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Multi-Oscillatory Control of Eclosion and Oviposition Rhythms in *Drosophila melanogaster*: Evidence from Limits of Entrainment Studies

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ABSTRACT

The eclosion and oviposition rhythms of flies from a population of *Drosophila melanogaster* maintained under constant conditions of the laboratory were assayed under constant light (LL), constant darkness (DD), and light/dark (LD) cycles of 10:10 h (T_{20}), 12:12 h (T_{24}), and 14:14 h (T_{28}). The mean ($\pm 95\%$ confidence interval; CI) free-running period (τ) of the oviposition rhythm was 26.34 ± 1.04 h and 24.50 ± 1.77 h in DD and LL, respectively. The eclosion rhythm showed a τ of 23.33 ± 0.63 h (mean $\pm 95\%$ CI) in DD, and eclosion was not rhythmic in LL. The τ of the oviposition rhythm in DD was significantly greater than that of the eclosion rhythm. The eclosion rhythm of all 10 replicate vials entrained to the three periodic light regimes, T_{20} , T_{24} , and T_{28} , whereas the oviposition rhythm of only about 24 and 41% of the individuals entrained to T_{20} and T_{24} regimes, respectively, while about 74% of the individuals assayed in T_{28} regimes showed entrainment. Our results thus clearly indicate that the τ

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and the limits of entrainment of eclosion rhythm are different from those of the oviposition rhythm, and hence this reinforces the view that separate oscillators may regulate these two rhythms in *D. melanogaster*.

Key Words: Circadian rhythm; Oviposition; Eclosion; *Drosophila melanogaster*; Limits of entrainment; Multiple oscillators.

INTRODUCTION

Circadian rhythms have been documented in a wide range of behavioral, physiological, and molecular processes in a variety of organisms including bacteria, fungi, plants, insects, birds, and mammals (reviewed in Zordan et al., 2000). Recent studies in many different organisms have shown that circadian timing systems are likely to be far more complex than thought earlier (Preitner et al., 2002). For example, there is now evidence that light input pathways for photoentrainment of circadian clocks may be a part of the central clock or under clock control (Emery et al., 1998), and overt rhythms may feed back on circadian clocks (Mrosovsky et al., 1992). Moreover, the circadian clock itself might be composed of several oscillators, such as putative “morning” and “evening” oscillators (Daan et al., 2001; Helfrich-Förster, 2000; Iglesia et al., 2000; Jagota et al., 2000; Pittendrigh, 1981) or separate master and slave oscillators (Pittendrigh and Daan, 1976). There is also evidence suggesting that circadian timing systems may consist of multiple oscillators, each controlling a different rhythm (Aschoff et al., 1967; Ebihara and Gwinner, 1992; Engelmann and Mack, 1978; Helfrich and Engelmann, 1987; Roenneberg, 1996; Sheeba et al., 2001a). In the alga *Gonyaulax polyedra*, two oscillatory subsystems with red/blue sensitive and blue sensitive photoreceptors control cell aggregation and bioluminescence and together fulfill the criterion for a true self-sustained circadian oscillator (reviewed in Roenneberg, 1996). In the moth *Pectinophora gossypiella*, egg-hatching, eclosion, and oviposition rhythms are controlled by separate oscillators (Pittendrigh and Minis, 1971), and three separate oscillators are believed to regulate the initiation of larval wandering, diapause induction and eclosion rhythms in the fleshfly *Sarcophaga argyrostoma* (Saunders, 1986).

Indications of multiple oscillators controlling different circadian rhythms have also come from studies on fruit flies of the genus *Drosophila*. The light pulse phase response curve (PRC) and the free-running period (τ) of eclosion and locomotor rhythms in *Drosophila pseudoobscura* were observed to differ (Engelmann and Mack, 1978). Similarly in *D. melanogaster*, eclosion, locomotor activity, and oviposition rhythms had significantly different τ , and the peak of oviposition and eclosion, as well as the onset of locomotor activity, occurred at different times under 24 h (12:12 h) light/dark (LD) cycles (Sheeba et al., 2001a). A parallel line of evidence supporting multi-oscillator organization for circadian clocks comes from studies on mutant *D. melanogaster* lines. In *ebony* mutants, adult locomotor activity was arrhythmic, whereas eclosion was rhythmic (Newby and Jackson, 1991). In *lark* mutants eclosion was arrhythmic but the locomotor activity rhythm was normal (Newby and Jackson, 1993). Though the observation of τ differences among rhythms (Engelmann and Mack, 1978; Sheeba et al., 2001a) strongly suggests the involvement



of different oscillators in governing various overt rhythms, evidence from studies on the phase relationship among rhythms (ψ) (Sheeba et al., 2001a), and the *ebony* and *lark* mutants (Newby and Jackson, 1991, 1993) suggests differences in output pathways among rhythms controlled by the same oscillator.

