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Developmental plasticity of the locomotor activity rhythm of *Drosophila melanogaster*

V. Sheeba [a], M.K. Chandrashekaran [b], A. Joshi [a], V.K. Sharma [b,*]

[a] *Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, P.O. Box 6436, Jakkur, Bangalore 560 064, Karnataka, India*

[b] *Chronobiology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, P.O. Box 6436, Jakkur, Bangalore 560 064, Karnataka, India*

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Abstract

We used four replicate outbred populations of *Drosophila melanogaster* to investigate whether the light regimes experienced during the pre-adult (larval and pupal) and early adult stages influence the free-running period (τ_{DD}) of the circadian locomotor activity rhythm of adult flies. In a series of two experiments four different populations of flies were raised from egg to eclosion in constant light (LL), in light/dark (LD) 12:12 h cycle, and in constant darkness (DD). In the first experiment the adult male and female flies were directly transferred into DD and their locomotor activity was monitored, while in the second experiment the locomotor activity of the emerging adult flies was first assayed in LD 12:12 h for 15 days and then in DD for another 15 days. The τ_{DD} of the locomotor activity rhythm of flies that were raised in all the three light regimes, LL, LD 12:12 h and in DD was significantly different from each other. The τ_{DD} of the locomotor activity rhythm of the flies, which were raised in DD during their pre-adult stages, was significantly shorter than that of flies that were raised as pre-adults in LL regime, which in turn was significantly shorter than that of flies raised in LD 12:12 h regime. This pattern was consistent across both the experiments. The results of our experiments serve to emphasise the fact that in order to draw meaningful inferences about circadian rhythm parameters in insects, adequate attention should be paid to control and specify the environment in which pre-adult rearing takes place. The pattern of pre-adult and early adult light regime effects that we see differs from that previously observed in studies of mutant strains of *D. melanogaster*, and therefore, also points to the potential importance of inter-strain differences in the response of circadian organisation to external influences.

Keywords: Circadian rhythms; Free-running period; Light/dark cycles; Life stages; *Drosophila*

1. Introduction

Almost all living organisms are known to possess endogenous timing systems which regulate various circadian (approximately a day) rhythms in behavioural and physiological functions (Zordan et al., 2000). These rhythms are most often studied in constant darkness (DD), and sometimes in constant light (LL), with temperature and all other factors that could possibly act as time cues kept constant (Saunders, 1982). Under such constant conditions the circadian pacemakers of organisms are believed to free-run expressing their endogenous periodicity referred to as the 'free-running period' (τ). For the same group of individuals, the free-running period measured in DD (τ_{DD}) and in LL (τ_{LL}) typically differs (Pittendrigh, 1960; Aschoff, 1979). We will consistently use τ to refer τ_{DD} throughout the paper unless explicitly stated otherwise. The τ of a circadian rhythm is often regarded as a rigid characteristic of a species, with the τ of individual animals being approximately normally distributed around the species mean, usually with a fairly small variance (Moore Ede et al., 1982).

At the same time, however, there are a few evidences that the τ of a circadian pacemaker varies in response to various environmental conditions, often reflecting residual effects of prior environmental conditions experienced, typically referred to as 'after effects' (Pittendrigh,

* Corresponding author. Tel.: +91-80-8462750; fax: +91-80-8462766.

E-mail address: vsharma@jncasr.ac.in (V.K. Sharma).

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1960; Sokolove, 1975; Christensen, 1978; Page and Block, 1980). After effects have been observed in rhythms monitored under DD after the animals were previously exposed to LL, or to LD cycles of varying photoperiod length, but the results have not been unequivocal in experiments using different rodent species (Pittendrigh, 1960) and insects (Sokolove, 1975; Christensen, 1978). However, the after effects of LD cycles are speculated to be of functional significance in helping organisms to perform various behavioural and physiological functions at appropriate times even when the environmental LD cycle is masked, for example due to cloud cover (Beersma et al., 1999).

