Egg Laying Rhythm in *Drosophila melanogaster*

Thesis

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By

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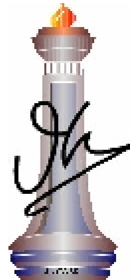

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December 2008

To

My Family

And

Friends

DECLARATION

I declare that the matter presented in my thesis entitled "**Egg Laying Rhythm in *Drosophila melanogaster*"** is the result of studies carried out by me at 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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CERTIFICATE

This is to certify that the work described in the thesis entitiled **"Egg laying rhythm in *Drosophila melanogaster*"** is the result of investigations carried out by **Mr. Manjunatha Thondamal** in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bagalore 560 064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

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Summary

Many behavioral and physiological processes in the fruit fly *Drosophila melanogaster* show robust circadian oscillation. Some of the best studied circadian rhythms include those in adult emergence, activity/rest, olfaction, mating and egg laying. Unlike most other circadian rhythms egg laying rhythm is unique and relatively less understood; it persists under constant light (LL), and also in the absence of the ventral lateral neurons which are known to be the circadian pacemakers for several other rhythms. Further, the expression of core clock genes such as *period* (*per*) and *timeless* (*tim*) in the ovaries do not show any oscillation. In this thesis, I have discussed the findings of some of my studies aimed at probing behavioural, neuronal and genetic mechanisms underlying egg laying rhythm in *Drosophila*.

We began by studying egg laying rhythm under temperature cycles with the objective of assessing whether it can synchronize to temperature cycles. We studied egg laying behavior in temperature cycles imposed under constant darkness (DD) and LL conditions. Results of our study suggest that temperature cycles synchronize circadian egg laying rhythm with the phase of oviposition peak occurring close to the onset of low temperature phase of temperature cycle. Also, the percentage entrainment of egg laying rhythm is significantly greater under temperature cycles compared to light/dark (LD) cycles suggesting that temperature cycle is a stronger Zeitgeber.

Next we tested whether electrical silencing of the pigment dispersing factor (PDF) expressing LNv has any effect on the persistence and entrainability of egg laying rhythm. For this we genetically manipulated the electrical properties of PDF-

expressing ventral lateral neurons (LNv) in flies by using pdf-GAL4 driver to express ion channels dORKΔ-C1 and Kir2.1 in a tissue-specific manner, and studied its effect on the egg laying rhythm in DD and LD. We found that while electrical silencing of LNv neurons abolished adult emergence and activity/rest rhythms, egg laying rhythm continued unabated under DD. However, electrical silencing of the LNv neurons significantly lengthens the circadian periodicity of egg laying rhythm. This suggest that although the electrical output from the LNv neurons may not be required for the persistence of circadian egg laying rhythm in DD, it is required for maintaining the clock periodicity close to 24 hr.

We also studied the role of mating in the regulation of egg laying rhythm to determine whether mating patterns have any effect on circadian egg laying rhythm. We used per^0w mutants (arrhythmic for mating behaviour) and its control *w* (rhythmic for mating behavior) to assay egg laying behavior. Our results indicate that presence of rhythmic female (*w)* invariably enhanced the percentage of flies that showed rhythmic egg laying behavior in DD, and percentage of flies that entrained to LD cycles in all the male-female combinations compared to the case when arrhythmic females (per^0w) were used. This suggests that the robustness in the persistence of egg laying rhythm in DD and its entrainability in LD is primarily driven by females.

Finally, we studied the expression of *logjam (loj)*, a gene essential for oviposition in *Drosophila*. The objective of this study was to find out whether expression pattern of this gene oscillates in ovaries. We used female *CantonS* flies and performed quantitative real-time PCR to quantify the mRNA levels of *loj* using primers which amplify a region of the gene which is common to all known transcripts of *loj*. Results of our studies showed that the mRNA expression of *loj* differs in a time

dependent manner; its expression level is significantly greater at Zeitgeber Time 0 (ZT0) compared to ZT12. This suggests that *loj* may have some role to play in the regulation of circadian egg laying rhythm in *Drosophila* apart from its role in oviposition behaviour.

Based on the results of our studies we conclude that egg laying rhythm in *Drosophila* is a complex circadian phenomenon whose underlying molecular mechanisms seem to be independent of the core clock genes and the circadian neural network that have been implicated in the regulation of other behavioural rhythms. It is likely that circadian egg laying rhythm in *Drosophila* is regulated by molecular mechanisms involving post-translational regulations of core clock genes and/or novel molecular mechanisms involving the gene *loj*. The circadian pacemaker for egg laying rhythm could be the peripheral oscillators in the ovaries.

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Chapter 1

Introduction

Chapter 1

1.1 Introduction to circadian rhythms

Most organisms posses biological timers in the form of circadian clocks. Organisms track time in their local environment by entraining these clocks to natural light/dark (LD) cycles. A large number of biological processes, both simple and complex, are oscillatory in nature; and occur with a 24 hr periodicity. Thus giving them a likeness to the geophysical cycles. These rhythms persist when isolated from environmental cycle, i e under constant laboratory conditions, with periodicities that are approximately 24 hr. Hence such oscillatory processes are termed circadian (Latin: *circa* -about, *dies* -day) rhythms. Circadian rhythms exhibit a stable periodicity, which remains unperturbed within physiologically tolerable ranges of temperature and nutrition. Thus they can be said to be temperature and nutrition compensated (Pittendrigh, 1960).

The *D. melanogaster* genome is complex, with ~13,500 genes (Adams *et al*., 2000), yet they are amenable to genetic manipulations of the molecular pathways that regulate biological processes. Due to this advantage *Drosophila melanogaster* has been extensively used in numerous studies for understanding the genetic and molecular underpinnings of circadian behaviors. In *Drosophila,* circadian rhythms in activity/rest (locomotor activity), adult emergence (eclosion), mating and egg laying (oviposition) behaviors have been used as read-outs for parsing the complex circadian clockwork (Saunders, 2002). Although a great deal about how oscillators work at the molecular level and which neuronal network are involved in regulation of some rhythms, relatively less is known about how the individual molecular oscillators, in the circadian pacemaker network, organize themselves to produce the rhythms. There is a large body of evidence to suggest that circadian clock architecture in *Drosophila* is multi-oscillatory; several behavioral and physiological behaviors are timed by separate sets of

oscillators. The basic rhythm generating machinery is cellular, and involves an elaborate network of neurons. While, some clarity exists regarding which clock genes and neurons work in different feedback loops and circadian networks to generate circadian signals for locomotor activity, olfactory and emergence rhythms in *Drosophila*, neurogenetic and molecular mechanisms that govern egg laying rhythm has thus far remain elusive. In this review, we are going to discuss studies related to egg laying rhythm in *Drosophila* and the molecular, neuronal and hormonal mechanisms that may govern egg laying rhythm.

1.2 Egg laying rhythm in *Drosophila*

Egg laying is a complex phenomenon, involving at least two separate physiological processes - vitellogenesis and egg-retention (Allemand, 1976 a, b). Periodic deposition of fertilized eggs involves series of events starting from the production of oocytes to egg laying on selected sites (Allemand 1976b, Yang 2008). Like several other insect species, in fruit flies (Drosophilidae) including *D. melanogaster* (Rensing and Hardeland, 1967; Gruwez *et al*., 1972; David and Fouillet, 1973; Allemand, 1976 a,b, 1977; Sheeba *et al*., 2001; 2001; Howlader *et al*., 2006), in its close relatives (Allemand, 1974), and in *Zaprionus* (Allemand, 1976c) egg laying behavior is found to follow a 24 hr patterns. Egg laying rhythm in *Drosophila* has been shown to free-run under DD with circadian periodicities, thereby ascertaining its endogenous nature (Allemand 1976, a,b, 1977; Sheeba *et al*., 2001; Howlader *et al*., 2006). Although the rhythm follows circadian patterns, the periods range between 22 hr and 30 hr. This is quite unusual for circadian rhythms, and raises some doubts about its circadian nature. Is this a circadian rhythm or some overt manifestation of an hourglass timer? It is possible that egg output is oscillatory because a wave of eggs mature and then they are laid. To demonstrate that a circadian timer governs egg laying rhythms it is necessary to show that the period of the rhythm remains more or less unchanged with increase/decrease of

temperature and nutrition within physiologically permissible range (Pittendrigh, 1960). If the period of the rhythm does not show temperature and nutrition compensation, it would suggest that we are dealing with physiological cycles of egg maturation that are likely to be sensitive to food and temperature. This was rigorously tested in a study by Howlader *et al*., (2006). In this study periodicity of egg laying rhythm was estimated in several fly lines at three different temperatures (20, 24 and 28 ^{o}C), and two levels of nutritional quality (high and low protein diets). The period of the rhythm of *Drosophila* belonging to several genotypes did not differ significantly under different temperatures. Furthermore, period of the rhythm in these lines also remained stable when assayed on protein diets with different yeast concentrations. This suggests that egg laying rhythm in *Drosophila* is temperature and nutrition compensated (Howlader *et al*., 2006).

Circadian clocks control a wide range of rhythmic physiological and metabolic processes in insects (Saunders, 2002). Some of these rhythms in turn induce rhythmicity in mating behavior, certain aspects of gonadal maturation, oogenesis and oviposition (Saunders, 2002). In a previous study the yellow fever mosquito *Aedes aegypti* showed a well-defined peak in egg laying rhythm both in the field and under laboratory conditions of alternating LD cycles of 12:12 hr (Haddow and Gillett, 1957). The egg laying peak was found to coincide with "lights-off" of the LD cycle. In a separate study, *A. aegypti* raised in constant darkness (DD) were also found to exhibit a weak rhythm in egg laying behavior when assayed under DD condition (Gillett *et al*., 1959). However, after an exposure to a single brief light pulse, a robust rhythmicity in egg laying behavior appeared, which disappeared as soon as the mosquitoes were transferred to constant light (LL). The results of these and other similar experiments (Gillett *et al*., 1961) indicate that egg laying rhythms in mosquitoes are partially depend ant on external time cues, and that in aperiodic condition (DD) a trigger is required to set the circadian pacemaker in motion. Similarly, the egg laying rhythm in the pink boll worm *Pectinophora*

gossypiella was found to be suppressed by light, although a transfer from LL to DD initiated the rhythm with a periodicity of 22.66 hr (Minis and Pittendrigh, 1968). The same pattern of effects was observed in the European corn borer *Ostrinia nubilalis,* in which egg laying rhythm disappeared in LL, and was reinitiated with a free running period of 22.8 hr in DD, after a LL to DD transfer (Skopik and Takeda, 1980). Although egg laying rhythm has been studied in many insects, most of these studies were performed on groups rather than on individuals (Saunders *et al*., 2002), and therefore it is not at all surprising that egg laying rhythms in majority of these studies did not persist for long enough time under constant conditions, and in many cases required a trigger to reinstate. There is often a large variation in the egg laying patterns of individual flies, it is likely that pooling data from a number of individuals would abolish circadian patterns merely due to statistical artefact. For instance, the analysis of egg laying data pooled across several flies maintained under LL and DD yielded no significant pattern, but when the number of eggs laid by individual females was analysed separately, at least 50% of them showed circadian rhythmicity in egg laying behavior (Sheeba *et al*., 2001). Thus the absence of robust circadian rhythmicity in egg laying behavior under constant conditions (DD and LL) reported in a few previous studies may be because of pooling of data from group of flies.

Although egg laying rhythm in *D. melanogaster* is of circadian nature, some of its characteristics are quite different from the two other better characterised circadian rhythms in the fly; the activity/rest and adult emergence rhythms. Under DD, the circadian period of egg laying rhythm (27.66 ± 2.16 hr; mean ± 95% Confidence Interval) is significantly greater than those of locomotor activity (24.73 ± 0.29 hr), and adult emergence (23.64 ± 0.00 hr) rhythms (Sheeba *et al*., 2001). Even the limits of entrainment of the egg laying rhythm were quite different from those of activity/rest and emergence rhythms (Paranjpe *et al*., 2004). Another striking difference between egg laying and other rhythms is that egg laying continues to be

rhythmic under LL (Sheeba *et al*., 2001), while activity/rest and emergence behavior become arrhythmic (Saunders *et al*., 2002). These studies thus suggest that separate timing systems could regulate egg laying and activity/rest, and emergence rhythms in *Drosophila*. At the same time it is has also been shown in a previous study that circadian period of egg laying and activity/rest rhythms are positively correlated in the *period* mutants of *Drosophila*, suggesting that common mechanisms involving the *per* gene govern the two rhythms (McCabe and Birley, 1998). This study makes it clear that egg laying in *Drosophila* is a *per*-controlled rhythm. The obvious corollary to this would be that the loss of function in per^0 flies should be able to prove unequivocally that the pacemakers of egg laying rhythm house mechanisms that are similar to those governing activity/rest and adult emergence rhythms. However, results from McCabe and Birley (1998) indicate otherwise.

At the molecular level the circadian clockwork of *Drosophila* is based on transcription-translational feedback loops comprising of the *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *Cycle* (*cyc*) genes (Cyran *et al*., 2003). At the physiological level the circadian pacemaker network consists of at least six groups of clock neurons (Blanchardon *et al*., 2001; Myers *et al*., 2003; Sheeba *et al*., 2008; Sheeba 2008). Persistence of activity/rest and emergence rhythms in *Drosophila* requires all the clock genes, and the ventral lateral neurons (LN_v), a set of pigment dispersing factor (PDF) expressing neurons in the fly brain (Ewer *et al*., 1992; Renn *et al*., 1999, Myers *et al*., 2003). However, it is not clear to what extent the clock genes and the LNv based circadian pacemakers are responsible for the regulation of egg laying rhythm.

While, our understanding of the circadian egg laying rhythm in *Drosophila* has and continues to increase phenomenally (reviewed in Howlader and Sharma, 2006), some key pending questions still remain: (a) Can egg laying rhythm be entrained by temperature cycles?, (b) Which is a stronger Zeitgeber for egg laying rhythm, LD cycles or temperature cycles?, (c)

Does mating have any role in triggering egg laying rhythm?, (d) What are the key factors underlying the genesis of the rhythm, copulation, transfer of sex peptides, or transfer of sperms?, (e) What is the genetic basis of egg laying rhythm?, (f) What is the neural basis of egg laying rhythm?, and (g) Is egg laying rhythm regulated by hormones?

1.3 Entrainment of egg laying rhythm

An environmental cue that can synchronize circadian clocks is called a Zeitgeber (time giver). As a result of entrainment the biological rhythm maintains an exactly 24 hr periodicity, with a stable phase-relationship with the Zeitgeber (Sharma, 2003; Dunlap and Loros, 2004; Sharma and Chandrashekaran, 2005). Studies have shown that light/dark cycles can entrain egg laying rhythm of flies although the percentage entrainment under light/dark cycles is low in most laboratory strains. *CantonS* flies show weak entrainment (~25%) for egg laying rhythm (Howladar *et al*., 2006). Entrainment to LD cycles requires rhythmic TIM expression in the pacemaker cells (Zheng and Sehgal, 2008). Weak entrainment to LD cycles may be due to the absence of *cryptochrome* and hence rhythmic expression of TIM in the ovaries. Allemand (1976 a,b) has shown that under LD 12:12 hr egg laying is rhythmic with a prominent peak occurring at the beginning of the dark phase. Also, the number of mature oocytes in ovarian egg chambers during the early and late vitellogenesis was rhythmic under LD 12:12 hr. This rhythm disappeared when the flies are transferred to DD. Studies on individual females from a population of *D. melanogaster* maintained under prolonged LL demonstrated that a substantial proportion of flies were rhythmic under LD 12:12 hr with peak oviposition coinciding with lights-off (Sheeba *et al*., 2001). Egg laying rhythm of only ~25% of the flies entrained to LD 10:10 hr, while the percentage was higher under LD 12:12 hr (~40%), and LD 14:14 hr (~75%), suggesting that egg laying rhythm entrains better to longer day lengths (Paranjpe *et*

al., 2004). Interestingly in a separate study Sheeba *et al*., (2001) showed that the circadian periodicity of flies emerging at different time of the day is different, and flies emerging in the evening maintain a significantly different (+4.00 hr as opposed to +1.50 hr in those emerging in the morning) phase-relationship with the LD cycles.

1.4 Does mating have any role in triggering circadian egg laying rhythm?

The sexually dimorphic behaviors associated with *Drosophila* reproductive success is clearly governed by the actions of multiple genes (Karr, 1996). The major reproductive behaviors of females are (1) receptivity to courtship followed by copulation, and (2) deposition of eggs, a behavior that is independent of the act of mating and is under voluntary control of the female. Females become receptive to courting males at about 8-12 hr after emergence (Karr, 1996). Males perform a sequence of five behavioral patterns to court females. First, males orient themselves while playing a courtship song by horizontally extending and vibrating their wings. Soon after, the male positions itself at the rear of the female's abdomen and attempts copulation (Karr, 1996). Females can reject males by moving away and extruding their ovipositor. The average duration of successful copulation is ~30 minutes, during which males transfer a few hundred very long (1.76 mm) sperm cells in seminal fluid to the female. Females store sperms, which may need to compete with sperms of other males to fertilize eggs (Karr, 1996). Virgin females are refractory to mating advances by males on the first day after emergence (Manning 1966, 1967). During this time the ovaries mature (Mahowald and Kambysellis, 1980; Lin and Spradling, 1993), cuticular pheromonal profiles change to make females more enticing to males (Jallon, 1984; Tompkins, 1984, 1998), and hormonal fluxes foster the development of female sexual receptivity (Manning, 1966). Unmated females retain mature eggs but eventually lay unfertilized eggs beginning at approximately the fifth day after emergence (Mahowald and Kambysellis, 1980).

