Circadian Rhythms In Five Wild Caught Species of *Drosophila*

Thesis

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By

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December 2009

.....to

My family

Uncle and Sister

DECLARATION

I declare that the matter presented in my thesis entitled "**Circadian Rhythms in Five Wild Caught Species of *Drosophila***" is the result of studies carried out by me at Chronobiology laboratory of the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Vijay Kumar Sharma and that this work has not been submitted elsewhere for any other degree.

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

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Place: Bangalore

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CERTIFICATE

This is to certify that the work described in the thesis entitled **"Circadian Rhythms in Five Wild Caught Species of *Drosophila*"** is the result of investigations carried out by Mr. M Muzafar Beigh in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Vijay Kumar Sharma

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Summary

Living organisms have evolved circadian clocks to time their physiological and behavioural activities to stay in tuned with the geophysical world. Signatures of such timing systems can be observed at various levels of complexity and organization. The fruit fly *Drosophila melanogaster* has been extensively used for understanding the structure and function of circadian clocks. Some of the best-studied circadian rhythms in *Drosophila* include those in adult emergence, activity/rest, mating, and egg laying behaviours. Many researchers have used different species of *Drosophila* to study variability in circadian rhythms in order to understand how circadian clocks have evolved. For my thesis, I have studied circadian rhythms in five wild caught species of *Drosophila* to investigate if closely related species have similar circadian rhythms. The flies belong to three separate genuses. *D. melanogaster and D. ananassae* belong to the subgenus Sophophora, while *D. malerkotliana* belongs to a complex subgroup within *D. ananassae*. *D. nasuta* belongs to the subgenus *Drosophila*, and species group Immigrans and *Zaprionus indianus* belongs to the genus Zaprionus and sub-genus Zaprionus and species group Armatus. These populations were maintained on cornmeal medium under constant light and at 25 °C temperature and ~90% relative humidity.

I began by studying the activity/rest rhythm of five wild caught species of *Drosophila*. The main objective of my study was to assay the activity/rest behaviour of five different species of *Drosophila*, and the distribution of activity/rest patterns in males and females under 12:12 hr light/dark (LD) cycles and in constant darkness (DD). The activity/rest behaviour of 32 flies (16 males and 16 females) was recorded in

Drosophila Activity Monitors, for the first 8 days under LD cycle and for subsequent 8 days under DD. The results of the experiment revealed species- and sex- specific differences. Males of all five species were found to show bimodal activity patterns, while the activity of the females is evenly distributed throughout day. A closer look of the activity profiles suggests sex-specific differences in the overall activity patterns with females showing higher activity than males. Furthermore, *D. ananassae* (both males and females) flies were found to be more active during morning hours than evening hours compared with other related species of *Drosophila.* The anticipation index (AI) for the "morning" and "evening" activity peaks in *D. melanogaster, D. annanasse, D. nasuta* was significantly different from those in *Zaprionus indianus*, while the AI in *D. melanogaster, D. ananassae, D. nasuta* and *D. malerkotliana* did not differ statistically. The morning peak in *D. ananassae* is higher than the evening peak, while in *D. melanogaster* and *D. malerkotliana* the evening peaks were higher than the morning peaks. The morning and evening peaks in *Zaprionus indianus* occurred on an average 2 hr after "dawn" and "dusk", respectively. The activity in males and females was higher during the light phase than dark phase, and females exhibited higher overall daytime activity than males. Analyses of the sleep data revealed that duration of sleep is shorter in females than in males. Further analysis revealed that *D. nasuta* females show higher sleep duration compared to other related species. Night time sleep in *D. ananassae* females was significantly greater than *D. melanogaster* and *D. malerkotliana* but lesser than *D. nasuta.* The circadian period could only be calculated for three of the five wild caught species - *D. ananassae, D. melanogaster* and *D. malerkotliana* because *D. nasuta* and

Zaprionus indianus were less active in DD. The circadian period of activity/rest rhythm is species and sex specific; with *D. ananassae* having significantly shorter periodicity than other two species of the same genus (*D. melanogaster* and *D. malerkotliana*). The circadian periodicity of the males is significantly shorter periodicity than females. The results from the activity/rest behaviour experiments suggest that male-female dimorphism in circadian phenotypes is consistent across a wide range of *Drosophila* species.

In my second experiment, I studied the mating rhythm in five wild caught species of *Drosophila*. Male-female pairs from each species were introduced in glass vials loaded with corn meal, and mating was assayed continuously for 20 minute, every 3 hours. The results of the assay indicate that the time course and waveform of mean mating frequency in different species, under LD cycles was significantly different; *D. ananassae* has its mating peak (with ~40% mating) during "dawn" (ZT00), *D. nasuta* (~70%) four hours later (ZT04), whereas *D. malerkotliana, D. melanogaster* and *Z. indianus* (~30 to ~40%) during mid-to-late afternoon hours with peaks of mating between ZT00 and ZT09. While most *Drosophila* species mate only during day, *D. malerkotliana* was found to mate even in the night. The results suggest that *D. ananassae* and *D. malerkotliana* which belong to the same sub-genus and species group mate at different times of the day - *D. ananassae* prefers morning hours, while *D. malerkotliana* evening hours, leading to temporal isolation.

Finally I studied the egg laying behaviour in only four of the five wild caught species of *Drosophila*. Male-female pairs were kept in glass vials with corn meal

medium. Two sets of twenty such vials were introduced in 12:12 hr LD conditions for the three days and subsequently transferred to DD for the egg-laying assay. To estimate the time course and waveform of egg-laying behaviour, the number of eggs laid over a period of two hours was counted for a minimum of seven days. The results suggest that *D. melanogaster* lays maximum number of eggs soon after "dusk" (~ZT12), *D. ananassae* and *D. nasuta* prior to "dusk" (~ZT10), whereas no such preference for egg-laying was observed in *Zaprionus indianus*. These results suggest that different species of *Drosophila* have different preference for time of egg-laying under 12:12 hr light/dark cycle. The percentage of flies in which egg-laying rhythm entrained to 12:12 hr LD cycles varied from one species to another – egg-laying rhythm in ~85.0% of *D. ananassae* flies, ~18.2% of *Zaprionus indianus* flies entrained to LD cycles, whereas the percentage was ~61.53% and ~40% in *D. nasuta* and *D. melanogaster, respectively.* These results indicate that entrainment of egg-laying rhythm to 12:12 hr LD cycles is highest in *D. ananassae* and lowest in *Zaprionus indianus,* suggesting that egg-laying rhythm of different species of *Drosophila* respond differently to LD cycles.

Based on the results of our studies we conclude that five wild caught species of *Drosophila* show species-specific differences in their circadian rhythms in activity/rest, mating and egg-laying behaviours. The results are in conformity with the findings from previous studies done in other species of *Drosophila*, and suggest that during the course of evolution different species of *Drosophila* evolve different clock mechanisms to regulate activity/rest, mating and egg-laying behaviours. While five wild caught species of *Drosophila* exhibit differences in their circadian phenotypes underlying activity/rest,

mating and egg-laying rhythms, the differences do not correlate to relatedness between species. In other words, in *Drosophila* even phylogenetically related species show significant differences in circadian phenotype. My study suggests that wild caught species of *Drosophila* living in sympatry may have evolved different circadian clock mechanisms for activity, mating and egg-laying rhythms over the course of time for better adaptability and survivorship.

Contents

Chapter 1

Introduction

1.1 Introduction

Earth's rotation around its axis causes predictable changes in the geophysical environment, thereby providing organisms with options to occupy appropriate spatio-temporal niches (Paranjpe and Sharma, 2005). To keep track of the daily changes in their environment living organisms have evolved mechanisms that can precisely measure passage of time on an approximately 24 hour scale. Such mechanisms serve as an intrinsic time keeping devise helping organisms track time in their local environment and are referred to as circadian clocks. Circadian rhythms persist in the absence of time cues depicting its endogenous nature and fine-tune physiological and metabolic activities to meet the needs of daily activities. Circadian rhythms are outwardly quite similar in most species but the genes that constitute the clock mechanisms are quite different (Brody *et al.,* 2004). Circadian clocks form one of the most fascinating adaptations to life on earth. From cyanobacteria to higher mammals, circadian clocks control nearly all life processes in a rhythmic manner. Processes such as emergence of adults from pupae in *Drosophila* (Pittendrigh, 1954), opening and closing of leaves and flowers to provide protection for more delicate tissues from the lower temperatures of night (Enright, 1982; Darwin, 1895), alertness, body temperature rhythms in mammals that causes to be active during the day and to sleep at night (Moore-Ede *et al.,* 1982) show rhythmic behaviour. Thus it can be rightly said that circadian clocks have evolved to control every life form in concerted manner in tune with 24 hr environmental cycles.

Drosophila has been used as a model organism to study circadian clocks since 1930. The pioneering work of Erwin Bünning and Colin S. Pittendrigh on fruit flies

(*Drosophila*) laid the foundation of the field of Chronobiology. Pittendrigh showed that wild species of *D. persimilis* show higher activity in the morning whereas *D. pseudoobscura* show higher activity during the evening hours (Pittendrigh, 1993). Early studies in *Drosophila* relied primarily on the adult emergence rhythm (eclosion) but now many behavioural and physiological rhythms can be monitored with ease and precision. Initially *D. pseudoobscura* was used as a model organism in almost all studies in chronobiology but the paucity of knowledge and tools of genetics in *D. pseudoobscura* prompted circadian researchers to turn to *D. melanogaster*. The use of a variety of molecular and biochemical techniques led to the discovery of genetic and molecular mechanisms underlying circadian rhythms in *Drosophila*. One such discovery was the identification of clock gene *period* (*per*) in *D. melanogaster* by Konopka and Benzer in the year 1971. Subsequently several other clock genes such as *timeless (tim), doubletime (dbt), clock (clk), cycle (cyc)* were identified, characterized, and incorporated into the model of molecular circadian clock machinery (reviewed in Zheng and Sehgal, 2008).

Circadian rhythms are driven by endogenous biological clocks and are synchronized to environmental cues (Simonetta *et al.,* 2009). Many behavioral rhythms such as activity/rest, mating and egg-laying rhythms are under the control of endogenous circadian clocks (Sheeba *et al.,* 2008). The various behaviours seen across different phylogenetic taxa shows differences in their rhythms for example, mating rhythm of *D. melanogaster* and *D. simulans* are antiphasic (Sakai and Ishida, 2001). Since mating in these two sympatric species is antiphasic, it might have led to temporal isolation by mating during the course of evolution. Related species of insects have

varying daily activity pattern; the circadian clock controlling activity pattern may have contributed in temporal isolation (Edery, 2000).

1.2 Speciation and Evolution

Speciation is the process through which one species diverges into different strains that ultimately become reproductively isolated and evolutionary independent (Santos *et al.,* 2007). During the process of speciation different kind of barriers get created hindering gene exchanges within the same group of species. These barriers may be physical, leading to spatial separation of subpopulations and resulting in “allopatric speciation”, or temporal separation, giving rise to “allochronic speciation”, or gene pools separated within the same region but by different habitats or other means (parapatric speciation), are often difficult to prove, since present reproductive isolation could have originated allopatrically with subsequent sympatry (“secondary contact”) (Mallet, 2006).

Any discussion of the genetics of speciation must begin with the observation that species are real entities in nature (Orr and Coyne, 1998). In simple terms species can be defined as a population of organisms interbreeding only with each other. Dobzhansky (1935) and Mayr (1942) proposed the biological species concept (BSC), which considers species to be group of populations reproductively isolated from each other by ‘isolating mechanisms’ that prevent gene exchange between them. Some scientists believe that speciation is the by-product of conventional evolutionary forces such as selection and drift, and evolution of species is simply an epiphenomenon of normal population-genetics processes (Coyne and Orr, 1998). The distinctive feature of the genetics of

speciation is, therefore, epistasis. This is mainly true for post-zygotic isolation but may also occur for other forms of pre-zygotic isolation (Coyne and Orr, 1998).

Speciation is the direct result of changes in the gene pool (Singh and Kulathinal, 2000). The common factor in all mechanisms of speciation is a reduction in gene flow between two populations, this starts the divergence and eventually speciation occurs (Santos *et al.,* 2007). Gene flow in allopatric models is reduced by geographical separation, whereas in sympatric or parapatric models are reduced by other means. Speciation is intimately associated with the evolution of reproduction-related traits, including those affecting hybrid incompatibility (postzygotic isolation) and species recognition (prezygotic isolation) (Servedio and Saetre, 2003). After subsequent sympatry (secondary contact), initially slight differences in mate recognition traits are exaggerated by selection in favor of pre-zygotic isolation through assortative mating (reinforcement theory) (Coyne, 1994). Pre-zygotic isolating mechanisms may aid in the evolution of sympatric species (Moehring *et al.,* 2006), while natural selection may help in the evolution of allopatry (Whitaker, 2006). For sympatric species, pre-zygotic isolation through natural selection evolves more rapidly between species that produce unfit hybrids (Hayashi and Kawata, 2002).

Some pre-zygotic (pre-mating) isolating examples are as follows:

1. Allopatric separation – For example, origin of different species of *Drosophila* species in Hawaii following volcanic eruption.
2. Ecological or habitat isolation (sympatric) – For example, the European mosquito *Anopheles* group consists of six morphologically indistinguishable

species. They are isolated reproductively as they breed in different habitats. Some breed in brackish water, some in running fresh water and some in stagnant fresh water and therefore, they never meet to breed.

3. Mechanical isolation - The reproductive organs of the sexes are not anatomically compatible and this impedes reproduction. Lack of pollen tube growth down style of a different plant species is an example.

4. Gametic isolation - Gamete transfer takes place but fertilization does not occur. Many species of *Drosophila* show an insemination reaction as a result of which sperm is killed in the vagina.

Some examples of post-zygotic isolating mechanism are as follows:

1. F1 hybrids inviable: Fertilization occurs but embryonic development does not take place. For example, in crosses between sheep and goats embryos are formed but cannot develop after certain developmental stages.

2. F1 hybrids infertile: The hybrids occur but do not produce functional gametes, For example, the offspring of a cross between female horse and male ass results in mule which is sterile.

Hybrid breakdown: F1 hybrids are viable and fertile, but F2, backcross or later-generation hybrids are inviable or infertile (Price and Bouvier, 2002). The most common post-zygotic isolation mechanism between populations of the phytophagous mite *Tetranychus urticae* is 'hybrid breakdown', i.e. when individuals from two different populations are crossed, F1 hybrid females are produced, but F2 recombinant male

offspring suffer increased mortality (Vala *et al.,* 2000). Two-spotted spider mites collected from two populations, one on rose and the other on cucumber plants, were infected with Wolbachia bacteria. These bacteria may induce cytoplasmic incompatibility in their hosts: uninfected females become reproductively incompatible with infected males (Vala *et al.,* 2000).

1.3 Evolution of Circadian Clocks

While the components of circadian clock molecular machinery appears to be different in many organisms, their functions bear remarkable degree of similarity across taxa (Sharma, 2003; Paranjpe and Sharma, 2005; Sheeba *et al.,* 2008). Several studies have attempted to understand the molecular evolution of clock genes within the family of Diptera (Kyriacou *et al.,* 2000). The *per* gene sequence of *Musca domestica* shows unusual characteristics and high degree of similarity with those in *D. melanogaster* (Piccin *et al.,* 1999). Phylogenetic analysis reveals that the Per-ARNT-Sim motif (PAS) and cytoplasmic localization domains (CLD) of *per* gene in *D. melanogaster* are more closely related to *Musca domestica* than with *D. pseudoobscura* and *D. virilis* (Piccin *et al.,* 2000). These results are surprising particularly because the time of divergence from the common ancestor of muscid and fruit flies was about 100 million years ago, whereas that of *D. melanogaster* from other fruit fly species was about 30-60 million years (Rosato and Kyriacou*,* 2001). This evolutionary profile of *per* gene nevertheless correlates remarkably well with the functional data (Rosato and Kyriacou*,* 2001). Interestingly, in a *per* gene rescue experiment in *D. melanogaster* introducing *per*

transgene from *D. pseudoobscura* resulted in poor recovery of rhythmic behavior, whereas robust rhythmicity was restored when *per* transgene from *Musca* was used (Piccin *et al.,* 2000). This suggests that speciation might have played a significant role in the divergence of clock gene sequences in species which are otherwise more closely related to each other, perhaps for better survival under a given geophysical environment (Rogers *et al.,* 2004).

Similarly divergence in *tim* has also been observed in a number of *Drosophila* species, suggesting that multiple genes in the clock network might have undergone changes during the course of evolution. Sequence variations in *tim* has been found in some *Drosophila* species, suggesting species-specific polymorphism at the putative translational start site of TIM protein (Costa and Kyriacou, 1998). The polymorphism in *tim* of *D. melanogaster* gave rise to a stop codon, and reduced the length of the TIM protein by 23 residues, and this truncated form of TIM is unique to *D. melanogaster,* and is not found in any other *Drosophila* species (Piccin *et al.,* 2001). The comparison of TIM protein in *D. melanogaster* and *D. virilis* revealed an overall identity of ~77%, which is much higher than the PER protein identity in these species (Myers *et al.,* 1997). Interspecific analysis of *per* expression patterns reveals evolutionary alterations in *per* regulation as well (Costa and Kyriacou*,* 1998). Therefore, it is believed that over the course of evolution, variation in different clock genes such as *per, tim* and *Clk,* fine-tuned the circadian rhythms in different species of *Drosophila* that in turn could have aided the emergence of new species.

