REVIEW ARTICLE

Egg-laying rhythm in *Drosophila melanogaster*

MANJUNATHA T., SHANTALA HARI DASS and VIJAY KUMAR SHARMA*

Chronobiology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P. O., Bangalore 560 064, India

Abstract

Extensive research has been carried out to understand how circadian clocks regulate various physiological processes in organisms. The discovery of clock genes and the molecular clockwork has helped researchers to understand the possible role of these genes in regulating various metabolic processes. In *Drosophila melanogaster*, many studies have shown that the basic architecture of circadian clocks is multi-oscillatory. In nature, different neuronal subgroups in the brain of *D. melanogaster* have been demonstrated to control different circadian behavioural rhythms or different aspects of the same circadian rhythm. Among the circadian phenomena that have been studied so far in *Drosophila*, the egg-laying rhythm is unique, and relatively less explored. Unlike most other circadian rhythms, the egg-laying rhythm is rhythmic under constant light conditions, and the endogenous or free-running period of the rhythm is greater than those of most other rhythms. Although the clock genes and neurons required for the persistence of adult emergence and activity/rest rhythms have been studied extensively, those underlying the circadian egg-laying rhythm still remain largely unknown. In this review, we discuss our current understanding of the circadian egg-laying rhythm in *D. melanogaster*, and the possible molecular and physiological mechanisms that control the rhythmic output of the egg-laying process.

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Introduction

Most organisms posses biological timers in the form of circadian clocks. Organisms track time in their local environment by entraining these clocks to abiotic and biotic environmental cycles. A large number of biological processes, both simple and complex, are oscillatory in nature, and occur with a 24 h periodicity, in harmony with the daily geo-physical cycle of day and night. These rhythms persist in organisms isolated from the environmental cycle (under constant laboratory conditions) with periodicities that are approximately 24 h. Hence, such oscillatory processes are termed circadian (*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).

*For correspondence. E-mail: vsharma@jncasr.ac.in.

The *Drosophila melanogaster* genome is large and complex, with 13,500 genes (Adams *et al*. 2000), yet the species is amenable to genetic manipulations of the molecular pathways that regulate biological processes. Due to this advantage, *D. melanogaster* has been extensively used in numerous studies for understanding the genetic and molecular underpinnings of circadian behaviours. In *Drosophila*, circadian rhythms in activity/rest (locomotor activity), adult emergence (eclosion), mating and egg-laying (oviposition) behaviours have been used as read-outs for parsing the complex circadian clockwork (Saunders *et al*. 2002). There is a large body of evidence to suggest that circadian clock architecture in *D. melanogaster* is multi-oscillatory; several behavioural and physiological rhythms are timed by separate sets of oscillators. Although the basic rhythm generating machinery is cellular, it involves an elaborate network of neurons. While some clarity exists regarding which clock genes and neurons govern circadian locomotor activity, olfactory and emergence rhythms in *D. melanogaster*, neurogenetic and molecular mechanisms that govern the egg-laying rhythm have

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thus far remained elusive. In this review, we discuss studies related to the egg-laying rhythm in *D. melanogaster* and the molecular, neuronal and hormonal mechanisms that may govern this rhythm.

The egg-laying rhythm in *D. melanogaster*

Egg-laying is a complex phenomenon, involving at least two separate physiological processes: vitellogenesis and egg-retention (Allemand 1976a,b). Periodic deposition of fertilized eggs involves a series of events starting from the production of oocytes to egg-laying on selected sites (Allemand 1976b; Yang *et al*. 2008). As in several other insect species, egg-laying behaviour is found to follow a 24 h pattern in fruit flies (Drosophilidae), including *D. melanogaster* (Rensing and Hardelande 1967; Gruwez *et al*. 1972; David and Fouillet 1973; Allemand 1976a,b, 1977; Sheeba *et al*. 2001a,b; Howlader *et al*. 2006) and related congeners (Allemand 1974), and in *Zaprionus* sp. (Allemand 1976c). The egg-laying rhythm in *D. melanogaster* has been shown to free-run with circadian periodicities under constant darkness (DD), thereby ascertaining its endogenous nature (Allemand 1976a,b, 1977; Sheeba *et al*. 2001b; Howlader *et al*. 2006). Although the rhythm follows circadian patterns, the periods range between 22 h and 30 h. 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 rhythm it is necessary to show that the period of the rhythm remains more or less unchanged with increase/decrease in temperature and nutrition, within a physiologically permissible range (Pittendrigh 1960). If the period of the rhythm does not show temperature-compensation 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 possibility was rigorously tested in a study by Howlader *et al*. (2006). In this study, periodicity of the egg-laying rhythm was estimated in several lines of *D. melanogaster* at three different temperatures (20, 24 and 28°C), and two levels of nutritional quality (high-protein and low-protein diets). The period of the egg-laying rhythm was relatively stable under different temperatures, and this was true for several genotypes. Further, the period of the rhythm in these lines also remained stable when assayed on protein diets with different yeast concentrations. This suggests that the egg-laying rhythm in *D. melanogaster* is indeed temperature-compensated and nutrition-compensated (Howlader *et al*. 2006).