A supporting line of evidence for separate oscillators governing different rhythms could in principle come from studies on the limits of entrainment of various circadian rhythms. Circadian clocks show a limited range of environmental periods to which they can entrain; this range constitutes the limits of entrainment. Therefore, a Zeitgeber (time-cue) with a period very different from τ of a particular circadian rhythm may not be able to cause entrainment (Moore-Ede et al., 1982). Circadian rhythms that show different limits of entrainment are likely to be governed by separate oscillators. In this article, we report the results of a study in which the limits of entrainment for eclosion and oviposition rhythms were assessed in a population of *D. melanogaster* by examining entrainment to LD regimes of different period lengths.

MATERIALS AND METHODS

The experimental flies were sampled from a large ($N \sim 1200$ breeding adults), outbred, laboratory population (LL-1) of *D. melanogaster* that was reared in the laboratory under constant light (intensity approximately 100 lux, constant temperature of 25°C ($\pm 1^\circ\text{C}$) and constant high humidity, at moderate larval and adult densities, on a 21-day discrete generation cycle. The origin and maintenance of these populations has been described in detail in Sheeba et al. (2000).

The eclosion rhythm was assayed by collecting eggs from the running culture and dispensing them into 50 glass vials (25 mm height, 9 mm diameter), containing about 6 mL of food medium, at a high density of ~ 350 eggs per vial. At such high density, the pre-adult development is staggered, and we could get a sufficient number of flies eclosing for more than 10 cycles. Ten vials each were subjected to the following regimes: constant light (LL), constant darkness (DD), and light/dark (LD) cycles of 10:10 h (T_{20}), 12:12 h (T_{24}), and 14:14 h (T_{28}). The intensity of light used in the LL regime and during the light phase of LD cycles was about 100 lux. Red light of wavelength greater than 650 nm was used for handling vials and observations in dark. When adults began to eclose, the vials were inspected every 2 h. Eclosing adults were collected, and the number of female flies in each vial eclosing during the preceding 2 h period was recorded. This process was continued for 10 consecutive days, or until most pupae had eclosed. The final sample size in each light regime was 10 vials.

Oviposition rhythm of females was assayed by collecting flies that emerged from low-density cultures derived from the running culture during the peak of eclosion and by transferring male–female pairs into vials containing ~ 3 mL food medium. Twenty such pairs were subjected to each of the five light regimes (LL, DD, T_{20} , T_{24} , and T_{28}). The flies were transferred to fresh food medium every 4 h and the number of eggs laid in each vial over the preceding 4 h period were recorded. This procedure was continued for 10 consecutive cycles in each light regime. In case of death or escape of males, they were replaced using flies from a mixed sex cohort



maintained as backups in all five light regimes. Only data from females living for the full 10 days were used for analysis, yielding final sample sizes of 18 or 19 females in all light regimes.

The time series data obtained for oviposition by individual females, and eclosion for individual vials, were subjected to Fourier spectral analysis using STATISTICA™ (Statsoft, 1995). Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). In the different light regimes, DD, LL, T_{20} , T_{24} , and T_{28} , the fraction of females or vials showing clear-cut rhythmicity, as well as the fraction of rhythmic females or vials that actually entrained to the imposed LD cycles, was estimated.

RESULTS

The mean ($\pm 95\%$ confidence interval; CI) τ of the eclosion rhythm was 23.33 ± 0.63 h in DD. Eclosion was not rhythmic in LL. In DD, 7 out of 10 vials showed a significant circadian rhythm. The frequency distribution of τ observed in DD for the eclosion rhythm is shown in Figure 1. Representative patterns of eclosion in DD are shown in Figure 2. In all 10 vials assayed in each periodic regime (T_{20} , T_{24} , and T_{28}), the eclosion rhythm entrained to the imposed periodicity, indicating that the limits of entrainment for eclosion rhythm in this population of *D. melanogaster* extends at least from 20 to 28 h (Figure 3). The peak of the eclosion rhythm occurred 5.38 and 2.82 h after dark to light transition under T_{20} and T_{24} regimes, whereas it occurred 3.29 h before dark to light transition in T_{28} regime (Figure 4). Representative patterns of eclosion rhythm in T_{20} , T_{24} , and T_{28} regimes are shown in Figure 4.