Once the female has mated, her behavior alters as she refuses further mating advances (Chapman *et al*., 2003). Proteins and other compounds in the male ejaculate affect the female's rate of ovulation, oviposition, and her receptivity to male mating overtures (Chen *et al*., 1988; Monsma and Wolfner, 1988; Aigaki *et al*., 1991; Kubli, 1992; Herndon and Wolfner, 1995; Wolfner 1997, 2002; Wolfner *et al*., 1997; Heifetz *et al*., 2000, 2001; Chapman *et al*., 2001; Fleischmann *et al*., 2001; Saudan *et al*., 2002). Mated females have been observed to retain eggs if they do not find ideal oviposition conditions (Grossfield, 1978). These post-mating responses were shown to be induced by factors synthesized in the reproductive tract of the adult male and transferred in the seminal fluid to the female during copulation. One of these factors, named Accessory gland peptide 70A (sex-peptide or SP), has been identified in *Drosophila*. It encodes a 36-amino-acid peptide that is synthesized in the accessory gland and is transferred to the female where it represses female sexual receptivity and stimulates oviposition (Chapman, 2000). Target sites for sex-peptide have been identified in female genital track, corpus allatum (CA) and antennal region in brain (Ding, 2003). The *logjam (loj)* gene is one of the few genes known to control female post mating behaviors (Carney and Barbara, 2003). The gene is named after its mutant phenotype; where it is seen that one or more mature eggs become lodged within the genital tract, causing a logjam of eggs within the female oviduct, and thus preventing further release of eggs. The *loj* gene is expressed in a variety of tissues, particularly in the adult central nervous system (CNS) and in developing eggs (Carney and Barbara, 2003). It is likely that sex-peptide after binding to CA triggers the expression *loj*, which is essential for oviposition in flies, and profiling of *loj* could reveal whether rhythmic expression of this gene (if it is oscillatory) is crucial for the regulation of egg laying rhythm.

1.5 What is the molecular basis of egg laying rhythm?

In fruit flies *D. melanogaster* the molecular clockwork consists of two basic helix–loop–helix (bHLH) transcription factors, CLOCK (CLK) and CYCLE (CYC), which bind to upstream E-boxes and activate the transcription of the *period* (*per*) and *timeless* (*tim*) genes as well as other genes such as *vrille (vri)* and *par domain protein 1* (*pdp1*) (Cyran *et al*., 2003). PERIOD and TIMELESS (PER and TIM) proteins associate with each other in the cytoplasm and the heterodimer is transported into the nucleus. The PER–TIM heterodimer then acts on the transcription factor complex CLK–CYC to inhibit the transcription of *per* and *tim* genes (for review see Hardin, 2005). A second feedback loop, which involves two transcription factors VRI and PDP1, regulate the transcription of *Clk* in a time dependent manner (Cyran *et al*., 2003; Glossop *et al*., 2003). Although, the molecular mechanisms through which PER-TIM represses the transcriptional activation of CLK-CYC are not yet clearly understood, some preliminary evidence point out towards posttranslational modification of clock proteins (Edery, 1999; Akten *et al*., 2003).

Two kinases, DOUBLETIME (DBT) and casein kinase II (CKII) have been implicated in the clock mechanisms that regulate the concentration of PER protein in the cytoplasm (Price *et al*., 1998; Kloss *et al*., 1998, 2001; Martinek *et al*., 2001; Lin *et al*., 2002). These kinases phosphorylate clock proteins in a time-dependent manner and affect their stability, a process that is believed to provide temporal gating in the nuclear localization of PER and TIM (Curtin *et al*., 1995; Dembinska *et al*., 1997; So and Rosbash, 1997; Kim *et al*., 2002; Shafer *et al*., 2002). Studies on egg laying rhythm in the *period* mutants of *Drosophila* (per^{+}, per^{s}, per^{0}, and per^{l}) have shown that all four genotypes show significant rhythmicity in egg laying rhythm. This suggests that for egg laying rhythm functional *period* gene may not be necessary

(McCabe and Birley, 1998). TIM also plays a key role in the photoentrainment mechanisms of the molecular clock, mediated through the circadian photopigment CRYPTOCHROME (CRY) (Helfrich-Förster, 2005). Finally, timed release of a neurotransmitter Pigment Dispersing Factor (PDF) by the clock neurons serves as an output signal for the downstream targets that are responsible for the regulation of behavior (Stanewsky, 2002).

Furthermore, the PER and TIM proteins are found to be constitutively expressed at high levels in the ovaries (Plautz *et al*., 1997; Hardin, 2005) and in follicle cells of developing oocytes, and their levels do not oscillate in the ovaries of *Drosophila* (Beaver *et al*., 2003). This suggests that circadian egg laying rhythm in *Drosophila* is governed by novel molecular mechanisms involving genes that have not yet been implicated in circadian clockwork (Howlader and Sharma, 2006). While the non-oscillatory nature of the core clock proteins may be due to the absence of positive feedback elements, *Clock* and *cycle* expression, or due to the absence of *Cryptochrome* (*Cry*) in the ovary (Beaver *et al*., 2003), what remains to be established is which mechanisms would be generating rhythmic signals for egg laying rhythm? Interestingly, ectopic expression of CRY in the ovaries resulted in circadian oscillation of the genes in the negative feedback loop of the molecular clock, i.e. *period* and *timeless* (Rush *et al*., 2006). Further, studies have also shown that loss of function *per* and *tim* mutant females showed reduced fecundity and fertility (Beaver *et al*., 2003). Therefore, it is likely that *per* and *tim* genes play a non-circadian role in the *Drosophila* ovary.

1.6 What is the role of *logjam* gene in the regulation of egg laying rhythm?

The post-mating responses in females, i e increased ovulation and oviposition and decreased receptivity, are regulated at least in part by products in the male ejaculate that are

transferred to females during mating (Chen *et al*., 1988; Monsma *et al*., 1988; Aigaki *et al*., 1991; Kubli *et al*., 1992; Herndon *et al*., 1995; Wolfner *et al*., 1997; Heifetz *et al*., 2000; 2001; Chapman *et al*., 2001; Fleischmann *et al*., 2001; Saudan *et al*., 2002). Carney and Barbara (2003) observed that mature eggs were inside the female genital tract, particularly in the uterus of the *loj* mutant flies. Further the egg laying deficit was primarily due to loss of a required signal rather than the loss of motor neuronal input to the genital tract muscles. This led them to hypothesise that *loj,* in addition to shuttling neurotransmitters to their release sites, might have a similar function as COP (coat protein complex) vesicle components, functioning in both the anterograde and retrograde secretory pathways of cytoplasmic transport of cellular components. (Bednarek *et al*., 1996; Fiedler *et al*., 1996). Hence the loss of *loj* function results in the subsequent loss of appropriate signal causing the egg to be lodged inside the uterus. Given that *logjam* appears to be responsible for egg laying behavior, it is likely that it may be rhythmically expressed in the ovaries and/or brain.

Studies have also shown that brain and ventral nerve chord (VNC) are important centres for processing gustatory, olfactory, and visual inputs and transforming this information into an appropriate behavioral outcome such as oviposition (Szabad *et al*., 1982). Exploiting mosaic animals egg laying behavior was mapped to the thorax (Szabad *et al*., 1982), which contains the VNC. Carney and Barbara (2003) also focused to a number of cells in the thoracic and abdominal ganglia of the VNC which expressed *logjam*. The brain also has a role to play in the regulation of egg laying, since decapitated or anaesthetized *Drosophila* females lay eggs as a reflex response (Grossfield, 1978). This makes VNC as the obvious target for the study of circadian pacemakers regulating egg laying rhythm.

Expression pattern of *loj* in adults involves its expression in midstage vitellogenic egg chambers (Carney and Barbara, 2003). The majority of the signal in these chambers is found in

follicle cells which provide nutrients and other components necessary for oocytes development and produces the outer coverings of the egg, the vitelline membrane and the chorion (Mahowald *et al*., 1980; Lin and Spardling, 1993). Studies suggest that the *loj* positive egg cells provide cues to the female's genital tract and musculature that aid proper egg release from the ovary and navigation through the genital tract to the uterus. This signalling mechanism is expected to either function prior to the formation of the vitelline membrane and chorion or to be a component of these protective coverings of the mature egg.

As noted above, the process of ovulation is affected in *loj* females. Since a mature egg is found in the uterus of essentially every mutant female, it appears that initial egg release is not affected. However, Carney and Barbara (2003) observed partially ovulated eggs in the upper portions of the lateral oviducts as well as multiple eggs in these portions of the genital tract. It was suggested that ovulation initially proceeds normally in *loj* females but the presence of unlayed eggs in the uterus disrupts the feedback loop that regulates ovulation. Therefore, *loj* mutant females have a weak ovulation defect that is a secondary consequence of the loss of oviposition behavior.

1.7 What is the physiological basis of egg laying rhythm?

The neuronal architecture underlying circadian rhythms in *Drosophila* has been extensively studies for several decades (for review see Sheeba *et al*., 2008; also see Sheeba 2008). The core pacemaker for activity/rest rhythm has been localized in the lateral ventral neurons (LN_v). The neuropeptide Pigment Dispersing Factor (PDF) is used by the LN_v neurons to communicate among each other and with other neurons in the circadian pacemaker network (Renn *et al*., 1999; Blanchardon *et al*., 2001; Sheeba *et al*., 2008). Core clock proteins expressed in the LN_v are essential for the maintenance of activity/rest and emergence rhythms

(Ewer *et al*., 1992; Myers *et al*., 2003). However, neural network underlying the egg laying rhythm in *Drosophila* is yet to be unravelled. In case of grasshoppers, the neural circuit has been identified to certain extent, and a large portion of the circuit is found to be completed by the end of embryonic development, well before it is needed for the behavior (Thompson *et al*., 1998). This suggests that some egg laying genes start functioning quite early during the development. Once the appropriate circuitry is established, signalling pathways should be able to initiate and sustain egg laying behavior at the appropriate age. The activity of the motor neurons that directly synapse on the uterine and oviductal muscles is likely to be controlled by descending inputs from the command inter-neurons in the brain, including the subesophageal ganglion (Thompson, 1986a). Local circuit inter-neurons in the posterior abdominal ganglion and sensory inputs from neurons in the ovaries and internal reproductive tract also are expected to function in activating and modulating egg laying behavior (Thompson, 1986b). This circuit should be extensively probed as it is likely that egg laying rhythm is governed by the neural network that involves reproductive system. Once the neural circuit is localised the next question would be to study what kind of mechanisms these pacemakers use to regulate egg laying behavior.

Recent study by Howlader *et al*., (2006) has demonstrated that in *D. melanogaster* flies where the LN_v neurons were genetically ablated circadian egg laying rhythm continue to persist under DD conditions. It was also shown that PDF mediated signaling is not required for the persistence of this rhythm in DD. This suggests that the LN_v neurons do not serve as the circadian pacemakers for egg laying rhythm in *Drosophila*. However, LNv ablated flies invariably showed a significantly shorter periodicity in egg laying rhythm compared to the wild type counterparts, which suggests that although LN_v neurons are not critical for the persistence of the rhythms under DD, they may still influence somehow the circadian period. Further, egg

laying behavior of *pdf*^{0} and *disconnected* (*disco*^{1}) mutant flies were also studied, and were found to be rhythmic, though with altered periodicity (Howlader *et al*., 2006). In the *pdf*^{01} flies the output signal from the LN_v based circadian pacemakers is absent (Renn *et al*., 1999), while in the *disco*^{1} mutants the clock's neural connections are impaired, and the LNv neurons that are left behind lack PER and PDF (Blanchardon *et al*., 2001). Therefore, it was quite rightly concluded that circadian egg laying rhythm in *Drosophila* is not under the control by the circadian neuronal circuitry that govern other circadian rhythms (Howlader and Sharma, 2006).

1.8 Is egg laying rhythm regulated by hormones?

It is likely that reproductive hormones themselves are central to the regulation of egg laying rhythm in *Drosophila* (Howlader and Sharma, 2006). Juvenile hormone (JH) and 20-hydroxy ecdysone (20HE) are hormones with known gonadotropic functions (Riddiford, 2008; Gruntenko and Rauschenbach, 2008). Both have also been implicated for their role in development (Riddiford, 1993). The Corpus Allata (CA) cells of the brain secrete JH, while 20HE, the major moulting hormone, is secreted from the prothoracic gland. Sex Peptide, a protienacious factor present in the seminal fluid, has been shown to activate JH synthesis in CA (Moshitzky *et al*., 1996). Proteins that are involved in signal transduction of 20HE JH interact with each other. This functions to mediate a communication between these hormones (Bitra and Palli, 2008). A balance between levels of JH and 20HE, brought about by the neurotransmitter dopamine, is vital for oogenesis. Ecdysone control of JH metabolism also occurs via dopamine (Gruntenko *et al*., 2005). An increase in JH titre leads to oviposition arrest while increased 20HE titres causes degradation of vitellogenic oocytes (Gruntenko and Rauschenbach, 2008). JH also plays a key role in regulating egg laying behavior under adverse condition such as starvation and heat stress (Raushenbach *et al*., 2005; Gruntenko *et al*., 2003).

JH acts by stimulating vitellogenic oocyte progression and inhibiting apoptosis. Cayre *et al*., (1996) proposed that JH might regulate egg laying behavior via polyamine metabolism in crickets. When anitsera against FMRFamide was injected in mated *Rhodinus prolixus* females a delay in oviposition was observed (Sevala *et al*., 1992). Based on the above understanding, we postulate that in *Drosophila* too, JH might mediate egg laying behavior via downstream amide/amine components. Since juvenile hormone analogue does not elicit increased oviposition and reduced receptivity, Sex-Peptide must have an additional, separate effect on these two post-mating responses (Soller, 1999). Further, application of the juvenile hormone (JH) analog methoprene is found to mimic the sex-peptide-mediated stimulation of vitellogenic oocyte progression in sexually mature virgin females (Soller, 1999). 20 HE is shown to deter oviposition and females avoid laying eggs in the presence of 20 HE (Calas *et al*., 2006; 2007). Apoptosis is induced by 20HE in nurse cells of egg chambers at physiological concentrations [$10^{(-7)}$ M] (Soller, 1999). 20-Hydroxyecdysone thus acts as an antagonist of early vitellogenic oocyte development. However, simultaneous application of JH analog protects early vitellogenic oocytes from 20-hydroxyecdysone-induced resorption. These results suggest that a fine balance between these hormones in the hemolymph regulates whether oocytes will mature or undergo apoptosis.

Oviduct contraction is an essential step in the process of egg laying behavior (Rodríguez-Valentín *et al*., 2006). Two neuroactive substances are known to be critical for oviduct contraction: octopamine (OA), a monoamine that inhibits oviduct contraction, and glutamate (Glu), a neurotransmitter that induces contraction. Modulation of oviduct contraction is known to occur via octopaminergic neurons of the thoracic abdominal ganglion (TAG) (Middleton *et al*., 2006; Rodríguez-Valentín *et al*., 2006). Flies lacking oviduct contraction, due to the disruption of the octopaminergic neural network that innervates the genital tract,

show absence of egg laying and sperm accumulation in the oviduct (Rodríguez-Valentín *et al*., 2006). Although, octopamine does not play a role in functioning or development of the circadian pacemaker, it influences features that are not under the direct control of the circadian pacemaker, such as reduction in period between daily onset and offset of locomotor activity, and an increase in the average expression of *per* mRNA in the brain of *Apis mellifera* (Bloch and Meshi, 2007). Recent studies have shown that octopamine is a sleep-promoting agent (Crocker and Sehgal, 2008). Protein kinase A (PKA) is a putative target of octopamine signalling, and has also been implicated in *Drosophila* sleep (Huang *et al*., 2007; Hildebrandt and Müller, 1995). However, the effect of PKA was not exerted in the mushroom bodies, a site previously associated with PKA action on sleep. These results suggest the existence of a novel pathway by which octopamine might regulate circadian rhythms in sleep/wake and egg laying behavior. The ability of octopamine in regulating oviduct contraction as well as a wake promoting signal suggests that it might play a role in the regulation of egg laying rhythm *Drosophila* (Fig. 1).

1.9 Possible mechanisms underlying egg laying rhythm

Egg laying rhythm is unique among the rhythmic behaviors exhibited by *Drosophila* because: (i) it is rhythmic under LL, and (ii) does not require LNv for its persistence under DD. The scenario is further complicated by a large number of regulatory mechanisms such as neuronal, hormonal, genetic, and nutritional and temperature signals. Therefore it is not surprising that the mechanisms underlying egg laying rhythms still remain elusive. Based on some recent understanding of egg laying behavior we propose a model that encompasses all the components underlying egg laying rhythm in *Drosophila* (Fig. 2).

Hormonal regulation of egg laying behavior in *Drosophila*

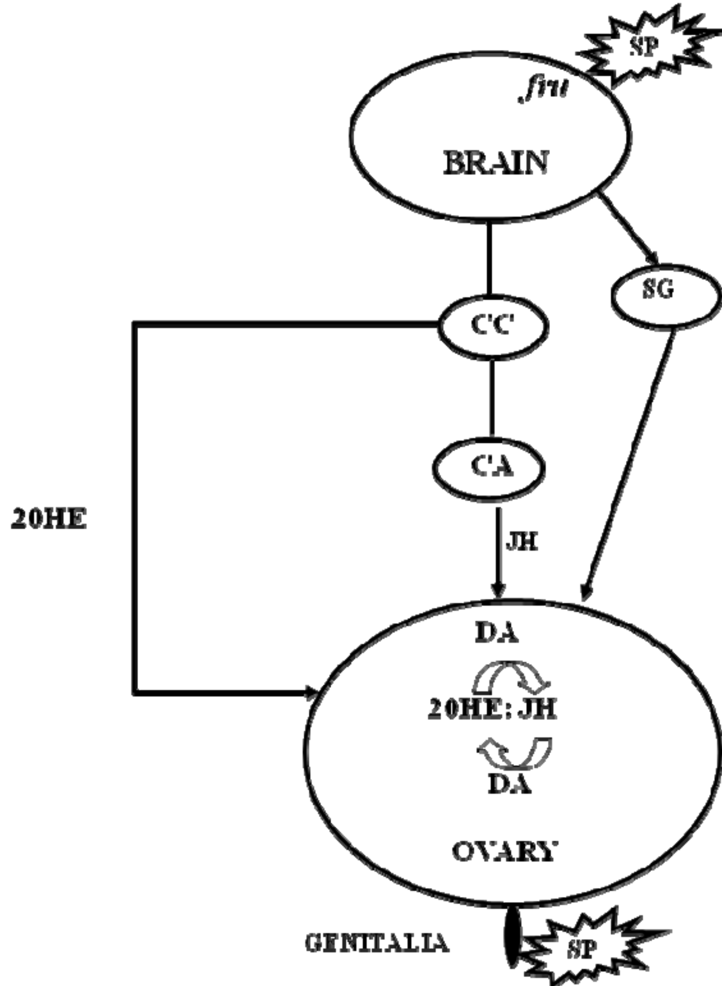


Fig. 1: Hormonal regulation of egg laying behavior in *Drosophila*. Sex Peptide (**SP**) binds to the *fruitless* neurons in the female brain. The unknown signal stimulates the Corpus Allata (**CA**) to secrete Juvenile Hormone (**JH**). 20 Hydroxyecdysone (**20HE**) is secreted by neurosecretory cells and is stored in the Corpus Cardiaca (**CC**). The balance between 20HE and JH is critical for driving oviposition behavior. The balance is mediated by dopamine (**DA**). Sex Peptide also binds at genitalia reducing receptivity to new mates. The activity of motor neurons that synapse on the uterine and oviductal muscles is likely to be controlled by subesophageal ganglion (**SG**).

