Ultradian rhythm unmasked in the *Pdf* clock mutant of *Drosophila*

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A diverse range of organisms shows physiological and behavioural rhythms with various periods. Extensive studies have been performed to elucidate the molecular mechanisms of circadian rhythms with an approximately 24 h period in both *Drosophila* and mammals, while less attention has been paid to ultradian rhythms with shorter periods. We used a video-tracking method to monitor the movement of single flies, and clear ultradian rhythms were detected in the locomotor behaviour of wild type and clock mutant flies kept under constant dark conditions. In particular, the *Pigment-dispersing factor* mutant (*Pdf^{01}*) demonstrated a precise and robust ultradian rhythmicity, which was not temperature compensated. Our results suggest that *Drosophila* has an endogenous ultradian oscillator that is masked by circadian rhythmic behaviours.

1. Introduction

Rhythmic activities are found in a wide range of organisms from unicellular organisms to mammals. Biological rhythms are classified by the length of periods. The periods of rhythmic activities range from subseconds to years (Rusak and Zucker 1975; Rapp 1979; Rensing *et al.* 2001; Hall 2005). Rhythms with periodicity longer than 1 day, referred to as infradian rhythms, are influenced by rotation of the earth or the moon. Circadian rhythms cycle with a period of about 1 day (24 ± 4 h) and can be synchronized by external stimuli like light and temperature. Rhythms with periodicity shorter than 1 day are referred to as ultradian rhythms. Ultradian rhythms are complicated, as various period lengths have been observed (Corner 1977; Wollnik 1989; Lloyd and Rossi 2008). However, nothing is yet known about the biological meaning of ultradian oscillations, including whether such oscillations can be influenced by external or internal signals.

An ultradian rhythm of locomotor activity in *Drosophila* was first reported in the arrhythmic mutant fly strain, *per^{01}* (Dowse *et al.* 1987; Dowse and Ringo 1987). Thereafter, ultradian rhythms were observed in wild-type flies under constant conditions (Power *et al.* 1995; Tomioka *et al.* 1998). All previous studies on the locomotor activity of *Drosophila* used a monitoring system that detects the movement of flies at one point in a measuring chamber with an infrared beam. In this study, a video recording system was used to analyse all fly movements. Initially, a burst of rhythmic locomotor activity during the night was observed (not shown), which prompted us to analyse such behaviour in detail. The time series data were analysed by the maximum-entropy method of the MemCalc software package, which is resistant to noise (Dowse *et al.* 1987; Dowse and Ringo 1987, 1989; Power *et al.* 1995; Levine *et al.* 2002) and has been used for time series analysis of various phenomena (Ohtomo *et al.* 1995, 1996a, b; Sumi *et al.* 1997a, b; Sawada *et al.* 1997). This software combines the

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maximum-entropy method for spectral analysis in the frequency domain and the nonlinear least squares method for fitting analysis in the reproduction of the power spectrum (Ohtomo *et al.* 1994). The use of this method to analyse walking behaviour permitted the detailed description of ultradian rhythms in *Drosophila melanogaster*. Ultradian rhythmicity was detected in circadian rhythm mutant strains as well as in a wild-type strain placed under constant dark or light conditions. Notably, robust ultradian rhythmicity was also detected in a clock output mutant, *Pigment-dispersing factor (Pdf^{01})*. Furthermore, this ultradian rhythmicity had different properties from those of the circadian system indicating that coupling of ultradian oscillations is not likely to underlie circadian rhythm.

2. Materials and methods

2.1 *Fly stocks*

Flies were grown with standard cornmeal-agar-glucose medium in an incubator at 25°C under a 12 h:12 h light:dark cycle. Adult male flies (3 to 6 days after emergence) were used in all behavioural experiments. Oregon-R (OR) was used as the wild-type strain, and *per^{01}*, *per^{s}*, *per^{L}*, *w tim^{01}*, *w per^{01};tim^{01}*, *Clk^{Jrk}*, and *y w;Pdf^{01}* clock mutant flies were also investigated. The *Clk^{Jrk}* mutant was a gift of Michael Rosbash (Brandeis University, Waltham, USA). The *per^{01}*, *per^{s}*, *per^{L}* and *y w;Pdf^{01}* mutants were a gift of Jeffrey C Hall (University of Washington, Seattle, USA). The *w tim^{01}* and *w per^{01};tim^{01}* mutants were a gift of Michael W Young (The Rockefeller University, New York, USA).