Circadian clocks control a wide range of rhythmic physiological and metabolic processes in insects (Saunders *et al*. 2002). Some of these rhythms in turn induce rhythmicity in mating behaviour, certain aspects of gonadal maturation, oogenesis and oviposition (Saunders *et al*. 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 light:dark cycles of 12:12 h (LD 12:12) (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 DD were also found to exhibit a weak rhythm in egg-laying behaviour 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 behaviour appeared, which disappeared as soon as the mosquitoes were transferred to constant light (LL). The results of these and other similar studies (Gillett *et al*. 1961) indicate that egg-laying rhythm in mosquitoes are partially dependent upon external time cues, and that a trigger is required to set the circadian pacemaker in motion in aperiodic conditions (DD). Similarly, the egg-laying rhythm in the pink bollworm *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 h (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 h in DD, after an LL to DD transfer (Skopik and Takeda 1980). Although egg-laying rhythm have 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 the majority of these studies did not detect self-sustained egg-laying rhythm under constant conditions, and in many cases a trigger was required to reinstate the rhythm. There is often a large variation in the egg-laying patterns of individual insects; it is therefore 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 behaviour (Sheeba *et al*. 2001b). Thus, the absence of robust circadian rhythmicity in egg-laying behaviour under constant conditions (DD and LL) reported in a few previous studies may be an artefact of pooling data across groups of flies.

Although the egg-laying rhythm in *D. melanogaster* is of circadian nature, some of its characteristics are quite different from the two better characterized circadian rhythms in this species: the activity/rest and adult emergence rhythms. Under DD, the circadian period of egg-laying rhythm (27.66 ± 2.16 h; mean ± 95% CI) is significantly greater than those of locomotor activity (24.73 ± 0.29 h), and adult emergence (23.64 ± 0.00 h) rhythms in the same populations (Sheeba *et al*. 2001b). Even the limits of entrainment of the egg-laying rhythm were different from those of activity/rest and emergence rhythms (Paranjpe *et al*. 2004). Another striking differ-

ence between egg-laying and other rhythms is that egg-laying continues to be rhythmic under LL (Sheeba *et al*. 2001b), while activity/rest and emergence behaviours become arrhythmic (Saunders *et al*. 2002). These studies, thus, suggest that separate timing systems regulate egg-laying, activity/rest, and emergence rhythms in *D. melanogaster*. At the same time, it is has also been shown in a previous study that the circadian periods of egg-laying and activity/rest rhythms are positively correlated in the period (*per*) mutants of *D. melanogaster*, suggesting that some common mechanisms involving the *per* gene govern the two rhythms (McCabe and Birley 1998). The obvious corollary to this would be that the loss of function in per^0 flies should be able to unravel if pacemakers of egg-laying rhythm house mechanisms that are different than those governing activity/rest and adult emergence rhythms. However, results from the study of McCabe and Birley (1998) indicate otherwise.

At the molecular level, the circadian clockwork of *D. melanogaster* 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). Persistence of activity/rest and emergence rhythms in *Drosophila* requires the presence of 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 LN_v based circadian pacemakers are responsible for the regulation of the egg-laying rhythm.

While our understanding of the circadian egg-laying rhythm in *D. melanogaster* continues to increase (reviewed in Howlader and Sharma 2006), some key questions still remain. (i) Can the egg-laying rhythm be entrained by temperature cycles? (ii) Which is a stronger time-cue (*Zeitgeber*) for the egg-laying rhythm, LD cycles or temperature cycles? (iii) Does mating have any role in triggering the egg-laying rhythm? (iv) What are the key factors underlying the genesis of the rhythm, copulation, transfer of sex peptides, or transfer of sperms? (v) What is the genetic basis of the egg-laying rhythm? (vi) What is the neural basis of the egg-laying rhythm? (vii) Is the egg-laying rhythm regulated by hormones?