Among insects, after effects of LL of varying intensities on τ of the locomotor activity rhythm have been studied in *Drosophila melanogaster*, and the kind of after effect seen was found to vary with *per* locus genotype (Konopka et al., 1989). In cockroaches, exposure of nymphal stages to LD cycles of varying periodicity affects not only the τ, but also the sensitivity to brief light pulse as can be observed in terms of modified light pulse phase response curve (PRC) in the adults (Barrett and Page, 1989; Page and Barrett, 1989). In these studies, animals raised as pre-adults in LD 11:11 h had significantly shorter τ and a reduced delay portion of the PRC, whereas those raised in LD 13:13 h had longer τ and reduced advance portion of the PRC, relative to animals raised in LD 12:12 h. However, the PRC of animals raised as nymphs in DD did not differ significantly from those raised in LD 12:12 h. Animals raised as nymphs in LL were also found to have significantly longer τ as adults, compared to animals raised as nymphs in DD (Page and Barrett, 1989). Circadian rhythms in adults have also been reported to be affected by photoperiod experienced in pre-adult stages in the cricket species *Gryllus bimaculatus* (Tomioka and Chiba, 1989a,b).

Effects on adult rhythms of light regimes experienced during the pre-adult stage have also been observed in *D. melanogaster* which, unlike cockroaches or crickets, undergoes complete metamorphosis wherein virtually all adult tissues are formed anew. In *Drosophila*, the circadian timekeeping mechanism is believed to function from the first larval instar (L1) onwards (Helfrich-Förster, 1995), and light pulses given during L1 can shift the phases of adult eclosion and locomotor activity rhythms (Kaneko et al., 1997). A recent study on wild type (Canton S) and *per* mutant *D. melanogaster* reared in LL, in DD, and in 24 h LD cycles with different ratios of light/dark; 4:20; 6:18; 12:12; 18:6 and 20:4 h, revealed that the light regime experienced during the pre-adult and early adult stages (egg, larval, pupal stages and first seven days as adult) affects τ of adult locomotor activity rhythms (Tomioka et al., 1997). Konopka et al. (1989) reported that the free-running period of the locomotor activity rhythm of per^S and per^L mutants of *D. melanogaster* (Canton-S strain) behaved in a reciprocal manner when they underwent a transfer from DD to LL or from LL to DD. However, the results of Tomioka et al. (1997) are opposite to those of Konopka et al. (1989): following rearing in LL during early stages of development, τ was decreased in per^+, per^S and per^{L1} homozygotes, relative to τ in control flies that were reared in DD throughout the pre-adult and adult stages. However, for the flies reared in LD 12:12, LD 8:16 and LD 4:20 h, Tomioka et al. (1997) did observe opposite responses of subsequently measured τ to the light regime experienced in per^S and per^{L1} homozygotes, as had been earlier reported by Konopka et al. (1989).

One drawback of the study by Tomioka et al. (1997) was that their experimental design, as the authors note, confounds the effects of light regime experienced during pre-adult stages with that experienced during the first 7 days of adult life. Consequently, one cannot unequivocally ascribe the observed effects of light regime on τ to the light regime experienced as pre-adults or as adults, and it may be that an interaction between light regime experienced at different life stages is responsible for the discrepancy between their results and those of Konopka et al. (1989). Another potential problem in generalising from the results of Konopka et al. (1989) and Tomioka et al. (1997) is that in both studies the wild type strain (per^+ homozygotes) was Canton S. Given the dramatic difference in the type of after effects seen in per^L and per^S homozygotes in both studies, it is difficult to formally rule out the possibility, however implausible it may seem, that the after effects seen in the Canton S strain reflect some aspect of its genotypic constitution other than the fact that the flies are homozygous for the per^+ allele.

In this paper, we report results from two experiments in which the effect of the light regimes experienced during the pre-adult (larval and pupal) stages and early adult stage on τ of the adult locomotor activity rhythm was examined. We used four replicate outbred populations of *D. melanogaster*, which have been maintained separately, i.e. without any gene flow among them, for over 600 generations. These are essentially 'wild type' flies that have not been actively selected for any mutations and have been maintained under laboratory conditions of LL, constant temperature (24±1°C) and humidity for more than 600 generations. In a series of two experiments, individuals from these populations were exposed to LL, DD and LD 12:12 h from the egg through the pupal stages, and the τ and the phase-angle difference (ψ) of the locomotor activity of the adults were assayed. In the first experiment the τ of the locomotor activity of the freshly emerging adults was assayed in DD immediately after eclosion, while in the second experiment, the locomotor activity of flies emerging in the three regimes was first monitored in LD 12:12 h for 15 days following which they were introduced to DD regime for up to 15

days and the τ of the locomotor activity of the adults was assayed for up to 15 days.