The oviposition rhythm showed a mean ($\pm 95\%$ CI) τ of 26.34 ± 1.04 h in DD and 24.55 ± 1.77 h in LL. The τ of the oviposition rhythm in DD did not differ significantly from that in LL ($p > 0.05$). However, τ of the oviposition rhythm in DD was significantly greater than τ of the eclosion rhythm in DD ($p < 0.001$). In LL and DD, the number of flies that exhibited circadian rhythm of oviposition were 14 out of 19 and 13 out of 18, respectively. The frequency distribution of τ for the oviposition rhythm in LL and DD is shown in Figure 1. Representative patterns of oviposition rhythms in LL and DD are shown in Figure 2. The number of flies exhibiting rhythmic oviposition, and among them, those that entrained to the imposed LD cycles, varied substantially among regimes. In a T_{20} regime, 17 out of 18 females showed rhythmic oviposition, but only 4 females out of the 17 rhythmic individuals entrained to the imposed periodicity of 20 h (Figure 3). In a T_{24} regime, 17 out of 19 females exhibited rhythmic oviposition, and 7 out of the 17 rhythmic females entrained (Figure 3). In a T_{28} regime, oviposition of 19 out of 20 females was rhythmic, and 14 out of the 19 rhythmic females showed entrainment (Figure 3). This suggests that the oviposition rhythm of majority of individuals entrain to T_{28} regime, indicating that the limits of entrainment for oviposition rhythm are closer to 28 h (Figures 5–7). The peak of the oviposition rhythm occurred around the light to dark transition under T_{24} and T_{28} regimes (Figures 6 and 7). Representative patterns of oviposition rhythm in T_{20} , T_{24} , and T_{28} regimes are shown in Figures 5–7, respectively.



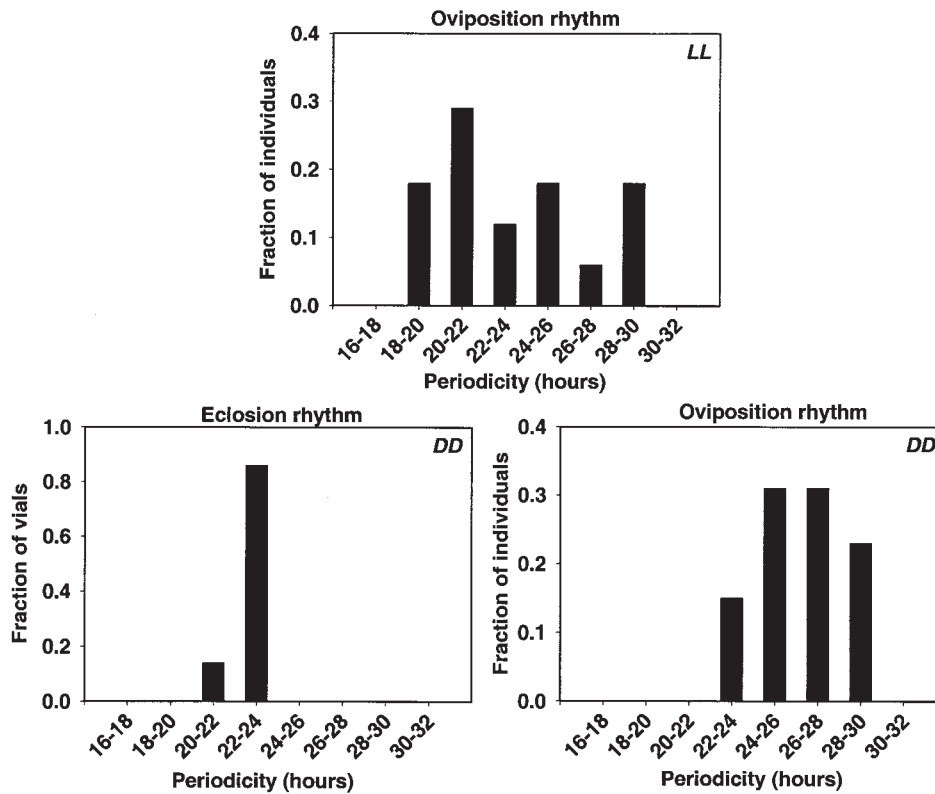


Figure 1. The upper panel represents the frequency distributions of τ values of oviposition rhythms in LL regime. The y-axis indicates the number of individuals exhibiting a certain periodicity, and the x-axis indicates the range of periodicities. The lower panel represents the frequency distributions of τ values of eclosion and oviposition rhythms in DD. The y-axis indicates the number of vials or individuals having a certain periodicity, and the x-axis indicates the range of periodicities.