2.2 *Behavioural analyses*

Sugar-agar (10% (wt) glucose, 1.5% agar) solution was added to a plastic Petri dish (30 mm diameter) to about 10 mm thickness, and a single fly was housed under a watch glass (28 mm diameter) placed on the agar medium. Flies could walk freely on the round agar surface. Twenty Petri dishes were placed together on an illumination box using LED lights. Infrared LEDs (TLN115A, Toshiba Co., Ltd, Japan) were used for illumination in the complete dark condition. This LED emits infrared light a wavelength longer than 880 nm. *Drosophila* cannot detect this wavelength (Yamaguchi *et al.* 2010). A 12 h light: dark cycle was produced by white LED (NSPW510DS, Nichia Corporation, Japan) illumination controlled by a timer. Images (480 × 640 pixels) were taken by an infrared-sensitive camera (WAT-902H, Watec Co., Ltd, Japan) once every 1 s for 24 h and stored on a PC. The video files were analysed using the MultiTracker plug-in on ImageJ software (*http://rsb.info.nih.gov/ij/*; NIH, USA). Raw data from MultiTracker correspond to the binary centric coordinates of an individual fly. Finally, walking distance was calculated from raw data using a custom-made program.

2.3 *Statistical analysis*

All data values represented the total walking distance of an individual fly for 1 min and were statistically analysed by maximum-entropy methods (MEM) using MemCalc software (GSM, Shibuya, Japan). First, each data point was divided by the average value of all data points to normalize the data. MemCalc was then applied to estimate the period of rhythmicity in walking behaviour. To calculate ultradian rhythmicity, walking data from 1 day (1440 min) was used. MemCalc software calculates the power spectral density (PSD) of each frequency component. Primary components with the biggest PSD were regarded as the rhythmicity of fly activities. For ultradian rhythms, periods shorter than 6 h, corresponding to Nyquist frequency of 12 h that is artificial lighting hours were the focus of interest. For 1-D continuous wavelet analysis, the MATLAB Wavelet Toolbox (MathWorks, Boston, USA) was used. The morlet wavelet was selected as the mother wavelet (Bracic 1998; Price *et al.* 2008).

3. Results

3.1 *Ultradian rhythms in per^{01} mutants*

per^{01} mutants were examined to investigate whether they demonstrated rhythms with ultradian periodicity. Mutant *per^{01}* flies showed ultradian rhythmicity under constant darkness (D:D) conditions as previously reported (figure 1) (Dowse *et al.* 1987). PSD analyses indicated that this ultradian rhythmicity ranged from 20 to 200 min. The mean ultradian period was about 1.5 h (figure 1c; table 1). A sine curve with 1.5 h primary component fit well to the walking pattern of a representative fly (figure 1d). More than 80 % of flies showed ultradian rhythms. The period varied in individual flies and over successive days. Data were also analysed using continuous wavelet analysis (CWA) (supplementary figure 1). This analysis visually displays changes in period length over experiment days as a wavelet scalogram. Almost all flies showed a similar pattern. The circadian rhythm remained until the first or the second day, and ultradian rhythms appeared after the second or the third day.

3.2 *Ultradian rhythms in Pdf^{01} mutants*

Several other clock mutants were examined to determine whether they show rhythms with an ultradian periodicity. All examined clock mutants demonstrated ultradian rhythms under D:D conditions with a high frequency (supplementary
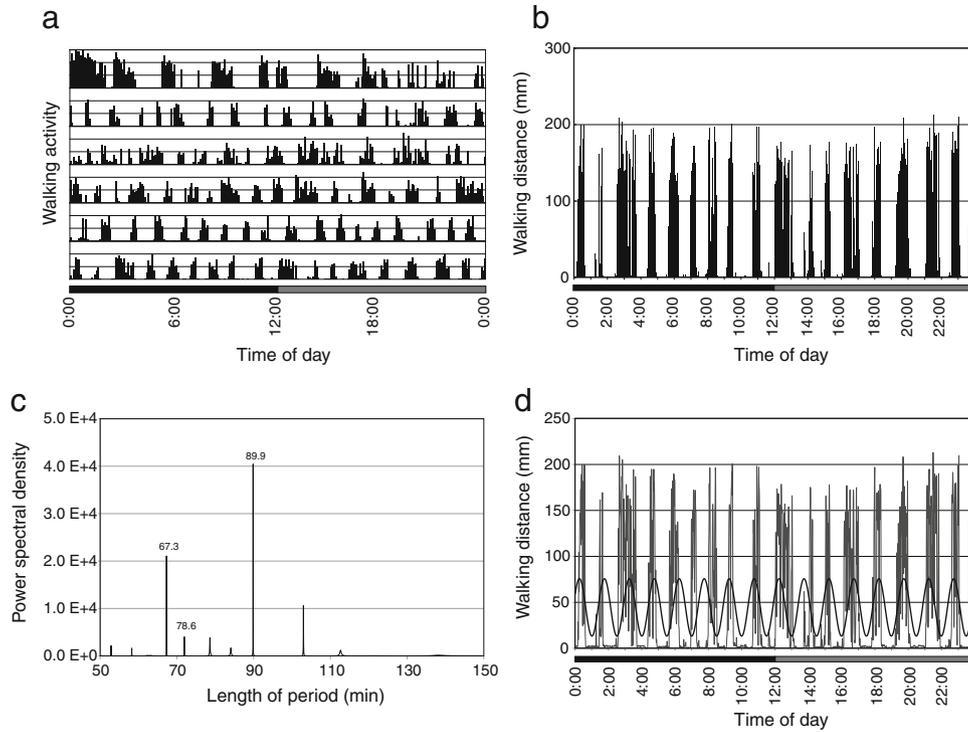
Figure 1. The ultradian rhythm of *period* mutants under constant dark conditions. (**a**) Typical locomotor activity of a *per^{01}* mutant fly under constant dark (D:D) conditions for 6 days at 25°C. All flies were first entrained to the D:L cycle. (**b**) Walking distance of a *per^{01}* mutant fly under D:D conditions at day 6. (**c**) The power spectral density distribution of the fly at day 6. The primary component is an element with peak at 89.9 min. The secondary component is an element with peak at 67.3 min. The y-axis is a logarithmic scale. (**d**) The fitting of a sine curve to the 89.9 min cycle.