1.4 Molecular Mechanism underlying circadian rhythms in *Drosophila*

At the molecular level circadian clock mechanism in *Drosophila* involves genes and their products. At the core of the molecular clockwork are transcriptional-translational feedback loops (TTFLs) involving transcripts and proteins of clock genes (reviewed in Sheeba *et al.,* 2008). In *Drosophila*, the molecular clock machinery is believed to comprise inter-locked transcriptional-translational feedback loops (TTFLs) (Glossop *et al.,* 1999; Cyran *et al.,* 2003; Yu and Hardin, 2006). The *per*, *tim* and *dbt* genes have been implicated as the components of the negative limb of the loop. Additional clock genes *Clk*, *cyc, vrille (vri)* and PAR domain protein (*pdp1*) - constitute the positive feedback loop. CRYTOCHROME (CRY) functions as an intracellular photoreceptor; however, there are indications of it being a part of the pacemaking mechanisms as well (Sheeba *et al.,* 2008). CRY adjusts the speed of the clock to 24 hr via light-dependent TIM-degradation. The basic TTL is remarkably similar across multiple phylogenetic classes, although some doubts have been raised about the centrality of the molecular machinery (Nakajima *et al.,* 2007; Tomita *et al.,* 2005; Hardin, 2006; Rust *et al.,* 2007). In *Drosophila,* the gene products of *per* and *tim* form the core of the TTFL in pacemaker cells. Levels of PER and TIM proteins and their mRNAs exhibit cyclic expression in pacemaker cells, with PER and TIM translocating into nucleus in a time-of-day dependent manner (Siwicki *et al.,* 1988; Hardin *et al.,* 1990). The transcription of *per* and *tim* is activated by two proteins CLK and CYC which heterodimerize and bind to the E-box promoters of *per* and *tim* genes, thus forming a feedback loop. The peaks of *per* and *tim* mRNAs occur at dusk and are followed by a subsequent increase in PER/TIM multimer roughly after 6 hrs (Hardin *et al.,* 1990; Hunter-Ensor *et al.,* 1996). This is partly mediated by the kinase DBT which

destabilizes PER by phosphorylating it. The PER/TIM heterodimer then enters the nucleus around midnight (Martinek *et al.,* 2001; Lin *et al.,* 2002).

The central feature of the TTFL model of circadian pacemaking mechanism assumes that DBT-PER and TIM complex represses *per* and *tim* transcription in the nucleus by binding to CLK and CYC transcription factors and by releasing CLK/CYC bound to the E-box sequences of the *per* and *tim* promoters (Kim and Edery, 2006). Therefore, in contrast to CLK and CYC, TIM and PER form the negative limb of the TTFL. TIM is gradually degraded by light through its interaction with the photosensitive CRY and eventually by proteosomal mechanisms (Naidoo *et al.,* 1999; Busza *et al.,* 2004). The loss of PER/TIM mediated repression frees CLK/CYC to begin a new cycle of the molecular clock work.

Although TTFL model is appealing in many ways, it has a number of unresolved inconsistencies (Sheeba *et al.,* 2008). The current TTFL model does not explain the persistence of overt behavioural rhythms when *clk* mRNA is expressed using *per* or *tim* promoters such that expression is no longer antiphasic to *per* and *tim* (Kim *et al.,* 2002). Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has been central to any research and circadian biology is paving the way for understanding gene expression in a time dependent manner.

Experimental Populations

2.1 The Experimental System

The arsenal of genetic techniques to manipulate *Drosophila* at molecular level gave circadian biologists a wealth of information about the behavioural and molecular mechanisms underlying innumerable cellular processes involved in circadian rhythms. The main advantage of *Drosophila* that has made it a model for understanding behavioural and molecular studies is that it can easily be reared and can be manipulated at the genetic level to a large extent (Bridges, 1916). The life-span of *Drosophila* is also relatively shorter than other higher organisms, and each of its life stages can be studied under laboratory conditions.

Drosophila is a holometabolous insect that undergoes complete metamorphosis through four different stages namely the egg, larvae, pupa and adult stages (**Fig. 1**). In our laboratory, several wild caught species of *Drosophila* are being reared at constant temperature of 25 ^{o}C and ~90% relative humidity, and at a light intensity of ~100 lux. These species complete the pre-adult stages in their life cycles at varying durations of time (Bharathi, 2006). The eggs are laid after 4-6 hr of mating, and eggs start hatching after about 24 hr. The *Drosophila* larvae go through three instars, the first, second and the third instars. The first and second instars last for about 24 hr each, the third instar for about 48 hr, and the pupal stage lasts for about 72 hr (Pittendrigh, 1974, 1981). The pre-adult development time for *D. melanogaster, D. ananassae, D. malerkotliana* is roughly about 8 days, and that of *D. nasuta* and *Zaprionus indianus* is roughly between 12 and 17 days.

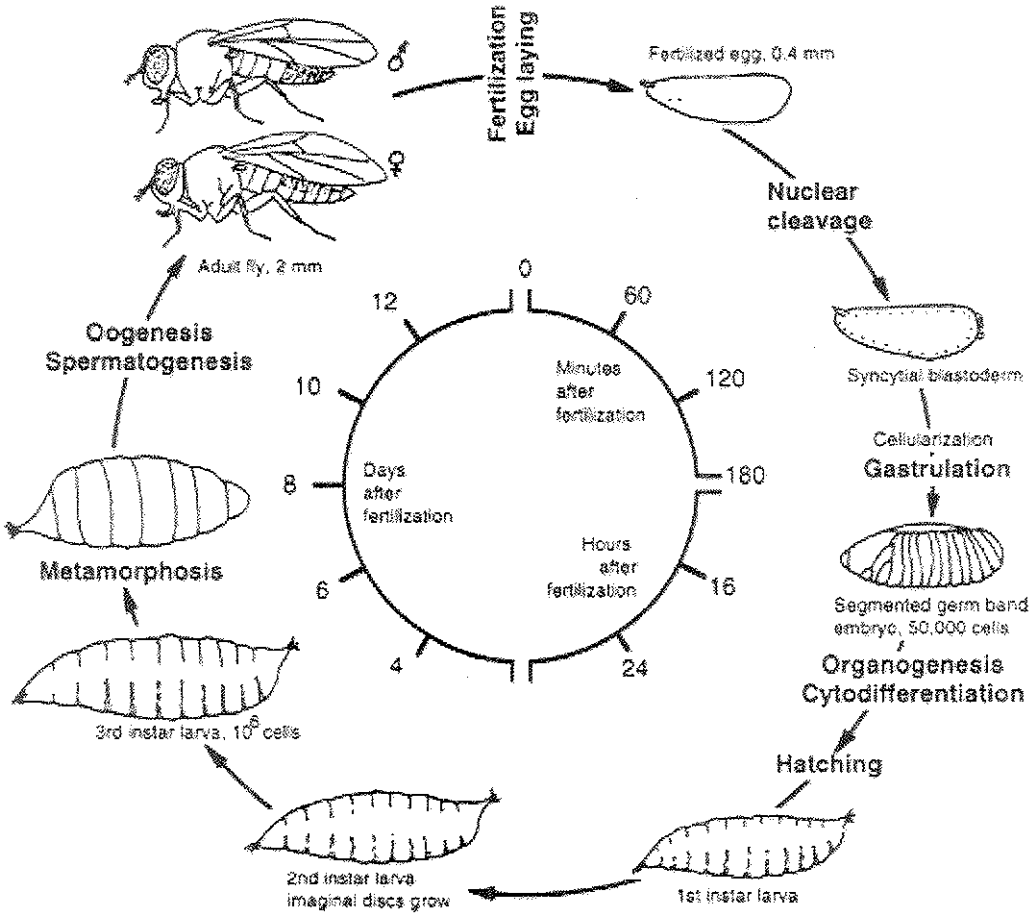


Fig. 1 Life Cycle of *Drosophila melanogaster* (Adapted from Wolpert *et al.*, 1998).

2.2 Population Setup for Different Species

Before starting any experiment on laboratory populations, a number of biotic and abiotic factors need to be taken into consideration. The important biotic factor that should be given paramount importance is founder population, i.e., number of individuals used to start a population. The founder population can affect the outcome of results drastically by founder effect, inbreeding depression and linkage disequilibrium as explained below. Various physical factors such as light, temperature and humidity also play a pivotal role in the outcome of the results. In natural conditions several factors change simultaneously making it difficult to understand the effect of environmental conditions on a particular behavior. To avoid any such confounding effect, experiments are often performed under controlled laboratory conditions where factors such as light, temperature, humidity are kept constant.

Some of the biotic factors that need to be taken into consideration before starting any laboratory populations are:

Founder effect:

The founder effect is the loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population. Ernt Mayr (1963) described this phenomena as the establishment of a new population by a few original founders (in an extreme case, by a single fertilized female) which carry only a small fraction of the genetic variation of the parental population (King *et al.,* 2002). The founder effect is quite effective in reducing the genetic variation because if a population is started with few individuals, chances are those alleles that will be helpful in survival of

the species, will be lost from the original population. The founder effect is often seen when a population passes through a 'bottleneck' in which only a few individuals survive and later expand into a large population when the conditions become favorable (Hill *et al.,* 2004). Further in a small population, the frequencies of the diseased genes may differ from the parental populations, i.e., frequencies of the diseased alleles increase in small population, which in turn may affect population in a negative way. Therefore, founder effect becomes an important factor that should be taken into consideration before any population is initiated. In our laboratory population of wild caught species, we started our population with large sample size of flies collected from the surrounding areas in Bangalore.

Inbreeding depression:

In a small population, mating between close relatives is common. Such inbreeding may lower the population's ability to survive and reproduce, a phenomenon called as inbreeding depression. Inbreeding depression can be defined as the loss of fitness that has often been observed within progeny produced by the mating of closely related individuals (Lynch and Walsh, 1998). Any reduction in population size would cause inbreeding depression, which can lead to artefactual correlations (Rose *et al*., 1996). Further in a large population natural selection weeds out the deleterious alleles and dominant non-deleterious alleles dominate recessive deleterious alleles and suppress their effects, more generally then in large populations, the odds are stacked against weakly deleterious mutations and so selection should be more efficient in large populations (Hurst, 2009). In small population this is not possible as few dominant non-

deleterious alleles will always be present and hence the threat to population getting wiped out is more. Thus to avoid any such threat, populations should be maintained in large sizes. In our laboratory, the wild caught species of *Drosophila* were maintained at a size of about 1000-1200 individuals per population.

Linkage Disequilibrium:

Linkage disequilibrium is the non-random association of alleles at two or more loci, not necessarily on the same chromosome (Ritchie *et al.,* 2007). It is different from linkage which describes the association of two or more loci on the same chromosome with limited recombination (Hey, 2004). Linkage disequilibrium is a situation in which some alleles or genetic markers occur less frequently than expected from a random formation of haplotypes from alleles based on their frequencies. When there is no such deviation and linkage disequilibrium is 0, the population is said to be in linkage equilibrium (Baker *et al.,* 1999). The level of linkage disequilibrium is influenced by a number of factors including genetic linkage, rate of recombination, rate of mutation, genetic drift, non-random mating and population structure. Therefore in order to take into account all the above factors in laboratory populations, it is essential to start populations from a sizeable number of individuals and maintain them in sizeable numbers.

2.3 Experimental Populations

Collection of flies

The five species of *Drosophila* used in my studies *D. melanogaster, D. ananassae, D. malerkotliana, D. nasuta* and *Zaprionus indianus* will be henceforth referred as **DM, DA,**

Table 1: Five wild caught species of *Drosophila* used in the study.

S.No.	Species	Place of collection	Time of collection	Starting populations
1.	*D. melanogaster*	Bangalore (Hebbal)	May - June 2005	~70
2.	*D. ananassae*	Bangalore (Vijnanpura)	May - June 2001	~300
3.	*D. nasuta*	Bangalore (Vijnanapura)	Oct - Nov 2001	~70
4.	*D. malerkotliana*	Bangalore (Vijananpura)	Oct - Nov 2001	~70
5.	*Z. indianus*	Bangalore (Hebbal)	May - June 2005	~70

DK, DN and **ZI,** respectively. The populations of *D. ananassae, D. malerkotliana and D. nasuta* flies were collected from orchards and domestic garbage dumps in different parts of Bangalore. A combination of banana traps and net sweepings, kept mostly during the morning and evening hours was used to collect flies, and each population was started after thorough examination of markers for each species (**Fig. 2**).

2.4 Identification and maintenance of flies

The wild caught flies were initially maintained in vials containing banana-jaggery medium before being established as regular laboratory populations. Each female fly was kept in a separate vial containing 10 ml of banana-jaggery medium and allowed to lay eggs. The emerging flies were examined for morphological markers on the male body. All the flies were examined and transferred in vials till sizeable population was established and once it reached 1200 flies per species they were transferred into Plexiglass cages. At pre-adult stages these populations were maintained at a moderate density of 60-80 eggs per vial. The adults upon emergence were transferred into Plexiglass cages (25 cm x 20 cm x 15 cm), and were fed on yeast paste. All species were maintained on a discrete generation cycle of 21 days. Initially the populations were maintained on banana-jaggery meal, and after a few generations they were shifted on cornmeal medium. The reason for this shift to cornmeal medium was that the banana-jaggery medium is moist and many adult flies drowned in the medium. The eggs collected from food plates were introduced in glass vials containing approximately 10 ml of cornmeal food. The cornmeal is prepared from corn powder, yeast, sugar, agar-agar

and charcoal, and was prepared according to the defined protocol (Bharathi, 2002). The cornmeal medium composition per litre is given in **Table 2**. All the five ingredients are mixed together in 1 litre water and stirred thoroughly in a mixer and boiled in a pressure cooker. The food is cooled till 60 ^{o}C. 10 ml of propionic acid and 1 g of p-hydroxy methyl benzoate is mixed with 10 ml of ethanol and is added to the cooked food and mixed thoroughly. About 0.5 g of charcoal is added to the food to give it a color, so that eggs collection is easy and that eggs can be distinguished from the food particles.

2.5 Taxonomic features of five wild caught species

The taxonomic features of five species of *Drosophila* maintained in our laboratory are:

Drosophila melanogaster (**Taxonomic ID: 7227: sub-genus *sophophora, melanogaster* species group, *melanogaster* sub-group**): *Drosophila melanogaster* belong to the genus Drosophilidae, sub-group *melanogaster*. They have brick red eyes, are yellow-brown in color, and have transverse black rings across their abdomen more prominently found in males than in females. The females have large bodies with average size of 2.5 mm, males are smaller and darker in colour with distinct black patch in the abdomen. Males have spiky hairs (claspers) surrounding anus and genitals to attach with females for mating.

Drosophila anananasse (Taxonomic ID: 7217: sub-genus sophophora, melanogaster species group, ananassae sub-group, species complex ananassae): *D. ananassae* flies are greyish in colour, and males and females are nearly equal in size. The males have distinguishable sex-comb and females have rounded abdomen prominently seen under

Drosophila ananassae
Sub-genus *sophophora*
melanogaster species group
ananassae sub-group
ananassae species complex

Drosophila nasuta nasuta
Sub-genus *Drosophila*
immigrans species group
nasuta sub-group
frontal sheen species
complex

Drosophila melanogaster
Sub-genus *sophophora*
melanogaster species group
melanogaster sub-group

Zaprionus indianus
Sub-genus *Zaprionus*
armatus species-group
vittiger sub-group
species

Drosophila malerkotliana
Sub-genus *sophophora*
melanogaster species group
ananassae sub-group
bipectinata species complex

Fig. 2: The pictures shown above are male and female flies of five species of *Drosophila* used in our study.

microscope. The females develop white rounded abdomen upon yeasting due to the accumulation of eggs and can be helpful in distinguishing them from males.

Drosophila malerkotliana (**Taxonomic ID: 30036: sub-genus *sophophora, melanogaster* species group, *ananassae* sub-group, bipectinata species complex**): *D. malerkotliana* flies are morphologically similar to other members of the *bipectinata* species complex and are indistinguishable from each other. In *D. malerkotliana*, the last three abdominal segments are darkly pigmented in males but not in females and the claspers are in two sets.

Drosophila nasuta nasuta (**Taxonomic ID: 42062, sub-genus *Drosophila*, *immigrans* species group, *nasuta* sub-group, frontal sheen species complex**): *D. nasuta* flies are large in size, red in color, and have shiny bristles and brown mark on the lateral side of thorax. Females are morphologically indistinguishable, while males have markings on their frons, females are larger than males and can easily be distinguished.

Zaprianus indianus (**Taxonomic ID: 76712, sub-genus *Zaprionus*, species group *armatus* and species subgroup *vittiger***): *Z. indianus* have yellow body color with distinct white stripes dorsally that extend from antennae to the tip of thorax and laterally to the wing base giving them a name as zebra flies. The body length is 3.5 mm. Males have narrow abdomen than females and have distinguishable sex-comb.

All these species exhibit different types of morphological characters differing in their color, size, and number of bristles. The most obvious morphological features distinguishing the species complex are abdominal pigmentation and sex comb morphology (Kopp *et al.,* 2007).