DISCUSSION

The limits of entrainment for a circadian clock depend upon the τ and the amplitude of the light pulse PRC, both of which are thought to reflect the properties of the circadian clock (Pittendrigh, 1981). The results of our experiments suggest that the limits of entrainment for the eclosion rhythm in *D. melanogaster* are different from those for the oviposition rhythm. The limits of entrainment for the eclosion rhythm in *D. pseudoobscura* are known to be quite large, from 18.5 to 28.5 h (Pittendrigh, 1966). In a separate study, a large range of entrainment was also reported for locomotor activity rhythm of *D. melanogaster* (Wheeler et al., 1993). In our study, too, the range of entrainment for the eclosion rhythm in *D. melanogaster* extends at least between 20 and 28 h.



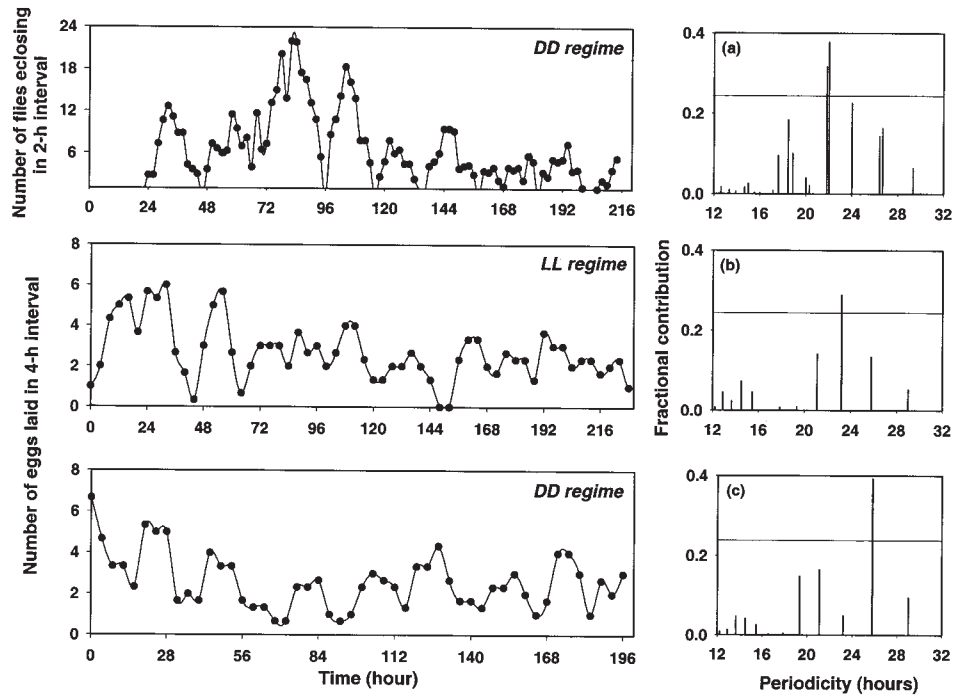


Figure 2. The upper panel represents time series data of eclosion in DD. The x -axis indicates time in hours and the y -axis indicates the number of flies eclosing during a 2 h interval. The corresponding periodogram (panel a) shows a significant contribution of a 23 h periodicity. The middle panel represents time series data of oviposition in LL. The x -axis indicates time in hours and the y -axis indicates the number of eggs laid during a 4 h interval. The corresponding periodogram (panel b) shows significant contribution of 24 h periodicity. The lower panel represents the time series data of oviposition in DD. The x -axis indicates time in hours, and the y -axis indicates the number of eggs laid during a 4 h interval. The corresponding periodogram (panel c) shows a significant contribution of a 26.8 h periodicity.

Earlier studies on the oviposition rhythm of wild type *D. melanogaster* reported a mean \pm SD τ value of 24.3 ± 2.7 h (McCabe and Birley, 1998), which is quite different from that we report in this article (26.34 ± 1.04 h). This could be due to the differences in the rearing conditions of the flies used in both studies or due to the fact that we used individual flies whereas McCabe and Birley used groups of flies. Such differences in τ may also arise due to pooling of data, because it is known that besides behavioral and social influences, mere pooling of individual oviposition data modifies the τ values strongly (Sheeba et al., 2001b).