2. Materials and methods

This study was conducted on four large ($N \approx 1500$ breeding adults), outbred, populations of *D. melanogaster* (LL-1…LL-4; first described by Sheeba et al. (2000)) which have been maintained under constant conditions of light (LL), temperature (24±1°C) and humidity at moderate larval and adult densities on a 21-day discrete generation cycle for about 35 generations. These populations were derived from ancestral populations that have been reared in the laboratory under similar conditions for more than 600 generations (JB-1…JB-4; first described by Sheeba et al. (1998)). For each experiment, eggs were collected from the running culture of each population in plexiglass cages ($25\times20\times15$ cm^3), by allowing females to lay eggs on food medium for about 2 h. For each population, exactly 50 eggs were collected into 24 vials (9.0 cm height×2.4 cm diameter) containing approximately 6 ml of banana–jaggery food. Eight vials from each population were kept under constant light (LL), 8 vials under light/dark cycles (LD 12:12 h), and 8 under constant darkness (DD).

The light phase in these treatments was achieved by means of fluorescent white light sources (approximate intensity 2.5 W/m^2 or 300 lx), whereas the dark phase was actually dim red light ($\lambda > 640$ nm), to facilitate observation and manipulation of flies without interrupting the dark phase. The continuous darkness (DD) in our experiments refers to continuous dim red light of wavelength greater than 640 nm. In all three light regimes, the vials were closely monitored once pupae darkened, and when peak eclosion occurred, flies that eclosed within a 4–5 h window were collected and males and females were separated. The virgin flies thus obtained were set up individually in glass tubes (80 mm height, 6 mm diameter) with sugar crystals at the bottom and cotton wicks moistened with water at the upper end (26 flies per sex per population per rearing light regime). The up and down movement of the flies was monitored by two pairs of infra-red emitters and sensors which were placed perpendicular to one another in such a manner that when a fly cuts the IR beams, the event was recorded by a computerised recording and display system. Activity was recorded in 5 min bins and each fly was monitored for up to 15 days in the first experiment and for up to 30 days in the second experiment.

The τ of locomotor activity in DD regime was estimated for each fly separately by using the slope of the regression line drawn to fit the time of onset of activity for at least six consecutive days. The values of τ for flies reared in all three light regimes for each sex were then used as data in a mixed model analysis of variance (ANOVA) in which replicate populations were treated as random blocks, and assay (with or without exposure to LD cycle during early adult stage), light regime and sex as fixed factors crossed with block; post hoc comparisons were done using Tukey's test. The ψ of locomotor activity rhythm for 'onset of activity' in LD 12:12 h, was estimated by averaging the time interval between onset of activity and 'lights-on' for about 10 consecutive days. The values of ψ for onset of activity for the flies of both sexes, reared in all three regimes were used in a mixed model ANOVA. Replicate populations were treated as random blocks while larval light regime and sex were fixed factors crossed with block. All statistical analyses were implemented using STATISTICA™ (STATISTICA™, 1995) for Windows Release 5.0 B (StatSoft Inc., 1995).

3. Results

The τ of the locomotor activity rhythm in flies reared under different light regimes during the pre-adult stages was found to range between 22.5 and 25.5 h (Fig. 1,

