used as the unit of analysis and hence, only the fixed factor could be tested for significance.

4.3. Results

4.3.1. Egg-hatching time assays: Since egg-hatching in *Drosophila* typically lasts for 18–24 h, and requires very high resolution of data to pick-up any differences between selected and control populations (Yadav and Sharma, 2013), we estimated this duration only under the most divergent regimes – LL, *T24* and DD. Following 40 generations of selection, egg-hatching waveform of FD was shifted towards lower values compared to BD under all the three regimes (Figure 4.1a) and the duration of egg stage in FD was reduced compared to BD by ~1.6 h in LL, ~1.6 h in *T24* and ~0.9 h under DD (Figure 4.1b). ANOVA revealed a statistically significant effect of L ($F_{2,6}$ = 104.21; $p < 0.0001$), S ($F_{1,3}$ = 12.38; $p < 0.04$), however, the effect of L × S interaction was statistically not significant ($F_{2,6}$ = 1.29; p = 0.34; Figure 4.1b; Table 4.1a). Egg-hatching time of FD was significantly shorter compared to BD under all the three light regimes (Figure 4.1b, Table 4.1a) and difference in egg-hatching time between FD and BD did not differ significantly among LL (1.61 h), *T24* (1.62 h) and DD (0.86 h) ($F_{2,6}$ = 1.29; p = 0.34, Figure 4.1c). Shortening of egg-hatching duration in FD under all three light regimes suggests that response of imposed selection for faster development overrides that of light regime (Figure 4.1c). Given that effect of light regime and selection interaction on egg-hatching duration did not reach

statistically significant levels, we repeated this assay once again at the 50th generation and found a consistent reduction in egg-hatching time of FD by ~1.1 h in LL, ~1.8 h in *T24* and ~1.6 h under DD (Figure 4.1d, e). ANOVA on the 50th generations egg-hatching time data revealed a statistically significant effect of L ($F_{2,6}$ = 17.83; p < 0.003), S ($F_{1,3}$ = 155.03; p < 0.001) and of L × S interaction ($F_{2,6}$ = 7.45; p < 0.02; Figure 4.1d, Table 4.1b). Post-hoc multiple comparisons using Tukey's test revealed that egg-hatching time of FD was significantly reduced compared to BD under all the three light regimes, however, the difference was least in LL (Figure 4.1e). ANOVA on the difference data revealed a statistically significant effect of L ($F_{2,6}$ = 7.45; p < 0.024; Figure 4.1d, e). A composite ANOVA of 40th and 50th generation data revealed a statistically significant effect of assay generation (G) ($F_{1,3}$ = 13.59; p < 0.035), L ($F_{2,6}$ = 48.1; p < 0.0002), S ($F_{1,3}$ = 90.14; p < 0.002) and G × L × S interaction ($F_{2,6}$ = 6.24; p < 0.03; Table 4.1c). Post-hoc multiple comparisons using Tukey's test revealed that the extent of speeding-up in egg-hatching time under all three light regimes was greater in the 50th generation assay compared to 40th generation (Figure 4.1d, e; Table 4.1c). Thus, consistently shortened egg-hatching time in FD compared to BD suggests a strong response of imposed selection and significant interaction of generations, light and stocks revealed that duration of egg stage depends upon the imposed light regimes and the extent of selection. Considering the absence of evidence for functional circadian clocks at egg stage, the difference in egg-hatching time between FD and BD stocks appears to be entirely light-mediated and thus clock-independent.

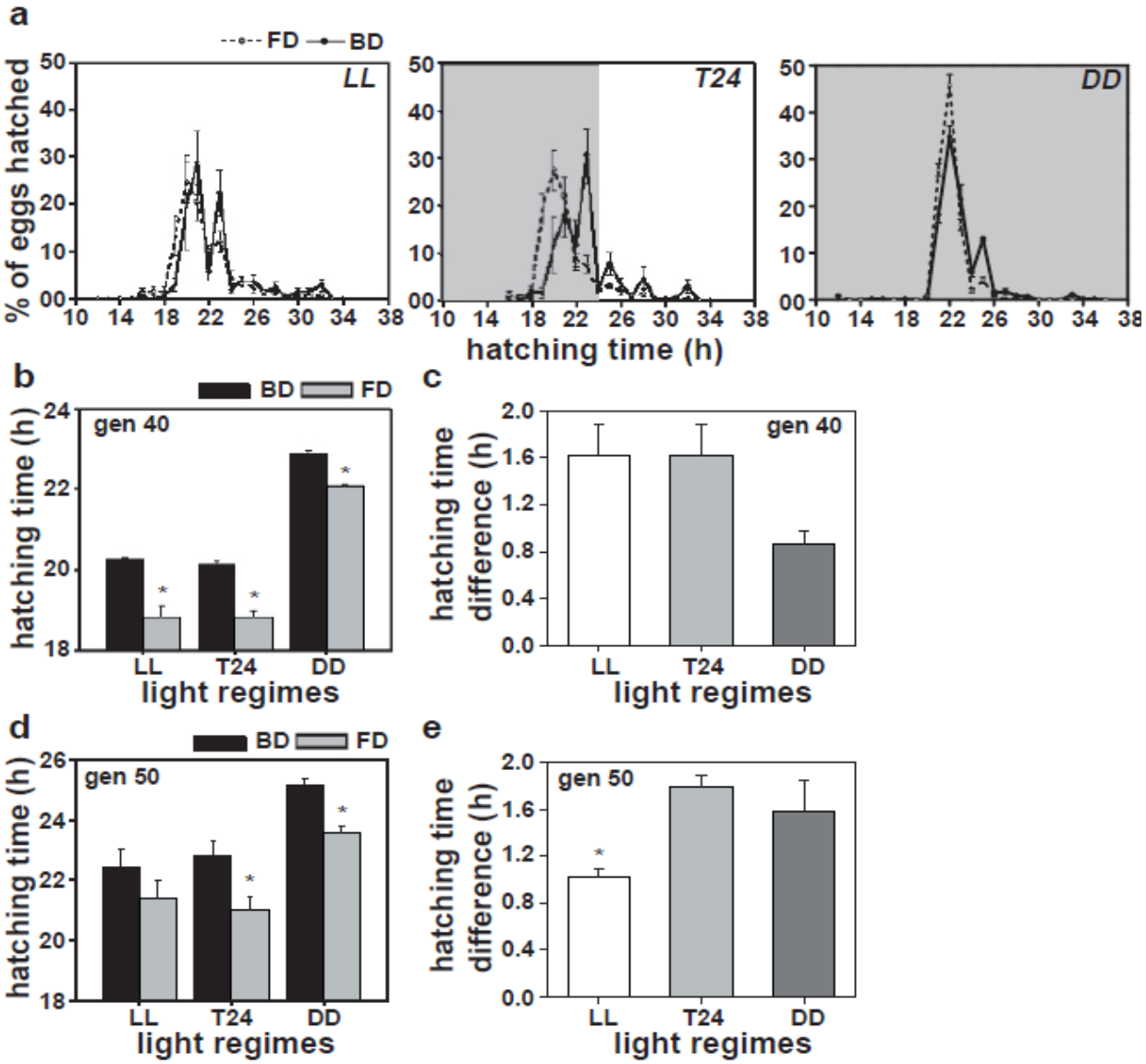


Figure 4.1. Light regimes affecting egg-hatching time: Waveforms showing differences in egg-hatching time between selected (FD) and control (BD) stocks under LL, *T24* and DD conditions (a). The mean egg-hatching time (time interval from egg-to-first instar larvae) of FD and BD stocks under LL, *T24* and DD conditions, showing significant difference under three different regimes after 40 and 50 generations of selection (b and d, respectively). Difference between egg-hatching time of FD and BD stocks under LL, *T24* and DD from assays done at the 40^{th} (c) and 50^{th} (e) generations showing light regime effect on egg-hatching time. The error bars in all the panels are standard error around the mean (SEM) and statistically significant differences are indicated by asterisks.

Table 4.1a. Results of ANOVA on egg-hatching time under LL, *T24* and DD (gen 40).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light (L)	2	23.67	6	0.227	104.21	0.000
Stocks (S)	1	8.8	3	0.711	12.38	0.039
L × S	2	0.19	6	0.147	1.29	0.341

Table 4.1b. Results of ANOVA on egg-hatching time under LL, *T24* and DD (gen 50).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light (L)	2	16.47	6	0.923	17.83	0.003
Stocks (S)	1	12.85	3	0.083	155.03	0.001
L × S	2	0.32	6	0.043	7.45	0.024

Table 4.1c. Results of ANOVA on egg-hatching time under LL, *T24* and DD (gen 40 and 50).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Generations (G)	1	60.29	3	4.434	13.59	0.035
Light (L)	2	39.81	6	0.827	48.10	0.000
Stocks (S)	1	21.47	3	0.238	90.14	0.002
G × L	2	0.33	6	0.32	1.02	0.416
G × S	1	0.19	3	0.56	0.34	0.599
L × S	2	0.51	6	0.13	1.14	0.379
G × L × S	2	0.36	6	0.06	6.24	0.034

4.3.2. Pupation and pigmentation time assays: Both pupation time (Figure 4.2) and wing-pigmentation time (Figure 4.3) of FD flies were reduced significantly compared to BD under all four regimes. The pupation-time difference between FD and BD under *T20*, *T24*, *T28* and DD was 9.12, 7.03, 9.01 and 12.51 h, respectively (Figure 4.2b, c). Under *T20*, FD pupated in dark and BD in light, while in *T28*, FD pupated in light and BD in the dark, however, under *T24*, the pupation in FD spanned over both the dark and light phases, while in BD it started towards the end of dark phase and was over soon after lights-on, similarly FD also pupates earlier than BD under DD (Figure 4.2a). ANOVA on pupation time data revealed a statistically significant effect of L ($F_{3,9} = 343.62$; $p < 0.0001$), S ($F_{1,3} = 117.18$; $p < 0.002$) and L × S interaction ($F_{3,9} = 5.36$; $p < 0.02$; Figure 4.2b, c; Table 4.2a). Post-hoc multiple comparisons using Tukey's test revealed that under all four light regimes, pupation time of FD was significantly reduced compared to BD. However, pupation time difference was shortest in *T24,* followed by *T20* and *T28* and greatest under DD (Figure 4.2c). ANOVA on the difference data revealed a statistically significant effect of L on the difference (BD-FD) in pupation time ($F_{3,9} = 5.36$; $p < 0.02$, Figure 4.2c), with the difference being in the order *T*24 < *T*20, *T*28 < DD. Thus, under entrained conditions clock-mediated difference in pupation time of FD and BD flies reduced, whereas under free-running conditions, clocks-mediated difference can be seen. These results thus suggest that circadian clocks regulate egg-to-pupal development time in fruit flies *D. melanogaster*.

The difference in wing-pigmentation time between FD and BD populations was about 10.7 h in *T20*, 12.1 h in *T24*, 12.5 h in *T28* and 18.1 h in DD (Figure 4.3). Under *T20*, FD wing-pigmentation occurred mostly during light and of BD partly in light and partly in dark,

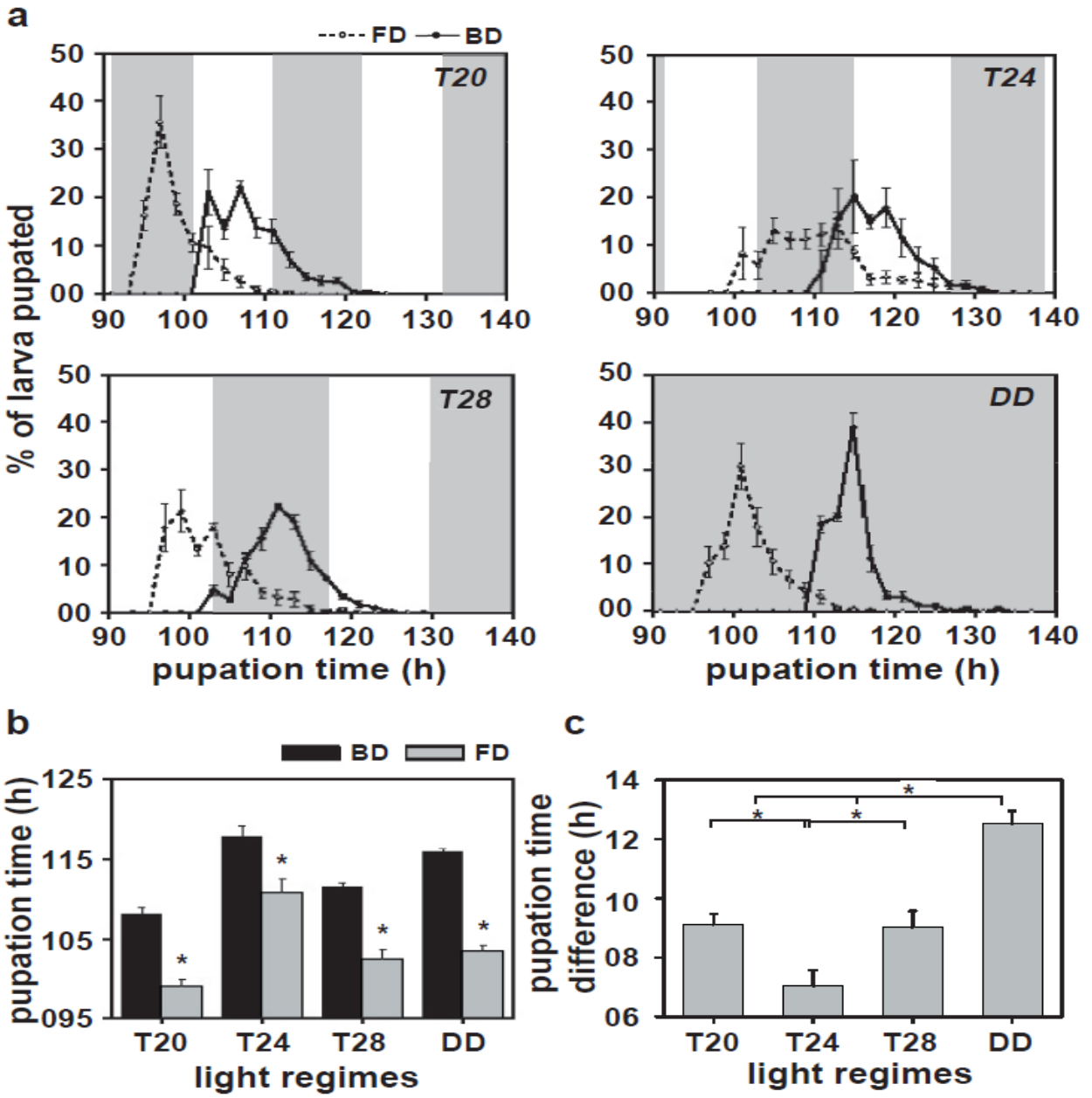


Figure 4.2. Light regimes affecting egg-to pupation time: Waveforms showing differences in pupation time between selected (FD) and control (BD) stocks under *T20*, *T24*, *T28* and DD conditions (a). The pupation time (time interval from egg-to-pupal formation) of FD and BD stocks under *T20*, *T24*, *T28* and DD, showing significant effect under different light regimes (b). Difference between the pupation time of FD and BD stocks under *T20*, *T24*, *T28* and DD conditions, showing light regime effect on egg-to-pupation duration (c). All other details are same as in Figure 4.1.

while in *T28*, FD starts pigmenting during dark and BD wing-pigmentation spanned partly in dark and partly in light (Figure 4.3a). Under *T24*, FD wing-pigmentation mostly occurred in dark, while in BD it started in the middle of light phase and under DD, FD pigmented earlier that BD (Figure 4.3a). ANOVA on wing-pigmentation time data revealed a statistically significant effect of L ($F_{3,9}$ = 151.07; $p < 0.0001$), S ($F_{1,3}$ = 519.86; $p < 0.0001$) and L × S interaction ($F_{3,9}$ = 13.46; $p < 0.001$; Figure 4.3b, c, Table 4.2b). Post-hoc multiple comparisons using Tukey's test revealed that under all four light regimes, wing-pigmentation time of FD was significantly shorter than BD (Figure 4.3b). However, wing-pigmentation time difference was shortest in *T20,* followed by *T24* and *T28* and maximum under DD (Figure 4.3c). ANOVA on the difference data revealed a statistically significant effect of L ($F_{3,9}$ = 13.46; $p < 0.001$; Figure 4.3c), which suggests that under entrained conditions such as *T20, T24* and *T28*, clock-mediated differences in pigmentation time of FD and BD flies is smaller than that under free-running condition (DD). These results thus suggest that circadian clocks partly regulate egg-to-wing-pigmentation duration in fruit flies *D. melanogaster.* Hence, larval and pupal (until wing-pigmentation) stages in *D. melanogaster* appears to be light-mediated and clock-dependent.

4.3.3. Egg-to-adult development time assays: After 40 generations of selection, the difference in pre-adult development time between selected and control populations was about 18.2 h in LL, 18.1 h in *T20*, 15.5 h in *T24*, 15.2 in *T28* and 18.3 h in DD (Figure 4.4b, c). Pre-adult developmental profiles of FD and BD stocks showed similar pattern under LL and DD, with FD emerging consistently earlier than BD (Figure 4.4a). Although development time was shorter under LL and *T20* than in *T24, T28* and DD, differences (~18 h) between stocks remained comparable to *T20* and DD but greater than *T24* and *T28* (~15 h) (Figure 4.4c). Under *T20*, both

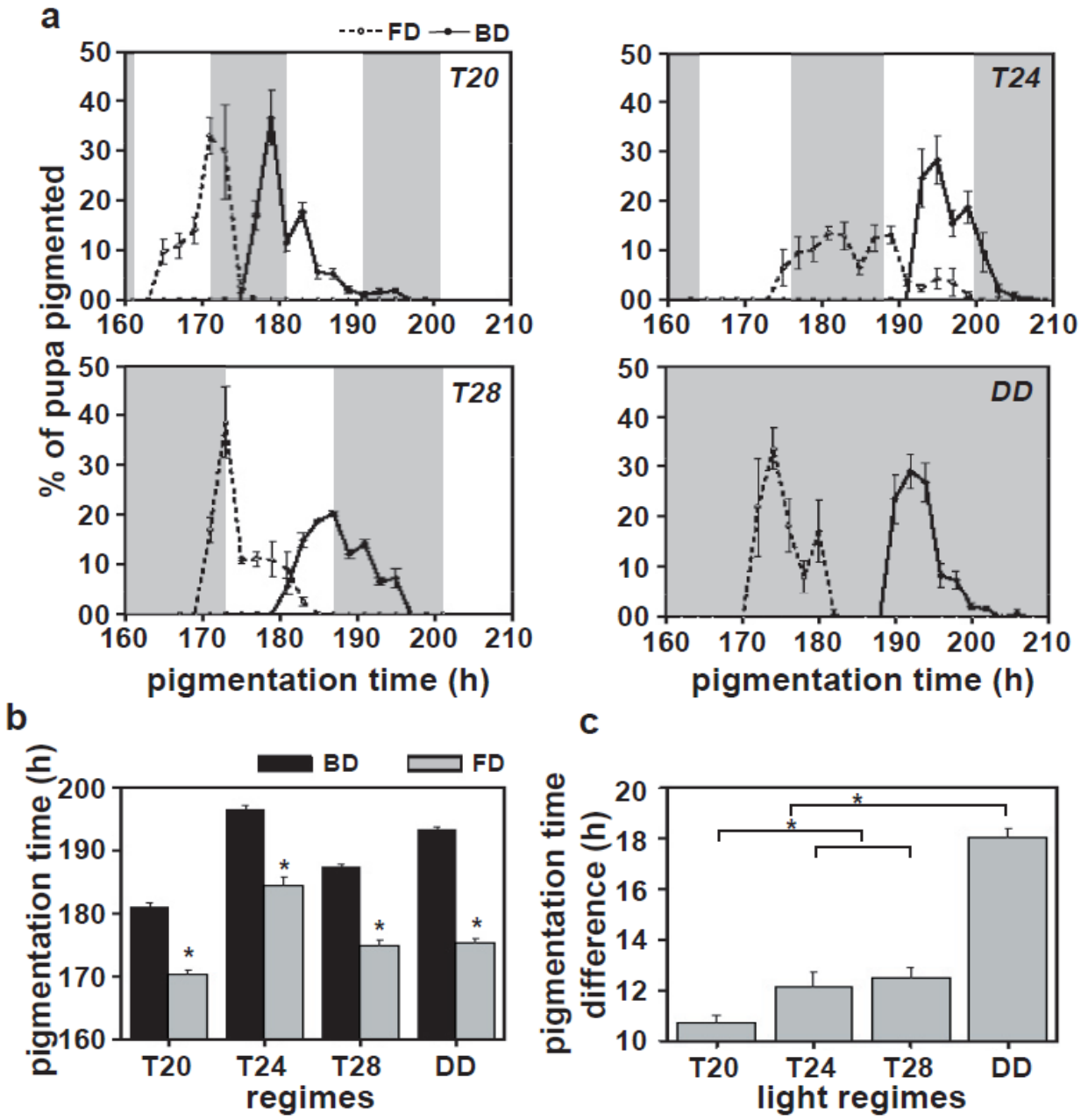


Figure 4.3. Light regimes affecting egg-to pigmentation time: Waveforms showing differences in pigmentation time of selected (FD) and control (BD) stocks under *T20*, *T24*, *T28* and DD (a). The wing-pigmentation time (time interval from egg-to-wing-pigment formation) of FD and BD stocks under *T20*, *T24*, *T28* and DD conditions (b). Difference between the wing-pigmentation time of FD and BD stocks assayed under *T20*, *T24*, *T28* and DD conditions, showing light regime effect on egg-to-wing-pigmentation duration (c). All other details are same as in Figure 4.1.

FD and BD flies emerged in the dark, while in *T24* in the light (Figure 4.4a). Under *T*28, FD flies started emerging late in the light phase and continued emerging during the dark phase, while BD flies started emerging in the dark and continued emerging during the light phase of the next cycle (Figure 4.4a). ANOVA revealed a statistically significant effect of L ($F_{4,12}$ = 62.04, p < 0.0001), S ($F_{1,3}$ = 285.68, p < 0.0004), however, the effect of L × S interaction was statistically not significant ($F_{4,12}$ = 1.16, p = 0.38; Figure 4.4b; Table 4.2c). Post-hoc multiple comparisons using Tukey's test revealed that under all five light regimes, egg-to-adult development time of FD was significantly shorter than BD. However, differences in pre-adult development time of the two stocks remained more or less comparable under constant conditions (LL and DD) and *T20* (Figure 4.4c). ANOVA on difference data revealed that effect of light was statistically not significant ($F_{4,12}$ = 1.16; p = 0.38; Figure 4.4c). These results thus suggest that although light regimes alter the overall pre-adult development time of *Drosophila*, difference in development time between selected and control populations under entrained conditions such as *T20*, *T24, T28*, and rhythm abolishing conditions (LL), is comparable to that under free-running condition (DD). Comparable magnitude of differences in total pre-adult development time between FD and BD among light regimes suggests that clock-mediated shortening of larval and pre-wing-pigmentation pupal durations is compensated by lengthening of development time during post-wing-pigmentation stages in fruit flies *D. melanogaster*.