Entrainment of the egg-laying rhythm

An environmental cue that can synchronize circadian clocks is called a *Zeitgeber*. As a result of entrainment, the biological rhythm maintains an exactly 24 h periodicity, with a stable phase-relationship with the *Zeitgeber* (Sharma 2003; Dunlap and Loros 2004; Sharma and Chandrashekaran 2005). Studies have shown that LD cycles can entrain the egg-laying rhythm of flies, although the percentage entrainment is low in most laboratory strains. Canton S flies show weak entrainment (~25%) for the egg-laying rhythm (Howlader *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 lack of rhythmic expression of TIM in the ovaries. Allemand (1976a,b) has shown that under LD 12:12 egg-laying is rhythmic, with a prominent peak at the beginning of the dark phase. Also, the number of mature oocytes in ovarian egg chambers during the early and late vitellogenesis is rhythmic under LD 12:12, although this rhythm disappeared when the flies were 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, with peak oviposition coinciding with lights-off (Sheeba *et al*. 2001b). Egg-laying rhythm of only ~25% of the flies entrained to LD 10:10, while the percentage was higher under LD 12:12 (~40%), and LD 14:14 (~75%), suggesting that egg-laying rhythm entrains better to longer day lengths (Paranjpe *et al*. 2004). Interestingly, in a separate study Sheeba *et al*. (2001a) showed that the egg-laying rhythm of flies emerging at different times of the day have different circadian period. Flies emerging in the evening maintain a significantly different phase-relationship with the LD cycles ($\sim$ +4.00 h as opposed to $\sim$ +1.50 h in those emerging in the morning).

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

The sexually dimorphic behaviours associated with *Drosophila* reproductive success are clearly governed by the actions of multiple genes (Karr and Pitnick 1996). The major reproductive behaviours of females are (i) receptivity to courtship followed by copulation, and (ii) deposition of eggs, a behaviour that is partially independent of the act of mating and is under voluntary control of the female. Females become receptive to courting males at about 8–12 h after emergence (Karr and Pitnick 1996). Males perform a sequence of behavioral patterns to court females. First, they orient themselves while 'singing' 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 and Pitnick 1996). Females can reject males by moving away and extruding their ovipositor. The average duration of successful copulation is $\sim$30 min, during which males transfer a few hundred very long (1.76 mm) sperm cells in seminal fluid to the female. Females store sperms to fertilize eggs (Karr and Pitnick 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; Tompkins *et al*. 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 behaviour 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 *et al*. 1997; Heifetz *et al*. 2000, 2001; Chapman *et al*. 2001; Fleischmann *et al*. 2001; Wolfner 2002; Saudan *et al*. 2002). Mated females have been observed to retain eggs if they do not find ideal oviposition substrates (Grossfield 1978). These post-mating responses were shown to be induced by factors synthesized in the reproductive tract of the adult male and transferred through the seminal fluid into the female during copulation. One of these factors, named accessory gland peptide 70A (sex-peptide or SP), has been identified in *D. melanogaster*. 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 *et al*. 2000). Target sites for SP have been identified in female genital tract, corpus allatum (CA) and antennal region in the brain (Ding *et al*. 2003). The *logjam* (*loj*) gene is one of the few genes known to control female postmating behaviours (Carney and Taylor 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 Taylor 2003). It is likely that SP after binding to CA triggers the expression of *loj*, which is essential for oviposition in flies, and temporal profiling of *loj* could reveal whether rhythmic expression of this gene (if it is oscillatory) is crucial for the regulation of egg-laying rhythm.

What is the molecular basis of egg-laying rhythm?

In *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). The 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 (reviewed by Hardin 2005). A second feedback loop, which involves two transcription factors VRI and PDP1, regulates 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 points towards post-translational modification of clock proteins (Edery 1999; Akten *et al*. 2003).

Two kinases, DOUBLETIME (DBT) and casein kinase Ie (CKIε) 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 the 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. 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 photo-entrainment 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 clock neurons serves as an output signal for the downstream targets that are responsible for the regulation of various rhythmic behaviours (Stanewsky 2002).

