A case for multiple oscillators controlling different circadian rhythms in *Drosophila melanogaster*

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Abstract

A population of the fruit fly *Drosophila melanogaster* was raised in periodic light/dark (LD) cycles of 12:12 h for about 35 generations. Eclosion, locomotor activity, and oviposition were found to be rhythmic in these flies, when assayed in constant laboratory conditions where the light intensity, temperature, humidity and other factors which could possibly act as time cue for these flies, were kept constant. These rhythms also entrained to a LD cycle of 12:12 h in the laboratory with each of them adopting a different temporal niche. The free-running periods \( (\tau) \) of the eclosion, locomotor activity and oviposition rhythms were significantly different from each other. The peak of eclosion and the onset of locomotor activity occurred during the light phase of the LD cycle, whereas the peak of oviposition was found to occur during the dark phase of the LD cycle. Based on these results, we conclude that different circadian oscillators control the eclosion, locomotor activity and oviposition rhythms in the fruit fly *D. melanogaster*.

Keywords: Locomotor activity; Eclosion; Oviposition; Circadian rhythms; Multiple pacemakers

1. Introduction

Almost all organisms studied so far have been found to possess an endogenous mechanism that regulates the timing of various physiological and behavioural processes. These physiological and behavioural processes occur with periodicities matching that of the geophysical environment (entrainment), and, under constant conditions in the laboratory, free-run with an endogenous periodicity which is often close to 24 h (circadian) (reviewed in Zordan et al., 2000). These circadian rhythms can be observed at various levels of biological organisation and complexity, ranging from mRNA concentrations within a cell, to the patterns of eclosion in populations of fruit flies.

In several organisms there exists evidence for multiple oscillators controlling different circadian rhythms. In the marine dinoflagellate *Gonyaulax polyedra*, which exhibits circadian rhythms in photosynthesis, cell aggregation, superoxide dismutase production, phototaxis, bioluminescence and cell division (Roenneberg, 1996), two oscillatory sub systems sensitive to different wavelengths of light, one controlling aggregation and the other controlling bioluminescence, have been demonstrated (Roenneberg, 1994). The locomotor activity of the Arctic ground squirrel showed a breakup of the activity band into two components, each of which free-ran with different periods when kept in constant light for several cycles (Pittendrigh, 1960) and a similar phenomenon was observed in hamsters kept in constant light for several days (Pittendrigh, 1974). In pinealectomised birds (Takahashi and Menaker, 1982), several separate oscillators in an individual could be independently entrained to light/dark (LD) cycles. Similarly, Ishizaki and colleagues showed that there are two independently light entrainable pacemakers in the saturnid moths, one in the forebrain and another in the prothoracic gland (Pittendrigh, 1993). In the flesh fly *Sarcophaga argyrostoma*, at least three distinct oscillators are believed to

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regulate initiation of larval wandering, diapause induction and pupal eclosion rhythms; adult locomotor activity, the deposition of cuticular growth layers on thoracic apodemes and duration of larval wandering, are also likely to be controlled by distinct oscillators (Saunders, 1986). The view emerging from these studies is that different cellular functions or groups of functions are likely to be controlled by separate circadian oscillators, and that these oscillators are coupled such that they influence each other, making up a multi-oscillator system (Roenneberg, 1996; Scully and Kay, 2000; Zordan et al., 2000).

In the fruit fly *Drosophila melanogaster*, circadian rhythmicity has been reported at various levels of organisation. A number of genes like *per*, *tim*, *clock* and *cry*, exhibit oscillations in the concentration of mRNA and/or their protein products (Zordan et al., 2000). In individual flies, locomotor activity and oviposition have been observed to be rhythmic (Helfrich, 1985), and pupal eclosion is also known to follow a circadian rhythm, indicating that the population as a whole can also act as a chronometer (Roenneberg, 1996; Scully and Kay, 2000; Zordan et al., 2000).