Possible mechanisms underlying egg laying rhythm in *Drosophila*

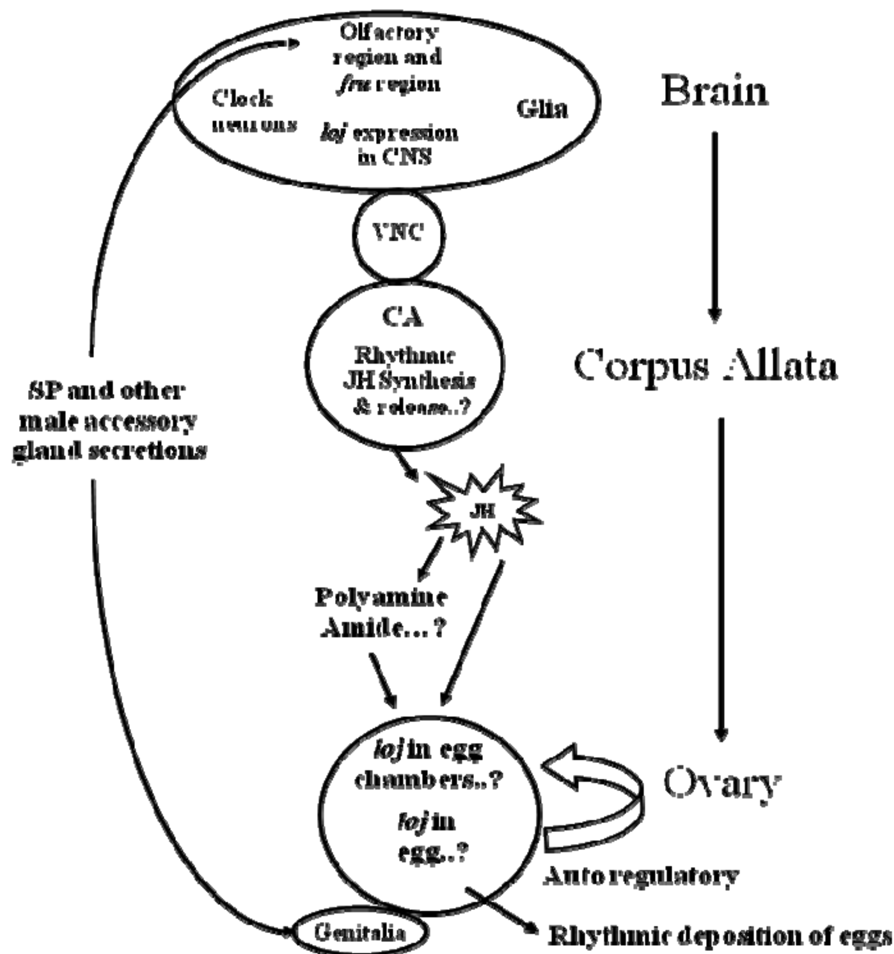
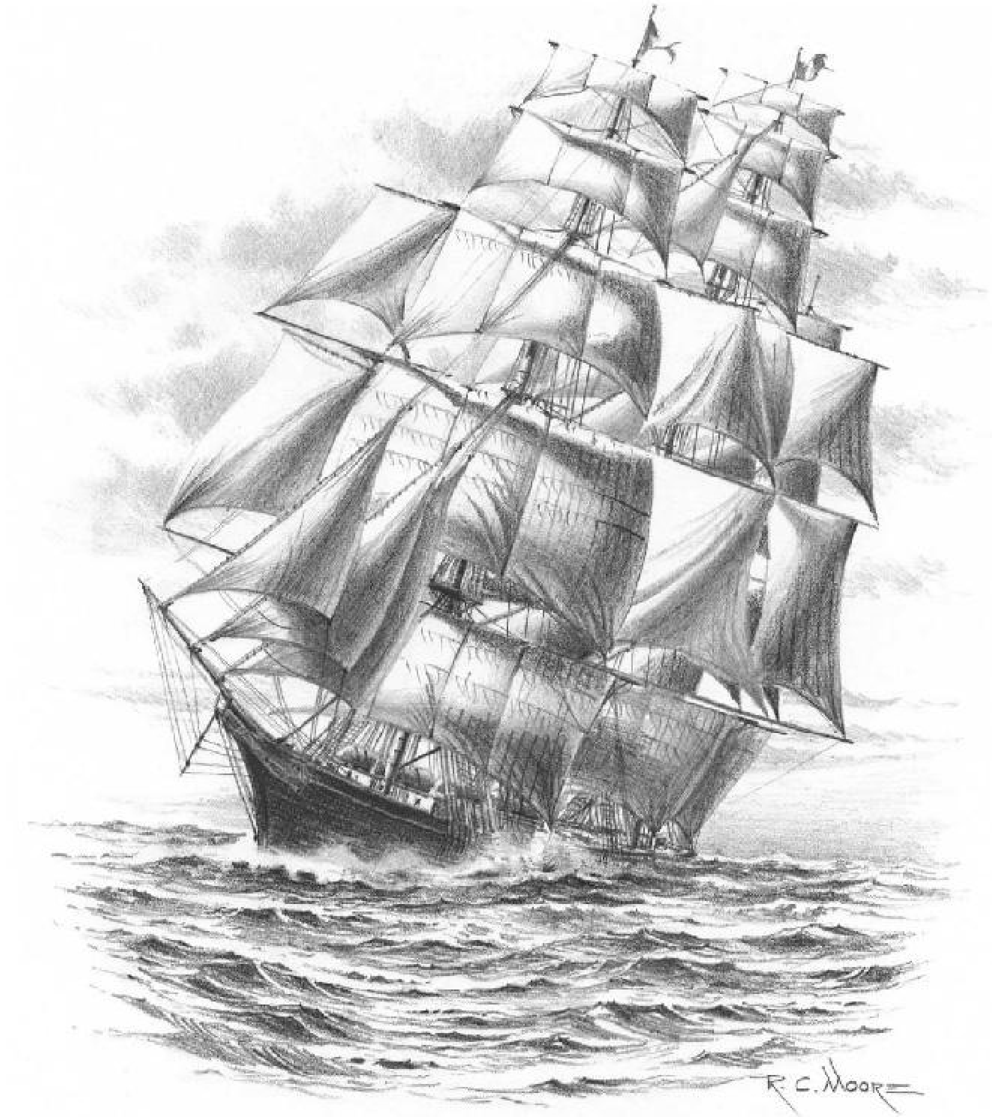


Fig. 2: Possible mechanisms underlying egg laying rhythm in *Drosophila*. Sex Peptide (SP) and other accessory gland secretions such as Ductus ejaculatory peptide have target sites in the fruitless/olfactory regions in the female brain. Upon binding, via downstream signals (yet unknown), they activate Juvenile Hormone (JH) synthesis in the Corpus Allata (CA). There are two possible downstream processes one via polyamines/amides and other through direct. This could activate logjam expression in the egg chamber and egg. The logjam expression might be oscillatory in nature. This in turn can cause rhythmicity in egg laying. It is possible that the peripheral oscillator present in the ovary might be autonomous in nature and hence this oscillator alone might be sufficient for the generation of egg laying rhythms.

Sex-peptide and other accessory gland secretions have target sites in the fruitless/olfactory regions in the female brain (Ding *et al*., 2003). Upon binding, via downstream signals (yet unknown), they might activate JH synthesis in the CA. There is evidence to suggest that the rate of JH biosynthesis in cricket follows a diurnal pattern (Zhao and Zera, 2004). JH might act on the ovaries either indirectly via polyamines/amides or directly. This in turn would activate *loj* expression in the egg chamber and in the eggs. If mature eggs are not expelled from the ovary, *loj* in the egg would send a signal to the ovary, which would prevent further synthesis of eggs. This suggests that *loj* expression should follow circadian oscillation. Given that *loj* is directly involved in egg output in *Drosophila*, one would expect egg laying behavior to be rhythmic almost mimicking *loj* expression profile. Alternatively, given that egg laying behavior is rhythmic in LL, it is likely that oscillators present in the ovary might regulate egg laying rhythm in a tissue autonomous manner. In other words peripheral oscillators located in the ovary alone might be sufficient for the persistence of egg laying rhythms, which may require phasic inputs from the core pacemakers in the brain in order to entrain to local LD cycles. Further, it is possible that the core clock genes such as *per* and *tim* that form the molecular machinery that regulate activity/rest and emergence rhythms, do not play any role in the maintenance of egg laying rhythms, and this rhythm may be governed by molecular mechanisms involving a novel sets of clock genes. There is also sufficient evidence to suggest that LNv based circadian pacemakers do not regulate the persistence of circadian egg laying rhythm in *Drosophila*, and that some yet identified neural network may be at work. These pacemakers could either be the CRY positive and PDF negative neurons in the fly brain such as the dLN, or some of the dorsal neurons (DN1, 2 and 3), or the single PDF negative small LN_v, or it could be Antennal neurons (AN). Finally, it is not entirely unlikely that all or some of the above processes may govern egg laying rhythms in *Drosophila* in a concerted manner.

Chapter 2



Entrainment of egg laying rhythm by temperature cycles

Chapter 2

Entrainment of egg laying rhythm by temperature cycles

2.1 Background

Life on our planet has been subjected to various geophysical cycles. With the exception of organisms that live in the depth of oceans, underground caves and rivers, or any similar aperiodic environment, most organisms have evolved strategies to exploit systematic variations in their environment. Although some of the rhythmic biological phenomena may be direct responses to environmental changes, many are overt manifestations of endogenous biological clocks. The most common of all these rhythms are the ones that recur with near 24 hr periodicity (circadian rhythms). Circadian rhythms maintain a stable period, which remains largely unaltered within physiologically tolerable ranges of temperature and nutrition (Pittendrigh, 1960). The natural cycles of light and darkness and of temperature synchronize these rhythms, in the absence of which they free-run, revealing their natural periodicity (free-running period), which is invariably close to but seldom equal to 24 hr.

Environmental stimuli that can synchronize circadian clocks are called "entraining agents" or time givers (zeitgebers). The consequences of entrainment are that the period of biological rhythm becomes equal to that of external stimuli, with a stable phase-relationship between the entraining cycle and biological oscillations. To establish that a time cue has truly entrained a free-running rhythm two criteria must be met (Pittendrigh, 1965; Sharma, 2003; Dunlap and Loros, 2004; Sharma and Chandrashekaran, 2005). First, the period of the overt rhythm must be equal to the period of the entraining cycle and the rhythm should maintain a stable phase-relationship with the Zeitgeber. Second, after return of the organism to constant

conditions, the free-running rhythm must continue with a phase determined by the entraining cycle. Although the free-running periods of circadian rhythms are stable over a physiological range of constant temperatures, any temporary non-recurring changes (step-up or step-down) in temperature affect the rhythm and cause phase shifts (Bunning and Tazawa 1957; Moser 1962).

In *Drosophila*, temperature cycles synchronize adult emergence (Pittendrigh, 1954), activity/rest rhythms (Wheeler *et al.*, 1993; Yoshii *et al.*, 2002, 2005; Glaser and Stanewsky, 2005), and molecular oscillations in the peripheral tissues (Glaser and Stanewsky, 2005) and brain neurons (Yoshii *et al.*, 2005). While light/dark (LD) cycles entrain activity/rest rhythm in a stable manner, temperature undoubtedly has a greater effect (Majercak *et al.*, 1999). Under low ambient temperatures, the morning peak of activity starts later and the evening peak earlier than what is observed under standard laboratory temperature of 25 ^{o}C. The molecular mechanisms underlying this change is believed to be the early accumulation of *per* and *tim* mRNAs, driven, respectively, by enhanced splicing of the 3' intron of *per* and by photo induction of *tim*. Collectively, these lead to the earlier accumulation of PER and TIM associated with the early activity peak (Majercak *et al*., 1999; Majercak *et al*., 2004; Chen *et al*., 2006).

Temperature cycles with difference of 3 °C imposed in constant darkness (DD) have been shown to synchronize activity/rest rhythm in *Drosophila* (Wheeler *et al.*, 1993). Other studies have shown that in wild-type flies previous synchronization to a light/dark (LD) cycles resulted in a robust evening peak of activity that anticipate the temperature transition when subjected to 12:12 hr 29:20 °C temperature cycles in DD, suggesting that circadian oscillators underlying evening activity are entrained by temperature cycles (Busza *et al.,* 2007). Recent studies have also shown that

combined LD and temperature cycles affect the phase of the *Drosophila* circadian rhythm at behavioral and cellular levels and some groups of clock neurons, that is, the LNs, entrain to the LD cycles, whereas others, the DNs and LPNs, entrain to temperature cycles (Miyasako *et al*., 2007). Although the organization and dynamics of the system have been studied in view of light entrainment (Pittendrigh and Daan, 1976; Helfrich-Förster, 2001; Stoleru *et al*., 2005; Rieger *et al*., 2006), little is known so far as to how it adapts to the natural environment where both light and temperature change simultaneously. Thus, the *Drosophila* system would contribute to understanding the circadian system in which temperature is known to be an important factor for phase regulation.

According to Sheeba *et al.* (2001a,b) egg laying behavior in *Drosophila* exhibits circadian rhythmicity under constant darkness (DD) and constant light (LL) conditions. Previous studies have also shown that circadian egg laying behavior in *Drosophila* entrains to LD cycles (reviewed in Saunders *et al.*, 2002). However, unlike other behavioral rhythms (adult emergence, and activity/rest rhythms), the percentage of flies in which egg laying rhythm entrained to LD cycles is considerably low in most laboratory strains of *Drosophila*. *CantonS* (CS) flies show weak entrainment (~25%) for egg laying rhythm (Howladar *et al*., 2006). Also, egg laying rhythm of only ~25% of the flies entrained to LD 10:10 hr, while the percentage is slightly higher in LD 14:14 hr (~75%), suggesting that egg laying rhythm entrains better to longer day lengths (Paranjpe *et al.* 2004). Till date, no systematic study has been done to investigate whether temperature cycles can serve as Zeitgeber for circadian egg laying rhythm in *Drosophila*. In this chapter we have discussed our study aimed at estimating the effect of temperature cycles on circadian egg laying rhythm in *Drosophila*.

2.2 Materials and methods

2.2 (a) Fly strains

Egg laying behavior was assayed in *Canton Special* (CS) strain of *D. melanogaster*. The flies were obtained from National Center for Biological Sciences, Bangalore. To acclimatize flies before monitoring egg laying behavior, freshly emerged flies were first kept under 12:12 hr LD cycles for two days with *ad libitum* food and constant temperature and humidity (25 ^{o}C and ~70% relative humidity).

2.2 (b) Behavioral studies

To monitor egg laying rhythm, 2-day-old male-female pairs were introduced into vials containing ~4 ml of food. Flies were synchronized to LD cycles for 2 days and then subjected to a temperature cycle of 29:25 ^{o}C (12:12 hr) in constant darkness (DD) or constant light (LL) and were compared with the two controls kept at 29 ^{o}C and 25 ^{o}C under DD or LL. After 24 hr, flies were transferred into fresh food every 2 hr and the number of eggs laid over the preceding 2 hr duration were counted for eight consecutive cycles. Dead males were replaced throughout the experiment with males kept in similar conditions. Dim red light of wavelength > 650 nm was used for transferring and handling flies.

2.2 (c) Statistical analysis

For egg laying rhythm assay, the periodicity was assessed by treating time series data collected over a period of eight days using Lomb Scargle periodogram analysis from the CLOCKLAB software (Actimetrics, Evanston, IL). The ratio of number of flies entrained to number of rhythmic flies was taken to calculate the percentage entrainment of egg laying rhythm in files. To compare the difference in

percentage rhythmicity, we first randomly selected 50% of rhythmic flies in each experimental and control setup and then calculated the percentage entrainment in such random selection. Three such selections were made to calculate the mean and variance and were tested for statistical difference.

2.3 Results

2.3 (a) Entrainment to temperature cycles under constant darkness (DD)

The egg laying rhythm in flies synchronize to the temperature cycle of 29:25 ^{o}C (12:12 hr) and free run under constant darkness at high (control 1, Fig 1.A) and low (control 2, Fig 1.B) temperatures. The egg laying peaks occur at the onset of low temperature phase (25 ^{o}C) (Fig 1.C). The mean free running period of egg laying rhythm was 22.21 ± 0.60 hr (mean ± SD) at high temperature and 24.01 ± 2.07 hr at low temperature. Also, ~93% flies were rhythmic at high temperature and ~88% at low temperature (Fig 3, Table 1). The temperature cycle of 29:25 ^{o}C yielded entrainment in ~87% flies (Fig 4).

2.3 (b) Temperature cycle in constant light (LL)

Even in constant light condition flies synchronize to temperature cycle of 29:25 ^{o}C (12:12 hr) and free run under constant light at high (Fig 2.A) and low (Fig 2.B) temperatures. The egg laying peaks occur at the end of high temperature phase (29 ^{o}C) of the temperature cycle (Fig 2.C and D). The mean free-running periodicity of egg laying rhythm was 22.55 ± 1.90 hr (mean ± SD) at high temperature and 25.32 ± 2.07 hr at low temperature. About 73% of the flies were rhythmic at high temperature and ~79% rhythmic at low temperature (Fig 4). The temperature cycle of 29:25 ^{o}C results in entrainment in ~43% flies (Fig 5).