4.4. Discussion

The timing of adult emergence in *Drosophila* depends upon a number of factors including developmental states, phase and period of circadian rhythms, and on external environmental conditions (Kyraicou et al., 1990; Qiu and Hardin, 1996; Paranjpe et al., 2005; Lone and Sharma, 2008). External LD cycles restrict adult emergence to a narrow window of time called "allowed

zone" or "gate" of emergence (Pittendrigh, 1954, 1966; Saunders, 1992, 2002b; Qiu and Hardin, 1996). Since in *Drosophila,* circadian rhythms are abolished in LL, gating of emergence is likely to be absent, therefore, developing individuals would enter subsequent developmental stages without any delay, and thereby speed-up pre-adult developmental stages (Lone and Sharma, 2008). On the other hand under LD cycles, development time is likely to be determined by some interaction between developmental states and circadian gating created by LD cycles, which is likely to be altered depending on the length of LD cycles and timing of light/dark phases (Qiu and Hardin, 1996; Paranjpe et al., 2005). Under DD, where circadian clocks free-run, development time would be determined by some interaction between developmental states and circadian clocks, and therefore development time of flies in this regime would be comparable to that in *T24*. Therefore, the pre-adult development time of *Drosophila* is expected to follow the trend of fastest development under LL then *T20* < *T24* or DD < *T28*. The results of our present study are consistent with the expectations and with earlier findings as it shows that mean pre-development time of males and females are shorter under LL and longer in *T24* and DD (Sheeba et al., 1999b; Paranjpe et al., 2005). As expected, flies take similar amount of time to develop under *T24* and DD (Figure 4.4b). Interestingly, lack of emergence gate under LL does not supersede the speeding-up of development under *T20*. Among the LD cycles, development time was shortest under *T20*, followed by *T24* and *T28*, with flies taking similar amount of time to develop under *T24* and *T28*. This hints at the possibility of a developmental threshold below and beyond which pre-adult development of *Drosophila* cannot be speeded up or slowed down any further, at least not by light, thus constraining the occurrence of correlation between development time and the period of light regime below and beyond a particular limit. Interestingly, development time of selected as well as control populations with small but

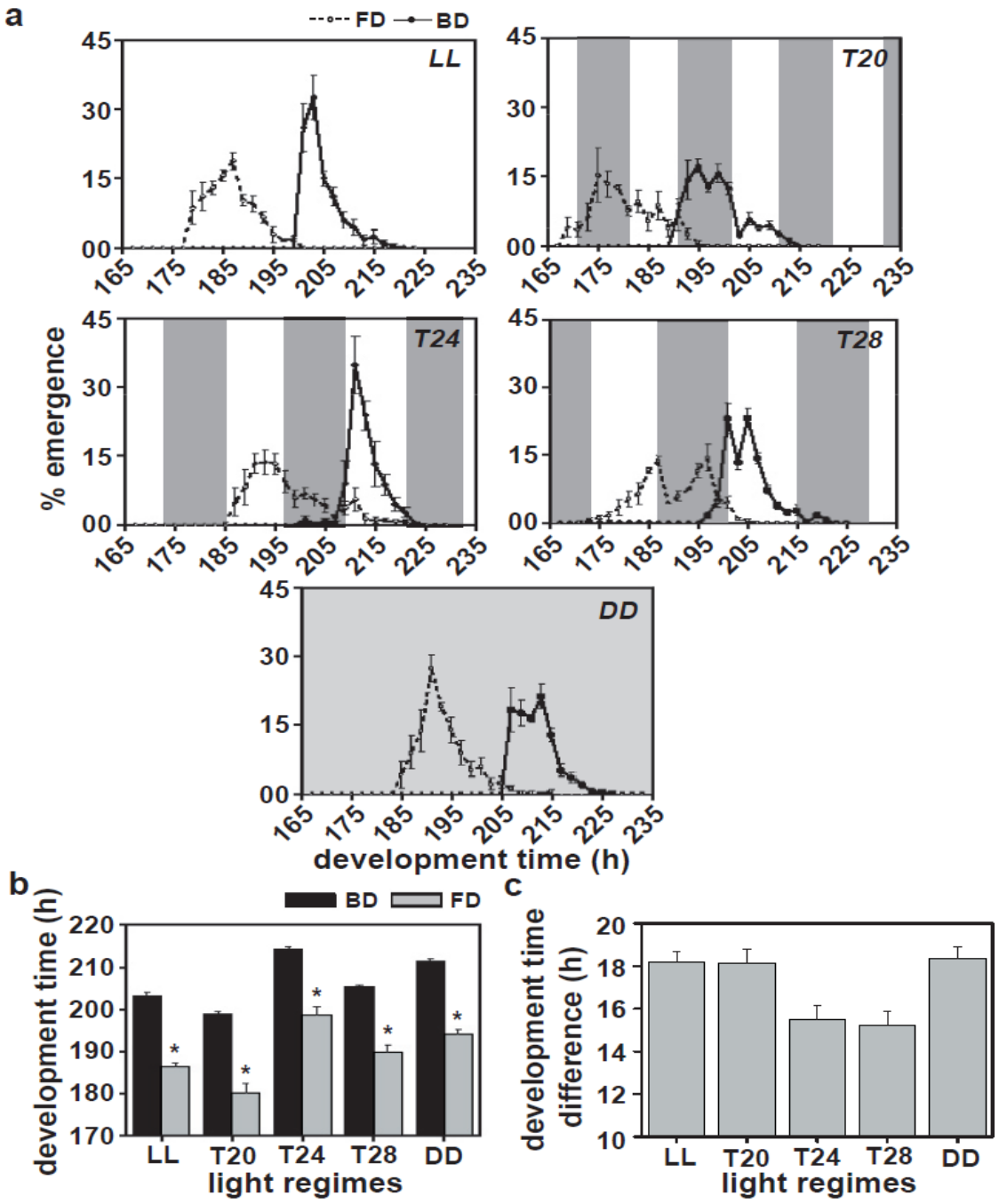


Figure 4.4. Light regimes affecting adult emergence duration: Waveforms showing difference in pre-adult development time of selected (FD) and control (BD) stocks under LL, *T20*, *T24*, *T28* and DD (a). The pre-adult development time (time interval from egg-to-adult emergence) of FD and BD stocks under LL, *T20*, *T24*, *T28* and DD conditions (b). Difference between pre-adult development time of FD and BD stocks under LL, *T20*, *T24*, *T28* and DD conditions showing light regime effect on egg-to-adult emergence duration (c). All other details are same as in Figure 4.1.

Table 4.2a. Results of ANOVA on pupation time under DD, *T20*, *T24* and *T28*.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light (L)	3	1178.73	9	3.43	343.62	0.0001
Stocks (S)	1	710.13	3	6.06	117.18	0.002
L × S	3	10.36	9	1.93	5.36	0.02

Table 4.2b. Results of ANOVA on wing pigmentation time under DD, *T20*, *T24* and *T28*.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light (L)	3	304.87	9	2.02	151.07	0.0001
Stocks (S)	1	1418.62	3	2.73	519.86	0.0001
L × S	3	21.06	9	1.56	13.46	0.001

Table 4.2c. Results of ANOVA on egg-to-adult development time assay under LL, DD, *T20*, *T24* and *T28*.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light (L)	4	389.52	12	6.28	62.04	0.0001
Stocks (S)	1	2917.83	3	10.21	285.68	0.0001
L × S	4	4.88	12	4.2	1.16	0.38

significant difference in clock period (~0.5 h) was significantly affected by light regimes, with FD flies developing consistently faster than BD in all light regimes. Thus, light regimes can modulate *Drosophila* developmental duration, but the extent does not reach levels achieved by selection because interaction between light regime and developmental states was not as strong as the effect of selection. Therefore, irrespective of the state of temporal organization, flies selected for faster pre-adult development evolved shorter egg-to-adult developmental duration compared to controls. This suggests that to a large extent, differences in development time between selected and control stocks are independent of the difference in their clock period. Since the observed development time difference between two different stocks comprises several developmental stages, it reveals that timing of developmental events in *D. melanogaster* depends upon both circadian clock-dependent and clock-independent components. In addition, since circadian clocks of faster developing and control flies at various developmental stages are entrained under periodic cycles such as *T20, T24* and *T28*, clock-dependent component of the differences between the stocks disappeared, whereas under free-running condition (DD), clock-dependent component of the difference persisted. Moreover, the difference in egg-to-adult development time between two stocks remains similar under all light regimes. This indicates that the effects of clock-independent components overrides to the light mediated egg-to-adult developmental difference (Figure 4.4c). These results thus suggest that circadian clocks partly regulate development time in *D. melanogaster*.

Our previous study had shown that under DD, developmental events such as egg-hatching, pupation and wing-pigmentation time of selected populations were also speeded-up proportionately in response to selection for faster pre-adult development (Yadav and Sharma, 2013). Similarly, in the present study, egg-hatching in FD was observed to be faster than BD in

LL, *T24* and DD, but difference in hatching duration between FD and BD was greater under DD, *T24* compared to LL (Figure 4.1e). Thus, irrespective of state of circadian clocks, eggs of FD flies hatched earlier than BD. Previous studies have reported that a single light pulse presented immediately after egg-hatching is sufficient to entrain the circadian clocks of *Drosophila* (Sehgal et al., 1992), suggesting that circadian clocks of flies are functional by as early as first instar larval stage. Consistently in the current study, lack of any light regime-mediated effect on the difference in egg-hatching time between FD and BD can be clearly seen, since this duration comprises of a stage before the appearance of functional circadian clocks, which suggests that egg-hatching time is entirely light-mediated and clock-independent.

The difference in pupal duration between selected and control populations was found to be greater under *T20* and *T28* than in *T24* (Figure 4.2c). This difference may be due to more efficient entrainment of circadian clocks of flies under *T24* compared to *T20* and *T28* (Figure 4.2b). *Drosophila* circadian clocks mature with succession of development under DD or even without exposure to zeitgebers (Sehgal et al., 1992) and exposure to light regimes are also known to play a key role in synchronizing the timing of emergence (Vallone et al., 2007). Earlier studies reported that egg-hatching in blood sucking bugs *Triatoma infestans* (Lazzari, 1991), in moth *Pectinophora gossypiella* (Minis and Pittendrigh, 1968), in mosquitos *Mansonia titillans* (Nayar et al., 1973) are 'gated' events. Similarly, pupation in fruit flies *Drosophila* (Rensing and Hardeland, 1967), in mosquitoes *Anopheles gambiae* (Jones and Reiter, 1975) and *Aedes taeniorhynchus* (Nayar, 1967a, b), and in *D. melanogaster* (Lorenz et al., 1989) pupation is also gated. Thus, until the wing-pigmentation stage that occurs after 7-8 d of egg-laying, circadian clocks are likely to have interacted with several gating events such as those in egg-hatching, pupation and wing-pigmentation. Therefore, such 'gated' stages and the transition time from one

stage to another can create constraints on developmental rate, which can cause reduction in proportional difference at pupation (Figure 4.2b) or wing-pigmentation stages (Figure 4.3b) under LD cycles.

Under DD, pupation and wing-pigmentation in control flies consistently began after these processes were almost completed in the selected flies (Figures 4.2a, 4.3a). This indicates that impact of selection for faster pre-adult development is much stronger on egg-to-wing pigmentation stages, however, the average difference in the duration of these stages between selected and control populations varied between entraining and free-running light regimes (Figures 4.2, 4.3; Table 4.2a, b). This suggests that the difference in time to pupation and wing-pigmentation between selected and control flies is a function of the difference in their clock period, and therefore interaction of light regimes with circadian clocks appears to be an important determinant for the timing of light-mediated and clock-dependent pre-adult events in *Drosophila*. Additionally, irrespective of the stock being studied, egg-to-pupation and egg-to-wing-pigmentation durations were shorter under *T20* compared to *T24* and *T28,* suggesting that it is possible to speed-up larval and pupal durations by maintaining flies under shorter LD cycles (*T20*), but not to slow them down beyond a particular limit (*T28*) (Figures 4.2b, 4.3b). Moreover, entrainment of circadian clocks under a variety of *T*-cycles can also result in such changes based on the correlation between clock period and the period of imposed *T*-cycles. Thus, the results of our study reveals that developmental stages of *D. melanogaster* (Figures 4.1-4.3) in particular larval, pupal (until wing-pigmentation) stages, are highly susceptible to LD regimes, as light regimes cause strong modulation of developmental duration of various pre-adult developmental stages. The time to egg-hatching, pupation, wing-pigmentation and egg-to-adult emergence was altered differently in the selected and control populations, which indicates that

difference in the status of stage-specific developing clocks as developmental stages differ physiologically from each other. Thus it is likely that clock-independent stage (egg) differ with partly clock-dependent larval stage, which in turn also differs with clock-dependent pupal stages. Moreover, wing-pigmentation in *Drosophila* is marked by formation of ocellar and bristle pigmentation, and is generally considered to be the last stage in development and thus, no further changes in pupae is expected (Harker 1965; Lorenz et al., 1989; Qiu and Hardin, 1996). However, significant light regime effect interacting with faster clocks does not seem to continue at least under LL and *T20* post-wing-pigmentation stage in the selected population as found in the current study. Thus, persistence of shortening of pre-adult durations in the selected populations compared to stages beyond wing-pigmentation, suggests that some developmental events occur even after wing-pigmentation, which probably compensate for the modulatory effect of imposed light regimes.

In *Drosophila*, ecdysone a steroid hormone released from prothoracic gland (Gilbert et al., 2002) triggers insect larval molting and adult emergence (Truman et al., 1983; Riddiford et al., 2010). Premature release of ecdysone speeds-up whereas delayed release slows-down development of tobacco hornworm *Manduca sexta* (L.) (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). During the larval stage, opening of a gate (lights-off at every night) is believed to be the signal that regulates the release of prothoracicotropic hormone (Rountree and Bollenbacher, 1986). Thus, it is suggested that the modulation in pre-adult development time may be (since conclusion is based on hornworm study) due to altered timing of prothoracicotropic hormone release which is primarily caused by alteration in the timing and duration of gate opening at different developmental stages. The steroid hormone ecdysone is known to set the timing of pre-adult stages by binding to nuclear hormone receptors, thus

regulating the expression of several developmental genes (Giebultowicz et al., 2008). It is therefore postulated that genes involved in various signaling pathways govern light regime-mediated developmental changes in *Drosophila* (Mensch et al., 2008). Although in the present study we did not estimate ecdysone level, given that both selected and control populations entrained to imposed LD cycles of different period length at egg (Figure 4.1a), egg-to-pupation (Figure 4.2a), and wing-pigmentation (Figure 4.3a) stages, provides an indirect evidence for the difference in the timings of ecdysone release which in turn could be the possible cause for the difference in gate durations at various developmental stages in selected and control populations.

Across successive stages of *Drosophila* development, circadian clocks undergo changes in its entrainability to LD cycles due to various "gated" developmental stages. Provided *Drosophila* development is a light regime-mediated clock-controlled event, stage-specific clock or light regime-dependent effects can only be observed by a manipulation of clock speed which in turn can be detected in terms of relative difference in development time across the light regimes. Our study reveals that unlike the egg stage, most pre-adult developmental stages are susceptible to changes in light regimes and their developmental rate varies in a light regime and clock-depended manner. However, it also appears that all else being equal pre-adult development of *Drosophila* cannot be slowed down beyond its developmental rate of LD12:12, at least not by lengthening the period of LD cycles.

4.5. Conclusions

Forty generations of selection for faster pre-adult development in *D. melanogaster* results in a decrease in pre-adult development time by ~19 h. We found that the pre-adult developmental stages of *D. melanogaster* are highly susceptible to various LD regimes and development time is likely to be determined by some interaction between developmental clocks and circadian gating

created by LD cycles. Our study clearly indicates that larval and pupal stages are light-mediated clock-dependent, suggesting that an interaction of light regimes and circadian clocks modulate timing of pre-adult events in *Drosophila.* Interestingly, our study indicates the possibility of some yet unknown mechanisms occurring post-wing-pigmentation, which probably compensates for the modulatory effect of imposed light regimes. Taken together our study suggests that most pre-adult stages in fruit flies *D. melanogaster* are light and clock-dependent, albeit by different extents.

Chapter 5

Environmentally-mediated modulations of developmental rate do not affect selection-mediated changes in pre-adult development time of fruit flies *Drosophila melanogaster*

5.1. Introduction

Organisms are exposed to variations in their native environment caused due to daily and seasonal cycles of nature, which they cope with by modulating their behaviour and physiological processes. The ability of organisms to adapt to thermal stress has profound effect on their evolutionary fitness (Huey and Bennett, 1990), which has also been demonstrated in a study on fruit flies *Drosophila melanogaster* (Hoffmann et al., 2003). Temperature affects several life history traits in *Drosophila* including its pre-adult development time, which has been shown to be negatively correlated with temperature (de Moed et al., 1998, 1999), with mean values of ~7 days at 28 °C, ~8 days at 25 °C, ~19 days at 18 °C and ~50 days at 12 °C (Ashburner et al., 2005). The pre-adult developmental duration in insects is also altered due to changes in a variety of other factors including light regimes (Kyriacou et al., 1990; Qiu and Hardin, 1996; Sheeba et al., 1999b; Paranjpe et al., 2005 Lone and Sharma, 2008; Lone et al., 2010), larval density (Joshi, 1997; Mueller, 1997), and nutrition (Sang, 1956; Carpenter and Bloem, 2002; Simpson et al., 2002; Warbrick Smith et al., 2009), which suggests a dependence of pre-adult developmental rate on environmental factors.

In fruit flies *D. melanogaster*, many biological processes such as adult emergence, locomotor activity, olfaction, mating and egg-laying are temporally regulated by circadian clocks (Krishnan et al., 1999; Sakai and Ishida, 2001; Howlader et al., 2006). In addition, several studies have shown that clock neurons in the *Drosophila* brain involved in the regulation of activity/rest rhythm are organized as morning and evening oscillators in which one group of neurons is responsible for the morning peak of activity and another group for the evening peak of activity under 12:12 h light/dark (LD) (Grima et al., 2004; Stoleru et al., 2004, 2005, 2007; Rieger et al., 2006; Sheeba et al., 2008). Circadian clocks have also been implicated in the

regulation of life history traits such as pre-adult development time and adult lifespan (Kyriacou et al., 1990; Klarsfeld and Rouyer, 1998; Paranjpe et al., 2005; Kumar et al., 2006; Lone and Sharma, 2008; Lone et al., 2010). In a previous study we had shown that the period of LD cycles alter the pre-adult development time with shorter cycles speeding up development and longer cycles slowing it down (Paranjpe et al., 2005). Links between circadian clocks and life history traits is also evidenced in a study on fruit flies *D. melanogaster* (Turner et al., 2011) when some of the genes influencing post embryonic development and metamorphosis were found to respond to selection for body size variation as allele frequencies of these genes were higher in the selected populations than in controls. Also, the list of genes with higher allele frequencies in the selected populations as compared to controls included some of the core clock genes, implying that polymorphism in clock genes is associated with natural variation in body size. The most convincing evidence for the role of circadian clocks in timing pre-adult development came from laboratory selection studies for faster and slower pre-adult development in melon flies *Bactrocera cucurbitae* (Diptera: Tephritidae; Miyatake, 1996, 1997a; Shimizu et al., 1997) and in fruit flies *D. melanogaster* selected for faster (Yadav and Sharma, 2013) or slower (Takahashi et al., 2013) pre-adult development, or morning and evening emergence (Kumar et al., 2006), which yielded populations bearing correlation between pre-adult development time and circadian period (τ).

Much like the LD cycles (Boothroyd et al., 2007; Yoshii et al., 2009), warm/cold (WC) cycles have also been shown to entrain circadian rhythms in fruit flies *D. melanogaster* (Wheeler et al., 1993; Tomioka et al., 1998; Yoshii et al., 2002, 2005, 2010; Glaser and Stanewsky, 2005; Boothroyd et al., 2007; Currie et al., 2009). For example, WC cycles with amplitude of 2–3 ^{o}C were able to entrain the activity/rest and molecular rhythms in *D. melanogaster* (Wheeler et al.,

1993). Thus, light and temperature are believed to be the two key zeitgebers for the circadian clocks of fruit flies *D. melanogaster*. While it is highly likely that flies exposed to multiple zeitgebers would exercise greater control over developmental rates, it would be interesting to examine the combined effect of zeitgebers on the pre-adult developmental duration.

In one of our previous studies on fruit flies *D. melanogaster*, we had shown that selection for faster pre-adult development yields populations with shorter egg to adult developmental duration (by ~29 h) and circadian clocks with shorter period (by ~0.5 h) (Yadav and Sharma, 2013; Chapter 3). It would be interesting to study the effect of manipulation in the rate of pre-adult development and/or clock period in these flies to examine its effects on the selection-mediated difference in pre-adult development time. In the current study, we exposed flies developing under continuous darkness (DD) to different ambient temperatures or to WC cycles to address this effect. The results revealed that although difference in pre-adult development time between the selected (FD) and control (BD) stocks is altered due to manipulations in developmental rate and/or clock period, relative difference in development time remains largely unchanged. These results imply that correlation between pre-adult development time and clock period breaks down under novel (non-native) environmental conditions.

5.2. Materials and Methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by a method described in detail in chapter 2.

Standardized populations were used for various assays described below.

5.2.1. Egg to adult (pre-adult) development time assay: For these assays, flies from the standardized populations were allowed to lay eggs on banana medium. In order to enhance the

egg-laying capacity of flies, two days prior to egg collection, live yeast paste was supplemented on the banana medium. Eggs were collected in DD under red light of λ greater than 650 nm, with the help of moistened fine brush under the microscope. Exactly 30 eggs were collected from the eggs laid in a 2 h window (08:00 to 10:00 h) and dispensed into vials containing ~10 ml of banana medium. The vials with developing flies were monitored daily for darkening of pupae. Once the pupae became dark, vials were regularly (every 2 h) monitored for the emergence of adult flies, and the number of males and females emerging from each vial in the preceding 2 h period was counted. The 2 hourly checks continued until all flies emerged out from each vial. From this data, we obtained pre-adult development time of each fly, which was used to calculate mean development time of each vial. Pre-adult development time (in h) of a fly was calculated as the duration between the midpoint of 2 h egg collection window and the midpoint of 2 h period during which the fly emerged as adult.

WC cycles of 12:12 h were created inside an incubator using a built in electronic timer driven thermostat, which stepped up and stepped down temperature to desired levels within ~20 min. Temperature and humidity inside the incubator were monitored continuously during the entire assay and were found to be stable. Development time of standardized $FD_{1\text{-}4}$ and $BD_{1\text{-}4}$ flies was assayed in three different sets of conditions. The first set of assays was performed under DD at three ambient temperatures 18, 25 and 29 °C. In the second set, assays were done under DD with 12:12 h WC cycles of 25:18 ^{o}C (WC1) or of 29:25 ^{o}C (WC2). In the third set, developing flies were exposed to WC2 in-phase with LD12:12 (WC-LD^{IP}: light phase with high temperature and dark phase with low temperature) or out-of-phase (WC-LD^{OP}: light phase with low temperature and dark with high temperature). In each set of assays, ten vials containing eggs from each replicate population were placed into incubators and maintained under previously

described conditions. Thus, a total of 240 vials (10 vials × 8 populations × 3 constant temperature regimes) for the first set, 160 vials (10 vials × 8 populations × 2 cyclic temperature regimes) for the second set, and 160 vials (10 vials × 8 populations × 2 cyclic light + temperature regimes) for the third set, were used in this assay.

***5.2.2. Egg to adult survivorship assay*:** In order to check whether cold and warm temperatures have any adverse effect on the physiological well being of flies, we performed a pre-adult survivorship assay on the FD and BD stocks. Pre-adult survivorship was calculated as the total number of adults emerging out of every vial divided by the total number of eggs dispensed into that vial (which was 30). Pre-adult survivorship of the FD and BD stocks was assayed under DD at 18, 25 and 29 ^{o}C. The experimental setup for survivorship assay was exactly similar to that of development time assay, except that vials were monitored for the next 5 to 6 days to estimate the total number of flies emerging out of each vial.

5.2.3. Activity/rest rhythm assay: After 55th generation of selection, the activity/rest rhythm of flies from $FD_{1\ 4}$ and $BD_{1\ 4}$ flies was monitored for a minimum of 11 days under DD at three different temperatures (18, 25 and 29 ^{o}C). For this assay, freshly emerged virgin adult males were introduced individually into activity tubes (0.5 cm inner diameter and 7 cm long) within 24 h of their emergence. These tubes contained corn meal based fly food medium sealed with paraffin wax at one end and cotton plug at the other, and were placed in *Drosophila* Activity Monitor (DAM) system from Trikinetics (Waltham, USA), kept inside environment controlled incubators, for recording activity/rest behaviour of flies under DD at three temperatures (18, 25 and 29 ^{o}C). Temperature and humidity inside the incubators were recorded every 5 min, during the entire assay, using *Drosophila* Environmental Monitor (DEnM; Trikinetics, USA) and were found to be stable. The DAM system is a simple computer aided device that uses a pair of

infrared emitter and sensor to detect the movements of flies in a narrow glass tube. From the recorded data, the τ of activity/rest rhythm was estimated using Lomb Scargle periodogram in CLOCKLAB software from Actimetrics (Wilmette, USA).

5.2.4. Statistical analyses: Mean pre-adult development time and survivorship data from replicate populations were used in mixed model analyses of variance (ANOVA) treating block (B) as random factor, and regime (R), temperature (T) and stock (S) as fixed factors crossed with block, hence only fixed factors could be tested for statistical significance. Post-hoc multiple comparisons were done using Tukey's honestly significant difference (HSD) test. The τ values were analyzed using ANOVA treating blocks as random factor, T and S as fixed factors followed by Post-hoc multiple comparisons using Tukey's HSD test. The τ values provided throughout the text are mean ± standard error around the mean (SEM). All statistical analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (StatSoft Inc., 1995).

5.3. Results

5.3.1. Effect of ambient temperature on pre-adult development time: Overall, flies took longer time to develop at cooler temperature (18 ^{o}C) and shorter at warmer temperature (29 ^{o}C) than at 25 ^{o}C (Figure 5.1a). ANOVA followed by Post-hoc multiple comparisons using Tukey's HSD test revealed that development time of both stocks was shortest at 29 ^{o}C, followed by 25 ^{o}C and longest at 18 ^{o}C, and that development time of FD stocks was significantly shorter than BD controls under all three temperatures (Figure 5.1a). ANOVA revealed a statistically significant effect of temperature (T), stock (S) and T × S interaction (see Table 5.1a).

However, the relative difference in pre-adult development time, [(BD-FD)/BD], between the two stocks remained unchanged with change in ambient temperature (Figure 5.1a). ANOVA showed that the effect of temperature and stock on the relative difference in the development

time between two stocks was statistically not significant (data in Table 5.1b); although on the absolute differences it was statistically significant.

5.3.2. Effect of different ambient temperature on pre-adult survivorship: Next we asked whether the temperature experienced during pre-adult developmental stages had any effect on the fitness of flies in terms of egg to adult survivorship. ANOVA revealed a statistically significant effect of T, S, while there was no significant T × S interaction (Figure 5.1b; Table 5.1c). Post-hoc multiple comparisons using Tukey's test revealed that warm temperature (29 ^{o}C) affects FD survivorship much more than that of BD controls. This suggests that warm temperature has adverse effect on the physiological well being of flies.