table 1). In particular, the most robust and accurate ultradian rhythms were observed in *Pdf^{01}* flies kept under D:D conditions (figure 2). All flies showed ultradian rhythmicity from day 2 (table 1). The variance of period length was almost constant across individual flies and across days. The period length was constant, ranging from 50 to 60 min. The

Table 1. The ultradian rhythm of *per^{01}* and *Pdf^{01}* mutants under constant dark conditions

Day		*per^{01}*				*Pdf^{01}*	
	n	No. ultradian rhythmic	Ultradian period length (min)	Relative PSD of ultradian (%)	n	No. ultradian rhythmic	Ultradian period length (min)
1	20	12 (60)	134.0 ± 12.9	7.6 ± 0.6	19	10 (52.6)	58.4 ± 3.0
2	20	20 (100)	71.8 ± 10.9	5.5 ± 0.7	19	19 (100)	56.0 ± 0.1
3	20	17 (85)	87.9 ± 17.3	3.8 ± 0.4	19	19 (100)	54.0 ± 1.6
4	20	16 (80)	101.9 ± 20.5	4.1 ± 0.5	19	19 (100)	58.6 ± 4.3
5	20	18 (90)	90.7 ± 13.9	3.8 ± 0.5	19	19 (100)	57.4 ± 1.0
6	20	18 (90)	70.8 ± 10.8	4.4 ± 0.6			
	Mean	16.8 (84.2)	92.8 ± 14.4	4.8 ± 0.5	Mean	17.2 (90.5)	57.0 ± 2.0

Day, day of recording; n, number of flies tested; PSD, power spectral density. Ultradian rhythmic indicates the number (%) of flies in each day that demonstrated ultradian rhythmicity with a period of less than 360 min. Ultradian period length indicates mean ± SEM of primary components. Relative PSD indicates the mean ± SEM of the PSD of primary components.
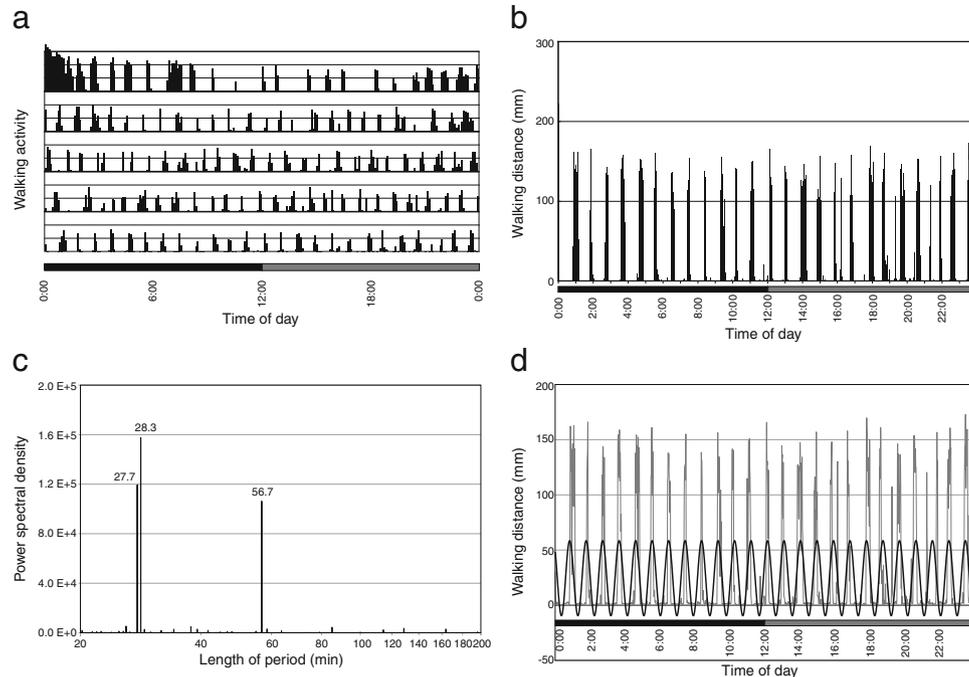
Figure 2. The ultradian rhythm of Pdf^{01} mutants under constant darkness. (**a**) Locomotor activity of a typical Pdf^{01} mutant fly for 5 days under D:D at 25°C. (**b**) Walking distance of the Pdf^{01} mutant fly at day 5. (**c**) The power spectral density (PSD) distribution of the fly at day 5. The primary component is an element with peak at 56.7 min. The secondary component is an element with peak at 28.3 min. The x-axis and y-axis are in logarithmic scale. Since the power of a spectrum is equal to the integral dimension of individual peaks, the height of peaks in this graph does not correspond to PSD. (**d**) The fitting of a sine curve to the 56.7 min cycle.