The oviposition rhythm in *Drosophila* has not been used to investigate the possible role of multiple oscillators in controlling circadian rhythms, even though the lack of cycling of *per* mRNA level in the ovary of *D. melanogaster* (Hardin, 1994; Plautz et al., 1997) suggests that oviposition may be under the control of a different circadian oscillator. It would therefore be interesting to investigate whether multiple oscillators control various circadian rhythms in *D. melanogaster*. For this purpose we chose one population level rhythm (eclosion), and two individual level rhythms (locomotor activity and oviposition), the underlying molecular mechanisms of which have been fairly well studied. Although, eclosion and locomotor activity rhythms have been investigated in some detail in the context of multioscillatory control of circadian rhythms, our main goal was to compare the circadian parameters of these two rhythms along with the relatively less studied oviposition rhythm in the light of the fact that most circadian rhythms share some common core molecular mechanisms.

Here, we report the results of experiments on a laboratory population of the fruit fly *D. melanogaster* which has been reared in alternating LD (12:12 h) cycles for ca 35 generations. The eclosion, oviposition and locomotor activity rhythms in these flies were assayed in DD and in LD 12:12 h, and the τ and phase angle difference (ψ) of these rhythms were recorded and compared with one another.

### 2. Materials and methods

The experimental flies were sampled from a large (*N*~1600 breeding adults), outbred, laboratory population of *D. melanogaster* that has been reared in LD 12:12 h for ~35 generations. This population is maintained in walk-in chambers at 25°C (±1°C), under constant humidity, at moderate larval and adult densities, on a 21-day discrete generation cycle. Eclosion rhythm was assayed by collecting eggs at high densities (~300 eggs per vial: 9 cm height×2.4 cm diameter) into vials containing ~6 ml of food medium. Twenty-four such vials were introduced both into DD and LD 12:12 h regimes. When adults began to eclose, the vials were monitored every 2 h and any eclosing adults were collected, and the number and sex of flies recorded. This was continued for 10 consecutive days, or until most of the pupae had eclosed. Oviposition rhythm of females was assayed by collecting flies that emerged during the peak of eclosion and introducing male–female pairs into vials containing ca 4 ml of food medium. The assay was initiated within 2 days after emergence of the flies. The flies were transferred to fresh food medium every 2 h and the number of eggs laid in each vial over the preceding 2 h was recorded. This procedure was continued for 10 consecutive days in LD 12:12 h and DD regimes. Twenty-four
male–female pairs were assayed per light regime. Locomotor activity rhythm was assayed using individual virgin male and female flies that eclosed during the peak of eclosion first under LD 12:12 h regime for 15 days and then under DD regime (n=25) for 15 days. The flies were transferred to the locomotor activity-monitoring set-up within 24–48 h after emergence. Single individuals were monitored using a set-up that uses infra red beams to detect the vertical movement of the fly in a narrow glass tube (0.6 cm inner diameter×2 cm height). The locomotor activity was monitored first in LD 12:12 h for 15 days and then in DD regime for another 15 days.

2.1. Statistical analyses

$\tau$ of locomotor activity rhythm was estimated using regression lines on the onset of activity for at least 6 consecutive days. The values of $\tau$ for the eclosion rhythm and oviposition rhythm were calculated by subjecting the time series data obtained from each vial in the eclosion rhythm assay and each female of the oviposition rhythm assay to Fourier spectral analysis using STATISTICA™ (Statsoft, 1995). Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). The value of $\psi$ for the eclosion rhythm in LD 12:12 h was estimated as the average time interval between peak eclosion and ‘lights on’ over 10 consecutive days. The value of $\psi$ for the oviposition rhythm in LD 12:12 h was estimated as the average time interval between peak oviposition and ‘lights on’ over 10 consecutive days, while the value of $\psi$ for the locomotor activity rhythm in LD 12:12 h was estimated by averaging the time interval between onset of activity and ‘lights on’ for over 10 consecutive days. Separate one-way analyses of variance (ANOVA) were carried out on the values of $\psi$ to test for any significant effect of the type of rhythm (eclosion, oviposition and locomotor activity). $\tau$ values of the locomotor activity and oviposition rhythms were compared using Student’s $t$-test. 95% confidence interval around the means was estimated to compare the $\tau$ values of eclosion and the other two rhythms (locomotor activity and oviposition).