5.3.3. ***Shortening of clock period (τ) at cold temperature:*** Given that circadian clocks are known to be temperature compensated, we sought to study the effect of rearing FD and BD flies under DD at three different temperatures (18, 25 and 29 ^{o}C) on the τ of activity/rest rhythm. Composite ANOVA revealed a statistically significant effect of T; however, the effect of S and T × S interaction was statistically not significant (Table 5.1d). Post-hoc multiple comparisons using Tukey's test revealed that the mean τ of flies was shorter at 18 and 29 ^{o}C compared to that at 25 ^{o}C but the shortening was significantly greater at 18 than at 29 ^{o}C (Figure 5.1c). When τ of FD and BD flies at different temperatures were analysed in separate ANOVA, at 25 ^{o}C the τ of FD flies (24.08 ± 0.04 h) was significantly shorter than that of BD (24.61 ± 0.08 h); whereas

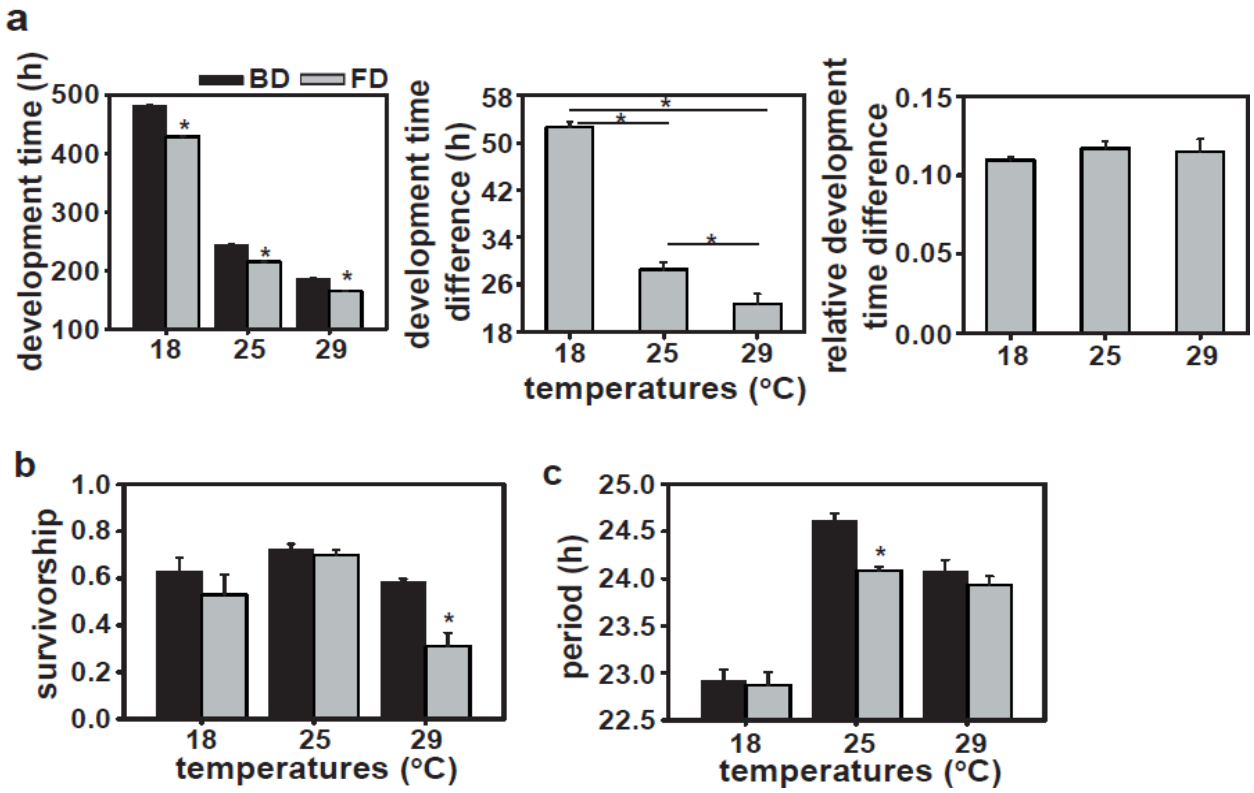


Figure 5.1. Temperature modulates pre-adult development and survivorship of flies: (a) Average development time and the difference (BD-FD) in pre-adult development time of the selected and the control stocks under constant darkness (DD) at 18, 25 and 29 ^{o}C, and the relative difference [(BD-FD)/BD] in pre-adult development time between the two stocks. (b) Average pre-adult survivorship of the selected and control stocks under DD at 18, 25 and 29 ^{o}C shows effect of temperature. (c) Mean free running period (τ) of the selected and control stocks at 18, 25 and 29 ^{o}C (under DD) shows the effect of temperature on τ. The error bars represent standard error around mean (SEM). Asterisks indicate statistically significant difference between the selected and control stocks or between two bars.

Table 5.1a. Results of ANOVA on the pre-adult development time data under constant dark (DD) at 18, 25 and 29 °C.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Temperature (T)	2	176475.14	6	8.89	19830.07	0.0001
Stock (S)	1	7028.19	3	1.74	4048.98	0.0001
Block (B)	3	3.91	0	0	--	--
T × S	2	543.21	6	4.45	121.96	0.0001
T × B	6	8.89	0	0	--	--
S × B	3	1.74	0	0	--	--
T × S × B	6	4.45	0	0	--	--

Table 5.1b. Results of ANOVA on the relative difference in pre-adult development time under constant dark (DD) at 18, 25 and 29 °C.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Temperature (T)	2	0.001	6	0.001	0.41	0.69
Block (B)	3	0.001	0	0	--	--
T × S	6	0.001	0	0	--	--

Table 5.1c. Results of ANOVA on the pre-adult survivorship data under constant dark (DD) at 18, 25 and 29 °C.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Temperature (T)	2	0.14	6	0.12	11.37	0.009
Stock (S)	1	0.11	3	0.01	11.35	0.04
Block (B)	3	0.01	0	0	--	--
T × S	2	0.03	6	0.01	3.35	0.11
T × B	6	0.12	0	0	--	--
S × B	3	0.01	0	0	--	--
T × S × B	6	0.01	0	0	--	--

Table 5.1d. Results of ANOVA on the circadian clock period (τ) data under constant dark (DD) at 18, 25 and 29 °C.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Temperature (T)	2	4.59	6	0.01	360.11	0.0001
Stock (S)	1	0.36	3	0.06	5.78	0.09
Block (B)	3	0.08	0	0	--	--
T × S	2	0.13	6	0.04	2.98	0.13
T × B	6	0.01	0	0	--	--
S × B	3	0.06	0	0	--	--
T × S × B	6	0.04	0	0	--	--

at 18 ^{o}C (FD-22.87 ± 0.14 h *vs.* BD-22.92 ± 0.11 h) and at 29 ^{o}C (FD-23.93 ± 0.09 h *vs.* BD-24.08 ± 0.11 h) the τ of FD and BD flies was not different. This suggests that selected flies have τ shorter than controls when reared at 25 ^{o}C, and this period difference is considerably reduced (or became non-existent) when flies were reared at low or high temperatures.

5.3.4. ***Effect of modulating of pre-adult developmental rate and clock period (τ) on selection-mediated difference in development time:*** To study the effect of manipulation of developmental rate and τ on the selection-mediated difference in pre-adult development time, we assayed the effect of two types of WC cycles presented under DD (12:12 h WC1 25:18 or WC2 29:25 ^{o}C) on the difference in development time between the two stocks. Emergence profiles under WC show that most FD and BD flies emerge during second half of the cold phase, just before low to high temperature transition (Figure 5.2a). ANOVA revealed a statistically significant effect of WC and S; however, the effect of WC × S interaction was statistically not significant (Table 5.2a). The mean development time of both stocks was shorter under WC2 compared to WC1 (Figure 5.2b). Post-hoc multiple comparisons using Tukey's HSD test revealed that in both the regimes FD flies developed significantly faster than BD controls. Furthermore, ANOVA on the relative difference between the development time of the two stocks showed a statistically significant effect of WC (Figure 5.2b). Post-hoc comparisons using Tukey's HSD test revealed that the relative difference in development time was significantly greater under WC2 compared to that under WC1 or DD (at 25 ^{o}C, Figure 5.2b). These results reveal that selection-mediated difference in development time persists, albeit with different rate, even when the clock period of flies is held at 24 h under WC1 and WC2.

5.3.5. ***Effect of manipulation of the phase of light/dark (LD) and warm/cold (WC) cycles on selection-mediated difference in development time:*** Thus far, we saw little or no effect of

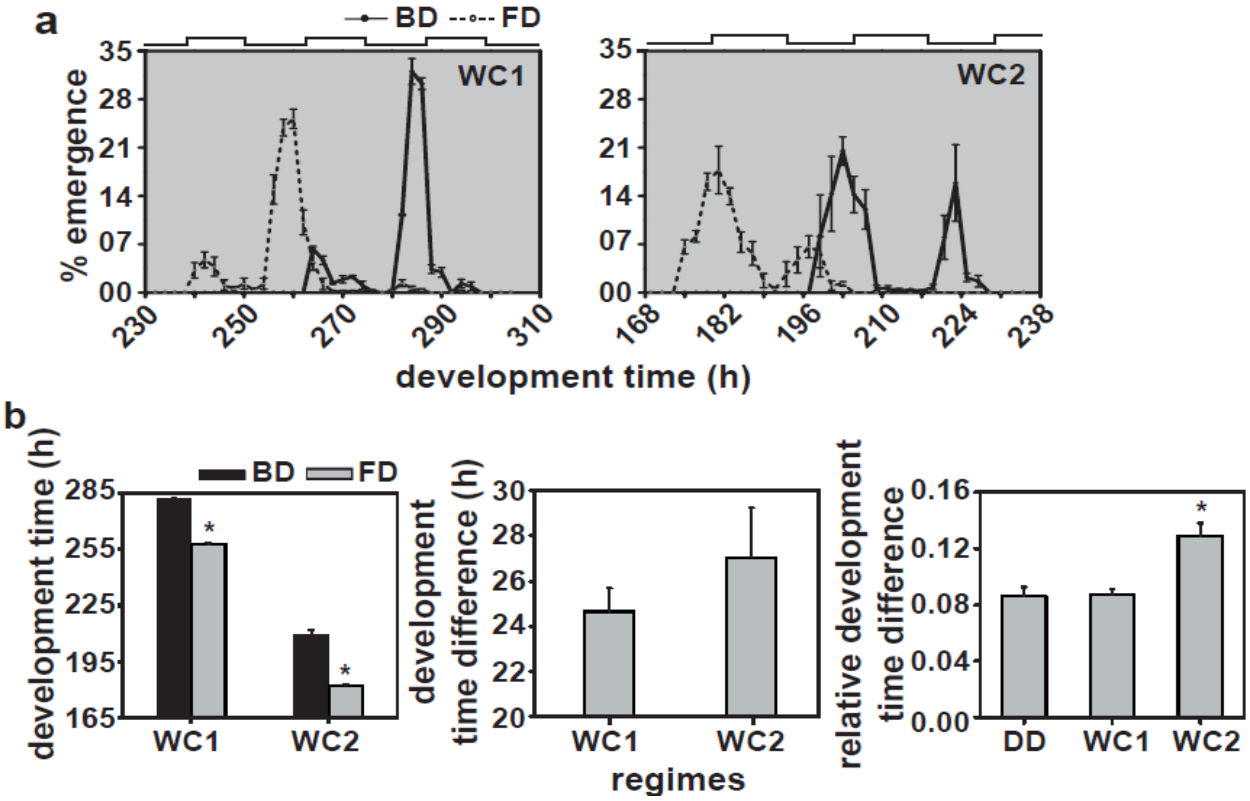


Figure 5.2. The emergence waveform of the selected stocks is altered by warm/cold (WC) cycles: (a) Waveform of adult emergence of the selected and control stocks under DD with 12:12 h WC1 and WC2 shows effect of WC cycles. (b) Average development time, selection-mediated difference in development time and relative difference [(BD-FD)/BD] in pre-adult development time between the two stocks under DD (at 25 ^{o}C) with 12:12 h WC1 and WC2. All other details are same as in Figure 5.1.

manipulation of developmental rate and/or τ on the selection-mediated difference in development time (except under WC2), so we decided to assay the development time of flies under WC2 superimposed with in-phase (WC-LD^{IP}) or out-of-phase (WC-LD^{OP}) LD cycles. Emergence profiles under WC-LD^{IP} shows that flies from both stocks emerge only during the cold and dark phase (Figure 5.3a). The BD stocks emerge towards the second half of the night with a prominent peak before lights on or low to high temperature transition, while emergence profile of the FD stocks was spread out in the dark with a relatively reduced peak before lights on or low to high temperature transition (Figure 5.3a). The emergence profile under WC-LD^{OP} shows that FD flies start to emerge in the dark phase disregarding the warm condition and continue to emerge in the light and cold phase, while BD flies emerge only (or mostly) during the light and cold phase (Figure 5.3a). This suggests that for emergence, both LD and WC cycles are important, however, stocks are normally maintained at 25 ^{o}C which is likely to have enabled WC2 to dominate over LD. ANOVA revealed a statistically significant effect of regime (R), S and R × S interaction (Table 5.2b). Post-hoc multiple comparisons using Tukey's HSD test revealed that pre-adult development time of BD stocks was significantly shorter under WC-LD^{OP} compared to that under WC-LD^{IP} (Figure 5.3b), while development time of FD stocks did not differ between these two conditions (Figure 5.3b). ANOVA on the relative difference in development time revealed that the effect of phase (P) was statistically significant (Figure 5.3c; Table 5.2c). These results suggest that while pre-adult development time of control flies depends upon the phase relationship between LD and WC cycles that of FD flies does not. Interestingly, the development time of FD stocks under WC-LD^{IP} or WC-LD^{OP} or WC2 remained comparable at ~175 h (Figure 5.3b), suggesting that LD cycles do not affect the developmental rate of faster developing flies.

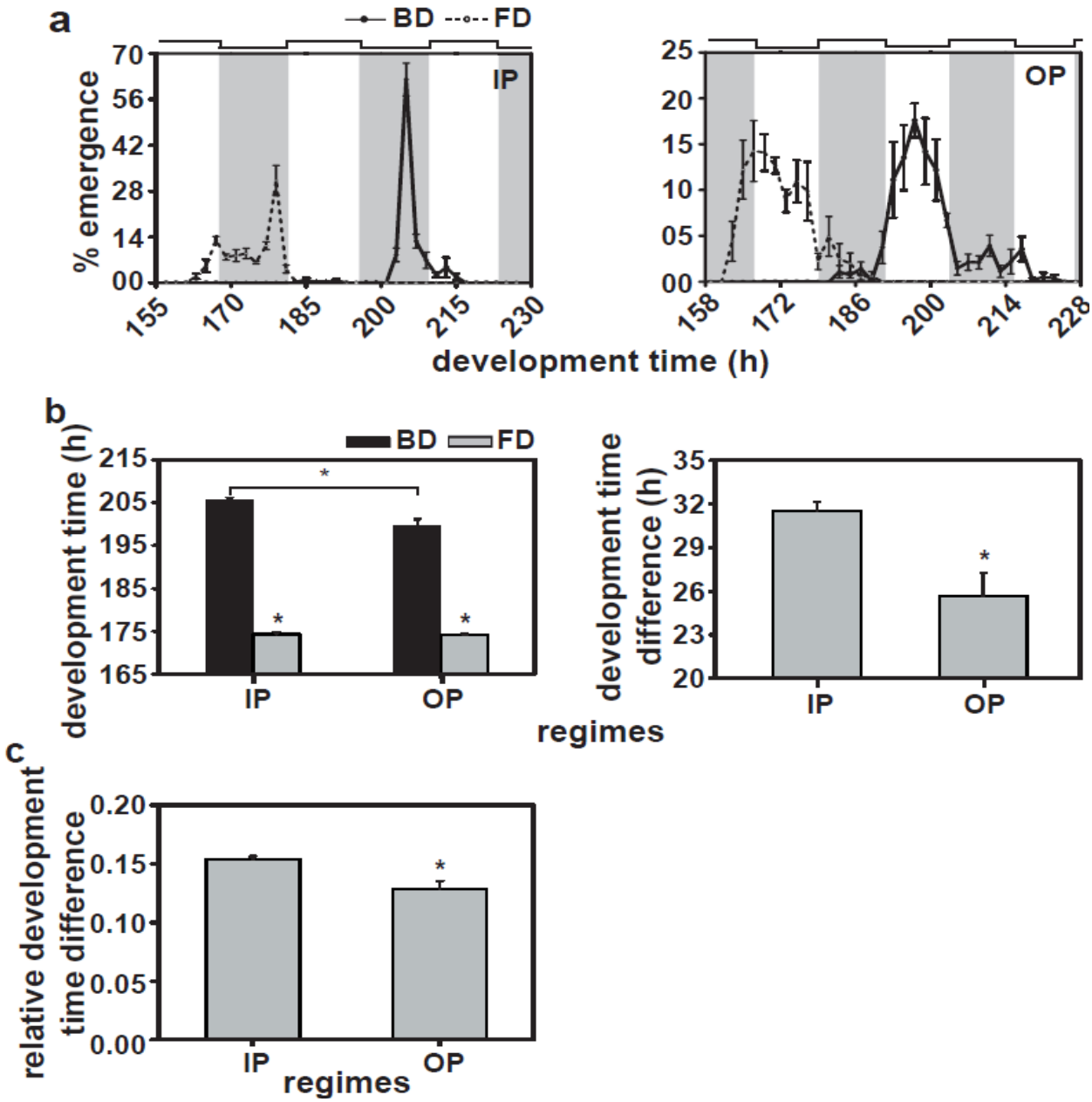


Figure 5.3. Effect of two zeitgebers under in-phase (IP) and out-of-phase (OP) warm/cold (WC) and light/dark (LD) cycles: (a) Average development time waveform of the selected and control populations under LD12:12 and WC-LD^{IP} (in-phase) and WC-LD^{OP} (out-of-phase). (b) Average development time and difference in average development time between the selected and control stocks under WC-LD^{IP} and WC-LD^{OP}. (c) Relative difference [(BD-FD)/BD] in pre-adult development time between the selected and control stocks under WC-LD^{IP} and WC-LD^{OP} conditions. All other details are same as in Figure 1.

Table 5.2a. Results of ANOVA on the development time data under warm/cold cycles (WC1 and WC2).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
WC cycle (WC)	1	21832.81	3	8.93	2444.85	0.0001
Stock (S)	1	2674.26	3	8.14	328.55	0.0004
Block (B)	3	4.07	0	0	--	--
WC × S	1	5.71	3	3.47	1.65	0.29
WC × B	3	8.93	0	0	--	--
S × B	3	8.13	0	0	--	--
WC × S × B	3	3.47	0	0	--	--

Table 5.2b. Results of ANOVA on the development time data under light/dark (LD12:12), warm/cold cycles (WC2) and WC cycles in-phase and out-of-phase with LD cycles (WC-LD^{IP} and WC-LD^{OP}).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Regime (R)	3	526.84	9	3.35	157.27	0.0001
Stock (S)	1	4991.64	3	4.67	1069.68	0.0001
Block (B)	3	20.59	0	0	--	--
R × S	3	92.51	9	5.01	18.49	0.0003
R × B	9	3.35	0	0	--	--
S × B	3	4.67	0	0	--	--
R × S × B	9	5.01	0	0	--	--

Table 5.2c. Results of ANOVA on the relative development time difference data under light/dark (LD), warm/cold (WC2) and in-phase and out-of-phase WC and LD cycles (WC-LD^{IP} and WC-LD^{OP}).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Phase (P)	3	0.004	9	0.001	23.21	0.0001
Block (B)	3	0.001	0	0	--	--
P × S	9	0.001	0	0	--	--

5.4. Discussion

5.4.1. ***Break down of correlation between development time and clock period (τ):*** By 55 generations of selection for faster pre-adult development, FD flies evolved faster rate of development and shorter τ (Yadav and Sharma, 2013; Chapter 3). At constant temperature of 25 ^{o}C, FD flies developed ~29 h faster and had τ shorter by ~0.5 h compared to BD controls, implying a positive correlation between development time and τ. The results of the present study revealed that flies selected for faster pre-adult development (FD) develop faster than controls (BD) under all the three temperatures tested (18, 25 and 29 ^{o}C), however, difference in the mean development time of the two stocks was greater at colder temperature (~52 h) and smaller at warmer temperature (~21 h), compared to that under the native rearing temperature of 25 ^{o}C (~29 h) (Figure 5.1a). While the rate of pre-adult development was altered with increase/decrease in ambient temperature, selection-mediated difference in development time between the FD and BD flies became highest at 18 ^{o}C and lowest at 29 ^{o}C, the τ of activity/rest rhythm underwent a significant reduction at high and low temperatures compared to that at 25 ^{o}C and selection-mediated difference in τ disappeared at those temperatures, indicating a temperature dependent break down of correlation between pre-adult development time and τ (Figure 5.1c). Such break down of correlation between development time and τ is not uncommon; as it was also reported in a long term selection study wherein fruit fly populations were subjected to selection for narrow gate of adult emergence (Kannan et al., 2012b; Mukherjee et al., 2012). While the selected flies took longer to develop as pre-adults compared to controls, the τ of their activity/rest rhythm was significantly reduced. Similarly, in a separate study on the adzuki bean beetle *Callosobruchus chinesis*, correlation between development time and τ was found to be lacking (Harano and Miyatake, 2011). While we do not yet know the reason behind

such break down of correlation between development time and τ, we speculate that it may be due to slowing down or speeding up of development by unusually low and high temperatures, mediated through clock independent mechanisms. It is possible that low temperature conditions induce diapause like situation similar to the ones observed in a previous study by Ames and Turner (2003), who reported severe (non-additive) slowing down of development indicating that flies prepare for diapause by altering its physiology in a manner that is not in line with "common temperature" observations. However, it is also possible that the breaking down of such correlations is a feature specific to these sets of populations.

Since circadian clocks are expected to start ticking right from the early stages of development the egg stage (Sehgal et al., 1992; Kaneko et al., 2000; Kowalska et al., 2010), selection-mediated difference in the pre-adult development time (~29 h), observed under DD at 25 ^{o}C is likely to comprise of clock independent (~24 h) and clock dependent components (~5 h, cumulative speeding up of clocks at the rate of 0.5 h/day in 10 days). However, even while being entrained under LD (Chapter 4), WC1 and WC2 cycles, the differences in development time between the FD and BD stocks were ~26 h, ~28 h and ~26 h, respectively, implying that the development time of fruit flies *D. melanogaster* is not exclusively under circadian clock control. Considering the same pace of the FD and BD clocks under entrained state, the clock dependent components will have little or no share in the divergence of development time except the difference that could be attributable to the phase difference in their emergence rhythms. Interestingly, the relative difference in development time remains largely unaffected by the manipulations in developmental rates and/or clock period, which suggests that despite the changes in the absolute difference in development time between the FD and BD flies at low or high temperatures, relative change in the rate of development remains unaltered (Figure 5.1a).

However, in artificial selection studies we do not expect correlated responses to be stronger than that for the trait under selection, and therefore it may not be appropriate to compare changes in pre-adult development time with those in τ.

5.4.2. ***Reduction in pre-adult survivorship at higher temperature:*** Rearing flies at temperatures below or above their normal rearing temperature is expected to significantly affect their fitness (Bonnier, 1926). As expected, we found a reduction in pre-adult survivorship at 29 ^{o}C compared to 18 and 25 ^{o}C, which differed significant between the FD and BD flies (Figure 5.1b). Thus, the results of our study are consistent with previous reports (Chippindale et al., 1997; Prasad et al., 2001) which showed a significant reduction in the pre-adult survivorship in the faster developing flies (Figure 5.1b; Table 5.1b).

5.4.3. ***Loss of robustness in temperature compensation:*** The rate of enzymatic reactions is expected to increase with temperature, often by more than two fold for a temperature increase by 10 ^{o}C. However, within biologically relevant range of temperature, τ of the circadian clocks whose underlying molecular machinery is comprised of biochemical reaction networks, has been found to be stable (temperature compensation; Pittendrigh, 1954). Clocks are temperature compensated not only in homeotherms but also in poikilotherms such as *Drosophila*, *Neurospora* and *Cyanobacteria* (Pittendrigh, 1954; Edmunds, 1988). It is believed that biochemical oscillators are able to keep their period unchanged in the face of variations in temperature, by striking a fine balance between the reactions that increase clock period with those that result in decrease in period (Kurosawa and Iwasa, 2005). Since temperature sensitivity of enzymatic reactions is controlled by activation energy of the reaction, which depends upon the three dimensional structure of the enzyme, a proper level of activation energy for every biochemical reaction is believed to have made the oscillator period robust against fluctuations in ambient

temperature (Kurosawa and Iwasa, 2005). However, circadian clock period is not entirely temperature compensated; rather the period varies slightly depending on the ambient temperature (Konopka et al., 1989). While, wild type strains of *Drosophila* display robust temperature compensation, long period mutant (per^L) show partial to complete loss of temperature compensation, perhaps due to the perturbation of the underlying temperature dependent intermolecular interactions governed by PER (Huang et al., 1995), or due to defects in the kinetics of molecular feedback loops (Gekakis et al., 1995). For example, in *D. melanogaster*, τ of activity/rest rhythm in the *per* mutants is altered due to temperature dependent splicing of *per*, which advances the steady state to higher level of mRNA and protein abundance (Majercak et al., 1999). Similarly, in our study, τ of activity/rest rhythm was reduced by ~1.5 h with a decrease in temperature by 11 ^{o}C (18 *vs.* 29 ^{o}C), indicating that clocks of these flies may not be temperature compensated (Figure 5.1c). While lowering or increasing of temperature speeded up clocks of both FD and BD flies, their τ values were comparable at 18 and 29 ^{o}C. Moreover, the period of FD and BD flies was significantly different at 25 ^{o}C compared to that at 18 and at 29 ^{o}C, though the difference did not reach statistical levels of significance when the effects of three temperatures were compared together (Figure 5.1c). These results indicate that the effect of selection on τ is unstable particularly at low or high temperature. Moreover, lowering of τ by ~1.5 h indicates that circadian clocks of such dark reared flies may not be temperature compensated.

5.4.4. ***Phase relationship between light and temperature cycles has significant effect on development time:*** The development time of fruit flies *D. melanogaster* is modulated by LD and WC cycles, owing to the entrainment of their developmental clocks to light or temperature cycles (Paranjpe et al., 2005; Folguera et al., 2010). To assess the relative importance of the two time

cues for the developmental clocks we exposed flies from FD and BD stocks to LD and WC cycles presented in-phase and out-of-phase. If light was more critical than temperature then flies would emerge during the light phase, whereas if temperature was more dominant, flies would emerge during the low temperature phase. The results revealed that when LD was presented in-phase with WC, flies emerged during dark which also coincided with the low temperature phase (25 ^{o}C) (Figure 5.3a), perhaps to avoid stressful effects of high temperature (29 ^{o}C), while under LD at constant 25 ^{o}C flies normally emergence in the light phase (Chapter 4). This is further confirmed when under WC-LD^{OP}, both FD and BD flies emerged during the low temperature phase even when light was present, indicating a clear dominance of temperature over light (Figure 5.3a). Although it appears that both temperature and light are equally important for the temporal regulation of development in *Drosophila*, flies tend to emerge only when the temperature was favorable, completely disregarding the light condition. These results can be taken to suggest that for the emergence clock of *Drosophila*, temperature is a more dominant Zeitgeber than light. These results are also consistent with the findings of a few previous studies, which showed that LD cycles may not be a key Zeitgeber for the circadian clocks of many insect species. For example, adult emergence rhythm of the leaf cutter bee *Megachile rotundata* (Tweedy and Stephen, 1970) and the tsetse fly *Glossina morsitans* (Zdarek and Denlinger, 1995) and feeding and digestion rhythms of the log infesting larvae of the beetle *Rhagium inquisitor* (Riba, 1976) showed a clear preference for temperature over light as Zeitgeber. However, in a separate study on the egg-laying rhythm in *D. melanogaster* both temperature and light were found to be equally important in regulating phase of the rhythm (Kannan et al., 2012a). Such comparisons of two different types of stimuli may not be appropriate as one has to take into account the strength of the stimuli, which cannot be scaled with respect to each other. For

example, weak temperature signals may prove to be less effective than light (under specified conditions), but stronger temperature stimulus might yield larger responses.

