Phenotypic and evolutionary effects of light on circadian clocks and related life history traits in *Drosophila melanogaster*

A Thesis
Submitted for the Degree of
Doctor of Philosophy

By
Dhanashree Paranjpe

To
Evolutionary and Organismal Biology Unit
Jawaharlal Nehru Centre for Advanced Scientific Research (A Deemed University)
Bangalore - 560064 (INDIA)

DECEMBER 2005

Dedicated

To my family and friends

For their unconditional support and infinite encouragement

CONTENTS

DECLARATION

I declare that the matter presented in my thesis entitled "Phenotypic and evolutionary effects of light on circadian clocks and related life-history traits in *Drosophila melanogaster*" is result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Center 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 the other investigators. Any omission, which might have occurred by oversight or error of judgment, is regretted.

Dhanashree Paranjpe

Place: Bangalore

Date: 16th Dec., 2005

Evolutionary and Organismal Biology Unit
JAWAHARLAL NEHRU CENTRE FOR
ADVANCED SCIENTIFIC RESEARCH
PO BOX 6436, JAKKUR, BANGALORE - 560 064
INDIA

16 December, 2005

CERTIFICATE

This is to certify that the work described in the thesis entitiled "Phenotypic and evolutionary effects of light on circadian clocks and related life history traits in *Drosophila melanogaster*" is the result of investigations carried out by Ms. Dhanashree A Paranjpe in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, 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, PhD
Associate Professor

Telephone: 91-80-22082755; FAX: 91-80-22082766

Acknowledgements

I thank my Ph.D. advisor Prof. Vijay Kumar Sharma for introducing me to the fascinating field of chronobiology. His valuable guidance, support and constructive criticism were useful throughout the work. I also wish to thank Prof. M. K. Chandrashekaran and Prof. Amitabh Joshi for their active interest and useful suggestions during the course of my work.

I am grateful to Prof. K. Vijayraghavan and Prof. V. Rodrigues, NCBS, Bangalore for kindly giving me permission to avail the facilities for recording ERG. I thank Prof. K. S. Krishnan, TIFR, Mumbai for useful discussions on ERG analysis and Prof. G. Hasan for letting me use the counter-current apparatus. Prof. M. Rosbash, Prof. P. Emery and Prof. J. Hall of Brandeis University, USA kindly donated mutant strains of flies used in my work. I am grateful for their kind donation.

All experiments that are part of this thesis involve efforts of my present and past labmates- Anitha, Shaliesh, Gitanjali, Ambika, Koustubh, Manjunath, Sreeja, and several summer research students. I am grateful for their timely help and patience.

Other present and past members of EOBU – Dr. Sheeba, Dr. Prasad, Dr. Mallikarjun, Sharmila, Sutirth, Shampa, Satish, Archana, Mohan, Anand, Raghavendra, Dr. Sujata, Snigdhadeep and numerous summer students always kept environment in the laboratory lively and made the work enjoyable. Shobana, Pushpa, Vinod, Neena, Gargi, Gautam, Ashish, Ramshankar, Rinky, Abhijit, Sugat have been great friends and made my stay at JNCASR memorable.

Finally I thank all my family members for their unconditional support and encouragement.

Summary

Circadian clocks regulate the timing of a number of key behavioral and physiological processes in a wide range of organisms (Dunlap et al., 2004). Its ubiquitous occurrence across levels of complexity and organization is often considered as an evidence for its adaptive significance (Sharma, 2003a). Organisms are thought to gain two types of adaptive advantages by having functional circadian clocks (i) "intrinsic advantage": gained by coordinating various metabolic processes in the internal milieu, and (ii) "extrinsic advantage": accrued by synchronizing behavioral and metabolic processes with the periodic external environment (Sharma, 2003a). Studies on free-living animals suggest that circadian clocks enhance the chances of their survival under natural environment (Paranjpe and Sharma, 2005). Studies in organisms living under constant conditions such as depth of the sea, and subterranean caves, suggest that these organisms may or may not exhibit circadian rhythms, and if they do, such rhythms may not entrain to periodic light/dark (LD) cycles (Sharma, 2003a). This raises an interesting and yet unanswered question whether organisms living under constant environments have functional clocks that are similar to those inhabiting periodic environments, and whether functional aspects of such timing systems such as sensitivity and ability to respond to LD cycles, ability to entrain different rhythms to a range of periodic environments, persist in these organisms. I have tried to address some of these questions in my thesis.

Previous studies on the adaptive significance of circadian rhythms suffer from one drawback or another; most studies lack population-level replication, true controls, and adequate control for the genetic composition of the populations (Sharma and Joshi, 2002). A number of studies have used mutants and highly inbred animals to address this issue, which in many ways limit the potential insights gained. Furthermore, often lifespan was used as the sole indicator of fitness, though it is well known that a fine balance between various life history traits, resulting from several genetic and environmental constraints, and trade-offs constitutes fitness of an organism (Loeschke, 1987; Stearns, 1992; Leroi et al., 1994). This suggests that a number of relevant fitness traits which could

directly or indirectly influence reproductive output of the organism should be taken into account to draw any meaningful conclusions about the adaptive value of circadian clocks. To empirically establish that circadian clocks have adaptive significance, systematic and rigorous studies which successfully address the above mentioned criticisms and demonstrates that (i) specific environmental conditions relevant to the trait have differential effect on the major fitness components, and (ii) the trait in question evolves differentially under those environments (Sharma and Joshi, 2002) needs to be carried out. I therefore decided to study the effect of light conditions on a number of key clock parameters and fitness components. I also investigated the long term (evolutionary) effects of rearing flies under different light conditions on the clock and clock-associated traits.

Using four large, out bred laboratory populations of *Drosophila melanogaster* reared under constant light (LL) for more than seven hundred generations (henceforth will be referred as LL populations) we studied behaviors such as adult emergence (eclosion), activity/rest (locomotor activity) and egg-laying (oviposition) under two constant environments, constant light (LL), constant darkness (DD), and five periodic LD cycles of 9:9 h (*T18*), 10:10 h (*T20*), 12:12 h (*T24*), 14:14 h (*T28*) and 15:15 h (*T30*). All three behaviors showed rhythmic patterns under DD with periods in circadian range and entrained to LD cycles ranging from *T18* to *T30*. The timing of peak of eclosion and the end of activity under LD cycles changed systematically with changing day length. This suggests that circadian rhythms of eclosion, locomotor activity and egg-laying persist in the LL populations, which confirms results of earlier studies from our laboratory done on these populations, about 100 generations ago (Sheeba *et al.* 1999a, 2001, 2002b). The fact that circadian rhythms in three key behaviours in the LL flies persist, and that the rhythms entrained stably to LD cycles with a wide range of periodicities, as well as other wild type flies do, suggests that circadian clocks have an intrinsic adaptive value. These results could also be taken to suggest that entrainment mechanisms are an integral and indispensable part of the circadian clock's pacemaking mechanisms.

We further investigated whether light regimes have differential effects on fitness components such as pre-adult development time, survivorship, lifespan, and reproductive output, and whether there is any role of circadian clocks in mediating such effects. We managed to speed up or slow down the *Drosophila* eclosion rhythm by keeping the flies in shorter and longer LD cycles, which in turn speeded up or slowed down the pre-adult development. This suggests that the period of LD cycles, and/or of the eclosion rhythms determines the duration of pre-adult development in *D. melanogaster*. However, as the period of LD cycles deviated from 24 h, the pre-adult survivorship was significantly reduced, suggesting that faster or slower development has a cost attached to it. Further, experiments under short and long photoperiods revealed that the duration of light and the light/dark ratio in the LD cycles also influence pre-adult development time in *Drosophila*. To the best of our knowledge, ours is the first detailed study of its kind on the effect of light regimes on pre-adult development time. The light regimes also had measurable effect on the adult fitness traits such as lifespan and reproductive output. Female flies, both virgin and mated, lived significantly shorter under constant environments (LL, DD) compared to the periodic environments (*T20*, *T24*, *T28*), which suggests that constant environments are deleterious for *Drosophila* females. Careful analysis of the reproductive output of both virgin and mated females revealed that the number of eggs laid by the flies was considerably higher under constant conditions compared to the LD cycles, which suggests that the reduction in the lifespan of females was primarily due to higher reproductive output and not due to some unknown deleterious effect of constant environment. On the other hand in virgin males, the light regime-dependent reduction of lifespan was primarily mediated through increased activity duration, whereas no such correlation was observed in mated males, and virgin and mated females, suggesting a sex-specific effect of environment, circadian clocks, and/or activity on the adult lifespan of *D. melanogaster*.

The next step towards establishing the adaptive significance of circadian clocks was to demonstrate that circadian clocks, and/or clock-associated features evolve differentially under constant and periodic light conditions. For this

purpose two new sets of populations were initiated from the LL populations (Sheeba, 2001) and reared for more than one hundred generations under DD and LD cycles of 12:12 h (henceforth will be referred as DD and LD populations, respectively). We evaluated the effects of rearing under different light conditions on general photic responses in the three populations after 100 generations. The clock's response to brief light stimuli in the DD flies was significantly reduced compared to the LL and LD flies. Such reduction in the photic response could have arisen due to impairment in light sensing mechanisms or due to the reduction in clocks' ability to respond to light stimuli. To examine the first possibility we recorded electro-physiological activity of fly retina by first exposing the flies to light stimuli and then recoding the retinal responses as electro-retinogram (ERG). The ERG records did not reveal any difference in light reception ability of the populations. A considerable fraction (~35-50%) of flies from all the three populations showed abnormal retinal responses, suggesting that light input was probably impaired in the LL populations to start with, and 100 generations of rearing under LD and DD regimes did not cause any measurable change. Subsequently, we decided to assay the behavioral photic responses of larvae and adults to estimate the clock's response to light stimuli. A greater fraction of larvae from the DD populations preferred darkness compared to the LL and LD populations, suggesting that rearing under DD makes flies more photophobic. Further, light or dark preference of larvae was found to be time dependent under DD in the LL and LD populations, suggesting that circadian clocks regulate the circadian larval photic response behaviour. The adult photic responses on the other hand were intensity dependent; at lower intensity of light, adults from the LL and DD populations showed time dependent photo-tactic behavior, suggesting that circadian clocks also regulate the adult photic-responses in *Drosophila*. At higher light intensity, photic response was not time dependent. At lower light intensity, adults from the DD populations showed significantly less photo-tactic behavior compared to those from the LL and the LD populations. These results suggest that rearing in DD makes flies more photophobic and their circadian clocks less responsive to light. These results are

surprising and their implications for fitness or adaptive advantage for circadian clocks under different light conditions are still unclear.

To confirm the role of circadian clocks in regulating the behavioral responses such as immediate response to a light stimulus, we studied adult photo-tactic responses in a number of fly strains. Adult photic-responses were time dependent in the wild type (CantonS and yw) flies, while the time-dependency was lost considerably in clock manipulated strains of flies (per^0, tim^0, clk^{irk}, cyc^0, cry^b, and gl^{60j}). Adult photic response was significantly reduced in the mutant with impaired photic-transduction (gl^{60j}) indicating that light-input pathways are important for rapid behavioral photo-responses in adults. Thus, our study clearly demonstrates that circadian clocks modulate behavioral photic responses in *D. melanogaster.* To the best of our knowledge, this is the first study of its kind demonstrating the role of circadian clocks in regulation of rapid behavioral response to light stimuli in *Drosophila* adults.

In conclusion, we could demonstrate that light influence a number of clock properties and related fitness components. In addition to such phenotypic effects, light also has a long-term evolutionary effect on the circadian clocks and the clock-related traits. Although, the adaptive advantage of such changes is not yet clear, it is a step forward towards the demonstration of adaptive significance of circadian clocks.

List of Publications

1. **Paranjpe DA**, Anitha D, Kumar S, Kumar D, Verkhedkar K, Chandrashekaran MK, Joshi A, Sharma VK **(2003)**: Entrainment of eclosion rhythm in Drosophila melanogaster populations reared for more than 700 generations in constant light environment. Chronobiol. Intl. 20: 977-987.
2. Padiath QS, **Paranjpe DA**, Jain S, Sharma VK **(2004)**: Glycogen Synthase Kinase 3β as a likely target for the action of Lithium on circadian clocks. Chronobiol. Intl. 21: 27-38.
3. **Paranjpe DA**, Anitha D, Joshi A, Sharma VK **(2004)**: Circadian clocks and life history related traits: Is pupation height affected by circadian organization in *Drosophila melanogaster*? J. Genet. 83:73-77.
4. **Paranjpe DA**, Anitha D, Joshi A, Sharma VK **(2004)**: Multi-oscillatory control of eclosion and oviposition rhythms in *Drosophila melanogaster*: evidence from limits of entrainment studies. Chronobiol. Intl. 21: 539-552.
5. **Paranjpe DA**, Anitha D, Chandrashekaran MK, Joshi A, Sharma VK **(2005)**: Possible role of eclosion rhythm in mediating the effects of light-dark environments on pre-adult development in *Drosophila melanogaster*. BMC Dev. Biol. 5: 5.
6. **Paranjpe DA**, Sharma VK **(2005)**: Evolution of temporal order in living organisms. J. Circadian Rhythms 3: 7.
7. Howlader G, **Paranjpe DA**, Sharma VK: Non-ventral lateral neuron based, non-PDF mediated clocks control circadian egg-laying rhythm in Drosophila melanogaster. J Biol Rhythms (in press).
8. **Paranjpe DA**, Anitha D, Sreeja G, Sharma VK: Effects of environmental light regimes on the adult life span of Drosophila melanogaster are mediated through clock period, activity duration and reproductive output. (in preparation)

Chapter 1

Introduction

1.1 Introduction to circadian rhythms

Earth's rotation around its axis causes predictable changes in the geophysical environment, which provides organisms with options to occupy a wide range of temporal niches. Most organisms place themselves suitably in such niches using time keeping system that can measure passage of time on an approximately 24 h scale (and hence are known as *circadian clocks*; *circa* = approximately; *dies* = a day) (Dunlap et al., 2004). Extensive studies over the past fifty years on a wide range of organisms have revealed some unique features of circadian clocks that delineate them from other biological oscillators. For example, circadian clocks have (i) inherent near 24 h periodicities, (ii) their periodicities are protected to a large extent from changes in temperature, nutrition and *pH* within physiologically permissible limits, and (iii) they can be made to oscillate with exactly 24 h period — a key property of circadian clocks known as *entrainment*, which enables organisms to keep track of time in their local environments. Circadian clocks enhance organism's chances of survival under periodic environments by enabling them to efficiently anticipate periodic events such as availability of food, light, mates, and presence of predators (Pittendrigh, 1993; Dunlap et al., 2004; Paranjpe and Sharma, 2005; Sharma and Chandrashekaran, 2005). Hence, it is not too surprising that a wide variety of organisms such as bacteria, fungi, fish, amphibians, reptiles, insects, mammals including humans, as well as plants possess circadian clocks that time their metabolic and behavioral processes.

1.2 Entrainment of circadian clocks to environmental cues

The issue of entrainment and its implications in temporal niche selection has remained central to circadian rhythm research since its inception. It is commonly believed that forces of natural selection act on the timing of a rhythmic behavior. Therefore, maintenance of stable and appropriate timing for behavioral and metabolic activities is considered as one of the most essential features of circadian clocks. This is especially true for organisms living under natural environments where light, temperature, humidity, food, predators and competitors show diurnal fluctuation. With the help of such periodic time cues circadian clocks keep track of local time by synchronizing various metabolic and behavioral processes with the external periodic environment (Pittendrigh and Daan, 1976; Daan, 2000; Roenneberg et al., 2003; Sharma, 2003a). For most organisms, periodic LD cycles are among the most reliable time cues (Dunlap et al., 2004), hence most studies on entrainment have dealt with photic entrainment. Entrainment of circadian clocks largely depends upon : free-running period (τ) of the clock, period of the entraining time cue (T), and clock's sensitivity to the stimuli of time cue typically plotted as phase response curve (PRC) (Beersma et al., 1999; Daan, 2000; Roenneberg et al., 2003; Sharma, 2003b). The τ and PRC are considered as invariant properties of circadian clocks as they are assumed to remain unchanged through out the entrainment process (Sharma, 2003b; Sharma and Daan, 2002). Yet, studies on insects and rodents have revealed that τ and PRC of circadian clocks are not as invariant as they are believed to be, because they often vary reflecting residual effects of prior

environmental experience typically referred as "after-effects" (Pittendrigh, 1960; Sokolove, 1975; Christensen, 1978; Page and Block, 1980; Sheeba et al., 2002a). For example, mice exposed to LD cycles continued to exhibit rhythmic locomotor activity under DD with τ close to the period of previously experienced LD cycles (Pittendrigh and Daan, 1976). Such after-effects can be thought to be of some functional significance to the organism, as they could help in maintaining a stable phase relationship (ψ), even when the perceived environmental cycles are masked due to cloud cover or behavioural changes (Pittendrigh and Daan, 1976; Enright, 1980; Kramm and Kramm, 1980; Beersma et al., 1999).

The ψ of a rhythm is known to change as a function of the period of LD cycles (T) (Pittendrigh and Daan, 1976; Pittendrigh, 1993), reflecting the ability of circadian clocks to adjust suitably to a wide range of LD cycles. Under shorter T cycles, the ψ is more negative while under longer T, ψ is more positive implying that as the length of the LD cycle increases, the behavior in question occurs earlier and earlier. Since ψ depends upon both τ and T, a relationship between τ, T and ψ would be expected to compensate for the day-to-day variations in τ as well as predictable (seasonal) changes in day lengths (Pittendrigh, 1981). In other words, systematic change in ψ as function of τ and T is believed to allow organisms to maintain an appropriate 'temporal niche' in the periodic environments. Therefore, it appears that circadian clocks have evolved entrainment mechanism to enhance its stability in ever fluctuating environments, a clock feature which could be critical for organism's survival in natural environment (Daan, 2000; Sharma, 2003b; Paranjpe and Sharma, 2005).

1.3 Circadian clocks and adaptive evolution

The phenomenon of adaptation is one of the central and important ideas in the field of biology (Pittendrigh, 1958). Phenotypic adaptation refers to changes in biological processes in response to the immediate set of environmental conditions occurring within the life time of an organism, while long term changes such as those in genetic composition of populations in response to the environmental conditions over several generations generally imply ultimate or evolutionary adaptation. For establishing empirically that a particular trait is "adaptive" rigorous criteria have been developed, which include demonstration that (i) specific environmental regimes relevant to the trait have differential effects on major fitness components, i.e. important life history traits such as life span, reproductive output which contribute to Darwinian fitness of an organism, and (ii) that the trait in question evolve differentially under these environmental regimes (Sharma and Joshi, 2002). Thus, it is apparent that systematic long term studies on laboratory populations could perhaps constitute the best approach for studying evolutionary adaptation (Rose et al., 1996; Joshi 1997; Sharma and Joshi , 2002).

Although circadian clocks are believed to have arisen as a result of adaptive evolution under periodic environments (Hastings et al., 1991) there has been hardly any rigorous and conclusive empirical study which meets the above mentioned criteria (Sharma 2003a). Given that circadian clocks are ubiquitous and are found in a wide range of organism at various levels of organization and complexity, it is often speculated that they must provide adaptive advantage to

their owners (Sharma 2003a). Organisms can be thought to gain adaptive advantage of possessing functional circadian clocks of two types - (i) "intrinsic advantage" gained by using circadian clocks to coordinate various cyclic metabolic processes within the internal milieu, and (ii) "extrinsic advantage" accrued by synchronizing various metabolic and behavioral processes with the periodic external environment (Sharma, 2003a). Further, it is believed that under periodic environments, precisely timed rhythmic activities confer greater adaptive advantage compared to randomly occurring activities, and in turn clocks that enable organisms to maintain such timings are selected for (Roenneberg et al., 2003; Sharma, 2003a). Therefore, the free running phenotype of circadian clocks is considered as an evolutionary outcome of forces of natural selection acting on entrained clocks (Hastings et al., 1991; Pittendrigh, 1993; Roenneberg et al., 2003; Sharma and Chandrashekaran, 2005). There is a general agreement among circadian biologists that circadian clocks have evolved under selection pressures comprising of periodic cycles of light, temperature and humidity, and at some later stage rhythmic activities of prey, predators, and dominant competitors might have provided additional selection pressures for it's fine tuning (Aschoff, 1965; Hoffmann, 1976; Hastings et al., 1991).

1.4 Adaptive significance of circadian clocks

Evidence from studies on free-living organisms

Circadian clocks regulate a number of key behaviors in a wide variety of organisms. For example, most insects emerge as adults from their pupal case

(an act known as *eclosion*) close to the "dawn", when humidity in the environment is the highest (Bünning, 1935; Kalmus, 1935; Pittendrigh, 1954). It is believed that by restricting eclosion to the early hours of the day, insects prevent desiccation, and thus enhance their chances of survival (Pittendrigh, 1958). Circadian clocks help organisms in timing their activity at species-specific time of the day, which enables them to find food and mates, escape predators, and avoid undue competition between sympatric species (Dunlap et al., 2004; Paranjpe and Sharma, 2005). Proximal advantages of possessing circadian clocks have been evaluated in a few field studies. For example, in a study on the guillemots (*Uria lomvia*), a greater percentage of fledglings jumping out of their nests at non species specific time fell prey to gulls compared to those that restricted their jumping behaviour during species specific time of the day (Daan and Tinbergen, 1980). Thus, timing jumping behaviour during evening hours, in synchrony with other juveniles resulted in greater chances of survival in the young fledglings (Daan, 1981). In ground squirrels living, the hypothalamus-based circadian clock — suprachiasmatic nucleus (SCN) — has been reported to play an important role in their survival under natural conditions. Under laboratory conditions, SCN ablated animals survived equally well as the controls (Ruby et al., 1998), but quickly fell prey to feral cats when released into a semi natural enclosure (DeCoursey et al., 1997). This suggests that although functional clocks may not be essential for survival under controlled laboratory conditions, they might become crucial under natural environment. In a subsequent marathon field study on the free living chipmunks *Tamias striatus*, DeCoursey and coworkers

demonstrated that the reduction in survival of the SCN ablated animals was primarily due to enhanced predation, perhaps due to increased nighttime restlessness (DeCoursey et al., 2000).

Circadian clocks play an important role in the regulation of day to day repertoire of social insects such as honeybees and ants. Social insect colonies are normally faced with a wide variety of challenges such as changing colony sizes, time of the year, food availability, predation pressure and changing climatic conditions. Survival of these colonies under such conditions requires a number of tasks to be performed simultaneously. Social insects seem to have evolved division of labor, a strategy, which in addition to enhancing efficiency of task management promotes biological evolution of complexity and diversity (Bourke and Franks, 1995). In a series of experiments, Robinson and coworkers demonstrated that social insects use circadian clocks to efficiently manage division of labor (Robinson, 1992). For example, in the colony of the Asian honeybee *Apis mellifera*, young workers (nurses) perform tasks that can be categorized as "nursing" practically around the clock without taking any rest (Moore et al., 1998), whereas the older honeybees (foragers) visit flowers to collect pollen and nectar in a rhythmic manner (Frisch, 1967). Thus, the honeybees use circadian plasticity to match the age dependent behavioral development, a phenomenon commonly known as age polytheism (Toma et al., 2000, Bloch et al., 2001). The role of circadian clocks has also been investigated in a few ant species. In ants, the virgin queens and males mate during nuptial flights, which occur at a species specific time of the day, during the mating

season (Hölldobler and Wilson, 1990; McCluskey, 1992). The virgin queens and males are believed to use circadian clocks to time their mating flight in order to encounter mating partners from the neighboring colonies (Hölldobler and Wilson, 1990; McCluskey, 1992). In the ant species *Camponotus compressus* the virgin queens and males were found to time their mating flights by maintaining a stable phase relationship with the environmental LD cycles, perhaps to facilitate cross breeding between colonies and to avoid inter species mating (Sharma et al., 2004a). In a separate study, some worker castes of this species, namely the major and media workers were found to show rhythmic activity/rest cycles, perhaps to time their day-to-day repertoire. Some major workers (the foragers) showed stable activity rhythms, while the rest (the soldiers) did not show any obvious sign of rhythmicity. The media workers, on the other hand, are task generalists; they were either found foraging most of the time or were restricted to their colonies, perhaps taking care of the queen and her brood (Sharma et al., 2004b). Interestingly, clock periods in the rhythmic media workers changed from less than 24 h to greater than 24 h, and *vice-versa*, which reflects a possible switching of activity patterns from nocturnal to diurnal or diurnal to nocturnal, suggesting that media workers are involved in shift work in the colony (Sharma et al., 2004c). The activity of minor workers on the other hand, neither entrained to light/ dark (LD) cycles nor did it show any sign of free-run in DD, which matches well with their role as nurses in the colony. The activity patterns of different castes of the ant species *C. compressus* thus seem to be well suited for the tasks assigned to them in a their colonies (Sharma et al., 2004b).

Migratory birds use circadian clocks to keep track of rapidly changing day lengths in order to navigate to a more favorable climate at a specific time of the year (Dunlap et al., 2004). European starlings use circadian clocks to compensate for changing position of the sun on long-distance journeys (Hoffmann, 1960). Monarch butterflies *Danaus plexippus*, use circadian clocks to undertake migratory flights every fall from northeastern America to their over-wintering grounds in central Mexico (Froy et al., 2003). Golden-mantled squirrels enter hibernation in autumn when day lengths begin to shorten and mean daily temperature starts to drop (Dunlap et al., 2004). It is believed that these animals use circadian clocks to measure day lengths in order to prepare themselves for hibernation at an appropriate time of the year. On average, hibernations last for about 7 months with periodic wake-up bouts for sustaining brain and kidney functions through long winters. These wake-up bouts are also regulated in part by circadian clocks (Dunlap et al., 2004), as SCN-ablation caused marked changes in the duration of wake-up bouts and the duration of hibernation (Ruby et al., 1996; 1998). Regular wake-up bouts are crucial even under hibernating conditions for rationing limited fat supply to last for the entire winter, as wake-up bouts are associated with muscular shivering and are metabolically expensive (Dunlap et al., 2004). These studies thus serve as a suggestive evidence for proximal advantages that circadian clocks could provide to their owners. However, they by no means should be taken to suggest that circadian clocks provide an evolutionary selective advantage.

Evidence from studies on latitudinal clines

Organisms living in temperate regions experience drastic changes in photoperiod and temperature, ranging from longer days and high temperatures in summer to very short days and subzero temperatures in winters. Organisms exposed to such extreme environmental conditions need to time their behaviors appropriately, and it is believed that circadian clocks play an important role in achieving that (Pittendrigh and Takamura, 1989). Studies on a number of strains of *D. littoralis* originating from a wide range of geographic locations at different latitudes, conducted to examine the clock properties of strains distributed over a wide range of geographical locations in order to understand how these species cope up with the seasonal changes, revealed a weak latitudinal trend in phase and period of eclosion rhythm (Lankinen, 1985). The northern strains had shorter τ and earlier phase of eclosion compared to the southern strains (Lankinen, 1985). Similarly latitudinal clines for phase and amplitude of eclosion rhythm were also reported in *D. auraria* (Pittendrigh and Takamura, 1989). Since amplitude of circadian rhythms respond to changes in photoperiod as well as temperature, it was concluded that these insects use circadian clocks to track seasonal changes in their environment (Pittendrigh et al., 1991). Latitudinal clines for adult diapause was reported in fifty-seven European populations of *D. littoralis*, where the northern populations responded to longer critical day lengths compared to the southern populations (Lankinen, 1985). In a separate study, clinal patterns in threonine-glycine (Thr-Gly) repeats were found at the *period* (*per*) locus in European and North African strains of *D. melanogaster* (Costa et

al., 1992) and *D. simulans* (Rosato et al., 1994). The northern strains showed higher frequency of $(Thr\text{-}Gly)_{17}$ compared to the southern strains $(Thr\text{-}Gly)_{20}$ (Costa et al., 1992; Rosato et al., 1994). Further studies on the locomotor activity rhythm in these populations done at two different temperatures (18 ^{0}C and 29 ^{0}C) revealed that circadian clocks of the $(Thr\text{-}Gly)_{20}$ variants had the most efficient temperature compensation ability, while this was not the case for the $(Thr\text{-}Gly)_{17}$ variants, and they showed period shortening at lower temperatures (Sawyer et al., 1997). Since clinal variation in phase and period are believed to have arisen as a result of natural selection, presence of such latitudinal clines can be taken as an indirect evidence for the adaptive evolution of circadian clocks (Pittendrigh and Takamura, 1989; Lankinen, 1985).

Evidence from fitness studies

The assumption that circadian clocks influence fitness traits had formed the basis of a number of studies aimed at addressing adaptive significance of circadian rhythms. Several studies had been carried out to investigate the role of circadian clocks in regulating life history traits such as pre-adult development time and adult lifespan. In one such study on the *per* mutants of *D. melanogaster*, which display circadian rhythms with widely different periodicities, pre-adult development time was measured under continuous dim light (LL), very bright continuous light (VLL), continuous darkness (DD), light/dark (LD) cycles of 12:12 h, and LD 12:12 h superimposed with temperature cycles (LD 12:12 T). Development time of the *per* mutants was positively correlated with the τ of their circadian clocks under DD, i.e. the per^S flies (τ = 19 h) developed faster than the

wild type flies (τ = 24 h), which in turn developed faster than the *per*^{L} flies (τ = 28 h) (Kyriacou et al., 1990). The correlation between development time and clock period remained unchanged under VLL, wherein flies are rendered arrhythmic (Konopka, 1972; Konopka et al., 1989). Moreover, development time and clock period showed positive correlation even under LD cycles, and in LD superimposed with temperature cycles (LD 12:12 T), wherein flies of different genotypes were entrained to a common 24 h periodicity. A positive correlation between development time and clock period observed even in absence of the overt rhythmicity under LL regime, and also under entrained conditions such as those prevailing under LD cycles suggests that *per* mutations have pleiotropic effects on clock period and pre-adult development time (Kyriacou et al., 1990). In a recent study in *D. melanogaster*, pre-designed to bypass such pleiotropic effects, we have demonstrated that the developmental time and the period of eclosion rhythm were positively correlated (i.e. faster eclosion rhythms were associated with faster development and slower eclosion rhythms were accompanied by slower development), which suggests that pre-adult development time in *Drosophila* is largely determined by the periodicity of LD cycles and/or of eclosion rhythm (Paranjpe et al., 2005). In a separate study on the melon fly (*Bactrocera cucurbitae*) that involved selection for faster and slower pre-adult development, faster developing lines were found to have faster circadian clocks and slower developing lines had slower circadian clocks (Miyatake, 2002). The timing of behaviors such as locomotion and preening was also significantly shifted to earlier hours of the day in faster developing lines

compared to the slower developing lines. The mating peaks in the faster developing lines occurred close to the dusk while most flies from the slower developing lines mated during the night (Miyatake, 1997). The τ of locomotor activity rhythm under DD was shorter (τ ~ 22.6 h) in faster developing lines and longer (τ ~ 30.9 h) in slower developing lines (Shimizu et al., 1997). Thus, selection on development time in this study resulted in changes in clock properties indicating some link between circadian clocks and life history traits. However, the robustness of such conclusion is limited by the fact that association between development time and period of circadian clocks in this as well as other previous studies shows very little effect of light regime.

Circadian clocks have also been implicated in the regulation of life span. In a study on the *tau* mutant hamsters, heterozygous (τ ~ 22 h) animals were found to live shorter under laboratory LD (14:10 h) cycles than the wild type animals (τ ~ 24 h), but on average the life span of homozygous animals (τ ~ 20 h) and wild type animals did not differ (Hurd and Ralph, 1998). Contradictory results were obtained in a similar study performed under DD conditions; here the homozygous animals were found to live significantly longer than the wild type controls, while the average lifespan of heterozygote animals did not differ from those of the wild type controls (Oklejewicz and Daan, 2002). Such differences in outcome of two otherwise similar experiments could arise due to the fact that the two studies were performed under different environmental conditions and environmental factors are known to influence both clock and life span to a great extent (Kyriacou et al., 1990; Hurd and Ralph, 1998; Sheeba et al., 2000;

Sharma and Joshi, 2002). In a separate study in fruit flies *D. melanogaster*, role of circadian clocks in determining life span was investigated. The lifespan of per^{T} (short period mutant, τ = 16 h), and per^{L} (long period mutant, τ = 29 h) mutants was considerably reduced compared to the per^{+} (wild type, τ = 24 h) flies, even when flies were maintained under LD cycles with periodicities closer to the endogenous periodicity of the mutant lines (Klarsfeld and Rouyer, 1998). This suggests that lifespan of *D. melanogaster* is not regulated by circadian clocks rather it is determined by the genotype of the flies, which suggests pleiotropic effect of *per* mutations on clock period and lifespan. Therefore, evidence in hand for the role of circadian clocks in controlling life-history traits is circumstantial and merely suggestive in nature.

Circadian clocks have also been implicated to play crucial role in the regulation of reproductive output of *D. melanogaster*. Studies on the loss of function mutants of some of the clock genes in *D. melanogaster* such as *period, timeless, cycle,* and *Clock* (per^{0}, tim^{0}, cyc^{0}, and Clk^{jrk}) revealed that a single mating among the clock-deficient phenotypes yields ~ 40% lesser progeny compared to the wild type flies (Beaver et al., 2002). In general, null mutants laid fewer eggs, out of which only a few developed as adults (Beaver et al., 2002). Further experiments on the per^{0} and the tim^{0} flies revealed that the amount of sperm released from the testes to seminal vesicles of males was also significantly reduced in the null mutants compared to the wild type flies (Beaver et al., 2002). Although, the loss-of-function mutants clearly had reduced reproductive output in *Drosophila* males and females, involvement of circadian

clocks remained unclear and intriguing. Interestingly, egg-laying is rhythmic in flies from a wide range of genotypes, transcripts of *per*, and protein levels of *per* and *tim* do not oscillate in the ovaries of *Drosophila* females (Hardin et al., 1994), and constitutively high level of PER and TIM proteins were found in the follicle cells of developing oocytes throughout the day (Beaver et al., 2003). Studies have also shown that PER and TIM interact in these follicle cells but do not translocate into the nucleus, thus leaving the clock mechanisms truncated (Beaver et al., 2003). It remained unclear for a long time as to what could be the functional role of the clock genes *per* and *tim* in the fly ovary. In a recent study, Beaver and co-workers (2003) demonstrated that lack of functional *per* and *tim* in virgin females results in significantly fewer mature oocytes in the per^0 and the tim^0 flies compared to the wild type flies. Rescue of clock function in per^0 mutants by ectopically expressing *per* in the lateral neurons alone did not enhance the production of mature oocytes, suggesting that *per* and *tim* may have non-clock like functions in the ovaries (Beaver et al., 2003).

Fitness components such as development time, reproductive output as well as circadian rhythms are multigenic traits, and the underlying genes may have pleiotropic effects (Sokolowski, 2001). Therefore, it is fair to speculate that mutations altering circadian phenotypes may simultaneously reduce reproductive fitness via mechanisms that could be independent of circadian machinery. Alternatively, manipulations in certain genes or processes closely associated with fitness traits may alter clock phenotype, through clock independent processes.