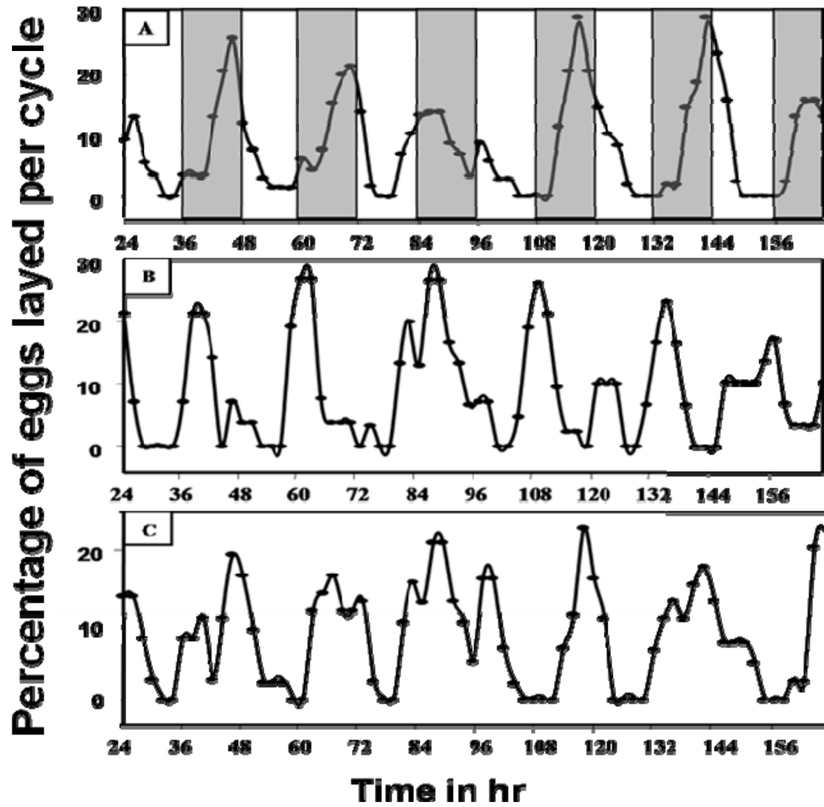


Fig. 1: Rhythmic profiles of egg laying behavior in temperature cycles under constant darkness. Flies synchronize to temperature cycle of 29:25 °C (12:12 hr) (Fig 1.A) and free-run in constant conditions (29 °C in Fig 1.B and 25 °C in Fig 1.C). The egg laying peaks occur during the onset of low temperature phase of the temperature cycle. Dark rectangular boxes in Fig 1.A represent the high temperature phase (29 °C) of temperature cycle. Values on y-axis represent percentage eggs laid by female per cycle.

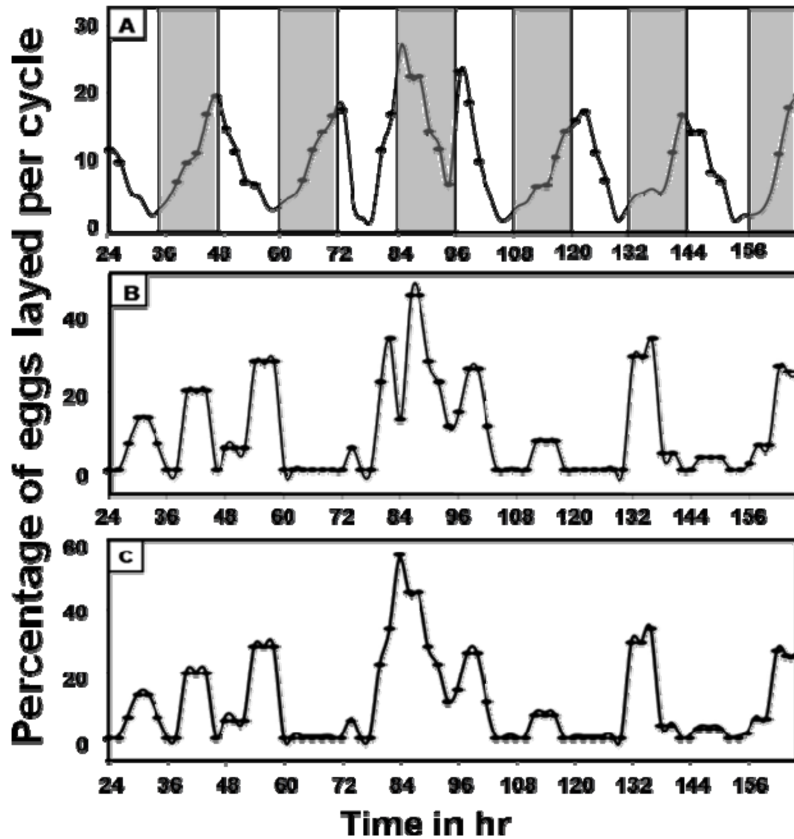


Figure 2: Rhythmic profiles of egg laying behavior in temperature cycle under constant light. Flies synchronize to the temperature cycle of 29:25 ^{o}C (12:12 hr) (Fig 2.A) and free-run in constant conditions (29 ^{o}C in Fig 2.B, and 25 ^{o}C in Fig 2.C). The egg laying peaks occurs at the end of high temperature phase of the temperature cycle. Dark rectangular boxes in Fig 2.A represent the high temperature phase (29 ^{o}C) of temperature cycle. Values on y-axis represent percentage eggs laid by female in one cycle.

Table1. Details of egg laying rhythm in temperature cycle

	Periodicity in (Hours) with 95% CI around the mean	% Entrained	% Arrhythmic
Control I(29 ^{0}C) in DD (n=16)	22.21 ± 0.60	-	7
Control I(29 ^{0}C) in LL (n=16)	22.55 ± 1.90	-	26
Control II(25 ^{0}C) in DD (n=16)	24.01 ± 2.07	-	12
Control II(25 ^{0}C) in LL (n=16)	25.32 ± 2.90	-	23
Temperature cycle in DD (n=16)	23.9 0± 3.32	87	7
Temperature cycle in LL (n=16)	23.8 0± 1.40	43	57
Light/Dark cycle (n=17)		25*	-

* Howlader et al., 2006

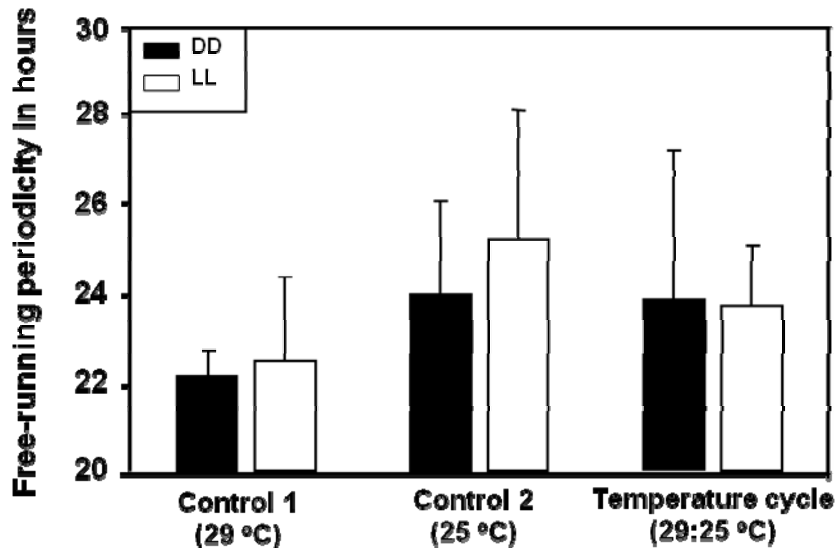


Fig. 3: Periodicity values of egg laying rhythm in constant darkness (DD) and constant light (LL) at different temperatures. Values on y-axis represent the period values of the egg laying rhythm. Error bars indicate the SEM with 95% Confidence Interval (95%CI) values around the mean.

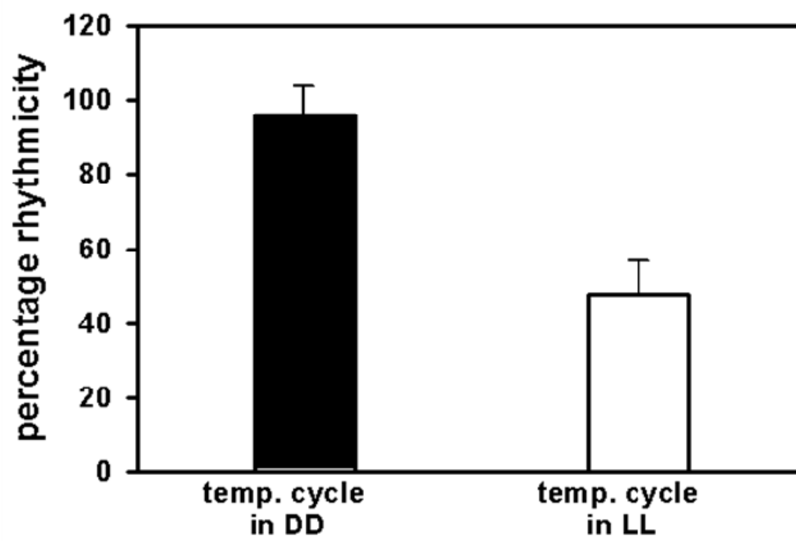


Fig. 4: Percentage rhythmicity of egg laying rhythm in temperature cycles under constant darkness (DD) and constant light (LL). The percentage egg laying rhythm is greater in flies under temperature cycle in DD compared to temperature cycle in LL.

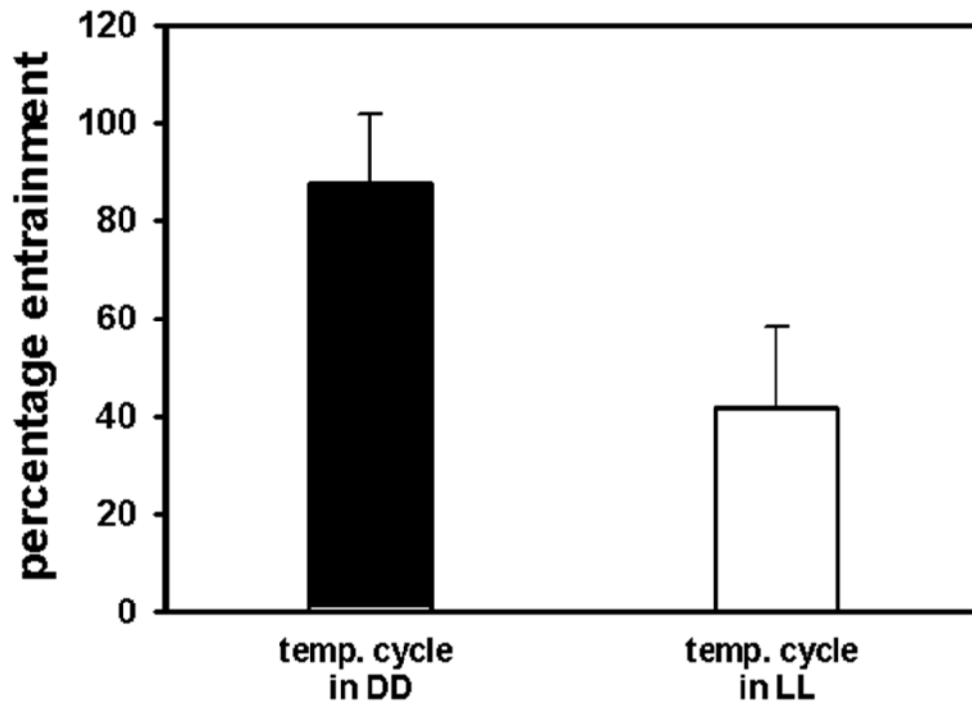


Fig. 5: Entrainment of egg laying rhythm to temperature cycles under constant darkness (DD) and constant light (LL). Egg laying rhythm of a greater percentage of flies entrains to temperature cycles under DD than under LL.

Although there is no difference in the free running periods of egg laying rhythm under DD and LL conditions ($p = 0.07$) (Fig 3), percentage rhythmicity under temperature cycle is greater in DD than in LL ($p < 0.01$) (Fig 4, Table 1). Also, the percentage entrainment was significantly greater ($p < 0.02$) in case of temperature cycle under DD (~87%) compared to temperature cycle in LL (~43%;) and LD cycles (~25%; Howlader *et al.*, 2006 and Fig 5, Table 1).

2.4 Discussion

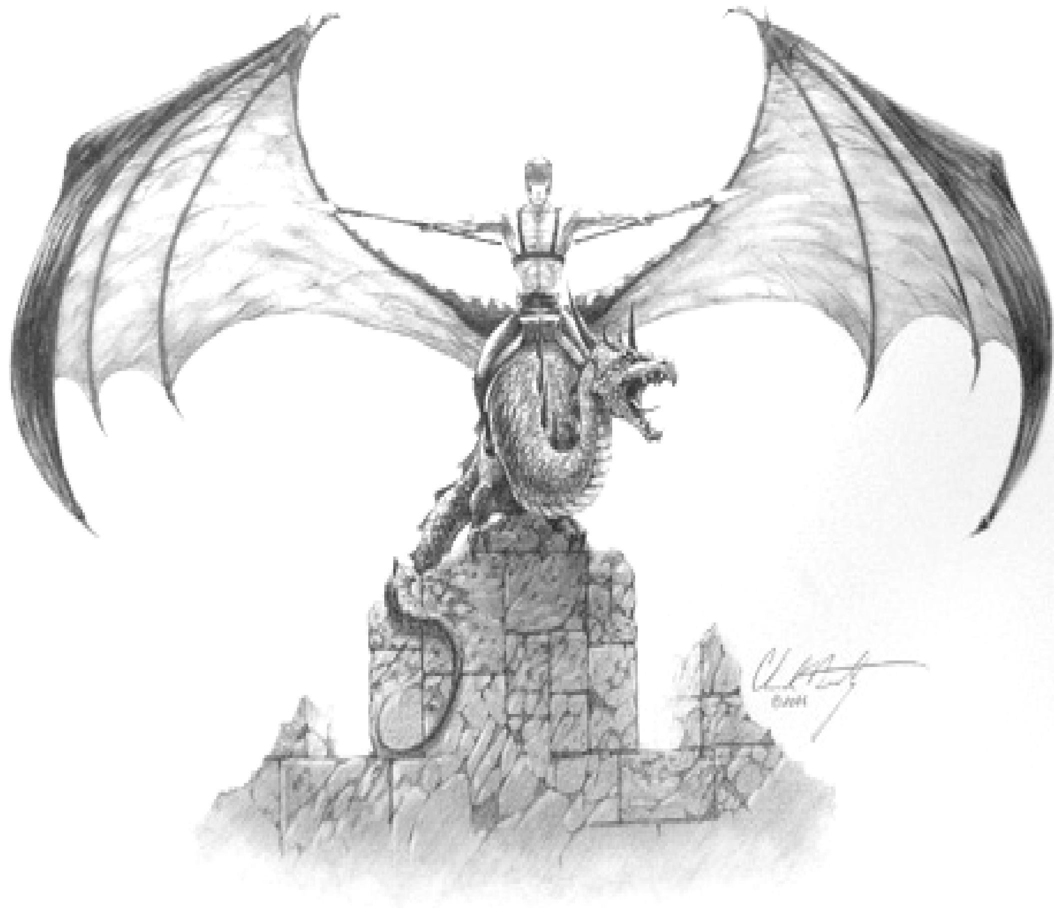
The results of our study reveal that temperature cycles are able to synchronize circadian egg laying rhythm in *Drosophila*. In both DD and LL conditions, egg laying rhythm in flies synchronizes to temperature cycle with the peak of the rhythm coinciding the onset of low temperature phase of the temperature cycle. Previous results (Howlader *et al.,* 2006) have shown that egg laying rhythm of only a small fraction of *CS* flies (~25%) entrain to LD cycles. The results of our present study suggests that temperature cycles are a more effective Zeitgeber for egg laying rhythm in *Drosophila* as compared to LD cycles. Recent studies also suggest that circadian neurons apart from the morning and evening cells are involved in the control of circadian behavior specifically when the temperature cycles are present. An evening cell - and morning cell - independent peak was observed under a long thermophase temperature cycle (Busza *et al.,* 2007). Although, temperature cycle synchronize circadian activity/rest rhythm slower than LD cycles, the ability to synchronize is greater when M (morning) cell oscillator is ablated or genetically manipulated (Busza *et al.,* 2007). However the cellular and molecular basis for the synchronizing mechanism of light/dark cycle and temperature cycle of the egg laying rhythm in *Drosophila* is yet to be elucidated.

In *Drosophila,* LD cycles have been shown to be strong synchronizing agent for circadian activity/rest and adult emergence rhythms. Whereas our results suggest that circadian egg laying rhythm of *Drosophila* entrains better to temperature cycles (Fig 5, Table 1). Weak entrainment of egg laying rhythm to LD cycles may be because of the fact that *timeless* (*tim*) protein is light insensitive in the fly ovaries (Busza *et al.,* 2007). This is thought to be due to the absence of circadian photoreceptor CRYPTOCHROME in the fly ovaries (Rush *et al*., 2006). Since ectopic expression of CRY in the ovaries causes light-dependent TIM degradation (Rush *et al.*, 2006), it would be interesting to see whether expression of CRY in the fly ovaries is sufficient to entrain egg laying rhythm to LD cycles.

The reduction in the percentage of entrainment to temperature cycles in LL compared to DD may be due to the fact that LL causes disruption of behavioral and molecular rhythms in *Drosophila* pacemaker system. It is possible that the molecular clockwork which is essential for entrainment mechanism in adult emergence and activity/rest rhythms is also required for synchronizing process of egg laying rhythm, and the absence of molecular oscillations in LL may also cause reduction in synchronizing ability of circadian egg laying rhythm.

Further studies need to be carried out to explore the mechanisms underlying temperature entrainment of circadian egg laying rhythm in *Drosophila*. Whether there are thermal receptors/temperature sensitive molecules in the fly ovaries is worth investigating. Another aspect to be studied is whether there are temperature sensitive mechanisms in the fly brain or ventral nerve chord (VNC), which can sense temperature signals from environment and transduce them to the ovaries which in turn cause rhythmic deposition of eggs.