2.6 Aims and Objectives

Southwood (1977) proposed that every organism's ecological strategies e.g. growth, activity, and reproduction – can be viewed as having selected to fit the spatial and temporal template of favorable characteristics to its habitat. Thus, the circadian rhythms exhibited by organisms can be viewed as a means by which periods of different activities are timed to coincide with the periods of suitable environment (Simunovic and Jaenike, 2006). *Drosophila* being an ecologically and biogeographically diverse genus with various species occupying diverse habitats, thus one might expect various species of *Drosophila* to vary adaptively in their circadian rhythms in a habitats-specific fashion (Simunovic and Jaenike, 2006). Previous studies have documented inter-species correlation in circadian rhythms in different strains of *Drosophila* e.g., activity/rest rhythm in different wild species of *Drosophila* (Helfrisch-Förster *et al.,* 2000). In my study, I examined patterns of various circadian behaviours such as activity/rest, mating and egg-laying rhythm in five wild caught species of *Drosophila.* The species examined in this study and the sites from which the experimental strains were collected are shown in **Table 1**. These species were chosen for study because all the populations were collected from surrounding, hence could give us good understanding of circadian rhythms in closely related species of *Drosophila.* In my thesis, I have studied circadian rhythms in these species of *Drosophila* to investigate if closely related species have similar circadian rhythms and also helped us to understand circadian rhythms in wild populations that are likely to reflect their adaptation to the natural conditions in which they are currently found. Given the great phylogenetic and ecological diversity of the genus *Drosophila*,

their standing as ideal model organisms for exploring circadian rhythms, the adaptive evolution and diversification of different circadian rhythms in natural population is plausible.

2.7 Method of Data Collection and Analysis

To study circadian rhythms of fruit flies, three different behavioral assays were carried out:

- *Locomotor activity rhythm*: Locomotor activities of flies were recorded individually under 12:12 hr LD conditions for a minimum of 8 days. The main purpose of the assay was to examine activity/rest patterns. The phases of the peaks are estimated in Zeitgeber Time (ZT), whereby the beginning of a 12:12 LD cycle (lights-on) is referred as ZT00 and, lights-off as ZT12. Locomotor activity behaviour of flies was recorded additionally under DD to estimate circadian periodicity. The locomotor activity data was analyzed by Lomb Scargle (LS) periodogram analysis using CLOCKLAB (Actimetrics, Evanston, IL).

- *Mating rhythm*: The mating behaviour of five species was assayed under 12:12 hr LD cycles and DD conditions. Five male-female pairs from each species were kept in long glass vials loaded with cornmeal food on flat trays in LD and DD condition, and mating related parameters were estimated every 3 hr for five consecutive days. The main purpose of assay was to look for time course and waveform of mean mating frequency in different wild caught species of *Drosophila*, under 12:12 hr LD cycles.

- *Egg-lying rhythm*: The egg lying behaviour of four wild caught species of *Drosophila* was assayed under 12:12 hr LD and DD conditions. The purpose of the experiment was to assay egg-laying rhythm in LD and DD conditions. Sixteen male and females pairs from each species were maintained under LD and DD and were given food change 2 hrs for 8 consecutive days.

2.8 Materials and Methods

One population each of the five species of flies was used in the three behavioral assays. The three species *D. ananassae, D. malerkotliana, D. nasuta* were collected from orchards and domestic garbage of various localities of Bangalore, while *D. melanogaster* and *Z. indianus* were collected from Hebbal farmland in Bangalore. The *D. ananassae* population were initiated from ~300 inseminated females collected during May-June 2001, while the other three populations were established from about 70 females each, during October-November 2001. The collections were done during "dawn" and "dusk" hours. The populations were maintained on 21 day generation cycle. Eggs were collected from each species by placing cornmeal food plate in a cage kept overnight. Approximately 30-40 eggs from *D. melanogaster, D. ananassae* and *D. malerkotliana* populations were collected and introduced in glass vials containing about 10 ml cornmeal food. Similarly 50-60 eggs were collected from *D. nasuta* and *Z. indianus* populations. The populations of *D. melanogaster, D. ananassae* and *D. malerkotliana* were maintained in 30 vials and each population was maintained at approximately

Table 2: Composition of cornmeal medium (per litre)

Ingredients	Amount (g)
Corn powder	100
yeast	40
sugar	40
Agar-agar	12
Charcoal	0.5

~1200 flies per population. The populations were kept on cornmeal medium throughout their life cycle which has similar nutritional value as that of banana-jaggery food.

Each population was maintained under constant light conditions in an incubator maintained at 25 ^{o}C and at a relative humidity of ~90% in small glass vials except *Z. indianus* which was maintained in long vials. All species were kept on cornmeal food from the time of egg collection till eclosion and thereafter. Since development time of wild caught species varies from one species to another, adults from each species were collected on different dates from the time of their egg collection. For example, it takes around 8 days for *D. melanogaster, D. ananassae* and *D. malerkotliana* to eclose after the time of egg collection; therefore adult flies were collected into Plexiglass cages on 10th or 11th day of egg collection. The collection of adults for *D. nasuta* and *Z. indianus* was done on the 14th to 18th day of egg collection as collection of these two species take much longer time to eclose than other species.

CHAPTER 3

Locomotor activity rhythm

3.1 Locomotor Activity Assay

Locomotion by animals is required for localization of food, mates, escape from predators, defense of territory, and in response to stress, and therefore is an integral component of animal behavior (Mackay *et al.*, 2000). Locomotor activity is performed by most insects and is found to be rhythmic in nature (Saunders, 1998). Many life forms anticipate environmental transitions, perform activities at biologically advantageous times of the day, and undergo characteristic seasonal change using endogenous circadian clocks which can be synchronized to external time cues (Delaunay *et al.,* 2000). Many day and night active species show bimodal (double-peaked) patterns of activity with high levels around dawn and dusk and reduced levels at noon and midnight (Aschoff, 1962, 1966). It appears that bimodality is an endogenous expression of the circadian clocks under 24 hr light/dark (LD) cycles. Animals living in arid environment show pronounced bimodal activity pattern with rest during the middle of the day, which is thought to be advantageous for their survival as it would be expected to reduce desiccation (Hinze *et al.*, 2006). However, it is found that organisms living in temperate climes also show bimodal activity patterns (Roberts, 1962; Wiedenmann, 1980, 1983; Chiba, 1971). Often the two peaks are of different heights, of which one is reduced or disappears after the animals are transferred to constant darkness (Engelmann and Mack, 1978). Pittendrigh and Daan (1976) proposed a model for the regulation of the rhythm in which the two activity peaks of rodents are thought to be controlled by two circadian oscillators with different properties. It was theorized that circadian rhythms are controlled by two independent, but coupled, Morning (M) and Evening (E) oscillators

(Pittendrigh and Daan 1976). Subsequent measurements of neuronal firing in the rodent brain sections that contains clock cells revealed two distinct subpopulations of neurons whose firing rhythms were out of phase, suggesting that these could be the "M" and "E" oscillators (Schwartz *et al.,* 2000). The clocks governing activity rhythm in *Drosophila* are also believed to comprise of two separate "M" and "E" oscillators (Stoleru *et al.,* 2004, 2005, 2007; Grima *et al.,* 2004). It is believed that a group of neurons that express PDF (LN_v) act as the "M" oscillators, and others located more dorsally (LN_d and DNs) serve as the "E" oscillators.

The locomotor activity rhythm in the fruit fly *Drosophila melanogaster* has been extensively studied and the underlying molecular and neural mechanisms have been elucidated to a large extent (Allada *et al.,* 1998; Rutila *et al.,* 1998; Sheeba *et al.,* 2008). Studies have shown that several sets of neurons in the fly brain are involved in regulating locomotor activity rhythm (Sheeba *et al.,* 2008). Genetic ablations of these neurons result in loss of locomotor activity rhythm under constant darkness (Renn *et al.,* 1999; Howlader *et al.,* 2006). We have monitored the locomotor activity behaviour of five wild caught species of *Drosophila* under 12:12 hour light/dark (LD) and constant dark (DD) conditions with the purpose of studying variations in their circadian phenotypes.

In *Drosophila,* males and females differ in their locomotor activity profile (Rosato *et al.,* 2000). Under 12:12 hr LD conditions, males show narrow distribution of activity around lights-on and lights-off and pronounced siesta in the middle of the day. The females show broader distribution of activity at light-on and lights-off and less siesta in

the day than males. Apart from gender, age of the flies is also known to affect locomotor activity pattern; flies become less rhythmic with age (Shaw *et al.,* 2000). A previous study from Helfrich-Förster's (2000) laboratory on three strains showed significant differences in the phase (morning peak), activity and period of locomotor activity rhythm in three strains of *Drosophila*. The sexual dimorphism in the phase of the morning peak under LD conditions suggests that the function of activity during morning and evening peak might be different. For example, during the morning peak males are active to find females (Helfrich-Förster, 2000). Other sex-specific differences are - shorter circadian period in males compared to females, and females showing higher activity levels in two out of three strains (Helfrich-Förster, 2000).

Sleep is ubiquitous in mammals and birds and it reflects a fundamental biological function that is as yet unknown (Campbell *et al.*, 1998). Flies subjected to 12:12 hr LD cycles exhibit sustained periods of activity and quiescence with approximately 90% of sleep occurring during night. Sleep like pattern has also been reported in *Drosophila* which declines with age (Shaw *et al.,* 2000). It is highest immediately after emergence and declines as flies age (Shaw *et al.*, 2000). Sleep-like state is also observed in the middle of the day which is referred to as 'siesta' (Hendricks *et al.,* 2008). It is more prominent during warmer summer days than winters (Young *et al.,* 2008). In our study we decided to study the sleep/wake cycle of five wild caught species of *Drosophila* living in sympatry.

3.2 Materials and Methods

Fly stocks: The five wild caught species - *D. melanogaster, D. ananassae, D. nasuta, D. malerkotliana* and *Z. indianus* were maintained in plexi glass cages, were kept on cornmeal food mixed with charcoal, and placed in incubators with continuous light conditions at approximately 25 ^{o}C temperature and about 90% relative humidity.

Activity tubes: 5 mm in diameter × 80 mm in length, 1 mm thick; usually locally available were used for recording the activity of flies and were placed in each channel in activity monitors.

3.3 Recording and analyzing data

We assayed locomotor activity behaviour of five species of *Drosophila* under 12:12 hr LD and DD. In order to study the locomotor activity behaviour of animals, we should be able to monitor their activity continuously over many days. To do this, continuous automation becomes obligatory. We used high throughput Drosophila Activity Monitors from Trikinectics, USA for this purpose. In these monitors, a single fly is placed in a small glass tube with food at one end and cotton plug at the other and continuous to-and-fro movement of flies is recorded using infra red emitters and receivers. A computer counts every time the infrared beam is broken, giving a measure of the level of activity of an individual fly. Activity counts are stored in a specific time slots called as bins - a bin is a small time interval that is associated with activity values (the number of times the fly crosses the infrared beam). As locomotor activity experiments are usually performed for 10 days or more, the probability of flies dying during an experiment increases with age. Therefore we normally use freshly emerged 2 to 3 days old flies for our assays.

Locomotor activity behaviour of flies was recorded individually under LD conditions for a minimum of 8 days. The phases of the peaks are estimated in Zeitgeber Time (ZT), whereby the beginning of a 12:12 LD cycle (lights-on) is referred as ZT00 and, of lights-off as ZT12. Locomotor activity behaviour of flies was recorded additionally under DD to estimate endogenous circadian periodicity. First few days of activity data was ignored while estimating the circadian period to eliminate any artifact due to the transfer of flies into DD. On an average 16 flies of each sex from each genotype was taken for the study of locomotor activity profile. In order to asses the reproducibility each experiment was repeated twice.

The phase-relationship of the morning and evening activity peaks with lights-on and lights-off in LD cycles was estimated as the average time interval between the morning peak and lights-on and between the evening peak and light-off. The phase-relationship values were considered to be negative if the peaks followed lights-on/ lights-off, and as positive if the peaks preceded them.

At the end of the experiments, raw data from each recorded fly was plotted as an actogram - a plot of activity as a function of time of the day, plotted one below another, chronologically for several days. Often the graph is double plotted by making the x-axis stretch to 48 hr. This allows ease in visualizing the activity patterns of the flies. Average was estimated under 12:12 hr LD and DD conditions for each fly. Periodogram analysis using Lombe Scargle (LS) test with $p = 0.05$ significance level was performed on the raw data to estimate period of the rhythm under DD conditions and also to assess entrainment under LD cycle.

3.4 Results

A. Locomotor activity profile of male and female flies under 12:12 hr light/dark cycle.

The graphs (**Fig. 1)** show locomotor activity pattern of five species of *Drosophila* under 12:12 hr LD cycles. Wild caught species of *Drosophila* show bimodal pattern of activity, displaying bouts of activity around lights-on and lights-off and 'siesta' in the middle of the day. Males of all five species are seen to show a pronounced siesta, while females of most species do not. *Z. indianus* flies also showed a clear bimodal pattern in their activity, albeit phase delayed by 4 hr.

B. Phase-relationship between activity onset/offset and lights-on/ off in five wild caught species of *Drosophila*

Clear differences in the phase-relationships were observed in different species. ANOVA on the phase-relationship data revealed a significant main effect of species ($F_{4,80}$ = 839.86; $p < 0.0001$), sex ($F_{1,80}$ = 55.55; $p < 0.0001$), species × sex interaction ($F_{4,80}$ = 39.73; $p < 0.0001$). Post-hoc multiple comparisons revealed that the activity peaks in *Z. indianus* males and females are significantly phase-delayed compared to the rest of the four species (**Fig. 2**).

C. Anticipation index of activity in five wild caught species of *Drosophila*.

The Anticipation Index (AI) of activity/rest rhythm relative to lights-on (AI_{on}) and lights-off (AI_{off}) were estimated under 12:12 LD cycle (**Fig. 3**). ANOVA on the AI data revealed a significant main effect of species ($F_{4,20}$ = 6.26; $p < 0.001$), while species × sex interaction was statistically not significant (**Table. 2**).

D. Cumulative day and night time activity

The analysis of the day and night time activity data revealed significant differences between day and night time activity as well as between male and female activity under 12:12 hr LD cycle. ANOVA on the cumulative day and night time activity data of males and females reveal a significant main effect of sex ($F_{4,10}$ = 41.60; p < 0.0001), phase (day and night time) ($F_{1,10}$ = 19.85, p < 0.0012), sex × phase interaction ($F_{4,10}$ = 5.086, p < 0.017). Multiple comparisons revealed that day time activity in both males and females is significantly greater than the night time activity, and females show significantly higher overall activity compared to males (**Fig. 4**). Statistical analysis revealed that day time activity of *D. ananassae* males is significantly greater than those of other closely related species (except *D. nasuta*) as well as from *Z. indianus*, similarly night time activity of *D. ananassae* males is significantly lower than other closely related species including *Z. indianus*. The day and night time activities of *Z. indianus* females differ significantly from the other four species of *Drosophila*.

E. Mid-day activity of five wild caught species of *Drosophila*

ANOVA on the mid-day activity data revealed a statistically significant effect of species ($F_{4,90}$ = 305.94; p < 0.0001), sex ($F_{1,90}$ = 186.86; p < 0.0001), and species × sex interaction ($F_{4,90}$ = 51.45, p < 0.0001). Multiple comparisons showed that the mid-day activity of *D. ananassae* (males as well as females) is significantly higher than other four species, and the mid-day activity of *D. melanogaster* males is significantly lower than those in the females (**Fig. 5**).

F. Sleep

Analysis of the sleep data indicates prominent siesta in males, which is more or less absent in females (**Fig. 6**). ANOVA revealed a significant main effect of species ($F_{9,20}$ = 24.53; $p < 0.0001$), sex ($F_{1,20}$ = 126.52; $p < 0.0001$), and sex x species interaction ($F_{9,20}$ = 4.89; $p < 0.0015$). Multiple comparisons revealed that night time sleep in males do not differ statistically among the closely related species; however, day time sleep in *D. nasuta* females is significantly greater compared to *D. malerkotliana* and *D. melanogaster*. Day time sleep duration in *Z. indianus* males and females is significantly greater compared to those in other species.

G. Circadian period

The locomotor activity behaviour of only three out of five species of *Drosophila* - *D. ananassae, D. melanogaster* and *D. malerkotliana* was found to be rhythmic under DD. ANOVA of the circadian periodicity data revealed a significant main effect of species ($F_{2,42}$ = 20.47; $p < 0.0001$), and sex ($F_{1,42}$ = 22.48; $p < 0.0001$), however, species × sex interaction was statistically not significant ($F_{2,42}$ = 1.612; p = 0.200). Multiple comparisons showed that the circadian periodicity of *D. malerkotliana* females is significantly greater than those of *D. ananassae* and *D. melanogaster*, and the circadian periodicity of *D. ananassae* males is significantly shorter than *D. malerkotliana* males but not from *D. melanogaster* males (**Fig. 6**).