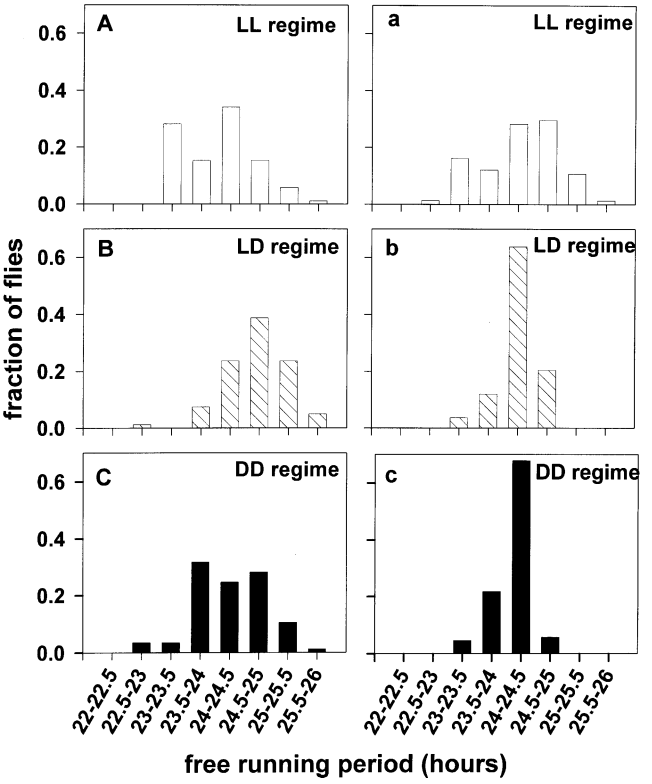

Fig. 1. Frequency distribution of free running period (τ) of locomotor activity rhythm in DD, of flies reared in three different light regimes (LL, LD 12:12 h and DD). The data on τ were averaged across population and sex for flies reared under different light regimes. (A)–(C) τ of flies assayed in DD immediately after emergence from the three different light regimes. (a)–(c) τ of flies after an exposure to LD 12:12 h regime for first 15 days after eclosion from three different light regimes.

Table 1
Mean free-running period (τ) of the locomotor activity rhythm of adult flies of both sexes, raised as pre-adults in three different light regimes (LL, LD 12:12 h, and DD) and assayed in constant darkness (DD). The confidence intervals (C.I.) are based on the variation between all the four replicate populations (LL1, LL2, LL3 and LL4)

Populations of flies	The free-running period (τ) in hours of male flies when raised as pre-adults in			The free-running period (τ) in hours of female flies when raised as pre-adults in		
	LL	LD	DD	LL	LD	DD
LL 1	24.336	24.485	23.946	24.577	24.317	23.770
LL 2	24.330	24.489	24.173	24.161	24.219	23.882
LL 3	23.800	23.868	23.450	24.288	24.145	23.834
LL 4	23.712	23.805	23.610	23.594	23.911	23.699
Mean	24.044	24.162	23.795	24.155	24.148	23.796
95% C.I.	0.533	0.599	0.519	0.656	0.275	0.127

Tables 1 and 2). The ANOVA on τ revealed significant main effects of block (replicate population) and light regime experienced during the pre-adult stages, but not of the type of assay (with or without exposure to LD cycles), or sex (Table 3). Post hoc comparisons using Tukey's tests revealed that mean τ of the locomotor activity rhythms of the flies raised as pre-adults in DD (23.95±0.11 h; mean ±95% C.I.) was significantly shorter than that of flies raised as pre-adults in LL (24.15±0.21 h; mean ±95% C.I.) which was in turn shorter than flies reared as pre-adults in LD (24.26±0.2 h; mean ±95% C.I.) ($p<0.05$ for all three comparisons) (Figs. 1–5). The marginal significance of the assay×light regime×sex interaction could be driven by the fact that females raised in LL from population LL-3 in the second experiment had lower mean τ as compared to those raised in DD regime (Table 2). The ANOVA on ψ did not reveal any significant main effect of light regime ($p=0.88$).

Table 3
Results of mixed model ANOVA on the τ of the locomotor activity of individual males and females of *D. melanogaster* raised in three light regimes (LL, LD and DD) as pre-adults for the two assays (with and without prior entrainment). Replicate populations were treated as random blocks, and the assays, light regimes and sex as fixed factors crossed with block. Since the analysis was performed on population means, the effects of block and interactions involving block, cannot be tested for significance

Effect	df	MS	*F*	*P*
Assay (A)	1	0.492	1.870	0.265
Block (B)	3	0.130	–	–
Light regime (L)	2	**0.389**	**43.104**	**<0.001**
Sex (S)	1	0.008	0.735	0.454
A×B	3	0.2623	–	–
A×L	2	0.046	1.437–	0.309
B×L	6	0.009	0.302	–
A×S	1	0.041	–	0.620
B×S	3	0.011	0.306	–
L×S	2	0.008	–	0.747
A×B×L	6	0.032	–	–
A×B×S	3	0.137	5.338	–
A×L×S	**2**	**0.0496**	–	**0.047**
B×L×S	6	0.026	–	–
A×B×L×S	6	0.009	–	–