Interestingly, 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 *D. melanogaster* (Beaver *et al*. 2003). This suggests that the circadian egg-laying rhythm in *D. melanogaster* is likely to be governed by novel molecular mechanisms involving genes that have not yet been implicated in the 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 discovered is what mechanisms generate rhythmic signals for egg-laying? Interestingly, ectopic expression of CRY in the ovaries results in circadian oscillation of the *per* and *tim* genes in the negative feedback loop of the molecular clock (Rush *et al*. 2006). Other studies have also shown that loss of function *per* and *tim* mutant females exhibit reduced reproductive output (Beaver *et al*. 2003). Therefore, it is likely that *per* and *tim* genes play a non-circadian role in the *D. melanogaster* ovary.

What is the role of *loj* in the regulation of the egg-laying rhythm?

The postmating 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 and Wolfner 1988; Aigaki *et al*. 1991; Kubli 1992; Herndon and Wolfner 1995; Wolfner 1997; Wolfner *et al*. 1997; Heifetz *et al*. 2000; Chapman *et al*. 2001; Heifetz *et al*. 2001; Fleischmann *et al*. 2001; Saudan *et al*. 2002). Carney and Taylor (2003) observed that mature eggs were lodged inside the female genital tract, particularly in the uterus of the *loj* mutant flies. Further, the egg-laying deficit seen in *loj* mutants is 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 hypothesize that *loj*, in addition to shuttling neurotransmitters to their release sites, might have a function similar to 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 *loj* appears to be responsible for egg-laying behaviour, it is likely that (i) it may be rhythmically expressed in the ovaries and/or brain; (ii) its post-transcriptionally or post-translationally modified in a rhythmic manner, (iii) it interacts with known circadian genes/proteins, and (iv) it is light sensitive. Future studies on the above aspects would help resolve the many questions regarding this unique circadian rhythm.

Previous studies have also shown that the brain and ventral nerve chord (VNC) are important centres for processing gustatory, olfactory, and visual inputs and transforming this information into an appropriate behavioural outcome such as oviposition (Szabad and Fajszi 1982). By exploiting genetically mosaic flies, egg-laying behaviour was mapped to the thorax (Szabad and Fajszi 1982) which contains the VNC. Further, Carney and Taylor (2003) determined that a number of cells in the thoracic and abdominal ganglia of the VNC express *loj*. The brain also has a role to play in the regulation of egg-laying, since decapitated or anaesthetized *D. melanogaster* females lay eggs merely as a reflex response (Grossfield 1978). This makes VNC an obvious target for the study of circadian pacemakers regulating the egg-laying rhythm.

The expression pattern of *loj* in adults involves its expression in mid-stage vitellogenic egg chambers (Carney and Taylor 2003). The majority of the signal in these chambers is found in follicle cells which provide nutrients and other components necessary for oocyte development, and produce the outer coverings of the egg: the vitelline membrane and the chorion (Mahowald and Kambysellis 1980; Lin and Spradling 1993). Other studies suggest that the *loj* positive egg cells provide cues to the female 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 mature eggs are found in the uterus of essentially every mutant female, it appears that initial egg release is not affected. However, Carney and Taylor (2003) observed partially ovulated eggs in the upper portions of the lateral oviducts as well as multiple eggs in portions of the genital tract. It was suggested that ovulation initially proceeds normally in *loj* females but the presence of unlaid 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 behaviour.

What is the physiological basis of the egg-laying rhythm?

The neuronal architecture underlying circadian rhythms in *D. melanogaster* has been extensively studied for several decades (for review see Sheeba *et al*. 2008). The core pacemaker for the activity/rest rhythm has been localized in the lateral ventral neurons (LN_v). 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 the activity/rest and emergence rhythms (Ewer *et al*. 1992; Myers *et al*. 2003). However, the neural network underlying the egg-laying rhythm in *D. melanogaster* is yet to be unravelled. In 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 behaviour (Thompson and Roosevelt 1998). This suggests that some egg-laying-related genes start functioning quite early during development. Once the appropriate circuitry is established, signalling pathways should be able to initiate and sustain egg-laying behaviour 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 interneurons in the brain, including the subesophageal ganglion (Thompson 1986a). Local circuit interneurons in the posterior abdominal ganglion, and sensory inputs from neurons in the ovaries and internal reproductive tract, are also expected to function in activating and modulating egg-laying behaviour (Thompson 1986b). This circuit should be extensively probed, as it is likely that the egg-laying rhythm is governed by the neural network that is involved in regulating the female reproductive system as a whole. Once the neural circuit is localized, the next question would be to study

what kind of mechanisms these pacemakers use to regulate egg-laying behaviour.