Shorter development time of BD flies in WC-LD^{OP} compared to WC-LD^{IP} with WC2 (29:25 ^{o}C) as temperature cycles could be due to the fact that under out-of-phase condition the two favorable phases for development (light and 25 ^{o}C) occur one after another, resulting in widening of the emergence profile, and faster developmental rate with shorter relative development time difference (Figure 5.3). On the other hand, emergence under in-phase condition is restricted to a particular phase (dark and 25 ^{o}C) resulting in a phase delayed emergence profile marked by a sharp peak. However, exposure to such zeitgebers at the emergence stage affects the development time of only the control flies (Figure 5.3b), which further indicates the refractoriness of faster developing (FD) flies to the environmental manipulations of pre-adult developmental rates. Interestingly, the difference in development time of the two stocks was higher when LD cycles were in-phase with WC cycles compared to when they were out-of-phase. This might be due to the fact that one of the two (morning or evening) oscillators governing the emergence rhythm is more strongly affected by external stimuli than the other. These results suggest that phases of the light and temperature cycles have significant effect on the pre-adult development time of fruit flies *D. melanogaster*.

5.5. Conclusion

The results of our study suggest that while selection-mediated reduction in development time was stronger at 18 ^{o}C and weaker at 29 ^{o}C, as compared to 25 ^{o}C, relative difference in development time of the two stocks remained unaffected. The τ of both FD and BD flies was reduced considerably under 18 and 29 ^{o}C, and in both these temperatures the selection-mediated differences in τ was diminished. Selection-mediated difference in development time also

persisted when the circadian clocks of flies were entrained under LD and WC cycles. Furthermore, the phase relationship of LD and WC cycles altered the rate of pre-adult development of BD flies, but had no effect on the development of FD flies, which continued to develop significantly faster than BD controls. Thus, our study suggests that although environmental temperature and/or light regimes significantly alter the rate of pre-adult development and/or circadian clock period, selection-mediated differences in development time of the two stocks remains largely unaffected but those in clock period was reduced significantly, suggesting a break down of correlation between pre-adult development time and τ.

Chapter 6

Lability of circadian clocks: Evidence from after-effect studies on exposure to cyclic environmental conditions in fruit fly populations selected for faster pre-adult development

6.1. Introduction

Circadian clocks regulate a variety of behavioural and metabolic processes in a wide range of organisms (Dunlap et al., 2004). These clocks entrain to light/dark (LD) or warm/cold (WC) cycles, free-run under constant darkness (DD), and are disrupted under constant light (LL) (Pittendrigh and Bruce, 1959; Highkin, 1960; Pittendrigh, 1960; Pittendrigh and Minis, 1972; Saunders, 2002c; Yoshii et al., 2005). The free-running period (τ) of these clocks is labile, and vary depending on the nature of prior environmental exposure (a phenomenon called "after-effects"; Pittendrigh, 1960, 1974; Sokolove, 1975; Christensen, 1978; Page and Block, 1980; Saunders, 2002c). Such after-effects have been found to persist for up to 98 d in some species of rodents (Pittendrigh and Daan, 1976). Prior exposure to LD cycles of a wide range of period lengths (Pittendrigh and Daan, 1976; Page and Block, 1980; Christensen and Lewis, 1982; Kenny and Saunders, 1991), LL (Pittendrigh and Daan, 1976), skeleton photoperiods (Pittendrigh and Daan, 1976), and phase-shifting light pulses (Pittendrigh and Daan, 1976) are all known to cause after-effects on circadian clocks. For example in crickets *Teleogryllus commodus*, prior exposure to continuous red light first lengthened and then gradually shortened τ of activity/rest rhythm (Sokolove, 1975). In weta *Hemideina thoracica*, prior exposure to long period LD cycles (e.g., LD8:23) significantly lengthened τ during the early days of DD, which eventually returned to its intrinsic value (Christensen and Lewis, 1982). Similarly in freshly emerged blowflies *Calliphora vicina*, prior exposure to short (LD4:20) to long (LD20:4) photoperiods for as long as 3-7 days caused an initial shortening of τ (< 24 h) before abrupt lengthening to greater than 24 h values after 6-10 days (Kenny and Saunders, 1991). Similarly, entrainment to different photophases or skeleton photoperiods, or phase-shifts caused by single light pulses, or exposure to LL was found to alter τ of activity/rest rhythm in mice *Mus musculus*

and in hamsters *Mesocricetus auratus* (Pittendrigh and Daan, 1976). Prior exposure to indoor room light (~200 lux) for 3 days attenuated the suppression of melatonin rhythm in humans (Smith et al., 2004). The τ of circadian rhythms in humans was found to vary according to the period length of LD cycles that they were exposed prior to the assays (Scheer et al., 2007; Czeisler and Gooley, 2007). Thus, it is clear that τ of circadian rhythms show history-dependent after-effects of light exposure in a wide variety of organisms. Although after-effects of exposure to cyclic environments have been reported for light regimes, to the best of our knowledge, such effects have never been shown for temperature cycles. On the contrary, large phase-shifts induced by cold temperature pulses, or exposure to 12:12 h WC cycles of 20:30 ^{o}C did not cause any detectable after-effect in the circadian activity/rest rhythm of cockroaches *Leucophaea maderae* (Page et al., 2001). It is believed that after-effects on circadian clocks help organisms in maintaining a stable phase-relationship with environmental cycles in the face of changing environmental and behavioural conditions (Beersma et al., 1999; Sheeba et al., 2002b).

Circadian clocks of some insect species are also developmentally plastic; exposure to LD cycles during pre-adult development alters the τ of activity/rest rhythm in *period* (*per*) mutant and wild-type flies of *Drosophila melanogaster* (Tomioka et al., 1997; Sheeba et al., 2002b). Exposure to LD cycles of varying period length, during the developmental stages not only modified the τ of activity/rest rhythm of adult cockroaches *Leucophaea maderae* but also altered their light induced phase response curve (Barrett and Page, 1989; Page and Barrett, 1989). Furthermore, it is known that rearing *Drosophila* populations under DD causes changes in its physiological and behavioural processes such as phototactic ability, quick copulation, longevity and fecundity (Mori and Yanagishima, 1959a, b; Mori, 1983; Katz and Minke, 2009; Izutsu et al., 2012). Ideally after-effect studies require a model organism, which has never been exposed

to any cyclic time cue. We therefore chose populations of fruit flies *D. melanogaster* selected for faster pre-adult development under DD (Yadav and Sharma, 2013; Chapter 3). The faster developing (FD) stocks evolved faster developmental rates (by ~29 h), and faster circadian clocks (τ ~0.50 h) than the controls (BD). Since these flies were never exposed to any daily cyclic time-cues for about 55 generations, we asked whether their circadian clocks would be affected by prior environmental experience. We also asked whether the difference in τ between FD and BD stocks would persist following exposure to a wide variety of cyclic environmental conditions including both light and temperature cues. These populations are best suited for after-effect studies on circadian clocks because of their small but consistent difference in circadian period not confounded by genetic background and age.

We assayed the activity/rest rhythm of FD and BD flies under DD after a week-long exposure to (a) either of LD cycles - 10:10 h (LD10:10), 12:12 h (LD12:12) or 14:14 h (LD14:14), or (b) 12:12 h WC of 25:18 ^{o}C (WC1) or 29:25 ^{o}C (WC2), or (c) LD cycles of 10, 100, 1000 lux light intensity (LD_{10}, LD_{100}, LD_{1000}), or (d) WC in-phase with LD12:12 cycles (WC1-LD^{IP}, WC2-LD^{IP}) or WC out-of-phase (WC1-LD^{OP}, WC1-LD^{OP}). Circadian clocks entrain readily to LD cycles within a narrow range of periodicity around 24 h (Pittendrigh and Daan, 1976; Winfree, 1980; Bordyugov et al., 2011). The frequency demultiplication hypothesis posits that circadian rhythms also entrain to LD cycles of period lengths that are multiples or submultiples of its period (Gwinner, 1973; Nisimura and Numata, 2002; Carmichael and Zucker, 1986). In the present study, we exposed fruit flies *D. melanogaster* to LD cycles with varying period length and light intensities, and to WC cycles either alone or in conjunction with LD cycles to manifest circadian entrainment of activity/rest rhythm. Our study involves two different stocks of fruit fly *D. melanogaster* with small but significant difference in τ of four FD

and four BD populations. Additionally, we used the loss of τ difference between FD and BD populations to assess the after-effects of exposure to cyclic conditions. The results provided interesting insights into cyclic environment-mediated after-effects on the τ of activity/rest rhythm in *Drosophila.* The results suggest that prior exposure to light and temperature cycles significantly changed the τ of FD and BD stocks, therefore clearly eliciting after-effects on circadian clocks. The difference in τ between selected and control stocks was reduced significantly following a week-long exposure to cyclic conditions, suggesting that circadian timing systems of *Drosophila* are plastic.

6.2. Materials and methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by a method described in detail in chapter 2.

Standardized populations were used for various assays described below.

6.2.1. Activity/rest rhythm assay: Flies from standardized populations of *D. melanogaster*, maintained under DD, were allowed to lay eggs for a period of 12 h on banana food. Sixty to eighty eggs were collected and dispensed into vials containing 6 ml banana food and introduced into a DD cubicle maintained at constant 25 °C temperature. Starting sixth day after egg collection, vials were regularly monitored for darkening of pupae. After pupae became dark, vials were continuously monitored for emergence, and newly emerging adult flies were collected and separated at regular intervals of 4-5 h. Only virgin males were transferred into locomotor activity tubes (8 cm long and 5 mm diameter) with corn meal at one end sealed with paraffin wax and plugged with cotton at the other end. Starting on the second day of emergence, activity/rest behaviour of flies from $FD_{1\text{-}4}$, and $BD_{1\text{-}4}$ stocks was monitored for a maximum of 7 cycles under

LD or WC cycles in a temperature-controlled incubator, and then shifted into DD at 25 ^{o}C for minimum of 11days. Physical conditions such as light intensity, temperature and relative humidity inside the incubators were recorded every 5 min during the entire assay using *Drosophila* Environmental Monitor, DEnM (Trikinetics, USA), and were found to be stable (temperature - 25 ± 0.5 ^{o}C, and relative humidity - 80-90%). The light phase of LD cycles was created with fluorescent white light of intensity ~100 lux, and red light of λ greater than 650 nm was used during the dark phase for the ease of assay. Activity tubes with fresh food were replaced 1day prior to the shift into DD. The activity/rest behaviour of virgin adult flies was recorded individually using *Drosophila* Activity Monitoring (DAM) system from Trikinetics, USA. The DAM system is a computer-aided device that uses a pair of IR emitter and sensor to detect movements of flies. The τ of the activity/rest rhythm under DD was calculated using Lomb Scargle Periodogram in CLOCKLAB from Actimetrics, USA. The locomotor activity assay under DD was performed in parallel with other assays described above and served as experimental control for comparison of change in τ and its difference between selected and control stocks.

6.2.2.Statistical Analyses: The τ data from various assays were subjected to mixed model analysis of variance (ANOVA), treating block (populations 1-4) as random factor and stock (FD and BD) as a fixed factor crossed with block. In all cases, block means were used as the unit of analysis and hence, only fixed factors could be tested for statistical significance. The after-effects of exposure to cyclic conditions on the τ of activity/rest rhythm was analyzed using ANOVA followed by post-hoc multiple comparisons using Tukey's honestly significant difference (HSD) test. All statistical analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (StatSoft Inc. 1995).

6.3. Results

6.3.1. Period (τ) shortening in faster developing flies: Average actograms of BD and FD stocks (Figure 6.1a) show that flies from both stocks free-run under DD albeit with different τ. Consistent with the results of our previous study (Yadav and Sharma, 2013), τ of activity/rest rhythm of FD (24.08 ± 0.04 h; mean ± SEM) was significantly shorter than that of BD (24.61 ± 0.08 h; $F_{1,3}$ = 48.17; p <0.006; Figure 6.1b), when adult flies were reared and recorded under DD. This assay was performed in parallel with other experiments described below and served as control for the comparison of change in τ and as baseline difference between FD and BD stocks.

6.3.2. After-effects of exposure to light/dark (LD) cycles: We estimated τ of activity/rest rhythm of flies exposed to 2 or 7 cycles of LD12:12 and compared it with that of flies which were exposed to DD (Figure 6.1b). The τ of BD flies exposed to 2 cycles of LD12:12 was significantly shorter than that of flies exposed to DD, however, τ of flies exposed to 7 cycles of LD12:12 was longer than that of flies exposed to DD (Figure 6.1b). In FD, the τ of flies exposed to 2 cycles of LD12:12 did not elicit any after-effects on τ as it was comparable to flies exposed to DD, but τ of flies exposed to 7 cycles of LD12:12 was significantly longer than that of flies exposed to 2 cycles of LD12:12 (Figure 6.1b). ANOVA revealed a statistically significant effect of number of cycles (N) ($F_{2,6}$ = 46.07; p <0.0002), stocks (S) ($F_{1,3}$ = 41.71; p <0.008) and N × S interaction ($F_{2,6}$ = 5.78; p <0.04; Figure 6.1b; Table 6.1a). Post-hoc multiple comparisons using Tukey's test revealed that in BD stocks, τ of flies exposed to 2 cycles of LD12:12 was significantly shorter than that of flies exposed to DD and that of flies exposed to 7

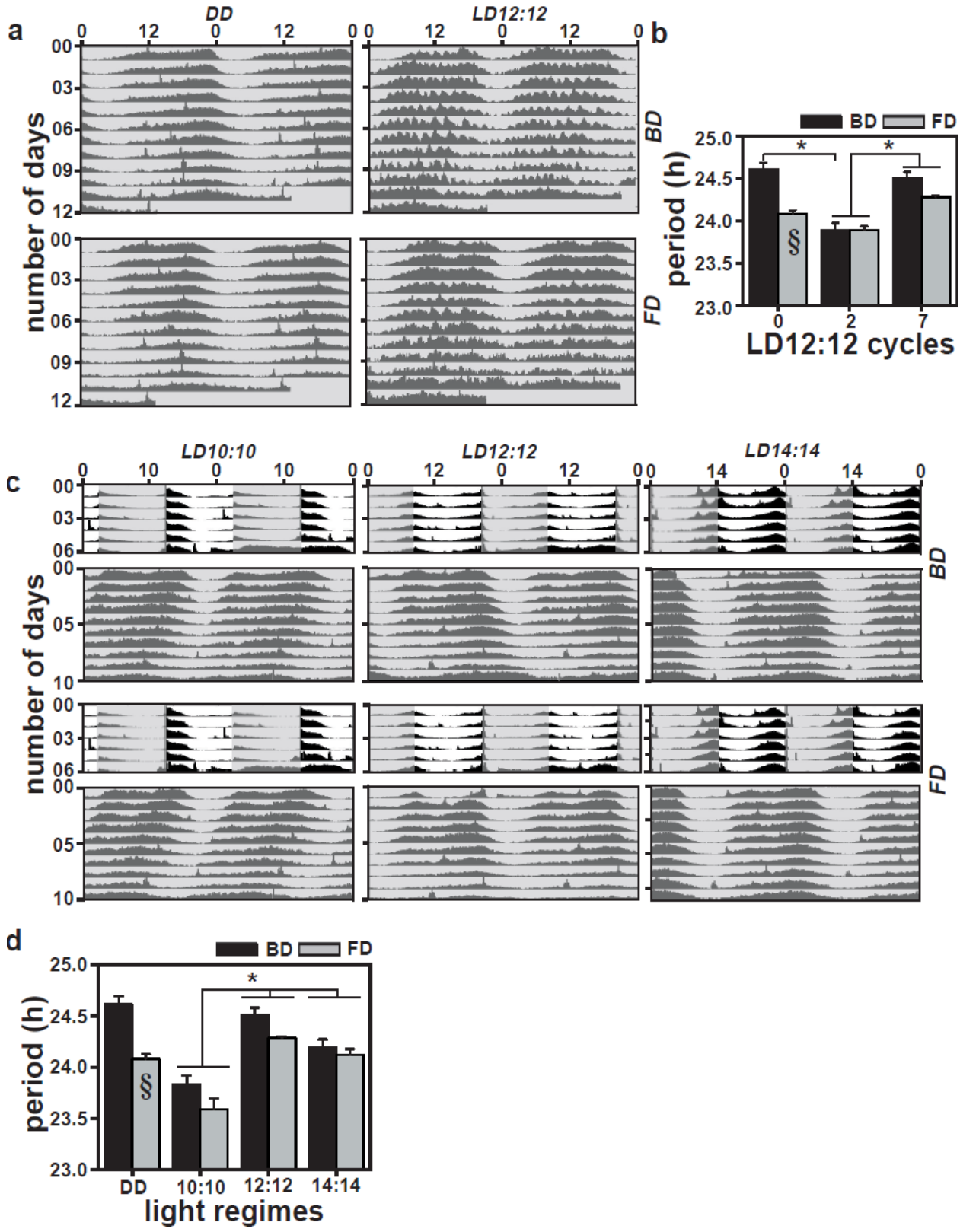

a
DD
LD12:12
number of days
BD
FD
b
BD
FD
period (h)
LD12:12 cycles
c
LD10:10
LD12:12
LD14:14
number of days
BD
FD
d
BD
FD
period (h)
light regimes
DD
10:10
12:12
14:14

Figure 6.1. After-effects of light/dark (LD) cycles: (a) Average double-plotted actograms showing activity rhythm of control (BD) and selected (FD) male flies under constant darkness (DD, left panel) and following 2 cycles LD12:12 (right panel). (b) Average free-running period (τ) of BD and FD stocks showing significant difference in DD exposed flies compared to those that were exposed to 2 and 7 cycles of LD12:12. (c) Average actograms showing entrainment under LD10:10 (left panel), LD12:12 (center panel) and LD14:14 h (right panel) and after-effects of 7 cycles of LD 10:10, LD12:12 and LD14:14 (lower panel) on BD and FD flies. Time (in h) is plotted along the *x*-axis and days of recording in chronological manner are plotted along the *y*-axis. The white and gray shaded regions represent light and dark phase of LD and completely gray shaded region represents DD regime of the assay. (d) The τ of BD and FD stocks under DD significantly differed with that following 7 cycle exposure of LD10:10, LD12:12 and LD14:14. Error bars are standard error around the mean (SEM). Asterisks indicate statistically significant differences and symbol "§" represents only statistically significant differences between FD and BD stocks marked in FD bar.

cycles of LD12:12. The τ of FD and BD flies exposed to LD12:12 for 2 or 7 cycles did not differ statistically, while it differed significantly in flies exposed to DD (Figure 6.1b). These results indicate that 2 or 7 cycle exposure of LD12:12 significantly alters τ of activity/rest rhythm in *Drosophila*.

Since we found that exposure to 2 or 7 cycles of LD12:12 caused after-effects on two different sets of four *D. melanogaster* populations, we decided to study such after-effects on the τ of activity/rest rhythm following exposure to LD cycles with different period lengths, such as LD10:10, LD12:12, and LD14:14. Average actograms of BD and FD stocks (Figure 6.1c) shows that flies from both stocks entrained to all imposed LD cycles and free-ran under DD. Analysis revealed that exposure to LD cycles resulted in after-effects on τ in a way that difference between the two stocks was abolished. ANOVA revealed a statistically significant effect of light regime (R) ($F_{3,9}$ = 53.67; p <0.0001) and S ($F_{1,3}$ = 12.57; p <0.04), however, the effect of R × S interaction was statistically not significant ($F_{3,9}$ = 2.90; p =0.09; Figure 6.1d; Table 6.1b). Post-hoc multiple comparisons using Tukey's test revealed that τ of flies exposed to LD10:10 was significantly shorter compared to that of flies exposed to LD12:12 and LD14:14 (Figure 6.1d). Following exposure to any of three types of LD cycles, the difference in τ between FD and BD stocks, seen in flies exposed directly to DD was diminished. Thus, our results clearly suggest after-effects of period of LD cycles on the τ of activity/rest rhythm. Since 2 cycle exposure shortened the τ of BD flies leaving that of FD unaffected, while 7 cycle exposure altered τ of both FD and BD flies, we chose 7 cycle exposure for further experiments.

6.3.3. After-effects of light/dark (LD) cycles with different light intensities: To study the role of zeitgeber strength in eliciting after-effects on τ, we recorded the activity under DD, of flies

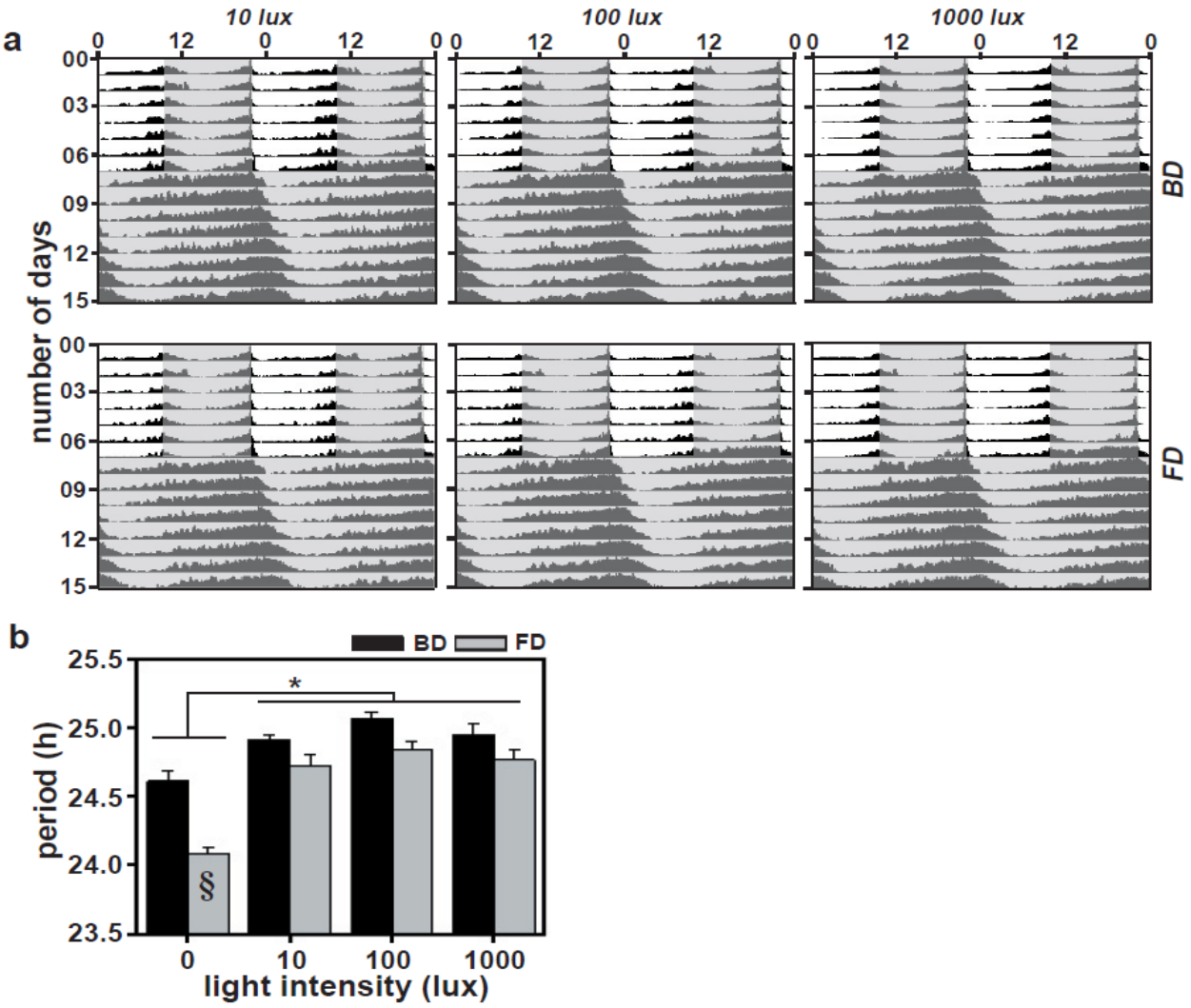


Figure 6.2. After-effects of exposure to light/dark (LD) cycles with different light intensities: (a) Average actograms showing after-effects of 7 cycle exposure to 12:12 h light/dark (LD12:12) with 10 (left panel), 100 (middle panel) and 1000 lux (right panel) intensities on BD and FD flies. (b) Average free-running period (τ) showing significant difference between τ of BD and FD stocks exposed to DD compared to that following 7 cycle exposure to LD12:12 with 10, 100 and 1000 lux. All other details are same as in Figure 6.1.

from FD and BD stocks, following exposure to 7 cycles of LD12:12 with 10 lux (LD_{10}), 100 lux (LD_{100}) and 1000 lux (LD_{1000}) intensities. Simultaneously, we recorded the activity/rest behaviour of those flies that had been exposed to DD. Average actograms of BD and FD stocks (Figure 6.2a) show that activity/rest rhythm of flies entrained to all three LD cycles and subsequently free-ran under DD. Compared to flies which were exposed to DD, exposure to LD cycles of all the three intensities lengthened τ of both FD and BD flies. ANOVA revealed a statistically significant effect of light intensities (I) ($F_{3,9}$ = 70.58; p <0.0001), S ($F_{1,3}$ = 19.59; p <0.02), and I × S interaction ($F_{3,9}$ = 4.63; p <0.032; Figure 6.2b; Table 6.1c). Post-hoc multiple comparisons using Tukey's test revealed that τ of flies exposed to 10, 100 and 1000 lux intensities was significantly longer than that of those exposed to DD (or 0-lux; Figure 6.2b). Additionally, the τ difference between FD and BD stocks was abolished following exposure to 7 cycles of LD (Figure 6.2b). These results suggest that while prior exposure to LD12:12 significantly lengthens the τ of activity/rest rhythm, it abolishes the difference in τ between FD and BD stocks.