4. Discussion

Our finding that τ of the adult locomotor activity of flies raised as pre-adults in LD 12:12 h is significantly

Table 2
Mean free-running period (τ) of the locomotor activity rhythm of adult flies of both sexes, raised as pre-adults in three different light regimes (LL, LD 12:12 h, and DD) and after emergence their locomotor activity rhythm was monitored in LD 12:12 h regime for first 15 days of the adult life and then in constant darkness (DD) for another 15 days. The confidence intervals (C.I.) are based on the variation between all the four replicate populations

Populations of flies	The free-running period (τ) in hours of male flies when raised as pre-adults in			The free-running period (τ) in hours of female flies when raised as pre-adults in		
	LL	LD	DD	LL	LD	DD
LL 1	24.086	24.319	24.127	24.158	24.35	24.033
LL 2	24.195	24.225	24.018	24.197	24.50	24.083
LL 3	24.457	24.425	24.109	23.760	24.225	24.187
LL 4	24.556	24.379	24.241	24.149	24.450	24.030
Mean	24.3232	24.3371	24.1238	24.0658	24.3813	24.0833
95% C.I.	0.350	0.137	0.145	0.326	0.193	0.117

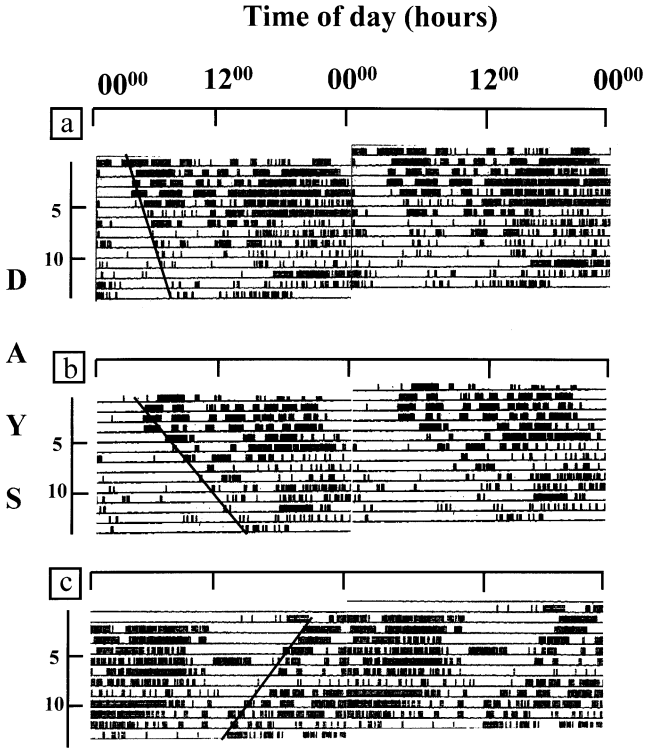

Fig. 2. (a)–(c) Representative locomotor activity records of male flies reared as pre-adults in three different light regimes (LL, LD and DD) and emerging adults were directly assayed in DD regime. (a) The fly was reared in LL regime and it exhibits a free-running period (τ) of 24.33 h; (b) the fly was reared in LD 12:12 h regime and it exhibits a τ of 24.78 h; (c) the fly was reared in DD regime and it exhibits a τ of 23.24 h. The abscissa represents time of day while ordinate represents the number of days. Thick bars indicate activity while horizontal lines indicate rest. The lines across the onsets of locomotor activity are indicative of the trend and were not used to calculate τ.