Evidence from circadian resonance studies

It is believed that at the very advent of early life forms on our planet several temporal patterns were present, however, only those that matched environmental periodicity managed to survive. Motivated by this thought, Pittendrigh and Bruce (1959) proposed the "circadian resonance hypothesis", according to which organisms with clocks periodicities matching those of cyclic environment would perform "better" compared to others with deviant periodicities. If circadian resonance was the driving force behind the evolution of circadian clocks, one would expect organisms with near-24 h clocks to have greater fitness advantage under a 24 h environment than in any other periodic or aperiodic environment. Indeed, fruit flies with near-24 h periodicities were found to live significantly longer under a 24 h LD cycle than either in 21 h (LD 10.5:10.5 h), 27 h (LD 13 5:13.5 h) LD cycles or under LL (Pittendrigh and Minis, 1972). Blowflies (*Phormia terranovae*) reared under 24 h LD cycles lived significantly longer in a 24 h LD cycle than under any other non-24 h LD cycles (von Saint Paul and Aschoff, 1978). On the other hand, in another study on the *per* mutants of *D. melanogaster*, lifespan of per^T (τ = 16 h), and per^L (τ = 29 h) males was significantly reduced compared to the wild type flies even under short and long LD cycles (Klarsfeld and Rouyer, 1998). Inferences on adaptive advantage based upon lifespan alone could be misleading as reproductive output in many organisms are known to bear an inverse relationship with lifespan (Bell, 1984; Partridge and Harvey, 1985; Stearns, 1992; Zwaan, 1999; Sheeba et al., 2000). This suggests that multiple fitness components should be taken into account to

assess adaptive significance (Sharma and Joshi, 2002). The most convincing and perhaps the only unequivocal demonstration of circadian resonance came from a study on the cyanobacteria *Synechococcus elongatus* (Ouyang et al., 1990). In this study, the growth rates of various strains of cyanobacteria with different clock periodicities were assayed while competing against each other. Under pure culture conditions in LL, all strains showed similar growth rates, but the results were different when the wild type (τ = 25 h) and two strains with mutations in the *KaiC* gene (τ = 23 h and τ = 30 h) were competed against each other in various combinations under different periodic environments. When two strains were mixed in approximately equal proportions and cultured under LD cycles of 11:11 h and 15:15 h, strains with clock period matching closely those of the LD cycle always out-competed strains with periods far removed from those of the LD cycles (Ouyang et al., 1990). These results were reexamined in strains having mutations on any of the three *Kai* genes (*Kai A*, *KaiB* and *KaiC*). The mutant strains thus obtained displayed circadian periodicities ranging between 22 h to 30 h, and in competition experiments, strains whose periodicity matched those of the LD cycles out-competed others with deviant periodicities. Thus, fitness advantages conferred to cyanobacteria via circadian resonance appear to be independent of the genotype but solely depend upon clock period (Woelfle et al., 2004). These results underscore the role of circadian clocks in fitness of organisms, however, are probably valid only under specific conditions such as periodic environments. Further studies are necessary to apply these results to other organisms or other environmental conditions.

Evidence from studies on organisms living under constant environments

An obvious corollary of circadian resonance hypothesis is that circadian clocks would be less advantageous to organisms living under constant environments such as depth of oceans, underground caves, or any other aperiodic environments (Sharma, 2003a). As a result, organisms living under seemingly constant environments might not have the ability to measure passage of time at least not on circadian time scale. Under constant environment where there is no apparent need to synchronize behavioral and physiological processes with the external environment functional clocks would be expected to confer no obvious adaptive advantage to their owners. In several laboratory evolution studies it has been shown that traits that do not confer any adaptive advantage to organisms are lost from the populations within 100-200 generations due to mutation accumulation and random genetic drift (Mueller, 1987; Service et al., 1988). Further, if the trait in question has some cost associated with it, the loss can be even faster (Rose et al., 1996; Joshi, 1997). Therefore, one would not expect to see rhythmic behaviors in organisms living under constant conditions. Further, even if one does such rhythmic processes may not have the ability to entrain to periodic environmental cycles, since these organisms do not experience any periodic environment and therefore there is no advantage of having entrainment mechanisms. Interestingly reports from studies on organisms living under constant conditions suggest that all the above mentioned possibilities could be true. For example, Amblyopsid fishes living under constant environments did not exhibit a circadian locomotor activity rhythm but showed rhythm in oxygen

consumption which could not be entrained to LD cycles (Poulson and White, 1969). Studies on eyeless crayfish revealed that these organisms either do not show any overt circadian rhythms or if they do, the periodicities were quite different from 24 hours (Blume et al., 1962). On the other hand, studies on the cave dwelling millipede *Blaniulus lichensteini* (Mead and Gilhodes, 1974) and *Glyphiulus cavernicolus* (Koilraj et al., 2000) reported persistence of circadian locomotor activity rhythm. In a separate study, working with four laboratory populations Sheeba and coworkers demonstrated that flies that had never experienced periodic environment for over 600 generations exhibit circadian rhythms of eclosion (Sheeba et al., 1999a), oviposition (Sheeba et al., 2001) and locomotor activity (Sheeba et al., 2002b). Further, the authors argued that these flies possess free-running circadian rhythms because clocks confer 'intrinsic adaptive advantage' (Sharma and Joshi, 2002; Sharma, 2003a). On the other hand, recent experiments on cyanobacteria *Synechococcus elongatus* suggested that having functional clocks may not be beneficial and might even be maladaptive under constant conditions (Woelfle et al., 2004). Arrhythmic mutant strains of cyanobacteria always out-competed the rhythmic wild type strain under constant conditions. The authors speculate that circadian clocks regulate metabolic processes in a manner which is not optimal under constant environment; therefore, functional clocks provide no adaptive advantage under constant environment and may even be detrimental to the organism's well being (Woelfle et al., 2004). These results, thus, contrast earlier predictions on intrinsic adaptive value of circadian clocks (Sharma and Joshi, 2002) and suggest that

circadian clocks may not confer any advantage to organisms inhabiting constant environments.

1.5 Rationale of the present study

The ubiquitous occurrence of circadian clocks across different levels of complexity and organization has been taken to suggest that they confer some adaptive advantage to organisms living on our planet (Sharma, 2003a). Organisms can be thought to gain two types of advantages by having functional circadian clocks - (i) "intrinsic advantage" gained by coordinating various cyclic metabolic processes within the internal milieu and (ii) "extrinsic advantage" accrued by synchronizing various metabolic and behavioral processes with periodic external environment (Sharma, 2003a). Although, there is hardly any empirical support for adaptive significance of circadian clocks, there has been several circumstantial evidence from studies on free-living animals suggesting extrinsic advantage of circadian clocks (Paranjpe and Sharma, 2005). Most studies suggest that synchronizing behavioral rhythms with the external cyclic environment is crucial for organisms living in wild and, therefore, lack of functional timing mechanisms can prove to be fatal (Daan and Tinbergen, 1980; Daan, 1981; DeCoursey et al., 1997). In contrast, under constant environments such as depth of the sea or subterranean caves, where there is no apparent need for organisms to synchronize their behaviors, clocks may not be of any adaptive advantage. The results of a number of studies on organisms living under constant conditions (Blume et al., 1962; Poulson and White, 1969; Mead

and Gilhodes, 1974; Koilraj et al., 2000; Sheeba et al., 1999a; 2001; 2002b) reveal that such organisms may not exhibit circadian rhythms at the first place and if they do its ability to entrain to LD cycles may be absent. This brings us to interesting and yet unanswered set of questions such as: (i) whether organisms living under constant environments have functional circadian clocks, and (ii) if they do, whether such clocks are able to entrain to periodic environments and respond to light stimuli similar to the clocks in organisms inhabiting periodic environments. In my thesis, using four large out bred laboratory populations of *D. melanogaster* reared under constant environment for more than seven hundred generations, I have tried to investigate whether environmental conditions, particularly light conditions, influence clock properties (Chapter 2, 5).

Most studies on adaptive significance of circadian rhythms suffer from one limitation or other. Some lack of population-level replication and others true controls and adequate control on the genetic composition of the populations, which in many ways limit the potential insights gained from such studies (Sharma and Joshi, 2002). Majority of the studies were carried out on mutants and highly inbred lines, which as we now know is likely to yield spurious genetic correlations between fitness components merely due to genetic drift (Mueller and Ayala, 1981). Further, a number of studies used life span as the sole indicator of fitness, though it is well known that a fine balance between various life history traits, resulting from several genetic and environmental constraints, and trade-offs, constitutes fitness of an organism (Loeschke, 1987; Stearns, 1992; Leroi et al., 1994). Moreover, for establishing empirically that circadian clocks have adaptive

value, systematic and rigorous studies need to be done, which should include demonstration that (i) specific environmental regimes relevant to the trait, in this case light regimes, have differential effects on fitness, and (ii) the trait in question evolves differentially under those environmental conditions (Sharma and Joshi, 2002). The first criterion mentioned above describes phenotypic effects of environment on the traits in question, which might help in estimating proximal adaptive advantage of the trait, while the second criterion describes long term effects, which might help in understanding evolutionary adaptive advantage of the trait. I therefore decided to investigate the effect of light conditions on a number of key clock parameters and related life history traits on four replicate out bred populations of *D. melanogaster* (LL1, LL2, LL3 and LL4) that had been reared under constant light, temperature and humidity conditions on a 21-day discrete generation cycle for more than 700 generations (Chapters 2, 3, 4).

The next step towards establishing the adaptive value for circadian clocks is to demonstrate whether circadian clocks and associated features evolve differentially under various environments, in this case light regimes. We have used populations of *D. melanogaster* reared for more than one hundred generations under two constant environments, LL and DD, and one periodic environment LD cycles of 12:12 h to study whether the light response characteristics of circadian clocks evolve under different light regimes (chapter 5).

Chapter 2

Studies on circadian parameters of the baseline populations

2.1 Introduction to the baseline populations

This section describes the ancestry and maintenance of the four baseline populations of *Drosophila melanogaster* used in our study. The baseline populations also served as ancestral populations for new sets of selection lines which were reared under different environmental conditions. The baseline populations (LL1..4) were derived from four separate laboratory populations (JB1..4) (described earlier in Sheeba et al., 1998; Sheeba, 2001). The four baseline populations (henceforth will be referred as LL populations) were maintained in our laboratory at constant temperature (24 ± 1^0C), constant humidity (~80-90%) and constant light (~100 lux intensity) on a 21-day discrete generation cycle. The populations typically consisted of ~1500 adults (roughly equal number of males and females) in a plexiglass cage (25 x 20 x 15 cm^3). For starting a new generation, the adult flies were provided with banana-jaggery food supplemented with live yeast paste for 2 days, after which they were allowed to lay eggs on a Petri plate with fresh banana food medium for about 14 hours. From these pertri plates, approximately 60-80 eggs were collected into glass vials (9 cm height x 2.4 cm diameter) containing ~6ml banana food in which larvae then developed into adults. Forty such vials were used for each population. Adult flies emerging from these vials were transferred to plexiglass cages on the 12^{th} day after egg-collection, which formed the breeding population for the next generation. For starting a new generation next set of eggs were collected after 21 days from the previous egg collection date and adults were discarded, thus maintaining a 21 day discrete generation cycle.

2.2 Background

As describe in the earlier section, LL populations used in this study were maintained under constant environment for several hundred generations. Under constant environments circadian clocks may not be of any use, and therefore one would not expect to see circadian rhythmicity in the LL populations. In contrast to such expectations, Sheeba and coworkers demonstrated that the LL flies do exhibit circadian rhythms of eclosion (Sheeba et al., 1999a), oviposition (Sheeba et al., 2001) and locomotor activity (Sheeba et al., 2002b) even after staying under constant environment for six hundred generations. Persistence of circadian rhythmicity does not guarantee that these populations will be able to synchronize to periodic environments, particularly those that have non-24 h periodicities. Sheeba and coworkers (1999a) had demonstrated that the LL populations were able to entrain their eclosion rhythms to LD cycles of 12:12 h. This is not entirely surprising as the τ of eclosion and activity/rest rhythms were close to 24 h, and entrainment of such rhythms could have been achieved as a result of weak sensitivity to light. Therefore, it remains to be seen whether LL populations have the ability to entrain to non-24 h LD cycles, as well as their other wild type counter parts do.

In this chapter, I have discussed the results of studies on the free-running and entrainment properties of circadian clocks of the LL flies by assaying three different behaviors—eclosion (act of adult emergence from the puparium), activity/rest (or locomotor activity) and egg-laying (or oviposition) rhythms under two constant conditions such as constant light (LL) and constant darkness (DD)

and five periodic conditions such as: light/ dark (LD) cycles of 9:9 h (*T18*), 10:10 h (*T20*), 12:12 h (*T24*), 14:14 h (*T28*) and 15:15 h (*T30*). In the following sections of this chapter I have discussed the clock properties of the baseline populations (LL1..LL4).

2.3 Properties of free-running and entrained eclosion rhythm

As eclosion occurs only once in the lifetime of an individual insect, rhythmic eclosion would require synchronized emergence of several adults from pupal case, hence this rhythm can be studied only at the population level. Earlier experiments on *D. pseudoobscura* and *D. melanogaster* populations revealed that eclosion rhythm is not just the consequence of completion of certain developmental stages but is controlled by endogenous circadian oscillators (Skopik & Pittendrigh, 1967). Interestingly eclosion rhythms can be phase shifted in a predictable manner by exposing the flies to light pulses during pre-adult stages (Pittendrigh and Skopik, 1970). In a series of experiments done on *D. pseudoobscura* populations, it was demonstrated that the phase relationship (ψ; defined as the time interval between the peak of eclosion and "lights-on") of the eclosion rhythm under LD cycles depends upon (i) the τ of the rhythm, (ii) periodicity of the LD cycles (*T*) and (iii) the PRC (Pittendrigh, 1981). The authors speculated that *Drosophila* brain is the locus of the driving oscillations (Pittendrigh and Skopik, 1970). Recent studies on *D. melanogaster* have demonstrated that the prothoracic gland (PG), a part of an endocrine structure known as ring gland in *Drosophila*, contains functional circadian clocks that time

eclosion, and that these clocks along with the ones located in ventral-lateral neurons (LNvs) are necessary for the overt expression of circadian rhythm of eclosion (Myers et al., 2003).

In an earlier study (Sheeba et al., 1999a) it was shown that the LL populations exhibit rhythmicity in eclosion with circadian periodicities that could be entrained to 24 h LD cycles. It would therefore be interesting to further investigate (i) whether the LL populations have the ability to entrain to non-24 h LD cycles, (ii) whether the phase of eclosion rhythm changes systematically under different LD cycles, similar to those reported for other wild type flies, and (iii) the range of LD cycles to which the eclosion rhythm show stable entrainment.

2.3a Materials and methods

Eclosion rhythm was assayed in all four baseline populations by collecting eggs in a short window (6 h) of time and dispensing them at high densities (~350 eggs per vial) into vials containing ~6 ml of food medium. At this density, the pre-adult development is staggered and we could get good number of flies eclosing for more than 10 cycles. Ten vials per population were introduced into each of the following regimes: LL, DD, *T18*, *T20*, *T24*, *T28* and *T30*. The light intensity used during the light phase of the LD cycle was approximately 100 lux. Red light of wavelength greater than 650 nm was used for observations and handling flies in darkness. Adult flies were collected every 2 h and their number and sex was recorded. This was continued for about 10 consecutive days. The time series data obtained for eclosion rhythm from each vial was subjected to Fourier

spectral analysis using STATISTICA™ (Statsoft, 1995). Statistical significance of observed peaks in the periodogram for eclosion was tested using modifications suggested by Siegel (1980). We then calculated the fraction of vials (out of10) in each population exhibiting rhythmic patterns in their eclosion under five different light regimes, and the percentage of vials in which eclosion rhythm entrained to the LD cycles. The ψ of the eclosion rhythm under LD cycles was estimated as the average time interval between peak of eclosion and 'lights-on' over 10 consecutive days. A mixed model ANOVA was carried out on the mean ψ of the eclosion rhythm in five periodic light regimes with population as random factor and light regime and sex as fixed factors, crossed with populations to understand whether ψ of the eclosion is different under five different LD cycles.

2.3b Results

About 90% vials from each of the four populations showed circadian rhythm in eclosion under DD conditions, while eclosion in only 18% vials followed circadian patterns under LL regime. The percentage of flies showing circadian rhythm of eclosion under DD was similar for the four populations. The mean τ of eclosion rhythm under DD, averaged across four independent populations was 22.85 ± 1.71 h (mean ± 95% confidence interval, CI). Representative eclosion patterns under two constant regimes (LL and DD) are shown in Figure 1. The frequency distribution of τ values under DD regime is shown in the Figure 2a.

Eclosion rhythm of most of the vials entrained to five periodic light regimes (*T18*, *T20*, *T24*, *T28* and *T30*). The representative patterns of eclosion rhythm

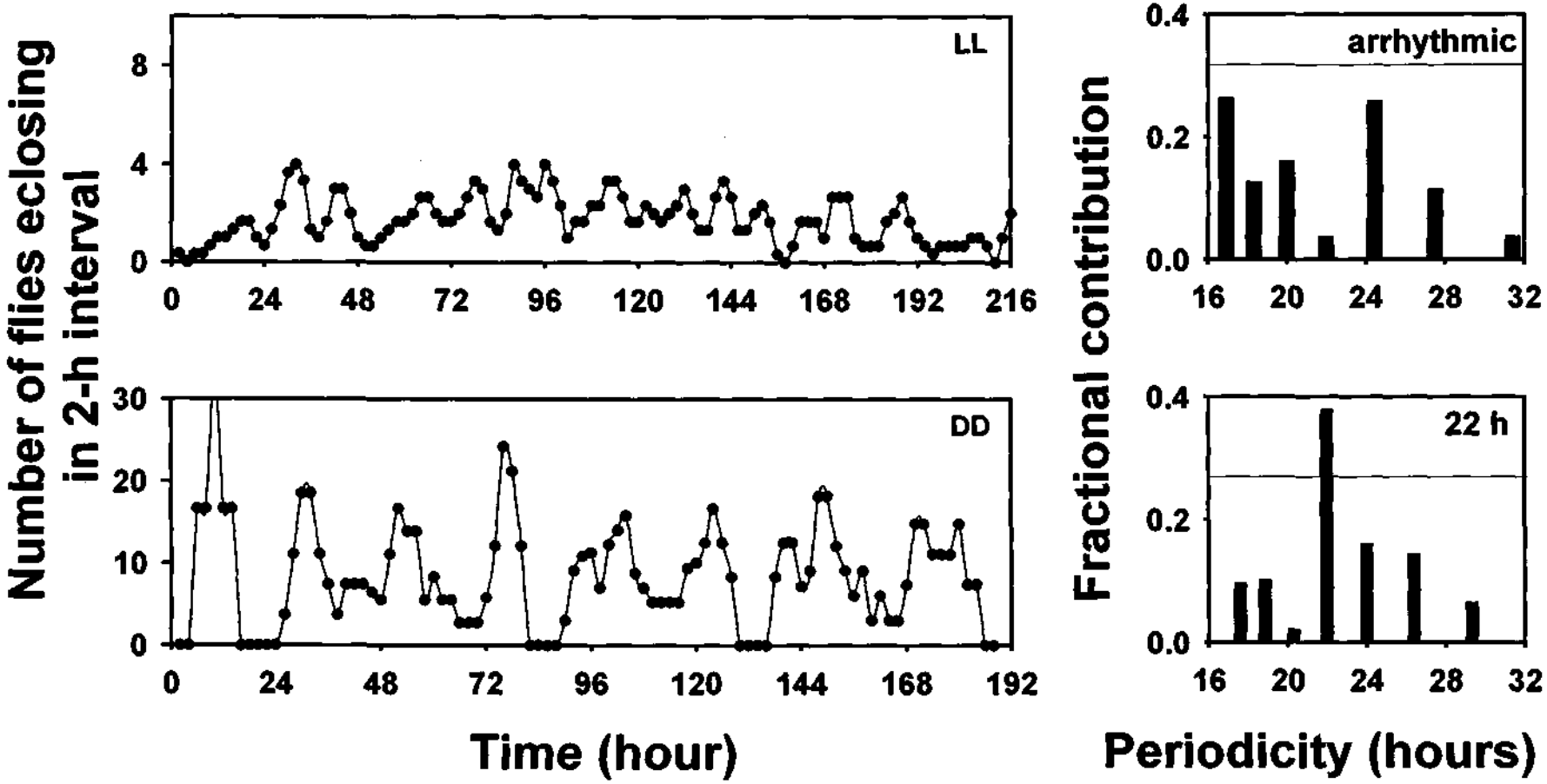

Figure 1: Eclosion was arrhythmic in LL but rhythmic under DD regime. For left hand panels, time in hours is plotted along x-axis and number of flies eclosing in 2 h intervals along y-axis. Upper left panel shows representative pattern of eclosion under LL conditions. Corresponding periodogram on the right shows that no periodicity in the circadian range made significant contribution to the periodgram. Lower left panel shows representative eclosion pattern under DD conditions. Corresponding periodogram shows that eclosion was rhythmic as 22 h period made significant contribution to the periodogram.

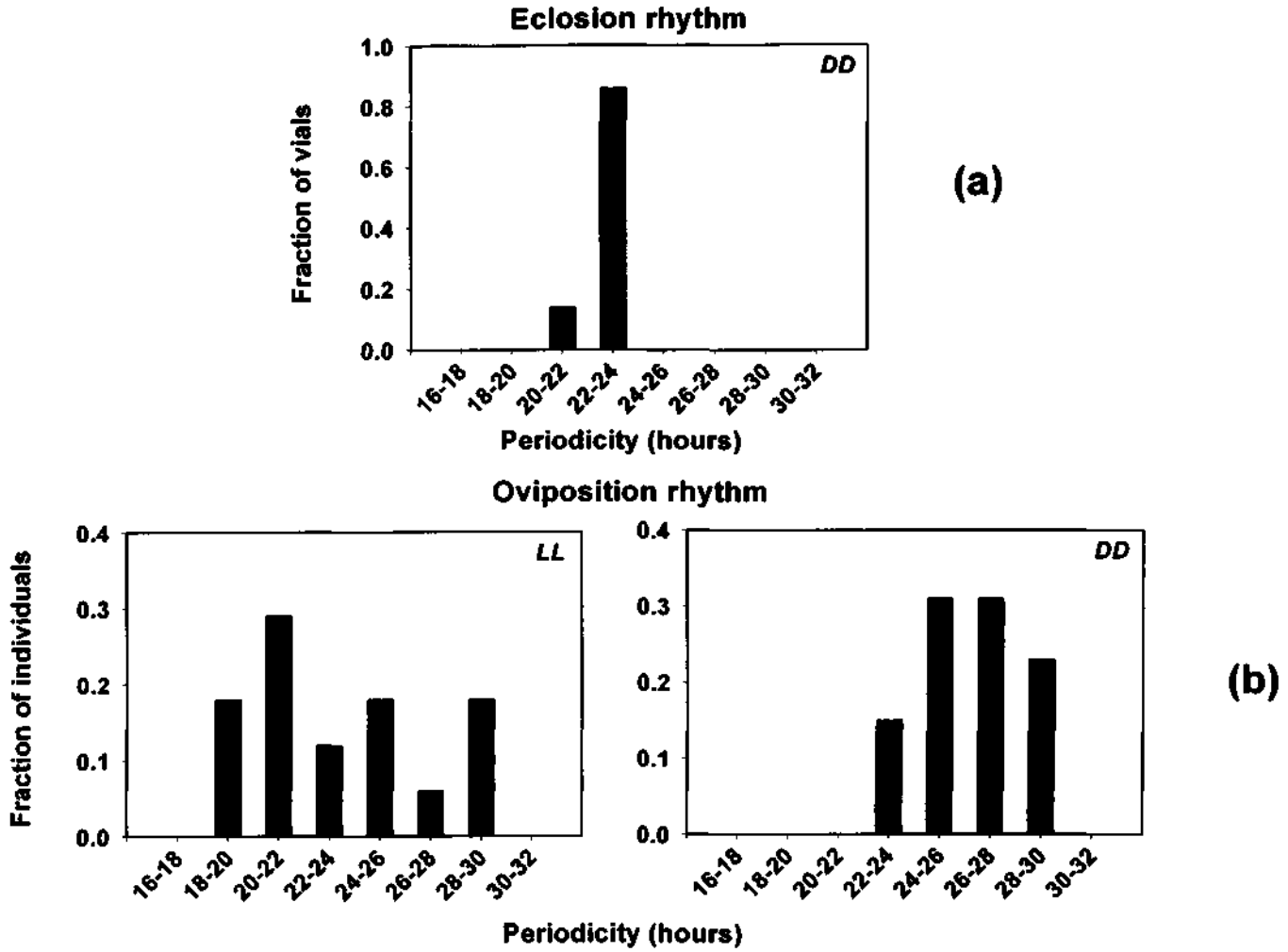

Figure 2:The upper panel (a) represents the frequency distributions of τ values of eclosion rhythm under DD regime. The y-axis indicates the fraction of vials (out of 10) showing circadian periodicity and the x-axis indicates the range of periodicities 16 h to 32 h. The lower panel (b) represents the frequency distributions of τ of oviposition rhythm under LL and DD regime. The y-axis indicates the fraction of individuals (out of 20) having a circadian periodicity and x-axis indicates the range of periodicities present in the data.

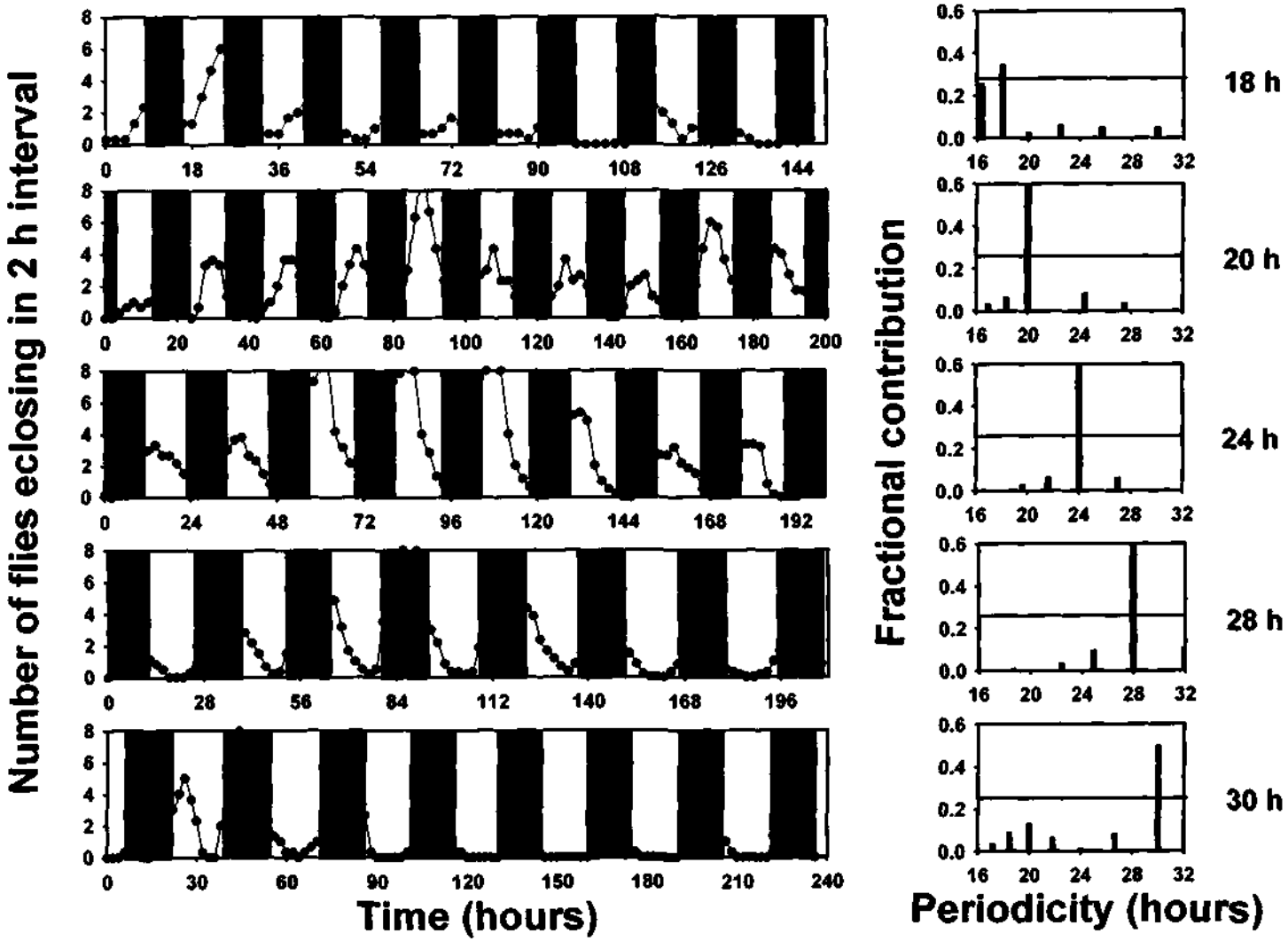

Figure 3: Eclosion rhythm entrained to LD cycles ranging from *T18* to *T30*. The left panels show representative eclosion patterns under different *T* cycles. Time in hours is plotted along x-axis while number of flies eclosing in 2 h intervals is plotted along y-axis. Right side panels show periodograms showing fractional contribution of different periodicities in the data.

Table 1: Mean phase relationship (ψ) of the eclosion peak relative to "lights-on" of the LL populations of *D. melanogaster* populations under different *T* cycles.

Light regime	Population	Phase relationship for males (hours)	Phase relationship for females (hours)
T18	**LL1**	**-7. 00**	**-5. 86**
	LL2	**-6. 11**	**-3. 00**
	LL3	**-7. 40**	**-4. 30**
	LL4	**-6. 50**	**-1. 60**
T20	**LL1**	**-8. 51**	**-7. 94**
	LL2	**-4. 38**	**-2. 57**
	LL3	**-5. 02**	**-3. 59**
	LL4	**-5. 60**	**-5. 40**
T24	**LL1**	**-3. 00**	**-3. 10**
	LL2	**-2. 60**	**-2. 50**
	LL3	**-2. 70**	**-3. 10**
	LL4	**-2. 80**	**-2. 80**
T28	**LL1**	**3. 99**	**3. 41**
	LL2	**3. 12**	**3. 22**
	LL3	**3. 32**	**3. 71**
	LL4	**2. 73**	**2. 86**
T30	**LL1**	**8. 40**	**8. 50**
	LL2	**8. 50**	**7. 50**
	LL3	**7. 10**	**8. 00**
	LL4	**6. 33**	**7. 40**

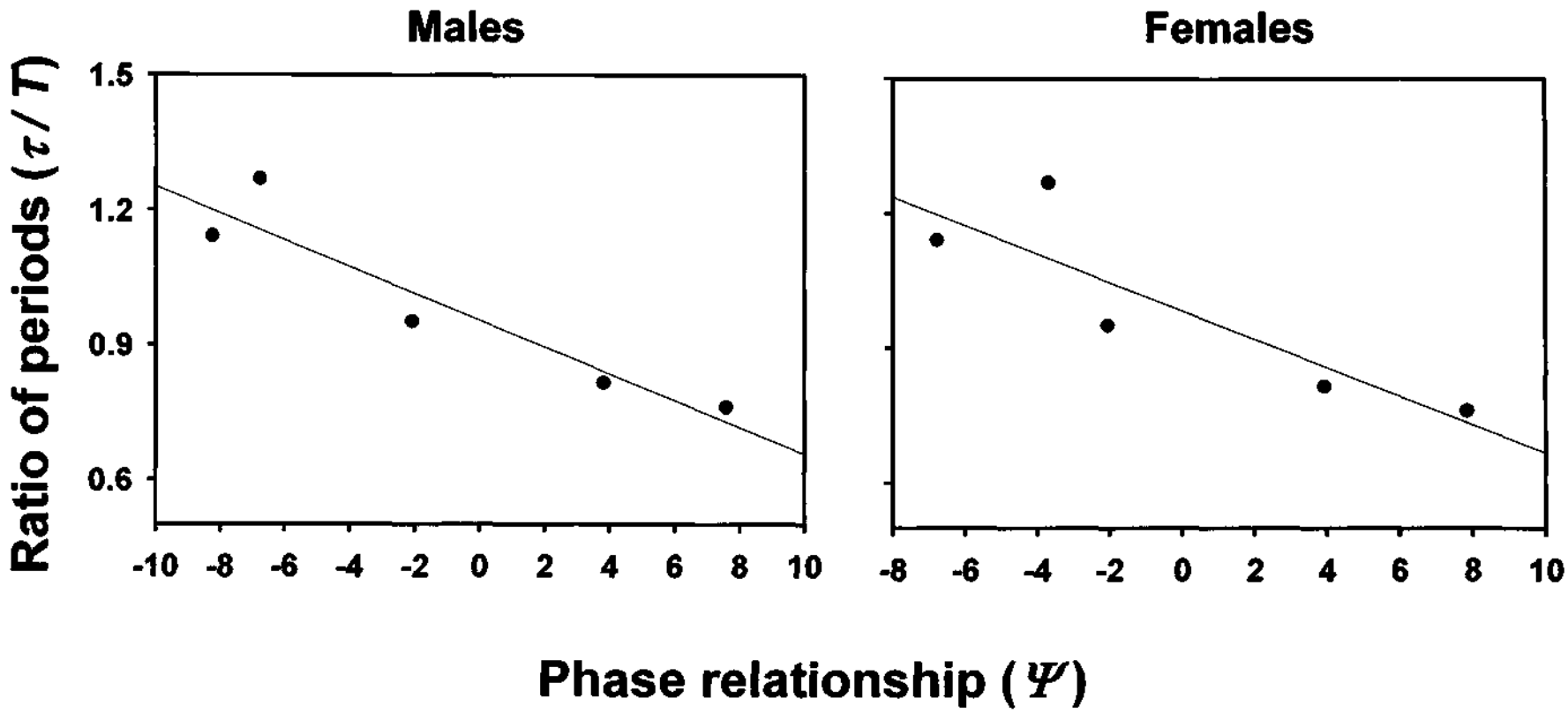

Figure 4: The ψ of eclosion rhythm changed systematically with increase in the length of LD cycles. The ψ values under different *T* cycles ranging from *T18* to *T30* are plotted along x-axis and the ratio of τ of eclosion rhythm to period of *T* cycle is plotted along y-axis. More negative ψ values were seen under shorter LD cycles and positive ψ values were observed under longer LD cycles.

under five periodic light regimes are shown in Figure 3. The results of ANOVA on the ψ values revealed significant main effect of light regime ($F_{4,30}$= 392.79, p < 0.001) and sex ($F_{1,30}$= 11.87, p < 0.05). The light regime x sex interaction was also significant ($F_{4,30}$= 4.13, p < 0.05), i.e. males had significantly smaller ψ than females in *T18* and *T20* regimes (Table 1). However, under *T24*, *T28* and *T30* regimes the ψ of eclosion rhythm of males and females did not differ significantly. Post-hoc multiple comparison using 95% CI around the mean revealed that the ψ of eclosion rhythm under *T20* (-7.52 h), *T18* (-5.22 h), *T24* (-2.06 h), *T28* (+3.88 h) and *T30* (+7.72 h) regimes were significantly different among each other (p < 0.001). The peak of eclosion occurred relatively earlier as the period of LD cycles increased from *T18* to *T30*. Under different *T* cycles, the ratio and ψ of the eclosion rhythm showed a systematic functional relationship, with more negative ψ values under shorter *T* cycles, and positive ψ values under longer *T* cycles (Figure 4). The nature of the relationship between (τ /*T*) and ψ was similar for both males and females.

2.4 Properties of free-running and entrained locomotor activity rhythm

The locomotor activity (activity/rest) behaviour is one of the best studied circadian rhythms in *D. melanogaster*. Day active organisms such as fruit flies show two distinct peaks of activity coinciding "dawn" and "dusk" with minimum or no activity during the dark hours, with most flies showing clear anticipation of dawn and dusk (Helfrich-Förster, 2000). Extensive studies over past few decades

have demonstrated that two sets of four-five neurons known as the ventro lateral neurons (s-LNv) that express a neurotransmitter - pigment dispersing factor (PDF) are considered to be the 'core circadian pacemakers' in the fruit fly (Helfrich-Förster, 2005). Genetic ablation of these neurons resulted in the loss of locomotor activity rhythm under DD (Renn et al., 1999). Although LNvs have been suggested to be necessary and sufficient for the persistence of locomotor activity rhythm in *Drosophila* (Frisch et al., 1994), dorsal lateral neurons (LNds), dorsal neurons (DN1s, DN2s, DN3s), and a few glial cells are also believed to contribute to the overt expression of the rhythm (Helfrich-Förster, 2005). The neurotransmitter PDF secreted by the LNvs are believed to coordinate the phase and amplitude of the oscillations in the pacemaker cells underlying locomotor activity rhythm in *Drosophila* (Lin et al., 2004).