6.3.4. After-effects of exposure to warm/cold (WC) cycles: Since temperature is also considered as an important zeitgeber for the circadian entrainment of *Drosophila* activity/rest rhythm, we exposed flies from FD and BD stocks to 7 cycles of WC1, or of WC2, and assayed the activity/rest rhythm under DD at 25 ^{o}C, to examine whether WC cycles cause any after-effects on the τ of activity/rest rhythm of FD and BD flies as was seen for LD cycles. Average actograms of FD and BD stocks (Figure 6.3a) show that flies entrained to WC1 and WC2 and free-ran under DD. Prior exposure to WC caused detectable after-effects on τ (Figure 6.3b). Following exposure to WC, τ of flies from FD stocks became similar to that of flies from BD stocks (Figure 6.3b). ANOVA revealed a significant effect of R ($F_{2,6}$ = 13.28; p <0.006), S ($F_{1,3}$

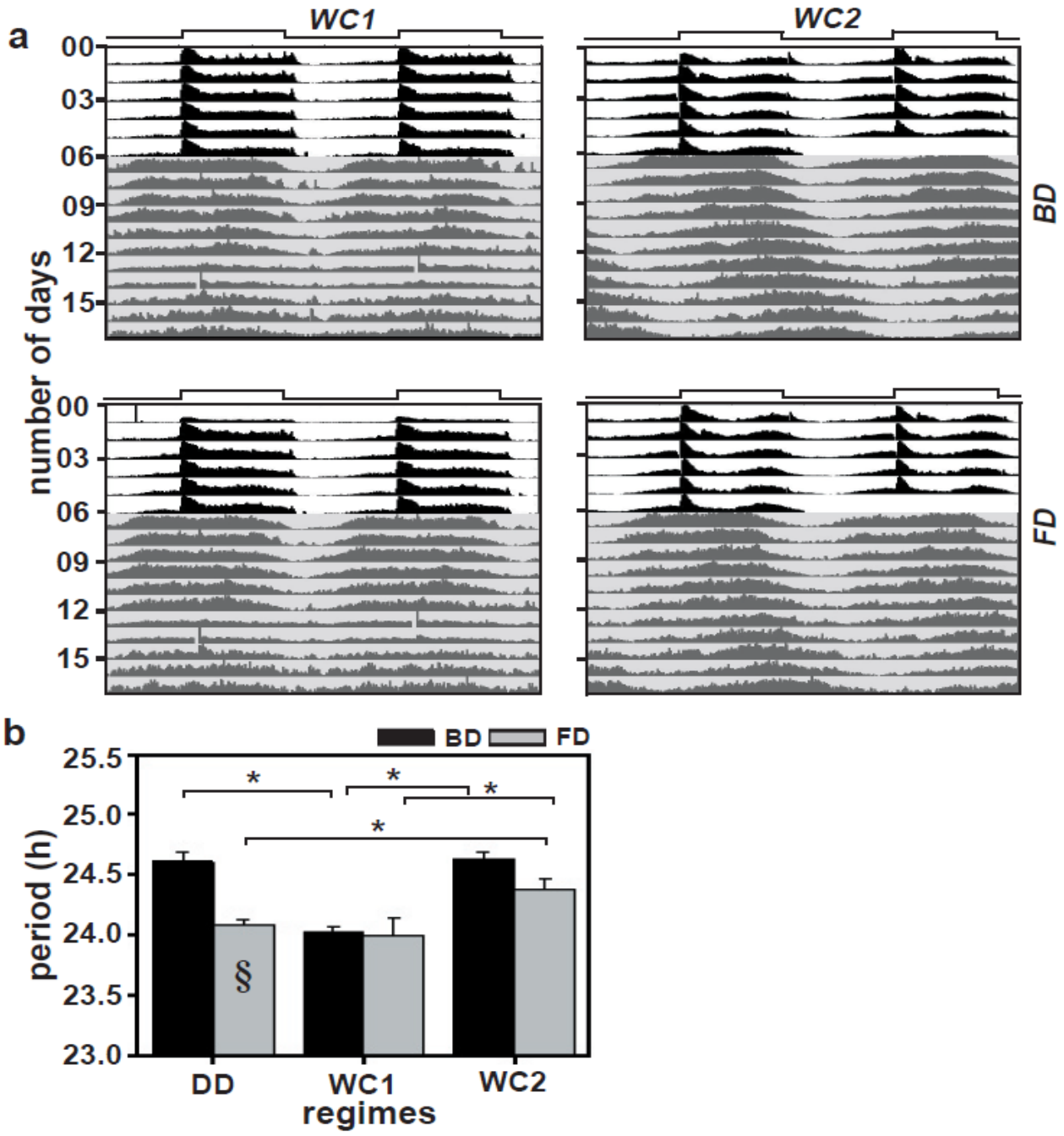


Figure 6.3. After-effects of warm/cold (WC) cycles: (a) Average actograms showing after-effects of 7-cycle exposure to 12:12 h warm/cold (WC) cycles of 18:25 ^{o}C (WC1) or of 25:29 ^{o}C (WC2) cycles on control (BD) and selected (FD) populations. Average actograms showing entrained and free-running activity/rest rhythm of flies exposed to WC1 or to WC2 cycles on BD and FD flies under DD. (b) Average free-running period (τ) showing significant difference between the τ of BD and FD stocks following exposure to WC1 and WC2 compared to DD. All other details are same as in Figure 6.1.

= 14.50; p <0.03) and R × S interaction ($F_{2,6}$ = 10.90; p <0.01; Table 6.1d). Post-hoc multiple comparisons using Tukey's test revealed that in BD stocks, exposure to WC1 significantly shortened τ compared to that of flies exposed to DD, while τ of FD flies remained unchanged (Figure 6.3b). Similarly, exposure to WC2 significantly lengthened the τ of FD flies compared to that of flies exposed to DD, while that of BD flies remained unchanged (Figure 6.3b). In addition, unlike flies exposed to DD, τ of FD and BD flies exposed to WC cycles did not differ. Thus, our results indicate the role of WC1 and WC2 in eliciting after-effects and suggest that WC cycles in general influence the circadian clocks and the τ of activity/rest rhythm in *Drosophila*.

6.3.5. After-effects of warm/cold (WC) cycles superimposed with in-phase (IP) or out-of-phase (OP) light/dark (LD) cycles: Since cycles of light and temperature are the two key zeitgebers for the circadian entrainment of *Drosophila* activity/rest rhythm, we subjected flies to 7 cycles WC1 or WC2, presented in-phase (WC1/WC2- WC-LD^{IP}), or out-of-phase with LD12:12 (WC1/WC2-WC-LD^{OP}). Average actograms show that flies from both stocks entrained to WC1-LD^{IP} or WC1-LD^{OP} (Figure 6.4a) and WC2-LD^{IP} or WC2-LD^{OP} (Figure 6.4b) and free-ran under DD. Following WC1-LD^{IP} exposure, FD flies had a slightly shorter τ compare to BD flies, while following exposure to WC1-LD^{OP}, τ of FD and BD flies did not differ (Figure 6.4c). Similarly following exposure to WC2-LD^{IP}, τ of FD and BD flies did not differ (Figure 6.4c). ANOVA revealed a statistically significant effect of R ($F_{4,12}$ = 81.05; p <0.0001), S ($F_{1,3}$ = 14.01; p <0.03), and R × S interaction ($F_{4,12}$ = 4.69; p <0.02; Figure 6.4c; Table 6.1e). Post-hoc multiple comparisons using Tukey's test revealed that τ of FD and BD stocks did not differ following exposure to WC1-LD^{IP} or WC-LD^{OP}, or to WC2-LD^{IP} or WC2-LD^{OP} conditions. Interestingly, WC2-LD^{IP} and WC2-LD^{OP} exposure significantly shortened the τ of both FD and BD flies

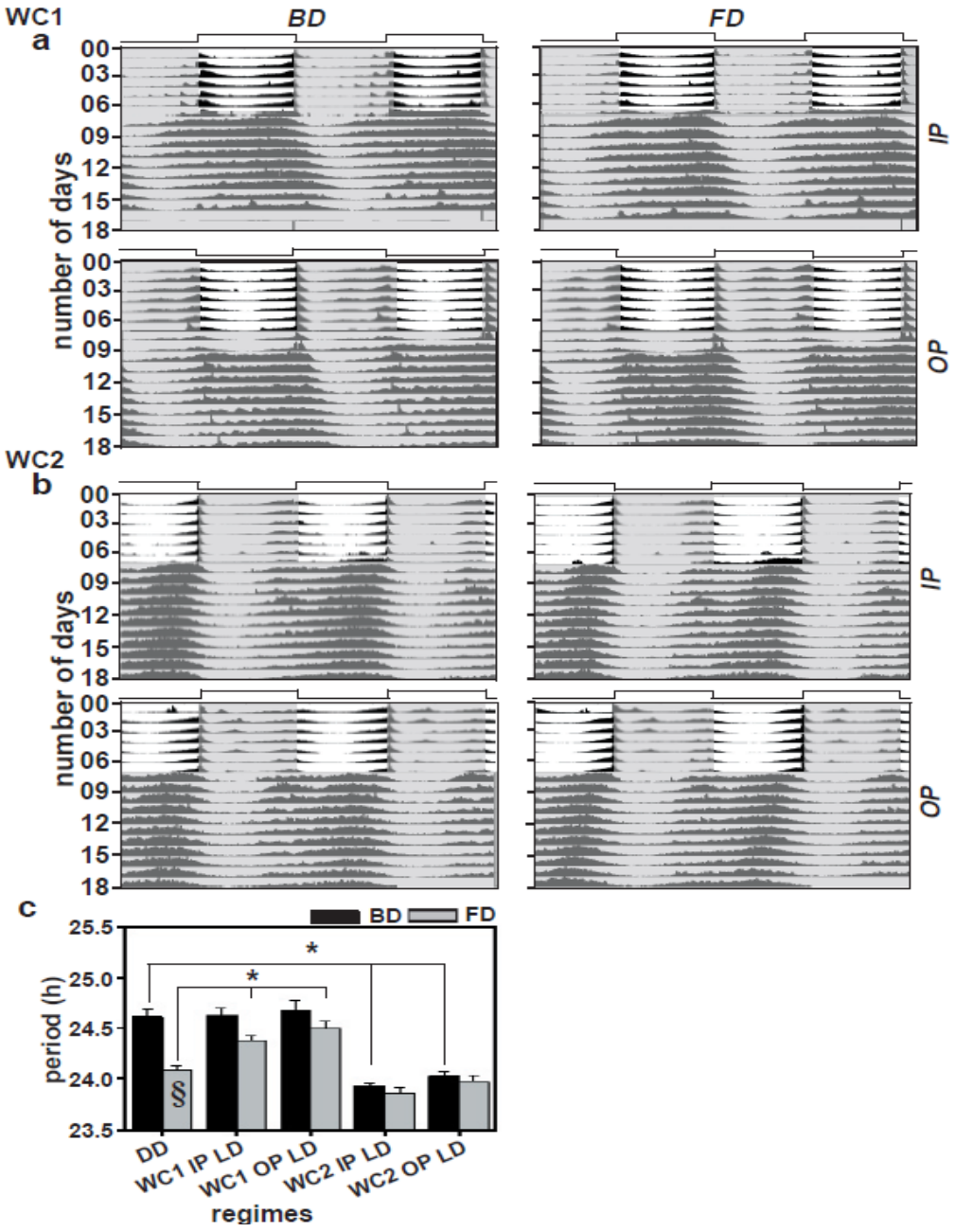


Figure 6.4. After-effects of warm/cold (WC) cycles superimposed with light/dark (LD) cycles: (a) Average actograms of BD and FD stocks showing entrained and free-running activity/rest rhythm of flies exposed to WC1 in-phase (WC1-LD^{IP}) and out-of-phase (WC1-LD^{OP}) with LD12:12. (b) Similarly, average actograms of BD and FD flies showing entrained and free-running activity/rest rhythm of flies exposed to WC2 in-phase (WC2-LD^{IP}) and out-of-phase (WC2-LD^{OP}) with LD12:12 cycles. (c) Average free-running period (τ) of BD and FD stocks showing significant difference between flies exposed to WC1-LD^{IP} or WC1-LD^{OP} compared to WC2-LD^{IP} and WC2-LD^{OP}, or DD. All other details are same as in Figure 6.1.

Table 6.1a. Results of ANOVA on circadian period (τ) data following exposure to DD, 2 and 7 cycles of light/dark (LD12:12) cycles.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Cycles (N)	2	0.622	6	0.014	46.074	0.0002
Stocks (S)	1	0.385	3	0.009	41.714	0.0075
N × S	2	0.136	6	0.024	5.778	0.0399

Table 6.1b. Results of ANOVA on circadian period (τ) data following exposure to light/dark (LD10:10, LD12:12, LD14:14) cycles.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light regimes (R)	3	0.783	9	0.015	53.673	0.0001
Stocks (S)	1	0.583	3	0.046	12.565	0.0382
R × S	3	0.069	9	0.024	2.901	0.0939

Table 6.1c. Results of ANOVA on circadian period (τ) data following exposure to light/dark (LD12:12) cycles of different light intensities.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Intensity (I)	3	0.586	9	0.008	70.576	0.0001
Stocks (S)	1	0.634	3	0.032	19.588	0.0214
I × S	3	0.054	9	0.012	4.627	0.0319

Table 6.1d. Results of ANOVA on circadian period (τ) data following exposure to warm/cold cycles (WC1 and WC2).

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Regimes (R)	2	0.503	6	0.038	13.284	0.0063
Stocks (S)	1	0.437	3	0.031	14.502	0.0318
R × S	2	0.123	6	0.011	10.901	0.0101

Table 6.1e. Results of ANOVA on circadian period (τ) data following exposure to warm/cold cycles (WC) inphase and out-of-phase to light/dark (LD12:12) cycles.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Regimes (R)	4	0.749	12	0.009	81.05	0.0001
Stocks (S)	1	0.471	3	0.034	14.01	0.0333
R × S	4	0.072	12	0.015	4.692	0.0164

compared to that of flies exposed to WC1-LD^{IP} or WC1-LD^{OP} and that of BD exposed to DD (Figure 6.4c; Table 6.1e). Thus temperature cycles in-phase or out-of-phase with LD are effective in causing after-effects on the τ of activity/rest rhythm. These results thus suggest that prior exposure to cyclic conditions such as LD cycles (of 2 or 7 cycles, period lengths, or intensities), WC cycles of different temperature range (25-18 or 29-25 ^{o}C), and WC-LD^{IP} or WC-LD^{OP}, entrain activity/rest rhythm of fruit flies *D. melanogaster* and cause long-lasting after-effects on the τ.

6.4. Discussion

Exposure to LD12:12 for as few as 2 cycles readily changed τ and elicited after-effects (Figure 6.1c). While, exposure of LD10:10 substantially reduced the τ of both FD and BD flies and abolished the selection-mediated difference between the FD and BD stocks, exposure of LD12:12 or LD14:14 only abolished the selection-mediated difference in τ (Figure 6.1d). Treatments affect the 'morning' or 'evening' oscillator differently (the reduced 'morning' component), predominantly the LD12:12 recordings of both BD and FD might indicate that the morning oscillator is reduced (Figure 6.1c). In our study exposure to as few as 2 cycles of LD was enough to elicit after-effects on the τ of activity/rest rhythm. These results are interesting in the light of the fact that most after-effects in circadian rhythms reported thus far were due to long-term exposure (Pittendrigh and Daan, 1976; Barrett and Page, 1989; Page and Barrett, 1989; Christensen and Lewis, 1982; Kenny and Saunders, 1991), which is likely to include confounding effects of age in addition to that of prior environmental conditions. Consistent with previous reports in rodents (Pittendrigh and Daan, 1976) and in cockroaches (Christensen and Lewis, 1982), exposure to short period LD cycles (LD10:10) shortened the τ of activity/rest rhythm compared to that of flies exposed to DD, however, exposure to long period LD cycles

(LD12:12 or LD14:14) did not lengthen the τ beyond what was seen in flies exposed to DD (Figure 6.1d). This could be due to stability of τ of laboratory dark reared populations (Yadav and Sharma, 2013; Chapter 3), which allows shortening of τ to a certain extent but does not permit lengthened beyond its natural values.

Exposing flies to 7 cycles of LD12:12 of different light intensities revealed the effectiveness of LD exposure in lengthening of τ and in abolishing the τ difference between BD and FD stocks. Since, flies from FD and BD stocks did not show any difference in τ after being exposed to LD12:12 with different intensities, it validates our previous results of efficacy of LD cycles to elicit after-effects (Figure 6.2b). Possibly, rearing flies in the dark for over 100 generations has enhanced the sensitivity of their circadian clocks to light, which might have caused a stable change in the components of the circadian pacemaker including light input pathways. However, the question of increased photosensitivity can also be addressed by other studies involving PRC or jet-lag experiments. Moreover, it would be interesting to study such after-effects in flies following exposure to LD12:12 of low light intensities to assess a range of LD cycles capable of eliciting after-effects on circadian clocks, hence the role of zeitgeber strength in causing after-effects. Such after-effects of zeitgeber exposure reveal plasticity of *Drosophila* circadian timekeeping systems. Thus, our study showed that LD cycles are unique in its ability to cause circadian entrainment and in eliciting long-lasting changes in the clock speed by modifying some elements of time-keeping system that either is unaffected or is affected with different routes or mechanism during circadian entrainment.

Temperature cycles with as low as 3 ^{o}C difference between warmer and colder conditions were found to entrain the activity/rest rhythm of *D. melanogaster* under DD (Wheeler et al., 1993), and even in LL wherein activity/rest behaviour of flies becomes arrhythmic (Yoshii et al.,

2002, 2005; Glaser and Stanewsky, 2005; Boothroyd et al., 2007). Although, both LD and WC cycles are known to act as zeitgeber for the activity/rest rhythm of *Drosophila* (Tomioka et al., 1998; Yoshii et al., 2002, 2005; Glaser and Stanewsky, 2005; Boothroyd et al., 2007; Boothroyd and Young, 2008), thus far there has been no report of after-effects of temperature cycles. Therefore, our study provides the first ever evidence for after-effects of temperature cycles on the circadian clocks in fruit flies *D. melanogaster*. Flies exposed to 7 cycles of WC 25:18 ^{o}C and 29:25 ^{o}C in otherwise DD condition altered τ of flies and reduced the τ difference between FD and BD stocks (Figure 6.3). This indicates that temperatures cycles are as effective, if not more, as light cycles in eliciting after-effects in the circadian activity/rest rhythm of fruit flies *D. melanogaster*. Currently we do not have any evidence for the equivalence of light and temperature, and therefore we cannot ascertain whether WC is more effective than LD in inducing after-effects on circadian clocks. Furthermore, exposure to WC1 reduced the τ of BD but not FD flies, whereas to WC2 increased τ of FD but not of BD flies. Such differential sensitivity of the stocks to WC cycles could be due to the fact that selection for faster development might have also resulted in altered sensitivity to temperature. This is particularly interesting because in a previous study on cockroaches, even 10 ^{o}C temperature difference (20:30 ^{o}C) in WC cycles failed to elicit any after-effects on τ activity/rest rhythm (Page et al., 2001). Thus, taken together these results suggest that temperature difference of ~4 ^{o}C not only entrain the circadian activity/rest rhythm of *Drosophila* (~3 ^{o}C in Wheeler et al., 1993), but also cause long-lasting after-effects on circadian clocks.

While all WC + LD conditions abolished the selection-mediated difference in τ, coupling of WC2 with LD12:12 in-phase or out-of-phase significantly reduced the τ of activity/rest rhythm in both FD and BD flies, while that of WC1-LD^{IP} or WC1-LD^{OP} lengthened the τ of only FD

flies (Figure 6.4; Table 6.1e). Light fluctuates along with temperature in a daily and seasonal manner under natural conditions, and both are known to regulate activity/rest and adult emergence rhythms in insects including *Drosophila* (Yoshii et al., 2010; Menegazzi et al., 2013). The fact that combinations of LD and WC cycles caused similar after-effects on τ, when presented in-phase or out-of-phase, suggests that after-effects of exposure to cyclic conditions is independent of the phase-difference between the cyclic cues. This suggests redundancy of LD and WC cycles at least with regards to causing a parametric change in circadian clocks. The results of our study also rule out any possible additive effect of the two zeitgebers in eliciting after-effects on τ of activity/rest rhythm. Our results are consistent with the findings of earlier studies which based on circadian entrainment, concluded that cooperation of the LD and WC is not a simple sum of their effects. Tomioka et al. (1998) showed that temperature and light cooperate to suppress activity in a certain phase and induce in the opposite phase. For example, at warmer temperatures flies tended to be more active during the dark phase, while at cooler temperatures they are more active during the daytime. On the other hand, Wheeler et al. (1993) argued that combination of light and temperature cycles would interact to synchronize rhythms more than the sum of these two kinds of stimuli. Such after-effects on τ of activity/rest rhythm may be due to the activation of morning or evening oscillators under cyclic conditions. This is consistent with the finding of a previous study which showed that light and temperature conditions interact together to favor either morning or evening oscillator (Cusumano et al., 2009). Since the period length of the entraining LD and WC regimes was close to that of the FD and BD flies, flies entrained readily (Figures 6.2-6.4), and therefore after-effects resulted in no shift in activity timing. Thus the selection-mediated response for faster pre-adult development on shortening of τ is abolished as a result of exposure to various cyclic conditions. Such after-

effects on circadian clocks are likely to help organisms in maintaining stable phase-relationship with their geophysical world in the face of fluctuations in their local environments. This suggests that circadian clocks have evolved a variety of mechanisms to enhance stability of time-keeping, which is likely to increase its chances of survival under ever fluctuating environments.

6.5. Conclusions

Our study suggests that LD and WC exposures result in after-effects on the τ of activity/rest rhythm in *Drosophila*. Prior exposure to zeitgebers altered the τ of both FD and BD flies and abolished the selection-mediated difference in τ. While, exposure to as few as 2 cycles of LD12:12 was enough to cause after-effects, LD cycles with shorter period turned out to be more effective. Similarly, exposure to WC1 shortened while WC2 lengthened the τ, however, when superimposed with LD12:12 the trend was reversed. LD and WC cycles superimpositions either in IP or OP caused similar after-effects on the circadian period, suggesting that after-effects of exposure to cyclic conditions is independent of the phase-difference between the cyclic cues. The results of our study also rules out any possible additive effect of the two zeitgebers in eliciting after-effects on τ of activity/rest rhythm. These results can be taken to suggest that exposure to LD and WC cycles elicit after-effects, which is capable of abolishing selection mediated difference in circadian period.

Chapter 7

Faster pre-adult development and faster running circadian clocks are associated with lower adult life history traits in fruit flies *Drosophila melanogaster*

7.1. Introduction

Circadian clocks rhythmically regulate most behavioral and metabolic processes in organisms ranging from bacteria to humans. It is believed that these clocks help organisms in scheduling their activity at a particular time of the day, which reduces competition and predation, while enabling them to find food and mates (Saunders, 2002a; Vaze and Sharma, 2013). In nature, fruit flies emerge as adults during the dawn when humidity in environment is high and temperature is low, perhaps to avoid desiccation under harsh environmental conditions prevailing during the daytime (De et al., 2012). Disruption of circadian timing system results in the reduction of reproductive output in the gypsy moth, *Lymantria dispar* (Giebultowicz et al., 1990) and fruit fly *Drosophila melanogaster* (Beaver et al., 2002, 2003), shortening of adult lifespan in *D. melanogaster* (Hendricks et al., 2003; Kumar et al., 2005), reduction in vegetative growth and survivorship in *Arabidopsis thaliana* (Dodd et al., 2005), and increase in the risk of predation in chipmunks *Tamias striatus* and ground squirrels *Spermophilus lateralis* (DeCoursey et al., 1997, 2000). Furthermore, circadian clocks in resonance with environmental light/dark (LD) cycles have been shown to enhance lifespan of fruit flies *D. melanogaster* (Klarsfeld and Rouyer, 1998; Pittendrigh and Minis, 1972), blow flies *Phormia terranovae* (von Saint Paul and Aschoff, 1978) and the competitive ability of cyanobacteria *Synechococcus sp*., in mixed cultures of two strains (Ouyang et al., 1998).

Starvation and desiccation resistance is often used to assess fitness (Service et al., 1985, 1988; Zwaan et al., 1991; Chippindale et al., 1996; Prasad and Joshi, 2003), which has led to the wide spread acceptance of the stress theory of ageing, which posits that pre-adult and adult fitness traits are correlated with extent of stress resistance (Parsons, 2003). In *D. melanogaster*, studies on selection for resistance to starvation and desiccation reported a correlated increase in the pre-adult development time (Chippindale et al., 1996; Harshman et al., 1999), also selection for extended lifespan was found to result in an increase in starvation

and desiccation resistance (Service et al., 1988; Graves et al., 1992), suggesting a positive correlation between lifespan and resistance to starvation and desiccation. Circadian clocks in *Drosophila* have also been found to be involved in the response to exogenous oxidative stress (Gorbacheva et al., 2005; Gachon et al., 2006; Lee and Edery, 2008) and in defensive response to exogenous stressors (Krishnan et al., 2008), which suggests the role of circadian clocks in stress resistance.