Chapter 3



Circadian egg laying rhythm persists in flies with electrically silenced pacemaker neurons

Chapter 3

Circadian egg laying rhythm persists in flies with electrically silenced pacemaker neurons

3.1 Background

The fruit fly *Drosophila melanogaster* has been successfully used in numerous studies as a model organism to understand the neurogenetic basis of circadian rhythms because of its amenability to genetic manipulation. Among the widely studied circadian rhythms in *Drosophila* are the rhythms in activity/rest (locomotor activity), adult emergence (eclosion), mating and egg laying (oviposition) behaviors (Saunders, 1982). For activity/rest and emergence rhythms, a large body of information is now available pertaining to the neuronal groups that regulate them and the genes comprising their rhythm-generating machinery. Various groups of neurons such as small and large ventral lateral neurons (sLN_v and lLN_v), dorsal lateral neurons (LN_d) and three groups of dorsal neurons (DN1, 2, and 3) in the *Drosophila* brain regulate circadian rhythms in activity/rest behavior (reviewed in Shafer *et al*., 2006). In addition, posterior lateral cortex (LPN) cluster of neurons that express clock genes are also believed to play some role in the rhythm regulation (Kaneko and Hall, 2000; Shafer *et al*., 2006). While glial cells could also be involved in generation of activity/rest rhythms, neurons are certainly necessary and sufficient for the maintenance of robust circadian rhythmicity (Ewer *et al*., 1992; Frisch *et al*., 1994). Most clock neurons send out processes to the dorsal protocerebrum (Helfrich-Förster and Homberg, 1993; Kaneko and Hall, 2000), which is connected to many areas of the brain, and also contains many neurosecretory cells. It is therefore likely that it is this area of the fly brain which receives output signals for behavior from different

clusters of clock neurons. The Pigment Dispersing Factor (PDF)-expressing LN_v neurons serve as the circadian pacemakers for activity/rest and emergence rhythms in constant darkness (DD) (Ewer *et al*., 1992; Renn *et al*., 1999; Blanchardon *et al*., 2001; Myers *et al*., 2003). The LN_v neurons serve as circadian pacemakers in DD, whereas DN and LN_d neurons serve as pacemakers under constant light (LL) conditions (Murad *et al*., 2007; Stoleru *et al*., 2007). At the molecular level, rhythmic transcription and translation of core clock genes *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*) are deemed to be necessary for the persistence of circadian rhythms in DD (Glossop *et al*., 1999; Cyran *et al*., 2003; reviewed in Hall, 2005). While, our previous studies have shown that egg laying rhythm in *D. melanogaster* persists in DD and LL with circadian periodicity (Sheeba *et al*., 2001; Paranjpe *et al*., 2004; Howlader *et al*., 2006) and the rhythm is temperature and nutrition compensated (Howlader *et al*., 2006), neural and molecular mechanisms underlying this rhythm are yet unknown.

Certain features of egg laying rhythm differ from those of the activity/rest and emergence rhythms. For example, neither the transcripts nor the protein products of the core clock genes *per* and *tim* oscillate in the ovaries of female *D. melanogaster* (Plautz *et al*., 1997; Beaver *et al*., 2003). Unlike the activity/rest and emergence rhythms, egg laying rhythm continues unabated under laboratory LL (Sheeba *et al*., 2001). Ablation of the PDF-expressing LN_v neurons abolishes rhythmicity in activity/rest and emergence behavior but not in egg laying behavior (Howlader *et al*., 2006). This led to the conclusion that non-LN_v based, non-PDF mediated circadian oscillators, such as those residing in LN_d, DNs, non-PDF-expressing 5th sLN_v, LPN or yet unknown cells in the fly brain, or in the ovaries function as circadian pacemakers for egg laying rhythm in *D. melanogaster* (Howlader and Sharma, 2006).

An important factor to be considered here is the electrical property of the clock neurons. Electrical excitability is a distinguishing property of functional neurons (Kandel *et al*., 2000). In fact, very early models for circadian clocks were based on feedback interactions between membrane ion transport systems and ion concentration gradients (Njus *et al*., 1974, 1976). Mechanisms for the regulated release of peptides from peptide-releasing neurons of insects have been reported to be similar in the case of eclosion and ecdysis triggering hormones (Ewer *et al*., 1997; Hewes, 1999). Multiple oscillators in the fly brain are synchronized and coordinated with each other largely due to the excitability of LN_v neurons, which in turn, leads to the cyclic release of PDF, the output circadian neuropeptide (Nitabach *et al*., 2006). Electrical silencing of LN_v neurons severely affects the adult activity/rest rhythm (Nitabach *et al*., 2002), and immediate photophobic response in larvae (Mazzoni *et al*., 2005), suggesting that electrical activity of LN_v neurons are necessary for the persistence of circadian activity/rest rhythm and for the transmission of rapid light signals. This raises the possibility that electrical activity of pacemaker LN_v neurons also regulate other behavioral rhythms such as those in mating and egg laying rhythm.

Although previous studies on egg laying rhythm have revealed that neither LN_v neurons nor its neurotransmitter PDF is essential for the persistence of egg laying rhythm in DD, we do not yet know what role the electrical properties of these neurons play in the regulation of the rhythm (Howlader and Sharma, 2006). In *Drosophila,* electrical silencing of pacemaker LN_v neurons by expressing modified *Drosophila* open rectifier for potassium (K^+) channel (dORKΔ-C1) or inward rectifier potassium channel (Kir2.1) severely impairs circadian activity/rest, and molecular oscillations (Nitabach *et al*., 2002). Recent studies have also shown that transgenic expression of a low activation threshold voltage-gated sodium channel in LN_v neurons results in the

disruption of circadian behavioral rhythms and clock protein oscillations in the transgenic flies (Nitabach *et al*., 2006; Sheeba *et al*., 2008). These studies suggest that electrical activity of LN_v neurons is an essential property of circadian pacemaker mechanisms.

In the present study, we asked whether the electrical properties of the PDF positive neurons influence egg laying rhythm. To do so, we assayed egg laying behavior in transgenic *D. melanogaster* flies expressing *dORK*Δ*-C1* or *Kir2.1* under 12:12 hr LD cycles and in DD. Both these constructs when expressed using the UAS/GAL4 system, cause silencing of the target neurons. These lines have been described in details in Nitabach *et al*. (2002), and the use of the UAS/GAL4 system is briefly described in the methods section of this chapter (described in detail in Duffy, 2002). The expression of either *dORK*Δ*-C1* or *Kir2.1* in LN_v results in behavioral arrhythmicity in DD as measured by locomotor activity assays and is accompanied by a complete run-down of molecular oscillations as indicated by the levels of PER and TIM proteins in LN_v neurons (Nitabach *et al*., 2002, 2005).

3.2 Materials and methods

3.2 (a) Fly strains

Targeted expression of *dORK*Δ*-C1, Kir2.1* or *dORK*Δ*-NC1* (control for *dORK*Δ*-C1*, where non-conducting potassium channels are introduced, and hence does not cause silencing of the neurons) was carried out using the UAS/GAL4 systems. The UAS/GAL4 system is a well-established method for targeted gene expression in *Drosophila.* GAL4, identified in *Saccharomyces cerevesiae* is a gene regulator induced by Galactose. It regulates the transcription of transcribed GAL1 and

GAL10 genes by binding to four related 17 base pair sites located in between these loci. These sites define an Upstream Activator Sequence (UAS), which is analogous to the enhancer element in eukaryotes. It is essential for the transcription and activation of GAL4 regulated genes. Therefore, gene expression can be manipulated by using tissue specific driver lines (Duffy, 2002). Egg laying behavior was assayed in, *pdf*GAL4, UAS-*Kir2.1* (parent lines as controls), *pdf*GAL4/UAS- *dORKΔ-NC1* (control line where non-conducting potassium channels are introduced in PDF expressing cells), *pdf*GAL4/UAS-*dORK*Δ-*C1*, *pdf*GAL4/UAS-*Kir2.1* (PDF expressing cells are silenced) (Nitabach *et al*., 2002). The fly strains were maintained under LD cycles of 12:12 hr (intensity of 100 lux during the light phase of the LD cycle), at 24 ± 1 ^{o}C temperature and ~70% relative humidity.

3.2 (b) Confirmation of targeted expression of dORKΔ-C1

We were able to check for the effectiveness of our UAS-*dORK*Δ-*C1* driver lines and their genetic crosses as these lines have a Green Fluorescence Protein (GFP) tag. UAS-*dORK*Δ-*C1* when used in conjunction with *pdf*GAL4 was expressed in the PDF-expressing LN_v (reviewed in Sheeba *et al*., 2008).

3.2 (c) Behavioral assays

Flies were anesthetized for a short duration of time using CO_2 and a pair of two-day old male and female was placed in a glass vial containing ~1 ml of food, and introduced into the each light regime for the egg laying rhythm assay. Twenty pairs of flies of each genotype were used in each light/dark regime. After 24 hr for acclimatization, oviposition rhythm was assayed by transferring the male-female pair into fresh food vials at 2 hr intervals, and the number of eggs laid over the preceding 2 hr was counted. This continued for a minimum of seven consecutive days under LD and DD regimes. Dead males were replaced throughout the experiment with virgin

males kept under similar light conditions. Dim red light of wavelength > 650 nm was used under DD for handling flies. The temperature and humidity throughout the experiment were maintained at 24 ± 1 ^{0}C and ~70%, respectively.

Freshly emerged, virgin males from all the populations were taken and activity/rest behavior was assayed for a minimum of 10 days under LD and DD. Activity was recorded in 5 min bins using the Drosophila Activity Monitoring (DAM) system of TriKinetics Inc., USA. Glass tubes of 0.5 cm diameter and 6 cm length, with cornmeal at one end and cotton plug at the other were used.

3.2 (d) Statistical analysis

For activity/rest behavior and egg laying rhythm assay, the periodicity under DD and entrainment under LD was assessed by treating time series data collected over a period of seven days using Lomb Scargle periodogram analysis from the CLOCKLAB software (Actimetrics, Evanston, IL).

3.3 Results

3.3 (a) Electrical silencing of LN_v neurons does not abolish egg laying rhythm

We achieved targeted silencing of PDF expressing LN_v neurons by driving the expression of *dORK*Δ*-C1* or *Kir2.1* in these neurons. The egg laying rhythms of such transgenic female flies and their respective controls were assayed under 12:12 hr LD and DD conditions in various transgenic lines of *D. melanogaster*. The results obtained for both these rhythms are summarized in Table 1.

The activity/rest rhythm of control and experimental flies are entrained to the imposed LD cycles. Under DD a high proportion (78 - 100%) of control flies show robust circadian rhythmicity in activity/rest behavior. As expected, flies with silenced PDF-expressing cells (*pdf*GAL4/UAS-*dORK*Δ-C_1 and *pdf*GAL4/UAS-*Kir2.1*) are

Table 1: Properties of egg laying rhythm under LD and DD

Genotypes	Percentage entrainment under LD	Strength of rhythm under LD	Percentage rhythmicity under DD	Strength of rhythm under DD	Free-running periodicity in hours
UAS-Kir 2.1	38 (n=15)	149.48 ± 35.27	91 (n=11)	122.23 ± 11.06	22.70 ± 0.92
pdf GAL4	55 (n=11)	184.80 ± 24.16	73 (n=11)	105.74 ± 13.77	22.71 ± 0.48
pdf GAL4/*UAS-Kir 2.1*	20 (n=11)	123.91 ± 18.50	75 (n=16)	133.63 ± 17.28	28.45 ± 1.29
pdf GAL4/*UAS-dORK*Δ*-NC$_1$*	25 (n=16)	140.38 ± 26.34	93 (n=14)	107.35 ± 22.66	24.40 ± 1.45
pdf GAL4/*UAS-dORK*Δ*-C$_1$*	16 (n=14)	124.45 ± 37.62	93 (n=15)	110.08 ± 25.59	28.39 ± 1.52

Table 1: Properties of egg laying rhythm of flies from different genotypes under light/dark (LD) and constant darkness (DD) conditions. Strength of rhythm was calculated by measuring amplitude of Lomb Scargle periodogram where amplitudes greater than 11.77 was considered to be statistically significant.

Activity profiles and actograms of flies from different genotypes

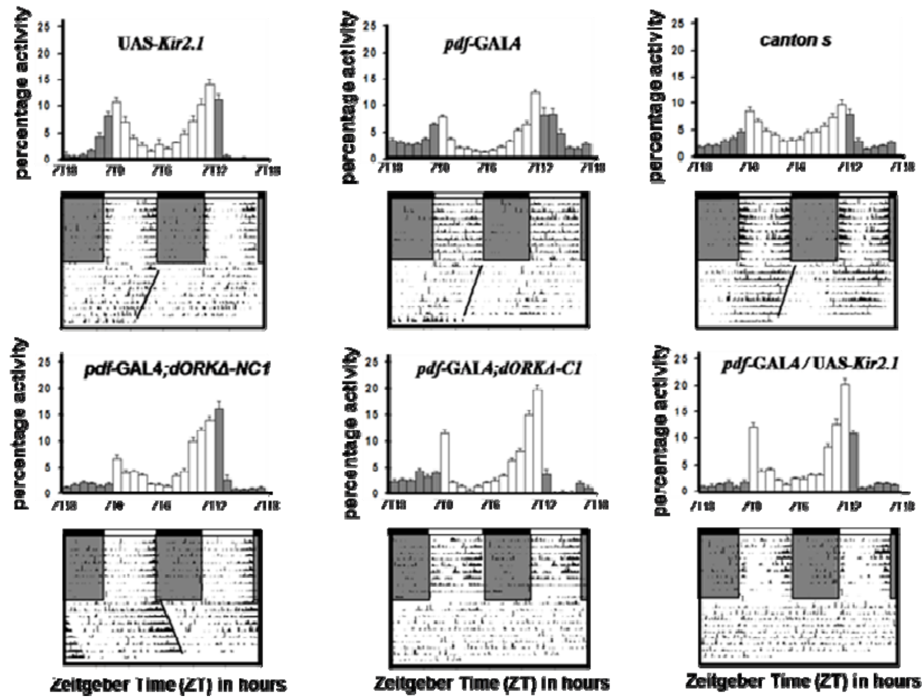


Fig. 1: Activity profiles (in light/dark - LD) and actograms (in both LD and constant darkness - DD) showing adult activity/rest patterns of flies wherein pacemaker neurons are electrically silenced by using either modified *Drosophila* Open Rectifier K^+ Channel (dORK) or inward rectifier K^+ channel (*Kir2.1).* Activity profiles shown are cumulative percentage activity of 16 flies from each genotype under LD. Dark bars and boxes in the activity profiles and actograms represent dark phase of the LD cycle.

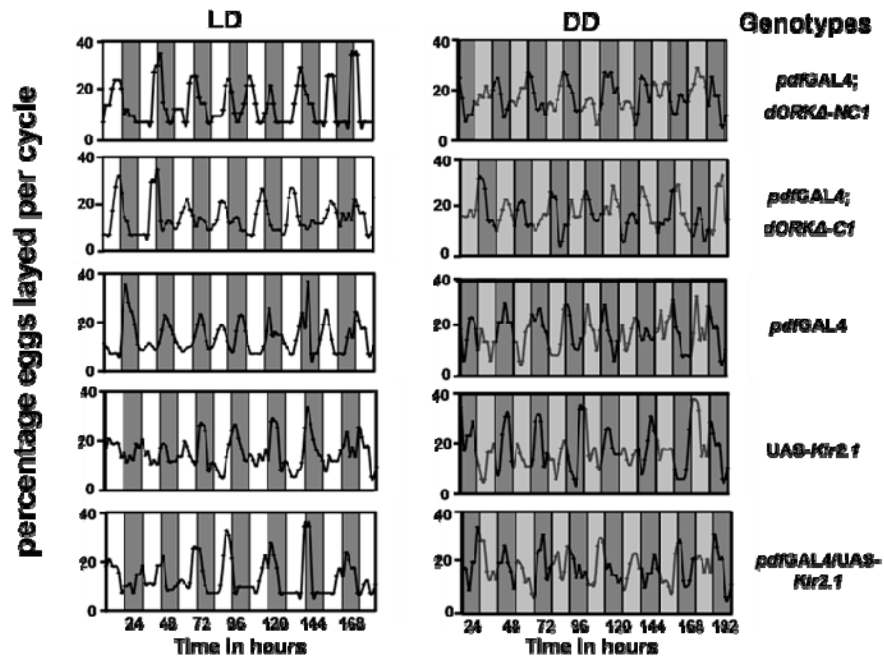


Fig. 2: Rhythmic profiles of egg laying rhythm in flies from different genotypes under light/dark (LD) and constant darkness (DD) conditions.

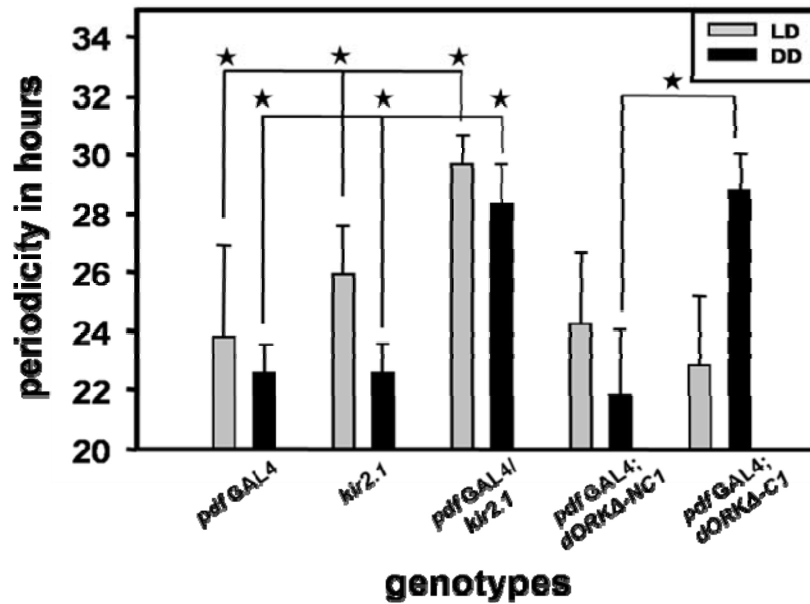


Figure 3: Circadian periodicity of egg laying rhythm in flies from different genotypes under constant darkness (DD) and those that free-run in presence of light/dark (LD) cycles. The free-running periodicity of egg laying rhythm in flies with electrically silenced ventral lateral neurons (LN_v) (*pdf*GAL4;UAS-*dORKΔ-C1 and pdf*GAL4/UAS-Kir2.1) is significantly longer compared to the controls (*pdf*GAL4, UAS-Kir2.1, and *pdf*GAL4;UAS- *dORKΔ-NC1*) ($p < 0.001$)

arrhythmic under DD (Fig 1). On the other hand, all lines including those that display arrhythmic activity/rest behavior in DD show robust circadian rhythmicity in egg laying behavior (Fig 2). The percentage of flies in which egg laying rhythm is synchronized to LD cycles or show circadian pattern under DD, does not vary much among the various genotypes. The percentage of rhythmic flies or those in which egg laying rhythm entrained to LD cycles is often lower than those for activity/rest behavior. Hence, to contrast with activity/rest rhythm, we have focused mainly on the comparisons of the presence or absence of circadian egg laying rhythm for each genotype and not so much on the actual percentage of flies that show synchronization or rhythmicity in the behaviors (Table 1).