3.5 Discussion

A. Locomotor activity profiles of male and female flies under 12:12 hr light/day (LD) cycle.

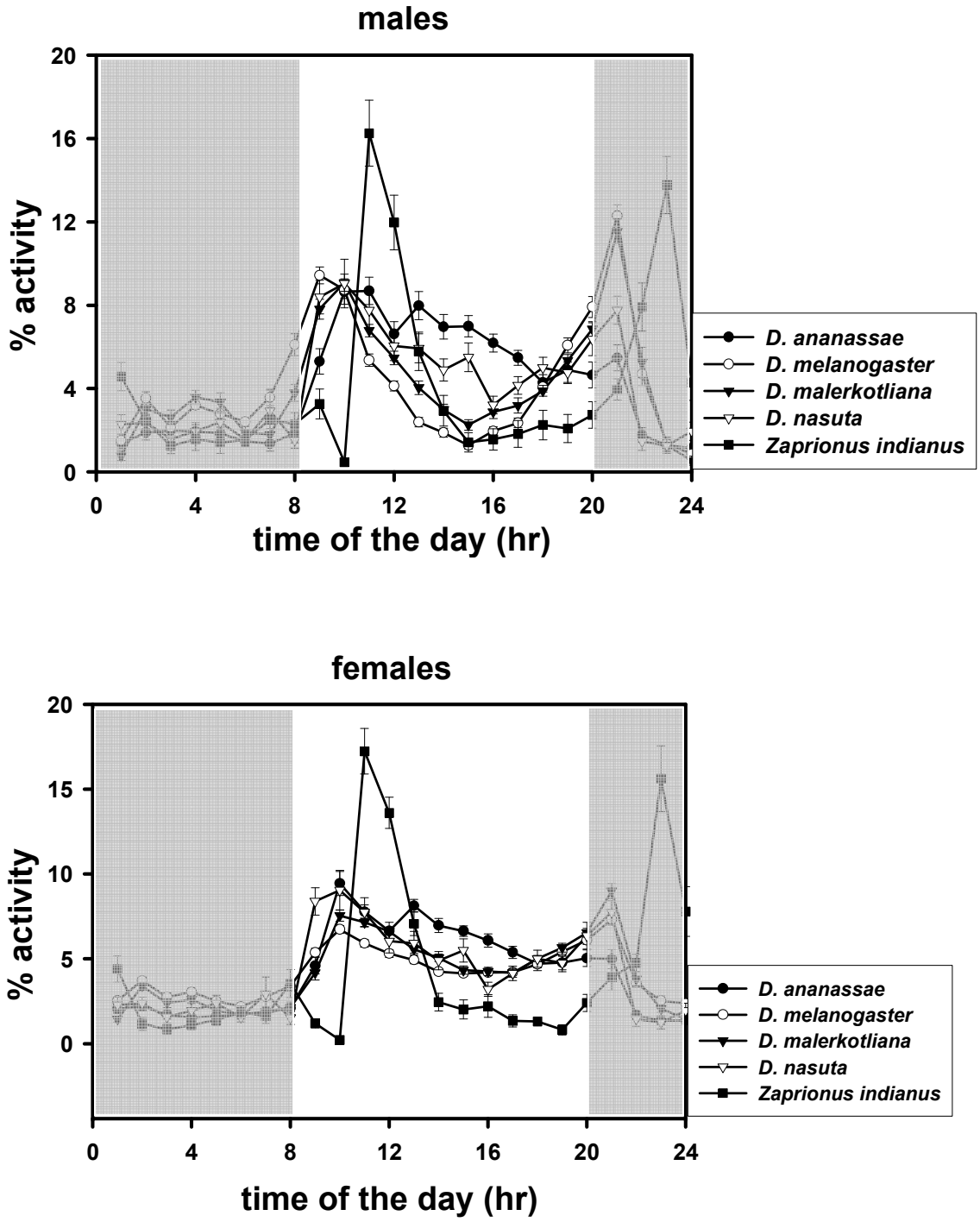


Fig. 1 Locomotor activity profiles of five wild caught species of *Drosophila* under 12:12 hr light/dark (LD) cycle**.** The activity profile show bimodality pattern with morning and evening peaks close to lights-ON and lights-OFF, respectively.

B. Phase-relationship of activity peaks in five wild caught species of *Drosophila*

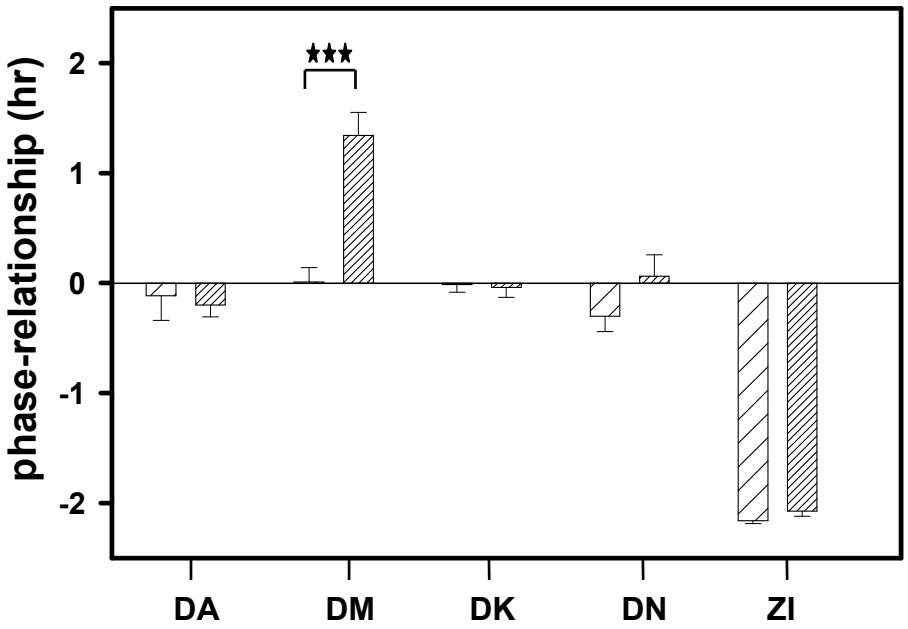


Fig. 2 The phase-relationship between activity peaks of different male and female wild species of *Drosophila.* Asterix (★) indicate significant difference in activity peaks in **DM** species. The x-axis represent species (**DA –** *D. ananassae*, **DM** – *D. melanogaster*, **DK** – *D. malerkotliana*, **DN** – *D. nasuta*, **ZI** – *Zaprionus indianus*) and y-axis represent phase-relationship (hr).

C. Anticipation index of five wild caught species of *Drosophila*.

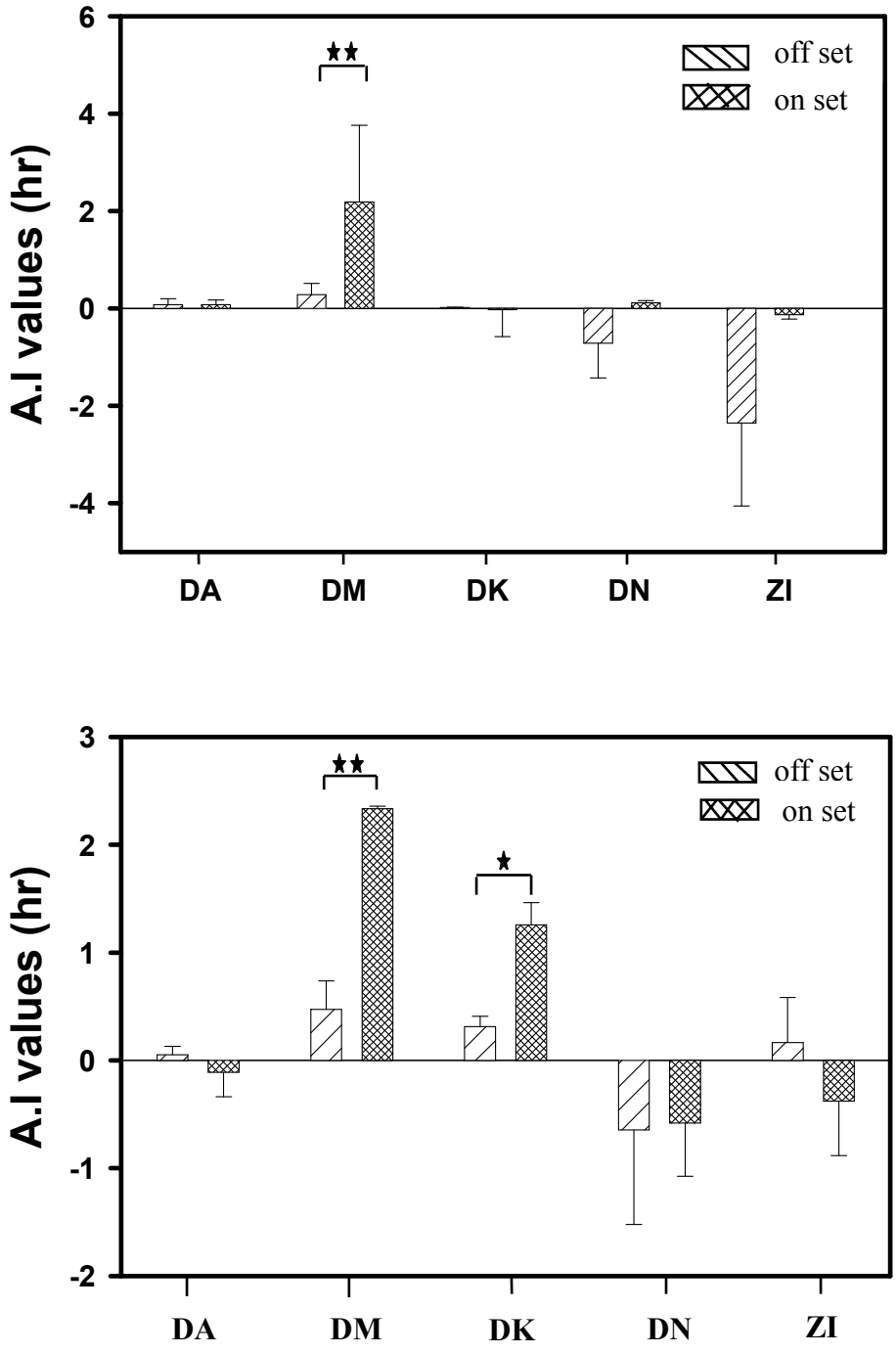


Fig. 3 Anticipation index of male (top) and female (bottom) flies of five wild caught species of *Drosophil*a under 12:12 hr light/dark cycle. Asterix (★) indicate significant difference in A.I in **DM** (males as well as females) and **DK** (females).

D. Cumulative day and night time activity

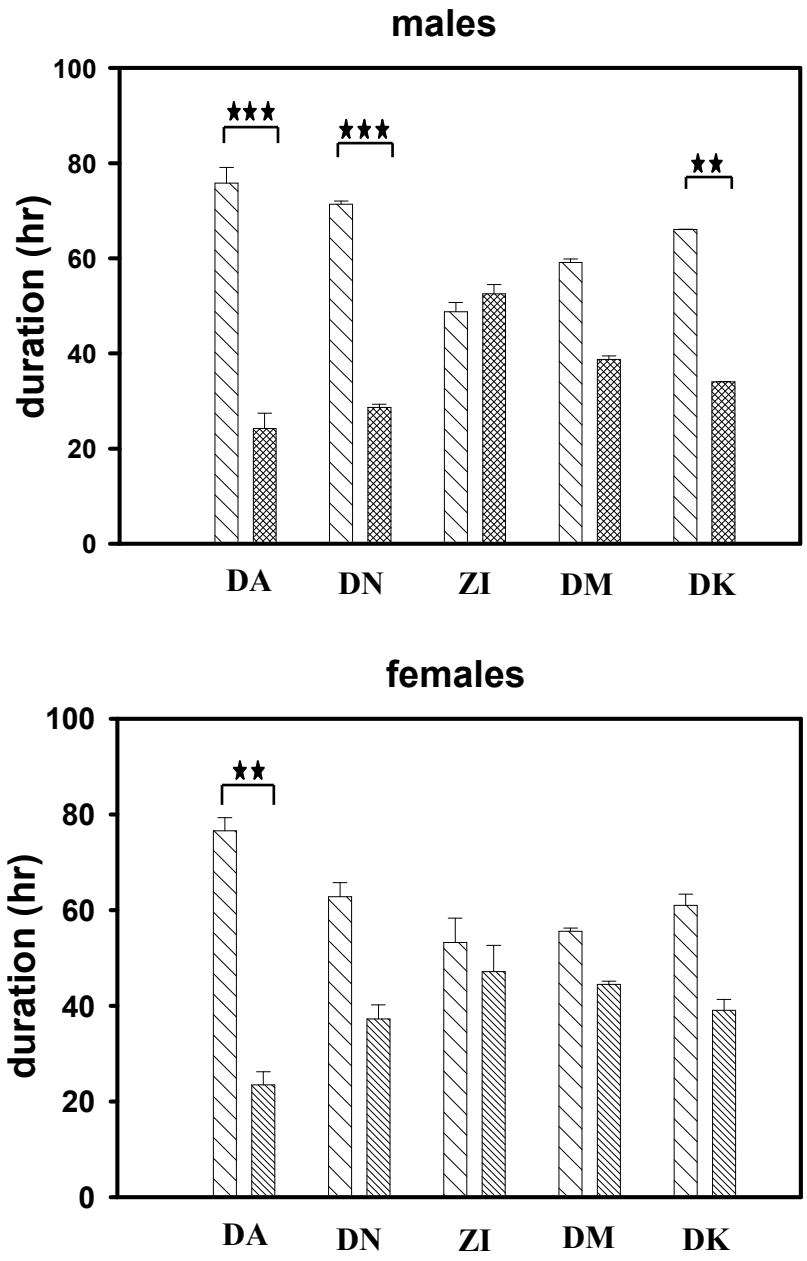


Fig. 4 The figures show day (▨) and night(▩) time activity of males and females species from five wild caught species of *Drosophila* under 12:12 hr light/dark cycles. Asterix (★) indicate significant difference of day and night time activities in **DA, DN, DK (males)** as well as significant difference in day and night time activity in **DA** (females).

E. Sleep duration for males and females

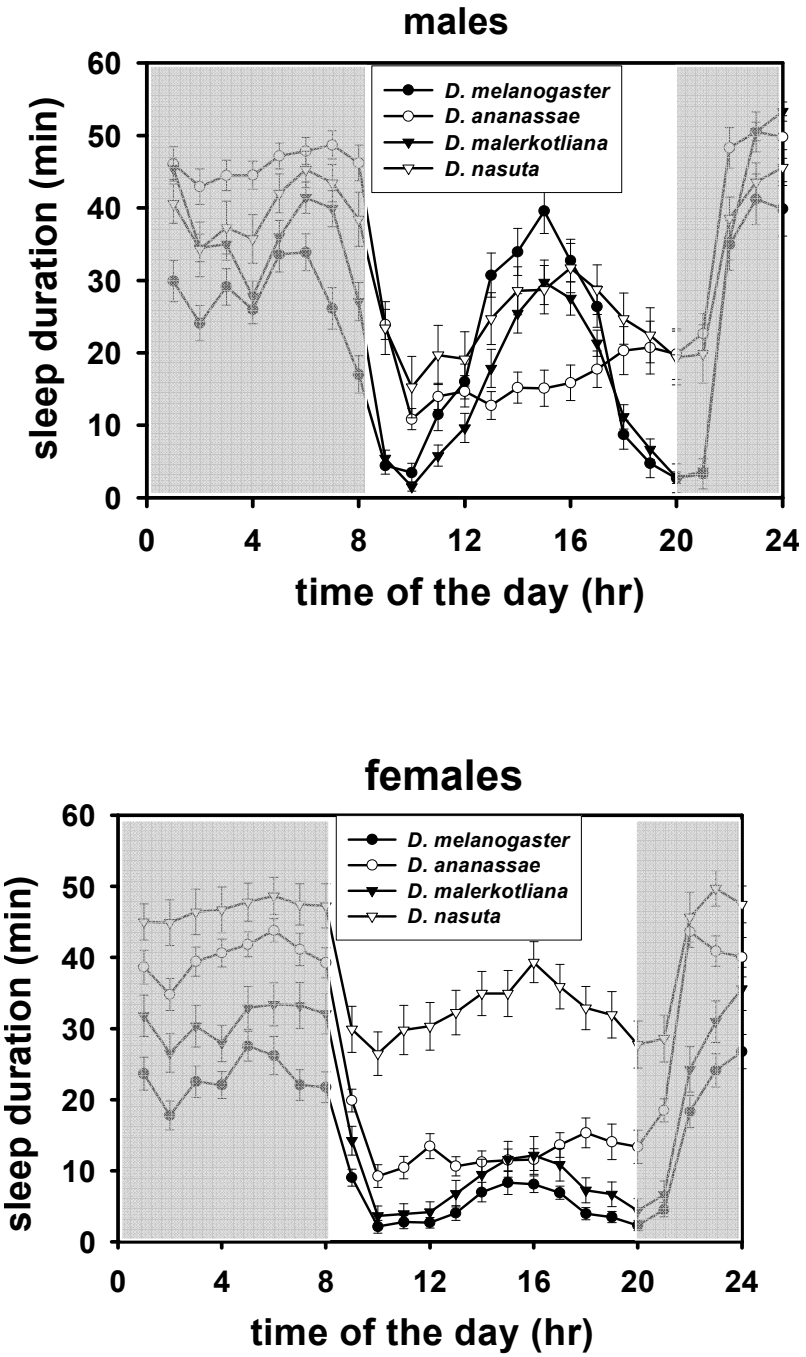


Fig. 6 The above graphs show the sleep duration of male and female wild caught species *Drosophila* respectively. The shaded and unshaded areas represent night and day time respectively.