In insects, circadian clocks have been implicated in the regulation of life history traits such as pre-adult development time (Takahashi et al., 2013; Yadav and Sharma, 2013) and adult lifespan (Miyatake, 1997a). Correlation between development time and circadian clocks was reported in laboratory selection studies on melon flies *Bactrocera cucurbitae* (Miyatake, 1997b; Shimizu et al., 1997) and fruit flies *D. melanogaster* (Kumar et al., 2006; Takahashi et al., 2013; Yadav and Sharma, 2013). In a study on fruit fly *D. melanogaster* populations, reduction in adult fitness traits such as fecundity and lifespan was reported in populations subjected to selection for faster pre-adult development and early reproduction (Chippindale et al., 2004), whereas studies on flies selected for slower development and delayed reproduction resulted in increased pre-adult development time (Chippindale et al., 1994) and adult lifespan (Partridge and Fowler, 1992; Djawdan et al., 1996). These results suggest a genetic correlation between pre-adult development time and adult lifespan, perhaps mediated via age at reproduction. In a separate study on melon flies *Bactrocera cucurbitae*, pre-adult development time and adult lifespan were found to be positively correlated (Miyatake, 1997a). However, a few studies in *Drosophila*, where laboratory selection strategy was employed to raise faster developing populations, adult lifespan of the selected flies was found to be comparable with the unselected controls (Zwaan et al., 1991), suggesting that lifespan can evolve independently of the pre-adult developmental time. Thus studies examining the links between circadian clocks and pre-adult and adult fitness traits

have always been studied separately, were often carried out in different model organisms, under varying selection or maintenance protocols, yielding contradictory and inconclusive evidence. It would therefore be interesting to comprehensively study the role of circadian clocks in the regulation of various life history traits to understand the adaptive significance of circadian clocks.

To examine the role of circadian clocks in the regulation of pre-adult and adult fitness traits, we used four large outbred faster developing (FD) populations of *D. melanogaster*. After 55 generations of selection, these populations evolved with significantly faster pre-adult development (~29 h; ~12%) and have circadian period (τ) of activity/rest rhythm ~0.5 h shorter than controls (BD) (Yadav and Sharma, 2013; Chapter 3). We analyzed the pre-adult survivorship, resistance to starvation and desiccation, body weight and body length, fecundity and adult lifespan in these flies to assess its pre-adult and adult fitness. The results revealed that faster developing flies with shorter clock period have evolved significantly higher fecundity per unit body weight, at the cost of reduced adult lifespan, suggesting a role for circadian clocks in the regulation of life-history traits.

7.2. Materials and methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by method described in detail in chapter 2. Standardized populations were used for various assays described below.

***7.2.1. Pre-adult survivorship assay*:** Pre-adult survivorship of both FD and BD flies was assayed at regular intervals of 5 generations. Flies from the standardized populations of $FD_{1\text{-}4}$, and $BD_{1\text{-}4}$ stocks were allowed to lay eggs for 2 h (08:00 to 10:00 h) on banana medium, of which exactly 30 eggs were dispensed into long glass vials containing ~10 ml banana

medium. Ten such vials for each replicate population were kept in DD. Thus, a total of 80 vials were used in this assay (10 vials×4 replicates×2 stocks). Eggs for this assay were collected under DD illuminated by red light of λ>650-nm. Pre-adult survivorship (in fraction) was estimated by dividing the total number of adults emerging from a vial by the total number of eggs dispensed in that vial.

***7.2.2. Adult lifespan assay*:** From the running culture of each standardized population, 60-80 eggs were collected and transferred into vials with 6 ml banana medium. After 7-8 days, freshly emerged virgin males were collected for 5-6 h, anesthetized with the help of CO_2 and separated under red light and introduced into DD or LD. Ten virgin males were placed in each vial containing ~6 ml of corn food and ten such vials were used for each population. Thus 40 vials (4 blocks×10 vials) of FD and 40 for BD were used for this assay under each of the light regimes (LD and DD). The light phase of LD was created using fluorescent white light of intensity ~100 lux, while during the dark phase red light of λ>650 nm was used. The vials were checked every day for deaths and flies were transferred into fresh food vials every third day.

***7.2.3. Fecundity assay*:** The experimental set-up was similar to that used for lifespan assay. However, instead of 10 virgin males, here one male-female pair was placed under DD into glass vials with 1 ml un-yeasted banana medium, which was replaced every 12 h by fresh vials containing banana medium. The number of eggs laid during early-life (3-5 days), mid-life (10-12 days) and late-life (20-22 days) stages were counted. The average number of eggs laid by a female at ages 3, 4 and 5 days was considered as the reproductive output of the fly at age of 4 days. Similarly the average number of eggs laid at ages 10, 11, 12 and 20, 21 and 22 days was considered as the reproductive output of the fly at age 11 and 21 days respectively.

***7.2.4. Dry weight assay*:** Eggs were collected from standardized populations at a density of 30 eggs/vial. Flies that emerged during the emergence peak which spanned ~6 h were collected from each vial, at 1 h intervals. Freshly emerged virgin males and females were separated, freezed and dried for 36 h at 70 ^{o}C and finally weighed in groups of 10 males or 10 females. Five groups of dried flies were chosen randomly from each FD and BD population and weighed.

***7.2.5. Body size/length assay*:** Collection of eggs, adult fly separation and experimental set-up was similar to that in dry weight assay. Briefly, freshly emerged virgin males and females collected every 1 h during the emergence peak (~6 h) were separated using CO_2 and head to abdomen length was measured for 30 males or 30 females per population without freezing. Body length of anesthetized flies was measured with the help of microscope provided with measuring scale (least count 0.1 mm) (Yucon Instrument Co. Limited, China).

***7.2.6. Starvation resistance assay*:** The experimental set-up was similar to that previously described for lifespan assay. The only difference here was that 10 virgin males or 10 virgin females were placed in vials without any food but were provided with 3 ml agar (1.2%) to prevent desiccation. Forty vials (4 blocks×10 vials) of FD and 40 of BD were used under each of the light regimes (LD and DD). These vials were checked every 1 h for deaths till the last death was recorded.

***7.2.7. Starvation and desiccation resistance assay*:** The experimental set-up was similar to that previously described for starvation resistance assay. The only difference was that flies were placed in completely dried vials, without any food medium or desiccating agent.

***7.2.8. Statistical analyses*:** For the lifespan assay, mean adult lifespan (in days) was used as data for a mixed model analysis of variance (ANOVA) in which replicate populations (Blocks-B) were treated as random factor, whereas stocks (Populations-P) and light regimes

(L) were treated as fixed factors crossed with blocks. In all cases, block average of replicate populations was used as the unit of analysis and hence, only the fixed factors could be tested for significance. Post-hoc multiple comparisons were done using Tukey's HSD test. Data from starvation and starvation + desiccation resistance assays were analyzed similarly. For the analysis of reproductive output, mean egg output data was used in a two-way mixed model ANOVA with stocks (P) and age (A) as fixed factors and blocks (B) as random factor. All statistical analyses were implemented on STATISTICA for Windows Release 5.0 B (StatSoft, 1995).

7.3. Results

***7.3.1. Pre-adult survivorship remains unchanged in faster developing flies*:** The pre-adult survivorship of FD and BD stocks was assayed under DD at regular intervals of 5 generations. The survivorship of FD and BD flies did not differ except at 10^{th} and 20^{th} generation assays (Figure 7.1a). ANOVA revealed a statistically significant effect of generation (G), but not of stocks (P) and G×P interaction (Figure 7.1a; Table 7.1a). Thus, selection for faster development did not affect pre-adult survivorship.

***7.3.2. Reduced dry weight and body size in faster developing flies*:** Dry weight at emergence was significantly reduced in both males and females from FD stocks compared to that of BD controls (Figure 7.1b). Dry weight of FD flies at emergence underwent a reduction by ~47% in males and ~45% in females compared to their respective BD controls. ANOVA revealed a statistically significant effect of Sex (S), Stocks (P) and S×P interaction. Post-hoc multiple comparisons using Tukey's HSD test revealed that dry weight at emergence of males was

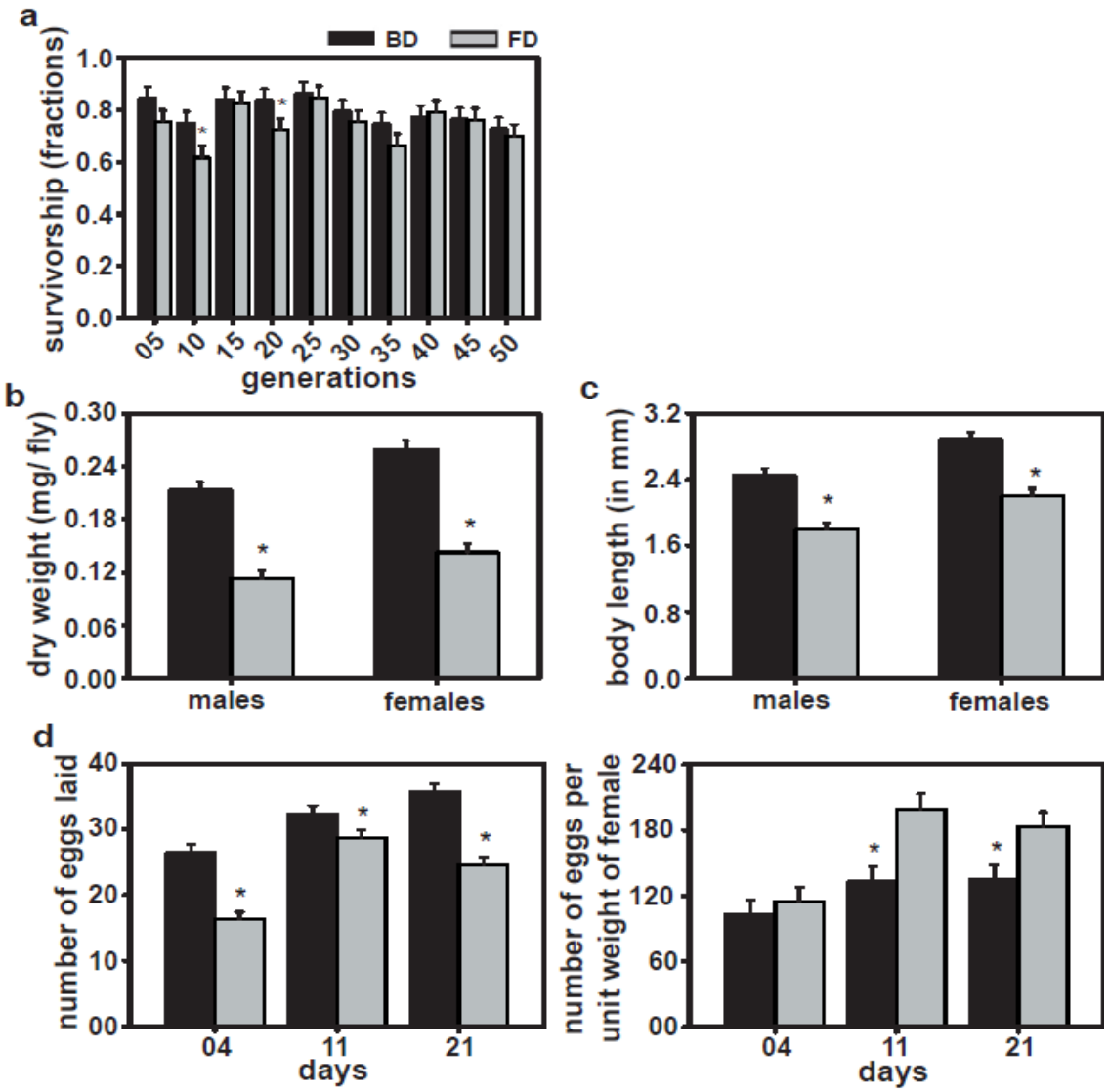


Figure 7.1. Pre-adult survivorship, dry weight and fecundity in FD stocks: The average pre-adult survivorship of selected and control populations under constant darkness (DD) across generations (a). Mean dry weight (b) and body length (c) at emergence of virgin males and females from FD and BD stocks shows significant effect of selection for faster development. Mean fecundity and mean fecundity per unit weight (d, left and right panels) of females from FD and BD stocks at early (4^{th} day), mid (11^{th} day) and late life stages (21^{st} day post-emergence). The error bars represents 95% confidence interval around the mean (95%CI) for visual hypothesis testing. Asterisks indicate statistically significant difference between selected and control populations.

Table 7.1a. ANOVA details of pre-adult survivorship assay under DD.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Generation (G)	9	0.024	27	0.003	7.47	0.0001
Stocks (P)	1	0.048	3	0.009	4.94	0.1127
Blocks (B)	3	0.007	0	0	--	--
G × P	9	0.005	27	0.004	1.25	0.308
G × B	27	0.003	0	0	--	--
P × B	3	0.009	0	0	--	--
G × P × B	27	0.004	0	0	--	--

Table 7.1b. ANOVA details of dry weight at eclosion.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Sex (S)	1	0.58	3	0.01	389.41	0.0003
Stocks (P)	1	4.71	3	0.09	54.59	0.0051
Blocks (B)	3	0.04	0	0	--	--
S × P	1	0.03	3	0.01	16.29	0.0274
S × B	3	0.01	0	0	--	--
P × B	3	0.09	0	0	--	--
S× P × B	3	0.01	0	0	--	--

Table 7.1c. ANOVA details of body size/length at eclosion.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Sex (S)	1	0.72	3	0.01	519.29	0.0002
Stocks (P)	1	1.79	3	0.01	541.39	0.0002
Blocks (B)	3	0.01	0	0	--	--
S × P	1	0.01	3	0.01	0.55	0.5137
S × B	3	0.01	0	0	--	--
P × B	3	0.01	0	0	--	--
S× P × B	3	0.01	0	0	--	--

Table 7.1d. ANOVA details of fecundity assay under DD.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Age (A)	2	240.81	6	8.15	29.55	0.0008
Stocks (P)	1	407.37	3	2.96	137.72	0.0013
Blocks (B)	3	35.87	0	0	--	--
A × P	2	9.54	6	2.63	3.63	0.0928
A × B	6	8.15	0	0	--	--
P × B	3	2.96	0	0	--	--
A × P × B	6	2.63	0	0	--	--

Table 7.1e. ANOVA details of fecundity per unit dry weight.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Age (A)	2	7907.71	6	289.09	27.35	0.0009
Stocks (P)	1	10668.33	3	1023.72	10.42	0.0483
Blocks (B)	3	1860.07	0	0	--	--
A × P	2	1588.06	6	91.85	17.29	0.0032
A × B	6	289.09	0	0	--	--
P × B	3	1023.72	0	0	--	--
A × P × B	6	91.85	0	0	--	--

significantly lower than that of females. Dry weight at emergence of FD males was significantly lower than BD controls, similarly that of FD females was significantly lower than BD controls (Figure 7.1b; Table 7.1b).

Body size/length at emergence of both males and females from FD stocks was significantly smaller compared to that of BD males and females (Figure 7.1c). ANOVA revealed a statistically significant effect of S and P, but not S×P interaction. Post-hoc multiple comparisons using Tukey's HSD test revealed that body length of males at emergence was significantly smaller than that of females. Body size at emergence of FD males was significantly smaller than BD males, similarly FD females were significantly smaller than BD controls (Figure 7.1c; Table 7.1c). These results suggest that selection for faster pre-adult development results in reduced body size and body weight at emergence in fruit flies.

***7.3.3. Higher fecundity per unit body weight in faster developing flies*:** After 50 generations of selection, the average egg output of FD females at all three life stages (early-life, mid-life and late-life) was lower compared to BD controls. ANOVA revealed a statistically significant effect of life stages (A) and stocks (P), however, the effect of A×P interaction was statistically not significant. Post-hoc multiple comparisons using Tukey's HSD test revealed that, at all three life stages fecundity of FD females was significantly lower than BD controls (Figure 7.1d; Table 7.1d). These results suggest that selection for faster pre-adult development results in reduced reproductive output in females.

Analysis of fecundity per unit weight revealed that except at the early-life stage fecundity per unit body weight of FD females was higher than that of BD controls. ANOVA revealed a statistically significant effect of A, P and A×P interaction. Post-hoc multiple comparisons using Tukey's HSD test revealed that except for early-life, fecundity per unit

body weight of FD females was significantly greater than that of BD controls (Figure 7.1d; Table 7.1e). These results suggest that although selection for faster pre-adult development results in reduced overall egg output, fecundity per unit body weight of the females from the faster developing stocks was significantly higher than controls.

***7.3.4. Reduced starvation resistance in faster developing flies*:** The FD flies had reduced starvation resistance compared to BD controls (Figure 7.2). ANOVA revealed a statistically significant effect of light regimes (L), stocks (P), sex (S) and L×P interaction, however, the effect of P×S and L×P×S interactions was not statistically significant (Table 7.2a). Post-hoc multiple comparisons using Tukey's test revealed that flies from both stocks had lower starvation resistance under LD compared to DD, which suggests that starvation resistance of dark-reared flies is significantly affected under novel light regime. Under both light regimes the starvation resistance of FD flies was lower than BD controls suggesting that selection for faster pre-adult development results in reduced resistance to starvation. Furthermore, females had significantly higher starvation resistance compared to males (Figure 7.2e, f).

***7.3.5. Reduced starvation and desiccation resistance in faster developing flies*:** The FD flies had reduced starvation and desiccation resistance compared to BD controls (Figure 7.3). ANOVA revealed a statistically significant effect of light regime (L), stocks (P), sex (S) and L×P, P×S interactions, however, the effect of L×P×S interaction was statistically not significant (Table 7.2b). Post-hoc multiple comparisons using Tukey's test revealed that flies from both stocks had higher resistance to starvation and desiccation under DD compared to LD. Additionally, under both LD and DD conditions starvation and desiccation resistance of FD females was significantly lower compared to BD controls, while that of males did not differ. Moreover, as expected, starvation and desiccation resistance of females was significantly higher than males (Figure 7.3). This suggests that LD adversely affects

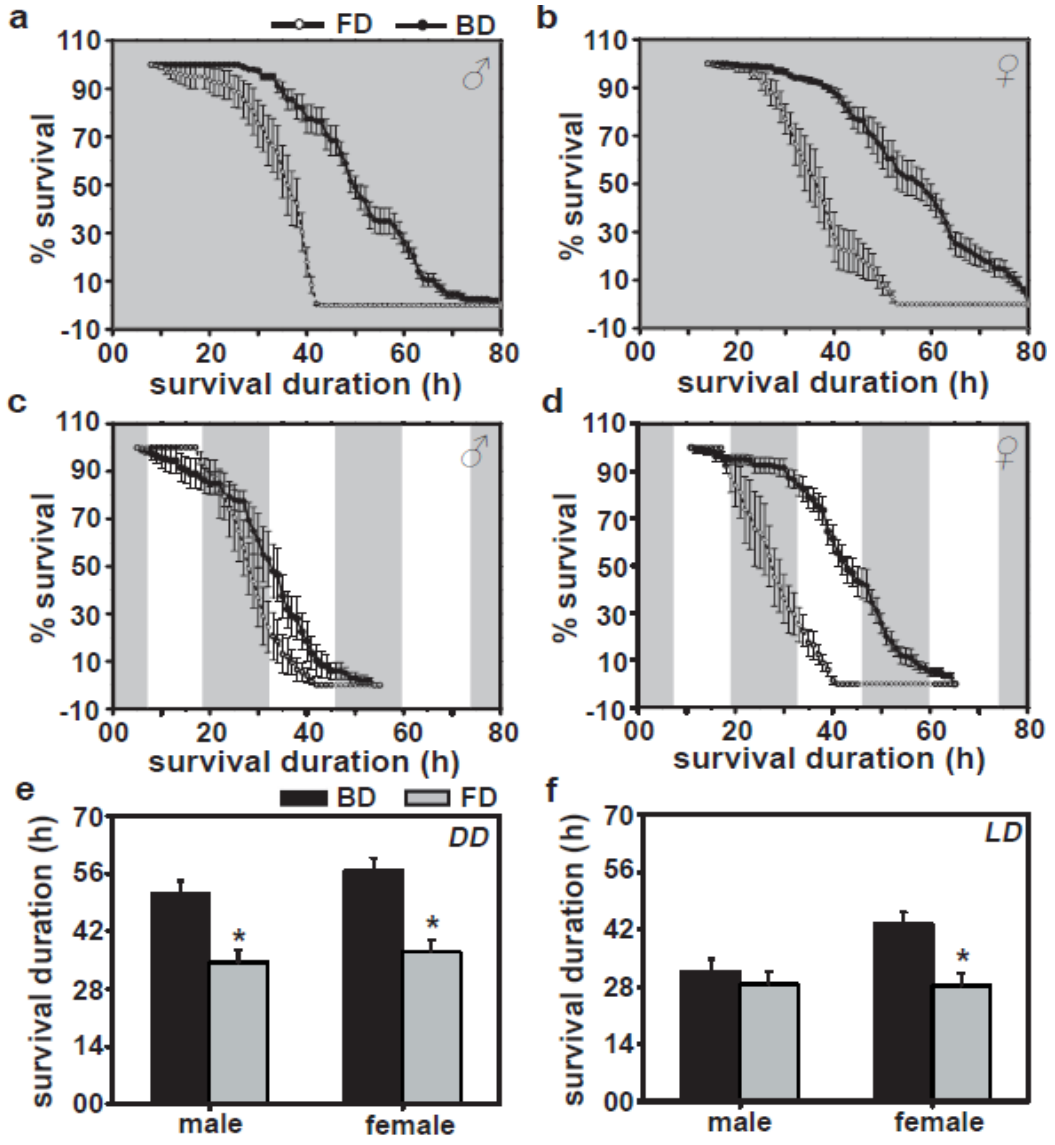


Figure 7.2. Reduced starvation resistance in FD stocks: Survivorship curves of virgin males and females from FD and BD stocks during starved condition under DD (a, b) and LD (c, d). Mean survivorship of virgin males and females under starved condition in DD (e) and LD (f). All other details are same as in Figure 7.1.

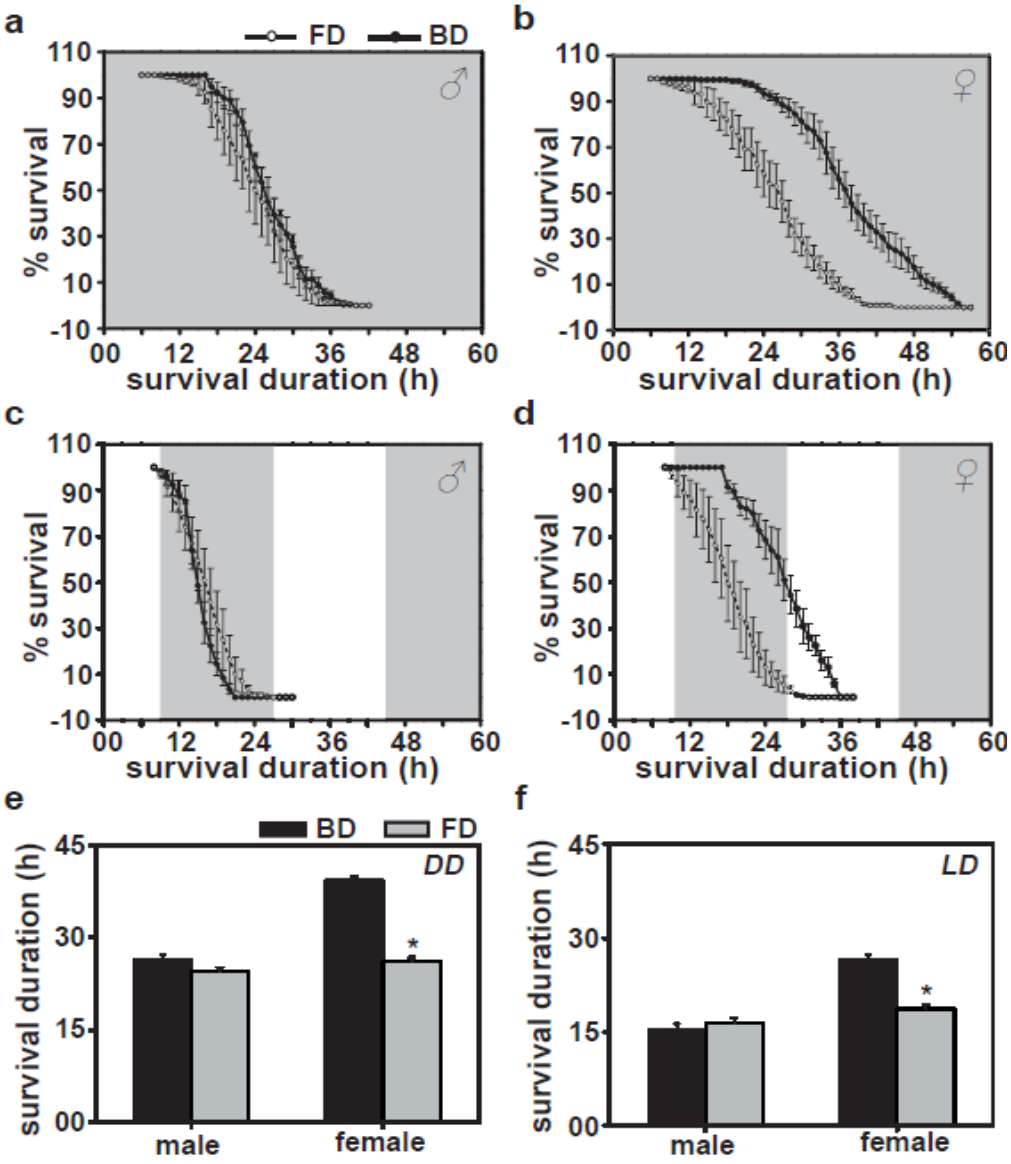


Figure 7.3. Reduced starvation and desiccation resistance in FD stocks: Survivorship curves of virgin males and females from FD and BD stocks during starvation and desiccation condition under DD (a, b) and LD (c, d). Mean survivorship of virgin males and females under starvation and desiccation condition in DD (e) and in LD (f). All other details are same as in Figure 7.1.