3. Results

The value of $\tau$ for the eclosion rhythm in DD was observed to be 23.64 h (Figs. 1 and 2), and was significantly shorter than the $\tau$ value of the locomotor activity rhythm which in DD was 24.73±0.29 h (mean ±95% CI) (Figs. 1 and 3). The $\tau$ value of the eclosion rhythm in DD was also significantly shorter than the $\tau$ value for the oviposition rhythm, which in DD was 27.66±2.16 h (mean ±95% CI) (Figs. 1 and 4a–c). The value of $\tau$ for the oviposition rhythm in DD was significantly greater than the $\tau$ value of the locomotor activity rhythm in DD (Fig. 1) ($p=0.02$). The value of $\tau$ for the eclosion rhythm in DD was observed to be similar for all the vials used in the assay. Inter-individual variations in $\tau$ in DD were recorded for the individual level rhythms with the $\tau$ value of the locomotor activity rhythm (SD of 0.29 h) showing smaller variability compared to the $\tau$ value of the oviposition rhythm (SD of 2.16 h) in DD. All three rhythms (eclosion, locomotor activity and oviposition) were found to entrain to LD 12:12 h (Figs. 4–6). The periods of the eclosion, locomotor activity and oviposition rhythms in LD 12:12 h were not significantly different from 24 h. The mean value of $\psi$ for the eclosion rhythm was found to be $-4.43±0.77$ h (Fig. 5) (mean±SD), while that of the oviposition rhythm was $-10.495±2.42$ h (Fig. 6) and that of the locomotor activity was $+3.64±2.74$ h (mean±SD) (Fig. 4a–c). Unlike $\tau$, the $\psi$ value of the eclosion rhythm did show some inter-vial variation (SD=0.77 h). However, these variations were less compared to the inter-individual differences in the $\psi$ value of locomotor activity and oviposition rhythms (SD = 2.74 and 2.42 h, respectively). One-way ANOVA on the $\psi$ value of all three rhythms revealed a significant effect of type of rhythm ($F_{3,27}=123.91$, $p<0.001$). Multiple comparison showed that the $\psi$ values for all three rhythms were significantly different from each other ($p<0.001$, for all three comparisons; Scheffe’s test) (Fig. 7). While the locomotor activity rhythm phase leads ‘lights-on’ of the LD 12:12 h cycle, the eclosion rhythm phase lags it and the oviposition rhythm is almost 180° out of phase compared to eclosion and oviposition rhythms (Fig. 7).
4. Discussion

In several rhythm mutants of D. melanogaster such as per, tim, and disco (Konopka and Benzer, 1971; Dushay et al., 1989), the locomotor activity and eclosion rhythms have been found to be affected in a similar manner. In another study, the τ values of the oviposition and activity rhythms of per mutants and wild type D. melanogaster have been found to be significantly correlated and it was concluded that the per gene influences the circadian rhythmicity of both activity and oviposition in a similar manner (McCabe and Birley, 1998). Several mutations, however, like ebony and lark affect only one of the two rhythms: eclosion and locomotor activity (reviewed in Jackson, 1993). While ebony mutants show arrhythmic adult locomotor activity and a normal eclosion rhythm, lark has an arrhythmic eclosion rhythm and periodic locomotor activity (Newby and Jackson, 1991, 1993), suggesting that these mutations are perhaps affecting clock-controlled genes (CCGs) and not the central pacemaker. These studies suggest that eclosion, locomotor activity and oviposition rhythms in D. melanogaster share some common pathways in the pacemaker regulatory mechanisms which when modified by mutation proportionally affect the rhythms. In a study on D. pseudoobscura (Engelmann and Mack, 1978) it was observed that the τ values and the PRC of the eclosion and locomotor activity rhythms were different suggesting that separate circadian pacemakers control these two rhythms. The τ value of the eclosion and locomotor activity rhythms were reported to be different in D. melanogaster (Helfrich, 1985). In the present study we also found that the values of τ and ψ for the eclosion, locomotor activity and oviposition rhythms of D. melanogaster were significantly different from each other. The fact that these rhythms can be modified proportionally in some mutants rules out the possible role of independent circadian pacemakers controlling these three rhythms. However, these results can be explained on the assumption that separate circadian oscillators, which do not form part of the common pacemaker mechanism, control the three rhythms (eclosion and locomotor activity and oviposition).