PSD of ultradian rhythmicity in Pdf^{01} flies was significantly higher than that of per^{01} flies ($P<0.0001$, t-test); these values were comparable to the PSD of circadian rhythm in wild-type flies (not shown). Notably, the secondary components of power spectral density in Pdf^{01} flies were nearly second harmonic to the value of primary components (figure 2c). Accordingly, a sine curve generated to represent primary period length completely fit the walking pattern (figure 2d). The period length of ultradian rhythms was fairly consistent in individual flies, but the phase of individual fitting curves was variable. The robustness of the ultradian period length between experiment days is clear from the scalogram (supplementary figure 2). Strong periodicity was consecutively noted in the scale from 40 to 50, which is equivalent to period length of 50 to 60 min.

3.3 *Ultradian rhythms in the wild-type fly under constant conditions*

Next, wild-type flies were examined for ultradian rhythms. Wild-type flies do not show circadian rhythmicity under constant illumination (Tomioka *et al.* 1998). Flies under constant light conditions (L:L) were found to demonstrate ultradian rhythms with high incidence after day 2 (figure 3; table 2). The proportion of wild-type flies showing ultradian rhythms was similar to those of clock mutants. The period length was about 1 h. The ultradian rhythm length of wild-type flies under constant illumination varied between days. These flies demonstrated a similar CWA scalogram pattern to per^{01} flies (supplementary figure 4). Circadian rhythms in wild-type flies disappeared immediately after moving to L:L conditions.

Although wild-type flies are exhibited circadian rhythms under D:D conditions, wild-type flies were examined to investigate their demonstration of ultradian rhythms under constant darkness. Approximately 5% of flies showed ultradian rhythms under D:D conditions (table 2). Wild-type flies kept under D:D conditions walked mainly during the subjective day (figure 4a; supplementary figure 5). Flies rested for a longer time during subjective night. To understand the influence of these two phases, short sampling segments were analysed (table 3). Interestingly, the incidence of ultradian rhythms with a 12 h sampling segment was higher than with a 24 h segment ($P<0.01$, t-test). Moreover, the mean ultradian period length between subjective day (51.8 min) and night (77.2 min) was significantly different ($P<0.05$, t-test).
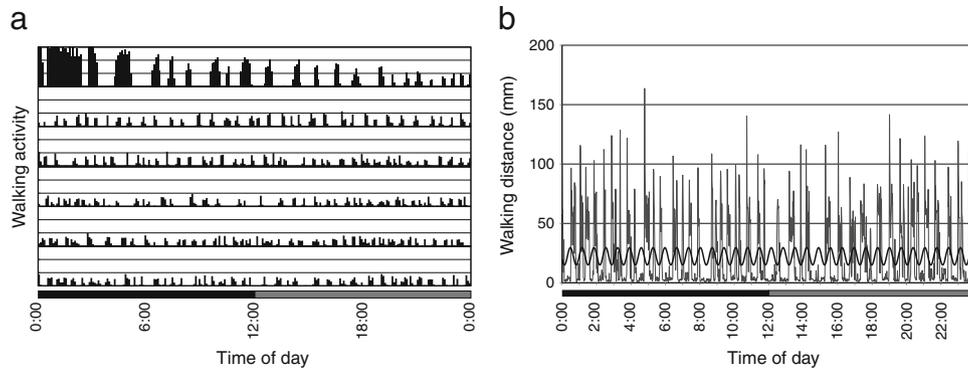
Figure 3. The ultradian rhythm of a wild-type fly under constant illumination. (**a**) Locomotor activity of a typical wild-type fly under constant light (L:L) conditions for 6 days at 25°C. (**b**) Walking distance of the wild-type fly at day 6, with the fitting of a sine curve to the 41.2 min cycle.