F. Midday activity of five wild-caught species of *Drosophila.*

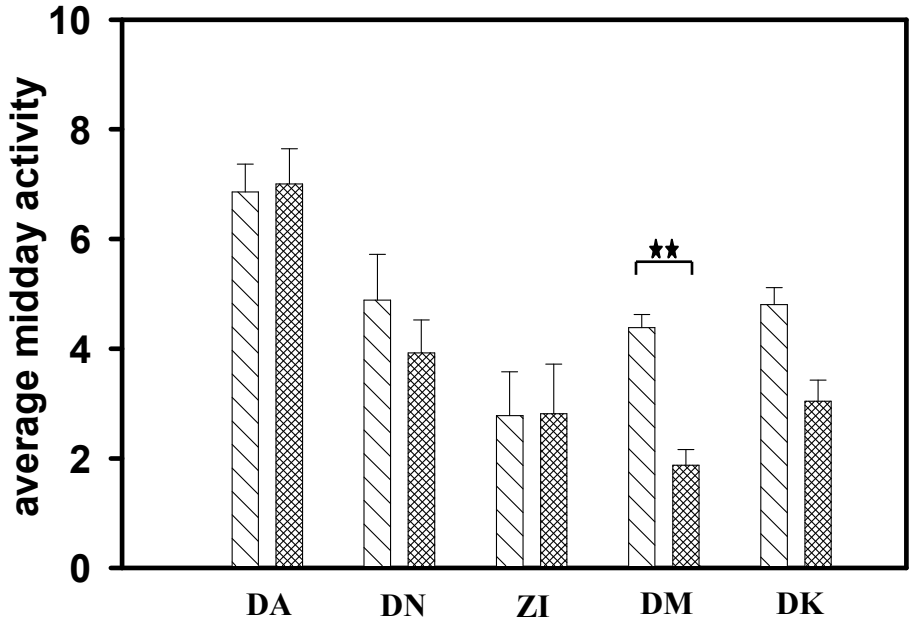


Fig. 5 Mid-day activity of five female (▨) and male (▩) wild species of *Drosophila* between ZT6-9 hrs. Midday activity varies between males and females in **DM**. Asterix (★) indicate significant difference in mid-day activity in **DM** (males and females) between ZT6-9 hrs.

G. Free-running period under constant darkness (DD).

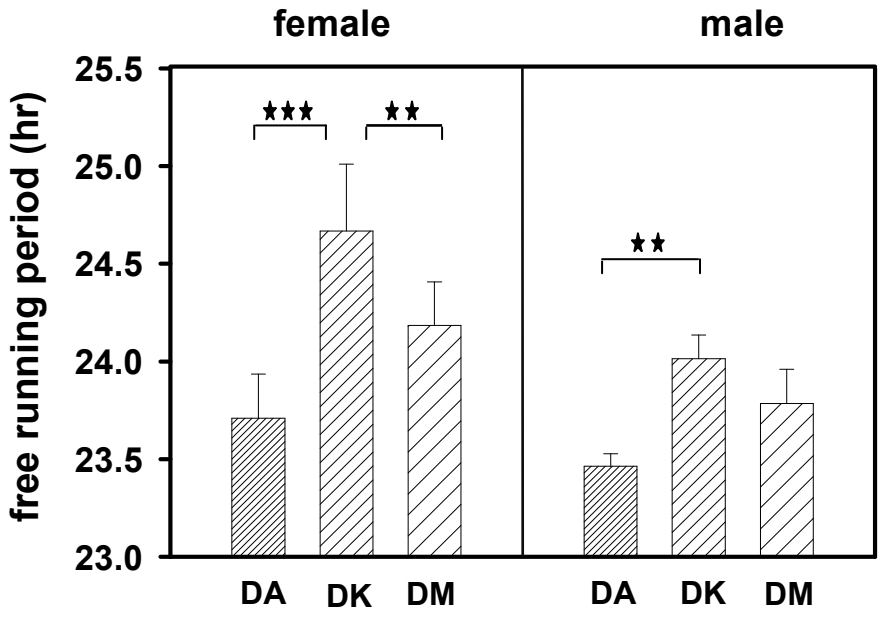


Fig. 8 Average free-running period of males and females from three wild caught species of *Drosophila* under constant dark conditions (DD). Symbols are same as previously mentioned.

H. Sleep duration for male and female flies of five wild caught species of *Drosophila* under 12:12 hr light/dark cycle.

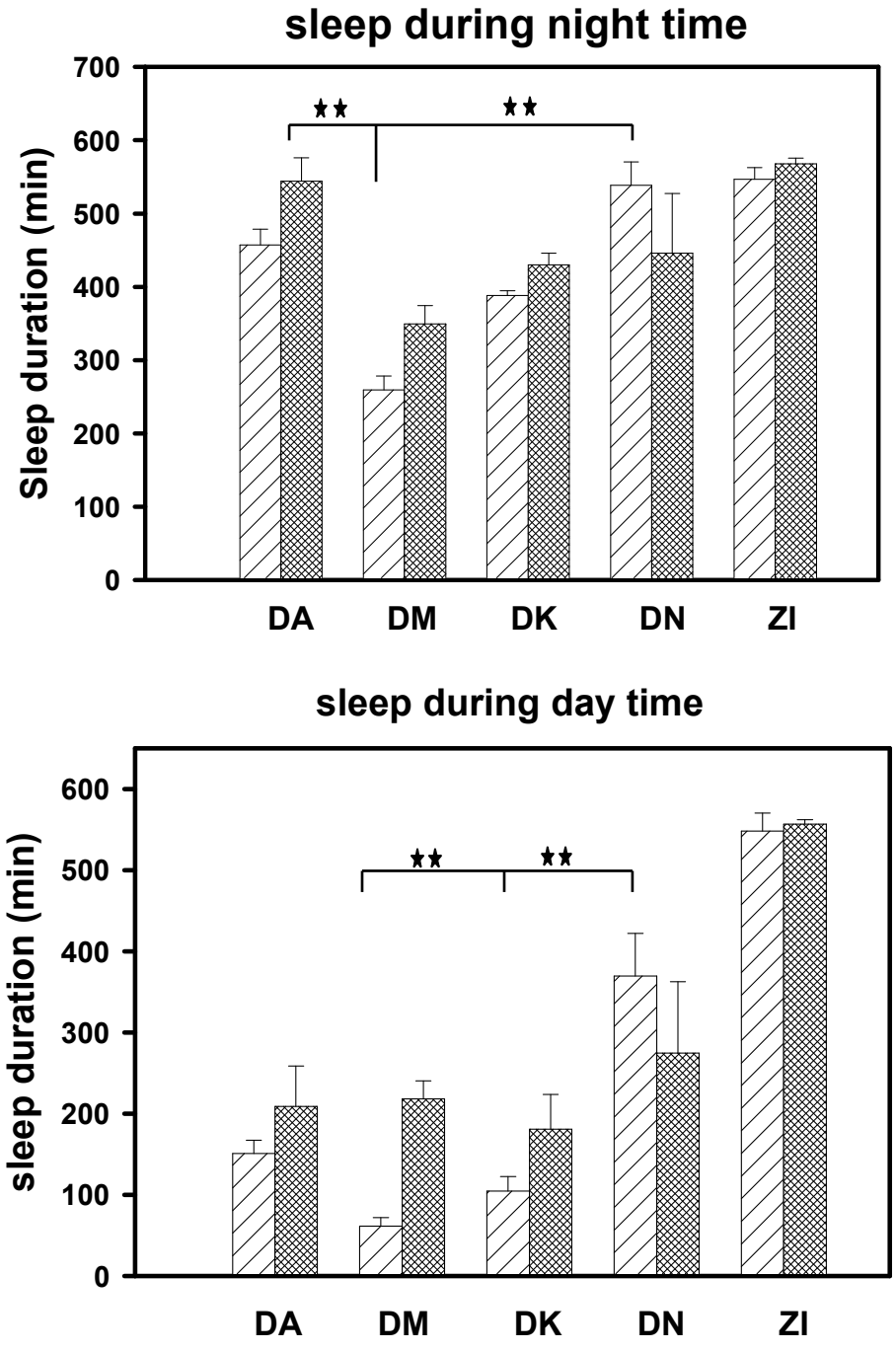


Fig. 7 Sleep duration of female (▨) and male (▩) flies of five wild caught species of *Drosophila* during night and day time respectively. The X-axis represent five species, y-axis represent sleep duration in minutes (min). Asterix (★) indicate significant difference of **DM** (female) with **DN** (male) and **DA** (male) in night time and **DN** (female) with **DK** (female) as well as with **DM** (females) as shown.

Table 1. Result of Analysis of Variance (ANOVA) on phase of activity/rest rhythm of five species of *Drosophila*. Analysis reveal significant main effect of species, sex and species sex interaction.

Summary of all effects on phase of activity/rest rhythm

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	208.09	805	0.248	839.87	**0.0001**
Sex (s)	1	13.76	805	0.248	55.56	**0.0001**
Sxs	4	9.84	805	0.248	39.73	**0.0001**

Table 2. Result of the Analysis of Variance (ANOVA) on the anticipation index of five species of *Drosophila*. ANOVA reveal significant main effect of species only.

Summary of all effects on anticipation index of five species of *Drosophila*

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	4.96	20	0.79	6.26	**0.001**
Sex (s)	3	2.29	20	0.79	2.89	0.061
Sxs	12	0.93	20	0.79	1.17	0.364

Table 3. Result of Analysis of Variance (ANOVA) on day and night time activities of five species of *Drosophila*. Analysis of variance reveal the significant main effect of species, time and interaction of both factors.

Summary of all effects on day and night activity of five species of *Drosophila*

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	10.7	10	0.26	41.61	**0.0001**
Phase (P)	1	5.1	10	0.26	19.85	**0.0012**
SxP	4	1.3	10	0.26	5.087	**0.016**

Table 4. Result of Analysis of Variance (ANOVA) on the midday activity data of five species of *Drosophila*. Analysis of variance reveal significant main effect of species, sex and interaction between species and sex.

Summary of all effects on midday activity of five species of *Drosophila*

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	38.23	90	0.12	305.94	**0.0001**
Sex (s)	1	23.35	90	0.12	186.86	**0.0001**
S x s	4	6.43	90	0.12	51.45	**0.0001**

Table 5. Result of the Analysis of Variance (ANOVA) on amount of total sleep of five species of *Drosophila*. Analysis of variance reveal significant main effect of species, sex and interaction between species and sex.

Summary of all effects on sleep of five species of Drosophila

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	9	66552.1	20	2713.52	24.53	**0.0001**
Sex (s)	1	343327	20	2713.52	126.52	**0.0001**
S x s	9	13286.3	20	2713.52	4.89	**0.0015**

Table 6. Result of Analysis of Variance (ANOVA) of free-running period data of three species of *Drosophila*. ANOVA reveal significant main effect of species and sex but no effect of their interaction.

Summary of all effects on free running period of three species of *Drosophila*

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	2	14.21	422	0.69	20.47	**0.0001**
Sex (s)	1	15.61	422	0.69	22.48	**0.0001**
S x s	2	1.12	422	0.69	1.61	0.2006

The locomotor activity rhythm in *Drosophila* has been extensively studied under various set of environmental conditions (Sheeba *et al.,* 2002). All previous studies on laboratory reared flies have shown that the rhythm is robust under DD and LD regimes but damps out in LL regime (Konopka *et al.*, 1989; Emery *et al.*, 2000). In our study we assayed locomotor activity behaviour of five wild caught species of *Drosophila* under 12:12 hr LD and DD conditions, and have also looked at the distribution of activity patterns of males and females.

The locomotor activity of both males and females followed bimodal pattern under LD (**Fig. 1)**. A closer look at the activity profiles revealed sex-related differences in the overall activity pattern with females showing more day time activity than males. Various environmental factors such as light, food, and temperature are known to influence circadian behaviour (Allada and Chung, 2009). For example, females show more robust rhythmicity than males and the main difference is seen in the locomotor activity pattern of flies kept under LD cycles with the activity of females being evenly distributed throughout the day (Helfrich-Förster, 2000). Locomotor activity is an essential part of foraging behaviour (Edery *et al.,* 2009; reviewed in Meunier et al., 2007), and females showing higher overall activity could be a result of enhanced foraging activity. It has been reported that the phase-relationship of activity rhythm differs among related *Drosophila* species as well as between males and females (Helfrich-Förster, 2000). Analysis of phase-relationship data revealed that *Z. indianus* show a negative phase-relationship compared to other species (**Fig. 2**). Furthermore, analyses by ANOVA also revealed statistically significant differences in the phase-

relationship of activity rhythm among closely related species. The anticipation indices of activity rhythm were different among several of the *Drosophila* species, however, the AI values of males and females do not differ statistically (**Fig. 3**). Comparison of the activity levels of five species revealed higher overall activity in *D. ananassae* (males and females) than other closely related species of the same genus (**Fig. 4**). Mid-day activity at ZT06-09 hrs revealed statistically significant difference between males and female of *D. melanogaster* species, while male-female differences in mid-day activity was not noticed in other related species (**Fig. 5**). The sleep architecture of *D. melanogaster* is sexually dimorphic, with females sleeping much less than males during day-time, presumably because reproductive success requires greater foraging activity by the female as well as the search for egg-laying sites (Issac *et al.,* 2009). The result of our study suggests that females sleep lesser during the day than males (**Fig. 7**). In *Drosophila*, often the evening activity peak is most prominent and it persists even under DD, while the morning peak is reduced considerably (Helfrich-Förster, 2000), which further suggests that mainly the evening component of activity constitutes the free-running rhythm, and therefore it is not a big surprise that the wild caught species which show higher evening activity peak also have longer circadian period. Our results clearly suggest that *D. melanogaster* and *D. malerkotliana* which have high evening peak show longer circadian period than the other closely related species such as *D. ananassae* (**Fig. 8**).

Chapter 4

Mating rhythm assay

4.1 Mating Rhythm

The physiological and behavioral activities of many organisms are restricted to specific times of the day. Most insects including *Drosophila* show circadian rhythmicity in courtship, mating and oviposition (Miyatake *et al.,* 1997). The mechanisms underlying circadian mating rhythm in *Drosophila* is believed to involve clock genes such as *per* and *tim*, and is primarily dictated by the females (Sakai and Ishida, 2001). The peak of mating occurs at different time of the day in separate *Drosophila* species, which might constitute one of the sources of reproductive isolation that allows organisms to avoid cross-breeding among species living in sympatry (Nishinokubi *et al.,* 2006). Previous studies also support the view that *per* gene plays an important role in the temporal reproductive isolation between closely related populations of *Drosophila* (Hall *et al.,* 1991; Tanimura *et al.,* 2002; Kyriacou *et al*., 2003). For example, *D. melanogaster* and *D. simulans*, two sympatric species, exhibit mating activity, out of phase with each other, *i.e.* the frequency of mating of one species is highest at a time of day when it is the least for the other and *vice versa*. Tauber (2003) reported that the period of locomotor activity rhythm is not causally related to that of mating rhythm, although the two rhythms appear to be manifestations of the same circadian clocks.

It has previously been reported that the circadian rhythm of mating activity differs among separate species of *Drosophila* (Sakai and Ishida, 2001). This is illustrated by the fact that females of *D. ananassae* species discriminate between males of its own species and its sibling species *D. pallidosa* by the male courtship songs (reviewed in Singh *et al.,* 2008). It was shown that as females gets older; she mates at a higher frequency than when younger.

Behaviors controlling the propensity to mate and remate can have large effects on fitness, because the decision to mate during a given period can have direct effects on current and future reproduction and hence fitness (Sgro *et al.,* 2000).

To elucidate whether the profile, period or robustness of mating rhythm varies in closely related species of *Drosophila*, we assayed mating rhythm in five wild caught species of *Drosophila*. Different components of mating behaviour such as mating profile, mean mating frequency, cumulative day and night time mating were quantified to analyze mating behavior.

4.2 Experimental Setup

Flies from each population of the five wild caught species of *Drosophila* were used for the mating behaviour assay. Males and females were maintained for 3 to 4 days under 12:12 hr LD cycle in separate vials loaded with cornmeal medium. After 3 to 4 days, five pairs of males-females were introduced into single long vial and five such vials per species were used to study mating behaviour.

Mating in *Drosophila* is known to vary with age, i.e., the percentage of mating in flies changes with age. For example, the mean mating frequency of a 3 day old fly shows no day and night time difference, while in a 9 day old statistically significant difference between day and night time mating is seen (Sakia and Ishida, 2001). Therefore, in our study we chose 3 to 4 day old flies maintained under 12:12 hr LD cycles.

In order to estimate different parameters of mating, data were recorded every 3 hr for 20 minutes. We primarily focused on the following three parameters:

(i) Tracking (chasing): Mating behavior in *D. melanogaster* starts with the male orienting itself towards and following the female, a behavior often referred as tracking. Tracking behavior is apparently guided visually, and therefore vision is extremely important for mating. Males discriminate strongly against females, which have ectopic male-specific pigmentation but are otherwise normal, hence vision seems to be essential for mating preference (Yamamoto *et al.,* 1999).

(ii) Attempt to mate: After tracking, males approach females and tap her abdomen; with his forelegs bearing gustatory as well as mechanosensory bristles which are believed to help the males in courtship. Tapping is followed by unilateral wing vibrations (courtship song), which sends auditory signal to females and involves the release of hydrocarbons which facilitate olfactory communication between males and females (Yamamoto, 1999). When the courting male is not rejected by the female, he turns to the back of the female and licks her genitalia with his probiscus, triggered by extrusion by the female of the ovipositor which is known to emit chemicals (Yamamoto *et al.,* 1999) signaling males for copulation.

(iii) Copulation: When the female is sufficiently receptive, she opens the vaginal plate for copulation. After stable copulation for 15-20 min, the male dismounts the female after genital uncoupling.

A score of 1 was given to every parameter noted during the 20 min observation interval and the total score was added for each of the above parameters, at every time point to give a score for the mean mating activity for each replicate vial. The whole procedure was repeated for five successive days.

4.3 Materials and Methods

***Fly Strains**:* Flies from each population of wild caught species of *Drosophila* was used for the mating rhythm assay. All the five species were maintained on cornmeal medium at approximately 25 ^{o}C and ~90% relative humidity in conditions of continuous light. Adult flies were collected on the 10th day of egg stage for the three species - *D. melanogaster, D. ananassae* and *D. malerkotliana,* while for *D. nasuta* and *Zaprionus indianus* adult flies were collected on around 15th day after egg collection as their pre-adult development timing is 13 to 17 days, this was repeated twice or thrice so as to get a sizeable population of flies.