A recent study by Howlader *et al*. (2006) has demonstrated that the circadian egg-laying rhythm persists in flies with ablated LN_v neurons. It was also shown that PDF-mediated signalling is not required for the persistence of this rhythm in DD. These results together suggest that the LN_v neurons are dispensable for the egg-laying rhythm. However, LN_v ablated flies invariably showed a significantly different periodicity in the egg-laying rhythm compared to their wild-type counterparts, which suggests that although LN_v neurons are not critical for the persistence of the egg-laying rhythm under DD, they may continue to influence the circadian period through as yet unknown mechanisms. Further, the egg-laying behaviour of pdf^{01} and *disconnected* ($disco^1$) mutant flies was also studied, and 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), whereas in the $disco^1$ mutants the clock's neural connections are impaired, and the LN_v neurons that are left behind lack PER and PDF (Blanchardon *et al*. 2001). Therefore, it was concluded that the circadian egg-laying rhythm in *D. melanogaster* is not under the control by the circadian neuronal circuitry that governs other well-studied circadian rhythms (Howlader and Sharma 2006).

Is the egg-laying rhythm regulated by hormones?

It is likely that reproductive hormones themselves are central to the regulation of egg-laying rhythm in *D. melanogaster* (Howlader and Sharma 2006). Juvenile hormone (JH) and 20-hydroxy ecdysone (20HE) are hormones with known gonadotropic functions (Riddiford 2008; Gruntenko and Rauschenbach 2008), as well as a major role in development (Riddiford 1993). The CA cells of the brain secrete JH, while 20HE, the major moulting hormone, is secreted by the prothoracic gland. The SP in the seminal fluid has been shown to activate JH synthesis in CA cells (Moshitzky *et al*. 1996). Proteins that are involved in signal transduction of 20HE and JH interact with each other, thus mediating communication between these hormones (Bitra and Palli 2008). A balance between the 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*. 2003). An increase in JH titre leads to oviposition arrest, whereas increased 20HE titres cause degradation of vitellogenic oocytes (Gruntenko and Rauschenbach 2008). JH also plays a key role in regulating egg-laying behaviour under adverse condition such as starvation and heat-stress (Raushenbach *et al*. 2004; 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 behaviour via polyamine metabolism in crickets. When anitsera against FMRFamide was injected into mated *Rhodinus prolixus* females, a delay in oviposition was observed (Sevala *et al*. 1992). Based on the above studies, we postulate that in *D. melanogaster* too, JH might mediate egg-laying rhythm via downstream amide/amine components. Since JH-analogues do not elicit increased oviposition and reduced receptivity, SP must have an additional, separate effect on these two post-mating responses (Soller *et al*. 1999). Further, application of the JH analogue methoprene is found to mimic the SP-mediated stimulation of vitellogenic oocyte progression in sexually mature virgin females (Soller *et al*. 1999). 20HE is known to deter oviposition, and females avoid laying eggs in the presence of 20HE (Calas *et al*. 2006, 2007). Apoptosis is induced by 20HE in nurse cells of egg chambers at physiological concentrations (10^{-7} M) (Soller *et al*. 1999). 20HE thus acts as an antagonist of early vitellogenic oocyte development. However, simultaneous application of JH analogue protects early vitellogenic oocytes from 20HE-induced resorption. These results suggest that a fine balance between these hormones in the hemolymph determines whether oocytes will mature or undergo apoptosis.

Oviduct contraction is an essential step in the process of egg-laying behaviour (Rodríguez-Valentin *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-Valentin *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-Valentin *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 also 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 *D. melanogaster* sleep (Hildebrandt and Muller 1995; Huang *et al*. 2007). However, the effect of PKA was not exerted in the mushroom body, 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 behaviour. The ability of octopamine to regulate oviduct contraction and function as a wake promoting signal suggests that it may play a role in the regulation of the egg-laying rhythm in *D. melanogaster* (figure 1).