3.4 *Correlation of circadian and ultradian rhythms in period mutant flies*

Kyriacou and Hall (1980) described the connection between an ultradian property found in courtship song and the circadian phenotype of the *period* gene (Kyriacou and Hall 1980; Lloyd and Rossi 2008). *period* mutant flies were examined for the presence of any correlation between ultradian and circadian rhythms in walking. The *per*-short (*per^s*) mutant demonstrated a shorter circadian periodicity and a shorter period length of courtship song interpulse intervals. Ultradian rhythms in walking activity were detected in *per^s* mutants under L:L conditions (91.7). The *per*-long (*per^L*) mutants also showed ultradian rhythms under L:L conditions (83.3%). The *per^s* and *per^L* mutants did not demonstrate ultradian rhythms under D:D conditions

(not shown). In comparing *per^s* and *per^L* with *per^01* mutants and wild-type flies under L:L conditions (figure 5), no significant difference was detected between the mean ultradian period length of *per^s* and *per^L* mutants (*t*-test). Moreover, no significant difference was observed among wild-type, *per^s*, and *per^L* flies (ANOVA).

3.5 *Temperature compensation of the ultradian rhythms in Pdf^01 mutant flies*

To determine whether the ultradian rhythms were temperature-compensated, the walking distances of *Pdf^01* flies, which demonstrated the most precise ultradian periodicity, were measured at different ambient temperatures (figure 6a). Almost all *Pdf^01* flies at each temperature

Table 2. The ultradian rhythm of wild-type flies under constant light and dark conditions

Day	n	No. ultradian rhythmic	Ultradian period length (min)	Relative PSD of ultradian (%)	n	No. ultradian rhythmic	Ultradian period length (min)
			L:L			**D:D**	
1	20	6 (30)	109.5 ± 36.2	5.3 ± 0.6	20	12 (60)	135.5 ± 17.3
2	20	16 (80)	74.3 ± 17.0	4.8 ± 0.5	20	5 (25)	98.2 ± 56.6
3	20	17 (85)	71.0 ± 17.6	3.8 ± 0.3	20	6 (30)	39.6 ± 5.9
4	20	18 (90)	38.2 ± 3.2	3.2 ± 0.3	20	3 (15)	66.3 ± 20.6
5	20	16 (80)	53.7 ± 12.5	3.4 ± 0.2	20	2 (10)	34.0 ± 10.4
6	20	14 (70)	35.2 ± 4.4	2.9 ± 0.3	20	0 (0)	N. A.
	Mean	14.5 (72.5)	63.7 ± 15.2	3.9 ± 0.4	Mean	4.7 (23.3)	74.7 ± 22.2

N. A., not available due to no flies showing ultradian rhythmicity.
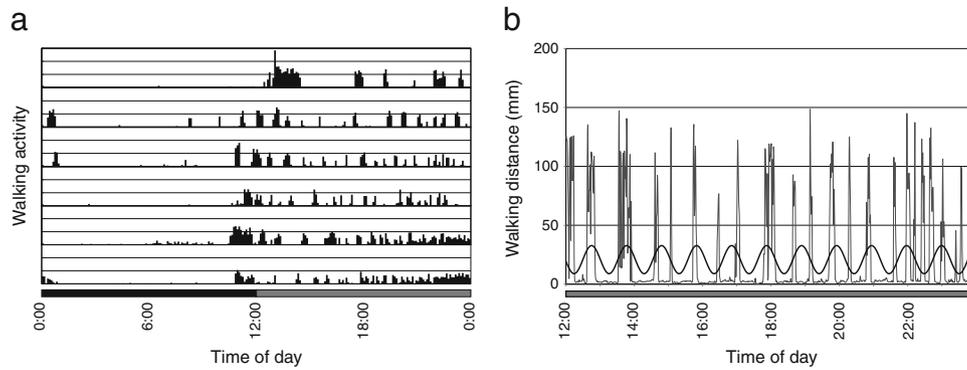
a

b

Figure 4. The ultradian rhythm of wild-type flies under constant darkness. (**a**) Locomotor activity of a typical wild-type fly under constant dark (D:D) conditions for 6 days at 25°C. (**b**) Walking distance of the wild-type fly during subjective day at day 3, with the fitting of a sine curve to the 61.4 min cycle.

showed ultradian rhythms after day 2. The period length of ultradian rhythms demonstrated clear temperature dependency. By analysing the variance of all data (ANOVA), the period length was found to stabilize after day 2. The average period length of ultradian rhythms after day 2 was calculated, and the plots were found to form a linear approximation (figure 6b). Additionally, temperature coefficient (Q_{10}) values for the period length of ultradian rhythms were calculated. The Q_{10} value for the range of 20–27.5°C was 2.68. The Q_{10} value for most chemical reactions lies from 2 to 3 (Hochachka and Guppy 1987), as opposed to the Q_{10} value of circadian rhythmic phenomena, which is approximately 1.