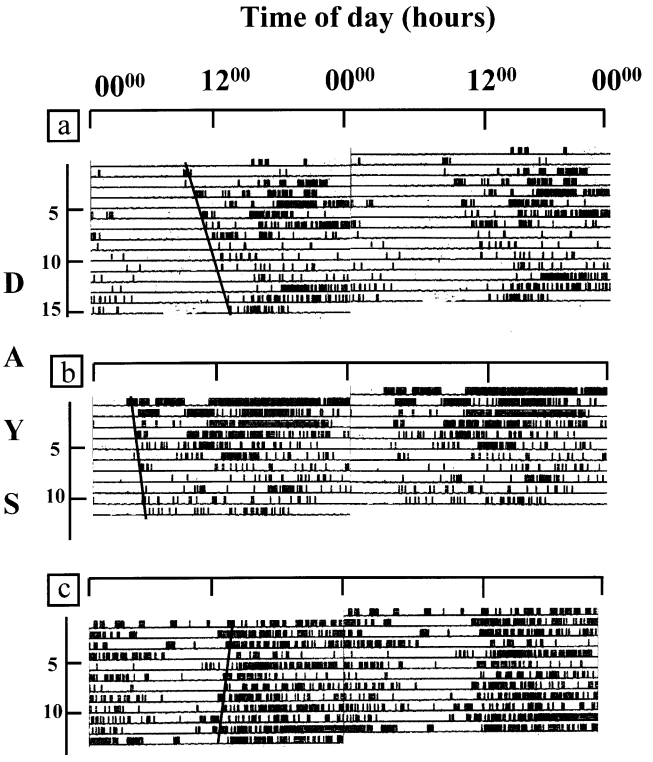

Fig. 3. (a)–(c) Representative locomotor records of female flies reared as pre-adults in three different light regimes (LL, LD and DD) and emerging adults were directly assayed in DD regime. (a) The fly was reared in LL regime and exhibits a free-running period (τ) of 24.22 h; (b) the fly was reared in LD 12:12 h regime and exhibits a τ of 23.71 h; (c) the fly was reared in DD regime and exhibits a τ of 23.6 h. Other details are the same as in Fig. 2.

greater than that of flies raised in DD (Figs. 1–5) is in concordance with the observations of Tomioka et al. (1997) for per^S and per^+ homozygotes. Konopka et al. (1989) reported that the free-running period of per^S homozygotes increased by about 0.6 h, whereas the period of per^L homozygotes decreased by about 1 h after they were transferred from DD to LL. The reverse was reported to occur when these flies are subjected to LL–DD transfer. Unfortunately, only the effects of LL and DD regimes during early adult stages were examined in the study by Konopka et al. (1989), ruling out a broader comparison across studies using *D. melanogaster*. Tomioka et al. (1997) reported that following rearing in LL; τ_{DD} was decreased in per^+, per^S and per^{L1} homozygotes, relative to τ_{DD} in control flies that were reared in DD throughout the pre-adult and adult stages. However, for the flies reared in LD 12:12, LD 8:16 and LD 4:20 h, the responses of subsequently measured τ to the light regime experienced in per^S and per^{L1} homozygotes were opposite. The main problem in generalising the observations made in both these experiments and that of ours is that besides differences in the experimental protocols the flies used in all these studies are different. The difference between τ of the locomotor activity rhythm of the flies raised in DD and LL observed in our experiments, is also in agreement with observations of Barrett and Page (1989) on cockroaches. In addition the results of our experiments further suggest that that both males and females are influenced by the pre-adult light regimes in a similar manner as no statistically significant main effect of sex was observed (Table 3).

The lack of a significant main effect of assay or interaction between assay (with or without exposure to LD 12:12 h) and the light regime experienced during pre-adult stages (LL, LD 12:12 h and DD) (Table 3) is remarkable as it indicates that the pattern of larval light regime effects on the value of τ of the locomotor activity rhythm is not altered by the intervening LD cycles during early adult stage. This suggests that the modifications in the circadian clocks during the pre-adult stages as reflected by the differences in the τ of locomotor activity rhythm of adult flies remain unaffected by the light regime experienced during early adult stage. We also observed that the phase relationship between the onset of locomotor activity and the lights-on in the LD cycle was not significantly different among the flies reared as