3.3 (b) Electrical silencing of LN_v neurons lengthens free running period of egg laying rhythm

The periodicity of circadian egg laying rhythm in flies with electrically silenced LN_v neurons was significantly greater compared to the controls (Figure 3, Table 1). ANOVA on the circadian period values revealed a statistically significant effect of genotype ($F = 16.84$, $df = 4$; $p < 0.001$). Post-hoc multiple comparisons using Tukey's test revealed that the periodicity of egg laying rhythm in flies whose LN_v neurons are electrically silenced is significantly greater than the controls.

3.4 Discussion

The present study is an attempt towards understanding the role of electrical signals from pacemaker neurons in the regulation of egg laying rhythm. The results clearly show that while there is no contribution from pacemaker neurons in the persistence of the rhythm in DD, there is a significant lengthening of circadian

periodicities in the electrically silenced flies as compared to the controls. This suggests that electrical properties of the LN_v neurons are required in the maintenance of free-running period to a near 24 hr (circadian) value. Previous studies from our laboratory on egg laying rhythm in flies with ablated PDF expressing LN_v neurons have shown no difference in circadian periodicity between experimental and control genotypes (Howlader and Sharma, 2006). However, these studies were done with a sampling interval of 4 hr. In the present study we have carried out experiments by sampling data (counting eggs) every 2 hr. Thus we expect greater resolution of estimates of free-running period in the present data set.

Taken together, the results of this study and others which examined the effect of modulation of electrical activity of circadian pacemaker neurons suggest that electrical activity of pacemaker LN_v neurons is crucial for maintaining circadian integrity in behavioral rhythms such as activity/rest and egg laying probably via the regulation of molecular clock oscillations in the circadian pacemaker neurons (Nitabach *et al*., 2002). Furthermore our study suggest that membrane electrical activity of LNv are also involved in keeping the free running period of egg laying rhythm within circadian range. It is possible that the egg laying rhythm is directly governed by peripheral oscillators in the ovaries, which are coupled to the circadian pacemaker neurons in the brain. In absence of PDF-expressing neurons, other circadian oscillators in the fly brain may take over as pacemaker for this rhythm. However, in the presence of PDF-expressing neurons coupling of these neurons with other circadian oscillators may take place, and electrical output from the PDF-expressing neurons may influence the phase of egg laying rhythm by exerting its dominance on non-PDF based circadian oscillators.

Recent studies have shown that flies with modified arborization of large PDF neurons that show higher density of PDF fibers in medulla, dorsal protocerebrum and accessory medulla, show a longer circadian period than controls (Wülbeck *et al*., 2008). Also, mutant flies that lack PDF and severely reduced optic lobe show reduced rhythmicity and a shorter period as compared to wild type flies. Also, following transgenic expression of sodium channels NaChBac in PDF expressing cells an internal desynchronization was observed in circadian locomotor rhythm (Nitabach *et al*., 2006; Sheeba *et al*., 2008). Our study shows that electrical silencing of PDF-expressing neurons increases the period length of circadian egg laying rhythm. In spite of the presence of PDF in the electrically silenced PDF-expressing neurons lack of electrical output is unable to maintain the periodicity of egg laying rhythm at near 24 hr value.

Since circadian rhythm in egg laying differs from the two other well-studied circadian rhythms namely the activity/rest and emergence rhythms, we asked if the electrical silencing of PDF expressing neurons has any role to play in the rhythm regulation. In this study we showed that targeted silencing of the PDF-expressing LN_v neurons does not abolish egg laying rhythm, however it considerably lengthens the free-running period of the rhythm. The fact that circadian egg laying rhythm persists in flies with electrically silenced LN_v neurons suggests that the master pacemakers for this rhythm are located outside the known circadian pacemaker circuit. These oscillators could either be those that reside in the glial cells, or some yet unexplored cells in the fly brain, or those located in some peripheral oscillators in the ovaries.

Chapter 4

Role of mating in the regulation of egg laying rhythm

Chapter 4

Role of mating in the regulation of egg laying rhythm

4.1 Background

In many insect species including *Drosophila*, a number of key processes such as adult emergence, activity/rest, olfaction, feeding, and mating have been shown to be under circadian regulation (reviewed in Saunders *et al*., 2002; Howlader and Sharma, 2006). These behaviors occur rhythmically even in absence of environmental time cues. Although we know a great deal about the molecular mechanisms underlying rhythmic behaviors, the links between gene regulation and downstream processes is far from clear.

Many insect species exhibit rhythmicity in mating behavior (Hardeland 1972; Ikeda, 1976; Smith 1979; Charlwood & Jones 1979; Ziv *et al.,* 1991; Marques & Waterhouse, 1994; Miyatake 1997), which is controlled by an endogenous circadian clock (Smith, 1979; Charlwood & Jones, 1979). The fruit fly *D. mercatorum* shows daily rhythm in mating activity under 12:12 hr light/dark (LD) cycles (Ikeda, 1976). Additionally several *Drosophila* species show daily rhythmicity in male courtship behavior under LD cycles (Hardeland, 1972). In *D. melanogaster*, wild type flies display robust circadian pattern in mating activity, while flies carrying loss of function mutations for the *period* (per^{01}) and *timeless* (tim^{01}) genes show arrhythmic mating (Takaomi and Norio, 2001). Interestingly, it is the genotype of the females that solely determines the pattern of mating in these flies. Circadian rhythm in mating is abolished when arrhythmic per^{01} or tim^{01} females are paired with rhythmic wild type males (Takaomi and Norio, 2001). *The disconnected* (*disco*) mutants that have severe defects in their optic lobes and lack lateral neurons (LN) show arrhythmic mating behavior. These results suggest that mating rhythm in *Drosophila* is under the control of circadian clocks (Takaomi and Norio, 2001). Also, an anti-phasic relationship is detected between the

circadian mating rhythm of *D. melanogaster* and that of its sibling *D. simulans* (Takaomi and Norio, 2001), suggesting species-specific variation in mating rhythm is caused perhaps by long term reproductive isolation. While circadian rhythm in mating is under the control of LNv-based, *per-tim* regulated circadian clocks, rhythmicity in its downstream process (egg laying) does not seem to be so (Howlader and Sharma, 2006). It is likely that insects have evolved "redundant" circadian pacemaking mechanisms to ensure that under adverse conditions rhythmicity in one of its most fundamental processes (egg laying) is unaffected. Hence it is likely that fruit flies have evolved to use mating as a trigger to coordinate processes responsible for circadian egg laying rhythm.

Previous studies have reported that mating and activity/rest behaviors in *D. melanogaster* tim^{01} mutants are arrhythmic (Takaomi and Norio, 2001). Also, tim^{01} flies carrying *tim* cDNA of *D. ananassae* exhibit rhythmic mating, however, the time course and waveform of mating rhythm differ significantly from those of *D. melanogaster* and *D. ananassae*. In *D. pseudoobscura* transformant line that express *D. pseudoobscura per* fused to the *D. melanogaster per* promoter, peak of mating rhythm occurs later than in *D. pseudoobscura* (Tauber *et al.,* 2003). These studies suggest that *per* plays a key role in reproductive isolation in *Drosophila* (Miyatake *et al*., 2002; Tauber *et al*., 2003).

Many insect species undergo behavioral and physiological changes at specific stages in their life cycle (Chen *et al.,* Monsma and Wolfner, 1988; Kubli, 1992). In *Drosophila,* the transcript of the sex-specific *fruitless (fru)* gene acts as a switch that determines male or female mating behavior (Arthur *et al.,* 1998; Demir & Dickson, 2005; Manoli *et al.,* 2005). After mating, *Drosophila* females show remarkable changes in their reproductive physiology and behavior (Wolfner, 2002). Production of eggs and its release are dramatically enhanced, and the female's tendency to re-mate is drastically reduced. Post-mating changes in females

are believed to be triggered by sex-peptide (SP), a 36-amino acid peptide (Chen *et al.,* 1988; Chapman *et al.,* 2003; Liu and Kubli, 2003; Gillott 2003), and are known to persist for fairly long time. Behavioral changes are also believed to be induced by seminal fluids and by the presence of stored sperm in the female spermatheca (referred as the ‘‘sperm effect’’) (Liu & Kubli, 2003; Chapman *et al*., 2003). Accessory glands present in the male reproductive tract secrete male seminal fluid proteins referred as accessory gland proteins (Acps) (Liu & Kubli, 2003; Chapman *et al*., 2003). Post-mating changes in female *Drosophila* have been categorized into two types, short- and long-term changes. The short-term effects are attributed largely to the rapid action of several Acps, which act before and during the storage of sperm. The long-lasting changes in female reproductive physiology require the presence of sperm and a host of mechanisms by which sperms act (Bloch *et al*., 2003). Taken together it is likely that rhythmic pattern in mating and hence the transfer of male ejaculate may regulate circadian egg laying rhythm in *Drosophila*.

Since, *Drosophila* females undergo dramatic changes in their reproductive behavior following mating, we asked if mating patterns have any effect on the robustness and persistence of egg laying rhythm. To address this we assayed egg laying rhythm in fruit flies maintained in different male-female genotypic combinations, wherein either male or female is arrhythmic (per^0w) for mating behavior. The main objective of our study was to assess whether mating behavior alone can account for circadian egg laying rhythm in *Drosophila*.

4.2 Materials and methods

4.2 (a) Fly strains

We used per^0w, and *w* flies in various male-female combinations to assay the effect of mating on egg laying rhythm. The male-female combinations used in this study were as follows:

***per^0w* ♂ × *w* ♀:** In this combination males are arrhythmic for activity/rest and mating behaviors while females are rhythmic for these behaviors.

***w* ♂ × *per^0w* ♀:** In this combination males are rhythmic for activity/rest and mating behaviors while females are arrhythmic for these behaviors.

***per^0w* ♂× *per^0w* ♀:** In this combination both males and females are arrhythmic for activity/rest and mating behaviors (henceforth this combination will be denoted as "per^0w").

***w* ♂ × *w* ♀:** In this combination both males and females are rhythmic for activity/rest and mating behavior (henceforth this combination will be denoted as "*w*").

4.2 (b) Behavioral studies

For the oviposition assay, freshly emerged (2-day-old) male-female pairs of different genotypic combinations were introduced into vials containing ~4 ml of food. Flies were maintained under LD cycles for 2 days and subsequently transferred into DD or LL conditions. After one day, the male-female pairs were periodically transferred into fresh food vial every 2 hr and the number of eggs laid over the preceding 2 hr duration were counted. This was continued for eight consecutive days. Dead males were replaced throughout the experiment with age matched males maintained in a similar condition. White fluorescent light of ~250 lux was used under LL and light phase of LD cycles. Dim red light of wavelength > 650 nm was used in DD as well as the dark phase of LD for transferring and handling flies.

4.2 (c) Statistical analysis

For egg laying assay, the periodicity was assessed by treating time series data collected over a period of eight days using Lomb Scargle periodogram analysis of the CLOCKLAB software (Actimetrics, Evanston, IL).

4.3 Results

4.3 (a) Percentage rhythmicity is greater in male-female combinations having rhythmic females

Although in all male-female genotypic combinations ~60% of the females are rhythmic for egg laying rhythm (Fig 4, Table 1), statistical analysis revealed that percentage rhythmicity is significantly greater in $per^{0}w$ ♂ × *w* ♀ and *w* ♂ × *w* ♀ pairs than in the rest of the combinations ($F = 19.53$, $p < 0.001$, $df = 3$). $per^{0}w$ ♂ × *w* ♀ yield 100% rhythmicity in LD as well as DD, whereas the percentage is ~67% in LL. The percentage rhythmicity in *w* ♂ × $per^{0}w$ ♀ pair is ~83% in LD and ~71% in DD, whereas in $per^{0}w$ ♂× $per^{0}w$ ♀ pair it is ~74% in LD and ~70% in DD (Fig 1, Table 1, 3). In LL, the percentage of flies that show rhythmicity in egg laying behavior in the *w* ♂ × *w* ♀ and $per^{0}w$ ♂ × *w* combination is comparable to those in the combinations where arrhythmic females were used (Fig 1, Table 1, 2).

4.3 (b) Male-female genotypic combinations have no measurable effect on the circadian periodicity of egg laying rhythm

ANOVA on the periodicity values revealed that the main effect of genotypic combinations ($F = 1.19$, $p > 0.3$, $df = 3$) and light regimes ($F = 1.22$, $p > 0.2$, $df = 2$) is statistically not significant (Fig 2, Table 3). The mean circadian periodicities of egg laying rhythm under LD, DD and LL in $per^{0}w$ ♂ × *w* ♀ combination are 25.05 ± 1.87 hr (mean ± SD), 25.10 ± 2.23 hr and 23.23 ± 2.37 hr, respectively, while those in *w* ♂ × $per^{0}w$ ♀ combination are 24.62 ± 1.49 hr, 24.73 ± 1.99 hr and 23.43 ± 2.96 hr, respectively (Fig 2, Table 1,3). The mean circadian

Details of egg laying rhythm of females maintained in different male-female genotypic combinations.

Genotype	% Rhythmic	FRP in hr	% Entrained in LD
per⁰w-LD	75	21.1 ± 2.5	8
w-LD	93	24.1 ± 2.3	29
Per⁰w-DD	69	24.1 ± 1.8	
w-DD	93	24.8 ± 1.7	
per⁰w-LL	69	23.3 ± 2.4	
w-LL	88	23.9 ± 2.7	
per⁰w ♂ X *w* ♀ - LD	100	25.0 ± 1.9	53
w ♂ X *per⁰w* ♀ - LD	87	24.6 ± 1.6	15
per⁰w ♂ X *w* ♀ - DD	100	25.1 ± 2.3	
w ♂ X *per⁰w* ♀ - DD	80	24.7 ± 2.0	
per⁰w ♂ X *w* ♀ - LL	69	23.2 ± 2.3	
w ♂ X *per⁰w* ♀ - LL	64	23.4 ± 2.9	

Table 1: Egg laying behavior females maintained in different male-female genotypic combinations assayed under light/dark (LD) cycles, constant darkness (DD) and constant light (LL). The variance in circadian periodicity (FRP) represents SEM values with 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing

Percentage rhythmicity in egg laying behavior of females in various male-female combinations under LD, DD and LL conditions.

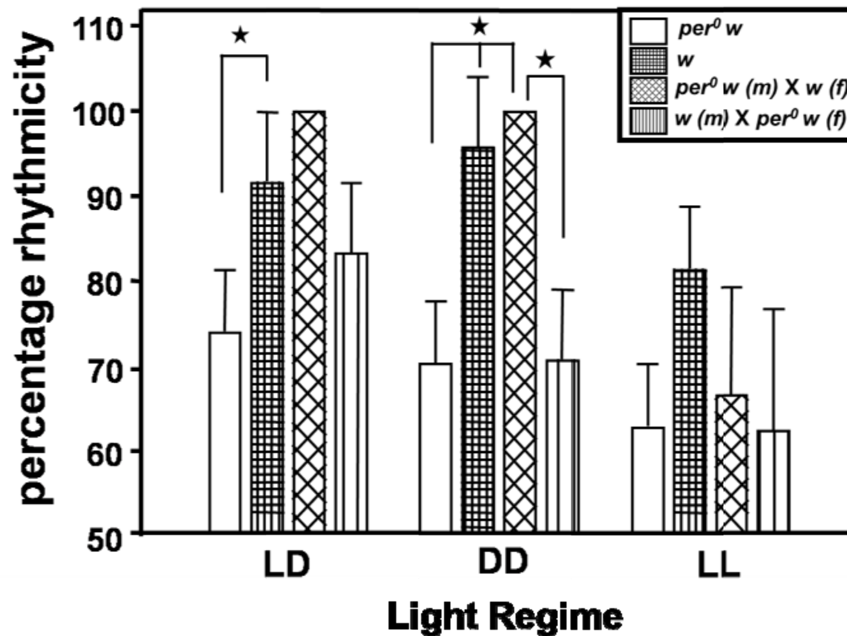


Fig. 1: Percentage rhythmicity of egg laying rhythm in females from different genotypic combinations. Percentage rhythmicity in egg laying behavior of females maintained under different genotypic combinations under light/dark (LD) cycle, constant darkness (DD) and constant light (LL). The presence of rhythmic females significantly enhances the percentage of females exhibiting circadian egg laying rhythmicity in LD and DD but not in LL. Error bars represent SEM with 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing.

Circadian periodicities of egg laying rhythm of females from different male-female genotype combinations under LD, DD and LL conditions.

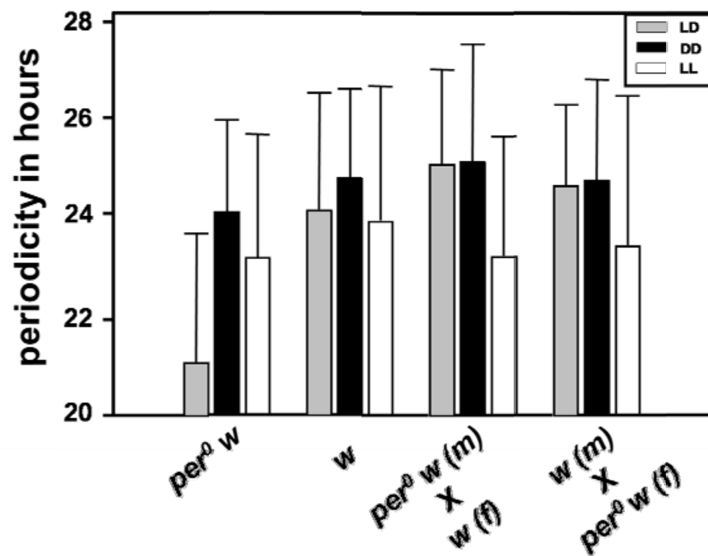


Fig. 2: Circadian periodicity of egg laying rhythm in females from different genotypic combinations. Circadian periodicity in egg laying rhythm of females from different male-female genotypic combinations maintained under light/dark (LD) cycle, constant darkness (DD) and constant light (LL). Error bars shown are SEM with 95% Confidence Interval (95%CI) around the mean for visual hypothesis testing.

Percentage entrainment in egg laying rhythm of females in different male-female combinations under LD cycles.