In an earlier study Sheeba and cowrkers (2002b) have shown that the LL populations exhibit circadian locomotor activity rhythm which is entrainable to a 24 h LD cycle. In the present study we have assayed the locomotor activity of virgin flies from the four LL populations under two constant conditions (LL and DD), and five periodic regimes ranging from *T18* to *T30*, in order to investigate the free-running and entrainment characteristics of the circadian locomotor activity clocks in the LL flies.

2.4a Materials and methods

The locomotor activity behaviour of individual flies from the four LL populations was recorded under LL, DD, *T18*, *T20*, *T24*, *T28* and *T30* regimes using

indigenously developed automated activity monitoring system. The activity monitoring system is a simple computer-aided device which uses two pairs of infrared (IR) emitters and sensors that detect the movements of flies in a narrow glass tube (0.6 cm inner diameter x 4 cm height) (Sharma, 2003c). An interruption of the IR beam creates an electrical signal which is amplified using an opto-amplifier unit and sent to an activity monitor which in turn sends the signals to a computer which stores the data that can be plotted as interruptions during five minute bins, in a chronological order, to give daily plot of activity called 'actogram' (Sharma, 2003c).

The period of the locomotor activity rhythm was calculated using a regression line drawn through the daily offsets of locomotor activity rhythm. The offset of activity was taken as phase reference point of the rhythm because generally in our flies, offsets show less cycle-to-cycle variability compared to the onsets (*Dhanashree Paranjpe, personal observation*). Data for a minimum of 7 cycles were used for period estimation. If period of the rhythm matched those of the LD cycles, the locomotor activity rhythm was considered to be under an entrained state. The ψ of the locomotor activity rhythm was defined as the time interval between lights-on and the onset of activity or lights-off and the offset of activity, under a given LD cycle. The duration of activity was calculated as the time interval between onset and offset of locomotor activity in each cycle.

2.4b Results

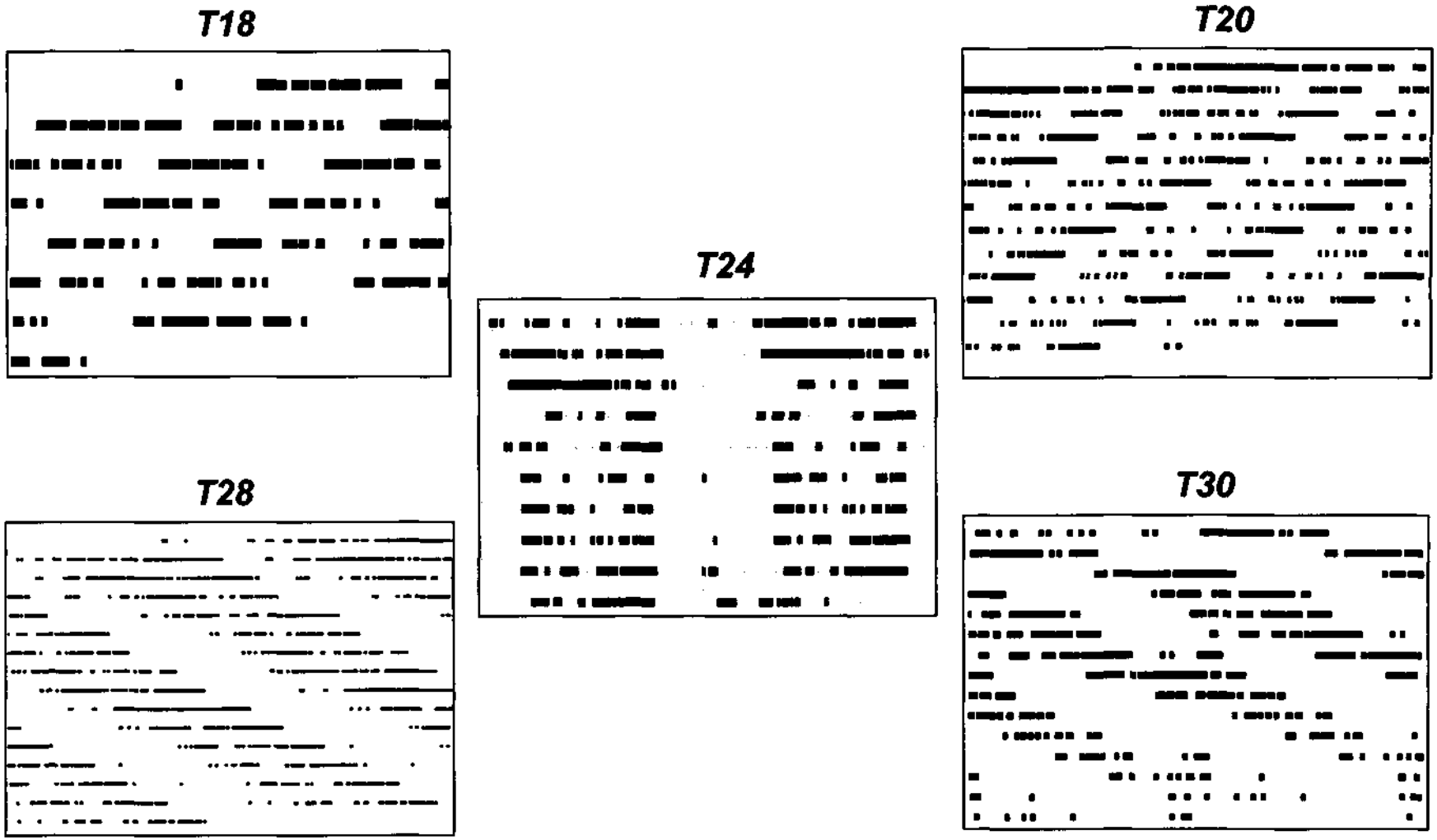

Figure 5: Activity-rest rhythm of LL populations of *D. melanogaster* entrained to a wide range of LD cycles. The panels represent the locomotor activity patterns under different LD cycles. Thick horizontal bars represent activity while empty spaces represent rest. Activity-rest data of subsequent days is arranged one below another chronologically to facilitate easier visualization of activity patterns.

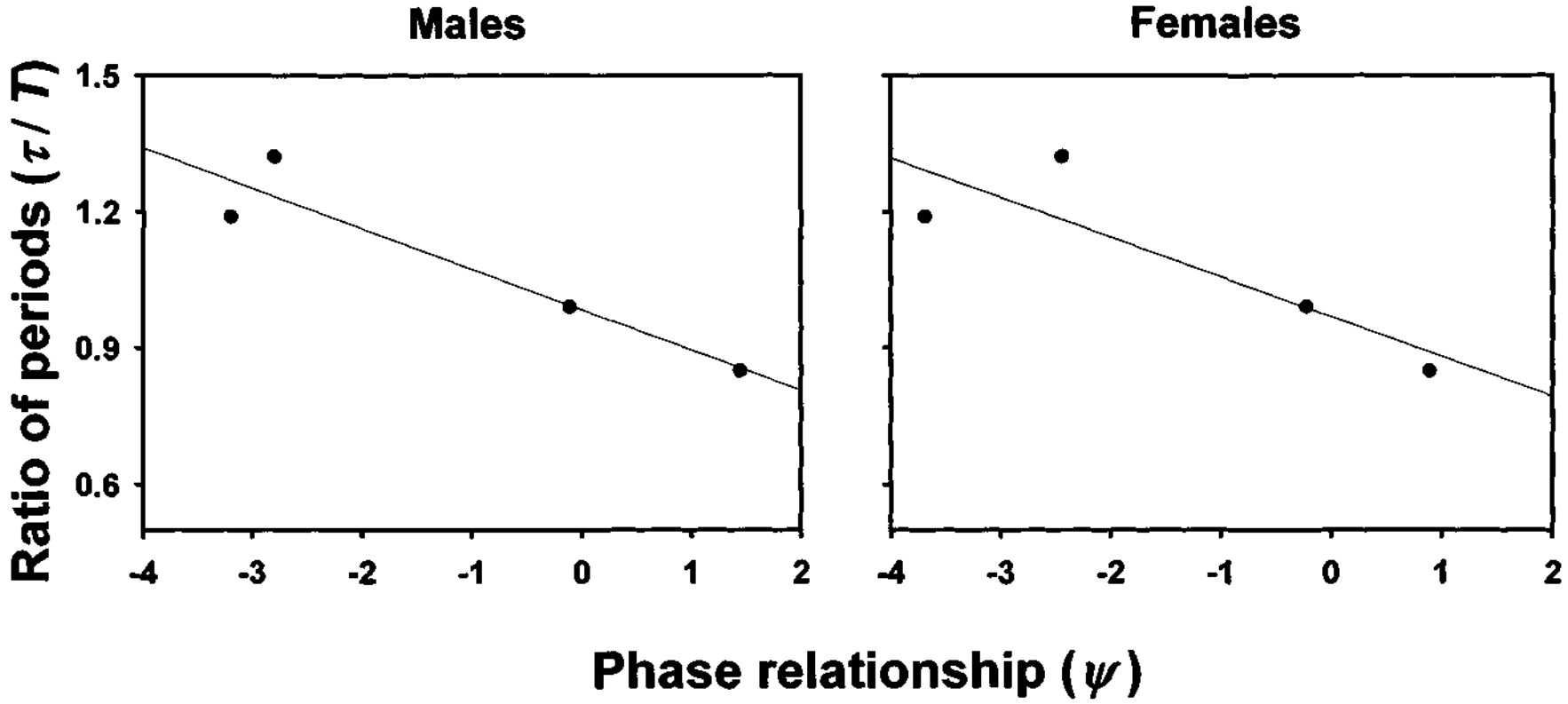

Figure 6: The ψ of activity-rest rhythm changed systematically with increase in the length of LD cycles. The ψ values under different *T* cycles are plotted along x-axis and the ratio of τ of activity-rest rhythm to period of LD cycles (*T*) is plotted along y-axis. More negative ψ values were seen under shorter LD cycles and positive ψ values were observed under longer LD cycles.

The locomotor activity was arrhythmic under LL conditions (light intensities ~10 lux), while under DD it free-ran with a mean τ of 23.79 ± 0.24 h (mean ± 95% CI). The average duration of activity under DD was 13.65 h. The locomotor activity rhythm entrained stably to *T18*, *T20*, *T24* and *T28* regimes (Figure 5), and the average duration of activity was 11.35 h, 13.35 h, 13.97 h and 16.88 h, respectively. The locomotor activity rhythm of only about 20% flies entrained to the *T30* regime, while about 70-85% flies showed entrainment under other *T* cycles. Further, the ψ of the offset of activity showed a systematic change with an increase in the period of the *T* cycles. The offsets of locomotor activity in both males and females occurred relatively later under *T18* and *T20* compared to the longer *T* cycles (Figure 6).

2.5 Properties of free-running and entrained egg-laying rhythm

Oviposition or egg-laying is believed to be the outcome of two separate physiological processes - vitellogenesis and egg retention (Allemand, 1976a; Allemand, 1976b). Rhythmic egg-laying involves a complex series of events leading to the deposition of eggs by the female (Allemand, 1976b). This behaviour is rhythmically exhibited by several species of insects including *D. melanogaster* (Allemand, 1976a; Allemand, 1976b; Gruwez et al., 1972; Rensing and Hardeland, 1967; David and Fouillet, 1973; Allemand, 1977; Sheeba et al., 2001). Earlier studies on *D. melanogaster* females demonstrated that the egg-laying rhythm persists in individual females sampled from one of the LL populations (Sheeba et al., 2001). The τ of the egg-laying rhythm under DD

ranged from 12.4 h to 25.84 h. Interestingly the rhythm persisted even under LL, whereas the locomotor activity and eclosion rhythms were abolished. The egg-laying rhythm of the LL flies entrained to LD cycles of 12:12 h, with the peak of egg-laying rhythm coinciding lights-off (Sheeba et al., 2001). Furthermore, the transcripts of *per* (Hardin, 1994), and the levels of PER and TIM proteins do not oscillate in the ovaries of *Drosophila* females (Saez and Young, 1988; Beaver et al., 2003); constitutive high level of PER and TIM proteins were found in the follicle cells of developing oocytes throughout the day. Previous studies have also demonstrated that PER and TIM interact in these follicle cells but do not translocate into the nucleus, thus leaving clock mechanisms truncated (Beaver et al., 2003). Thus, the mechanism(s) underlying egg-laying rhythm in *Drosophila* appear to be different than those regulating locomotor activity and eclosion rhythms.

Majority of earlier studies were confined to assaying egg-laying behavior under LD 12:12 h, but the behaviour of egg-laying rhythm under non-24 h LD cycles has not been reported. Therefore, we decided to study the egg-laying rhythm of the LL populations focusing mainly on free-running and the ability to entrain to non-24 h LD cycles.

2.5a Materials and methods

Oviposition rhythm of females from the LL1..LL4 populations was assayed by collecting flies that emerged from low density cultures derived from the running stocks and by introducing male-female pairs into vials containing ~3 ml food

medium. Twenty such pairs were set up in each of the five light regimes (LL, DD, *T20*, *T24*, and *T28*). The flies were transferred to fresh food medium every 4 h and the number of eggs laid in each vial over the preceding 4 h period was counted. This was continued for ten consecutive cycles under each light regime. In case of death or escape of males, replacement was made from flies from a mixed sex cohort maintained as backups in the respective light regimes. The data from females living for the full ten days were used for the analysis, yielding a final sample size of 18 to 19 females in each light regime.

The time series data obtained for the egg-laying behaviour was subjected to Fourier spectral analysis using STATISTICA™ (Statsoft, 1995). Statistical significance of observed peaks in the periodogram was tested using modification suggested by Siegel (1980). We estimated the percentage of females (out of 20) that showed rhythmic egg-laying behaviour under LL, DD, *T20*, *T24* and *T28*, as well as the percentage of rhythmic females whose egg-laying rhythm entrained to the imposed LD cycles.

2.5c Results

The egg-laying rhythm had a mean (± 95% CI) τ of 25.42 ± 0.98 h under DD, and 24.63 ± 0.68 h under LL. The τ of the egg-laying rhythm under DD did not differ significantly compared to those under LL regime ($p > 0.05$). But the τ of the egg-laying rhythm under DD regime was significantly greater than the τ of the eclosion rhythm ($p < 0.001$) and locomotor activity ($p < 0.001$) rhythms under DD. In LL and DD regimes, the percentage of flies that exhibited circadian egg-laying

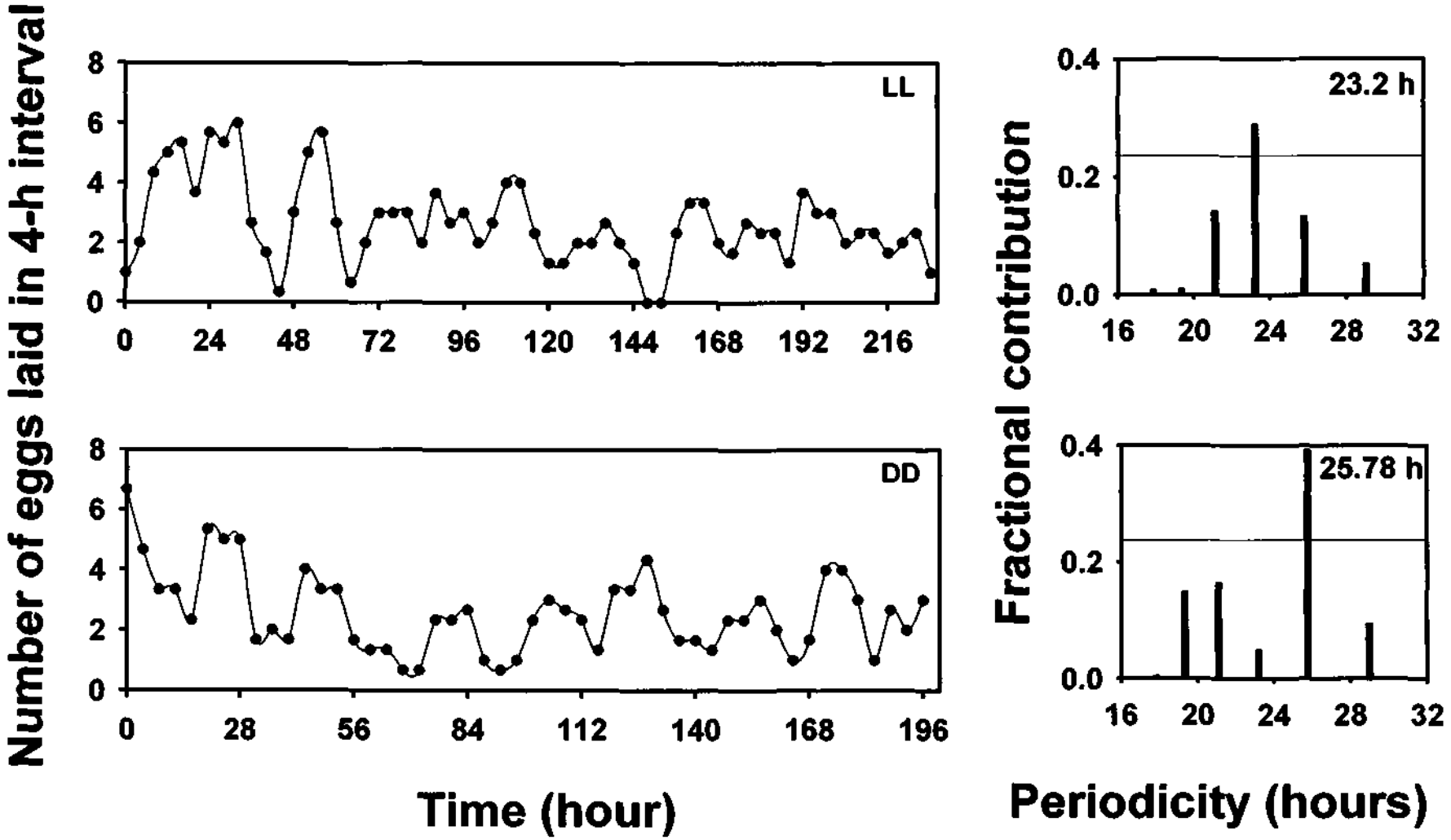

Figure 7: Oviposition was rhythmic under LL and DD regimes. For left panels, time in hours is plotted along x-axis and number of eggs laid in 4 h intervals along y-axis. Upper left panel shows representative pattern of oviposition under LL conditions. Corresponding periodogram on left side shows that oviposition was rhythmic and a period of 23.2 h made significant contribution in the time series data. Lower left panel shows representative oviposition pattern DD conditions. Corresponding periodogram shows that oviposition was rhythmic and a period of 25.78 h made significant contribution to the periodogram.

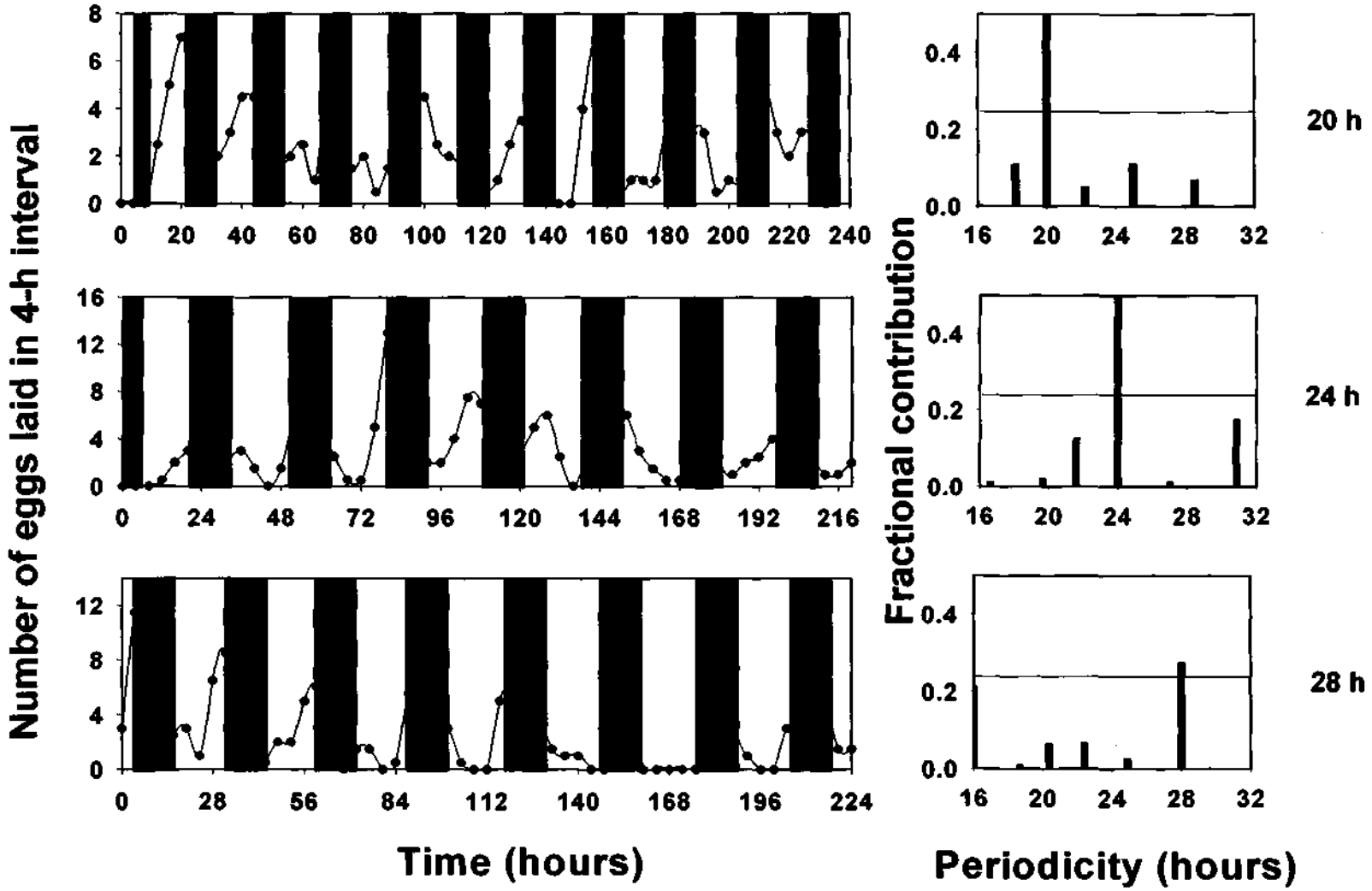

Figure 8: Oviposition rhythm of females from LL populations entrained to a range of LD cycles between *T20* to *T28*. The left side panels show representative patterns of oviposition under different *T* cycles, wherein, time in hours is plotted along x-axis while number of eggs laid in 4 h intervals are plotted along Y-axis. Right side panels show periodograms with fractional contribution of different periodicities in the data.

rhythm was 77.12 ± 8.12 (mean ± 95% CI) and 76.77 ± 7.04, respectively. The frequency distributions of τ values under LL and DD are shown in the Figure 2b. Representative patterns of egg-laying rhythm under LL and DD regimes are shown in the Figure 7. The number of flies that exhibited rhythmic egg-laying, and among them, those in which the egg-laying rhythm entrained to the imposed LD cycles, varied substantially among the light regimes. The peak of the egg-laying rhythm invariably occurred close to lights-off under all the light regimes. In *T20* regime, 90.28 ± 12.07 percent females showed rhythmic egg-laying, out of which 22.74 ± 3.66 percent flies entrained to the imposed periodicity of 20 h (Figure 2b, 8). In *T24* regime, 84.61 ± 8.75 percent females exhibited rhythmic oviposition, out of the rhythmic females 35.26 ± 11.92 percent entrained to the imposed LD cycles (Figure 2b, 8). In *T28* regime, egg-laying of 89.47 ± 14.59 percent females was rhythmic, out of which 57.75 ± 28.04 percent showed entrainment to 28 h LD cycles (Figure 2b, 8). This suggests that the oviposition rhythm of greater percentage of individuals entrain to *T28* regime, indicating that the limits of entrainment for oviposition rhythm are closer to 28 h as opposed to eclosion or locomotor activity rhythms.

2.6 Conclusions

The eclosion in majority of the vials followed circadian patterns under DD regime with a mean τ of 22.85 ± 1.71 h (mean ± 95%CI). The locomotor activity rhythm of the LL flies free-ran with an average τ of 23.79 ± 0.24 h (mean ± 95%CI) under DD, while the τ of the egg-laying rhythm was 25.42 ± 0.98 h (mean ± 95%CI).

This suggests that circadian rhythms of eclosion, locomotor activity and egg-laying persist in the LL flies that were maintained for several hundred generations under constant environment, albeit with different periodicities.

Eclosion rhythm of all the vials from all the four LL populations entrained to a wide range of LD cycles (*T18*, *T20*, *T24*, *T28* and *T30*) (Figure 3), whereas the locomotor activity rhythm of about 70-85% of flies entrained to LD cycles with periodicities ranging between 18 and 28 h, and only about 20% flies showed entrainment to *T30* regime. Egg-laying rhythm of about 23%, 35% and 58% females entrained to *T20*, T24 and T28 regimes, respectively. The fact that oviposition rhythm of only 23% females could entrain to *T20* regime suggest that *T20* falls close to the limits of entrainment of egg-laying rhythm. Similarly, given that locomotor activity rhythm of only 20% flies entrained to *T30* regime, suggests that *T30* falls close to the limits of entrainment of locomotor activity rhythm. However, the eclosion rhythm of the LL populations did not reach its limits of entrainment as it entrained to a wide range of LD cycles (from *T18* to *T30*). Therefore, the results of our experiments clearly suggest that the LL flies possess functional clocks that can generate robust circadian rhythms of eclosion, locomotor activity and egg-laying, which has photoentrainment abilities similar to their wild type counterparts.

Earlier studies on the LL populations, about 100 generations ago, had shown that the eclosion, oviposition and locomotor activity rhythms free-ran under DD conditions and entrained to *T24* regime (Sheeba et al., 1999a, 2001, 2002b). We show that the LL populations entrain even to non-24 h LD cycles

such as *T18*, *T20*, *T28* and *T30*. Moreover, as the period of the LD cycle increased from *T18* to *T30*, the peak of eclosion and the offset of activity shifted earlier. Thus, the ψ of peak of eclosion and offset of activity showed a functional relationship with the ratio τ /*T*, similar to those reported for three mammalian species - *P. maniculatus, P. leucopus* and *M. auratus* (Pittendrigh and Daan, 1976) and for the fruit flies *D. pseudoobscura* (Pittendrigh, 1993). Such systematic changes in the ψ of circadian rhythms under a range of LD cycles suggest that the LL populations can adjust to a range of day lengths while maintaining a specific timing for their behaviors. The functional relationship between τ / *T* and ψ is believed to compensate for the day-to-day variations in τ as well as predictable seasonal changes, and thereby, help organisms in maintaining a stable ψ with respect to the periodic environment (Pittendrigh, 1981). In other words, it is believed that systematic changes in ψ as function of τ /*T* under different LD cycles allow organisms to maintain an appropriate 'temporal niche' in their natural environments.

Our results demonstrate that the LL populations possess free-running circadian clocks and also have the ability to entrain its rhythmic behaviours to a wide range of LD cycles maintaining a stable ψ, which suggests that the LL flies have circadian clocks similar to other wild type flies. This leads us to an intriguing question as to why circadian clocks and its entrainment mechanisms have been preserved in the LL populations that have not been exposed to periodic conditions for more than 700 generations. One can argue that perhaps 700 generations is not long enough time for a trait such as circadian rhythms to

disappear. However, as mentioned earlier, in several laboratory evolution studies it has been shown that traits that do not confer any adaptive advantage to organisms are lost from the populations due to mutation accumulation and random genetic drift within 100-200 generations (Mueller, 1987; Service et al., 1988). Further, if the trait in question has some cost to it, the loss can be even faster (Rose et al., 1996; Joshi, 1997). Therefore, the possibility that the clock mechanisms have been retained in the LL populations neutrally for 700 generations seems to be unlikely. This suggests that circadian clocks provide some intrinsic adaptive value to organisms living under constant environments. Recent evidences in *Drosophila* have shown that the molecular feedback loops involving mRNA and protein products of genes like *period* (*per*), *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *doubletime* (*dbt*), *cryptochrome* (*cry*), *slimb, vrille, shaggy* and *pdp1* form the core mechanism of circadian clocks (Glossop et al., 2003; Stanewsky, 2003; Williams & Sehgal, 2001). Many of these clock genes have also been shown to have more than one role to play in clock mechanisms and in different metabolic processes that have nothing to do with the clock's rhythm generating machinery (Sokolowski, 2001); e.g. *Vrille* (*vri*) gene that encodes a repressor of *Clock* transcription. The *cry* gene is also a target for VRI repression and homozygous loss-of-function mutants of *vri* are found to be lethal (Glossop et al., 2003), suggesting a broader pleiotropic role of *vri* in the cellular metabolism. This however does not explain why the LL populations display photoentrainment ability as good as other wild type flies. We speculate that the photoentrainment mechanisms of circadian clocks are an integral part of the

clock's pacemaking process and therefore are as indispensable as the circadian clocks themselves (Paranjpe et al., 2003). Indeed, CRY in *Drosophila* is known to act as circadian photopigment, as well as a key component of the circadian pacemaking mechanisms (Stanewsky et al., 1998; Emery et al., 2000, Krishnan et al., 2001), thus suggesting that entrainment mechanisms could be an integral part of circadian clockwork. Therefore, the persistence of circadian clocks and the ability to entrain to a wide range of LD cycles, in the LL populations could be a consequence of maintaining metabolic processes that are not directly related to circadian clock mechanisms, and the ability to entrain to a wide range of LD cycles could to be an indirect consequence of maintaining the pleiotropic genes in the populations.

Chapter 3

Studies on the components of fitness in the baseline populations- Pre-adult traits

3.1 Background

Circadian clocks have been implicated in the regulation of life history traits such as pre-adult development time and adult lifespan (Kyriacou et al., 1990; Hurd and Ralph, 1998; Klarsfeld and Rouyer, 1998). It is generally believed that faster clocks speed up development and shorten adult lifespan, while slower clocks slow down development and lengthen lifespan. We assayed a number of pre-adult and adult life history traits in the baseline populations under different light regimes to study how light regimes influence them, and whether circadian clocks play any role in mediating such effects. In this chapter, I shall discuss the results of our studies done on the pre-adult life history traits such as pre-adult development time, pre-adult survivorship, and pupation height of the baseline populations.

3.2 Pre-adult development time and survivorship of the baseline populations

The role of circadian clocks in the pre-adult development of *Drosophila melanogaster* has been studied in the *per* mutants of *D. melanogaster* (Kyriacou et al., 1990). The pre-adult development time of the *per* mutants was positively correlated with the τ of their locomotor activity rhythm, i.e. the per^S flies (τ = 19 h) developed faster than the wild type flies (τ = 24 h), which in turn developed faster than the per^L flies (τ = 28 h) (Kyriacou et al., 1990). A positive correlation between development time and clock period under dim LL, very bright LL and in LD cycles suggests that the mutations in the *per* gene has pleiotropic effects on

the period and the pre-adult development time. Thus, the genotype that enables the clocks to run faster or slower, also cause speed up and or slow down pre-adult development. In order to bypass such pleiotropic effects one would need to assay development time in flies sampled from similar genetic background under short and long day lengths, wherein the circadian clocks would be entrain by speeding up or slowing down oscillations.

In order to investigate the role of circadian clocks in the regulation of pre-adult fitness traits we assayed pre-adult development time and pre-adult survivorship of the four LL populations of *D. melanogaster* under seven different light regimes: LL, DD, *T18*, *T20*, *T24*, *T28* and *T30*.

3.2a Materials and methods

From the running culture of each population (LL1..4), eggs laid on banana medium over a 2 h window were collected for the assay. Exactly 30 eggs were dispensed into 8 dram vials containing ~ 6 ml banana food and 50 such vials were set up from each population. Ten vials from each population were introduced into LL, DD, *T18*, *T20*, *T24*, *T28* and *T30*. Thus a total of 280 vials were set up for the assay (10 vials × 4 populations × 7 light regimes). These vials were introduced into five LD regimes at 20:00 h, when lights went off simultaneously in all of them. The vials were monitored for eclosion of adult flies after the pupae became dark. Eclosing adults were collected every 2 h, sexed, and counted. Fluorescent white light of intensity ~100 lux was used during light phase, whereas during dark phase red light of λ >650 nm was used.

Temperature and relative humidity in the all assay environments monitored continuously using a Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co, LTD, were found to be comparable.

Pre-adult development time in hours was calculated as the duration between the midpoint of the egg collection window, and the midpoint of the 2 h period during which eclosion occurred. The mean development time for a particular sex in a particular light regime was used as data in a mixed model analysis of variance (ANOVA), in which replicate populations were treated as random blocks, and light regime and sex were treated as fixed factors. Pre-adult survivorship was calculated as the fraction of eggs that successfully developed into adults in each vial. The mean pre-adult survivorship values were used as data in a mixed model ANOVA, with replicate populations as random blocks, and light regime as a fixed factor. The eclosion profiles of flies under different light regime development time data of individual flies from all four replicate populations were pooled and compared using Kruskal-Wallis test.

3.2b Results

ANOVA revealed a significant main effect of light regime on the pre-adult development time ($F_{6,18}$ = 311.70, p < 0.001) (Figures 1, 2). The mean development time of males and females was shortest under LL, followed by *T18*, *T20*, DD, and *T24, T28* & *T30*, in that order (Figures 1, 2; Table 1, 2). Multiple comparisons using 95% CI around the mean indicated that the development time of flies under most light regimes were significantly different from each other,

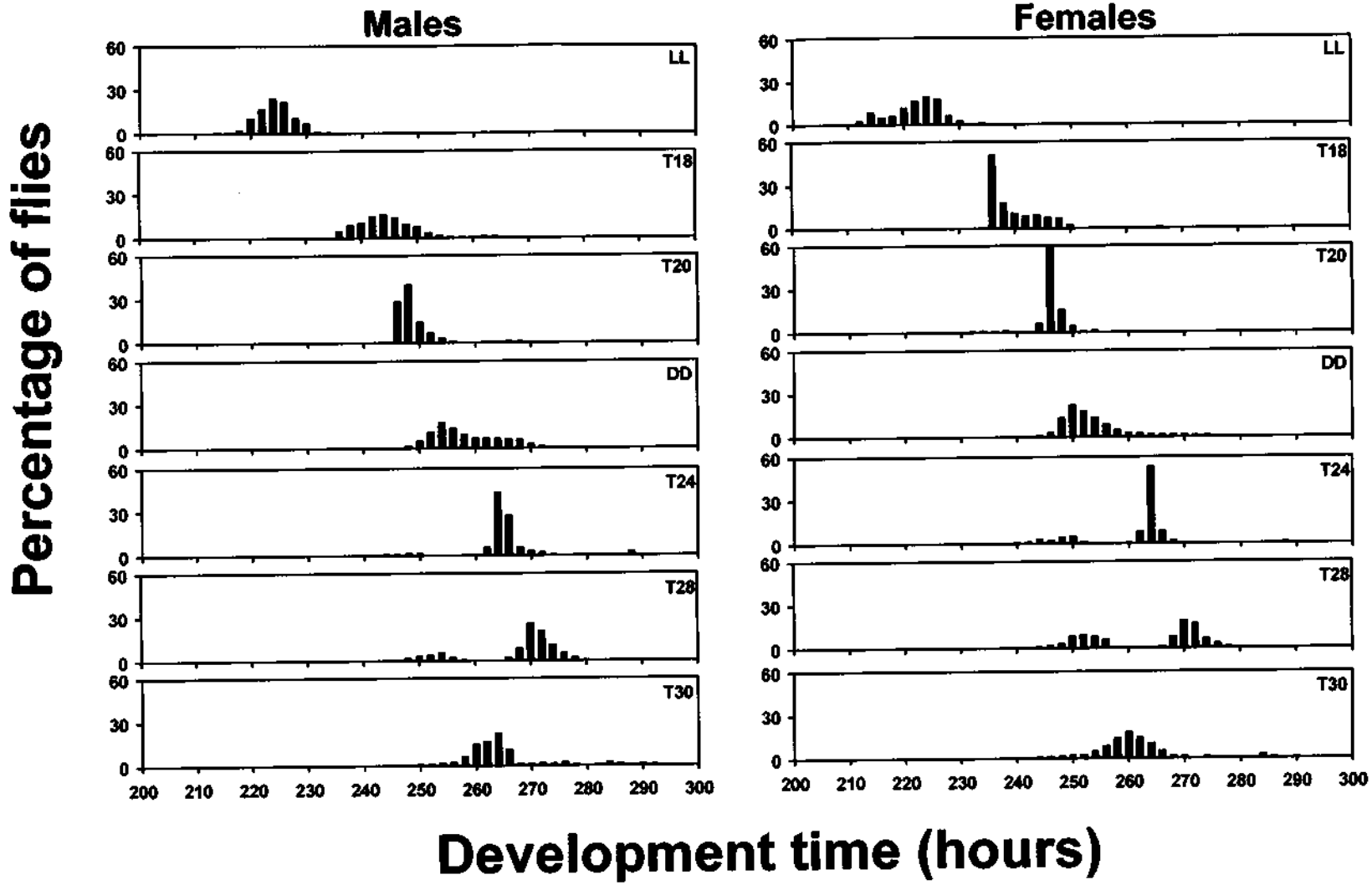

Figure 1: Eclosion profile of LL populations under seven different light regimes. The x-axis represents pre-adult development time in hours and y-axis represents percentage of flies. The left side panels show eclosion profiles of males under various light regimes, while those on right side show eclosion profiles of females. Pre-adult development time was shortest under LL followed by *T18*, *T20*, DD in that order and longest in *T24, T28* and *T30* regimes.