4. Discussion

4.1 *Detection of ultradian rhythmicity*

All previous studies concerning the ultradian rhythms of locomotor activity in *Drosophila* employed an apparatus that used an infrared beam to detect the movement of single flies (Dowse *et al.* 1987; Hall 2005). This method is limited in that it monitors fly movement at only one point in the measuring chamber when a fly crosses the beam. Furthermore, these studies collected fly activity data in rough intervals with 6 or 60 min bins. For data analyses, correlograms,

Table 3. The ultradian rhythm of wild-type flies under D:D conditions analysed with shorter sampling segment

Day	n	No. ultradian rhythmic	Ultradian period length (min)	Relative PSD of ultradian (%)
1	20	13 (65)	119.4 ± 22.5	9.1 ± 1.1
	20	16 (80)	99.7 ± 15.1	9.6 ± 1.0
2	20	10 (50)	84.0 ± 11.3	6.2 ± 0.6
	20	16 (80)	72.1 ± 20.5	6.2 ± 0.5
3	20	16 (80)	83.1 ± 17.4	6.0 ± 0.4
	20	15 (75)	37.8 ± 5.5	5.3 ± 0.5
4	20	8 (40)	78.1 ± 35.0	5.4 ± 0.4
	20	16 (80)	36.4 ± 5.2	4.6 ± 0.4
5	20	10 (50)	71.1± 31.4	4.8 ± 0.5
	20	10 (50)	32.0 ± 3.8	4.9 ± 0.6
6	20	5 (25)	27.2 ± 9.4	3.6 ±0.5
	20	8 (40)	32.9 ± 3.9	4.9 ± 0.5
	20	10 (50)	124.4 ± 45.1	5.2 ± 0.8
	Mean	11.9 (58.9)	69.1 ± 17.4	5.8 ± 0.6

The ultradian rhythm was calculated with a short sampling segment (12 h) using the same data in figure 4. Odd rows indicate subjective night; even rows indicate subjective day.
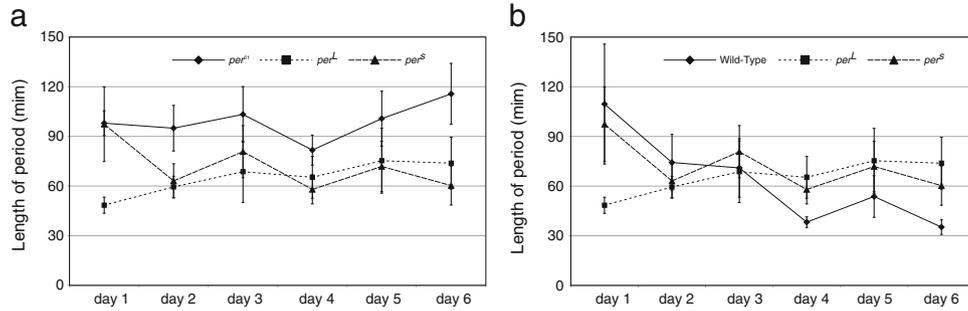
Figure 5. Comparison of ultradian rhythm length among *period* mutants. (**a**) Changes in period length over 6 days among *per^01^*, *per^s^*, and *per^L^* mutants at 25 °C. Closed rhomboids indicate the mean period length in *per^01^*; squares on dotted line, *per^L^*; triangles on dashed line, *per^s^*. (**b**) Changes in period length over 6 days among wild-type, *per^s^*, and *per^L^* mutants at 25 °C. Closed rhomboids indicate the mean period length in wild-type flies.

chi-square periodograms, or maximum-entropy spectral analysis (MESA) using the autoregressive (AR) model were employed after low-pass or high-pass filtering, which was necessary to simplify the periodicity calculation process (Levine *et al.* 2002).

These treatments may limit the accurate analysis of ultradian rhythms, because ultradian rhythms have periodicities within the fluctuation that may be caused by chaotic signals. When the frequency can be predicted, filtering is an effective technique to remove noise caused by high-frequency and low-frequency signals. However, if the frequency cannot be predicted, intact data should be used when possible. To overcome these problems, a video recording system was used to monitor all movement of individual flies every second (Zimmerman *et al.* 2008). In addition, MemCalc software was used to analyse raw locomotor data without filtration.

MemCalc overcomes the disadvantages of conventional spectral analysis in the frequency domain and the difficulty of nonlinear least squares methods in the time domain to detect dominant periodicities (Ohtomo *et al.* 1994).

Previous studies defined ultradian rhythms as having a period shorter than 20 h. In this study, ultradian rhythms with a period shorter than 6 h, corresponding to Nyquist frequency of 12 h that is artificial lighting hours, were investigated.