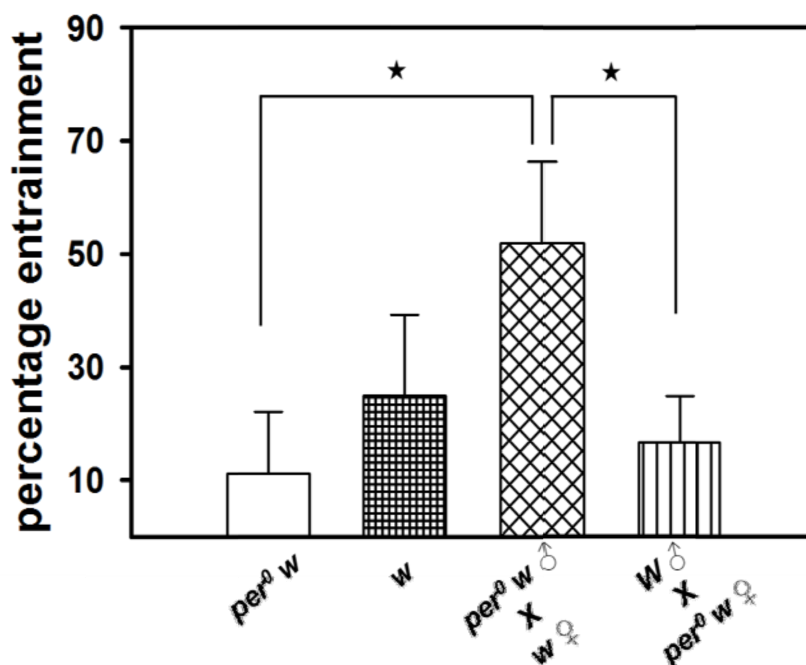


Fig. 3: Percentage entrainment to light/dark (LD) cycles of egg laying rhythm in females from different genotypic combinations. Percentage entrainment to LD cycle of egg laying rhythm in females maintained under different male-female genotypic combinations. A greater percentage of entrainment was observed in females from combinations where a rhythmic female is paired with either rhythmic or arrhythmic male. Error bars shown are SEM with 95% Confidence Interval (95CI) around the mean for visual hypothesis testing.

Egg laying profiles of flies of different male-female genotype combinations

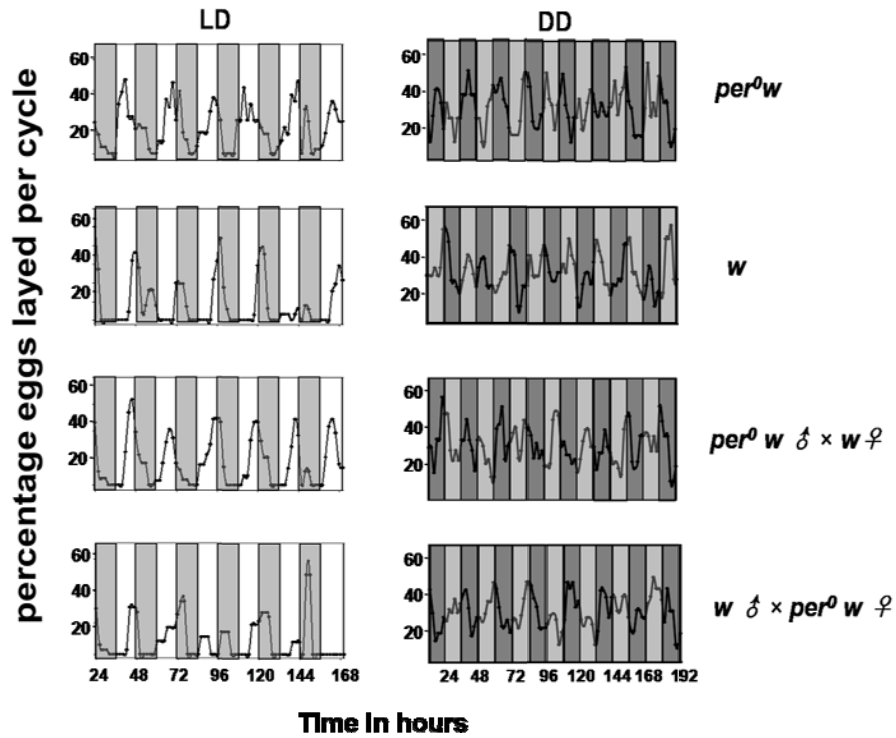


Figure 4: Representative egg laying profiles of females maintained in different male-female genotypic combinations under 12:12 hr light/dark (LD) cycles and constant darkness (DD). Dark boxes represent dark phases in LD cycles and subjective nights in DD.

Results of ANOVA on the percentage rhythmicity data

Summary of all Effects on percentage rhythmicity data

Effect	*df* Effect	MS Effect	*df* Error	MS Error	*F*	*p*-level
Genotype (G)	3	19.381	125	16.285	1.190	**>0.312**
Light regime (L)	2	19.970	125	16.285	1.226	**>0.296**
G × L	6	8.7691	125	16.285	0.538	**>0.778**

Table 2: Results of ANOVA performed on percentage rhythmicity data. The results yielded a significance difference in percentage rhythmicity in egg laying rhythm across genotypes ($p < 0.001$) and light regime ($p < 0.001$).

Results of ANOVA on the circadian periodicity data

Summary of all Effects on periodicity data						
Effect	d*f* Effect	MS Effect	*df* Error	MS Error	*F*	*p*-level
Genotype (G)	3	1052.58	24	54.387	19.353	**<0.000**
Light regime (L)	2	1232.8	24	54.387	22.667	**<0.000**
G × L	6	155.910	24	54.387	2.866	**<0.030**

Table 3: Results of ANOVA performed on the circadian periodicity values of egg laying rhythm in flies maintained in different male-female genotypic combinations. The periodicity values across genotypes and light regimes did not differ statistically.

Results of ANOVA on percentage entrainment data

Summary of all Effects on percentage in LD entrainment						
Effect	*df Effect*	MS Effect	*df* Error	MS Error	*F*	*p*-level
Genotype	3	978.009	8	116.383	8.403	**<0.008**

Table 4: Results of ANOVA performed on the percentage entrainment data. The results revealed a statistically significant difference in percentage entrainment to light/dark (LD) cycles of egg-laying rhythm across genotypes ($p < 0.007$).

periodicities of egg laying rhythm under LD, DD and LL in *per*^{0}*w* ♂× *per*^{0}*w* ♀ combination are 21.12 ± 2.55 hr, 24.10 ± 1.81 hr and 23.2 ± 2.43 hr, respectively, while those in *w* ♂ × *w* ♀ combination are 24.14 ± 2.32 hr, 24.10 ± 1.81 hr and 23.2 ± 2.43 hr, respectively (Fig 2, Table 1, 3).

4.3 (c) Egg laying rhythm of a greater percentage of females in *per*^{0}*w* ♂ × *w* ♀ combination entrained to LD cycles

ANOVA on the percentage entrainment data revealed a statistically significant effect of genotypic combinations ($F = 8.40$, $p < 0.01$, $df = 3$). Among different male-female genotypic combinations under LD cycles, the percentage of females in which egg laying rhythm entrained to LD cycles is greater in *w* ♂ × *w* ♀ and *per*^{0}*w* ♂ × *w* ♀ combination than in *per*^{0}*w* ♂× *per*^{0}*w* ♀ ($p < 0.007$) and *w* ♂ × *per*^{0}*w* ♀ ($p < 0.01$) combinations (Fig 3, Table 1, 4). Egg laying rhythm of ~52% females in *per*^{0}*w* ♂ × *w* ♀ combination showed entrainment to LD cycles as compared to ~11% and ~17% in *per*^{0}*w* ♂× *per*^{0}*w* ♀ and *w* ♂ × *per*^{0}*w* ♀ combinations, respectively. The percentage entrainment in *w* ♂ × *w* ♀ and *per*^{0}*w* ♂ × *w* ♀ and *per*^{0}*w* ♂× *per*^{0}*w* ♀ and *w* ♂ × *per*^{0}*w* ♀ combinations did not differ significant among each other (Fig 3, Table 1, 4).

4.4 Discussion

The present study examines whether mating patterns have any effect on the persistence and entrainment ability of egg laying rhythm in *D. melanogaster* females. It is well known that clock genes control rhythmicity in *Drosophila* mating behavior and that mating rhythm is especially attributed to females. We carried out experiments involving male-female pairs where either male or female is with or without functional circadian clocks. We found that egg laying rhythm is not abolished in any of the male-female genotypic

combinations. Presence of functional clocks in females significantly enhances the percentage of flies that exhibit rhythmic egg laying in DD and entrainment in LD. These results are consistent with previously reported findings on mating behavior in *Drosophila*, wherein circadian rhythm in mating is found to be abolished when loss of function *per*^{0} mutant females were paired with wild type males (Takaomi and Norio, 2001). A careful analysis of our results suggests that presence of functional circadian clocks in females significantly enhances the robustness of circadian egg laying rhythm. The decrease in robustness of the rhythm in combinations where arrhythmic female is present, may be due to lack of *per* expression in the ovaries, which is known to lower reproductive output in both males and females; loss of functional clock mutations in *per* and *tim* decreases the number of sperms and mature oocyte production (Beaver *et a*l., 2003). Ectopic expression of PER in loss of function *per* mutants confirmed that the decrease in robustness in egg laying rhythm is indeed due to the non-circadian function of *per* in the ovaries.

Percentage rhythmicity in DD and percentage entrainment in LD is reduced in females maintained in male-female combination wherein arrhythmic females were present. Unlike mating rhythms, rhythmicity in egg laying is observed in a sizable percentage of females maintained in all types of genotypic combinations. This suggests that mechanisms underlying persistence of egg laying rhythm are different from those underlying activity/rest and mating, however, presence of circadian clocks in females contributes to the robustness of egg laying. Previous studies have shown that mating in *Drosophila* is driven by two separate mechanisms, one that is clock driven and the other that is driven by environmental LD cycles (Takaomi and Norio, 2001). Previous studies in *Drosophila* have shown that circadian egg laying rhythm persists in flies without functional LNv based circadian clocks (Howlader *et al*., 2006). It has also been shown that flies, especially males, primarily use olfactory cues for

mating (Hall, 1994; Jallon, 1984; Cobb & Jallon, 1990), and olfactory response in *Drosophila* exhibit robust circadian patterns even in LNv ablated flies (Krishnan *et al.*, 1999).

Although percentage rhythmicity and percentage entrainment of egg laying rhythm is greater in females maintained in male-female combinations where females themselves are rhythmic, circadian periodicity did not differ among females. This suggests that the basic underlying mechanisms governing egg laying rhythm in *Drosophila* is, to a certain extent, independent of a functional circadian clock involving *per*. However, presence of *per* certainly enhances the robustness of the rhythm, suggesting that though core clock genes may not be involved in the generation of egg laying rhythm, they certainly exert some phase control to make the rhythm more robust. The results of our present study further suggest that, functional *per* gene is not necessary for the persistence of egg laying rhythm, because ~69% of per^0w females exhibit circadian egg laying rhythm, mechanisms governing this rhythm are likely to be partly *per* dependent.

Chapter 5

Role of logjam in the regulation of circadian egg laying rhythm in Drosophila

Chapter 5

Role of *logjam* in the regulation of circadian egg laying rhythm in *Drosophila*

5.1 Background

In eukaryotic and certain prokaryotic organisms circadian oscillators are governed by auto-regulatory feedback loops in gene expression (Dunlap, 1999). In *Drosophila* many genes have been identified that are necessary for circadian feedback loop function, among which five genes have crucial role in maintaining circadian rhythmicity: *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), and *doubletime* (*dbt*) (Hardin *et al*., 1990; Sehgal *et al*., 1994; Darlington *et al*., 1998; Bae *et al*., 1998; Allada *et al*., 1998; Kloss *et al*.,1998) of which three — *per*, *tim*, and *Clk* — express rhythmically. The *per* and *tim* mRNA levels peak at early evening — between Zeitgeber Time 13 to 16 (ZT13 to ZT16), and *Clk* mRNA levels peak between late night and early morning (ZT23 to ZT4) (Hardin *et al*., 1990; Sehgal *et al*., 1994; Darlington *et al*.,1998; Bae *et al*., 1998; Allada *et al*., 1998; Sehgal *et al*., 1995). Transcription factors CLK and CYC which contain basic helix-loop-helix–PAS domain form heterodimers and mediate the activation of *per* and *tim* transcription by targeting E-box regulatory elements of the sequence CACGTG in their promoters (Bae *et al*., 1998; Kloss *et al*., 1998; Hao *et al*., 1995; Gekakis *et al*., 1998). The *per* and *tim* mRNA levels peak early in the evening (ZT13 to ZT16), however their protein levels peak late in the night (ZT18 to ZT24) (Zerr *et al*., 1990; Edery *et al*., 1994). This delay results from the initial destabilization of PER by DBT dependent phosphorylation, followed by stabilization of PER due to dimerization with TIM (Kloss *et al*., 1998). After reaching peak levels PER-TIM

dimers move into the nucleus to form complex with dCLK-CYC (Lee and Edery, 1997), which eventually results in transcriptional repression of *per* and *tim* (by deactivation of CLK-CYC) (Darlington *et al*., 1998).

per and *tim* genes have nearly identical spatial expression patterns in the adult brain; they are expressed in the photoreceptor cells of the compound eyes, ocelli and extra retinal eyelets, in many glial cells, and in the lateral neurons (LNs) and dorsal neurons (DNs) (Ewer *et al*., 1992; Frisch *et al*., 1994; Helfrich-Forster, 1998; Zerr *et al*., 1990). Rhythmic expression of *per*, *tim* and other well established circadian genes in these neurons is essential for circadian oscillations of overt rhythms (Myers *et al*., 2003). Core clock genes are also shown to be expressed in many peripheral tissues including gut, excretory system and testes (Liu *et al*., 1988; Saez and Young, 1988; Hege *et al*., 1997; Plautz *et al*., 1997). PER and TIM are also seen in alimentary tract, rectum, fat body renal (Malpighian) tubules and parts of the reproductive system such as ovaries (Giebultowicz and Hege, 1997; Giebultowicz *et al*., 2001). In *Drosophila*, sexually dimorphic behaviors associated with reproductive success are clearly governed by the action of multiple genes, some of which function as dedicated components in the somatic sex-determination hierarchy (Burtis, 1993; McKeown, 1994; Cline and Meyer, 1996). Two genes, *doublesex* (*dsx*) and *fruitless* (*fru*) have been shown to be regulated by the upstream components of signalling pathway required for the somatic sex-determination hierarchy (Baker and Ridge, 1980; McKeown *et al*., 1988; Nagoshi *et al*., 1988; Ito *et al*., 1996; Ryner *et al*., 1996). Another gene called *dissatisfaction* (*dsf*) was shown to interact genetically with the signalling pathway genes but is not regulated directly by known upstream members of

the hierarchy (Finley *et al.*, 1997, 1998). All three genes (*dsx, fru*, and *dsf*) code DNA binding proteins (Burtis *et al*., 1991; Ito *et al*., 1996; Ryner *et al*., 1996; Finley *et al*., 1998). The genes that act downstream of *dsx* and *fru*, and might be necessary for the regulation of reproductive behaviors in the fly, are yet to be known.

Although hardly anything is known about the oviposition circuitry in *Drosophila*, studies in grasshopper have shown that different aspects of the oviposition phenomenon such as maturation, ovulation and transmittal of eggs through the genital tract and its release are regulated by necessary neural circuitry (Thompson and Roosevelt, 1998). The passage of eggs through the oviduct to the uterus and its final release is referred as "oviposition proper" and the genes that regulate these processes as "oviposition genes" (Carney and Barbara, 2003). Among these genes, "*logjam*" has been shown to have the strongest phenotype because its loss of function causes complete cessation of the oviposition phenomenon (Carney and Barbara, 2003).

logjam (loj) is expressed in a variety of tissues, particularly in the adult central nervous system (CNS), ovaries including follicle cells and developing eggs (Carney and Barbara, 2003). Sequence database search and transcripts structure determination have shown that *loj* has at least 6 different transcript classes (Carney and Barbara, 2003). The different transcript classes of *loj* and their sequence length are as follows.

Transcript class	Sequence length in nucleotides (nt)
Class I	1085
Class Ia	1105
Class II	1139
Class III	1180
Class IV	868
Class V	829

The predicted *loj* protein shares identity with the EMP24/GP25 family of cytoplasmic vesicles membrane proteins. These 24 kD proteins were isolated as components of coat proteins (COP) and have functions in mediating molecular transport between endoplasmic reticulum and Golgi membranes (Schimmoller *et al*., 1995; Stamnes *et al*., 1995; Boltz *et al*., 2006). In *loj* mutants both ovulation and oviposition are severely affected. Carney and Barbara (2003) argue that loss of signals from the CNS as well as eggs is responsible for the disruption of oviposition behavior in the *loj* mutant females and that the disruption of ovulation is a secondary effect of the mutation. Females homozygous for the P insert for loj^{00898} and a deficiency (*Df*) chromosome that uncovers the mutation (loj^{00898}/Df) show sterility (Carney and Barbara, 2003). This is because one or more mature eggs become lodged within the female genital tract causing "logjam" of eggs and thus preventing the release of eggs (Carney and Barbara, 2003). Enhancer trap and *in situ* hybridization experiments suggest that signals required for proper oviposition are derived from *loj* expression in the fly brain ventral nerve chord (VNC), and developing eggs (Carney and Barbara,

2003). The altered ovulation and oviposition phenotypes in *loj* mutants may be a consequence of the failure of eggs to signal their intentions to the reproductive tract, indicating that signalling from eggs is an active process required for successful oviposition behavior.

Although several genes have been identified in *Drosophila* that control sexual dimorphism and female reproductive success, the genes that regulate circadian egg laying rhythm remain yet unexplored. Since the transcript and protein levels of core clock genes do not oscillate in ovaries, it would be interesting to study the molecular genetic mechanisms underlying egg laying rhythm. Systematic studies need to be performed to find out whether the genes that regulate female reproductive success have any role to play in the regulation of circadian egg laying rhythm. Since *loj* is essential for egg laying behavior, we wanted to study whether expression pattern of this gene oscillate in the ovary of the fly. For this, we estimated *loj* mRNA expression at two time points ZT12 (middle of the oviposition peak which is usually between ZT10 and 14) and ZT0.