Table 7.2a. Starvation resistance assay under DD and LD 12:12.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light Regimes (L)	1	1133.59	3	12.08	93.81	0.0024
Stocks (P)	1	1497.96	3	23.95	62.54	0.0042
Sex (S)	1	185.76	3	3.75	49.55	0.0059
Blocks (B)	3	39.94	0	0	--	--
L × P	1	170.66	3	0.58	294.85	0.0004
L × S	1	5.53	3	9.36	0.59	0.4981
P × S	1	110.63	3	29.56	3.74	0.1485
L × B	3	12.08	0	0	--	--
P × B	3	23.95	0	0	--	--
S × B	3	3.75	0	0	--	--
L × P × S	1	38.41	3	5.54	6.93	0.0781
L × P × B	3	0.58	0	0	--	--
L × S × B	3	9.36	0	0	--	--
P × S × B	3	29.56	0	0	--	--
L × P × S × B	3	5.54	0	0	--	--

Table 7.2b. Starvation and desiccation assay under DD and LD 12:12.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light Regimes (L)	1	761.64	3	3.84	198.51	0.0008
Stocks (P)	1	246.21	3	19.41	12.68	0.0378
Sex (S)	1	381.97	3	1.17	327.29	0.0004
Blocks (B)	3	37.49	0	0	--	--
L × P	1	33.63	3	2.47	13.61	0.0346
L × S	1	0.59	3	4.69	0.13	0.7456
P × S	1	199.42	3	1.65	121.01	0.0016
L × B	3	3.84	0	0	--	--
P × B	3	19.41	0	0	--	--
S × B	3	1.17	0	0	--	--
L × P × S	1	2.56	3	0.59	4.36	0.1279
L × P × B	3	2.47	0	0	--	--
L × S × B	3	4.69	0	0	--	--
P × S × B	3	1.65	0	0	--	--
L × P × S × B	3	0.59	0	0	--	--

starvation and desiccation resistance of dark-reared flies and selection to faster development reduces resistance for starvation and desiccation.

***7.3.6. Reduced lifespan in faster developing flies*:** The adult lifespan of FD flies was shorter than BD controls by ~10 days under DD and ~9 days in LD (Figure 7.4a-c). ANOVA revealed a statistically significant effect of light regimes (L), stocks (S), however, the effect of L×S interaction was statistically not significant (Table 7.3a). Post-hoc multiple comparisons using Tukey's test revealed that the mean lifespan of flies from both the stocks was shorter under LD compared to DD, and the lifespan of FD flies was significantly shorter compared to BD controls (Figure 7.4a-c). This suggests that LD adversely affects lifespan of dark-reared flies, and faster development causes reduction in adult lifespan.

To confirm our results on adult lifespan, we repeated this assay once again after 10 generations; however, this time lifespan was assayed only under DD. The results revealed that the mean lifespan of virgin FD males was shorter than BD controls (Figure 7.4d, e). ANOVA followed by post-hoc multiple comparisons using Tukey's test revealed that the mean lifespan of FD flies was significantly shorter than BD controls (Figure 7.4d, e; Table 7.3b). This suggests that selection for faster pre-adult development results in reduction in mean adult lifespan.

7.4. Discussion

There have been several studies that reported a genetic correlation between circadian clocks and adult fitness traits in fruit flies *D. melanogaster* (Klarsfeld and Rouyer, 1998; Beaver et al., 2002, 2003). Although, a few previous studies implicated fitness consequences of changes in circadian rhythm, most of these studies examined correlations between clock period and one or two fitness traits at a time, which makes it difficult to judge the true

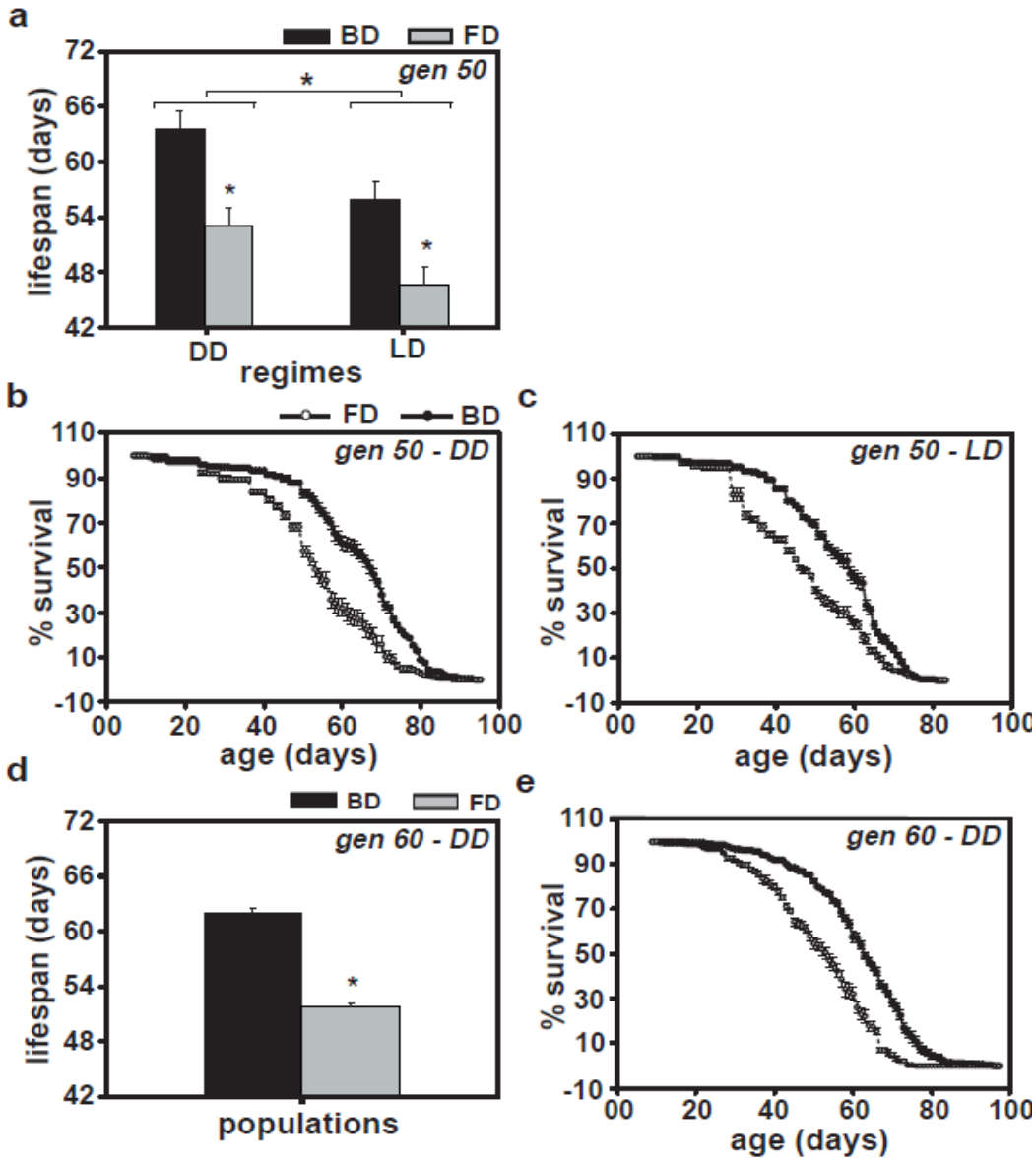


Figure 7.4. Reduced adult lifespan in FD stocks: Mean adult lifespan under DD and LD of virgin males from FD and BD stocks after 50 generations of selection (a) and their survivorship curves under DD and LD (b, c). Mean adult lifespan under DD of virgin males from the two stocks (d) and their survivorship curves (e) after 60 generations of selection. All other details are same as in Figure 7.1.

Table 7.3a. Life span under DD and LD 12:12 h generation 50.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Light Regimes (L)	1	195.66	3	3.12	62.82	0.004
Stocks (S)	1	389.08	3	12.43	31.31	0.012
Blocks (B)	3	6.08	0	0	--	--
L × S	1	1.57	3	12.79	0.123	0.749
L × B	3	3.12	0	0	--	--
S × B	3	12.43	0	0	--	--
L × S × B	3	12.79	0	0	--	--

Table 7.3b. Life span under DD generation 60.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	212.42	3	11.95	17.78	0.024
Blocks (B)	3	2.45	0	0	--	--
S × B	3	11.95	0	0	--	--

relationship between various life history traits. The FD flies which developed considerably faster and have shorter clock period, served as a good model system to study such correlations and therefore we decided to examine various pre-adult and adult fitness traits in these flies. Our analyses revealed that faster pre-adult development and shorter τ in FD flies is associated with compromised adult fitness traits such as lower starvation and desciccation resistance, and reduced fecundity and adult lifespan. However, pre-adult survivorship of FD and BD flies did not differ, indicating no association between circadian clocks and pre-adult survivorship (Figure 7.1a). Correlation between τ and adult fitness traits provides evidence for the role for circadian clocks in the regulation of fitness traits in fruit flies *D. melanogaster*.

In fruit flies *D. melanogaster*, pre-adult development time and pre-adult survivorship are known to be correlated (Chippindale et al., 1997; Prasad et al., 2000), and selection for faster pre-adult development has been found to result in reduction of pre-adult survivorship (Chippindale et al., 1997; Prasad et al., 2000). In our study, we found that FD flies evolved reduced stress resistance, fecundity and lifespan, however, pre-adult survivorship was not affected. The results of our study suggest that, while faster pre-adult development evolved at the cost of adult fitness components, it did not affect pre-adult fitness. The evolution of faster pre-adult development without any reduction in the pre-adult survivorship in our study is in stark contrast with previous selection studies (Chippindale et al., 1997; Prasad et al., 2000), where populations were additionally under selection for early or very early reproduction therefore, such additional selection pressures could be the cause of reduced pre-adult survivorship in these studies. However, our results are in line with those of Zwaan and co-workers (1995a) study that showed no difference in pre-adult survivorship between faster developing and control flies. Our study thus suggests that selection for faster pre-adult

development is not always accompanied by a cost in terms of pre-adult survivorship unless it is associated with early reproduction.

In *D. melanogaster,* daily fecundity increases during the first 2–4 days post emergence, and remains high for the next 20 days, and thereafter declines gradually (Rose, 1984; Novoseltsev et al., 2002). However, laboratory populations maintained on a 21 day discrete generation cycle were found to evolve higher daily fecundity, peaking 10–12 days post emergence, which corresponded to the age of egg collection for that population (Sheeba et al., 2000). In the present study, we found a gradual increase in fecundity between 4th and 21st day post emergence (Figure 7.1d). In addition, similar to the finding of reduced fecundity throughout the life by Zwaan and coworkers (1995b), we observed that the overall fecundity of FD females was significantly lower compared to BD controls (Figure 7.1d). This was observed across all the three age categories, which suggests a trade-off between faster development and reproductive output of females, in contrast to increased early-life fecundity in faster developing populations of melon fly, *Bactrocera cucurbitae* (Miyatake, 1997b). There is a reasonable body of evidence from studies in *D. melanogaster*, to suggest that increase in reproductive output comes at the cost of reduced lifespan (Partridge et al., 1987, 1999; Chippindale et al., 1993; Djawdan et al., 1996; Sgrò and Partridge, 1999). However, in our study we found that both lifespan and fecundity reduced in the FD flies which prompted us to test, whether simultaneous reduction in fecundity and lifespan in FD flies is a result of greater fecundity per unit weight of the FD flies. Interestingly, FD flies showed a higher fecundity per unit weight, suggesting that higher fecundity per unit weight in the faster developing flies came at a cost of reduced lifespan (Figure 7.1d). Apart from this, the difference in the nature of maintenance regime, environment interaction and inadvertent selection (particularly selection for late-life fecundity which might have caused extended lifespan) could also be possible reason for different trends seen in current study compared to

several previous studies (Prasad and Joshi, 2003). Therefore, in order to address the issue of cost of reproduction, it would be required to estimate lifelong fecundity, and a better assessment of such relationships would be in mated flies rather than virgins.

In *D. melanogaster*, greater body weight is associated with higher adult fitness (Chippindale et al., 1996; Harshman et al., 1999). Furthermore, most previous studies on selection for faster pre-adult development revealed a correlated reduction in adult body size and/or body weight (Chippindale et al., 2004; Prasad and Joshi, 2003). Consistently, in the current study, body size and body weight of FD flies at emergence was smaller compared to BD controls (Figure 7.1b, c), suggesting that pre-adult development time and adult body size and body weight are positively correlated traits in fruit flies *D. melanogaster*. Moreover, associations between body size with reproductive success (Zwaan et al., 1995a) and body size with fecundity are also evident in *Drosophila* (Robertson, 1957, Prasad and Joshi, 2003). Similarly, an association between body size with desiccation resistance also exists (Rose, 1984) and bigger flies are expected to have a smaller exposed surface area for water loss relative to their weight (Hoffmann and Parsons, 1993). Hence body size-resistance and body size-fecundity relationship are important parameters for better assessment of *Drosophila* life history traits.

Starvation resistance in insects is known to be closely associated with adult fitness and is often found to co-vary with adult lifespan in laboratory populations of fruit flies *D. melanogaster* (Service et al., 1988; Zwaan et al., 1991; Graves et al., 1992) and in natural populations of butterflies *Bicyclus anynana* (Pijpe et al., 2008). In many species of *Drosophila*, laboratory selection for extended lifespan resulted in an increase in resistance to starvation and desiccation (Service et al., 1985, 1988; Graves et al., 1992; Chippindale et al., 1994). Similarly, populations selected for increased resistance to starvation and desiccation had significantly higher level of metabolic reserves, such as lipids (Chippindale et al., 1996;

Djawdan et al., 1997, 1998), carbohydrates (Graves et al., 1992; Chippindale et al., 1998), water content, dry weight, and increased development time (Chippindale et al., 1996, 1998). Previous studies also revealed an association between starvation resistance and energy reserves, particularly between starvation and carbohydrate metabolic reserves (Djawdan et al., 1997, 1998). It would therefore be interesting to examine such relationships in the faster developing flies. The results of our study suggest that FD flies have reduced starvation and/or desiccation resistance compared to controls (Figures 7.2, 7.3). Furthermore, shorter larval durations (Yadav and Sharma, 2013), reduced stress resistance, body size/body weight and slower larval feeding rate in faster developing flies (Prasad and Joshi, 2003) suggests that these flies are poor in resource acquisition. Additionally, we observed that starvation resistance of both stocks was reduced under LD compared to DD (Figure 7.2). Interestingly, starvation and desiccation resistance in males from both stocks did not show any difference across light regimes whereas females showed reduced starvation and desiccation resistance in LD compared to DD. These results suggest that this trait is unlikely to be clock-dependent, but metabolic reserves acquired by males or females would play a greater role (Figure 7.3). Such light regime-mediated differences in resistance to desiccation and starvation could also be due to the fact that our stocks are normally reared under DD, and therefore non-native regimes might cause adverse effect on stress resistance.

In insects, lifespan is significantly reduced due to deleterious effects of light including LL and non 24 h LD cycles (Pittendrigh and Minis, 1972; von Saint-Paul and Aschoff, 1978), and DD is reported to stimulate defense system and enhance adult lifespan (Shostal and Moskalev, 2013). Hence, we expected enhanced lifespan in DD compared to LD cycles. In the present study, we did observed a small but significantly higher lifespan of flies from both stocks under DD compared to LD (Figure 7.4a-c). This is consistent with the results of Allemand et al. (1973), which reported longer lifespan in dark-reared flies under DD

compared to LD or LL. Shortened lifespan in FD flies is largely selection-mediated and clock-independent because clocks in FD and BD flies run with similar pace under LD and therefore clock-mediated differences would reduce considerably when assayed under entraining conditions such as LD. The results revealed that LD regime equally affect the lifespan of FD and BD flies, and selection-mediated differences persist under both the regimes. In a separate study, faster developing populations of butterfly *Bicyclus anynana* were found to have longer lifespan compared to the slower developing lines, implying a negative genetic correlation between pre-adult developmental time and adult lifespan (Pijpe et al., 2006). This is contrary to the findings of the laboratory selection studies for faster pre-adult development in melon flies *Bactrocera cucurbitae* (Miyatake, 1997a) and fruit flies *Drosophila* (Chippindale et al., 2004).

Positive correlation between pre-adult development time and adult lifespan in our study is consistent with those of two previous studies (Prasad and Joshi, 2003; Chippindale et al., 2004), where populations selected for faster pre-adult development and early reproduction were reported to have shorter lifespan. However, unlike these studies, the observed reduction in lifespan in our study seems entirely to be due to selection on pre-adult development without any contribution of age at reproduction. The fact that faster developing flies also have shorter clock period suggests a role of circadian clocks in the regulation of life history traits in fruit flies *D. melanogaster*. However, traits such as development time, fecundity, lifespan and clock period are polygenic, and the underlying genes may have pleiotropic effects. Therefore, it is likely that mutations altering circadian phenotypes (genes) may also alter life history traits. Additionally, pleiotropic effects raise an interesting situation in the context of circadian clocks regulation of adult lifespan. Role of circadian clocks in the regulation of reproductive output is pivotal to the issue of adaptive significance of circadian clocks. Our study demonstrates the involvement of circadian clocks in the regulation of life

history traits of wild-type populations of *D. melanogaster*. Furthermore, the results suggest that light regimes and circadian clocks play a key role in the regulation of physiological stress.

7.5. Conclusions

The results of our study suggest that flies selected for faster pre-adult development under DD have reduced adult fitness traits such as fecundity, body size and weight, stress resistance and lifespan. While overall reproductive output of faster developing females was lower than controls their egg output per unit body weight was significantly higher. However, selection for faster development did not accompany any detrimental effects on pre-adult fitness traits. Results from stress resistance and adult lifespan assays suggest that light regimes adversely affect starvation and/or desiccation resistance and adult lifespan. These results indicate that selection for faster development results in correlated reduction in adult fitness traits suggesting a possible role of circadian clocks in the regulation of life history traits in fruit flies *D. melanogaster.*

Chapter 8

Circadian clocks in faster developing fruit flies *Drosophila melanogaster* populations age faster than controls

8.1. Introduction

With increasing age, metabolic functions in an organism slow down; ability to perform various tasks declines and subsequently the risk of death increases (Jones and Grotewiel, 2011). With advancing age, amplitude of rhythms, ability to entrain to environmental cycles and to coordinate various behavioural and physiological cycles are reduced considerably resulting in several old age disorders (Turek et al., 1995; Pandi-Perumal et al., 2010). Age-related disruptions of circadian clocks in humans cause sleep problems such as reduced sleep duration and sleep quality (Mirmiran et al., 1992), which results in depression (Rodin et al., 1998) and increased risk of early death (Carskadon et al., 1982; Rumble and Morgan, 1992; Huang et al., 2002). In fruit flies (Bushey et al., 2010) and humans (Kripke et al., 2002; Tamakoshi and Ohno, 2004; Ferrie et al., 2007; Hublin et al., 2007), individuals with shorter sleep duration were found to have shorter lifespan than those with long sleep duration. Disruption in circadian rhythms, phenocopying ageing, leads to shortening of adult lifespan in *Drosophila* (Kumar et al., 2005), and premature ageing (Kondratov et al., 2006; Lee, 2006) and increased old-age mortality in mammals (Hurd and Ralph, 1998; Kondratov et al., 2006). Studies in mammals have shown that manipulations of the circadian clock mechanisms by the knock-out of specific clock genes leads to reduced lifespan (Davidson et al., 2006; Yu and Weaver, 2011). Disturbance in circadian rhythm causing sleep disorders, which is commonly observed during normal ageing process, is found to be exacerbated in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington disease (Wu and Swaab, 2007; Sterniczuk et al., 2010a,b). Furthermore, genetic or environmental manipulations of circadian clocks yields altered circadian phenotypes resulting in reduced survivorship in

Drosophila (Hendricks et al., 2003), hamsters (Hurd and Ralph, 1998; Oklejewicz and Daan, 2002) and *Arabidopsis* (Dodd et al., 2005). Taken together, these studies suggest that functional circadian clocks promote lifespan in the face of a variety of homeostatic challenges.

Drosophila as a model organism has greatly aided studies on ageing (Helfand and Rogina 2003) and age-associated problems in circadian time-keeping including the regulation of sleep (Hendricks et al., 2000; Shaw et al., 2000; Koh et al., 2006). Ageing in *Drosophila* has been characterized in terms of reduction in adult lifespan, fecundity, negative geotaxis (Miquel et al., 1976; Gargano et al., 2005), exploratory activity (Le Bourg, 1983), innate odor aversion and attraction (Cook-Wiens and Grotewiel, 2002), memory (Tamura et al., 2003), circadian rhythmicity (Driver, 2000), cardiac function (Paternostro et al., 2001) and increase in heart rate (Wessells and Bodmer, 2004). In a study on the sni^{1} mutants, flies reared in constant light (LL), which is known to disrupt circadian rhythms, were found to live significantly shorter than controls (Price et al., 1995). Such reduction in lifespan resulting from the disruption of circadian clocks strongly suggests that this phenotype is caused by the loss of rhythmicity, although one cannot rule out non-circadian clock mediated pleiotropic effects of *per* gene in ageing, because its protein is unstable under LL (Price et al., 1995). In a separate study on *D. melanogaster*, with increasing age the strength of sleep/wake rhythm was found to decrease and sleep became fragmented (Koh et al., 2006). It was possible to speed-up or slow-down such changes in sleep/wake cycles merely by manipulating the ambient temperature that decreased or increased adult lifespan, suggesting that ageing of circadian clocks is a function of its physiological rather than chronological age (Koh et al., 2006).

Thus, along with several disparate physiological dysfunctions, impaired circadian rhythms seem to be a conserved feature of ageing in a wide variety of organisms including *Drosophila*. It would be interesting to examine if there is a cause-effect relationship between circadian clocks and organismal ageing.

Developmental theory of ageing considers ageing as a part of development following differentiation or growth (Lints, 1978), and some studies in the past have shown that rates of pre-adult development and of ageing are correlated (Lints and Lints, 1969; Lints, 1988; Chippindale et al., 1994, 2004; Zwaan et al., 1995; Miyatake, 1997b, 1998; Prasad, 2003). In insects, faster pre-adult development and reduced adult lifespan is associated with shorter clock period (τ) (Miyatake, 1997a; Shimizu et al., 1997; Yadav and Sharma, 2013), whereas slower development and extended lifespan is associated with longer τ (Kyriacou et al., 1990; Miyatake, 1997a; Shimizu et al., 1997; Takahashi et al., 2013; Klasfield and Rouyer, 1998), suggesting that circadian clocks are involved in the regulation of development and ageing of organisms.

In the current study we asked whether the rate of pre-adult development and ageing in fruit flies *D. melanogaster* is concurrent with age-related changes in their circadian clocks. We used four outbred populations of fruit flies *D. melanogaster*, maintained under constant darkness (DD) which have been subjected to selection for faster pre-adult development ($FD_{1\text{-}4}$). After 55 generations of selection, faster developing flies started emerging ~29 h earlier and had τ ~0.5 h shorter than controls (Yadav and Sharma, 2013). We performed life-long recording of activity/rest behaviour under DD of flies from selected (FD) and control (BD) populations. Additionally, we assayed adult lifespan of these flies. The results revealed that period lengthening, reduction in power of

rhythmicity and onset of arrhythmicity occurred much earlier in the faster developing flies compared to controls, suggesting that circadian clocks in fruit flies *D. melanogaster* age in parallel with the organism.

8.2. Materials and methods

This study was done on four replicate populations of Baseline Developing Control (BD) and Faster Developing (FD) populations of *D. melanogaster* (origin and maintenance described in detail in chapter 2) that were standardized by a method described in detail in chapter 2.

Standardized populations were used for various assays described below.

8.2.1. Adult lifespan assay: Lifespan was assayed only on virgin male flies. Adult males and females were separated under DD after anesthetizing freshly emerged flies using CO_2. Red light (λ>650-nm) was used to facilitate fly handling under DD. Groups of ten virgin males from each of the populations were dispensed into vials with 6 ml of corn food, and a total of 10 such vials were set for each of the FD and BD populations. The number of dead flies was recorded every day and flies were transferred into fresh food vials on every 5^{th} day. The lifespan of individual fly was calculated as the number of days of survival post emergence. Thus, average lifespan for a group of flies in a vial was calculated as average lifespan of 10 flies in that vial. Similarly, average lifespan of 10 vials was taken as the mean lifespan of population. Physical conditions such as temperature and relative humidity in the cubicles were monitored continuously using Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co. LTD, Japan, and were found to be constant throughout the assay.

***8.2.2. Locomotor activity assay*:** Activity/rest rhythm of individual virgin males from FD and BD populations were recorded life-long under DD, to assess age-related changes in their circadian rhythm. For locomotor activity monitoring, 32 randomly chosen flies from each population were loaded on the 2nd day post emergence into 8 cm long and 5 mm wide glass activity tubes. One end of the tube had corn food and the other end was blocked with cotton plug, which allowed air circulation. Physical conditions such as temperature (25 ^{o}C) and relative humidity (80-90%) inside the recording cubicles were monitored every 5 min using *Drosophila* Environmental Monitor, DEnM (Trikinetics, USA) and were found to be stable during the entire assay. Locomotor activity was recorded as the frequency of IR beam cuts per five minutes, using Trikinetics *Drosophila* activity monitors. The raw activity data was used to estimate various properties of circadian activity/rest rhythm. To avoid artifacts in activity due to handling of flies, data collected for the first 5 days was excluded from the analyses. Dead flies during the first five days were replaced by age-matched back-up flies maintained under DD, after which the experimental setup remained undisturbed except for regular food change on every 5th day. Age-related changes in circadian activity/rest rhythm were assessed by estimating - (1) period, (2) robustness, (3) onset of arrhythmicity, and (4) average activity per circadian cycle.