4.2 Circadian and ultradian rhythms of locomotor behaviours in Drosophila

Ultradian rhythms were observed in *period* mutants kept under various conditions, as previously reported (Dowse *et al.* 1987; Power *et al.* 1995; Tomioka *et al.* 1998). The
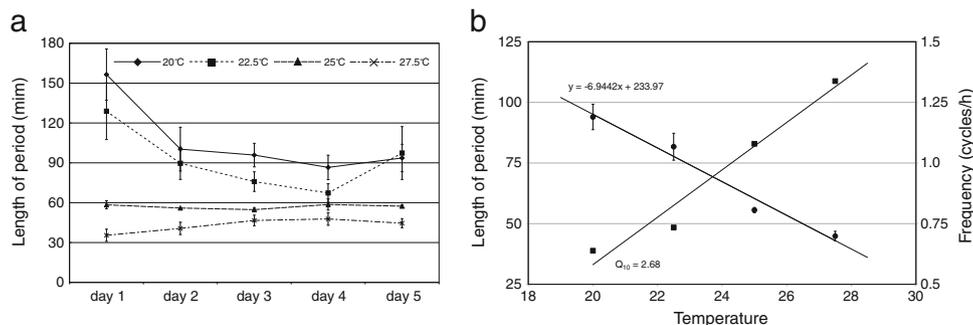

Figure 6. Temperature compensation of ultradian period length in *Pdf^01^* mutants. (**a**) Changes in period length across days and at different temperatures. Closed rhomboids indicate the mean period length at 20°C; squares on dotted line, 22.5°C; triangles on dashed line, 25.0°C; X symbols on dash-dotted line, 27.5°C. (**b**) The correlation between ultradian rhythm period length and temperature. The x-axis indicates temperature. The left y-axis indicates mean period length over 5 days; the right y-axis indicates mean frequency of ultradian rhythms. The Q_{10} value was calculated on the web (*http://www.csupomona.edu/~seskandari/physiology/physiological_calculators/Q10.html*).

present study also revealed that several clock mutants show ultradian rhythms under constant dark conditions (table 1; supplementary table 1). These results suggest that ultradian locomotor behaviours are masked by circadian behaviour. This hypothesis is strongly supported by the finding that wild-type flies kept under L:L conditions demonstrate high occurrence rates of ultradian rhythms comparable to those of clock mutants (table 2; Power *et al.* 1995; Tomioka *et al.* 1998). Ultradian rhythms are also prominent in rats under long-term light exposure when circadian rhythms disappear (Albers *et al.* 1981). Circadian rhythm is thought to have evolved for organisms to predict time and prepare for changes in the environment. Thus, circadian rhythm may be robust compared to other rhythmic phenomena. To uncover ultradian rhythms, the impact of circadian rhythm must be minimized.

In addition to the ultradian rhythms unmasked in the absence of circadian rhythms, wild-type flies under D:D conditions that maintained circadian periodic behaviours demonstrated ultradian rhythmicity (figure 4; table 3; supplementary figure 5). Ultradian rhythms are may persist whether or not a circadian rhythm is normal. Like circadian rhythm, the ultradian rhythm may be a fundamental rhythm. Although no ultradian rhythms were observed in wild-type flies under D:L conditions in this work, flies may have a masked ultradian rhythm. As we can see the ultradian periodicity in the wavelet scalogram with scale around 90 of wild-type flies in morning and evening (supplementary figure 6).

The ultradian rhythms of locomotor activity did not differ among *period* mutants (figure 5; Power *et al.* 1995). This result indicates that the ultradian rhythm is independent of the circadian rhythm, at least with respect to the action of the *period* gene. Kyriacou suggested that the *period* and *timeless* genes may play a role in the neurogenesis of the courtship song cycle (Kyriacou and Hall 1980; Lloyd and Rossi 2008). However, the present findings demonstrate that the *period* gene is not involved in the ultradian rhythm of locomotor activities.

4.3 *Ultradian oscillators in Drosophila*

The results of this study indicate that *Drosophila* possess innate ultradian rhythms and suggest the existence of endogenous ultradian oscillators. Power *et al.* (1995) proposed the following three hypotheses concerning the relationship between circadian and ultradian oscillators: (1) ultradian and circadian oscillators coexist; (2) ultradian rhythms result from desynchronization of the circadian oscillator; and (3) circadian rhythm is produced by the coordination of multiple ultradian oscillators. They favoured the third hypothesis, which was also supported by mathematical modeling (Dowse and Ringo 1987; Barrio *et al.* 1997; Paetkau *et al.*

2006). However, we believe that this hypothesis is unlikely, since the circadian molecular oscillator requires approximately 24 h to pass through one cycle. The second hypothesis can also be excluded, since wild-type flies showed ultradian rhythmicity under D:L and D:D conditions in spite of retaining circadian rhythmicity. Our results support the first hypothesis. Although not all flies demonstrated an ultradian rhythm, remarkably, ultradian rhythms could be observed in all strains examined and under any conditions. Thus, ultradian oscillators could exist independently of circadian oscillators.