Immunohistochemical studies of the expression of per in the brain during the larval and pupal stages showed that a small portion of the ventral lateral neurons (LNvs) with small stomata showed per cycling throughout metamorphosis starting from first instar onwards. The LNvs with large stomata, which developed when 50–60% of metamorphosis is complete, showed weak cycling in per
Fig. 3. Time series data of number of eggs laid by two representative females in continuous darkness (A, B). The corresponding periodograms show a significant contribution of 26 h and 30.22 h periodicity (a, b).

until eclosion. Dorsal LNds and glial cells showed cyclic per expression after about 50% of pupal development. These results suggest that while a certain subset of neurons (LNvs with small stomata) determines pupal ecylosion, all the LNts and the glial cells control adult activity rhythms (Helfrich-Förster, 1996). Therefore the observed differences in the rhythm parameters of the eclosion and the locomotor activity rhythms may be due to the progressive change in the composition of pacemakers that regulate these two rhythms.

In one of the first attempts to probe the possible endogenous control of the oviposition rhythm in D. melanogaster, groups of flies that were reared in DD were found to be arrhythmic when assayed in DD (Allemand, 1977). Moreover, a transfer from LD cycle of 12:12 h to DD did not elicit rhythmicity in oviposition, although a rhythm in vitellogenesis was found to persist. The lack of rhythmicity in oviposition in DD in the studies of Allemand (1977), could be due to the fact that oviposition was assayed on groups of females. Further, a study of rhythmicity using groups might not unequivocally reveal the nature of the rhythm of the individuals that constitute the group, especially if there is high variation in the periodicity exhibited by the individuals. In a previous study we have demonstrated circadian oviposition rhythm in individual females of D. melanogaster assayed in the DD regime. We have also shown that pooling time series data of individual females could obliterate the oviposition rhythm in DD regime (Sheeba et al., 2001).

The mRNA of the period gene, as well as its protein product PER, exhibit circadian oscillation in various tissues in the body of D. melanogaster and has been shown to be necessary for locomotor activity and eclosion rhythms (Hardin, 1994). The level of per mRNA and its protein product do not show any oscillation in the ovarian tissues (Hardin, 1994; Plautz et al., 1997). Further, PER is primarily cytoplasmic only in the ovary, while in all other body tissues and in tissues of the head, it is nuclear (Hardin, 1994). Hence some distinctive properties of ovarian tissues in terms of PER protein are evident, suggesting that in the ovary some genes other than per and its protein products may perhaps play a role in the generation of the overt oviposition rhythm. However, it is more likely that oviposition rhythm is controlled by the central nervous system and not by the ovarian tissues. This can be demonstrated by assaying oviposition rhythm in the flies that express per only in the brain. The fact that these three rhythms differ significantly from each other in two major circadian para-
Fig. 4. (a–c) Three locomotor activity records of flies for the first 15 days in LD 12: 12 h cycle, followed by the next 15 days in continuous darkness. The phase angle difference expressed as the average time difference between the onset of locomotor activity and ‘lights on’ was 4.77, 0.077 and 5.88 h, respectively, while free running period is 24.59, 24.66 and 24.25 h, respectively. The lights in the LD cycle were switched on at 0800 h and switched off at 2000 h.
The results of our experiments suggest possible role of different oscillators controlling various circadian rhythms, studies comparing the circadian parameters of the three rhythms (eclosion, locomotor activity and oviposition) in short and long period Drosophila mutants should be carried out to demonstrate unequivocally the multioscillatory organisation of the circadian timing system.
Fig. 7. Frequency distribution of the phase angle difference in LD 12:12 h cycle for eclosion, oviposition and locomotor activity rhythms. The x-axis represents class intervals with a range of 2 h starting from \(-16\) to \(+8\) h.

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