The oviposition rhythm of a greater proportion of flies entrained to $T28$ as compared to $T20$ and $T24$ regimes, which suggests that the range of entrainment for the oviposition rhythm is closer to 28 h. Furthermore, it appears that the closer the average τ of the oviposition rhythm to the periodicity of the LD cycle, the larger



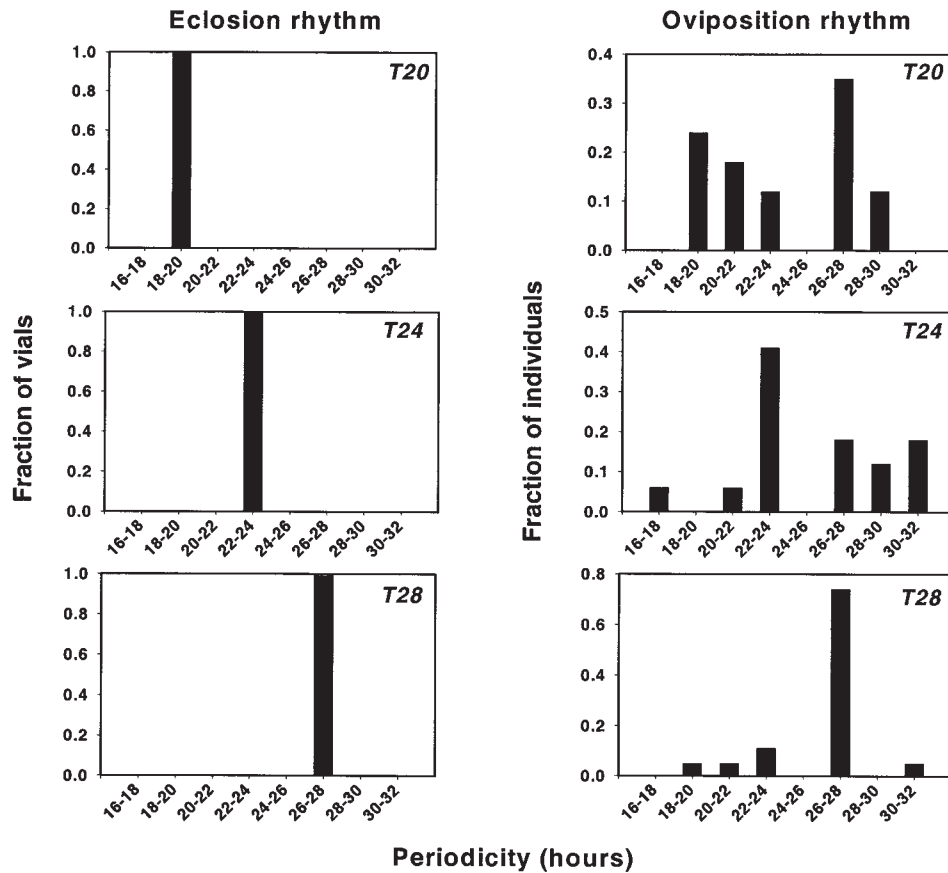


Figure 3. Left-side panels represent the frequency distribution of period values of the eclosion rhythm in T_{20} , T_{24} , and T_{28} regimes. The y -axis indicates the number of vials having a certain period and x -axis indicates the range of periodicities. The right-side panels represent the frequency distribution of the period values of the oviposition rhythm in T_{20} , T_{24} , and T_{28} regimes. The y -axis indicates the number of individuals having a certain period, and x -axis indicates the range of periodicities.

is the fraction of flies showing entrainment. The results of our experiments are also in accordance with the finding that, in a population ancestral to the one used in the present study, a majority of the individuals assayed showed rhythmic oviposition in LL, DD, and LD 12:12 h regimes (Sheeba et al., 2001b).