4.4 Results

A. Mating profile of five wild caught species of *Drosophila* under 12:12 hr light/dark cycle.

The mating curves obtained for each 3 hourly observation during the entire four to five day period for five wild caught species of *Drosophila* was normalized using the total number of matings observed during 4 to 5 day period. The average number of mating in 3 hr interval was plotted as a function of time (Fig. 1). The figures illustrate that the peaks of mating vary among the closely related species. *D. melanogaster, D. ananassae,* and *D. nasuta* show robust rhythmicity in mating behaviour with higher mating during the day time and low during the day time (**Fig. 1 A, B, C**), *Z. indianus* show high and low mating activity every alternate day (**Fig. 1 D**) and *D. malerkotliana* shows no preference for time of mating (**Fig. 1 E**).

B. Average mating frequency in five wild caught species of *Drosophila* under 12: 12 hr light/dark cycle.

The mating activity of five wild caught species of *Drosophila* under 12:12 hr light/dark cycles, lights-on at 8:00 hr (ZT00) and lights-off at 20:00 hr (ZT12) and was found to vary from one species to another **(Fig. 2).** *D. ananassae* show higher mating frequency (~42%) at ZT00, and *D. nasuta* show higher mating (~70%) at ZT03. *D. melanogaster* show high mating activity between ZT00 and ZT06, while mating activity in *D. malerkotliana* is high (~20-25%) between ZT03 and ZT09. *Zaprionus indianus* show higher mating activity between ZT03 and ZT06.

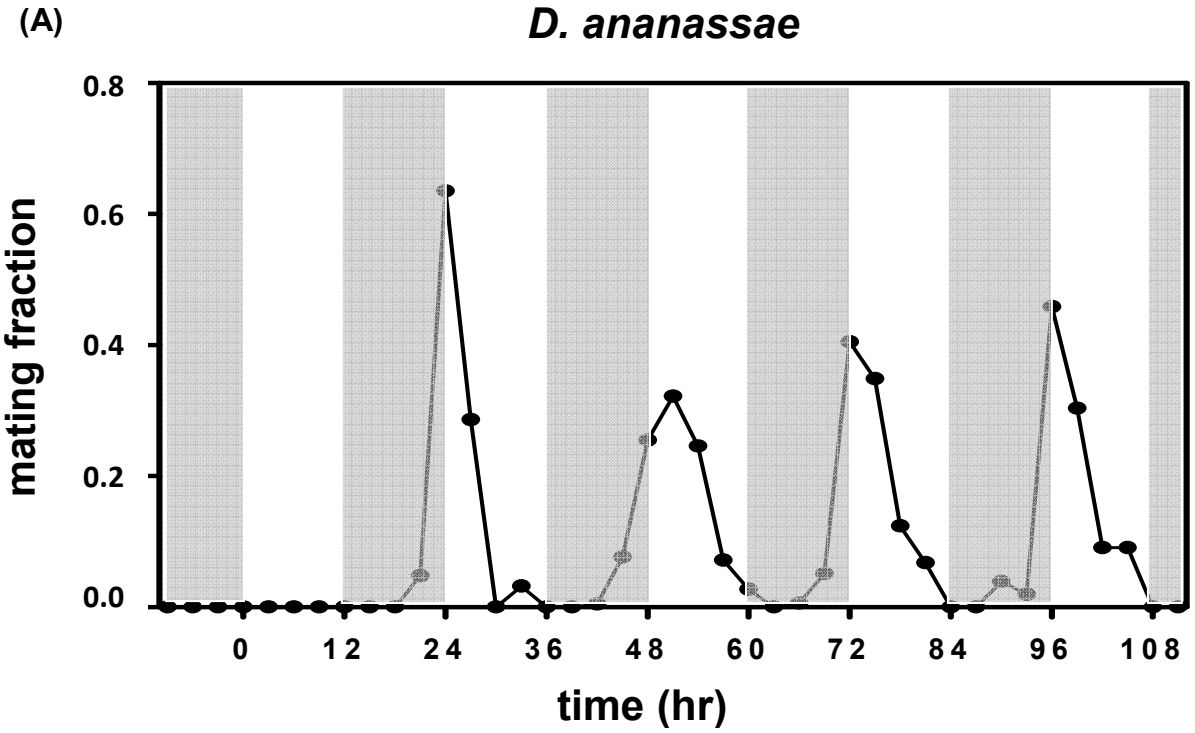
ANOVA on the average mating frequency data revealed statistically significant effect of species ($F_{4,96}$ = 127.53; p < 0.0001), phase ($F_{7,96}$ = 102.7978; p < 0.0001), and species x phase interaction ($F_{28,96}$ = 34.7809, p < 0.0001). Post-hoc multiple comparisons revealed that the mating activity of *D. nasuta* at ZT03 is higher than other wild caught species. Further, the night time mating activity of *D. malerkotliana* is significantly higher than those of the other wild caught species of *Drosophila*.

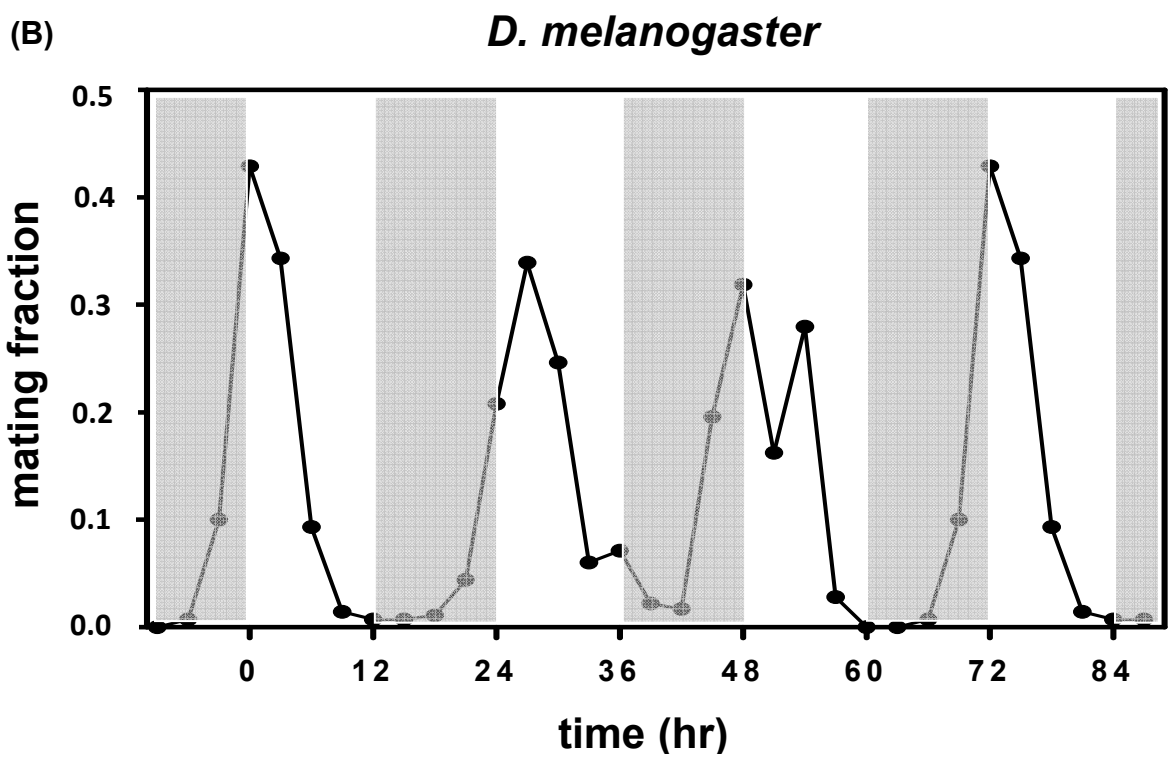
C. Cumulative day and night time mating under 12:12 hr light/dark cycle.

In order to study the average mating activity under 12:12 hr LD cycle, we calculated the average mating frequency during the day and night time separately. Mating in all five species is clearly higher during the day time and mean mating frequency is seen to vary from ~65 to ~97% in different species **(Fig. 3)**.

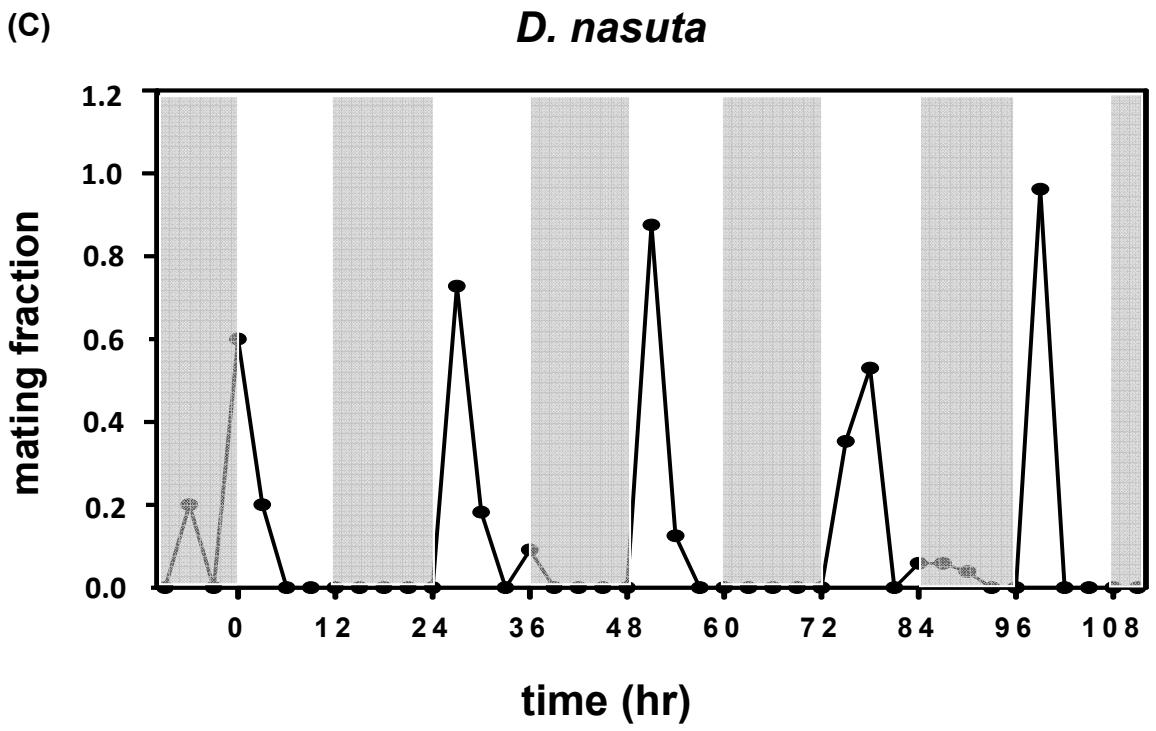
D. nasuta, D. ananassae, D. melanogaster and Zaprionus indianus mate primarily during day time (~90%), only ~10% mating occurs during the night time, however in *D. malerkotliana*

1. **Mating profile of five wild caught species of *Drosophila* in light/dark (12:12 hr) cycle.**

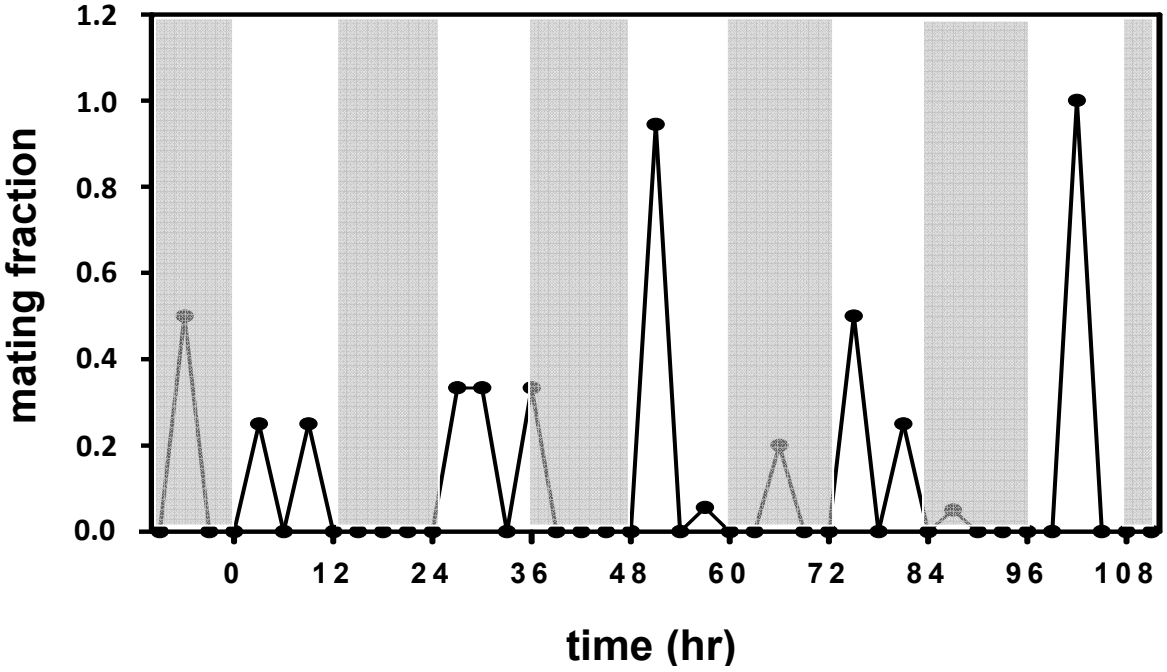




(C)


D. nasuta
mating fraction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
12
24
36
48
60
72
84
96
108
time (hr)

(D)


Z. indianus
mating fraction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
12
24
36
48
60
72
84
96
108
time (hr)

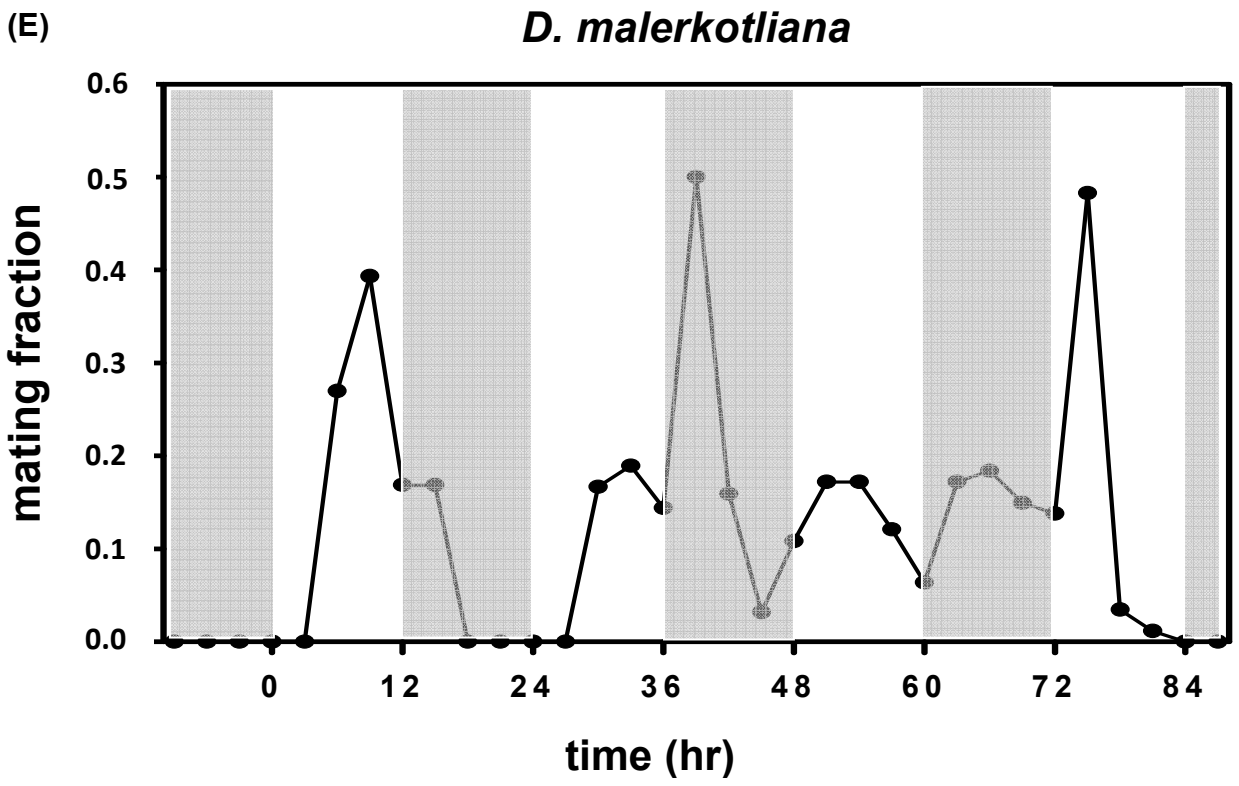


Fig. 1 Profiles of mating behavior under light/dark (LD) cycle (12:12 hr) in five wild caught species of *Drosophila,* shaded areas indicate dark phase whereas unshaded area represent light phase. The x-axis represents time in hours (hr) and y-axis represent mating fraction (average mating divide by total number of mating at each time point).