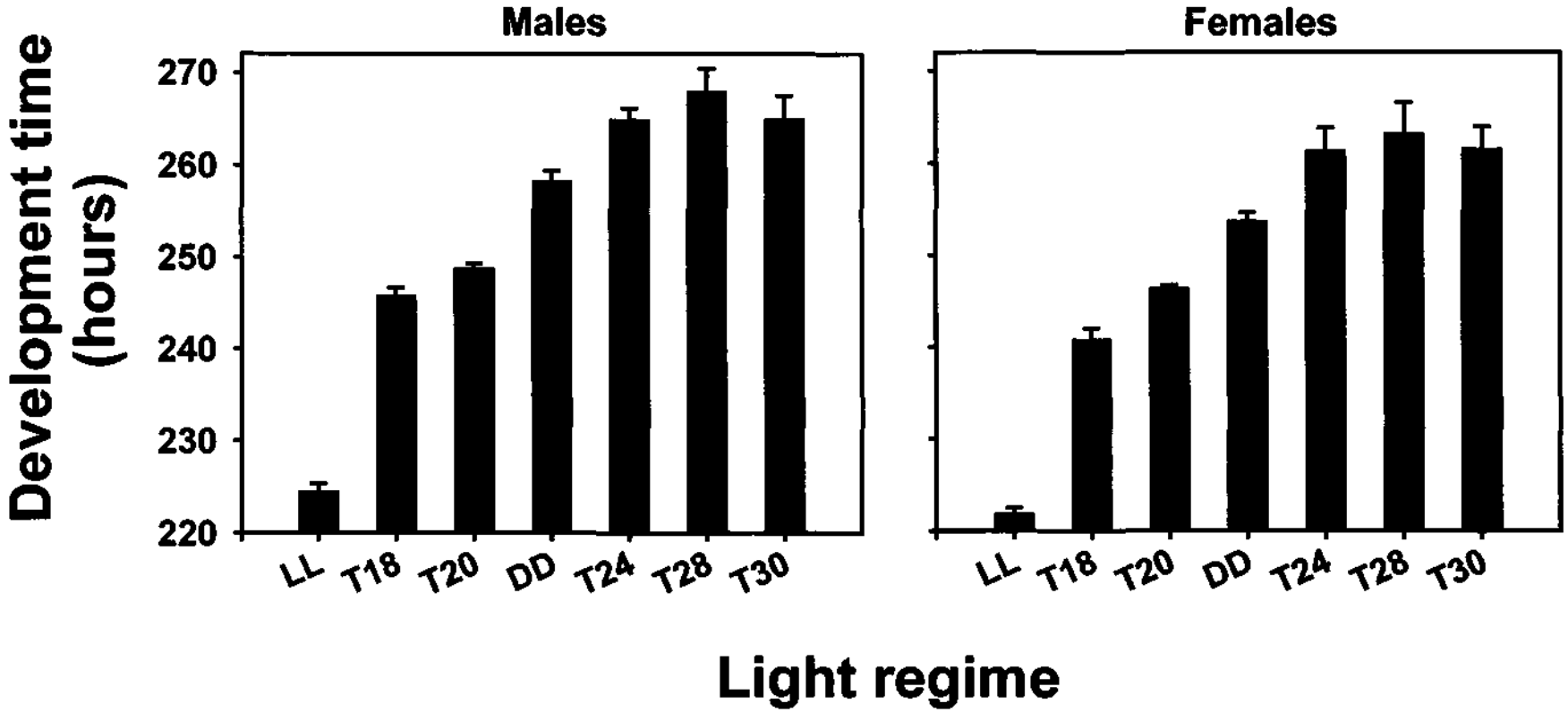

Figure 2: Mean pre-adult development time under seven light regimes. Light regimes are plotted along x-axis and mean development time plotted along y-axis. Left panel shows development time of males under various light regimes and right panel shows same for females. Pre-adult development time was shortest under LL followed by *T18*, *T20*, DD in that order and longest in *T24*, *T28* and *T30* regimes for both males and females.

Table 1: Results of ANOVA on pre-adult development time of four laboratory populations of *D. melanogaster*

	df Effect	MS Effect	F	p-level
Light regime	4	2129.47021	2411.969	0.0000
sex	1	135.976563	607.8455	0.0001
regime x sex	4	7.10910606	6.563789	0.0048

Table 2: Mean pre-adult development time of four laboratory populations of *D. melanogaster* assayed under seven different light regimes

Light Regime	Sex	Pre-adult development time (hours)
LL	m	224.37
LL	f	221.91
DD	m	258.32
DD	f	253.63
T18	m	245.80
T18	f	240.83
T20	m	248.63
T20	f	246.72
T24	m	263.96
T24	f	261.07
T28	m	265.32
T28	f	258.83
T30	m	253.64
T30	f	245.73

except for *T24*, *T28* and *T30*. ANOVA also revealed a significant main effect of sex ($F_{1,3}$ = 151.84, $p < 0.05$), and light regime x sex interaction ($F_{6,18}$ = 7.85, $p < 0.001$). Multiple comparisons using 95% CI around the mean showed that the females developed faster than the males under all the seven light regimes, and the male-female differences in development time was greatest under *T28* and *T30* regime, followed by DD, *T24*, LL, *T18* and *T20* regimes. Eclosion appeared to be bimodal under *T24*, *T28* and *T30*, and the bimodality was more prominent in females than in males (Figure 1). In order to compare the waveforms of eclosion under different light regimes Kruskal-Wallis test was performed. The test showed that the light regimes had a significant effect on the eclosion profile of males [H (6, N = 3443) = 2582.053, $p < 0.001$] and females [H (6, N = 3717) = 2722.088, $p < 0.001$]. The Kruskal-Wallis test thus further reconfirmed our results from the ANOVA. Although eclosion waveforms under no two light regimes were similar, the dissimilarity was more striking under *T24, T28* and *T30*. The test also revealed that mean development time of males and females was shortest under LL, followed by *T18, T20*, DD, *T24, T28* and *T30*, in that order.

The periodicity of eclosion rhythm under DD, *T18, T20, T24, T28* and *T30*, were 23.5 h, 18 h, 20 h, 24 h, 28 h and 30 h, respectively, while eclosion was arrhythmic under LL (Paranjpe et al., 2003). The peak of eclosion rhythm under different light regimes in the eclosion rhythm assay (section 2.3c; see also Paranjpe et al., 2003) matched closely the peak of eclosion observed in our development time assay (Figure 1). Further, the mean development time of the males and females under six light regimes (DD, *T18, T20, T24, T28* and *T30*)

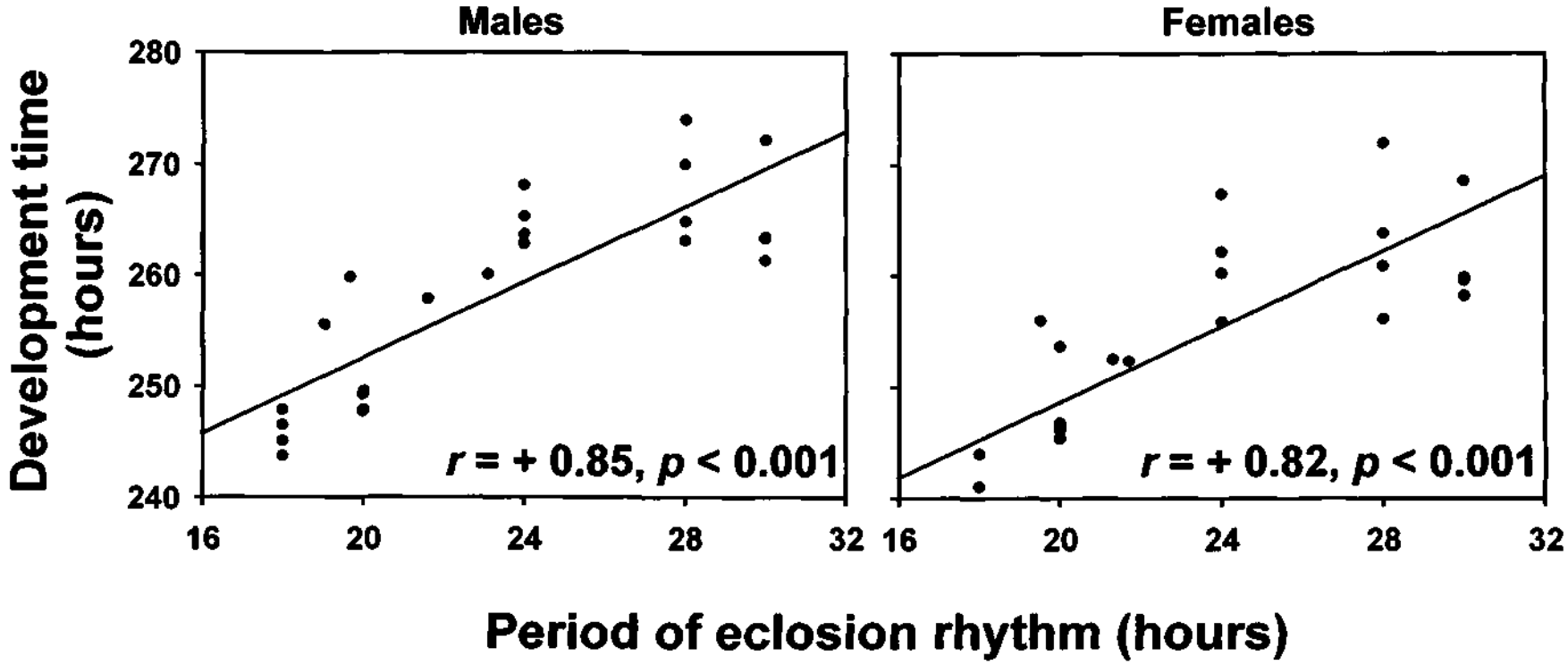

Figure 3: Correlation between pre-adult development time and the period of eclosion rhythm. Pre-adult development time was positively correlated with the period of eclosion rhythm ($r = + 0.85$, $p < 0.001$; $r = + 0.82$, $p < 0.001$) suggesting that rhythms regulate pre-adult development time in *D. melanogaster*.

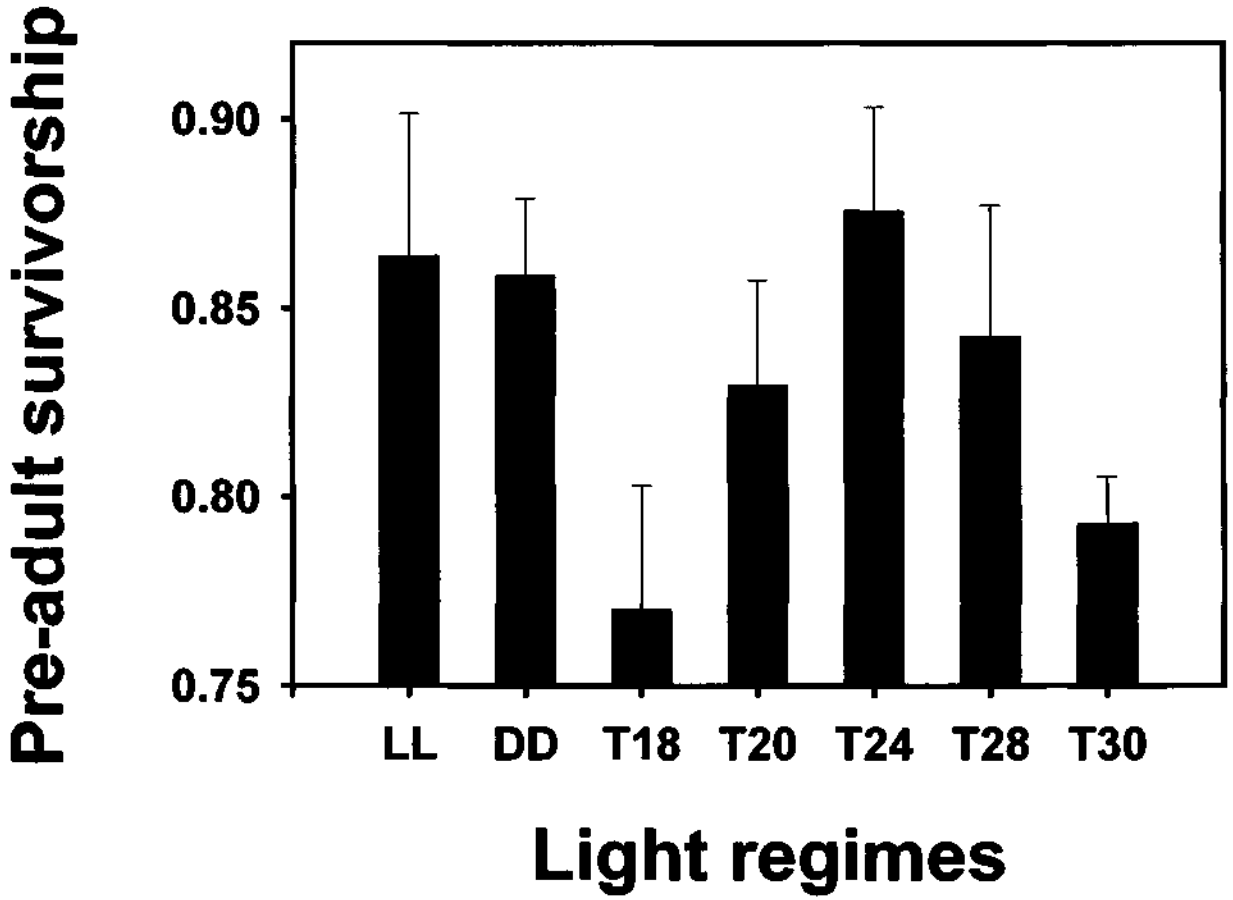

Figure 4: Pre-adult survivorship under seven light regimes. Pre-adult survivorship was least under *T18* and *T30* followed by *T20*. Pre-adult survivorship under LL, DD, *T24* and *T28* regimes was comparable.

showed a significant positive correlation with the mean period of eclosion rhythm under the corresponding environments (r = +0.85, $p < 0.001$ & +0.82, $p < 0.001$; Figure 3).

The ANOVA on the pre-adult survivorship data revealed a significant main effect of light regime ($F_{6,18} = 7.80$, $p < 0.001$). Multiple comparisons using 95% CI around the mean showed that while pre-adult survivorship under LL, DD, *T24* and *T28* regimes was comparable, they were significantly different than those under *T20*, *T18* and *T30* regimes (Figure 4).

3.2c Discussion

In *D. melanogaster* the circadian clocks that regulate adult eclosion are located in the prothoracic gland and in the ventral lateral neurons (Myers et al., 2003), and it is believed that these clocks play a key role in the regulation of pre-adult development (Qui and Hardin, 1996). Under environments wherein eclosion is arrhythmic, such as bright LL, the development time is solely determined by the developmental state of a fly, and under such a situation pre-adult development time would reflect the minimum time required by the fly to undergo different stages of pre-adult development. On the other hand, environments such as DD and LD cycles, wherein eclosion is rhythmic, an interaction between the developmental state of the fly and the eclosion clock would determine the duration of pre-adult development, and the developmental time would then be expected to be different than those under LL. In a previous study on the four *Drosophila* populations (JB1..4) reared under LL (the ancestral populations of the

flies used in the present study), Sheeba and coworkers (1999b) had reported that the development time of the LL reared flies was shortest under LL regime, followed by *T24*, and DD. In the present study too development time was shortest under LL, followed by *T18*, *T20*, DD, *T24*, *T28* and *T30*, in that order. As opposed to LL, eclosion under DD is gated in a circadian manner, and as a result flies took longer to develop compared to that in LL. We believe that the slight discrepancy in the results of the two studies could be due to the fact that different set of flies (about 100 generations apart) with different clock periods were used in the two experiments. It is important to note here that the τ of eclosion rhythm of JB populations under DD was greater than 24 h, whereas those of LL populations was less than 24 h. Thus, consistent with our speculations, JB populations with τ > 24 h took longer to develop under DD compared to T24 regime, whereas LL populations with τ < 24 h developed faster in DD compared to *T24*.

The mean development time under six different light regimes (*T18*, *T20*, DD, *T24*, *T28* and *T30*) showed a significant positive correlation with the mean period of eclosion rhythm under the corresponding light regimes; i.e. shorter period of eclosion rhythm under *T18* and *T20* was associated with faster pre-adult development, followed closely by DD, whereas flies took longest to develop under the light regimes where the periods of eclosion rhythms were 24 h, 28 h and 30 h, suggesting that development in *D. melanogaster* is regulated by the periodicity of the LD cycles and/or eclosion rhythm. These results are in agreement with the findings of previous studies, wherein the duration of pre-adult

development was reported to be positively correlated with clock period (Kyriacou et al., 1990; Shimizu et al., 1997; Miyatake, 1997). A subtle but significant difference between the outcome of present study and those of previous studies is that, the correlation between developmental time and eclosion period in our study is clearly mediated via the periodicity of LD cycles and/or of eclosion rhythm, whereas in previous studies, it appears to depend upon the genotype of the flies.

Although development time in our study was greater under *T28* and *T30* compared to *T24*, the differences did not reach levels of statistical significance, possibly due to some interactions between the developmental states, the period of LD cycles, and the eclosion profiles. A careful analysis of eclosion profiles under *T24, T28* and *T30* regimes revealed that the eclosion under *T24, T28* and *T30* was bimodal. To complicate the matter further eclosion patterns of the females had a greater propensity towards bimodality than the males. Kruskal-Wallis test confirmed that eclosion profiles of the male and the female flies under *T24, T28* and *T30* were significantly different (Figure 1).

A positive correlation between period of eclosion rhythm and pre-adult development time suggests that speeding up or slowing down of pre-adult development could be a physiological manifestation of the developmental or eclosion clock. Moreover, the mean development time under *T28* and *T30* did not increase proportionately relative to *T24*, in fact in females, it actually decreased under *T28* and *T30* regimes compared to *T24* indicating that development time under *T24* might be the longest. Thus, there appears to be a limit to which the development of *Drosophila* can be slowed down by lengthening clock period. On

the other hand, it appears that we have not yet reached the limit for fastest development. However, it is interesting to note that manipulating development time by making it faster or slower using eclosion rhythm or LD cycles has a cost attached to it. The pre-adult survivorship under *T18* and *T30* was significantly reduced compared to those under *T20* which in turn was significantly less compared to those under *T24* and *T28* regimes. Therefore, the extent of deviation of the period of LD cycles and/or eclosion rhythm from 24 h seems to have an adverse effect on the pre-adult survivorship. Since pre-adult survivorship is an important life history trait and a good indicator of overall fitness, our results could be taken as an validation for circadian resonance hypothesis which states that organisms with clock periods matching those of the cyclic environment perform "better" compared to others with deviant periodicities (Pittendrigh and Bruce, 1959).

In summary, our study clearly demonstrates that the pre-adult development of *D. melanogaster* can be speeded up or slowed down, albeit within a range of day lengths, without increasing the cost in terms of pre-adult survivorship, but speeding up or slowing down beyond a limit becomes detrimental. Adverse effects of speeding up development on other life history traits have been reported earlier in life history evolution studies in *D. melanogaster* (Chippindale et al., 1997; Prasad et al., 2001). In these studies, directional selection for faster development in *D. melanogaster* populations lead to the reduction in a number of life history traits such as pre-adult survivorship, pupation height, larval growth rates (Chippindale et al., 1997; Prasad et al.,

2000), and larval feeding rates and dry weights at eclosion (Nunny, 1996; Chippindale et al., 1997; Prasad et al., 2001) as a correlated response to imposed selection pressure.

Although, our study suggests that periodicity of the light regimes, and/or eclosion rhythm has measurable effect on two key pre-adult life history traits, it is not yet clear whether such effects could be merely due to differences in the photoperiods, and not due to the periodicity of the LD cycles. Therefore, we decided to investigations the effect of photoperiods on the pre-adult development time of the LL populations by exposing flies to different photoperiods in a LD cycle of 24 h period.

3.3 Pre-adult development time and survivorship of baseline populations under different photoperiods

It is clear from the results of our previous experiments (described in the section 3.2) that the duration of pre-adult development is regulated by the length of LD cycles and/or the period of the eclosion rhythm. It is also possible that the duration of pre-adult development in *D. melanogaster* is determined by the light (photo-period) or dark (scoto-period) rather than the total length of LD cycles. To examine these possibilities we assayed the pre-adult development time of the four LL populations under different photoperiodic regimes such as LD cycles of 9:15 h, 10:14 h, 12:12 h, 14:10 h and 15:9 h. Although, the duration of photoperiod was increased from 9 h to 15 h, thus, mimicking the duration of light under different *T* cycles (*T18* to *T30*), total length of the LD cycles in these experiments was kept constant at 24 h. If the duration of light or darkness is

crucial for the regulation of development time, one would expect that development time under a given photoperiods (or scoto-period) would be similar to that under the corresponding *T* cycle. If the total length of *T* cycle is crucial in the determination of the duration of pre-adult development, the development time under different photoperiods would be comparable to those under *T24* regime.

3.3a Materials and methods

The protocol used in the pre-adult development time assay was essentially similar to those described for other assays under the section 3.2a, except that from each of the four baseline LL populations ten vials containing exactly 30 eggs (10 vials x 4 populations) were introduced into LD cycles of 9:15 h, 10:14 h, 12:12 h, 14:10 h and 15:9 h. Lights went off in the photoperiodic regimes at exactly 2000 hrs. The vials were then regularly monitored for eclosion of adult flies after the pupae became dark. Eclosing adults were collected every 2 h, sexed, and counted. Pre-adult development time in hours was calculated as the duration between the midpoint of the egg collection window and the midpoint of the 2 h period during which eclosion occurred. The mean development time of a particular sex in a particular light regime was used as data in a mixed model analysis ANOVA, in which replicate populations were treated as random blocks, and light regime and sex of the fly were treated as fixed factors.

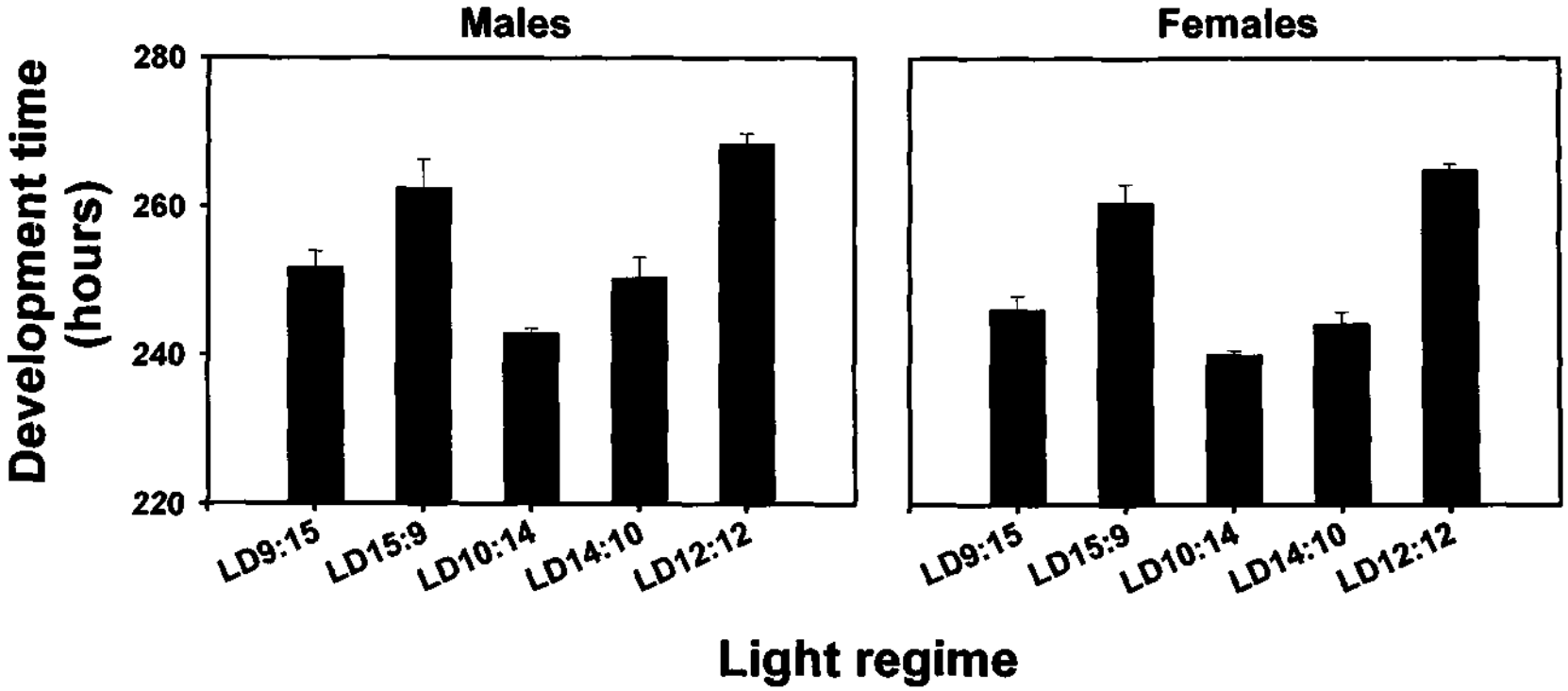

Figure 5: Pre-adult development time under different photoperiods. Pre-adult development time was significantly different under five photoperiods indicating that part of the development time differences in various LD cycles is due to differences in the duration of light as well as the ratio of light: dark in LD cycles.

3.3b Results

The results of the ANOVA revealed significant main effect of light regime (F = 25.90; p< 0.001; Figure 5) and sex (F = 115.40; p< 0.05) on the pre-adult development time. Post-hoc multiple comparisons using 95% CI around the mean revealed that the flies took longest to develop under LD 12:12 h followed by LD 15:9 h, and the least under LD 14:10 h, LD 10:14 h & LD 9:15 h photoperiods. Thus, the development time decreased with a decrement in the duration of the photoperiod except when the LD regime was 12:12 h.

3.3c Discussion

The photoperiods in our study were chosen such that the duration of the photoperiod could mimic the light phase under various *T* cycles (*T18* to *T30*). The results of our study showed that the pre-adult development time differed significantly under photoperiods ranging from 9 h to 15 h. The flies took the longest to develop under LD 12:12 h, followed by LD 15:9 h, LD 9:15 h, LD 14:10 h and the least under LD 10:14 h photoperiods. Thus, in contrast to our expectations the development time of the flies observed under different photoperiods neither matched their duration of pre-adult development under corresponding *T* cycles, nor to those under *T24* regime, rather it was intermediate, which suggests that besides the photoperiod the light : dark (L:D) ratio may also play an important role in the regulation of pre-adult development in *D. melanogaster*. To the best of our knowledge ours is the first detailed study of

its kind on the effects of light regimes on the pre-adult development time of *Drosophila*.

3.4 Pupation height of baseline populations

Pupation height, the height above the food medium at which *Drosophila* larvae in laboratory culture vials pupate, is genetically variable (Markow, 1979; Bauer, 1984), sensitive to various environmental factors such as the texture of food (De Souza et al., 1970), temperature, relative humidity and larval density (Sokal et al., 1960; Joshi and Mueller, 1993), and is known to change as a correlated response to selection on a variety of life-history traits (Mueller and Sweet 1986; Joshi and Mueller, 1996; Chippindale et al., 1997; Prasad et al., 2001). It has been suggested that pupation height in *Drosophila* reflects energy expended by larvae during the post-feeding wandering phase (Chippindale et al., 1997). This notion is supported by the finding that pupation height tends to decrease in populations selected for rapid development, as part of a syndrome of reduced energy expenditure in the pre-adult stages (Chippindale et al., 1997; Prasad et al., 2001).

Earlier studies on light regime effects on pupation behavior in *D. melanogaster* have shown that *D. melanogaster* larvae tend to pupate higher under DD than under LL (Markow, 1979; Schnebel and Grossfield, 1986), and when given a choice between illuminated and dark sites, prefer to pupate in dark sites (Rizki and Davis, 1953; Markow, 1981; Manning and Markow, 1981). These findings led to the view that light has an inhibitory effect on the wandering of post-feeding larvae and that greater pupation height of *D. melanogaster*

populations in the dark is perhaps an adaptation enabling larvae to avoid bright places with enhanced risk of heat stress, desiccation or predation (Markow, 1979; Manning and Markow, 1981; Schnebel and Grossfield, 1986). If this is correct, one can predict that pupation height of *D. melanogaster* under different LD regimes should be intermediate between that under LL and DD conditions. In any LD regime, depending on the phase at which larvae begin wandering, they will experience either light or darkness for some period of time. Thus, at most, the pupation height in an LD regime could equal that in either DD or LL, but should not be more extreme than that in these two constant conditions. On the other hand, if circadian clocks are involved in some way in gating or otherwise affecting the initiation of wandering or pupariation, or the duration of wandering, pupation height could be affected by LD regimes in many ways, potentially differing from the prediction made above. In *D. melanogaster*, it is not clear if pupariation is under circadian control (Bakker and Nelissen, 1963; Pittendrigh and Skopik, 1970), although circadian control of pupariation has been demonstrated in a few species of scaptodrosophilids (Rensing and Hardeland, 1967; Eeken, 1974). To understand how light regimes influence pupation height we measured pupation height in four populations of *D. melanogaster* under LL, DD, and five different LD regimes.

3.4a Materials and methods

From the running culture of each baseline population, eggs laid over 2 h window on banana-jaggery food medium were collected for the assay. Exactly 30 eggs

each were dispensed into 8 dram vials containing ~ 6 ml food. Forty such vials were set up for each population. Eight vials of each population were introduced into each of five light regimes: LL, DD, *T18*, *T20*, *T24*, *T28* and *T30*. Thus, a total of 280 vials were set up (10 vials × 4 populations × 7 light regimes). The vials were introduced in the assay light regimes at 20:00 h, when the lights went off in all LD regimes simultaneously.

Fluorescent white light of intensity ~100 lux was used for the light phase, whereas during the dark phase a red light (λ > 650 nm) was used for ease of observation and handling of vials. Red light with wavelengths > 650 nm is considered to be 'safe light' in dark phase as these wavelengths of light are not perceived by the *Drosophila* circadian system (Chandrashekaran et al., 1973). Temperature and relative humidity in all light regimes was continuously monitored using a thermo-hygrograph (Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co, LTD). Temperature was maintained at 25^{0}C (± 1^{0}C) and relative humidity at ~70% (± 5 %).

After all the individuals eclosed, pupation height was measured as the distance from the surface of food medium to the point between the anterior spiracles of the pupa, with any pupa touching the surface of the medium being given a pupation height of zero (Mueller and Sweet, 1986). Individual pupation heights were used as data for a mixed model nested analysis of variance (ANOVA) in which replicate populations were treated as random blocks, light regime as a fixed factor crossed with block, and vial as a random factor nested within the light regime × block interaction. Multiple comparisons among mean

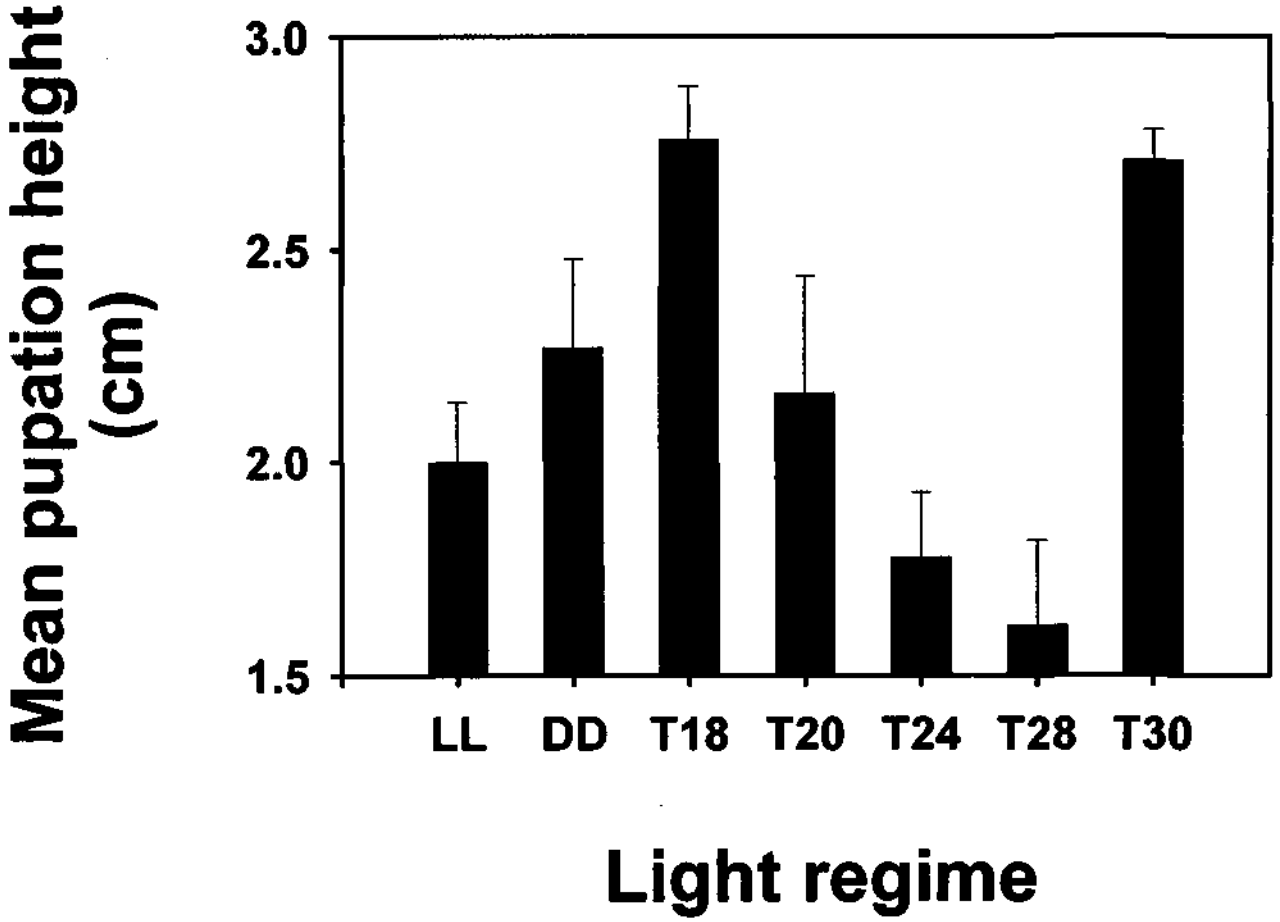

Figure 6: Pupation height under seven different light regimes. Larvae from LL populations climbed higher under DD compared to LL regime. Under periodic regimes, the pupation height decreased with increase in the length of LD cycles with exception of *T30* regime.

pupation height in different light regimes were done using Fisher's LSD test. All analyses were implemented on STATISTICA™ for Windows Release 5.0 B (StatSoft Inc., 1995).