The differences in τ and limits of entrainment for the eclosion and oviposition rhythms observed in the present study support the view that different oscillators may regulate eclosion and oviposition rhythms in *D. melanogaster*. However, rhythms with different limits of entrainment can only be judged to be governed by different oscillators if the strength of the Zeitgeber is similar for both rhythms. Although we assayed both rhythms under similar Zeitgeber conditions, differences in light



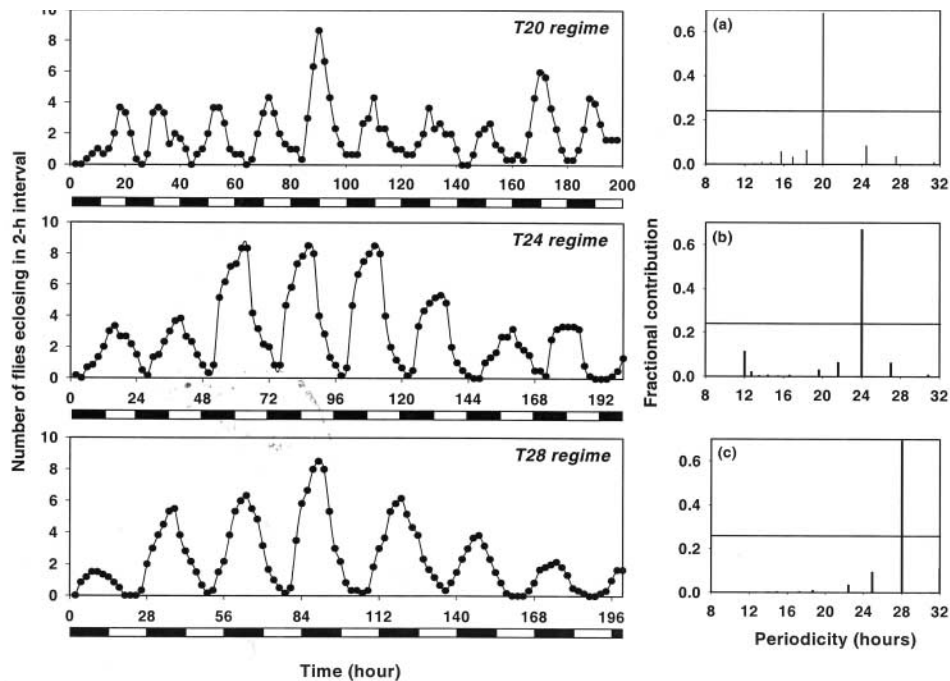


Figure 4. Time series data of eclosion in three representative vials in T_{20} , T_{24} , and T_{28} regimes, respectively. The dark horizontal bars below the x -axis indicate duration of darkness, and the hollow bars indicate duration of light in the light/dark cycle. The corresponding periodograms (a, b, c) show significant contributions of 20, 24, and 28 h periodicities.

sensitivity of the pupal and adult circadian systems cannot be ruled out completely. Further, eclosion and oviposition rhythms occur sequentially and not concurrently, and therefore, it cannot be argued that the same circadian clock controls both eclosion and oviposition rhythms in *Drosophila*. Perhaps the circadian clock that controls the eclosion rhythm during the pupal stage undergoes developmental changes resulting in small differences in circadian parameters in the adults. However, it is now established that a subset of ventral lateral neurons (LNvs) determines pupal eclosion rhythm, whereas all LNs plus some glial cells control adult activity rhythm (Helfrich-Förster, 1996). More recent evidence in *D. melanogaster* has further established that the prothoracic gland (PG) contains functional clocks which time eclosion and that these clocks together with the ones located in (LNvs) are necessary for the overt expression of circadian eclosion rhythm (Myers et al., 2003). Therefore, eclosion and locomotor activity rhythms in *Drosophila* are under the control of separate oscillators, if not clocks, and are not manifestations of the same oscillator undergoing developmental changes. Although we do not have similar evidence for the oviposition rhythm in the light of the results of our previous study (Sheeba et al., 2001a), the results of our experiments suggest that eclosion, locomotor activity, and oviposition rhythms in *D. melanogaster* are controlled by separate oscillators. Further, the *per* mRNA (Hardin, 1994; Liu et al., 1988) and its protein (Saez and