To estimate period, activity data was divided into five stretches of 10 day intervals which represented different age categories - such as, 11–20 (young), 18–27 (adult), 25-34 (middle-age), 32-41 (old) and 39–48 day (very old), and τ was calculated for each age category using Lomb Scargle Periodogram in CLOCKLAB, Actimetrics, USA. The mean τ for each age category was calculated as average of that age category.

Additionally, to assess finer age-related changes, τ was estimated for successive sliding windows of 10 cycles starting 11^{th} day post emergence. In parallel with the activity recording, we assayed the lifespan of adult flies kept individually in activity tubes (60-70 flies per population), this lifespan was in addition to the assays done in flies kept in vials.

Robustness of activity/rest rhythm was assessed by estimating power of the rhythm, for each age category of fly using Lomb Scargle Periodogram in CLOCKLAB. The day of onset of arrhythmicity was estimated by visual assessment of individual fly actograms which was subsequently confirmed using Lomb Scargle Periodogram. We defined onset of arrhythmicity when activity/rest rhythm did not show any pattern for at least 7 consecutive cycles. However in case of death of fly, 3-5 cycles before death were taken as cut-off. The first cycle among arrhythmic cycles was taken as the day for onset of arrhythmicity and the average day of onset of arrhythmicity across all flies in that population was taken as mean age of onset of arrhythmicity for a given population. Total activity for different age categories of flies per circadian cycle was estimated from the raw activity data (excluding activity of arrhythmic flies) across 10 days.

***8.2.3. Statistical analyses*:** From the primary data, mean adult lifespan (in days) was estimated and used for data in analysis of variance (ANOVA) considering replicate populations (blocks-B) as random factor and stocks (S) as fixed factor crossed with block. Block means were used as unit of analysis and hence, only the fixed factor could be tested for statistical significance. Post-hoc multiple comparisons of mean lifespan data were done using Tukey's honestly significant difference (HSD) test. All analyses were implemented on STATISTICA for Windows Release 5.0 B (StatSoft, 1995). Data from activity/rest rhythm assays was also subjected to composite mixed model ANOVA,

treating populations (block$_{1-4}$) as a random factor and stocks (FD and BD) as fixed factor. In all cases block means were used as replicates. The effect of age on periodicity of activity/rest rhythm (during 5 different age categories) was analyzed using τ values of those categories in two-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test.

8.3. Results

8.3.1. Shorter adult lifespan in faster developing (FD) flies: We assayed lifespan of FD and BD flies under DD, simultaneously in vials (ten flies/vial) and in activity tubes (solitary fly/tube). The results revealed that after 75 generations of selection FD flies live under both the assay conditions significantly shorter than BD controls (vials: FD = 45.09 ± 2.73 days (mean ± SEM) and BD = 62.60 ± 1.34 days; tubes: FD = 36.05 ± 1.78 and BD = 50.1 ± 0.82 days, Figure 8.1a). ANOVA followed by post-hoc multiple comparisons using Tukey's test revealed that mean lifespan of flies from both stocks was shorter in tubes than in vials, however, under both conditions FD flies lived for significantly shorter than BD controls (Figure 8.1a, b; Table 8.1a, b). These results suggest that virgin males from FD stocks live shorter than controls.

8.3.2. Life-long recording of activity/rest behaviour under constant darkness (DD):

Individual males from FD and BD stocks were assayed life-long under DD for their activity/rest behaviour and (1) clock period, (2) robustness of rhythm, (3) onset of arrhythmicity, and (4) activity per circadian cycle, were estimated in virgin males of different age categories.

***8.3.2.1. Period of activity/rest rhythm becomes longer with age*:** Representative double-plotted actograms of individual dark-reared virgin males from FD and BD stocks show

that period of the FD flies undergo age-related changes earlier than the BD controls, whose period remains more or less stable with age (Figure 8.2a). Complex rhythmic patterns including splitting of activity to complete arrhythmicity are observed much earlier in the FD flies compared to the BD controls (Figures 8.2a, 8.3a, b). Analysis of life-long activity data revealed that τ increases with age in the FD flies but not in the BD controls (Figure 8.2a-c). Analyses revealed greater variation in τ across different age groups in the FD flies (0.37 ± 0.06 h; mean ± SEM) compared to BD controls (0.21 ± 0.03 h). At younger stage, τ of FD flies was significantly shorter (23.68 ± 0.07 h) than BD controls (24.21 ± 0.09 h), however, with increasing age the difference in τ between the two stocks gradually diminished and eventually the trend became reversed with FD flies exhibiting longer period than BD controls (Figure 8.2b). ANOVA on the τ data across five age categories revealed a significant effect of age (A) and A × S interaction, while the effect of stock (S) was statistically not significant (Figure 8.2c; Table 8.2a). Post-hoc multiple comparisons using Tukey's test revealed that τ of young FD males was significantly shorter than that of old and very-old FD males, while τ of BD males did not differ across all five age categories (Figure 8.2c). Regression analyses of τ across different age categories revealed that regression coefficient in FD flies (R^2 = +0.86, $p <$ 0.001) was higher than that in BD controls (R^2 = +0.39, p = 0.08, Figure 8.2d). Thus, τ of FD flies shows significant change with age but that of BD controls remains largely stable.

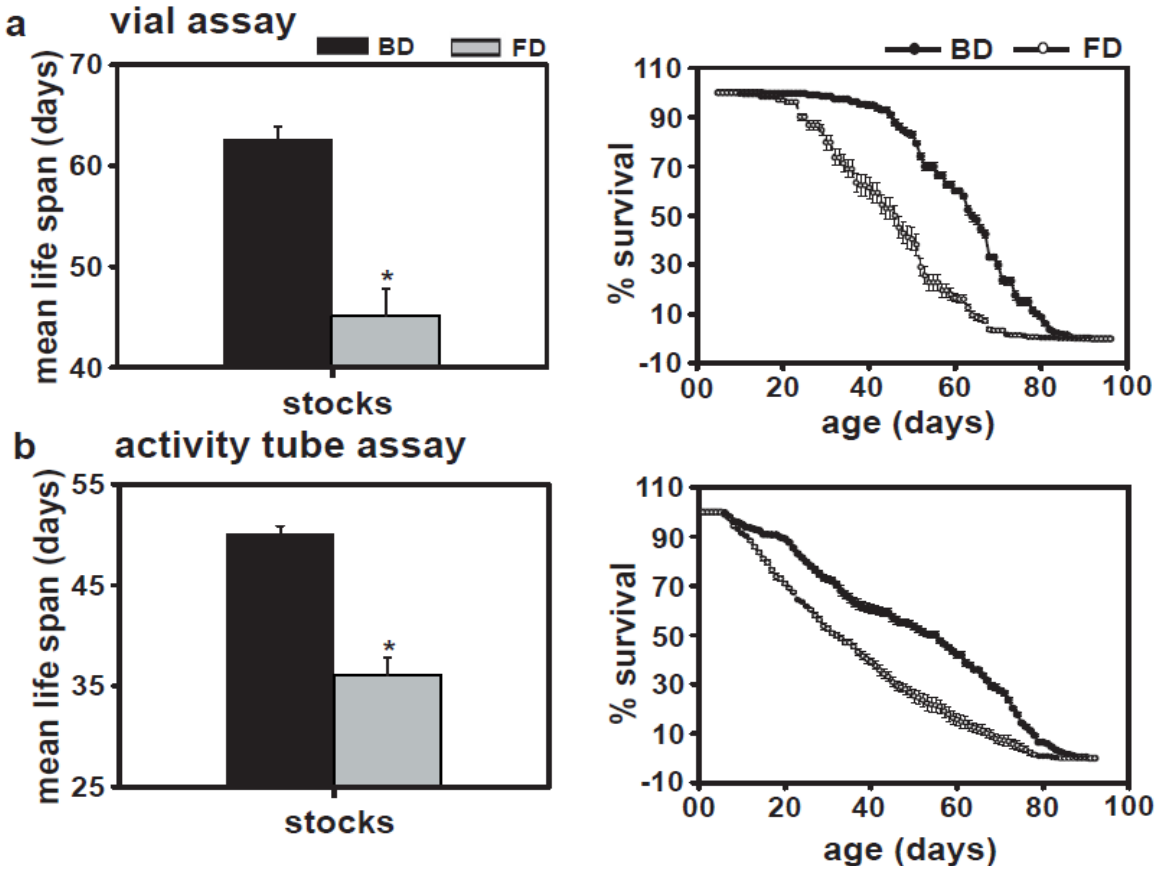


Figure 8.1. Age-dependent of mean lifespan in faster developing (FD) flies: Mean lifespan of faster developing (FD) males was significantly shorter than controls (BD) (a, b, left panels). Survivorship curves show shortening of adult lifespan in virgin males from FD populations compared to BD controls, when assayed in vials (a, right panel) or in activity tubes (b, right panel). The error bars represent standard error around the mean (SEM). A total of 10 vials (10 flies per vial) and 60-70 tubes were used for each of the four replicate populations. Asterisks indicate statistically significant difference between the FD and BD populations.

Table 8.1a. Results of ANOVA on lifespan data in glass vials.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	613.52	3	31.46	19.51	0.0216
Blocks (B)	3	5.46	0	0	--	--
S × B	3	31.46	0	0	--	--

Table 8.1b. Results of ANOVA on lifespan data in activity tubes.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	392.79	3	11.71	33.53	0.0102
Blocks (B)	3	3.55	0	0	--	--
S × B	3	11.71	0	0	--	--

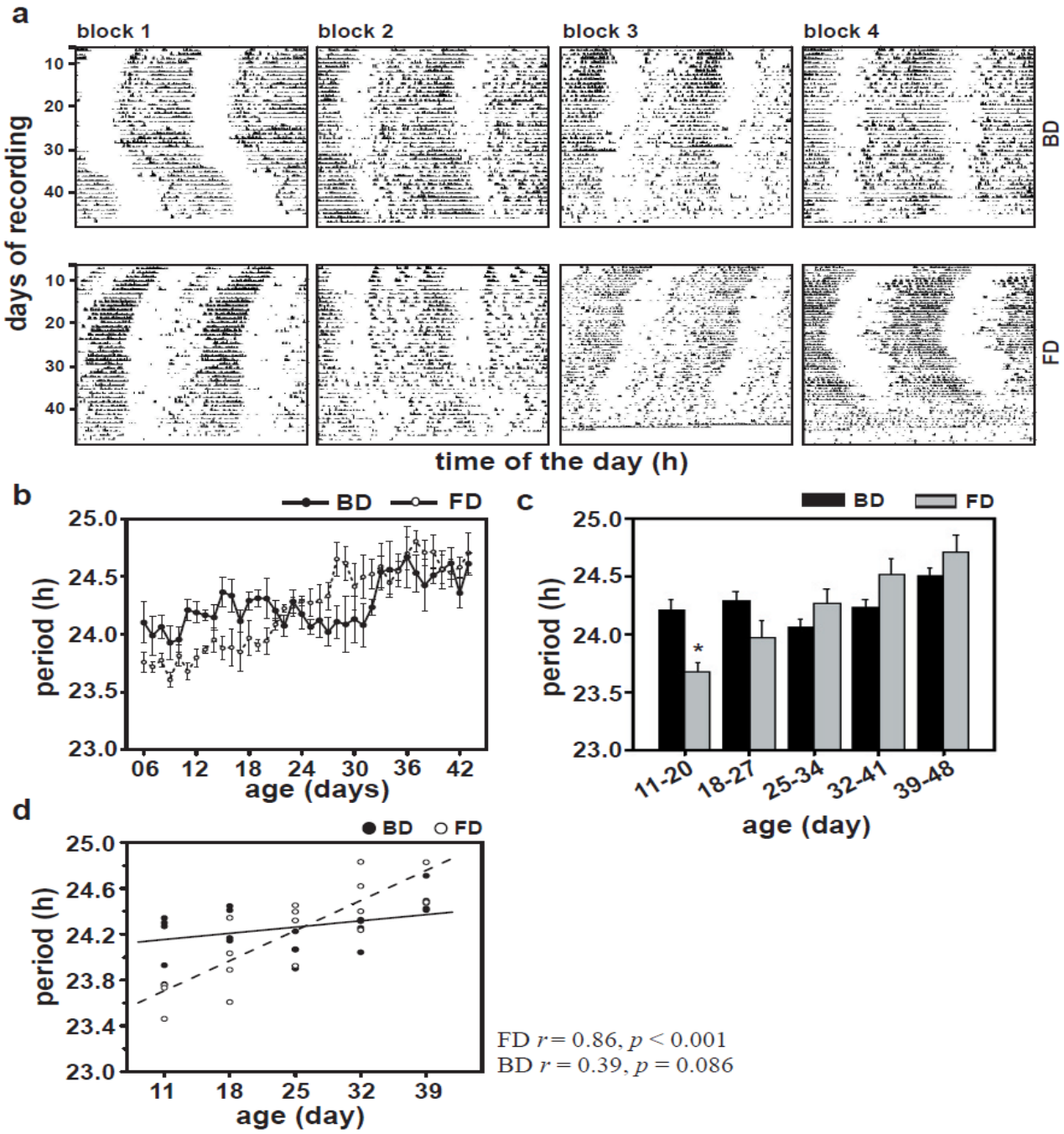


Figure 8.2. Age-dependent lengthening of clock period (τ) in faster developing (FD) flies: Representative double plotted actograms of activity/rest data, recorded life-long under DD from virgin males of faster developing (FD) and control (BD) flies (a). The *x*-axis represents time of the day and *y*-axis age of the flies. Mean clock period (τ) of activity/rest rhythm of flies from FD and BD populations shows age-dependent change (b, c). Regression lines across mean clock period of FD and BD flies at different life stages show that τ of FD flies change more rapidly than that of BD controls (d). In this assay a total of 32 flies were used for each replicate population. All other details are same as in Figure 8.1.

***8.3.2.2. Aging causes arrhythmicity in activity/rest behaviour*:** ANOVA revealed that the day for onset of arrhythmicity in FD males was significantly earlier (31.32 ± 1.66 days; mean ± SEM) compared to BD controls (49.96 ± 1.56 days; Figure 8.3a, b; Table 8.2b), such that ~50% of arrhythmicity in FD flies occurred by the time they were ~32 day old, while in BD flies it occurred when they were ~48 days old. Thus, onset of arrhythmicity occurred earlier in FD males compared to BD controls.

***8.3.2.3. Age-related loss of robustness in activity/rest rhythm*:** Robustness of activity/rest rhythm (power of the rhythm) in flies first increased then gradually decreased with age, however there was no difference between the FD and BD stocks (Figure 8.3c). ANOVA on the power of rhythm revealed a statistically significant effect of A and A × S interaction, but no effect of S (Figure 8.3c, Table 8.2c). Post-hoc multiple comparisons using Tukey's test revealed that power of rhythm was significantly higher in adult flies compared to young and very-old flies (Figure 8.3c). These results suggest that ageing causes reduction in the robustness of activity/rest rhythm in both FD and BD male flies.

***8.3.2.4. Age-related reduction in activity levels*:** Analysis of activity levels revealed that the total activity per circadian cycle diminished gradually with age in both the stocks (Figure 8.3d). ANOVA done on the average activity per cycle at different age categories revealed a statistically significant effect of A and A × S interactions, however, the effect of S was statistically not significant (Table 8.2d). Post-hoc multiple comparisons using

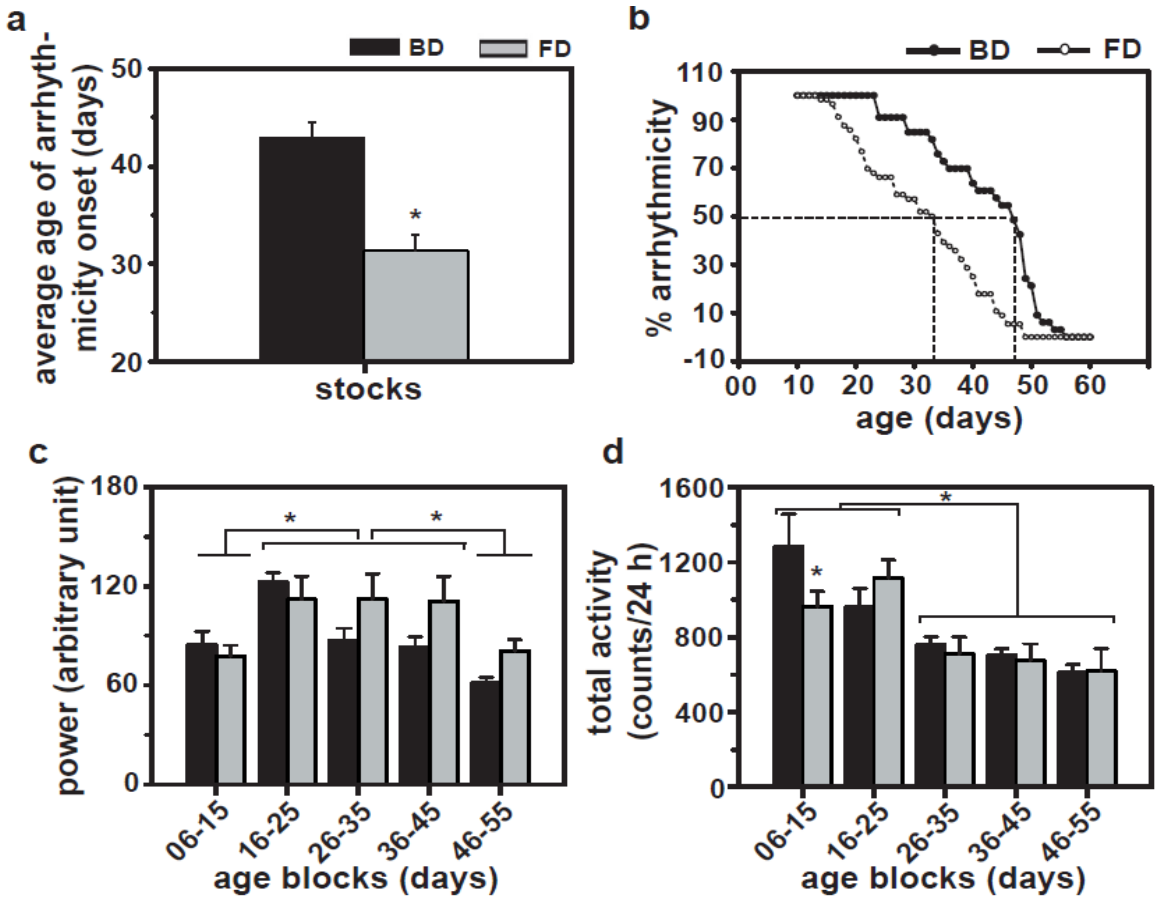


Figure 8.3. Significant age-dependent changes in circadian phenotypes of faster developing (FD) populations: Onset of arrhythmicity in faster developing (FD) populations (a) and its waveform (b) occur earlier compared to control (BD) populations. Mean power of activity/rest rhythm (c) and total activity (d) of FD and BD flies shows age-dependent reduction at older ages. All other details are same as in Figure 8.1.

Table 8.2a. Results of ANOVA on lifelong period change data.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Age (A)	4	0.52	12	0.04	12.49	0.0003
Stocks (S)	1	0.01	3	0.02	0.51	0.5271
Blocks (B)	3	0.07	0	0	--	--
A × S	4	0.28	12	0.05	6.11	0.0064
A × B	12	0.04	0	0	--	--
S × B	3	0.02	0	0	--	--
A × S × B	12	0.05	0	0	--	--

Table 8.2b. Results of ANOVA on onset age of arrhythmicity data.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Stocks (S)	1	270.87	3	5.37	50.48	0.0057
Blocks (B)	3	15.42	0	0	--	--
S × B	3	5.37	0	0	--	--

Table 8.2c. Results of ANOVA on lifelong power of rhythm data.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Age (A)	4	0.007	12	0.001	15.38	0.001
Stocks (S)	1	0.003	3	0.002	0.93	0.406
Blocks (B)	3	0.005	0	0	--	--
A × S	4	0.002	12	0.001	1.23	0.328
A × B	12	0.001	0	0	--	--
S × B	3	0.002	0	0	--	--
A × S × B	12	0.001	0	0	--	--

Table 8.2d. Results of ANOVA on total activity data.

Effect	*df*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Age (A)	4	408346.2	12	19098.79	15.38	0.001
Stocks (S)	1	21469.99	3	206850.41	0.93	0.406
Blocks (B)	3	21600.57	0	0	--	--
A × S	4	59031.11	12	13488.45	1.23	0.328
A × B	12	19098.79	0	0	--	--
S × B	3	206850.41	0	0	--	--
A × S × B	12	13488.45	0	0	--	--

Tukey's test revealed that young and adult flies were significantly more active compared to old or very-old flies, and activity of young FD males was lower than that of young BD males (Figure 8.3d; Table 8.2d). Thus, ageing results in significant reduction in activity levels in old or very-old flies, irrespective of their genotype. But the greatest difference in activity levels between the two stocks occurs only in the youngest age group.

8.4. Discussion

Selection for faster pre-adult development in fruit flies *D. melanogaster* results in significant reduction in adult lifespan (Figure 8.1) which can be taken to suggest that faster developing flies probably invest less in their somatic tissue development than controls. Moreover, previous studies have shown that life history traits such as development time and adult lifespan are clock-regulated; faster developing flies with shorter τ have relatively shorter lifespan than controls. Apart from the positive correlation seen in many species, negative correlation between development time and adult lifespan has also been reported in natural population of butterfly *Bicyclus anynana* (Pijpe et. al., 2006), suggesting that adult lifespan is a species-specific trait which may also depend on the pre-adult developmental conditions. Our findings of shorter lifespan in faster developing populations is contrary to those of Zwaan et al. (1995) and Nunney (1996), found it to be not significantly different from their controls. However, our results are consistent with those of Chippindale et al. (2004) and Prasad (2003), who reported reduced lifespan in faster developing populations. Thus, the observed reduction in faster developing fruit flies *D. melanogaster* populations, suggests a possible connection between development time and adult lifespan.

According to the developmental theory of ageing the rate of pre-adult development and ageing in *Drosophila* are causally correlated (Chippindale et al., 1994; Zwaan et al., 1995), therefore FD flies are expected to age faster than BD controls. We find that ageing in flies is also associated with noisy activity/rest rhythm, and τ lengthening (Figure 8.2). Further there was a tendency towards greater variation in τ of the FD flies compared to BD controls, which suggests τ is less stable in FD relative to their controls. Consistently, previous studies have also shown lower stability of τ and complex rhythms such as splitting to be associated with older age (Turek et al., 1995; Pandi-Perumal et al., 2002). Because of age-dependent effects on circadian clocks, selection-mediated difference in τ was reduced at later life stages (Figure 8.2b). Additionally, there was an early onset of arrhythmicity in the FD flies compared to BD controls, suggesting that circadian clocks are linked with physiological well-being of organisms. The FD flies showed age-related changes in their circadian activity/rest rhythm much earlier than BD controls which may be causally related with reduced lifespan. In previous studies, some clock null mutants have been shown to live significantly shorter than wild-type controls (Hendricks et al., 2003), which was taken to suggest the importance of proper functioning of circadian clocks for the physiological well-being of organisms. Our results are consistent with those observed in aged mice (Valentinuzzi et al., 1997) and insects (Watari and Arai, 1997) including fruit flies (Rakshit et al., 2012; Luo et al., 2012).

Genetic or environmental manipulations affect circadian clocks and thus accelerate ageing, which results in shortening of lifespan in the *tau* mutant hamsters (Hurd and Ralph, 1998; Oklejewicz and Daan, 2002) and fruit flies (Klarsfeld and

Rouyer, 1998). In several animals including humans, circadian rhythms have been found to become less robust and noisier with age which is believed to be detrimental to health (Turek et al., 1995; Pandi-Perumal et al., 2010). Moreover, loss of circadian rhythms result in several health problems including progression of neurodegeneration (Rezaval et al., 2008) and accelerated ageing (Krishnan et al., 2009, 2012). In the current study, FD flies showed significantly earlier loss of circadian rhythmicity compared to BD controls (Figure 8.3a), suggesting that early disruption of circadian rhythm in FD flies could be a possible cause for shorter lifespan and faster ageing.

Amplitude of rhythms, entrainability and ability to coordinate various behavioural or physiological oscillations are affected considerably at old age. We found that power of rhythm (Figure 8.3c) and total activity (Figure 8.3d) in flies was reduced with age consistent with previous reports (Le Bourg, 1987; Rezaval et al., 2008; Rakshit et al., 2012). Thus, selection for faster pre-adult development yields flies with reduced activity and robustness in activity/rest rhythm associated with shorter lifespan. Similarly, consistent with a previous study (Le Bourg, 1987) our results revealed that lower activity level particularly during old age is associated with shorter lifespan, however, the results of our study rules out any possible correlation between development and activity level since at most life-stages both FD and BD flies showed comparable activity levels (Figure 8.3e, f). Age-related changes in activity are associated with sleep fragmentation, a marker of old age (Koh et al., 2006) which can also be seen in actograms of FD flies (Figure 8.2a). Therefore, our results show age-related changes in circadian rhythm which occur earlier in the faster developing flies compared to their controls.

While it is commonly believed that shortening or lengthening of adult lifespan is a marker of faster or slower ageing respectively, age-dependent reduction in survival probability (excluding the condition of externally imposed causes of death) can also be taken as readout of ageing. Although there can be several possible causes of reduction in lifespan, the age-dependent changes in circadian activity/rest rhythm in the faster developing flies can be taken as one among them. Our results suggest that faster developing flies live significantly shorter and the markers of age-related changes in circadian rhythms in them set-in earlier than controls, which suggests that ageing of circadian clocks in fruit flies *D. melanogaster* is a function of physiological rather than chronological age.

8.5. Conclusions

Our results suggest that selection for faster pre-adult development results in reduction in adult lifespan. Faster developing flies manifest age-dependent changes in their clock properties such as early onset of arrhythmicity, less robust rhythms and early period lengthening earlier than controls. These changes reveal circadian dysfunction in faster developing flies as one of the possible reasons for faster ageing in fruit flies *D. melanogaster*.

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