The difference in ultradian rhythms between wild-type flies and clock mutants provide significant insight into the ultradian oscillator. One important finding is the variation of period length observed across fly strains. The presence of variation suggests that either one ultradian oscillator with unstable periodicity controls locomotor behaviour, or two or more ultradian oscillators exist and control the rhythm coordinately. If one stable ultradian oscillator controlled locomotor behaviour, period length should be equal across fly strains. However, period length differed not only across fly strains but also in individual flies across days. The difference in period length across fly strains could be caused by variation in genetic background, but individual differences and variations across experimental days cannot be explained by genetic background or mutations. Hence, if one ultradian oscillator controls locomotor behaviour, the oscillator should have a feature of unstable periodicity. If multiple ultradian oscillators control locomotor behaviour, variations in the period length of ultradian rhythms could be explained by assuming the coupling of multiple oscillators, each with a different phase, to produce the output rhythm.

Ichikawa (2009) reported an ultradian firing rhythm with a cycle of about 35 min in neurosecretory cells of the silkmoth, *Bombyx mori*. Similar ultradian oscillator neurons may exist in the *Drosophila* brain. The locomotor center of *Drosophila* is located in the central complex of the brain (Kahsai *et al.* 2010). Ultradian oscillator neurons may be located in the central complex or connected via upstream neurons (figure 7). The application of neuronal activity imaging technology to the fly brain for a long duration may lead to the identification of the ultradian pacemaker.

4.4 *Ultradian rhythm in the Pdf mutant*

Recent studies revealed that *Pdf*-positive neurons integrate multiple types of information including circadian rhythms, geotaxis, and sleep regulation (Mertens *et al.* 2005; Chung *et al.* 2009). The precision of ultradian rhythms in the *Pdf*[01] mutant is more prominent than in other strains. One possibility underlying this difference is that *Pdf*-positive neurons
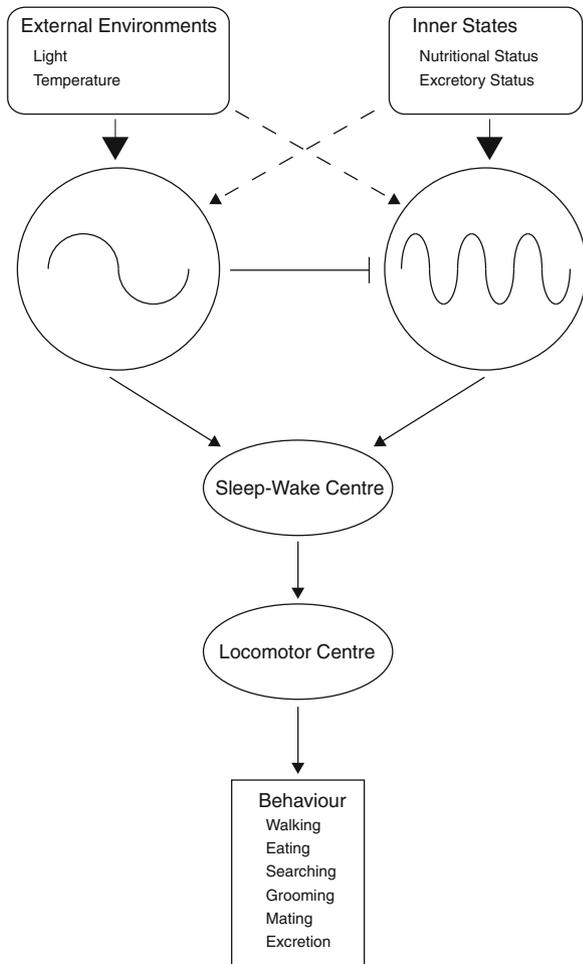
Figure 7. A model of circadian and ultradian rhythms. A scheme for controlling rhythmic behaviours from input to output.

integrate outputs from multiple putative ultradian oscillators, which are unmasked by the dissociation of outputs resulting from the mutation. As a result, Pdf^{01} mutant flies locomote with precise rhythmicity generated by one ultradian oscillator. In future studies, we would like to dissect this system using genetic tools available in *Drosophila,* for example by silencing a particular set of *Pdf*-positive neurons.

Circadian rhythms are known to be temperature compensated. However, the ultradian period of the Pdf^{01} mutant was found not to be temperature compensated. This result is not compatible with the hypothesis that circadian rhythms are formed by coupling of ultradian oscillations. Since the Q_{10} value of ultradian period length in Pdf^{01} mutants ranged from 2 to 3, some metabolic process involving enzymatic activities may govern the endogenous ultradian rhythm (Xu *et al.* 2008). The ultradian oscillator might be related to feeding or a digestion cycle. This possibility can be investigated by changing the metabolic status of flies through administration of chemicals or by directly observing their feeding behaviour.

Ultradian rhythms do not correspond to any known physical cycle in the environment, and their general functions have not yet been demonstrated. The ultradian rhythm of the Pdf^{01} mutant, which differs from other ultradian rhythms in its precise cycle, is likely to be useful for revealing the mechanisms of ultradian phenomena.

Acknowledgements

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