3.4b Results

Light regime had significant effect on pupation height ($F_{6,18}$ = 70.24, p < 0.001), with the least mean pupation height in *T24* and *T28*, and the greatest mean pupation height in DD, *T18* and *T30* regimes (Figure 6). Mean pupation height in LL was significantly lower than that under DD while under *T20* regime the pupation height was significantly less compared to *T18*, followed by *T24* and *T28* regimes (Figure 6).

3.4c Discussion

While our observation that pupation height in LL was less than that in DD confirms earlier observations (Markow, 1979), the fact that pupation height in *T24* and *T28* is significantly less than that in LL clearly suggests that there is more to light regime effects on pupation height than just a behavioral inhibition by light. If light suppresses the tendency of larvae to climb high up the walls of vials for pupation, it is hard to see why pupation height in *T24* and *T28* should be less than that in LL. At most, depending on the phase of initiation of wandering relative to lights on or off, larvae in *T24* and *T28* could experience light throughout their wandering period and, consequently, would be expected to have pupation heights similar to those seen under LL. If larvae in *T24* and *T28*

experienced darkness for part of their wandering stage, one would expect pupation height in these regimes to be intermediate between that seen in LL and DD.

At the very least, it is clear that these results cannot be explained by the behavioral inhibition of pupation height by light, as suggested by Markow (1979). There is clearly some more complex effect of light regime on pupation behavior at work here, and there may well be some involvement of the circadian organization in determining pupation height. The trend of pupation height decreasing with increasing length of LD cycles tempts us to conclude that period of LD cycles and/or clock might be involved in determining the pupation height, but for the exception of results under *T30* regime which goes against this speculation. Therefore, we cannot conclude about the exact form of involvement of LD cycles and/ or clocks. It would probably require not only more studies but also a better understanding of the genetic control of the initiation of wandering and pupariation, and the length of the wandering phase to explain such results. Overall, the results of our study indicate that LD regime may impinge upon the complex genetic regulation that results in the initiation of wandering and pupariation in a subtle way, suggesting that further investigation of light regime or circadian clock effects on pupation need to be done.

3.5 Conclusions

Pre-adult development time and circadian rhythm are both multigenic traits, and genes involved in regulating development time as well as circadian rhythms are

known to have pleiotropic effects (Kyriacou et al., 1990). Our study pre-designed to bypass such pleiotropic effects demonstrates a possible role of the periodicity of LD environment and/or of eclosion rhythm in determining the duration of pre-adult development. Pre-adult development can be accelerated or decelerated to certain extent by speeding up or slowing down circadian clocks by maintaining eggs under LD cycles of different period. Additionally, the duration of light in the LD cycles and/or the L:D ratio appears to be an important in determining the pre-adult development time in *D. melanogaster*. Taking into account the results of our experiments under different *T* cycles and photoperiods, it appears that the duration of pre-adult development in *D. melanogaster* essentially depends on several factors such as the period and phase of rhythm, developmental state, the interaction between the phase of eclosion rhythm and the phase of the LD cycles, the total length, L:D ratio and length of photoperiod in the LD cycles.

The pre-adult survivorship decreases as the period of LD cycles and/ or clock deviates from 24 h. Although, the role of clocks in determining pupation height remains unclear, pupation height also seems to be affected by light regimes.

In summery, our results underscore the importance of incorporating circadian organization explicitly while dealing with life-history traits. Laboratory studies of various fitness components in *D. melanogaster* are often conducted under LL, LD 12:12 h, LD 16:8 h, and sometimes even under fluctuating LD regimes wherein the timing of lights on and off is a function of when people enter or leave the laboratory. It is only now clear that light regimes have significant

effect on most life-history traits in *D. melanogaster* (Sheeba et al., 1999b, 2000, Paranjpe et al., 2005), and given that over 100 genes involved in a variety of functions are transcribed in a circadian manner in *D. melanogaster* (McDonald and Rosbash, 2001), it is even more likely that many other life-history traits will turn out to be sensitive to light conditions.

Chapter 4

Studies on components of fitness in the baseline populations- Adult traits

4.1 Background

In the previous chapter we have shown that light regimes have measurable effects on the pre-adult fitness components such as development time, pre-adult survivorship, and that the effects could partly be mediated via circadian clocks. Furthermore, it is known that light regimes influence important adult fitness traits such as life span and reproductive output (Sheeba et al., 2000). The evidence so far for the role of biological clocks or light regimes in regulating life span of is inconsistent as discussed in details in the chapter 1 (See section 1.6). We decided to study the effect of light regime on the adult fitness components such as lifespan, fecundity, using four LL populations in order to investigate the role of circadian clocks.

4.2 Lifespan of baseline populations under different light regimes

Living systems derive primary benefits of having circadian clocks by appropriately timing their behavioral and metabolic processes with respect to their external as well as internal environments (Aschoff, 1965; Hastings et al., 1991; Pittendrigh, 1993; Sharma, 2003a). There exist a number of empirical studies both from the field as well as laboratory conditions, demonstrating that failure in keeping local time can prove to be fatal for organisms living in periodic environments. For example, in a study on the guillemot fledglings, lack of maintenance of appropriate timing for jumping behavior enhanced the predation of the fledglings by gulls (Daan & Tinbergen, 1980). In a separate study it was demonstrated that squirrels (Decoursey et al., 1997) and chipmunks (Decoursey

et al., 2000) became more susceptible to predation when rendered arrhythmic due to the ablation of their circadian pacemaker (the suprachiasmatic nucleus, SCN). The lifespan was also found to be reduced considerably when organisms are maintained under constant light (LL), or under periodic light/dark cycles (LD) whose periodicities do not match the period of the organism's clocks (Pittendrigh and Minis, 1972; von Saint Paul and Aschoff, 1978). Any mismatch between the environmental and circadian periods (Ketellapper, 1960; Pittendrigh and Minis, 1972; von Saint Paul and Aschoff, 1978; Klarsfeld and Rouyer, 1998; Ouyang et al., 1998), and disturbances in the LD cycles (Halberg and Cadotte, 1975; Halberg et al., 1977; Nelson and Halberg, 1986), significantly reduced the growth and survival. These studies suggest that environmental light conditions and/or the circadian clocks play a major role in the determination of lifespan. However, the mechanism(s) underlying such effects still remains to be unraveled.

In mammals, a number of rhythms such as those in sleep, body temperature, and heart rate are disrupted during senescence (Sharma, 2001). With age, amplitude of rhythms, ability to coordinate various behavioral and physiological processes, and the ability to synchronize them to environmental cycles decrease considerably (Sharma, 2001). A few studies have also shown that certain kinds of manipulations in the circadian clocks, especially those that alter rhythms either by genetic, physiological or environmental modifications cause reduction in lifespan (Hendricks et al., 2003; Cirelli et al., 2005; Kume et al., 2005). For example, in a recent study on two wild type populations of *D. melanogaster* it was shown that under DD conditions arrhythmic flies live

significantly shorter than the rhythmic flies (Kumar et al., 2005). Although the primary cause of reduction in lifespan was not examined in this study, the authors argued that the reduction in lifespan of the arrhythmic flies was primarily due to circadian dysfunction (general malfunctioning of circadian clocks). In a separate study on the *cycle* (*cyc*^{0}) mutant of *D. melanogaster* that showed arrhythmic activity/rest patterns under DD, male *cyc*^{0} flies lived significantly shorter than the wild type males, while *cyc*^{0} females, *per*^{0} and *tim*^{0} males and females lived equally well as the wild type flies. This suggests that the loss of function mutation in the *cyc* gene causes disruption of circadian rhythms, and/or sleep, as well as sex specific reduction in lifespan (Hendricks et al., 2003). The authors argued that lack of sleep, and/or enhanced levels of activity during the otherwise rest period of the subjective cycle cased reduction in lifespan in the arrhythmic flies. In a study on the *Shaker* (*Sh*) mutants which had a point mutation in the gene encoding for the voltage-dependent potassium channels, the mutant flies were found to sleep considerably less compared to the wild type flies, but showed normal sleep homeostasis and normal response to sleep deprivation (Cirelli et al., 2005). The *Sh* mutants too had reduced lifespan compared to their genetic controls, but unlike the *cyc*^{0} flies, *Sh* mutants did not have any noticeable defect in their activity/rest rhythm. This suggests that the reduction in lifespan in the *Sh* mutants appears to be mainly due to lack of sleep, and/or due to pleiotropic effects of the mutation that impairs voltage-dependent potassium channels and not due to circadian dysfunction. In a recent study on the *fumin* (*fmn*) mutants of *D. melanogaster* that displayed increased activity

levels, lower sleep arousal threshold, and reduced rest rebound in response to sleep deprivation (Kume et al., 2005), lifespan of both males and females was similar to their genetic controls, in spite of the fact that *fmn* flies were more active than their controls. These results are therefore in sharp contrast to earlier observations in cyc^0 and *Sh* mutants, where reduction in lifespan always associated with higher levels of activity. Since *Sh* is a voltage-activated potassium channel with widespread expression in the nervous system, and *cyc* is a broadly expressed basic helix-loop-helix transcription factor, mutations in these genes might have more general impact on the physiology leading to shortening of lifespan (Kume et al., 2005). On the other hand, *fmn* is a mutation in *Drosophila* dopamine transporter (dDAT) gene that affects only the dopaminergic neurons, and therefore, might have less severe impact on other physiological processes (Kume et al., 2005). Therefore, it appears that in addition to circadian dysfunction, sleep deprivation, enhanced activity levels, and pleiotropic effects of different mutations such as *cyc*, *Sh* could also contribute to the reduction of lifespan in *Drosophila*.

It also appears that a number of factors that are directly or indirectly related to the circadian timing systems such as the clock period, activity levels, phase relationship with respect to external and internal cycles, and the reproductive output, contribute one way or other in the determination of lifespan in *D. melanogaster.* Previous studies also serve to elucidate complex, multi-faceted interactions between environmental light regimes, physiological processes, circadian clocks and life history traits. However, none of these studies

so far dealt with the factors that could be potentially mediating the effects of light regimes on physiological well being in an integrated and systematic manner. In order to investigate the effect of environmental light conditions on the adult life span of *D. melanogaster*, we assayed the adult lifespan of virgin and mated males and females in the four LL populations under two constant environments (LL and DD) and three periodic environments (*T20*, *T24* and *T28*).

4.2a Materials and methods

From the running culture of each of the LL populations, eggs laid on banana medium over a 2 h window were collected for the lifespan assays. From each population, approximately 50-60 eggs were collected into each of 24 vials. The vials were kept under LL until the adults eclosed. Freshly eclosed males and females were collected in vials at a density of either 8 males or 8 females per vial. Five vials of males and five vials of females from each population were introduced in each of the following light regimes - LL, DD, *T20*, *T24*, and *T28*. Flies were provided with fresh food every alternate day and the vials were checked every day for deaths. This procedure was continued until all the flies were dead. The protocol used to assay lifespan of reproducing flies was similar to those used for virgin flies except that in this case each vial contained 4 males and 4 females. Ten such vials were set up per population in each light regime.

The lifespan data of virgin and mated flies was then used to calculate the mean adult lifespan (in days) for each population under each light regime. The mean lifespan data was treated in a mixed model ANOVA where replicate

populations were treated as random blocks and light regime and sex were treated as fixed factors crossed with block.

4.2b Results

The results of ANOVA on the lifespan data revealed a significant main effect of the reproductive status (virgin *vs* mated) ($F_{1,3}$ = 183.78, p < 0.001), and the light regime ($F_{4,12}$ = 28.44, p < 0.001) on the adult lifespan. In addition, interactions between reproductive status and sex ($F_{4,12}$ = 6.73, p < 0.05), reproductive status and light regime ($F_{1,3}$ = 18.9, p < 0.05), sex and light regime ($F_{4,12}$ = 20.6, p < 0.001), and reproductive status, sex, and light regime ($F_{4,12}$ = 3.51, p < 0.05), also turned out to be statistically significant (Table 1). Multiple post-hoc comparisons using 95% CI around the mean revealed that virgin males lived the longest under *T20*, followed by constant environments (LL and DD), and the shortest under *T24* and *T28* regimes (Figure 1). The lifespan of mated males was comparable under all light regimes except LL. Under LL their lifespan was significantly shorter compared to all other light regimes (Figure 1). Multiple post-hoc comparisons using 95% CI around the mean revealed that both virgin and mated females lived significantly shorter under constant conditions (LL and DD) compared to the LD cycles, whereas among LD cycles lifespan of the mated females was shorter under *T20* and *T24* compared to *T28* (Figure 1). Thus, the overall pattern of lifespan suggests that flies of both sexes live significantly shorter under constant environments compared to periodic environments. The survivorship curves for virgin and mated males and females are shown in the

Table 1: Analysis of Variance for life span of LL populations under different light regimes

	df Effect	MS Effect	*F*	*p* -level
Reproductive status	1	6364.71	183.78	0.0008
Light regime	4	173.07	28.44	0.0000
Reproductive status x Light regime	4	27.23	18.9	0.0044
Reproductive status x sex	1	809.12	6.73	0.0224
Light regime x sex	4	136.64	20.6	0.0000
Reproductive status x Light regime x sex	4	31.61	3.51	0.0406

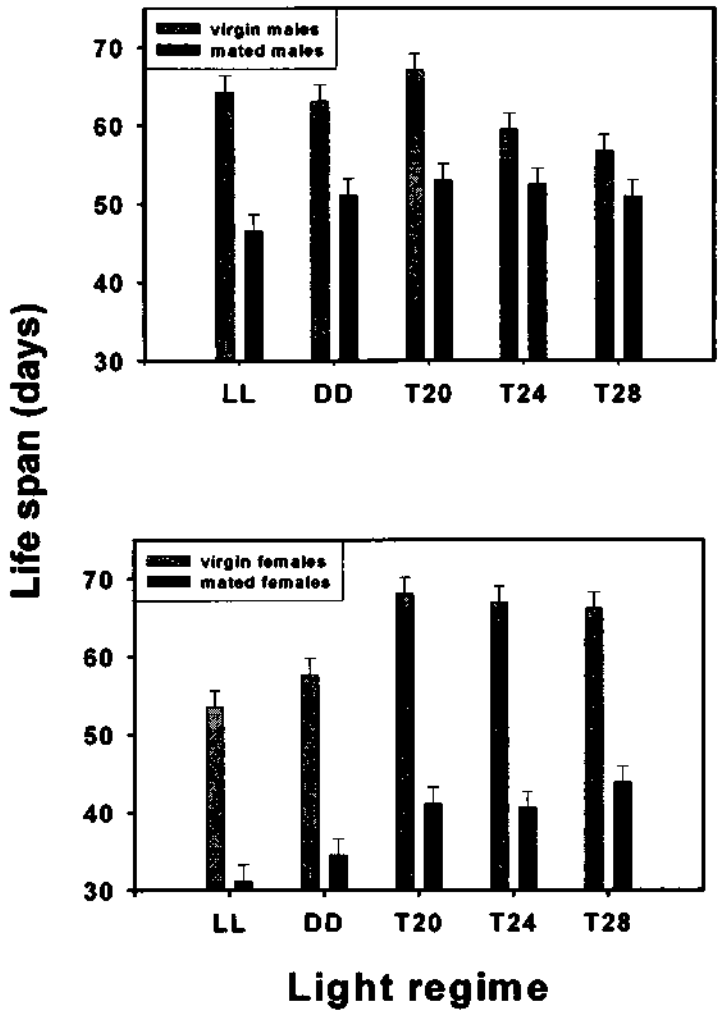

Figure 1: The mean life span of virgin and mated males under different light regimes is shown in the upper panel while the mean life span of virgin and mated females is illustrated in the lower panel. The grey bars represent life span of virgin flies while black bars represent those of mated flies. Virgin males lived longer under LL, DD and *T20* regimes compared to *T24* and *T28* regimes. Life span of mated males was significantly shorter in LL compared to all other regimes. Virgin and mated females lived longer under periodic conditions compared to constant conditions.

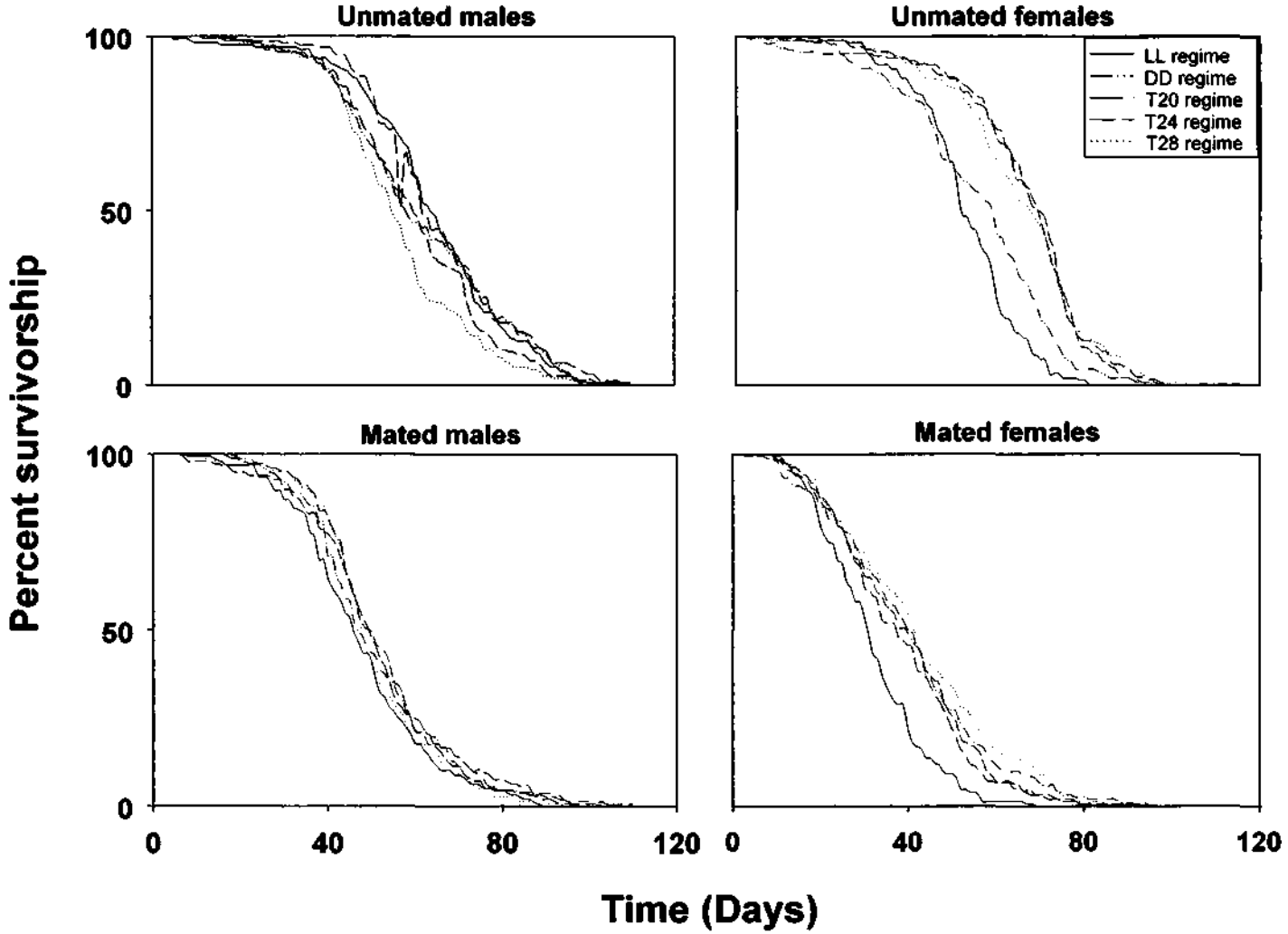

Figure 2: Survivorship curves for virgin and mated males and females under different light regimes. Percent survivorship is plotted along the x-axis and time in days along the y-axis. Survivorship curves for mated males and females were steeper under all light conditions compared to those for virgin males and females indicating faster mortality in flies due to mating. Survivorship curves for mated males suggest that their life span did not differ under different light regimes, while those for mated females differed considerably under different light regimes.

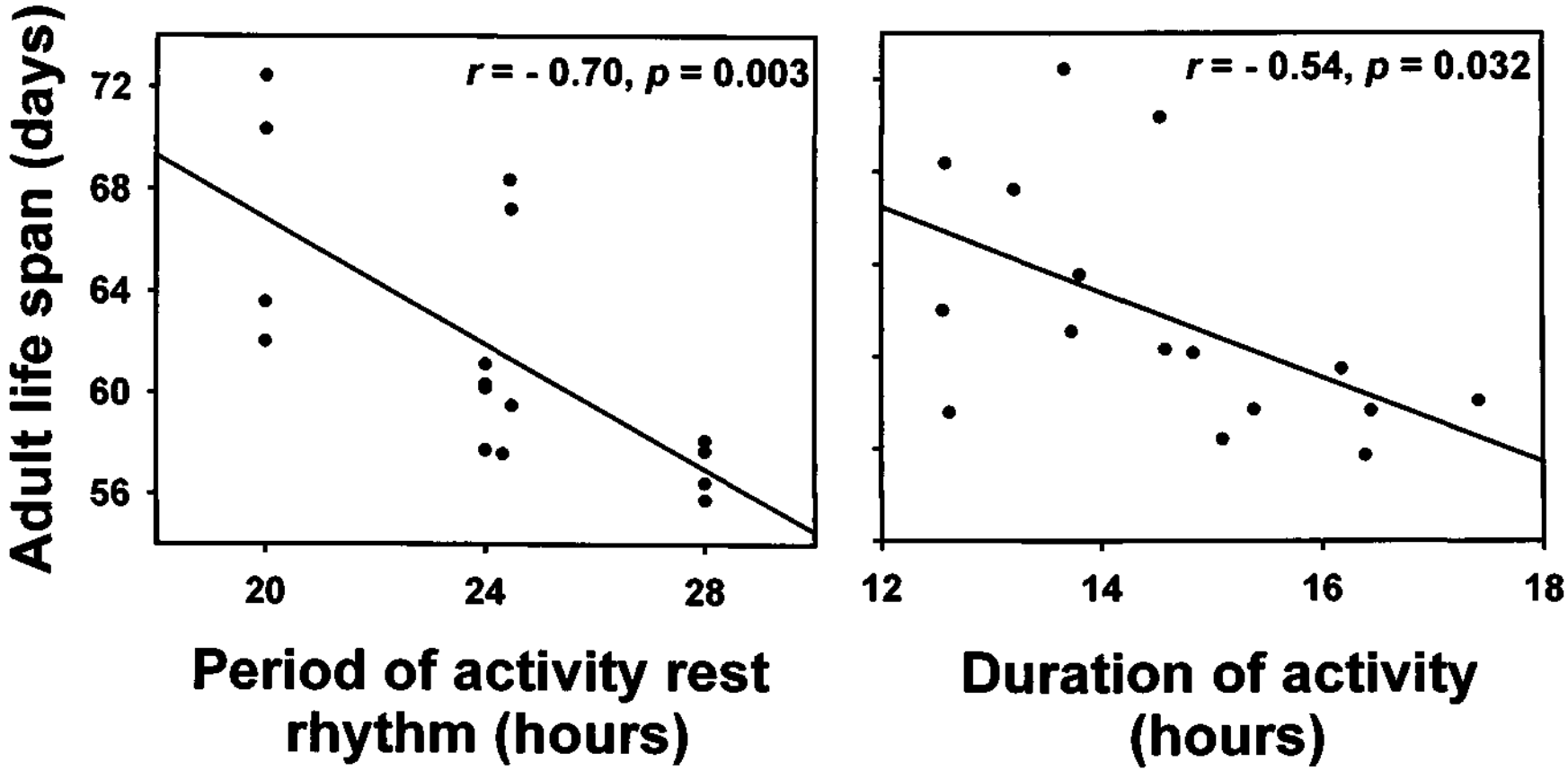

Figure 3: Left panel shows correlation between life span of virgin males and period of activity-rest rhythm, while the right panel shows correlation between life span and the duration of activity. A negative correlation in both cases implies that longer period of activity/rest rhythm and longer duration of activity were associated with a reduction in life span in the virgin males. No such correlation was seen in case of virgin and mated females.

Figure 2. The survivorship curves for the mated males and females were steeper under all light regimes compared to those for the virgin males and females, indicating that faster mortality in flies is a consequence of mating. The survivorship curves for the mated males suggests that their lifespan of virgin males did not differ much under different light regimes, while those for mated females differed considerably under different light regimes. The lifespan of virgin males was negatively correlated with the period of the LD cycles and/or the locomotor activity rhythm (r = -0.70, p < 0.004), and with the duration of activity (r = -0.54, p < 0.035) (Figure 3), suggesting that flies whose clocks ran faster and produced shorter activity durations in the activity/rest cycles live longer than those whose clocks ran slower and yielded longer activity durations. In case of virgin females, lifespan and clock period (r = -0.18, p = 0.51) as well as lifespan and duration of activity (r = +0.29, p = 0.28) did not show any correlation.

4.3 Fecundity of the baseline populations under different light regimes

It has been convincingly documented in numerous laboratory as well as field studies on a number of vertebrate and invertebrate species that lifespan is inversely correlated with reproductive output (Bell, 1984; Partridge and Harvey, 1985; Reznick, 1992; Stearns, 1992; Zwaan, 1999). In *Drosophila* too, cost of reproduction has been suggested to be primarily due to courtship, mating and egg production (Partridge and Harvey, 1985; Partridge et al., 1987; Chippendale et al., 1993, 1997a). Working with wild type populations of fruit flies Sheeba and coworkers (2000) showed that lifespan of reproducing flies from four populations

of *D. melanogaster* was significantly shorter under LL compared to LD 12 : 12 h and DD regimes. In reproducing females, the number of eggs laid (fecundity) and the rate of aging were considerably higher in flies maintained under LL compared to those kept in DD regime. The reduction in adult lifespan of the mated female under LL regime was suggested to be partly due to higher reproductive output (Sheeba et al., 2000). This suggests that LL may not be as deleterious as reported in earlier studies (Sharma and Joshi, 2002), but the results of those studies could be masked by an artifact of considering lifespan alone as measure of fitness. In other words taking lifespan alone to assess fitness could be incomplete, and often misleading, and one must estimate reproductive output in order to have a complete idea of the fitness advantages.

In earlier studies role of circadian clocks in reproductive output was investigated in great depth using mutants and wild type *D. melanogaster.* Studies on the loss of function mutants of *D. melanogaster* such as per^0, tim^0, cyc^0, Clk^{irk} revealed that a single mating among clock-deficient phenotypes resulted in ~ 40% lesser progeny compared to the wild type flies (Beaver et al., 2002). Further, flies with null mutation in clock genes laid fewer eggs compared to the wild type flies, out of which only a small fraction successfully developed into adults (Beaver et al., 2002). Experiments on the per^0 and tim^0 flies revealed that the amount of sperm released from the testes to seminal vesicles in males was also reduced significantly in null mutants compared to the wild type flies (Beaver et al., 2002). Extending the line of studies further, Beaver and co-workers (2003) demonstrated that the loss of function mutations in *Drosophila* resulted in

significantly fewer mature oocytes in per^0 and tim^0 flies compared to the wild type controls, and a rescue of clock functions by ectopically expressing *per* in the central pacemaker neurons of per^0 mutants LNs did not enhance the production of mature oocytes (Beaver et al., 2003), suggesting that *per* and *tim* may have non-clock like functions in the ovaries (Beaver et al., 2003). This suggests that the interaction of light regimes, circadian clocks and life history traits might be much more complex than thought earlier.

In order to investigate the effect of environmental light conditions on the fecundity of *D. melanogaster*, we assayed reproductive output of virgin and mated females in four separate populations of flies under two constant environments (LL and DD), as well as three periodic environments (*T20*, *T24* and *T28*). We planned to include virgin females in our study because virgin females of *Drosophila* are known to lay unfertilized eggs (*personal observation*).

4.3a Materials and methods

From the running culture of each of LL populations, eggs laid on banana medium over a 2 h window were collected for various assays. From each population, approximately 50-60 eggs per vial were collected into 24 vials. The vials were kept in LL until the adults eclosed. Freshly emerged male-female pairs were introduced into vials containing approximately 2 ml banana food. Twenty such pairs from each population were introduced in LL, DD, *T20*, *T24* and *T28*. Every day nearly at nearly the same time, the fly pairs were transferred into fresh food vials and the number of eggs laid by each female over the preceding 24 h

duration was counted. This was repeated everyday until all the females died. In the event that a male died during the assay, the dead fly was replaced by another male of the same age from a backup cohort maintained under the same light regime. The protocol used for the assay of lifetime fecundity of the virgin females was similar to those used for the mated flies except that each vial in this case contained a single virgin female. Fluorescent white light source (intensity ~ 100 lux) was used during the light phase of the LD cycles and in LL, whereas red light of λ >640 nm was used during the dark phase of LD cycle and DD. In addition, the lifespan of the virgin and mated females was estimated during the lifetime fecundity assay to have a separate confirmation of the results from the lifespan experiments (described under the previous section).

The total number of eggs produced by an individual female over its lifetime was calculated from the lifetime fecundity assay and the mean across 20 females was calculated for each population under a given light regime. Daily egg production per female was calculated by dividing total number of eggs laid by a female over its entire life time by the total number of days the fly had survived. The population means for daily fecundity, total fecundity and lifespan of the virgin and mated flies were used as data in separate mixed model ANOVA where light regime was treated as fixed factor and block was treated as random factor. Population means of the daily fecundity and the lifespan of the mated females in five light regimes were subjected to correlation analysis using STATISTICA^{TM} (Statsoft, 1995).

4.3b Results

The results of ANOVA on the fecundity data of the mated females revealed a significant main effect of light regime on the lifetime fecundity ($F_{4, 12} = 3.7$, $p < 0.05$), on the daily fecundity ($F_{4, 12} = 15.15$, $p < 0.001$), and on the adult lifespan ($F_{4, 12} = 3.39$, $p< 0.05$) (Figure 4). Post-hoc multiple comparisons on the fecundity data revealed that total fecundity under LL and DD was significantly greater than that under *T24* and *T28* regimes. Although the total fecundity under *T20* was higher than those under LL and DD but the differences did not reach statistical levels of significance. Post-hoc multiple comparisons of the daily fecundity data suggest that the daily fecundity of the mated females was significantly greater under constant conditions (LL, DD) compared to the periodic regimes (*T20*, *T24*, and *T28*) (Figure 4). The lifespan of mated females used in the fecundity assay showed a similar pattern as seen earlier in the lifespan assay. The mated females lived significantly shorter under LL and DD compared to *T20* and *T28* regimes. Although, the lifespan of reproducing females under *T24* was greater than those under LL & DD, the differences did not reach statistical levels of significance. Further, the lifespan of the mated females was negatively correlated to the daily fecundity ($r = -0.93$, $p < 0.05$), i.e., females that laid more eggs per day lived significantly shorter than those that laid fewer eggs. The lifespan of the mated females under five different light regimes, from two separate sets of experiments (lifetime fecundity assay and lifespan assay) did not differ significantly ($F_{1, 3} = 0.88$, $p = 0.42$), suggesting that the outcome of the two assays were comparable.

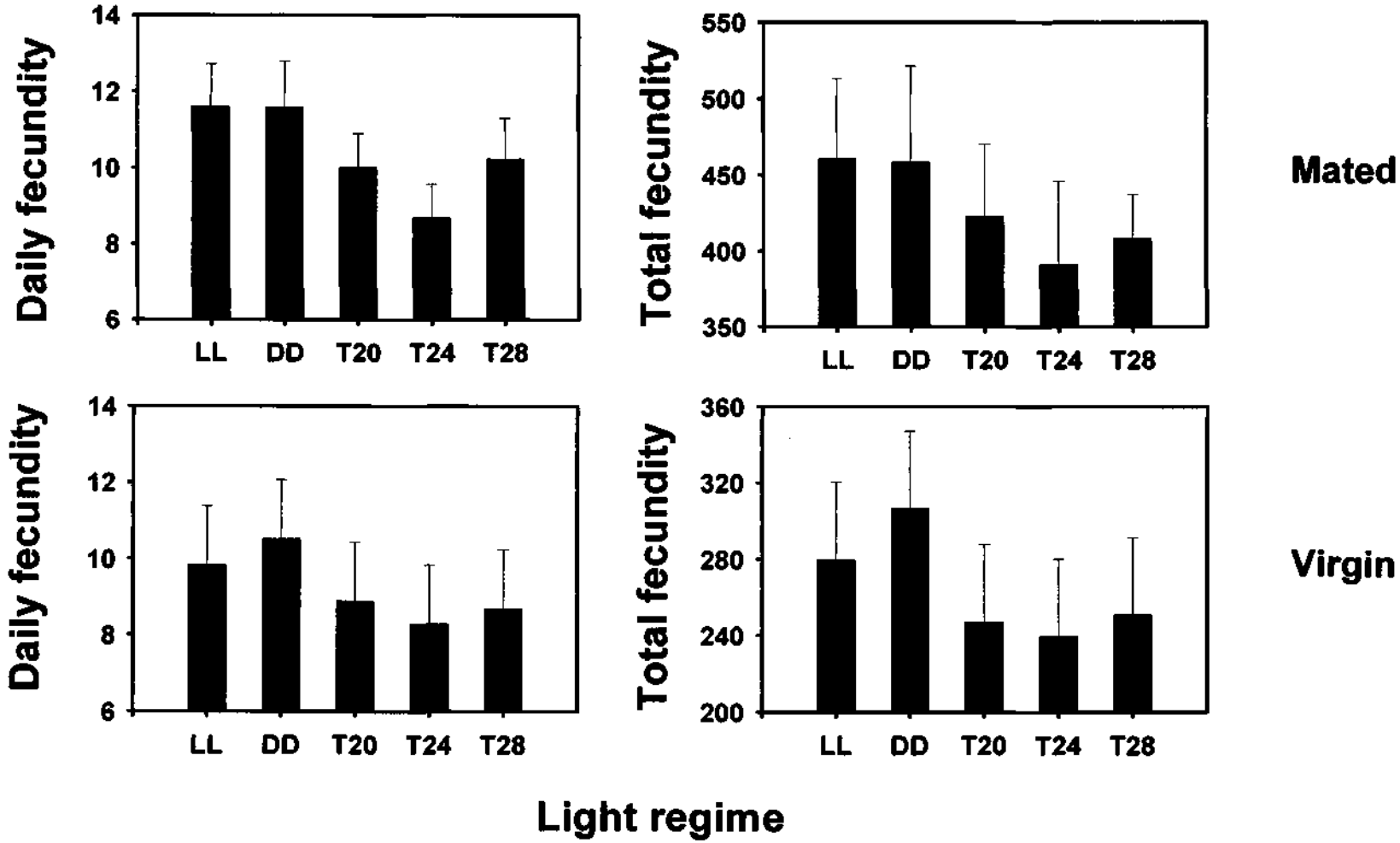

Figure 4: Reproductive output (daily and total fecundity) of both virgin and mated females was greater in LL and DD compared to periodic regimes. Left side panels show daily fecundity (average number of eggs laid per day by a female) and the left panels show total (lifetime fecundity) under different light regimes. Upper panels show reproductive output for mated females and lower panels shoe that for virgin females.