Possible mechanisms underlying the egg-laying rhythm

The egg-laying rhythm is unique among the rhythmic

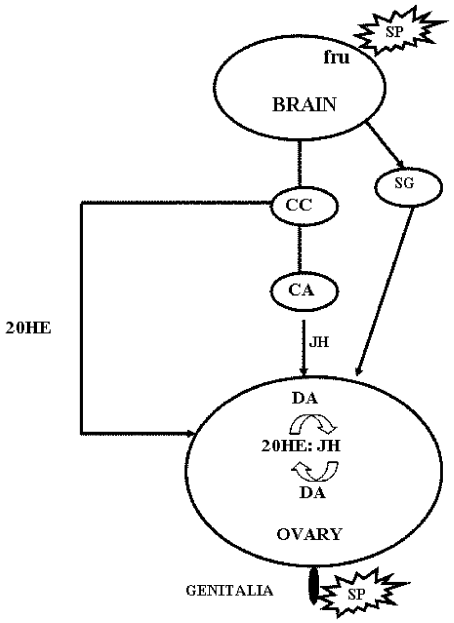

Figure 1. Hormonal regulation of egg-laying behaviour in *D. melanogaster*. Sex peptide (SP) binds to the *fruitless* neurons in the female brain. An unknown signal stimulates the corpus allata (CA) to secrete juvenile hormone (JH). 20 Hydroxyecdysone (20HE) is secreted by neurosecretory cells and is stored in the corpus cardiaca (CC). The balance between 20HE and JH is critical for driving oviposition behaviour. The balance is mediated by dopamine (DA). SP 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).

behaviours exhibited by *D. melanogaster* because: (i) it is rhythmic under LL, and (ii) does not require LN_v for its persistence under DD. The scenario is further complicated by a large number of regulatory mechanisms such as neuronal, hormonal, genetic, nutritional and temperature signals. Therefore, it is not surprising that the mechanisms underlying the egg-laying rhythm still remain elusive. Based on some recent studies of egg-laying behaviour, we propose a model that encompasses all the known components underlying egg-laying rhythm in *D. melanogaster* (figure 2).

SP 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 may 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 *D. melanogaster*, one would expect egg-laying behaviour to be rhythmic, almost mimicking the *loj* expression profile. Alternatively, given that egg-laying behaviour 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 rhythm, 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 which regulates activity/rest and emergence rhythms, do not play any role in the maintenance of egg-laying rhythm, and that this rhythm may be governed by molecular mechanisms involving a novel set of clock genes. There is also sufficient evidence to suggest that LN_v based circadian pacemakers do not regulate the persistence of circadian egg-laying rhythm in *D. melanogaster*, and that some yet to be 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

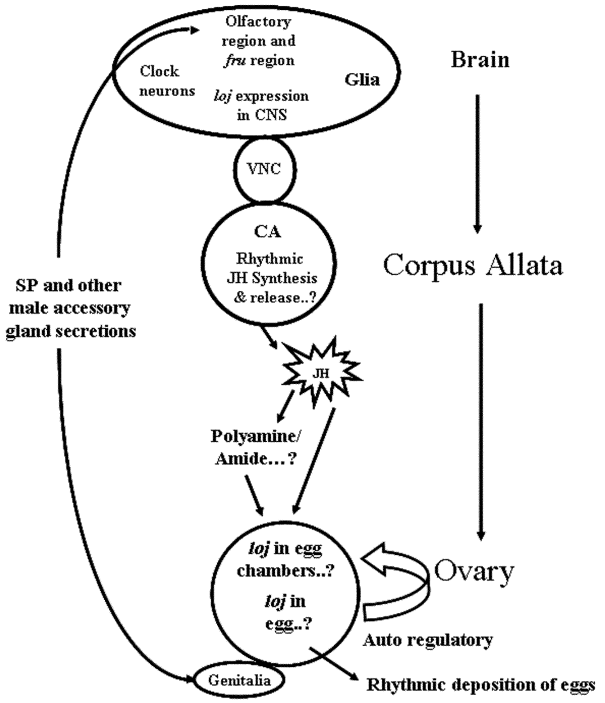

Figure 2. Possible mechanisms underlying the egg-laying rhythm in *D. melanogaster*. Sex peptide (SP) and other accessory gland secretions such as ductus ejaculatory peptide have target sites in the *fruitless*/olfactory regions in the female brain. Upon binding, via downstream signals (yet unknown), they activate juvenile hormone (JH) synthesis in the corpus allata (CA). There are two possible downstream processes, one direct and the other via polyamines/amides. 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 rhythm.

small LN_v, or the antennal neurons (AN). Finally, it is not entirely unlikely that all or some of the above processes may govern egg-laying rhythm in *D. melanogaster* in a concerted manner.

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