2. Mating frequency in five wild caught species of *Drosophila* under light/dark (12:12 hr) cycle.

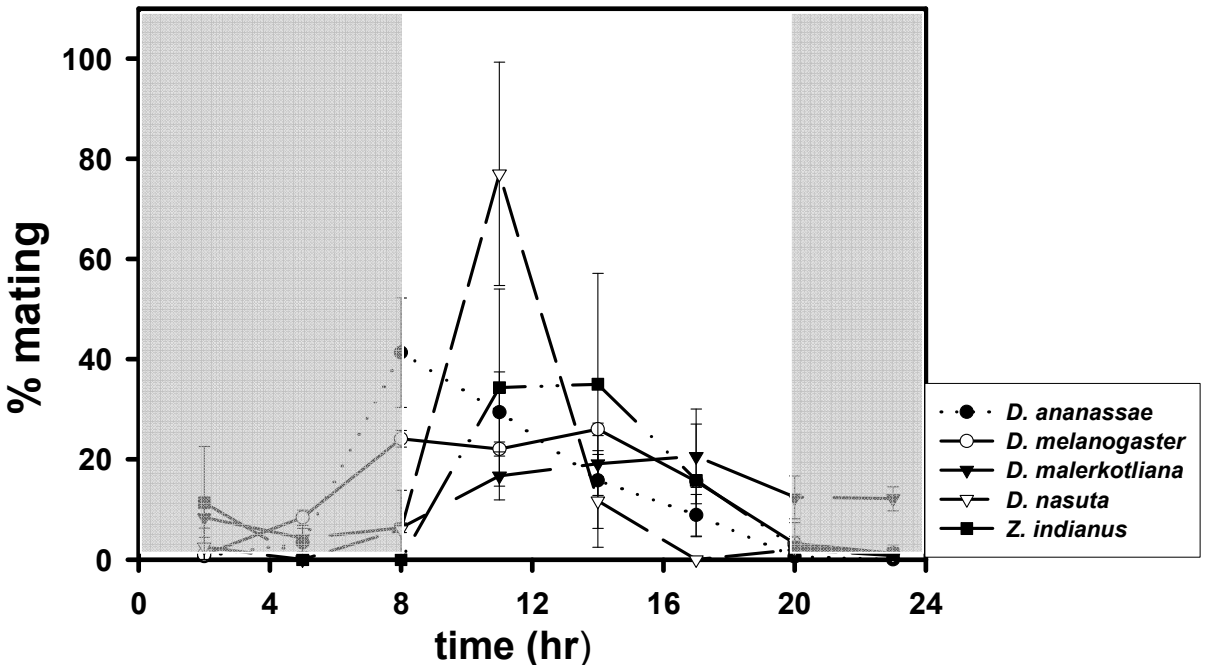


Fig. 2 Frequency of mating activity under 12:12 hr LD cycles in five wild caught species of *Drosophila* averaged across 5 days. The x-axis represent time in hours (hr) and y-axis represent % mating.

3. Cumulative day and night time mating under 12:12 hr light/dark (LD) cycle.

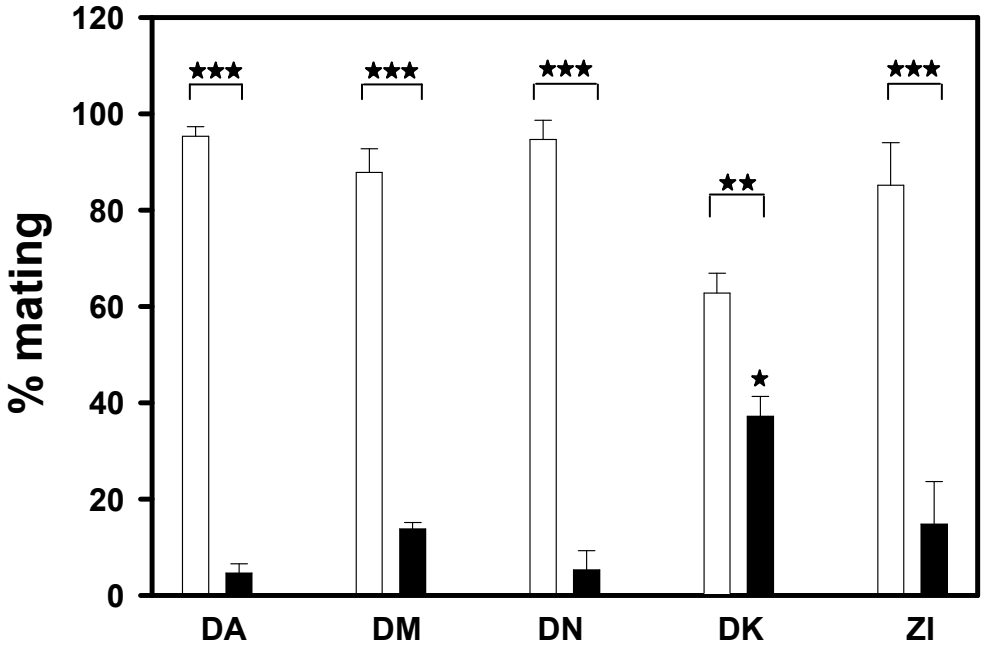


Fig. 3 Frequency of mating during day ☐ and night ■ time under 12:12 hr LD cycle for five wild caught species of *Drosophila.* The asterix (★) represent significant difference of night time mating in *D. malerkotliana* with other wild species of *Drosophila*. (**DA** - *D. ananassae,* **DM** - *D. melanogaster***, DN** - *D. nasuta,* **DK** - *D. malerkotliana,* **ZI** - *Zaprionus indianus*).

Table 1: Result of ANOVA on the percentage mating data under 12:12 hour light/dark (LD) cycle. Analysis of Variance reveal significant main effect of species, time and interaction between species and time.

Summary of all effects on percentage mating frequency of five species of *Drosophila*

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	370.19	960	2.9	127.53	**0.0001**
Time (T)	7	298.4	960	2.9	102.79	**0.0001**
SxT	28	100.96	960	2.9	34.78	**0.0001**

Table 2: Result of ANOVA on the percentage mating in day and night time under 12:12 hour light/dark cycle. Analysis of Variance reveal significant effect of species, time and interaction between species and time.

Summary of all effects on cumulative mating during day and night time

Variable	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p* -level
Species (S)	4	48083.5	240	661.46	72.692	**0.001**
Time (T)	1	145271	240	661.46	219.62	**0.001**
SxT	4	32081.2	240	661.46	48.5	**0.001**

maximum (~70%) mating occurs during the day and ~30% mating is observed during the night time.

ANOVA on the average cumulative mating activity data revealed a significant main effect of species ($F_{4,24}$ = 72.69, p < 0.0001), phase of mating ($F_{1,24}$ = 219.62, p < 0.0001) and species x phase interaction ($F_{4,24}$ = 48.50, p < 0.00). Post-hoc multiple comparisons revealed that cumulative mating activity during day time in five wild caught species of *Drosophila* is significantly higher than those during night time, with all species except *D. malerkotliana* showing ~90% day time mating. Further analyses revealed significantly higher cumulative night time mating in *D. malerkotliana* compared to other wild caught species of *Drosophila*.

4.5 Discussion

Insect mating is usually rhythmic (Miyatake *et al.,* 1997). Variations in timing of mating in insects reduce direct competition between species for food and other resources and lead to reproductive isolation among species living in sympatry (Saunders, 1982). It helps in preventing cross-breeding between species leading to allochronic speciation (Mallet, 2006). The species-specific differences in mating timing in *Drosophila* are thought to be clock-controlled (Sakai and Ishida, 2001). It has been reported that species-specific differences in circadian mating rhythm is dictated by females, and may affect daily pattern of mating activity (Sakai and Ishida, 2001). In *Drosophila* behavioral characteristics of mating, habitat, and breeding season vary in a species-specific manner thus creating species-specific barrier.

Mating profile of five wild caught species of *Drosophila* revealed difference in mating activity peaks. *D. ananassae* and *D. melanogaster* is seen to show robust mating rhythm relative to other related species of *Drosophila* (**Fig. 1**). Further analyses of the mating data revealed that mating frequency under 12:12 hr light/dark cycle varies from one species to another (**fig.2**). *D. ananassae* is found to show higher average mating frequency at ZT00, *D. nasuta* is found to mate most frequently at ZT03, while other closely related species such as *D. malerkotliana* show higher mating frequency during the middle of the day (ZT03-09), despite the fact that the these species are phylogenetically close to each other. The results further suggest that light/dark cycle is a stronger Zeitgeber for *D. ananassae* than its other closely related species. Rhythmic profiles of different species indicate species-specific pattern of mating with some species showing peak in mating at different time of the day, others on alternate days.

The cumulative mating activity data also revealed that mating is invariably higher during day time and low during night time. In most species ~90% mating is observed during the light phase of light/dark cycle (**Fig. 3**). Mating during day time might be advantageous for females as they can concentrate their activity on finding suitable substrate for egg deposition during the later part of the day (reviewed in Sisodia and Singh, 2005). In summary, the results of our study suggest that mating is time specific and it occurs preferentially during the day for many species. These results are in conformity with the previous studies done on other strains (Hardeland, 1972).

Chapter 5

Egg-laying rhythm

5.1 Egg-laying Rhythm

Circadian clocks are known to control a number of activities in insects; one such fundamental activity is the egg-laying rhythm and several species of insects exhibit this behavior including *Drosophila melanogaster* (Rensing and Hardelande, 1967; Gruwez *et al.,* 1972; Allemand, 1976a; Sheeba *et al.,* 2001a). Egg-laying rhythm in *Drosophila* entrains stably to a wide range of light/dark (LD) cycles, and free-runs under constant darkness (DD) and constant light (LL) with circadian periodicities, thereby indicating its endogenous origin (Allemand 1976a, b; Sheeba *et al.,* 2001b; Howlader *et al.,* 2006). Further studies have shown that the circadian period of egg-laying rhythm in *D. melanogaster* remains unchanged under different ambient temperatures and nutrition levels, suggesting that this rhythm is temperature and nutrition compensated (Howlader *et al.,* 2006).

Egg-laying is a complex phenomenon, involving at least two separate physiological processes – vitellogenesis and egg-retention (Allemand, 1976a,b). Periodic deposition of fertilized eggs involves a series of events starting from the production of oocytes to egg-laying on the selected sites (Allemand 1976b, Yang 2008). Although egg-laying rhythm in *D. melanogaster* is of circadian nature, some of its characteristics are quite different from the two well characterized circadian rhythms - activity/rest and adult emergence (reviewed in Howaldar and Sharma 2006; Manjunatha *et al.,* 2008). Under DD, the circadian period of egg-laying rhythm (27.66 ± 2.16 hr; mean ± 95% CI) is significantly greater than those of activity/rest activity (24.73 ± 0.29 hr), and adult emergence (23.64 ± 0.00 hr) rhythms (Sheeba *et al.,* 2001b). Even the limits of entrainment of the egg-laying rhythm are different from other two rhythms (Paranjpe *et al.,* 2004). Another striking difference between egg-laying and other rhythms is

that egg-laying rhythm continues unabated under LL conditions (Sheeba *et al.,* 2001b), while activity/rest and emergence behaviors become arrhythmic (Saunders *et. al.,* 2002). Although we know a great deal about the molecular mechanisms regulating activity/rest and emergence rhythms in *Drosophila*, the same cannot be said about egg-laying rhythm (Howladar *et al.*, 2006; Manjunatha *et al.*, 2008).

Oviposition site preference is an important aspect of non-sexual behavior of adult *Drosophila* females (Grossfield, 1978). It is closely related to fitness since *Drosophila* larvae have low mobility and therefore their survival depends largely on the choice of oviposition sites by the female parent (Sisodia and Singh, 2005). The oviposition behavior in *Drosophila* is strongly influenced by light and dark conditions (Singh *et al.,* 2005). Ohnishi (1977) found that females of *D. melanogaster, D. lutescens* and *D. virilis* lay more eggs during the light phase than in the darkness suggesting that day time is preferred for egg-laying in at least some species of *Drosophila* (Sisodia and Singh, 2005). On the contrary, several other studies (Allemanda, 1976a,b, Sheeba et al., 2001; Howladar et al., 2006), have shown that under 12:12 hr LD cycle egg-laying is rhythmic, with a prominent peak at the beginning of the dark phase. Sheeba and coworkers (2001b) assayed the egg-laying rhythm in individual flies for the first time, and found out that data averaged across all the flies under LL and DD regimes yielded no statistically significant pattern, whereas when the number of eggs laid by individual females was analyzed, at least 50% of them showed rhythmic egg-laying behaviour (Sheeba *et al.,* 2001b).

The neuronal architecture underlying circadian rhythms in *D. melanogaster* has been extensively studied for past several years (Sheeba *et al.,* 2008). The core pacemaker for the

circadian activity/rest rhythm has been localized in the lateral ventral neurons (LN_V). Core clock proteins expressed in the LN_V are believed to be essential for the maintenance of the regulation of activity/rest and emergence rhythms (Ewer *et al.,* 1992; Myers *et al.,* 2003). However, the neuronal network governing egg-laying rhythm in *Drosophila* is yet to be unraveled. Gross ablation experiments with insects have generally been done with a view towards partitioning the relative control of behavior among portions of Central Nervous System (CNS). Among several species of *Drosophila* was tested; only *D. melanogaster* was capable of egg-laying after decapitation (Sisodia and Singh, 2005). Therefore, it seems that egg-laying rhythm is controlled by peripheral oscillator; however, further investigations are needed to decipher the precise mechanism. At the level of physiology, it is known that functional LN_vs are necessary for the persistence of activity/rest rhythm in *Drosophila*, while LN_vs together with prothoracic gland (PG) are essential for the persistence of rhythmic eclosion in *D. melanogaster* (Myers *et al.,* 2003). The studies have also shown that the targeted (genetic) ablation of LN_vs and loss of function mutation of PDF abolish activity/rest and emergence rhythms, whereas egg-laying rhythm continues unabated (Howlader *et al.,* 2006). This suggests that egg-laying rhythm of *D. melanogaster* is controlled by non-PDF mediated non-LN_v based circadian oscillators (Howlader *et al.,* 2006). Although the LN_vs are considered as the central pacemaker for activity/rest and emergence rhythms in *Drosophila*, there is also sufficient evidence to suggest that autonomous peripheral oscillators that may or may not be directly under the control of the LN_v regulate circadian rhythms (Howlader *et al.,* 2006). For example, in *D. melanogaster* circadian pacemakers are found in non-innervated peripheral organs such as malphigian tubules (Giebultowicz and Hege, 1997). Taking cues from the earlier studies done on different

laboratory strains of *D. melanogaster,* we decided to study the egg-laying rhythm in four wild caught species of *Drosophila* under 12:12 hr LD cycle and in DD.

5.2 Experimental Setup

In order to assay egg-laying behaviour in four wild caught species of *Drosophila*, freshly emerged flies from each species were first kept under 12:12 hr LD cycles for two days with *ad libitum* food and at constant temperature and humidity (25 ^{o}C and ~90% relative humidity). To monitor egg-laying behaviour, 2 to 3 day old male-female pairs were introduced in a vial with ~3 ml of banana-jaggery food. Two sets of twenty such vials were kept under 12:12 hr LD cycles for three days, followed by a transfer to fresh vials containing small amount of food. Sixteen pairs of flies from each species were used for the assay, while few mixed cohorts of flies under similar conditions were maintained separately in case a fly died or escaped during assay. The egg-laying rhythm in *D. malerkotliana* could not be assayed due to moisture in the vials used for assaying egg-laying rhythm which led to the death of flies.

5.3 Materials and Methods

Each population of wild caught flies was maintained on cornmeal medium throughout their life cycle at constant temperature of 25 ^{o}C and relative humidity of ~90%. For assaying egg-laying behaviour each pair of males and females was kept as virgins in separate vials containing ~3 ml of banana-jaggery food. Banana-jaggery food was used for egg-laying assay because eggs can easily be counted and differentiated from food particle than on cornmeal food. Sixteen male-female pairs from each species were kept in LD conditions for 3 days and were subsequently

transferred into vials containing fresh food either in LD or DD conditions. After every 2 hr, eggs laid in each vial over the preceding 2 hr period were counted. The counting of eggs was done under microscope using cool light source from *Leica* (*Leica Microsystems*, Germany). The process continued for a minimum of 10 days under respective light regimes. 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 alive for the full ten days were used for the analysis, yielding a final sample size of 12 to 13 females in each light regime. Data from all the females was pooled and the average number of eggs laid in 2 hr interval was plotted.

5.4 Results

A. Representative time series data for egg-laying rhythm of individual flies from four wild caught species of *Drosophila* under 12:12 hr light/dark cycle.

The analyses of the time series data revealed that the phase of egg-laying rhythm differs among the four wild caught species of *Drosophila* (**Fig. 1**). The peak of egg-laying rhythm in *D. melanogaster* under 12:12 hr LD cycle occurs after "light-off", in *D. ananassae* and *D. nasuta* it occurs during the light phase, whereas no such timing preference for was seen in *Z. indianus,* and these flies laid eggs without any time preference. The data egg-laying behaviour under LD cycles suggests that even related species of *Drosophila* have different day/night preference for egg-laying behaviour (**Fig. 1**).