Results of ANOVA on the fecundity data of the virgin females revealed a significant main effect of light regime on the total fecundity ($F_{4, 12} = 4.52$, $p < 0.05$) and on the daily fecundity ($F_{4, 12} = 3.33$, $p < 0.05$) (Figure 4). Post-hoc multiple comparisons on fecundity data revealed that total fecundity as well as daily fecundity of virgin females under DD was significantly greater than those under *T20*, *T24* and *T28* regimes. Although the total fecundity under LL was higher than that under periodic regimes, the differences did not reach statistical level of significance. Moreover, the virgin females produced significantly less number of eggs compared to the mated females under all light regimes ($F_{1, 3} = 11.08$, $p < 0.05$). Nevertheless, the patterns of total and daily fecundity were similar under all five light regimes.

4.4 Discussion

In our study, mated flies of both sexes lived significantly shorter than the virgin flies irrespective of the light regimes they were assayed in, suggesting that mating is deleterious for *Drosophila*. In virgin males, lifespan was negatively correlated with the period of the locomotor activity rhythm and the duration of activity, i.e., with increasing period length and increasing duration of activity starting from *T20* and DD to *T28*, the lifespan of virgin males gradually reduced, suggesting that lifespan of *D. melanogaster* virgin males is modulated by the periodicity of LD cycles and/or activity rhythm or the duration of activity. The lifespan of virgin females, however, did not show any correlation either with the period of the LD cycles, or locomotor activity rhythm or with the duration of

activity, suggesting a sex specific effect of locomotor activity rhythm on the lifespan, quite similar to those reported in an earlier study on the *cyc°* mutants (Hendricks et al., 2003), wherein the *cyc°* males were reported to live shorter under LD and DD regimes compared to the wild type controls, whereas the *cyc°* females lived as long as the wild type females. Sex specific differences in lifespan have also been reported in the *per* mutants of *D. melanogaster*, where lifespan of the short period (per^T) and the long period (per^L) males under short (16 h) and long (24 h) LD cycles was reported to be significantly shorter than those of the wild type males, but the lifespan of the mutant females did not differ significantly from those of the wild type females (Klarsfeld and Rouyer, 1998). In our study, the lifespan of mated males neither appears to depend upon the period of the LD cycles and/or activity rhythm nor on the duration of activity, with a sole exception of LL regime, where flies remain active through out the day. Similarly, in an earlier study on four ancestral populations of the flies used in the present study, mated males were reported to live shorter under LL regime compared to *T24* and DD regimes (Sheeba et al., 2000). In the present study, the lifespan of the virgin males was longer under *T20*, DD and LL compared to *T24* and *T28* regimes. Thus, the light regimes seem to have measurable effect on the lifespan of virgin males but not of the mated males. Further, it appears that the differences in lifespan of the virgin males under different light regimes were abolished following courtship and mating, which suggests that cost of sex in *Drosophila* males could also depend upon environmental conditions (Figures 1, 2). Reduction in the lifespan due to courtship and mating has also been reported

in several earlier studies in *D. melanogaster* (Partridge et al., 1987; Luckinbill et al., 1988; Service, 1989). Besides the fruit flies *D. melanogaster* (Partridge and Fowler, 1990), cost of sex has also been reported in tsetse fly *Glossina morsitans* (Clutton-Brock and Langley, 1997), seed beetle *Callosobruchus maculatus* (Crudgington and Siva-Jothy, 2000), and the bed bug *Cimex lectularius* (Stutt and Siva-Jothy, 2001).

In our present study, the lifespan of virgin and mated females was shorter under constant environments (LL, DD) compared to periodic environments (*T20*, *T24* and *T28*). Interestingly the reduction in lifespan under LL and DD in the reproducing females was negatively correlated with reproductive output. The flies that laid more eggs lived significantly shorter (as under LL and DD) compared to those that laid fewer eggs (as under *T20*, *T24* and *T28*). Such correlations between lifespan and reproduction have also been extensively documented in life history evolution literature (Bell, 1984; Partridge and Harvey, 1985; Stearns, 1992; Zwaan, 1999). If the reduction in lifespan of mated females under LL and DD regimes is due to higher egg production, lifespan of virgin females under constant and periodic light regimes would be expected to be comparable. Surprisingly, virgin females too were found to live significantly shorter under constant environments (LL, DD) than the periodic environments (*T20*, *T24*, *T28*). How do we explain this? As we already know that even virgin *Drosophila* females too lay unfertilized eggs, we assayed the egg-output of the virgin females. To our surprise we found that the general pattern of egg-output in virgin females was comparable to those of the mated females (Figure 4). Thus, it appears that

higher egg out-put of virgin females under LL and DD compared to periodic regimes, quite similar to those observed for mated females, have resulted in the reduction of lifespan in the virgin females. Therefore, it appears that in females egg-production alone can cause significant light regime-dependent reduction in adult lifespan. In other words, reduction of lifespan under LL and DD in *Drosophila* females appears to be largely due to higher egg-output under these regimes, and not due to mating *per se*. Thus, by assaying the egg-output of the virgin females under different light regimes we were able to tease apart the cost of mating and cost of egg-production in *D. melanogaster* females.

Male-female asymmetry in cost of sex has been quite convincingly demonstrated in an early study in dung beetles *Onthophagus binodis* (Kotiaho and Simmons, 2003). This study revealed that female beetles did not incur any cost of sex, whereas males do. In females, courtship and/or mating did not cause any obvious deleterious effects, but egg-production and brood provisioning lead to significant reduction in their lifespan (Hunt et al., 2002). Therefore, cost of sex in males appears to be mediated through different sub-processes of reproduction such as courtship, competition for the mates, mating or sperm production, while in females it appears to be mediated through mating, egg-production, egg-laying, and brood provisioning (Kotiaho and Simmons, 2003). The results of our study suggests that the effects of light regimes on the lifespan of virgin males and females are mediated through different processes in *D. melanogaster*, for instance, in virgin males it is mediated through the period of locomotor activity rhythm and/or duration of activity, but in the virgin females it is routed through

egg-output. Mating considerably reduces the lifespan of both sexes; the light regime-dependent reductions in males are abolished post-mating, whereas although lifespan of the mated females is considerably reduced compared to the virgin females the light regime-dependent differences still persist.

Our study, thus, clearly demonstrates the effects of light regimes and/or biological rhythms and reproduction on the adult lifespan of fruit flies *D. melanogaster.* Such effects of light regimes on life history traits have been reported earlier for pre-adult development time (Paranjpe et al., 2005). In an earlier study from our laboratory, we had reported that the effect of light regimes on duration of pre-adult development is partly mediated through circadian clocks. It is possible that light regimes simultaneously influence biological rhythms and lifespan giving rise to coincidental rather than causal correlations between clock phenotypes and life history traits. Moreover, circadian rhythms as well as life history traits are regulated by a number of genes and their complex interactions, and therefore it is quite possible that light regimes and/or circadian clocks regulate life history traits in different ways by potentially complex mechanism(s) (Paranjpe et al., 2005). Our study evaluates factors influencing lifespan such as period of LD cycles and/or biological rhythms, activity duration, and reproductive output. It also highlights the necessity to examine multiple fitness traits in order to understand the intricate details underlying the interactions between light regimes, circadian clocks and life history traits.

Chapter 5

Behavioral and physiological studies on flies reared under constant and periodic light regimes

5.1 Background

The information of light is conveyed to the circadian clocks in *D. melanogaster* via a number of modes such as the compound eyes, the ocelli, an extra-retinal structure called the Hofbauer-Buchner (H-B) eyelet (Helfrich-Förster, 2001; 2002), and directly by the circadian photo-pigment CRYPTOCHROME, which is expressed in the LNs and in a number of other body tissues (Emery et al., 2000). The compound eyes of *D. melanogaster* that convey information of light to the LNs are among the sites of *per* transcription (Liu et al., 1988; Saez and Young, 1988) and translation (Siwicki et al., 1988). A decrease in visual pigmentation was noticed within several hours of light onset when the flies were exposed to LD cycles (Stark et al., 1988). Moreover, studies on the visual system of housefly *Musca domestica* and fruit fly *D. melanogaster* (Pyza and Meinertzhagen, 1993; 1995; 1999), revealed that the lamina axons of L1 and L2 that receive photic input by the way of innervations from photoreceptors in the overlying compound eye, change their volume in a rhythmic manner (Pyza and Meinertzhagen, 1995) and the rhythm persists under constant conditions (Pyza and Meinertzhagen, 1999). Chen and coworkers (1992) studied visual sensitivity in the *per* mutants under LD cycles and under DD by recording electro-physiological response of retina (plotted as electro-retinogram or ERG). In their study visual sensitivity of the *w per*$^{+}$ flies measured under LD cycles started decreasing about 8 h before lights-on and about 4 h later it decreased by almost 75%, and these cycles continued under DD, but were less pronounced (Chen et al., 1992). The *per* mutants such as *per*^{S}, *per*^{L} and *per*^{01} showed rhythmic visual sensitivity under LD cycles while the cycles of visual sensitivity under DD, if any, were subtle (Chen et

al., 1992). Rhythmic structural changes in lamina axons, cycling of visual pigments and rhythm of visual sensitivity suggest that light input via compound eyes may not be upstream to the core clock mechanisms as was thought earlier but might be under the control of clock. Therefore, it is likely that light regimes that are known to modulate circadian rhythms might also influence the light input for circadian clocks as well as other clock associated features such as sensitivity of fly's retina to light stimuli, and clock's response to light stimuli.

In the previous chapters we have seen that the light regimes modulate rhythmic behaviors (Chapter 2) as well as pre-adult (Chapter 3) and adult (Chapter 4) fitness components. As discussed in the chapter 2, persistence of free-running rhythms does not guarantee that various other features of functional clocks are retained in the LL populations. The LL populations have never experienced any LD cycles for more than seven hundred generations, and have always been maintained under constant light of moderate intensity (~100 lux). There have been no systematic study on the effect of prolonged exposure to light on either the visual sensitivity, or on the sensitivity or response of clock to light stimuli, and it is quite possible that the response of LL populations to light may be different than the flies that are normally reared under periodic LD cycles. We therefore decided to study the photic-response of the LL populations at behavioral as well physiological levels to understand how the rearing light regimes affect the clock mechanisms or behaviors that may not be directly under the control of circadian clocks. For the sake of comparison, we used populations reared for more than hundred generations under DD and LD cycles of 12:12 h.

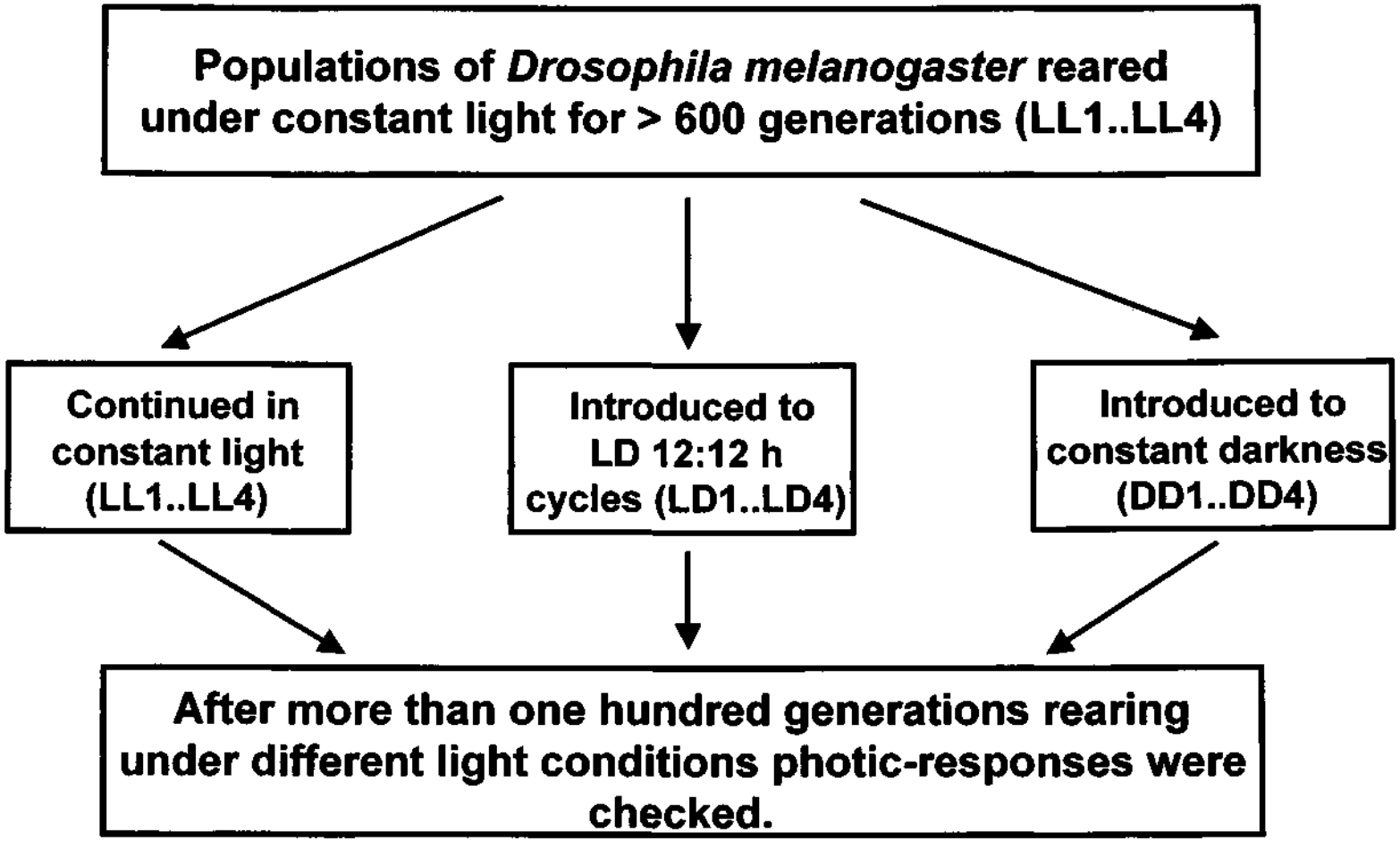

Figure 1: Schematic representation of protocol used for derivation and maintenance of different sets of populations from baseline LL populations.

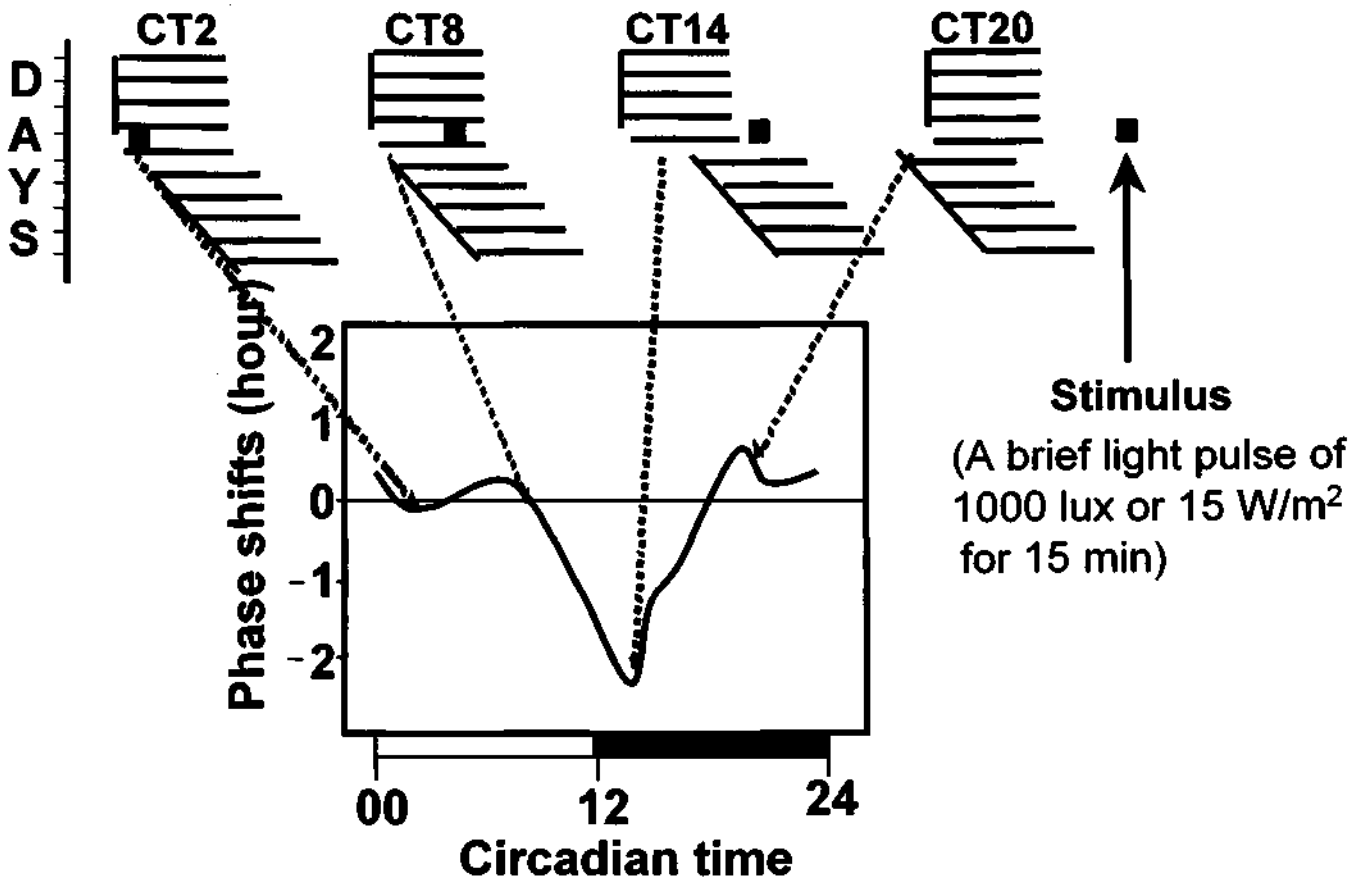

Figure 2: Protocol for plotting Phase Response Curve (PRC). See detailed protocol in text. The graph represents typical phase response curve obtained by giving a brief light stimulus. Subjective time or phase at which the stimulus is given is plotted along x-axis and response of circadian clock as phase shift in hours is plotted along y-axis. The light bar below graph indicates subjective day (circadian time 0 to 12) and dark bar indicates subjective night (circadian time 12 to 24). A brief light stimulus at CT14 usually elicits maximum phase delays while that at CT20 usually elicits maximum phase advances.

Two sets of four populations were derived from each of the baseline populations (Sheeba, 2001) (Figure 1). One set of four populations was maintained under LD cycles of 12:12 h (the LD populations), while the other set was kept under DD (the DD populations) for about hundred generations. All photic-response experiments described in this chapter were carried out on the LL, LD and DD populations.

5.2 Light induced PRC

We have seen in one of the earlier chapters (chapter 2) that the LL populations have the ability to entrain to a wide range of LD cycles. For stable entrainment to occur, circadian clocks must respond to light stimuli in a periodic manner (Sharma and Chandrashekaran, 2005; Sharma and Daan, 2002). Such phase-dependent responses of circadian clocks to entraining stimuli are usually documented in the PRC. These plots enable us to estimate circadian clock's response to brief perturbations, independently of the overt rhythmicity. For day active organisms such as *Drosophila*, active part of the activity/rest cycles under constant conditions is considered to be subjective day, and the period of inactivity as subjective night. Usually organisms are more sensitive to light stimuli during the subjective night compared to the subjective day. Light stimulus presented during the early subjective night usually phase delays the rhythm, while light exposure during the late subjective night causes phase advances (Figure 2). Photic response of circadian clocks can be measured by estimating phase shifts after giving light stimuli at different phases in the circadian cycle. In

order to study the effects of rearing light conditions on photic response of circadian oscillator, we compared the PRCs of LL, LD and DD populations by estimating phase shifts at two different phases. We chose: CT14 and CT20 (CT= circadian time or subjective time) because at these phases the clocks of *Drosophila* usually elicit maximum phase delays and phase advances, respectively.

5.2a Materials and methods

To rule out the possibility that the differences, if any, between the LL, LD and DD populations occur due to the parental rearing conditions and not due to the underlying genetic differences, all the populations were subjected to a common rearing conditions (standardization) for one generation before the actual experiment was done. For standardization, eggs laid on banana medium over a 2 h window from the running culture of each of the LL, LD and DD populations were collected into glass vials. From these populations, approximately 50-60 eggs were collected into 24 vials. The vials were kept under LD 12:12 h (*T24*) regime until the adult flies eclosed. Twelve days after the egg collection, adult flies were transferred to plexiglass cages. These caged populations were referred to as 'standardized' populations. From the standardized populations, eggs laid on banana medium over a 2 h window were collected for the PRC experiments. From each of the standardized populations (3 rearing conditions x 4 replicate populations), approximately 50-60 eggs were collected into each of 10 vials. The vials were kept under *T24* regime until the adult flies eclosed. Freshly

eclosed males were used for recording locomotor activity using the Drosophila Activity Monitoring system, TriKinetics Inc, Waltham, MA, USA. The locomotor activity of the males was recorded under LD 12:12 h conditions for the first ten days. After ensuring that most flies show stable entrainment to these conditions, they were shifted to DD conditions. On the second day under DD, different sets of flies from each population were exposed to a brief light pulse of ~ 1000 lux intensity and 15 minutes duration at CT14 (2 h after the extrapolated lights-off time when the flies were under *T24* conditions) (n = 20) and CT20 (8 h after the extrapolated lights-off time when the flies were under *T24* conditions) (n= 20). The light intensity was measured using Photo Meter (Model LI-250, LI-COR, USA). As control, one set of flies from each population (n= 20) were handled in the similar manner as the experimental flies except that they were not exposed to any light pulses. Following light pulse exposure, locomotor activity of the experimental and control flies was recorded for about 10 days under DD.

The offset of activity was taken as phase reference point to calculate the period of the rhythm because generally in case of *D. melanogaster* offsets show less cycle-to-cycle variability compared to the onsets. The τ of the locomotor activity rhythm was calculated using regression lines drawn through the offsets of activity over at least seven cycles under DD. The τ values thus obtained for each population were used as data for a mixed model ANOVA using STATISTICA™ (Statsoft, 1995), where the rearing conditions (LL, T24 and DD) were treated as fixed factor and the replicate populations were treated as random factor. Phase shifts were calculated using data of at least seven cycles under *T24* followed by

at least 7-8 cycles under DD conditions using method described by Sharma and Daan (2002). The phase shift values thus obtained for each population in each treatment (control, CT14 and CT20) were used as data for a mixed model ANOVA where the rearing conditions were treated as fixed factor and the replicate populations were treated as random factor.

5.2b Results

The results of ANOVA on the τ values revealed that the τ of locomotor activity rhythm of flies from all populations under all rearing conditions was comparable ($F_{2,6} = 0.18$, $p = 0.84$). The ANOVA on the phase shift values revealed that phase shifts obtained for different treatments (control, CT14 and CT20) were significantly different from each other ($F_{2,6} = 377.29$, $p < 0.001$). Post-hoc multiple comparisons using 95% CI around the mean revealed that the phase shifts obtained under all the three treatments were significantly different from each other; at CT14 flies from all populations showed phase delays (conventionally written as negative phase shifts), while at CT20 they showed phase advances (conventionally written as positive phase shifts). Magnitude of phase shifts in the control flies were significantly smaller compared to those at CT14 and CT20. Representative locomotor activity records of flies from LL, LD and DD populations that received different treatments (control, CT14, CT20) are shown in the Figure 3. The main effect of interaction between treatments and rearing conditions on the phase shift values was statistically significant ($F_{4,12} = 3.52$, $p < 0.05$). Post-hoc multiple comparisons using 95% CI revealed that flies

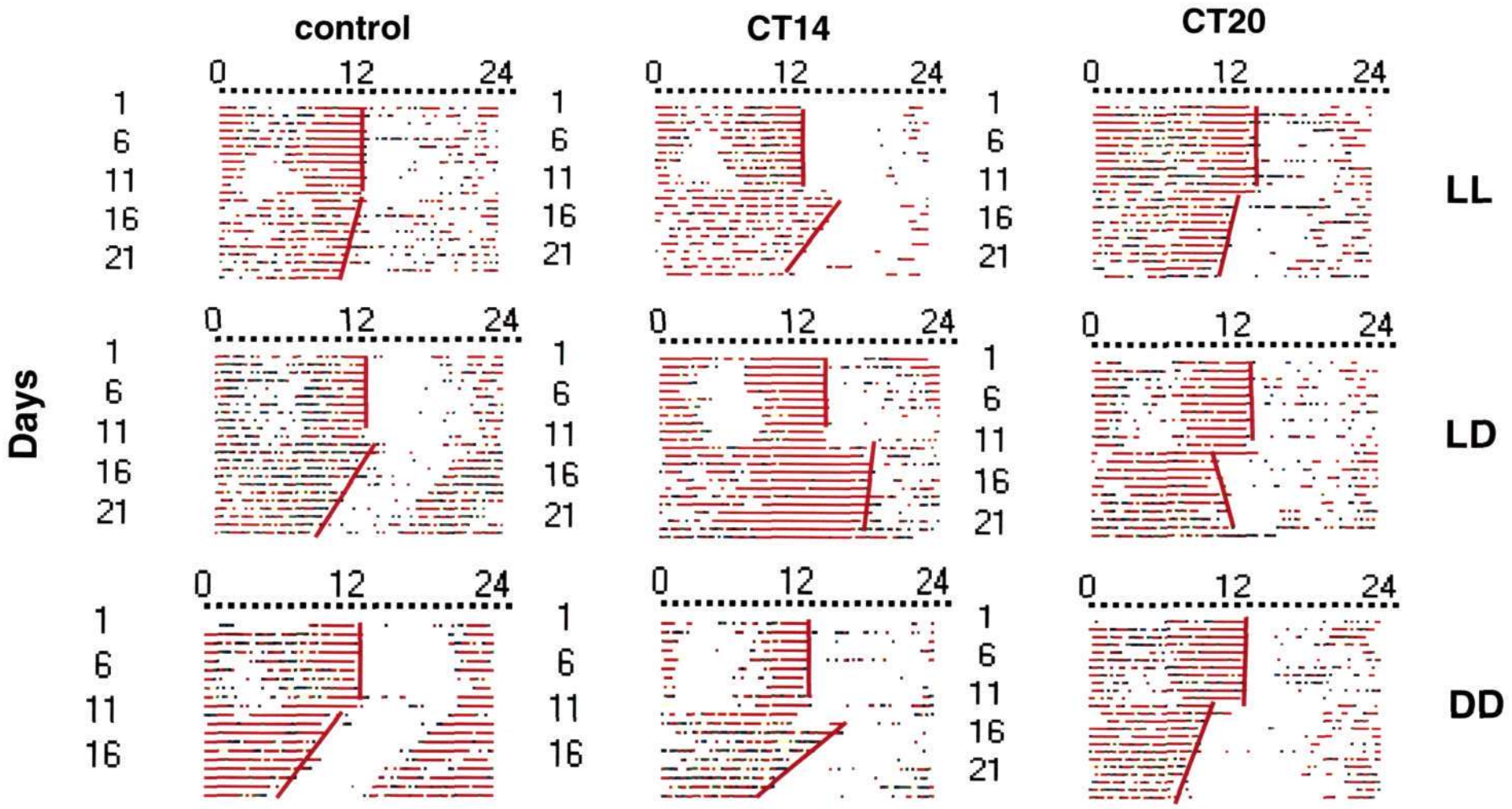

Figure 3: Representative recordings of locomotor activity of individual flies from LL, LD and DD populations. The locomotor activity was first recorded in LD 12: 12h cycles for 10 days after which flies were shifted to constant darkness. Light pulse of 1000 lux intensity was given at CT14 and CT20 for 15 minutes and the activity was monitored for subsequent 10 cycles. The thick vertical bars indicate a regression line across activity offsets. A brief light pulse at CT14 elicited phase delay, that at CT20 elicited phase advance while flies exposed to control treatment elicited minimal phase shifts, if any.

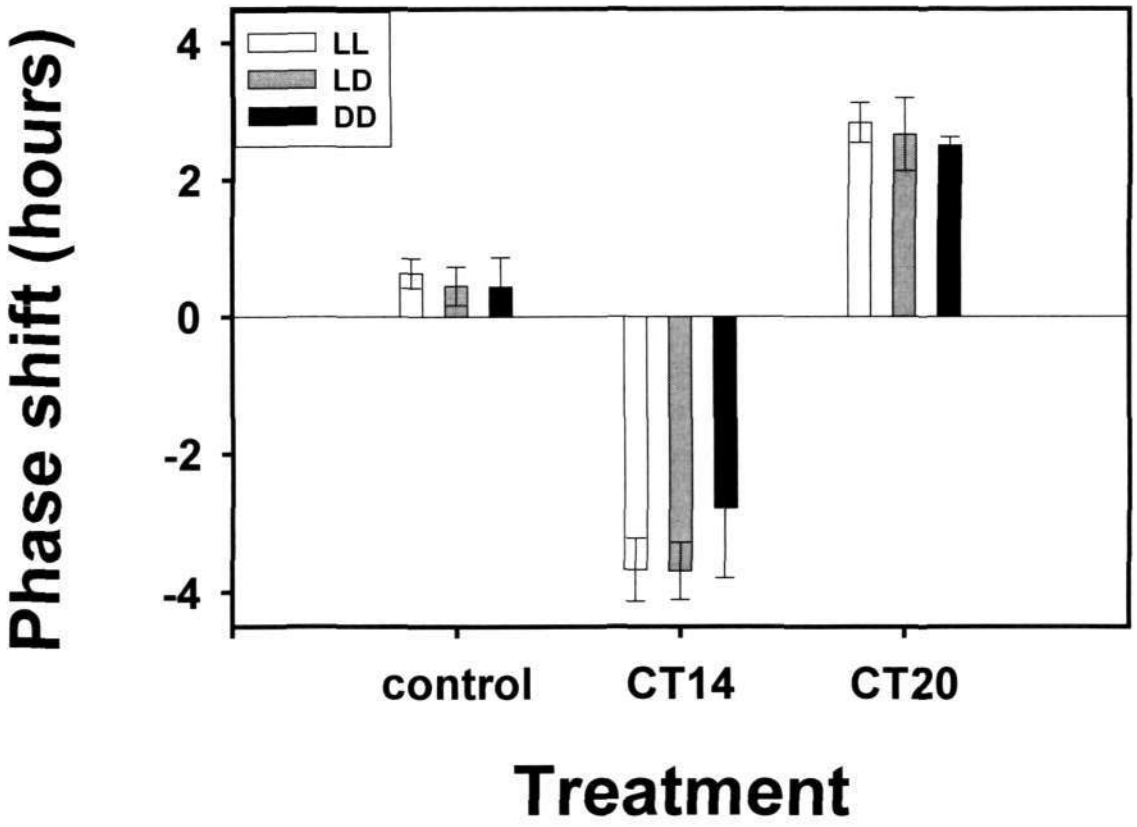

Figure 4: Phase shifts in LL, LD and DD populations. Light pulse treatments given to the flies are plotted along x-axis and phase shifts obtained for flies from LL, LD and DD populations are plotted along y-axis. Control flies showed very small phase shifts. Phase shifts in DD populations were less in magnitude compared to LL and LD populations at CT14 and CT20.

from the DD populations that received light pulse at CT20 showed significantly smaller phase advances compared to the flies from the LL populations, while phase shifts of flies from LD populations were comparable to those of LL and DD populations. At CT14, the phase shifts were comparable across all populations, although DD populations showed a tendency towards smaller phase delays than the LL and LD populations (Figure 4).

5.3 Electro-physiological response of the flies reared under different light conditions

The compound eyes of *Drosophila* consist of hundreds of smaller units called as ommatidia. A single ommatidium is formed of six peripheral and two central photoreceptor cells. The six peripheral photoreceptors R1-R6 are usually sensitive to low intensity and shorter wavelengths of light, while central photoreceptor cells R7-R8 are sensitive to high intensity and longer wavelengths of light (Chapman, 1998). Electro-retinogram (ERG) is an extra-cellular record of light-evoked membrane action potentials of retinal photoreceptor cells (Stark and Wasserman, 1974). In most diurnal insects, ERG consists of three characteristic components when recorded with the electrode on the cornea or in the receptor cell layer; positive on - and negative off - transient potentials track the onset and the offset of stimulus, respectively, while an asymptotic negative wave is sustained for the entire duration of the stimulus (Stark and Wassermann, 1974). The on - and off - transients arise from the R1-R6 connections in the first optic neuropil, the lamina ganglionaris, and are indicative of synaptic transmission from the photoreceptor cells to the laminar neurons, while the slow depolarization

wave is primarily a response of the photoreceptor cells to light (Heisenberg, 1971; Stark, 1972). If synaptic transmission between the laminar neurons and the photoreceptor cells is blocked, the on - off transients are preferentially lost (Palladino et al., 2002).

As stated in the earlier section (section 5.2b), the DD populations showed a tendency towards smaller phase shifts which could be due to two reasons: (i) the light input pathways of the DD flies might be impaired, or (ii) the photic response of the circadian clocks of the DD flies might have been be reduced significantly compared to the LL and LD flies. To investigate the first possibility we decided to measure the visual sensitivity by recording ERG of 4-6 days old flies from the LL, LD and DD populations at a specific time of the day.