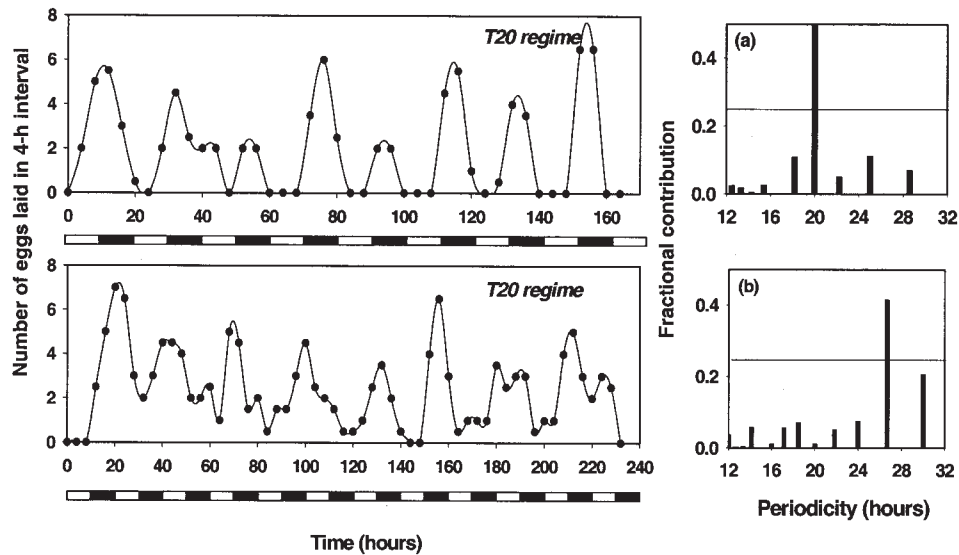


Figure 5. Upper panel shows time series data of a representative female for which the oviposition rhythm was entrained to a T_{20} regime. The corresponding periodogram (a) shows a significant contribution of the 20 h periodicity. Lower panel shows time series data of a representative female whose oviposition rhythm did not entrain to T_{20} regime. The corresponding periodogram (b) shows a significant contribution of a 26.67 h periodicity. The dark horizontal bars below the x -axis indicate duration of darkness, and the hollow bars indicate duration of light.

Young, 1988) did not display circadian oscillation in the ovary, which suggests that the core clock mechanism may not be involved in regulating oviposition rhythm in *D. melanogaster*. Since oviposition in our flies is rhythmic under LL, whereas most other circadian rhythms are not, one may also argue that mechanisms that control oviposition rhythm may be refractory to light. Our data clearly do not support this possibility as we demonstrate that the oviposition rhythm in a sizeable percentage of our flies does entrain to T_{20} , T_{24} , and T_{28} regimes. Further, the fact that oviposition rhythm persists even under LL, while both eclosion and locomotor activity rhythms are abolished, suggests that the oviposition rhythm may be under the control of mechanisms that are capable of generating rhythmic signals independent of the core clock genes such as *per* and *tim*. This seems to be the case, since we found in a preliminary study that the females of the *period* (per^0) and the *timeless* mutants (tim^{01}) of *D. melanogaster* show robust rhythmicity of oviposition (Gitanjali Howlader, Dhanashree Paranjpe, Vijay Kumar Sharma, unpublished data).

On the other hand, a study of oviposition and locomotor activity rhythms of four *period* mutants in *D. melanogaster* (per^s , per^0 , per^L) and per^+ revealed a significantly positive correlation between the τ values of the two rhythms, suggesting that both rhythms were under the control of a common underlying mechanism



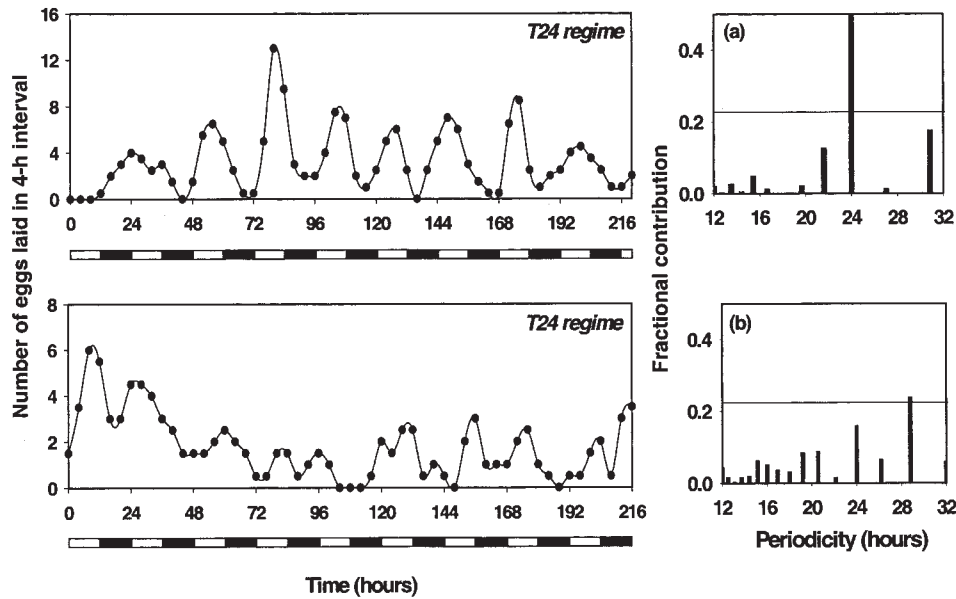


Figure 6. Upper panel shows time series data of a representative female for which the oviposition rhythm was entrained to a T_{24} regime. The corresponding periodogram (a) shows a significant contribution of 24 h periodicity. Lower panel shows time series data of a representative female whose oviposition rhythm did not entrain to T_{24} regime. The corresponding periodogram (b) shows a significant contribution of 30 h periodicity. The dark horizontal bars below the x-axis indicate duration of darkness and the hollow bars indicate duration of light.