5.2 Materials and Methods

5.2 (a) Fly strains

Canton S (*CS*) flies were used for expression profiling study of *loj* gene in the ovaries. 1-2 days old virgin females were synchronized to 12:12 hr LD cycles for 3 days. On the fourth day of LD, a group of 30 females were sacrificed at two ZT time points (ZT0 and 12) and were divided into two biological replicates of 15 flies each at each ZT. Ovaries from each biological replicate were collected and were used as tissue homogenate for the isolation of RNA.

5.2 (b) Isolation of total RNA

Ovary samples were homogenized in 800 µl of TRIzol reagent (Chomczynski P and Mackey K, 1995) and were incubated for 5 min at 15 to 30 ˚C to permit the complete dissociation of nucleoprotein complexes. To this, 160 µl of chloroform was added and the tubes were shaken vigorously for 15 sec followed by an incubation at 30 ˚C for 2 minutes. The tubes were then centrifuged at 12,000 rpm for 15 min at 4 ˚C. RNA in the upper aqueous phase was collected in a fresh tube. In order to precipitate the RNA, 400 µl of isopropyl alcohol was added and the tube was incubated at 30 ˚C for 10 minutes. RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4 ˚C. RNA pellet obtained was further washed with 75% ethanol prepared in DEPC treated water. This was followed by centrifugation at 7,500 rpm for 5 min. After carefully aspirating out ethanol from the tubes, the pellet was briefly air-dried and dissolved in 20 µl of DEPC water and stored at -20 ˚C until use. 2 µl of isolated RNA was taken and the RNA concentration was determined at 260 nm using Nanodrop™. The ratio 260/280 was measured to evaluate the purity of the RNA.

5.2 (c) cDNA synthesis

The total RNA obtained was reverse transcribed for the efficient synthesis of first strand cDNA. We made use of a recombinant M-MuLV Reverse Transcriptase which exhibits lower RNase H activity than AMV reverse transcriptase. Due to this feature, full-length cDNA can be synthesized from RNA templates that are up to 9 kb long. The recombinant RiboLock™ RNase Inhibitor, effectively protects RNA template from degradation.

The oligo$(dT)_{18}$ which is used as a primer anneals selectively on the poly(A) tail of mRNA. The first strand of cDNA can be directly used as a template in PCR. Degenerate primers based on conserved sequences from region and designed to specifically reverse transcribe were used (Sequence given below). RNA/primer mixtures were prepared in 0.2 ml DEPC treated PCR tubes as mentioned below:

Component	Sample	RT control
RNA	1.0 µg	-
Oligo $(dT)_{18}$ primer	2.0 µl	2.0 µl

Each sample was incubated at 70 °C for 5 min and immediately chilled on ice for 2 min. Then, the following mixture was prepared in the indicated order:

Component	Reaction
5X RT buffer	4.0 µl
RNase inhibitor	0.5 µl
10 mM dNTP mix	2.0 µl
DEPC treated water	to 20.0 µl

The mixture was incubated at 37 ˚C for 5 minutes followed by the addition of reverse transcriptase (1.0 µl) to each tube except the RT control and further incubated for 60 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 15 min and then chilled on ice. The tubes were centrifuged briefly and concentration of the cDNA

was determined using Nanodrop™ before proceeding with amplification by PCR using specific primers.

5.2 (d) PCR amplification of cDNA

For PCR amplification of the *loj* gene, primers which are common for all 6 transcripts of *loj* were used. The following gene specific primers were used to amplify *loj*:

Exon	Primer sequences		Product Size (bp)
Common for all transcript classes of *logjam*	**Fwd**	**5' -** GCTGGCAAGCTGGTCAACAT **- 3'**	**224**
	Rev	**5'** - CGATGACGACTGTGGGCTTG - **3'**	

The primers were used at a concentration of 5 µM. The dNTPs at a concentration of 200 µM were used and the final volumes were made to 25 µl with MilliQ water. Following are the constituents used for PCR amplification of *loj*.

Constituents	For 1 reaction
Buffer	2.5 µl
1.5mM $MgCl_2$	2 µl
Forward Primer (5µM)	1 µl
Reverse primer (5µM)	1 µl
dNTP (10mM)	2.5 µl
Taq polymerase	1 µl
Template (40ng/µl)	3 µl
MilliQ water	12 µl

The amplification of the cDNA (template) was carried out using a Peltier Thermal Cycler (PTC – 200; MJ Research). All the PCR reactions were carried out in 0.2 ml eppendorf tubes with the following reaction conditions:

Step 1 (Initial denaturation)	:	94 ˚C – 2 min
Step 2 (Denaturation)	:	94 ˚C – 1 min
Step 3 (Annealing)	:	60 ˚C – 45 sec
Step 4 (Extension)	:	72 ˚C – 45 sec
Step 5	:	Go to cycle 2 (35 times)
Step 6 (Final extension)	:	72 ˚C – 1 min
Step 7	:	4 ˚C - 10 min

To analyze the amplicons of PCR product agarose gel electrophoresis was used. The agarose was dissolved in 1X TBE buffer and was pre-stained by mixing it with 1 µl of ethidium bromide and poured on to the sealed gel template fitted with a comb. After solidification, the gel was immersed in 1X TBE running buffer in a horizontal electrophoresis tank. The PCR product mixed with equal volume of loading dye (2 µl of amplicon + 2 µl of loading dye) was loaded on the slots with the first slot serving as a reference with the standard DNA marker (100 bp/200 bp ladder) to evaluate the size of the amplicon. After optimizing the experiment conditions, PCR run showed the amplification of specific products (Fig 1).

PCR products of *logjam* (*loj*) at ZT0 and ZT12

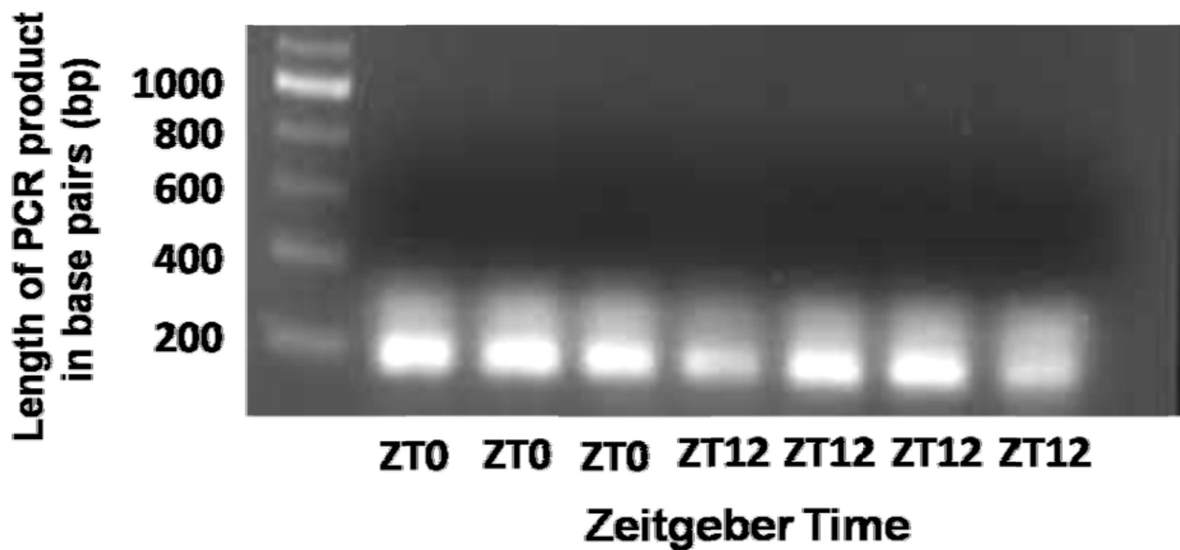


Fig. 1: Agarose gel electrophoresis showing bands of PCR products of *logjam (loj)* at two time points - Zeitgeber Time 0 and 12 (ZT0 and ZT12).

Amplification and melting curves of *actin* and *loj* genes

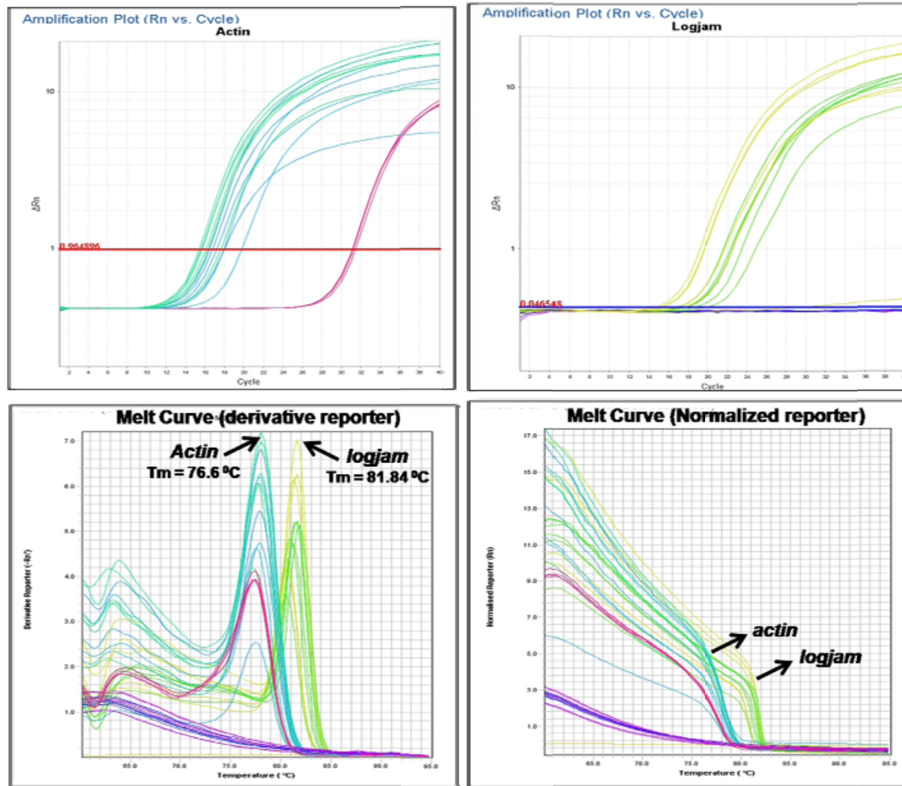


Fig. 2: Amplification and melting curve plots of *actin* and *logjam* (*loj*) genes. The melting curves show two distinct peaks at 76.6 ^{0}C (for *actin*) and 81.84 ^{0}C (for *logjam*) which confirm that specific products are amplified.

***loj* expression at ZT0 and ZT12**

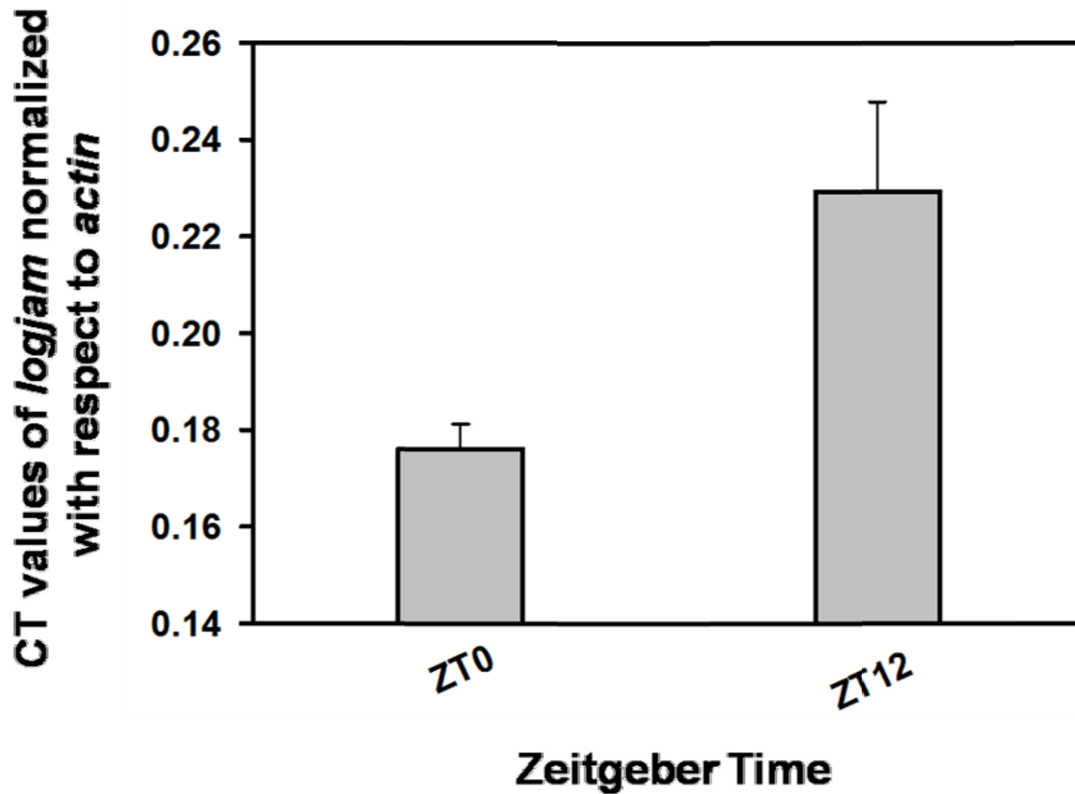


Fig. 3: Threshold cycle (CT) values of mRNA levels of *logjam* (*loj*) normalized with respect to *actin* at two time points - Zeitgeber Time (ZT0 and ZT12). Lower CT value of *loj* at ZT0 reflects that its expression levels are higher at ZT0 than at ZT12 ($p < 0.01$).

5.2 (e) Quantitative Real Time-PCR (qRT-PCR)

For relative quantitative detection of products (real-time PCR), cDNAs were amplified with primers directed to a region common to all known *loj* transcripts using ABI SYBR® green PCR master mix for quantitative real-time PCR. ABI PRISM 7700 sequence detection system (PE Biosystems) was used to detect the amplified products. The *ß-actin* gene which shows constitutive expression was used as an endogenous control to compare expression pattern of *loj* at two different time points (ZT0 and 12). Negative control for the reaction included the reactions that lacked cDNA template or primers as well as no reverse transcriptase enzymes to test for genomic DNA contamination. Results from the primer set (used for amplification of genes from cDNA sample) were used for statistical comparisons of the samples.

5.3 Results

5.3 (a) Expression level of *logjam* shows diurnal oscillation

The SYBR-Green I chemistry was used for qRT-PCR. The melt curve run in ABI Step One Plus showed fluorescence peak (-Rn) for *loj* at around 81.84 ˚C and for *actin* at 76.6 ˚C. The melting temperature of reaction product confirmed that the amplicons are of *loj*, thus confirming the specificity of *loj* primers and the robustness of our experiment (Fig 2).

ANOVA (Statistica™, 1995) was done for the obtained CT values of *loj* normalized by *actin*. Statistical analysis of normalized CT values of *loj* mRNA levels revealed that the mRNA expression levels of *loj* at ZT0 and ZT12 differ significantly ($F = 29.5$, $p < 0.01$, $df = 1$). The expression level of *loj* was significantly higher at

ZT0 than ZT12 (Fig 3).

5.4 Discussion

Since *loj* is an essential gene for oviposition in *D. melanogaster* we asked whether the expression levels of *loj* in ovaries oscillate in a circadian manner. The results reveal that mRNA levels of *loj* differ significantly in the ovaries between the two time points tested (ZT0 and 12); *loj* expression is significantly higher at ZT0 compared to ZT12 (Fig. 3). Previous studies have shown that, in *loj* loss of function mutant normal levels of egg laying can be restored when *loj* is expressed in the adult CNS using CNS specific GAL4 driver (Carney and Barbara, 2003). This suggests that a *loj* signal from the fly brain is sufficient to drive output for egg laying behavior. Studies have also shown that levels of *per* and *tim* mRNA and proteins do not oscillate in the fly ovaries, which suggests that *per* and *tim* genes may have a non-circadian role in ovaries (Plautz *et al*., 1997; Beaver *et al*., 2003). Further, ovarian TIM is not sensitive to light; this is thought to be due to the absence of circadian photoreceptor CRYPTOCHROME in the ovaries (Brandy *et al*., 2005). Studies have shown that the core clock genes *per* and *tim* are regulated differently in the follicle cells compared to the clock cells, and non-circadian regulation of these genes in ovaries control fitness related phenotypes in *Drosophila* (Beaver *et al.*, 2003). Though the transcript and protein levels of core clock genes do not oscillate in ovaries it is likely that their post-transcriptional/post-translational modifications might be under circadian control. This along with rhythmic modifications in their protein structure may affect their function in a circadian manner. Also, it is likely that clock genes in the ovaries may regulate egg laying rhythm by interacting with the genes that directly regulate oviposition

behavior. Therefore we hypothesize that circadian egg laying rhythm in *Drosophila* is governed by the following mechanisms: (i) involving post-transcriptional/post-translational modifications of core clock mRNA and proteins in ovaries, (ii) interaction of one or more core clock genes with *loj* or other similar genes, (iii) involving yet to be identified clock genes, (iv) involving a combination of some or all of the above processes.

Although oviposition rhythm peaks towards the end of light phase of the LD cycle (ZT10 to 14), we were surprised to find that mRNA levels of *loj* is low at ZT12 and high at ZT0. This suggests two possibilities, either (i) there is a significant delay in *loj* protein accumulation, or (ii) a delay due to post-transcriptional/post-translational processes. Further studies needs to be carried out to investigate whether protein levels of *loj* oscillate in a manner similar to its transcript, and maintain a near anti-phasic relationship with its transcript. Since flies with loss of function mutation for *loj* are sterile or lay very few eggs it is difficult to perform behavioral assays on such flies. An alternative approach would be to assay egg laying behavior in flies where molecular oscillation in *loj* is disrupted by over expression of *loj* in ovaries and adult CNS.

In conclusion, since core clock genes *per* and *tim* do not oscillate in ovaries, we hypothesize that novel circadian genes might govern the egg laying rhythm in *Drosophila* and *loj* could be a crucial part of such circadian machinery. *loj* expression in a subset of CNS and ovaries- may be regulated by sex-determining genes and that such a regulation could be masked by high levels of *loj* in tissues where *loj* is not controlled by such genes (Carney and Barbara, 2003). Although many sex-determination genes in *Drosophila* are expressed at early stages of development and do not have any role in female reproductive processes, *loj* turns out to be a crucial

gene in female reproductive process. While our study suggests some role for this gene in circadian egg laying rhythm in *Drosophila*, further studies should be carried out in a systematic manner to confirm this.

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