5.3a Materials and methods

As described under the section 5.2a, the standardized populations were used to record the ERGs. From the standardized populations, eggs laid on banana medium over a 2 h window were collected for the experiment. From each of the standardized populations (3 rearing conditions x 4 replicate populations), approximately 50-60 eggs were collected into 5 vials. The vials were kept under *T24* regime till the adults eclosed. Freshly eclosed males and females were transferred into fresh food medium and were kept under LD cycles of 12:12 h for 3 days. The flies were finally transferred to DD on the fourth day after eclosion and the ERGs were recorded at CT14. For recording the electro-physiological activity of fly retina, an oscilloscope (Hewlett Packard 54603B, 60 MHz) was

used along with a 1X2-700 dual intracellular pre-amplifier (Cornerstone by DAGAN) to amplify the signals by about 10 times. For the actual recording 4-6 day old flies were anaesthetized on ice for 15-20 min. An anaesthetized fly was then embedded in modeling clay block with its head and thorax free. This restricted the movements of the fly when it wakes up. The silver wire electrodes were dipped in Sodium Hypochlorite solution prior to the recordings for at least 10 minutes. Thin borosilicate glass capillaries (1 mm outer diameter x 0.58 mm inner diameter, Harvard Apparatus LTD) were pulled with Narashige vertical puller (Heater setting 4.5, magnet setting 2) and were filled with 3M KCl solution using Microfil Syringe needle (Precision Instruments, model MF34G-5) ensuring that there is no air bubble in the capillary. The glass capillaries filled with electrolyte solution were then fixed to the silver wire microelectrodes used for recording the response. The ground microelectrode was then positioned such that the tip of glass capillary pierced thorax of the immobilized flies without killing it. The recording microelectrode was then positioned such that the tip of glass capillary just touched the retina. After fixing the electrodes, the flies were dark adapted for about 5 min. A white light (Dichroic halogen-cold light source) with average intensity of 50 lux was flashed on fly retina with a frequency of 3 sec (on-off pulse) and the changes in action potential of the retinal photoreceptors were traced on the oscilloscope with display time 1.0 sec/unit and a delay of 5 sec (50.0 mV/ unit). 10 flies from each of populations (5 males and 5 females) were used for recording. Thus, totally 120 flies were recorded (10 flies x 3 rearing conditions x 4 replicate populations). All flies were recorded at 24 ^{0}C. The ERG

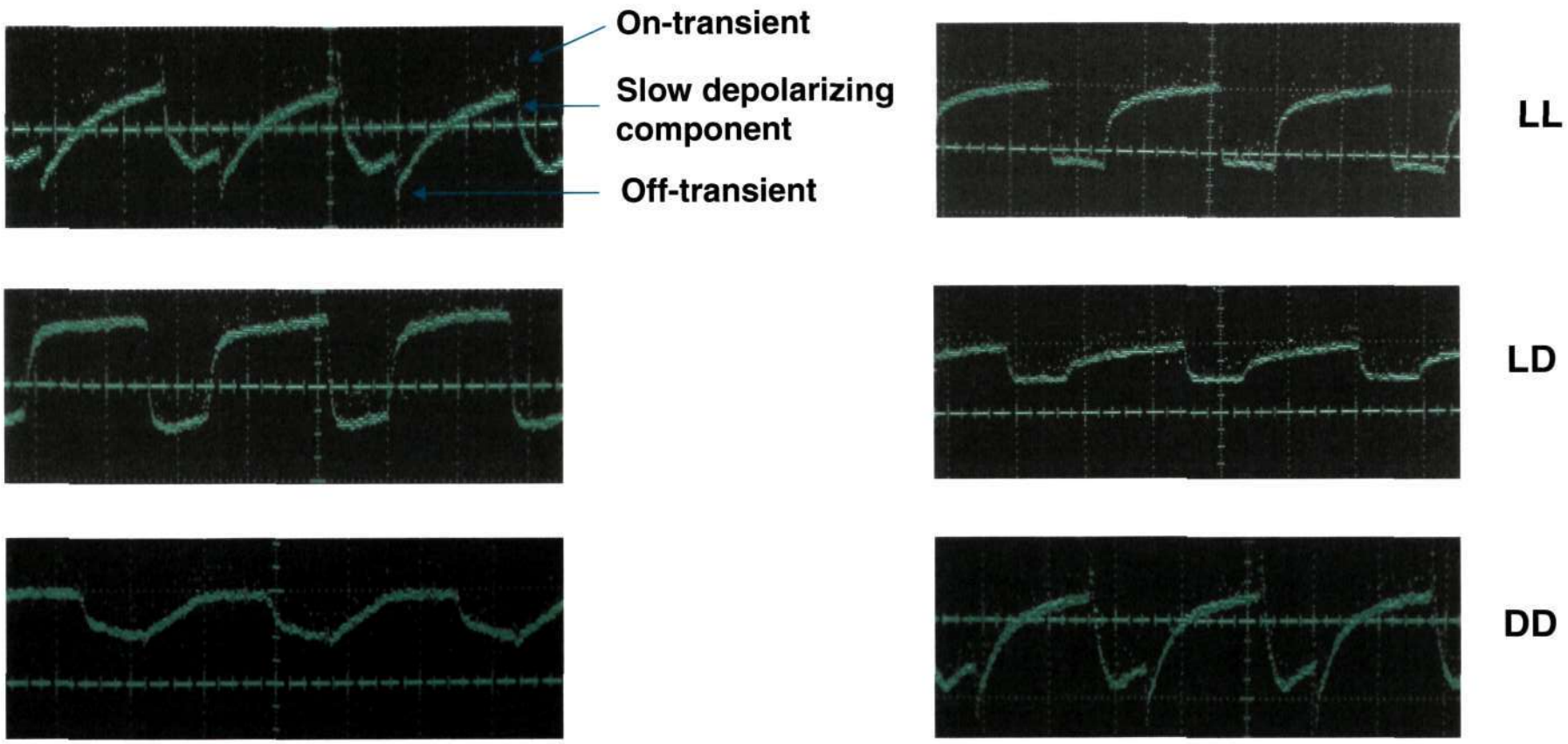

Figure 5: Representative Electro-retinograms (ERGs) of flies from LL, LD and DD populations. A large variation was seen in the ERG patterns recorded for flies across different rearing conditions. A considerable fraction of flies from all populations showed absence of on- and off transients, while some additionally showed abnormal shape/ slope of slow depolarizing component. Percentage of flies showing such abnormal patterns was comparable across rearing conditions.

traces obtained were photographed from a fixed distance at 3X magnification using Nikon Coolpix3100 digital camera (resolution 3.2 megapixels) and the photographs were used for further analysis.

5.3 b Results

A large variation was seen in the ERG patterns of the flies from the three populations (LL, LD and DD). About 35-50% of flies across all the rearing conditions (LL, LD, and DD) showed complete absence of on - off transients (Figure 5). In addition, in a considerable proportion of flies the slow depolarizing component of ERG trace was either abnormal in shape or the slope of the depolarizing wave was reduced giving rise to almost flat traces (Figure 5). The percentage of flies showing such abnormal patterns was also similar across all the three rearing conditions. The amplitude of the ERG records measured as the difference between the highest and lowest point of ERG trace in mV was also comparable across the three rearing conditions ($F_{2,6}$ = 0.82, p = 0.48). The amplitudes of ERG records in the male and female flies did not differ significantly ($F_{1,3}$ = 1.99, p = 0.25).

5.4 Photic-response of larvae

The *Drosophila* larvae have only one visual structure – the BO, which comprises of 12 photoreceptors located on both sides of the head. The BO axons project into the central brain where they terminate at the dendrites of the four LNs in each brain lobe (Kaneko et al., 1997; Malpel et al., 2002, Mazzoni et al., 2005).

The BO is also necessary for the third instar foraging larvae for its visual tasks such as avoiding light (Sawin-McCormack et al., 1995). A recent study on the visual and circadian systems in the *Drosophila* larvae demonstrated that the pacemaker neurons not only receive light information from the BO, but also relay this information to produce a rapid photo-phobic response (Mazzoni et al., 2005). The larvae of the *yw* genotype (controls) showed maximum response (i.e. the highest photo-phobicity) towards the end of the subjective night (CT0), and the lowest response towards the end of the subjective day (CT12). The time-dependent response was abolished in the per^0 and cyc^0 mutants (Mazzoni et al., 2005). Further, clock manipulated larvae were found to be defective in light avoidance behaviour, quite similar to the larvae that lacked all the photoreceptor cells, suggesting that circadian clocks may regulate larval photic-responses in *D. melanogaster* (Mazzoni et al., 2005). Since a considerable fraction of adult flies from the LL, LD and DD populations showed impairment of electro-physiological response of the retina (See section 5.3b), we decided to check whether photic-response of the larvae in terms of general light avoidance behavior from the LL, LD and DD populations was altered due to differences in rearing conditions.

5.4a Materials and methods

For measuring larval the photic-response, eggs laid on banana medium over 2 h were collected from the standardized populations (See section 5.2a). The midpoint of egg collection window was taken as 0 h. The plates were kept under LD 12:12 h condition for 48 h and then transferred to DD. The early third instar

larvae (85 h from mid point of egg collection window) were picked up from the banana medium with the help of number '0' soft brush and washed with PBS for ~30 sec. A petri-plate (8.5 cm diameter) with 20 ml of 1.5% agar-agar (Qualigens) was used for the assay with half of plate and corresponding half of lid covered with black electrical tape from outside to block light. Twenty larvae washed with PBS from each population were then placed on the agar plate on the boundary of light and dark halves. The plates were illuminated from above using Philips cool light tubes such that the intensity at the level of petri-plate was ~100 lux. The light intensity was measured using Photo Meter (Model LI-250, LI-COR, USA). The plates were left at room temperature for 15 min, at the end of which the number of larvae in each half was counted. The photic response was checked at two time points, CT2 and CT14. As a control, twenty larvae from each population were subjected to similar treatments without the light exposure and the number of larvae in each half of the plate was counted at the end of 15 min. This provided us with an idea of the random movement of larvae in absence of light stimulus on the agar surface. Six trials were done for each population at each time point (6 trials x 2 time points x 3 rearing conditions x 4 replicate populations).

The fraction of larvae in the two halves of the plate was calculated for both control and light treatments. The population mean of the control trials was subtracted from those for light treatment to get the 'relative fraction of larvae in light'. The relative fraction of larvae in light portion of the agar plate was used as data in a mixed model ANOVA where time point and rearing conditions were

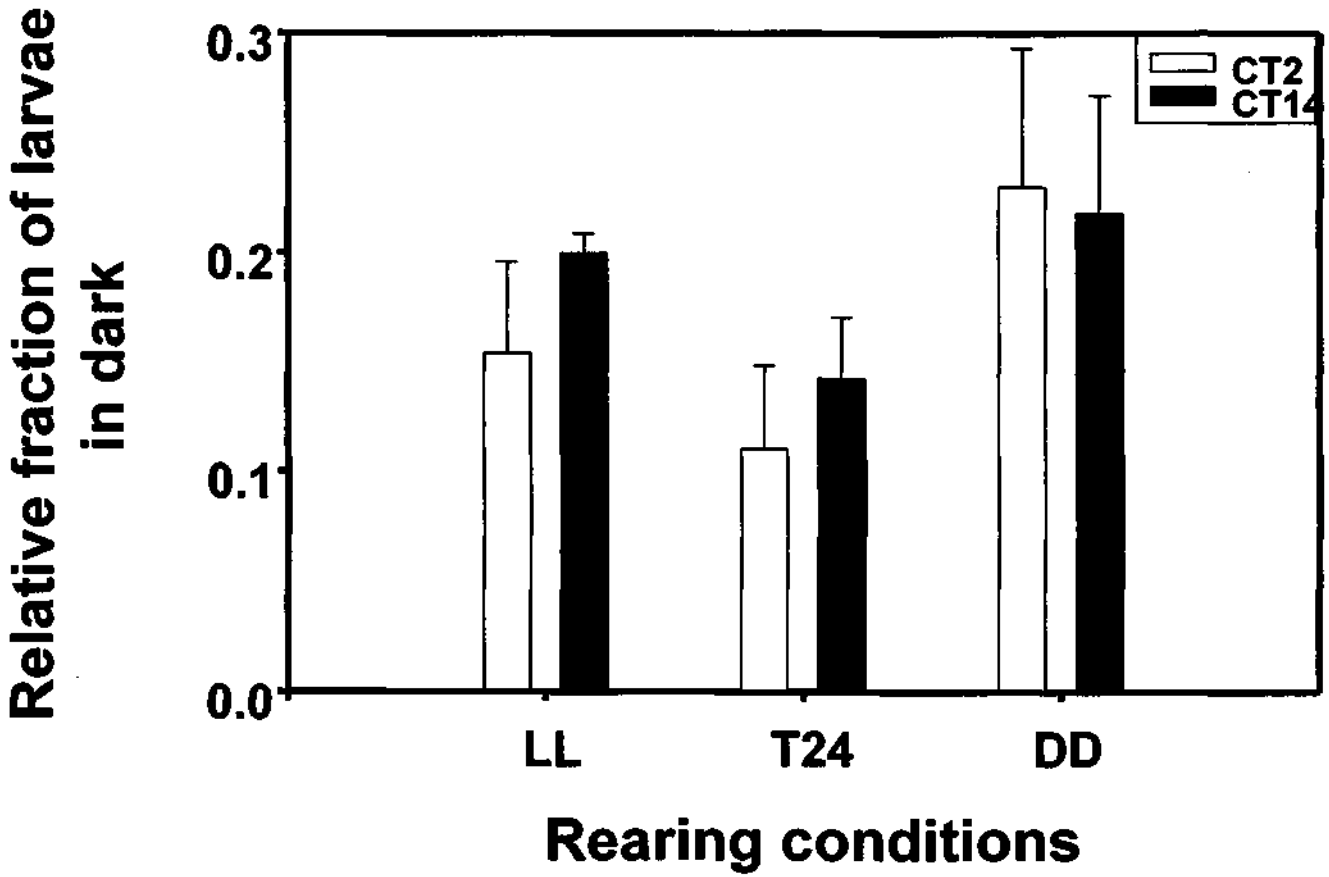

Figure 6: Larval photo-response. Rearing conditions from which larvae were picked up for the assay are plotted on x-axis and relative fraction of larvae found in dark portion of the petri-plate at the end of 15 min are plotted on y-axis. Light bars represent results of trials done at CT2 and dark bars indicate results of trials done at CT14. Greater fraction of larvae from DD populations preferred dark than LL and T24 larvae. The dark preference was not time-dependent.

treated as fixed factors while replicate populations were treated as random factor.

5.4b Results

The results of ANOVA done on the relative fraction of larvae found in the dark portion of the plate revealed a significant main effect of rearing conditions on the photo-phobicity ($F_{2,6} = 6.41$, $p < 0.05$). Post-hoc multiple comparisons using 95% CI around the mean showed that a significantly greater fraction of larvae from the DD populations preferred darkness compared to those from the LL and LD populations (Figure 6). The relative photic-response was similar at CT2 and CT14 for all populations across all three rearing conditions ($F_{1,3} = 3.75$, $p = 0.15$).

5.5 Photic-response of adults

Most species of *Drosophila* studied so far show an escape reaction from light; the flies when disturbed start flying towards light (Dobzhansky et al., 1974). The movement of flies towards light (photo-taxis) is a trait that has been traditionally used for laboratory selection studies on several species of *Drosophila* such as *D. melanogaster, D. pseudoobscura, D. persimilis,* and *D. subobscura* (Dobzhansky et al., 1974 and references therein). Populations of *D. pseudoobscura* collected from the wild were photo-neutral on average. However, when subjected to directional selection for positive or negative photo-taxis gave rise to populations having high or low photo-taxis scores, respectively, within 10 or more

generations (Dobzhansky et al., 1969). Although ecological roles of such responses to light are still little known it is believed that photic-responses are adaptive (Dobzhansky et al., 1974).

The clock responses to light stimuli, measured as phase shifts, were weaker in the DD populations compared to the LL and the LD populations (section 5.2b), and a considerable fraction of adults from all the three (LL, LD and DD) populations showed impairment of electro-physiological response of retina (See section 5.3b). This suggests that even though the light input was impaired to certain extent in all the three populations, the fraction of flies showing impairment of retinal response was comparable across all rearing conditions. We decided to compare the photic-response of adult flies from the LL, LD and DD populations in order to test whether the photic-response is impaired in the DD populations.

The results of a recent study on the loss of function mutants of *D. melanogaster* suggested that the larval photo-responses are regulated by the circadian clocks (Mazzoni et al., 2005). Therefore, it is possible that clock also modulates photo-responses in adult flies. To investigate the role of circadian clocks in the light response behaviour of the adults we assayed the adult photic-responses of LL , LD and DD populations at two time points CT2 and CT14 using flies from all three rearing conditions. Further, to confirm the role of clocks and clock genes in the rhythmic photic-response behaviour, we used various mutants and control flies. The clock mutants used in our experiments had loss-of-function

mutations in some of the core clock genes or in the genes in the light input pathways.

5.5a Materials and methods

Photic-response of the LL, LD and DD flies was checked using a countercurrent apparatus and using the protocol described by Benzer (1967). For the adult photic-response experiments on the LL, LD and DD populations, eggs laid on banana medium over 2 h were used. From each standardized population (See section 5.2a) 50-60 eggs were collected in each of the five vials and the vials were then kept under LD 12:12 h cycles until the adults eclosed. Freshly eclosed flies were grouped into six groups of 20 flies each (mixed male-female groups) under CO_2 anesthesia. Six such groups from each population were then kept under *T24* regime for 3 days after which they were transferred to DD. In the second cycle of DD, photic-responses of the LL, LD and DD flies were checked at CT2 and CT14, at two ambient light intensities (100 lux and 1000 lux). Fluorescent white light (Rechargeable white lantern, Hitachi, Japan) was used as light source and the light intensity at the level of experimental glass vials was adjusted by altering the distance between the counter-current apparatus and the light source. The light intensity was measured using Photo Meter (Model LI-250, LI-COR, USA). Eleven glass test tubes (9 cm height x 2.4 cm diameter) were joined by a sliding Lucite rack (five on top and six on bottom)(Figure 7). One group of twenty flies was transferred to the 0^{th} tube. The apparatus was laid horizontally in front of a fluorescent white light source that was kept horizontal

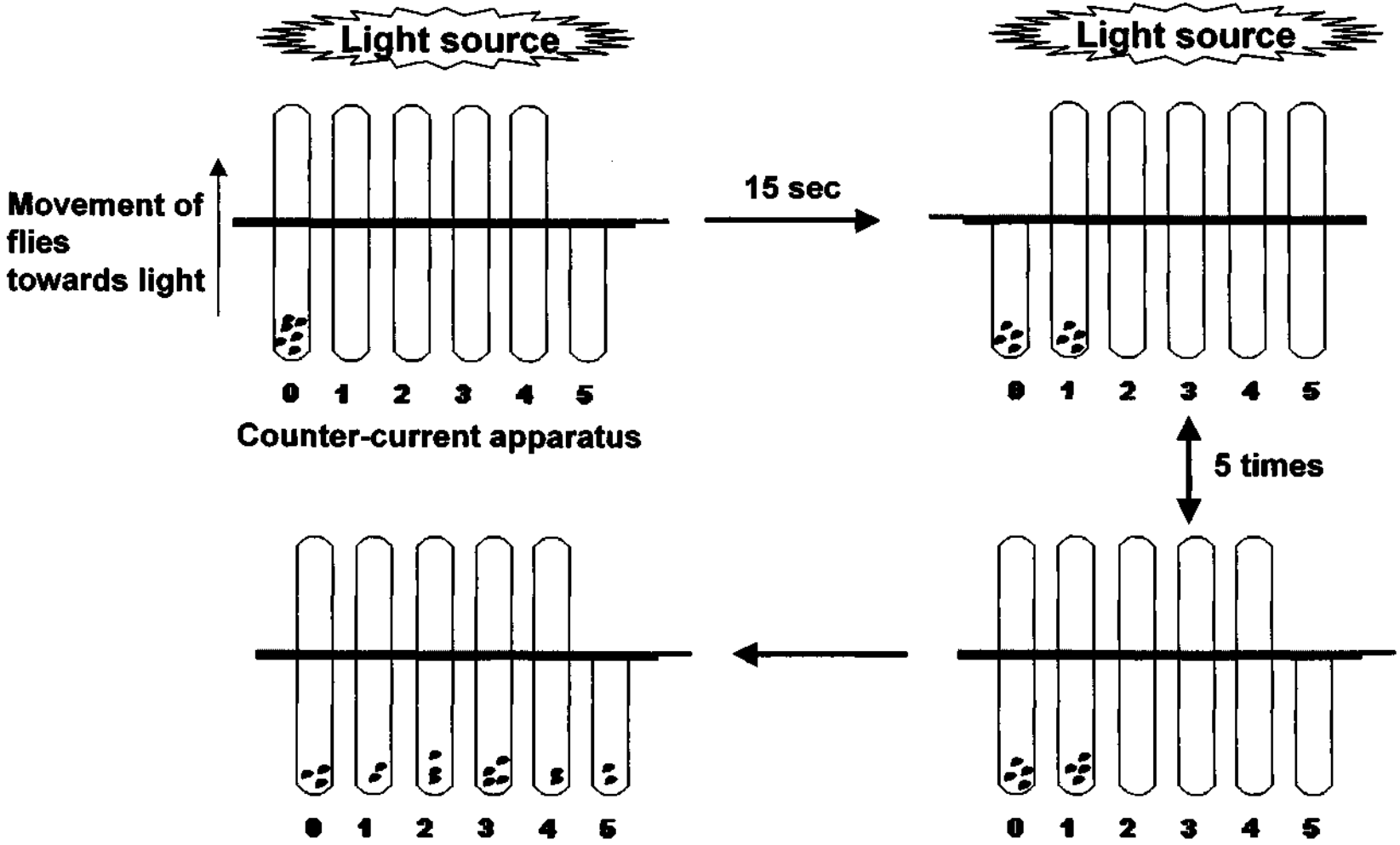

Figure 7: Schematic representation of protocol used for measuring photic-response of adult flies from LL, LD and DD populations. A group of flies is introduced in 0^{th} tube of counter-current apparatus. The light source is switched on and flies are allowed 15 sec to move towards to light. The tubes are then slid horizontally and flies tapped down, thus, transferring flies that moved towards light in 15 sec to adjacent tube. This is repeated five times and the fraction of flies in each tube to at the end of five runs is counted to give a response index.

and perpendicular to the tubes. The light was then switched on and the flies were given 15 sec to respond by moving toward the light before being transferred to the neighboring tube. After five such runs, the number of flies in all six tubes was counted and used for calculating response index. The flies that were initially placed in the 0^{th} tube had five chances to pass towards the tube adjacent to it. If the flies that are present in the 0^{th} tube pass through all the tubes, then at the end of 5^{th} trial they would find themselves in sixth tube. These flies would have responded and moved towards light all five times. Thus, the flies that reach tube 4 would have succeeded four times in going towards the light source. Six trials were performed for each population of flies at each time point (6 trials x 2 light intensities x 2 time point x 4 replicate populations). As control, six groups from each population were run through counter current apparatus at both time points (CT2, CT14) in the absence of light keeping rest of the experimental conditions similar.

The response Index (RI) for each trial was calculated and compared across time points and rearing conditions. To calculate the response index the numbers of flies that were present in each tube at the end of the experiment were taken as n_0, n_1, n_2, n_3, n_4, and n_5. The total score for the group of 20 flies used in one trial would be $(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4) + (5 \times n_5)$. The total score is then scaled to the maximum score that can be obtained if all flies reached fifth tube, i.e., $5 \times (n_0 + n_1 + n_2 + n_3 + n_4 + n_5)$. Thus, response index is calculated as

$$\frac{(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4) + (5 \times n_5)}{5 \times (n_0 + n_1 + n_2 + n_3 + n_4 + n_5)}$$

Response index values calculated from the control trials were subtracted from the RI values from the experimental trials of each population at both time points. Relative Response Index (RI) values thus obtained for each population (100 lux and 1000 lux) was used as data in mixed model ANOVA where rearing conditions and time points were used as fixed factors and replicate populations were taken as random factor.

To investigate the role of circadian clocks in the regulation of adult photic-responses, mutant flies that had defect in light input pathways (cry^b, gl^{60J}) or in the core clock mechanism (per^0, tim^0, cyc^0, clk^{irk}) were used to measure the photo-responses at CT2 and CT14 using 100 lux intensity of light. CantonS, *yw* flies were used as controls. The response index values were used as data for ANOVA whereas genotype and time points were considered as fixed factors.

5.5b Results

The results of ANOVA done using the response index data revealed that the photic-responses of adults from the LL, LD and DD populations were intensity dependent ($F_{1, 3} = 898.02$, $p < 0.001$). Post-hoc multiple comparisons using 95% CI showed that the responses at 1000 lux intensity were significantly greater than those at 100 lux (Figure 8). ANOVA further revealed a significant main effect of rearing conditions on the response index ($F_{2, 6} = 23.84$, $p < 0.05$). The relative response index values for the DD populations were significantly smaller compared to the LL and the LD populations indicating that adults from the DD

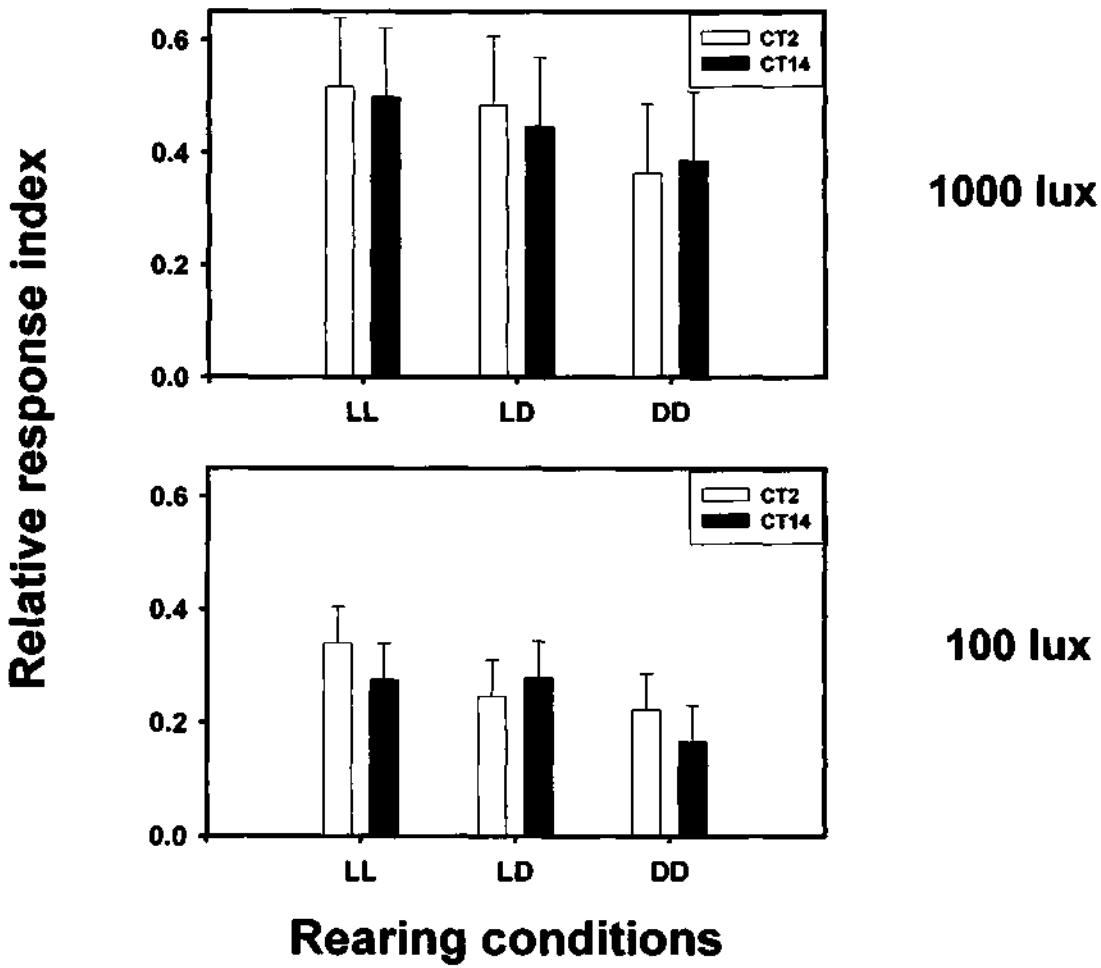

Figure 8: Photo-response of adult flies from LL, LD and DD populations. Rearing conditions of the populations are plotted on x-axis and relative response index is plotted on y-axis. Light bars represent results of trials done at CT2 and dark bars indicate results of trials done at CT14. Adults from DD populations showed significantly less photo-tactic behavior compared to LL and LD populations. The photo-responses were intensity dependent and time-dependent in LL populations at 100 lux.

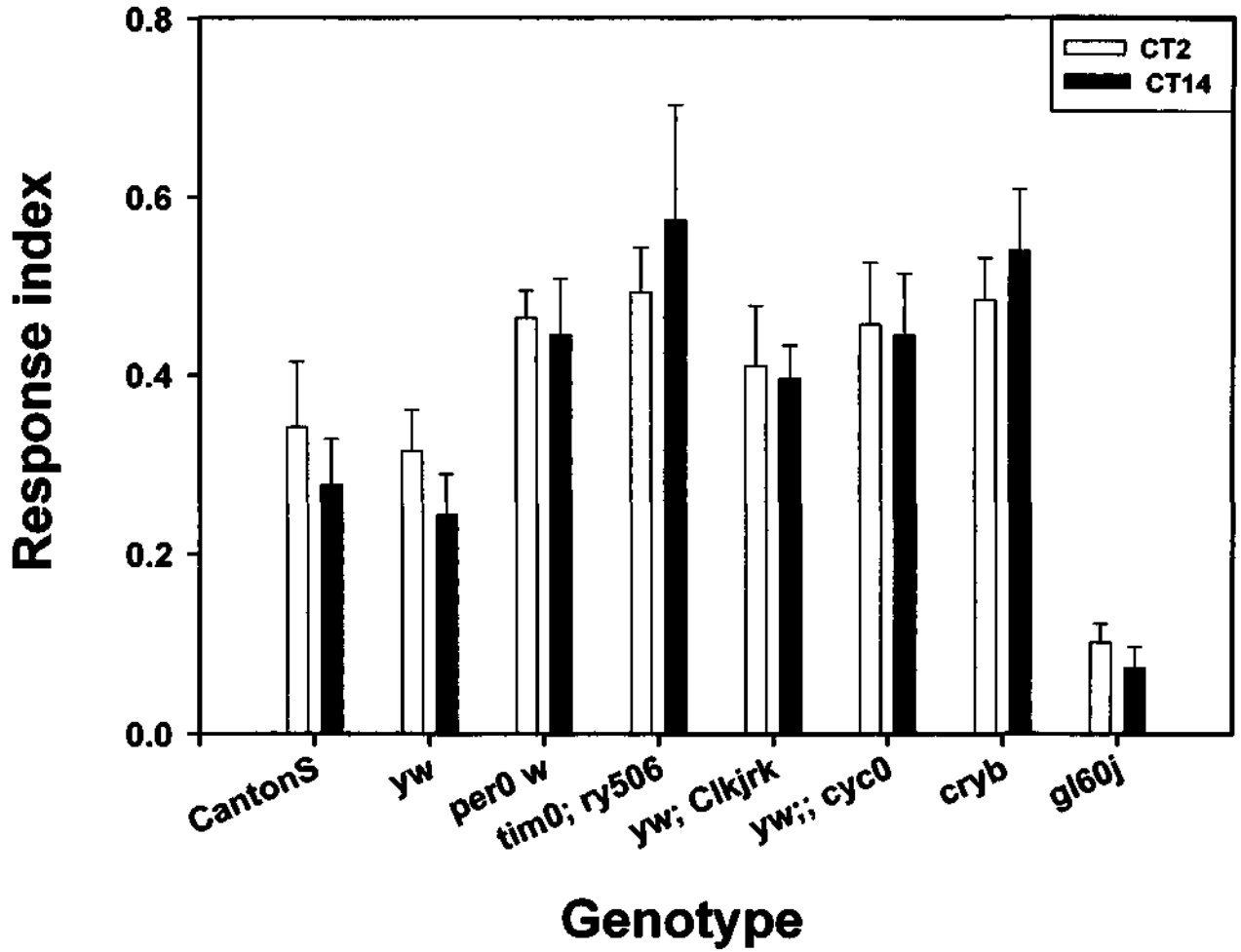

Figure 9: Adult photo-responses in clock mutants. Genotypes used for the experiment are plotted on x-axis and response index values plotted on y-axis. Adult photo-responses were time dependent in control genotypes, such time-dependence was absent in the loss-of-function mutants in core clock genes and light input pathways.

populations show less photic-response compared to the LL and the LD flies (Figure 8). Moreover, the relative response indices calculated at 1000 lux intensity ($F_{1, 18}$ = 0.07, p = 0.79) were comparable at both time points (CT2, CT14), while at 100 lux intensity the response was time dependent in LL populations (Figure 8). In summary, DD flies show less response to light intensities of 1000 as well as 100 lux at both time points compared to LL and LD flies.

ANOVA on the response index values of various clock mutants revealed a significant main effect of genotype ($F_{7, 76}$ = 48.27, p < 0.001). Post-hoc multiple comparisons using Scheffe's test showed that CantonS, *yw*, per^0, tim^0, cyc^0, clk^{Jrk} and cry^b showed significantly higher RI (p < 0.001) compared to the gl^{60J} flies (Figure 9). ANOVA on the CantonS and the *yw* flies followed by multiple comparisons using Scheffe's test revealed that the flies from both the genotypes showed significantly greater photic-response at CT2 than at CT14 ($F_{1, 19}$ = 6.13, p < 0.05) (Figure 9). Thus, the adult photic-response of the control flies was time dependent, while the time-dependency was lost in the per^0, tim^0, cyc^0, clk^{Jrk} and cry^b flies.

5.6 Conclusions

Light is one of the prominent time cues for circadian clocks that can influence behavior and physiology of the organisms to a great extent. It is often speculated that light/dark fluctuations in the environment have acted as one of the primary selection pressures for adaptive evolution of circadian clocks (Hastings et al.,

1991). However, there has been hardly any evidence for the role of LD cycles in the evolution of circadian clocks. We used *D. melanogaster* populations reared under environments that differed merely in terms of light conditions. Earlier studies from our laboratory using the same populations, about 100 generations ago had shown that some of the clock properties such as free-running period, phase-relationship were altered in the LL, LD and DD populations in about 30 generations of rearing under different light conditions (Sheeba, 2001). This lead us to think that, in addition to the clock properties, some of the clock-associated features such as response to light might have been altered in these populations due to rearing for many generations under different light conditions. To verify this conjuncture, we measured photic-responses of circadian clocks at behavioral and physiological level. The results of our experiments demonstrate that responses of circadian clocks seem to be reduced in DD populations to certain extent as evident by smaller phase shifts obtained for DD populations at both time points (CT2 and CT14) compared to the LL and LD populations. Little is known about the extent to which the clock mechanisms are involved in the behavioral responses. However, comparatively smaller phase shifts in DD populations indicate that sensitivity of the circadian clock was altered due to rearing under DD. The smaller phase shifts values obtained for the DD populations could, in principle, result from reduced sensitivity to light stimuli or due to differences in the ability to respond to light. We investigated both the possibilities by measuring light sensitivity of fly retina and ability to respond to light stimuli using flies from all three rearing conditions.

The two light input pathways in *Drosophila* (ocular and extra-ocular) appear to be redundant as the locomotor activity rhythm of flies lacking either functional CRY or functional visual system could still be synchronized by LD cycles, whereas flies lacking both could not be synchronized (Helfrich-Förster et al., 2001). Although we do not have any knowledge about the fraction of total light information reaching the core circadian pacemaker via retina or via CRY protein, it is generally believed that the retinal photo-receptors receive and relay a considerable amount of light information to the core pacemaker. We measured the response of retinal photo-receptors of the flies from the LL, LD and DD populations. The electro-physiological response of the retina suggests that about 35-50% of flies from populations reared under all three conditions (LL, LD, DD) had abnormal ERG response. Therefore, it appears that the light input pathways might be impaired in all the three populations to certain extent. Since the LD and the DD populations have been derived from the LL populations, these results suggest that the light input was probably impaired in the LL populations to start with, and 100 generations of rearing under LD and DD regimes did not cause any measurable change. Further, the larvae as well as the adults from the DD populations showed greater preference for darkness, compared to the LL and the LD populations. In other words, although the retinal photo-receptors were impaired in comparable fraction of adults across all rearing conditions, behavioral photic-responses were reduced in a greater percentage of individuals from the DD populations compared to the LL and the LD populations. This motivated us to speculate that the light input pathways downstream to the retinal photo-receptors

might have been affected and/or the levels of CRY expression might be reduced in the DD flies. Alternatively, the TIM protein degradation might be reduced to a greater extent in the DD flies compared to the LL and the LD flies as TIM is known to interact with CRY in the presence of light after which TIM is sent for degradation (Busza et al., 2004).

In a recent study on the clock mutants of *D. melanogaster* the authors suggested that the clock neurons transmit and modulate visual information that controls rapid behavioral responses in larvae (Mazzoni et al., 2005). In this study the photic-response was not measured. Given that the larvae and adult photo-entrainment mechanisms for the circadian clocks in *Drosophila* differ considerably we decided to assay the photic-responses in adult. To study the involvement of circadian clocks in adult photic-responses, we used a number of clock mutants that had mutations in the core clock genes or in the genes involved in the light input pathways. Interestingly, the wild type (CS and *yw*) flies showed time-dependent photo-tactic response which was absent in the clock mutants, illustrating the fact that circadian clocks regulate the rapid behavioral responses in *Drosophila*. Our results are consistent with those observed in *D. melanogaster* larvae (Mazzoni et al., 2005), where larvae showed time-dependent preference for darkness in control strains which was absent in the clock mutants. In our study, the role of circadian clocks in adult photic-responses is further strengthened by the fact that photo-tactic responses were not time-dependent in the cry^b mutants, where the photo-pigment dedicated for light input to circadian clocks is non-functional; however, the magnitude of photic-responses were

comparable to the wild type strain. Mutation in the *glass* gene, an important transcription factor in the photo-transduction pathway (Helfrich- Förster, 2005) reduced the photic-responses to a great extent. Moreover, the time-dependency of light response was lost in the *glass* null mutant, suggesting that both the light input pathways might be important in determining the time-dependent photic-responses in the adult flies. This is, to the best of our knowledge, the first study of its kind demonstrating the involvement of circadian clocks in adult photic-response behaviour in *D. melanogaster*.