driven by the *period* gene (McCabe and Birley, 1998). Studies on the melon fly *Bactrocera cucurbitae* revealed that populations subjected to selection for short or long development times over many generations also evolved differences in the phasing of several rhythms such as courtship, mating, locomotion, and preening (Miyatake, 1997; Miyatake and Shimizu, 1999). Together, these results support the view that many rhythms are controlled by a common underlying mechanism. Recent molecular studies also suggest that the molecular feedback loops involving the cycling mRNA and protein levels of clock genes constitute the core mechanism of circadian clocks, which is believed to be common to most circadian rhythms, though some additional elements downstream of the central pacemaker play a role in controlling overt rhythms in different tissues or organs (Williams and Sehgal, 2001). How do we then reconcile these two apparently contradictory lines of evidence?

It is possible that different rhythms in *D. melanogaster*, such as eclosion and oviposition, are actually controlled by different oscillators that share some components. If pacemakers controlling circadian rhythms consist of more than one oscillator (Daan et al., 2001; Pittendrigh and Daan, 1976; Pittendrigh, 1981), it is likely that an oscillator comprising *per* and *tim* based transcription-translation loops



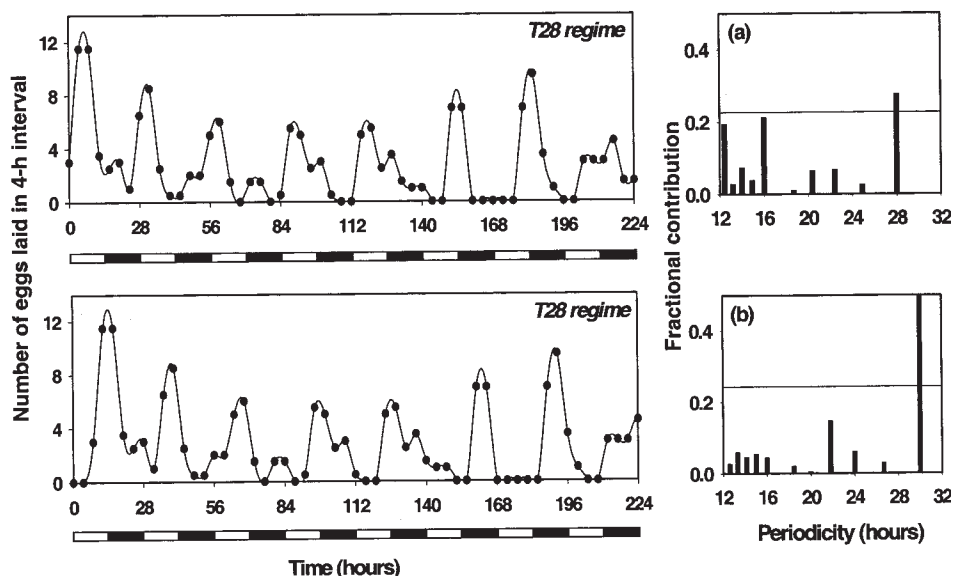


Figure 7. Upper panel shows time series data of a representative female for which the oviposition rhythm was entrained to T_{28} regime. The corresponding periodogram (a) shows a significant contribution of 28 h periodicity. Lower panel shows time series data of a representative female whose oviposition rhythm did not entrain to T_{28} regime. The corresponding periodogram (b) shows a significant contribution of 30 h periodicity. The dark horizontal bars below the x-axis indicate duration of darkness and the hollow bars indicate duration of light.

is a common part of the pacemakers controlling different rhythms. At the same time, other oscillator(s) that constitute the pacemaker might vary when controlling different rhythms. Such a scenario could in principle explain the findings that different rhythms show different τ and limits of entrainment, but are nevertheless affected similarly by mutations at loci such as *per*. Overall, it seems clear that more detailed studies of the structure of pacemakers controlling different rhythms are required.

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