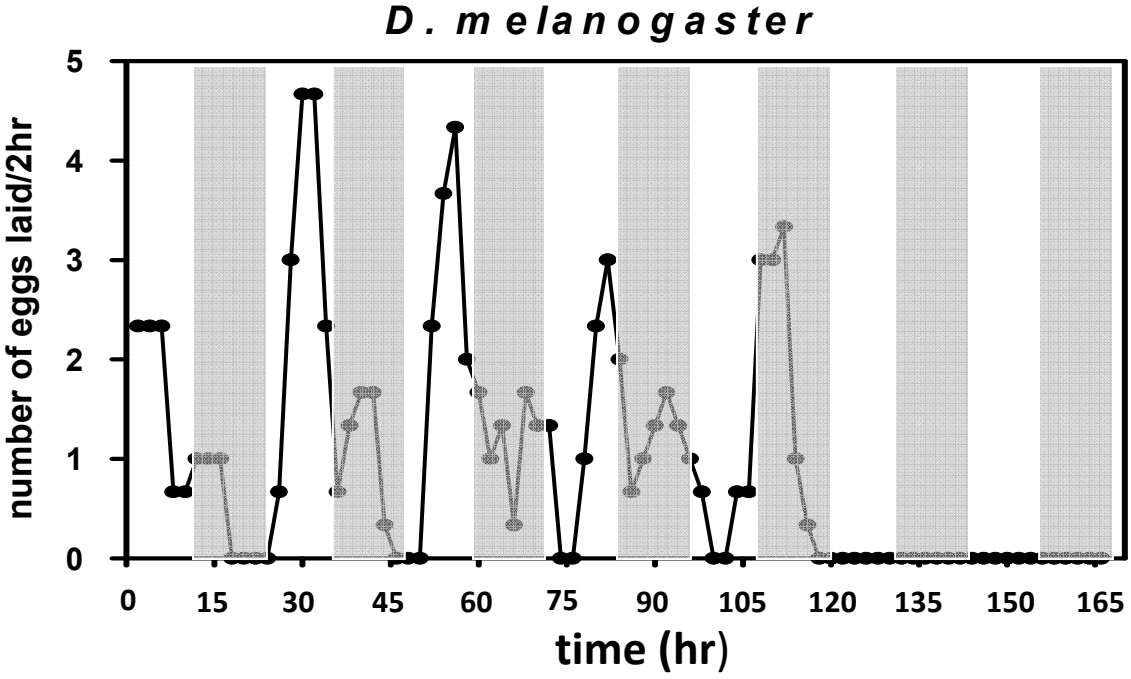
B. Circadian period of egg-laying rhythm of four wild caught species of *Drosophila* under constant dark conditions.

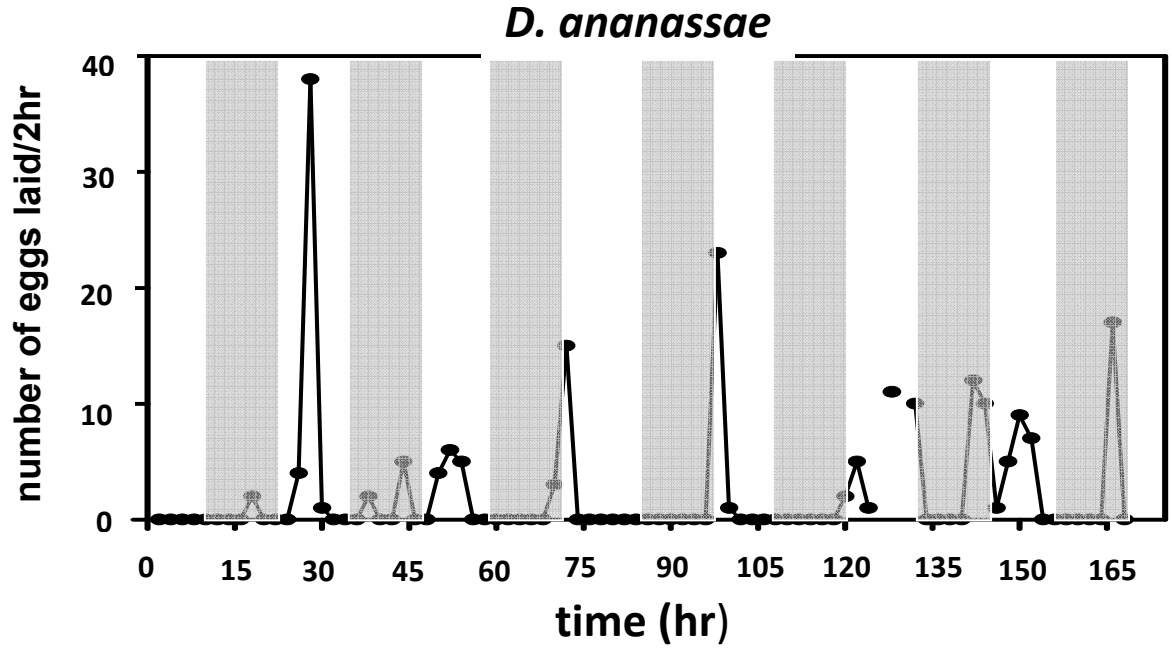
The circadian period of egg-laying rhythm in DD was calculated for each species, and was found to differ. The mean circadian period of egg-laying rhythm in *D. ananassae, D. melanogaster, D. nasuta* and *Z. indianus* is 23.43 ± 0.91 hr (mean ± SD), 23.8 ± 1.69 hr, 23.41 ± 1.34 hr and 21.68 ± 2.93 hr respectively (**Fig. 2**). The periodicity of *Z. indianus* is significantly shorter than those of *D. melanogaster* and *D. nasuta* ($p < 0.001$), however, it did not differ statistically from *D. ananassae* ($p > 0.05$). ANOVA on the circadian period data revealed a significant effect of species ($F_{3,22} = 8.15$; $p < 0.001$), and post-hoc multiple comparisons revealed that the circadian period of egg-laying rhythm in closely related species of genus *Drosophila* do not show significant difference from each other, whereas those of the distantly related species *Z. indianus* is significantly shorter than the rest.

C. Percentage entrainment of egg-laying rhythm under 12:12 hr light/dark (LD) cycle.

Percentage of flies in which egg-laying rhythm entrained under 12:12 hr LD cycles was estimated for each of the four wild caught species of *Drosophila*. Analyses of the data revealed that percentage entrainment of egg-laying rhythm vary even in closely related species - with *D. ananassae* entraining the most (~80%) among the Drosophila genus (**Fig. 3**). ANOVA revealed a statistically significant effect of species ($F_{3,37} = 26.82$, $p < 0.001$). The percentage of flies in which egg-laying rhythm entrained to 12:12 hr LD cycles varied from one species to another – egg-laying rhythm in ~85.0% of *D. ananassae*, ~18.2% of *Z. indianus*, ~35% *D. nasuta*, and ~60% *D. melanogaster* flies entrained to LD cycles. These results indicate that entrainment of egg-laying rhythm to 12:12 hr LD cycles is highest in *D. ananassae* and lowest in *Z. indianus.* This suggests that egg-laying clock of different species of *Drosophila* respond differently to LD cycles.

A. Representative time series data for egg-laying rhythm of individual fly from four wild caught species of *Drosophila* under 12:12 hr light/dark cycle.





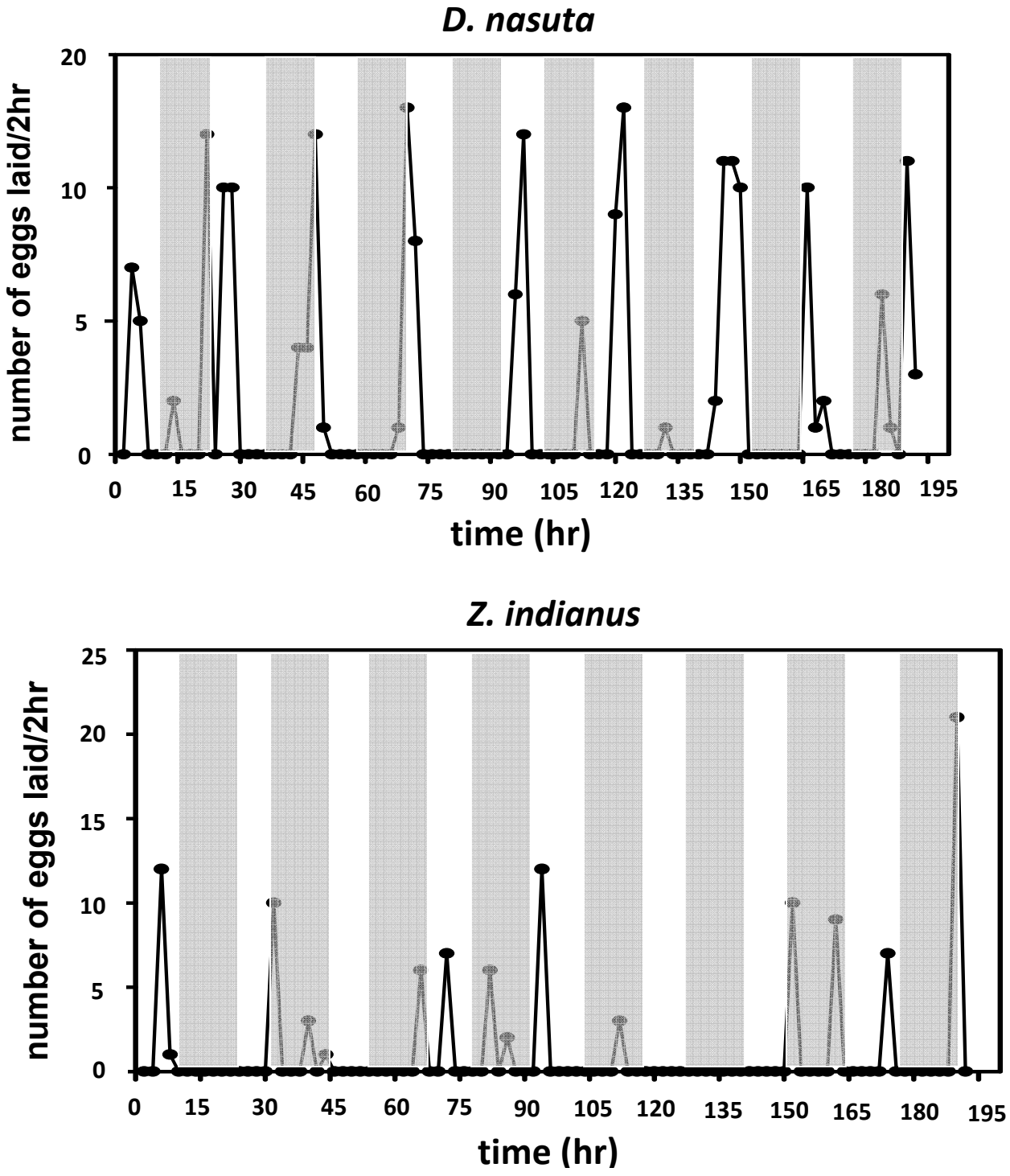


Fig. 1 Time series data of egg-laying of a representative fly under 12:12 hr light/dark cycle. The x-axis represent time in hours (hr) and y-axis represent number of eggs laid. Shaded and unshaded areas represent dark and light phases of light/dark cycle respectively.

B. Circadian period of egg-laying rhythm of four wild caught species of *Drosophila* under constant dark conditions (DD).

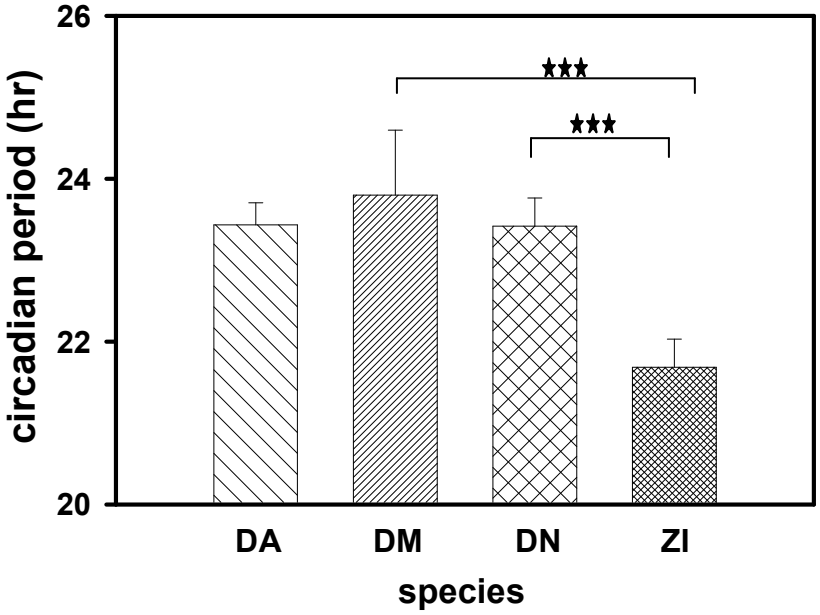


Fig. 2 Circadian period (hr) of egg-laying rhythm of four wild caught species of *Drosophila,* estimated under constant darkness (DD). The x-axis represent species (**DA** - *D. ananassae,* **DM** - *D. melanogaster***, DN** - *D. nasuta,* **ZI** - *Zaprionus indianus*) and y-axis represent circadian period (hr).

C. Percentage entrainment of egg-laying rhythm under 12:12 hr light/dark (LD) cycle.

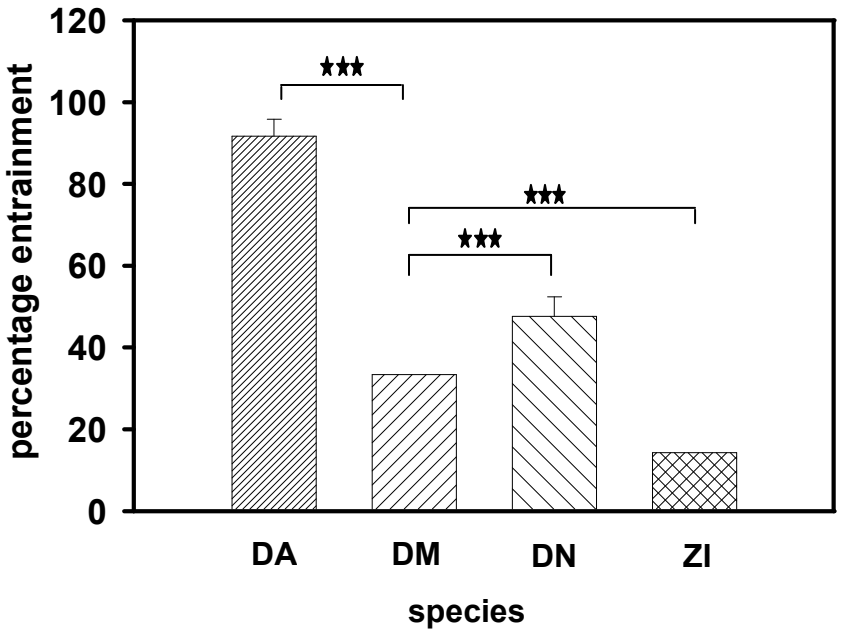


Fig. 3 Percentage entrainment of egg laying rhythm in females to light/dark cycles of different wild caught species of *Drosophila*. The x-axis represent different species (**DA** - *D. ananassae,* **DM** - *D. melanogaster***, DN** - *D. nasuta,* **ZI** - *Zaprionus indianus*) and y-axis represent percentage entrainment under 12:12 hr light/dark cycle.

5.5 Discussion

Among the circadian phenomena that have been studied so far in *Drosophila*, the egg-laying rhythm is unique, and relatively less explored. Unlike most other circadian rhythms, this rhythm persists in LL, and the circadian period is considerably greater than most other rhythms (Manjunatha *et al.,* 2008). We assayed egg-laying rhythm in four wild caught species of *Drosophila* under LD cycles and DD conditions. The results of the study suggest that *D. melanogaster* females lay maximum number of eggs after lights-off and *D. ananassae* and *D. nasuta* during the light phase. The egg output of each species is also found to differ from one species to another with *Z. indianus* laying least number of eggs than other wild caught species of *Drosophila*. Analysis of our data revealed that circadian periodicities for egg-laying rhythm varied among wild caught species of *Drosophila.* The results suggest that four wild caught species of *Drosophila* entrained under 12:12 hr LD cycle maintain different time course in LD cycle (**Fig. 1**).

The circadian period of egg-laying rhythm in closely related species (*D. melanogaster, D. ananassae* and *D. nasuta*) does not differ statistically, whereas the circadian period of *Z. indianus* is significantly shorter than the other three species. This suggests that the circadian clock regulating egg-laying rhythm has diverged among these four *Drosophila* species. The percentage of entrainment of egg-laying rhythm to LD cycles is also found to vary among the four species indicating a possible effect of light on the egg-laying rhythm (**Fig. 2**). Earlier studies had reported that LD cycles entrain egg-laying rhythm in *Drosophila*, however, percentage entrainment is found to be low in laboratory strains - with *CantonS* flies showing weak

entrainment of ~25% (Howlader *et al.,* 2006). The results of our study suggest that *D. ananassae* show highest entrainment to 12:12 hr LD cycles (~85%), followed by *D. melanogaster* (~60%), and *D. nasuta* (~35%), and then *Z. indianus* (~18%) (**Fig. 3**). Thus our results are in conformity with previous findings reported for laboratory strains of *Drosophila* (Howlader *et al.,* 2006). We noticed no clear time preference for egg-laying in *Z. indianus* (**Fig. 1**). This may be the reason behind poor entertainment and shorter circadian periodicity seen in this species (**Figs. 2 and 3**). The above results further indicate that *Drosophila* has possibly evolved diverse mechanisms for the regulation of fundamental life processes such as egg-lay rhythm so as to avoid competition for resources especially during pre-adult development.

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