The results of our studies clearly demonstrate that the rearing conditions have significantly altered photic-responses of larvae and adults at least at the behavioral level, and have also affected the light response properties of the underlying circadian clocks. It is important to note here that the differences in the photic-responses measured in different assays are genetic and not due to memory of the parental environment since the populations from all rearing conditions were exposed to common environmental conditions for one generation, prior to the actual experiments. Moreover, we have used wild type large, out bred, replicate populations which greatly reduce the probability that we might have seen such results by chance or due to inbreeding or due to pleiotropic effects of any mutation(s). In summary, light conditions appear to have long term evolutionary effects on the clock associated features such as sensitivity or response to light stimuli. Although, the mechanism(s) by which the light responses are modulated by circadian clocks is not yet clear, it seems to play a

significant role in the regulation of time-dependent behavioral photic-responses in the fruit flies *D. melanogaster*.

References

Allemand R. Les rythmes de vitellogenese et d' ovulation en photoperiode LD 12 :12 de *Drosophila melanogaster*, *J. Insect Physiol.* **22**, 1031-1035 (1976a).

Allemand R. Influence de modifications des conditions lumineuses sur les rythmes circadiens de vitellogenesis et d' ovulation chez *Drosophila melanogaster*, *J. Insect Physiol.* **22**, 1075-1080 (1976b).

Allemand R. Influence de l' intensite d' eclairment sur l' expression du rythme journalier d' oviposition de *Drosophila melanogaster* en conditions lumineuses LD 12 :12, *C R Acad. Sci. Paris D* **284**, 1553-1556 (1977).

Aschoff J. The phase angle of entrainment. In *Circadian Clocks.* Eds Aschoff J. Amsterdam, North-Holland Publications, p. 262-276 (1965).

Bakker K, Nelissen FX. On the relations between the duration of the larval and pupal period, weight and diurnal rhythm in emergence in *Drosophila melanogaster*, *Entomol. Exp. Appl.* **6**, 37-52 (1963).

Bauer SJ. Sex differences in pupation site choice in *Drosophila melanogaster*, *Dros. Inf. Ser.* **60**, 58 (1984).

Beaver LM, Gvakharia BO, Vollintine TS, Hege DM, Stanewsky R, Geibultowiz JM. Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA* **99**, 2134-2139 (2002).

Beaver LM, Rush BL, Gvakharia BO, Geibultowiz JM. Noncircadian regulation and function of clock genes *period* and *timeless* in oogenesis of

Drosophila melanogaster, *J. Biol. Rhythms* **18**, 463-472 (2003).

Beersma DGM, Daan S, Hut RA. Accuracy of circadian entrainment under fluctuating light conditions: Contributions of phase and period responses, *J. Biol. Rhythms* **14**, 320-329 (1999).

Bell G. Evolutionary and non-evolutionary theories of senescence, *Am. Nat.* **124**, 600-603 (1984).

Benzer S. Behavioral mutants of *Drosophila* isolated by counter-current distribution, *Proc. Natl. Acad. Sci. USA* **58**, 1112-1119 (1967).

Bloch G, Toma DP, Robinson GE. Behavioral rhythmicity, age, division of labor and period expression in the honeybee brain, *J. Biol. Rhythms* **16**, 444-456 (2001).

Blume J, Bünning E, Günzler E. Zur Aktivitatsperiodik bei Hohlentieren, *Naturwissenschaften* **49**, 525 (1962).

Bourke AFG, Franks NR. *Social Evolution in Ants*. Princeton, New Jersy, Princeton University Press (1995).

Brower LP. Monarch butterfly orientation: missing pieces of a magnificent puzzle, *J. Exp. Biol.* **199**, 93-103 (1996).

Bünning E. Zur Kenntniss der endogonen Tagesrhythmik bei insekten und pflanzen, *Ber. Dt. Bot. Ges.* **53**, 594-623 (1935).

Busza A, Emery-Le M, Rosbash M, Emery P. Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception, *Science* **304**, 1503-1506 (2004).

Cashmore AR, Jarillo JA, Wu YJ, Liu D. Cryptochromes: Blue light receptors for

plants and animals, *Science* **284**, 760-765 (1999).

Chandrashekaran MK, Johnsson A, Engelmann W. Possible "dawn" and "dusk" roles of light pulses shifting the phase of circadian rhythm, *J. Comp. Physiol.* **82**, 347-356 (1973).

Chapman RF. *The insects: Structure and function.* 4th Edition, Eds. Chapman RF, Cambridge University Press, Cambridge, UK, (1998).

Chen D, Christianson SJ, Sapp R, Stark WS. Visual receptor cycle in normal and period mutant *Drosophila*: Microspectrophotometry, electrophysiology, and ultrastructural morphometry, *Visual Neurosci.* **9**, 125-135 (1992).

Chippindale AK, Leroi AM, Kim SB, Rose MR. Phenotypic plasticity and selection in *Drosophila* life-history evolution: I. Nutrition and the cost of reproduction, *J. Evol. Biol.* **6**, 171-193 (1993).

Chippindale AK, Leroi AM, Saing H, Borash DJ, Rose MR. Phenotypic plasticity and selection in *Drosophila* life-history evolution: II. Diet, mates and the cost of reproduction, *J. Evol. Biol.* **10**, 269-293 (1997a).

Chippindale AK, Alipaz JA, Chen HW, Rose MR. Experimental evolution of accelerated development in *Drosophila*. 1. Developmental speed and larval survival, *Evolution* **51**, 1536-1551 (1997b).

Christensen ND. The circadian clock of *Hemediena thoracica* as a system of coupled oscillators, *M. Sc Thesis*. University of Auckland, New Zealand (1978).

Cirelli C, Bushey D, Hill S, Huber R, Kreber R, Ganetzky B, Tononi G. Reduced sleep in *Drosophila Shaker* mutants, *Nature* **434**, 1087-1092 (2005).

Clutton-Brock T, Langley P. Persistent courtship reduces male and female longevity in captive tsetse flies *Glossina morsitansmorsitans* Westwood (Diptera: Glossinidae), *Behav. Ecol.* **8**, 392-395 (1997).

Costa R, Peixoto AA, Barbujani G, Kyriacou CP. A latitudinal cline in a *Drosophila* clock gene, *Proc. R. Soc. Lond. B* **250**, 43-49 (1992).

Crudgington HS, Siva-Jothy MT. Genital damage, kicking and early death, *Nature* **407**, 855-856 (2000).

Daan S. Adaptive daily strategies in behavior. In *Handbook of Behavioral Neurobiology. Volume 4.* Eds. Aschoff J, Plenum Publishing Corporation, p. 275-298 (1981).

Daan S. Colin Pittendrigh, Jürgen Aschoff and the natural entrainment of circadian systems, *J. Biol. Rhythms* **15**, 195-207 (2000).

Daan S. Tinbergen JM. Young guillemots (*Uria lomvia*) leaving their Arctic breeding cliffs: A daily rhythm in numbers and risk, *Ardea* **67**, 96-100 (1980).

David J, Fouillet P. Enregistrement continue de la ponte chez *Drosophila melanogaster* et importance de conditions experimentales pour l' etude du rythme circadien d' oviposition, *Rev. Comp. Anim.* **7**, 197-202 (1973).

DeCoursey PJ, Krulas JR, Mele G, Holley DC. Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure, *Physiol. Behav.* **62**, 1099-1108 (1997).

DeCoursey PJ, Walker JK, Smith SA. A circadian pacemaker in free-living chipmunks: essential for survival? *J. Comp. Physiol. A* **186**, 169-180

(2000).

De Souza HML, Da Cunha AB, Dos Santos EP . Adaptive polymorphism of behavior evolved in laboratory populations of *Drosophila willistoni*, *Am. Nat.* **104**, 175-189 (1970).

Dobzhansky T, Spassky B, Sved Y. Effects of selection and migration on geotactic and phototactic behavior of *Drosophila*. II *Proc. Roy. Soc. Lon. B Biol. Sci.* **173**, 191-207 (1969).

Dobzhansky T, Judson CL, Pavlovsky O. Behavior in different environments of populations of *Drosophila pseudoobscura* selected for phototaxis and geotaxis, *Proc. Natl. Acad. Sci. USA* **71**, 1974-1976 (1974).

Dunlap JC. Molecular bases for circadian clocks, *Cell* **96**, 271-290 (1999).

Dunlap JC. Fundamental properties of circadian rhythms. In *Chronobiology: Biological Timekeeping*. Eds. Dunlap JC, Loros JJ, DeCoursey PJ. Sunderland, Massachusetts, USA: Sinauer Associates, Inc. Publishers, p. 67-105 (2004).

Eeken JCJ. Circadian control of the cellular response to β-ecdysone in *Drosophila lebanonensis*. I. Experimental puff induction and its relation to puparium formation, *Chromosoma* **49**, 205-217 (1974).

Emery P, So WV, Kaneko M, Hall JC, Rosbash M. CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity, *Cell* **95**, 669-679 (1998).

Emery P, Stanewsky R, Hall JC, Rosbash M. A unique circadian-rhythm Photoreceptor, *Nature* **404**, 456-457 (2000).

Enright JT. *The Timing of Sleep and Wakefullness.* Springer-Verlag, Berlin (1980).

Fleury F, Allemand R, Vavre F, Fouillet P, Boulétreau M. Adaptive significance of a circadian clock: temporal segregation of activities reduces intrinsic competitive inferiority in *Drosophila* parasitoids, *Proc. R. Soc. Lond. B* **267**, 1005-1010 (2000).

Frisch K von. *The Dance Language and Orientation of Honeybees.* Harvard University Press, Cambridge, Massachusetts (1967).

Frisch B, Hardin PE, Hamblen-Coyle MJ, Rosbash M, Hall JC. A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system, *Neuron* **12**, 555-570 (1994).

Froy O, Gotter AL, Casselman AL, Reppert SM. Illuminating the circadian clock in Monarch butterfly migration, *Science* **300**, 1303-1305 (2003).

Glossop NRJ, Hardin PE. Central and peripheral circadian oscillator mechanisms in flies and mammals, *J. Cell Sci.* **115**, 3369-3377 (2002).

Glossop NRJ, Houl JH, Zheng H, Fanny SNG, Dudek SM, Hardin PE. VRILLE feeds back to control circadian transcription of clock in *Drosophila* circadian oscillators. *Neuron* **37**, 249-261 (2003).

Gruwez G, Hoste C, Lints CV and Lints FA. Oviposition rhythm in *Drosophila melanogaster* and it's alteration by a change in the photoperiodicity, Experientia **27**, 1414-1416 (1972).

Halberg F, Cadotte L. Increased mortality in mice exposed to weekly 1800 shifts of lighting regime LD 12:12 beginning at 1 years of age., *Chronobiologia* **2**, 26 (1975).

Halberg F, Neloson W, Cadotte L. Living routine shifts simulated on mice by weekly or twice-weekly manipulation of light-dark cycle, *Proc. XII Int. Soc. Chronobiol.* 133-138 (1977).

Hall JC. Molecular neurogenetics of biological rhythms, *J. Neurogenet.* **12**, 115-81 (1998).

Hardin PE. Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators, *Mol. Cell. Biol.* **14**, 7211-7218 (1994).

Hastings JW, Rusak B, Boulos Z. Circadian rhythms: The physiology of biological timing. In *Neural and Integrative Animal Physiology.* Eds. Prosser CL. Wiley-Liss, Inc, p. 435-545 (1991).

Heisenberg M. Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*, *J. Expt. Biol.* **55**, 85-100 (1971).

Helfrich-Förster C. Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster* –Sex specific differences suggest a different quality of activity, *J. Biol. Rhythms* **15**, 135-154 (2000).

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R. The circadian clocks of fruit flies is blind after elimination of all known photoreceptors,

Neuron **30**, 249-261 (2001).

Helfrich-Förster C, Edwards T, Yasuyama K, Wistozky B, Schneuwly S, Stanewsky R, Meinertzhagen IA, Hofbauer A. The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function, *J. Neurosci.* **22**, 9255-9266 (2002).

Helfrich-Förster C. Neurobiology of the fruit fly's circadian clock, *Genes Brain Behav.* **4**, 65-76 (2005).

Hendricks JC, Lu S, Kume K, Yin JC, Yang Z, Sehgal A. Gender dimorphism in the role of cycle (BMAL1) in rest, rest regulation, and longevity in *Drosophila melanogaster*, *J. Biol. Rhythms* **18**, 12-25 (2003).

Hoffmann K. Experimental manipulation of the orientational clock in birds, *Cold Spring Harb. Symp. Quant. Biol.* **25**, 379-387 (1960).

Hoffmann K. Adaptive significance of biological rhythms corresponding to geophysical cycles. In *The Molecular Basis of Circadian Rhythms.* Eds. Hastings JW, Schweiger HG. Abakon Verlagsgesellschaft, Berlin, p. 63-75 (1976).

Hölldobler B, Wilson EO. *The Ants* Springer-Verlag, Berlin Heidelberg, New York (1990).

Hunt J, Simmons LW, Kotiaho JS. A cost of maternal care in the dung beetle *Onthophagus taurus*? *J. Evol. Biol.* **15**, 57-64 (2002).

Hurd MW, Ralph MR. The significance of circadian organization for longevity in the golden hamster, *J. Biol. Rhythms* **13**, 430-436 (1998).

Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A, Golden SS,

Johnson CH, Kondo T. Expression of a gene cluster *KaiABC* as a circadian feedback process in cyanobacteria, *Science* **281**, 1519-1523 (1998).

Johnson CH, Golden SS. Circadian programs in cyanobacteria: Adaptiveness and mechanism, *Ann. Rev. Microbiol.* **53**, 389-409 (1999).

Joshi A. Adaptive evolution and the footprints of history, *Curr. Sci.* **72**, 944-949 (1997).

Joshi A, Mueller LD. Directional and stabilizing density-dependent natural selection for pupation height in *Drosophila melanogaster, Evolution* **47**,176-184 (1993).

Joshi A, Mueller LD. Density-dependent natural selection in *Drosophila*: trade-offs between larval food acquisition and utilization, *Evol. Ecol.* **10**, 463-474 (1996).

Kalmus H. Periodizität und autochronie (ideochronie) als zeitregelnde eigenschaffen der organismen, *Biologia Generalis* **11**, 93-114 (1935).

Kaneko M, Helfrich-Forster C, Hall JC. Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling, *J. Neurosci.* **17**, 6745-6760 (1997).

Ketellapper HJ. Interaction of endogenous and environmental periods in plant Growth, *Plant Physiol.* **35**, 238-241 (1960).

Klarsfeld A, Rouyer F. Effects of circadian mutations and LD periodicity on the life span of *Drosophila melanogaster*, *J. Biol. Rhythms* **13**, 471-478

(1998).

Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, Wesley C, Young MW. The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase I-epsilon, *Cell* **94**, 97-107 (1998).

Koilraj AJ, Sharma VK, Marimuthu G, Chandrashekaran MK. Presence of circadian rhythms in the locomotor activity of a cave dwelling millipede *Glyphiulus cavernicolus sulu* (Cambalidae, Spirostreptida), *Chronobiol. Int.* **17**, 757- 765 (2000).

Konopka RJ. Circadian clock mutants of *Drosophila melanogaster, PhD thesis.* California Institute of Technology (1972).

Konopka RJ, Pittendrigh CS, Orr D. Reciprocal behavior associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants, *J. Neurogenet.* **6**, 1-10 (1989).

Kotiaho JS, Simmons LW. Longevity cost of reproduction for males but no longevity cost of mating or courtship for females in the male-dimorphic dung beetle *Onthophagus binodis, J. Insect Physiol.* **49**, 817-822 (2003).

Kramm KR, Kramm DA. Photoperiodic control of circadian activity rhythms in diurnal rodents, *Int. J. Biometeorol.* **24**, 65-76 (1980).

Krishnan B, Levin JD, Lynch MKS, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE. A new role for *cryptochrome* in a *Drosophila* circadian oscillator, *Science* **411**, 313-317 (2001).

Kumar S, Mohan A, Sharma VK. Circadian dysfunction reduces life span in *Drosophila melanogaster, Chronobiol. Int.* **22**, 641-653 (2005).

Kume K, Kume S, Park SK, Hirsh J, Jackson FR. Dopamine is a regulator of arousal in the fruit fly, *J. Neurosci.* **25**, 7377-7384 (2005).

Kyriacou CP, Oldroyd M, Wood J, Sharp M, Hill M. Clock mutations alter developmental timing in *Drosophila*, *Heredity* **64**, 395-401 (1990).

Lankinen P. Genetic variation of circadian eclosion rhythm, and its relation to photoperiodism in *Drosophila littoralis*, *PhD Thesis.* University of Oulu, Department of Genetics (1985).

Leroi AM, Chippindale AK, Rose MR. Long term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster* 1. The role of genotype by environment interaction, *Evolution* **48**, 1244-1257 (1994).

Lin Y, Stormo GD, Taghert PH. The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system, *J. Neurosci.* **24**, 7951-7957 (2004).

Liu X, Lorenz L, Yu Q, Hall JC. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*, *Genes Dev.* **2**, 228-238 (1988).

Loeschke v. Genetic constrains on adaptive evolution and the evolution of genetic constrains. In *Genetic Constrains on Adaptive Evolution*. Eds. V. Loeschke, Springer Verlag, Berlin, p. 1-3 (1987).

Luckinbill LS, Grave JL Jr, Tomkiw A, Sowirka O. A qualitative analysis of some life history correlates of longevity *Drosophila melanogaster*, *Evol. Ecol.* **2**, 85-94 (1988).

Malpel S, Klarsfeld A, Rouyer F. Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during

Drosophila brain development. *Development* **129**, 1443-1453 (2002).

Manning M, Markow TA. Light-dependent pupation site preferences in *Drosophila*. II. *Drosophila melanogaster* and *Drosophila simulans*, *Behav. Genet.* **11**, 557-563 (1981).

Markow TA. A survey of intra- and inter-specific variation for pupation height in *Drosophila*, *Behav. Genet.* **9**, 209-217 (1979).

Markow TA. Light-dependent pupation site in *Drosophila*, *Behav. Neural. Biol.* **31**, 348-353 (1981).

Mazzoni EO, Desplan C, Blau J. Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response, *Neuron* **45**, 293-300 (2005).

McCluskey ES. Periodicity and diversity in ant mating flights, *Comp. Biochem. Physiol.* **103A**, 241-243 (1992).

McDonald MJ, Rosbash M. Microarray analysis and organization of circadian gene expression in *Drosophila*, *Cell* **107**, 567-578 (2001).

Mead M, Gilhodes JC. Organization temporella de l' activite locomotrice chez un animal cavernicole *Blaniulus lichensteini* Bröl. (Diplopoda), *J. Comp. Physiol.* **90**, 47-52 (1974).

Miyatake T. Correlated responses to selection for developmental period in *Bactrocera cucurbitae* (Diptera: Tephritidae): Time of mating and daily activity rhythms, *Behav. Genet.* **27**, 489-498 (1997).

Miyatake T. Pleiotropic effect, clock gene, and reproductive isolation, *Popul Ecol.* **44**, 201-207 (2002).

Moore D, Angel JE, Cheeseman IM, Fahrbach SE, Robinson GE. Time keeping in the honey bee colony: integration of circadian rhythms and division of labor, *Behav. Ecol. Sociol.* **43**, 147-160 (1998).

Mueller LD. Evolution of accelerated senescence in laboratory populations of *Drosophila*, *Proc. Natl. Acad. Sci.* **84**, 1974-1977 (1987).

Mueller LD, Ayala FJ. Fitness and density-dependent population growth in *Drosophila melanogaster*, *Genetics* **97**, 667-677 (1981).

Mueller LD, Sweet VF. Density-dependent natural selection in *Drosophila*: evolution of pupation height, *Evolution* **40**, 1354-1356 (1986).

Myers EM, Yu J, Sehgal A. Circadian control of eclosion: Interaction between a central and peripheral clock in *Drosophila melanogaster*, *Curr. Biol.* **13**: 526-533 (2003).

Nelson W, Halberg F. Schedule shifts, circadian rhythms and lifespan of freely-feeding and meal-fed mice, *Physiol. Behav.* **38**, 781-788 (1986).

Nikaido SS, Johnson CH. Daily and circadian variation in survival from ultraviolate radiation in *Chlamydomonas reinhardtii*, *Photochem. Photobiol.* **71**, 758-765 (2000).

Nunny L. The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: an example of a fitness trade-off, *Evolution* **50**, 1193-1204 (1996).

Oklejewicz M, Daan S. Enhanced longevity in *tau* mutant Syrian hamsters, *Mesocricetus auratus*. *J. Biol. Rhythms* **17**, 210-216 (2002).

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH. Resonating

circadian clocks enhance fitness in cyanobacteria, *Proc. Natl. Acad. Sci. USA* **95**, 8660-8664 (1990).

Page TL, Block GD. Circadian rhythmicity in cockroaches: effects of early post-embryonic development and aging, *Physiol. Entamol.* **5**, 271-281 (1980).

Palladino MJ, Hadley TJ, Ganetzky B. Temperature sensitive paralytic mutants are enriched for those causing neurodegeneration in *Drosophila, Genetics* **161**,1197-1208 (2002).

Paranjpe DA, Anitha D, Kumar S, Kumar D, Verkhedkar K, Chandrashekaran MK, Joshi A, Sharma VK. Entrainment of eclosion rhythm in *Drosophila melanogaster* populations reared for more than 700 generations in constant light environment, *Chronobiol. Int.* **20**, 977-987 (2003).

Paranjpe DA, Anitha D, Chandrashekaran MK, Joshi A, Sharma VK. Possible role of eclosion rhythm in mediating the effects of light-dark environments on pre-adult development in *Drosophila melanogaster, BMC Dev. Biol.* **5**, 5 (2005).

Paranjpe DA, Sharma VK. Evolution of temporal order in living organisms, *J. Circa Rhytms* 3, 7 (2005).

Partridge L, Harvey PH. Cost of reproduction, *Nature* **316**, 20-21 (1985).

Partridge L, Green A, Fowler K. Effects of egg production and of exposure to males on female survival in *Drosophila melanogaster, J. Insect Physiol.* **33**, 745-749 (1987).

Partridge L, Fowler K. Non-mating costs of exposure to males in female *Drosophila melanogaster, J. Insect Physiol.* **36**, 419-425 (1990).

Pittendrigh CS. On temperature independence in the clock controlling emergence time in *Drosophila*, *Proc. Natl. Acad. Sci. USA* **40**, 1018-1029 (1954).

Pittendrigh CS. Perspectives in the study of biological clocks. In *Perspectives in marine biology*. Eds. Buzzati-Traverso AA, University of California Press, p. 239-268, (1958).

Pittendrigh CS. Circadian rhythms and the circadian organization of living Systems, *Cold Spring Harb. Symp. Quant. Biol.* **25**, 159-184 (1960).

Pittendrigh CS. Biological clocks. In *Science in the Sixties*. Eds. Arm DL. University of New Mexico, Office of Publications, Albuquerque, p. 96-111 (1965).

Pittendrigh CS. Biological Clocks: the functions, ancient and modern, of circadian oscillations. In *Science and the Sixties. Proc. Cloudcraft Symp Air Force Office of Sci. Res.*, p. 96-111 (1965).

Pittendrigh CS. Circadian systems. I. The drive oscillation and its assay in *Drosophila pseudoobscura*, *Proc. Natl. Acad. Sci.* **58**, 1762-1767 (1967).

Pittendrigh CS. Circadian organization and the photoperiodic phenomena. In *Biological clocks in seasonal reproductive cycles*. Eds. Follet BK, Follet DE , Scientenhnica, Bristol, p. 1-35 (1981).

Pittendrigh CS. Temporal organization: Reflections of a Darwinian clock-watcher, *Ann. Rev. Physiol.* **55**,17-54 (1993).

Pittendrigh CS, Bruce V. Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In *Photoperiodism and Related Phenomenon in Plants and Animals*. Eds. Withrow RB,

American Association of Advancement of Sciences, Washington DC, p. 475-505 (1959).

Pittendrigh CS, Minis DH. Circadian systems: Longevity as a function of circadian resonance in *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA* **69**, 1537-1539 (1972).

Pittendrigh CS, Daan S. A functional analysis of circadian pacemaker in nocturnal rodents. IV. Entrainment: pacemaker as clock, *J. Comp. Physiol. A* **106**, 291-331 (1976).

Pittendrigh CS, Skopik SD. Circadian systems, V. The driving oscillation and the temporal sequence of development, *Proc. Natl. Acad. Sci. USA* **65**, 500-507 (1970).

Pittendrigh CS, Kyner WT, Takamura T. The amplitude of circadian oscillations: Temperature dependence, latitudinal clines and the photoperiodic time measurement, *J. Biol. rhythms* **6**, 299-313 (1991).

Pittendrigh CS, Takamura T. Latitudinal clines in the circadian properties of a pacemaker, *J. Biol. Rhythms* **4**, 217-235 (1989).

Poulson TL, White WB. The cave environment, *Science* **105**, 971-981 (1969).

Prasad NG, Shakarad M, Gohil VM, Sheeba V, Rajamani M, Joshi A. Evolution of reduced pre-adult viability and larval growth rate in laboratory populations of *Drosophila melanogaster* selected for shorter development time, *Genet. Res.* **76**, 249-259 (2000).

Prasad NG, Shakarad M, Anitha D, Rajamani M, Joshi A. Correlated responses to selection for faster development and early reproduction in *Drosophila*:

the evolution of larval traits, *Evolution* **55**, 1363-1372 (2001).

Pyza E, Meinertzhagen IA. Daily and circadian rhythms of synaptic frequency in the first visual neuropil of the housefly's (*Musca domestica* L.) optic lobe, *Proc. Roy. Soc. London B* **254**, 97-105 (1993).

Pyza E, Meinertzhagen IA. Monopolar cell axons in the first optic neuropil of the housefly, *Musca domestica* L., undergo daily fluctuations in diameter that have a circadian basis, *J. Neurosci.* **15**, 407-418 (1995).

Pyza E, Meinertzhagen IA. Daily and rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*, *J. Neurobiol.* **40**, 77-88 (1999).

Qui J, Hardin PE. Developmental state and the circadian clock interact to influence the timing of eclosion in *Drosophila melanogaster*, *J. Biol. Rhythms* **11**, 75-86 (1996).

Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*, *Cell* **99**, 791-802 (1999).

Rensing L and Hardeland R J. Zur Wirkung der circadianen Rhythmik auf die Entwicklung von *Drosophila, J. Insect Physiol.* **13**, 1547-1568 (1967).

Reznick D. Measuring the costs of reproduction, *Trends Evol. Ecol.* **7**, 42-45 (1992).

Rizki MTM, Davis CG Jr. Light as an ecological determinant of interspecific competition between *D. willistoni* and *D. melanogaster*, *Am. Nat.*

87, 389-392 (1953).

Robinson GE. Regulation of division of labor in insect societies, *Ann. Rev. Entomol.* **37**, 637-665 (1992).

Roenneberg T, Daan S, Merrow M. The Art of Entrainment, *J. Biol. Rhythms* **18**, 183-194 (2003).

Rosato E, Peixoto AA, Barbujani G, Costa R, Kyriacou CP. Molecular polymorphism in the period gene of *Drosophila simulans*, *Genetics* **138**, 693-707 (1994).

Rosato E, Kyriacou CP. Flies, clocks and evolution, *Phil. Trans. R. Soc. Lond. B* **356**, 1769-1778 (2001).

Rose MR, Nusbaum TJ, Chippindale AK. Laboratory evolution: the experimental wonderland and the Cheshire Cat Syndrome. In *Adaptation*. Eds. Rose MR and Lauder GV, Academic press, New York, p. 221-241 (1996).

Ruby NF, Dark J, Heller HC, Zucker I. Ablation of suprachaismatic nucleus alters timing of hibernation in ground squirrels, *Proc. Natl. Acad. Sci. USA* **93**, 9864-9868 (1996).

Ruby NF, Dark J, Heller HC, Zucker I. Suprachiasmatic nucleus: role in circannual body mass and hibernation rhythms of ground squirrels, *Brain Res.* **782**, 63-72 (1998).

Sawin-McCormack EP, Sokolowsky MB, Campos AR. Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development, *J. Neurogenet.* **10**, 119-135 (1995).

Saez L, Young MW. *In situ* localization of the *per* clock protein during

development of *Drosophila melanogaster*, *Mol. Cell. Biol.* **8**, 5378-5385 (1988).

Sawyer LA, Hennessy JM, Peixoto AA, Rosato E, Parkinson H, Costa R, Kyriacou CP. Natural variation in a *Drosophila* clock gene and Temperature compensation, *Science* **278**, 2117-2120 (1997).

Schnebel EM, Grossfield J. The influence of light on pupation height in *Drosophila*, *Behav. Genet.* **16**, 407-413 (1986).

Service PM. The effect of mating status on life span, egg laying and starvation resistance in *Drosophila melanogaster* in relation to selection on longevity, *J. Insect Physiol.* **35**, 447-452 (1989).

Service PM, Hutchinson PW, Rose MR. Multiple genetic mechanisms for the evolution of senescence in *Drosophila melanogaster*, *Evolution* **42**, 708-716 (1988).

Sharma VK. Do biological clocks age like their owners? *Proc. Indian Natl. Sci. Acad.* **B67**, 373-388 (2001).

Sharma VK. Adaptive significance of circadian clocks, *Chronobiol. Intl.* **20**, 901-919 (2003a).

Sharma VK. Period responses to Zeitgeber signals stabilize circadian clocks during entrainment, *Chronobiol. Int.* **20**, 389-404 (2003b).

Sharma VK. Simple computer aided device for monitoring activity of small mammals and insects, *Biol. Rhythm Res.* **34**, 3-12 (2003c).

Sharma VK, Chandrashekaran MK. Zeitgebers (Time cues) for biological clocks, *Curr. Sci.* **89**, 1136-1146 (2005).

Sharma VK, Daan S. Circadian phase and period responses to light stimuli in two

nocturnal rodents, *Chronobiol. Int.* **19**, 659-670 (2002).

Sharma VK, Joshi A. Clocks, genes and evolution: the evolution of circadian organization. In *Biological Clocks*. Eds. Kumar V, Narosa Publishers, New Delhi and Berlin, Springer-Verlag, p. 5-23 (2002).

Sharma VK, Lone SR, Goel A. Clocks for sex: loss of circadian rhythms in ants after mating? *Naturwissenschaften* **91**, 334-337 (2004a).

Sharma VK, Lone SR, Goel A, Chandrashekaran MK. Circadian consequences of social organization in the ant species *Camponotus compressus*, *Naturwissenschaften* **91**, 386-390 (2004b).

Sharma VK, Lone SR Mathew D, Goel A, Chandrashekaran MK. Possible evidence for shift work schedules in the media workers of the ant species *Camponotus compressus*, *Chronobiol. Int.* **21**, 297-308 (2004c).

Sheeba V. Probing the adaptive significance of circadian rhythms using *Drosophila melanogaster*, Ph. D. Thesis. Manipal Academy of Higher Education, India (2001).

Sheeba V, Madhyastha NAA, Joshi A. Oviposition preference for novel versus normal food resources in laboratory populations of *Drosophila melanogaster*, *J. Biosci.* **23**, 93-100 (1998).

Sheeba V, Sharma VK, Chandrashekaran MK, Joshi A. Persistence of eclosion rhythms in populations of *Drosophila melanogaster* after 600 generations in an aperiodic environment, *Naturwissenschaften* **86**, 448-449 (1999a).

Sheeba V, Sharma VK, Chandrashekaran MK, Joshi A. Effect of different light regimes on pre-adult fitness in *Drosophila melanogaster* populations

reared in constant light for over six hundred generations, *Biol. Rhythm Res.* **30**, 424-433 (1999b).

Sheeba V, Sharma VK, Shubha K, Chandrashekaran MK, Joshi A. The effect of different light regimes on adult life span in *Drosophila melanogaster* is partly mediated through reproductive output, *J. Biol. Rhythms* **15**, 380-392 (2000).

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. Persistence of oviposition rhythm in individuals of *Drosophila melanogaster* reared in an aperiodic environment for several hundred generations, *J. Exp. Zool.* **47**, 1217-1225 (2001).

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. Developmental plasticity of the locomotor activity rhythm of *Drosophila melanogaster*, *J. Insect Physiol.* **48**, 25-32 (2002a).

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. Locomotor activity rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment. *Naturwissenschaften* **89**, 512-514 (2002b).

Shimizu T, Miyatake T, Watari Y, Arai T. A gene pleiotropically controlling developmental and circadian periods in the melon fly *Bactrocera cucurbitae* (Diptera: Tephritidae), *Heredity* **79**, 600-605 (1997).

Siegel FJ. Testing for periodicity in a time series, *Am. Stat. Asso.* **75**, 345-348 (1980).

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC. Antibodies to the *period* gene product of *Drosophila* reveal diverse issue distribution and

rhythmic changes in the visual system, *Neuron* **1**, 141-150 (1988).

Skopik SD, Pittendrigh CS. Circadian systems II. The oscillation in the individual *Drosophila* pupa; its independence of developmental stage, *Proc. Natl. Acad. Sci.* **58**, 1862-1869 (1967).

Sokal RR, Ehrlich PR, Hunter PE, Schlager G. Some factors affecting pupation site of *Drosophila*, *Ann. Entomol. Soc. Amer.* **53**, 174-182 (1960).

Sokolove PG. Locomotory and stridulatory circadian rhythms in the cricket *Teleogryllus commodus*, *J. Insect Physiol.* **21**, 537-558 (1975).

Sokolowski MB. *Drosophila*: Genetics meets behavior, *Nat. Rev. Genet.* **2**, 879-890 (2001).

Stal LJ, Krumbein WE. Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. Grown under alternating light-dark cycles, *Arch. Microbiol.* **143**, 76-71 (1985).

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC. The *cry*^{b} mutation identifies Cryptochrome as a circadian photoreceptor in *Drosophila*, *Cell* **95**, 681-692 (1998).

Stanewsky R. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals, *J. Neurobiol.* **54**, 111-147 (2003).

Stark WS. Transient and receptor potentials in the electroretinogram of *Drosophila*, *Vision Res.* **12**, 1771-1775 (1972).

Stark WS, Wasserman GS. Wavelength specific ERG characteristics of pigmented- and white-eyed strains of *Drosophila*, *J. Comp. Physiol.* **91**, 427-441 (1974).

Stark WS, Sapp R. Eye color pigment granules in wild type and mutant *Drosophila melanogaster*, *Can. J. Zool.* **66**, 1301-1308 (1988).

STATISTICA™ Statistica Vol III; Statistica II. Statsoft Inc., Tulsa, OK, USA (1995).

Stearns SC. *The Evolution of Life Histories.* Oxford University Press, New York (1992).

Stutt AD, Siva-Jothy MT. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularis*, *Proc. Natl. Acad. Sci. USA* **98**, 5683-5687 (2001).

Toma DP, Bloch G, Moore D, Robinson GE. Changes in *period* mRNA levels in the brain and division of labor in honeybee colonies, *Proc. Natl. Acad. Sci. USA* **97**, 6914-6919 (2000).

Trajano E, Manno-Baretto L. Free running locomotor activity rhythms in cave dwelling catfishes *Trichomycterus sp.* from Brazil, *Biol. Rhythms Res.* **27**, 329-335 (1996).

Von Saint Paul U, Aschoff J. Longevity among blowflies *Phormia terraenovae R.D.* kept in non-24-hour light-dark cycles, *J. Comp. Physiol. A* **127**, 191-195 (1978).

Williams JA, Sehgal A. Molecular components of the circadian system in *Drosophila*, *Ann. Rev. Physiol.* **63**, 729-755 (2001).

Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH. The adaptive value of circadian clocks: An experimental assessment in cyanobacteria, *Curr Biol.* **14**, 1481-1486 (2004).

Zwaan BJ. The evolutionary genetics of aging and longevity. *Heredity* **82**, 589-

597 (1999).