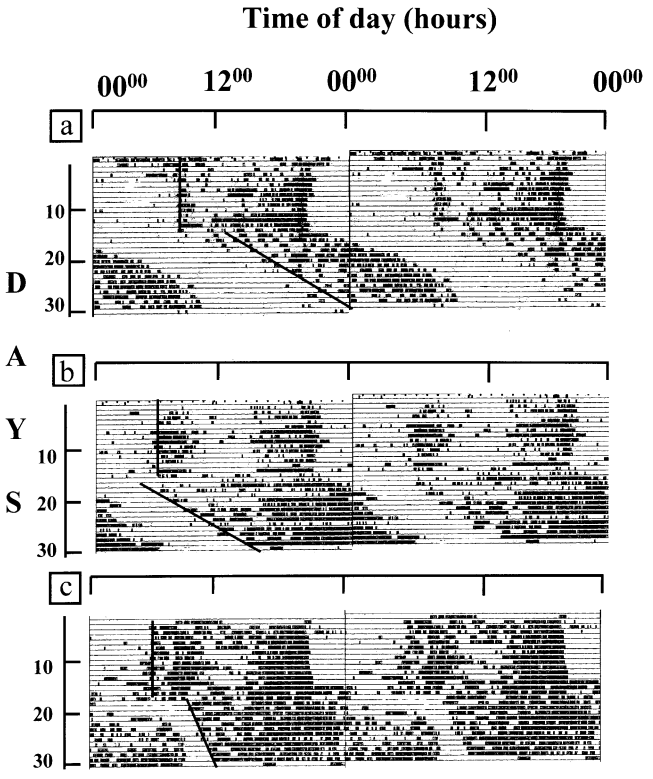

Fig. 4. (a)–(c) Representative locomotor records of male flies reared as pre-adults in three different light regimes (LL, LD and DD) and emerging adults were assayed for the first 15 days in LD 12:12 h regime followed by the next 15 days in DD regime. (a) The fly was reared in LL regime and exhibits a phase-angle difference (ψ) of 0.0 h for onset of activity followed by a free-running period (τ) of 24.91 h; (b) the fly was reared in LD 12:12 h regime and exhibits a ψ of +2.1 h followed by τ of 24.78 h; (c) the fly was reared in DD regime and exhibits a ψ of +2.55 h followed by a τ period of 24.19 h. Other details are the same as in Fig. 2. The lines across the onsets of locomotor activity are indicative of the trend and were not used to calculate the ψ or τ.

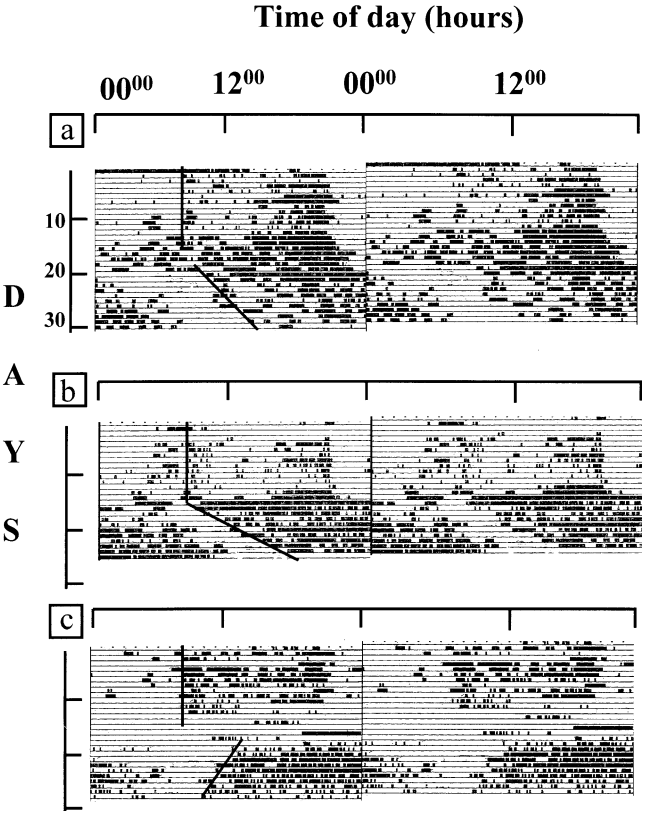

Fig. 5. (a)–(c) Representative locomotor records of female flies reared as pre-adults in three different light regimes (LL, LD and DD) and emerging adults were assayed for the first 15 days in LD 12:12 h regime followed by the next 15 days in DD regime. (a) The fly was reared in LL regime and exhibits a phase-angle difference (ψ) of -0.29 h for onset of activity followed by a free-running period (τ) of 24.46 h; (b) the fly was reared in LD 12:12 h regime and exhibits a ψ of 3.0 h followed by a τ of 24.97 h; (c) the fly was reared in DD regime and exhibits a ψ of -0.5 h followed by a τ of 23.66 h. Other details are the same as in Figs. 2 and 4.

pre-adults in LL, LD 12:12 h and DD (Figs. 4 and 5). This suggests that although manipulating the developmental light conditions can modify the τ_{DD} of the circadian clocks, the phase-angle difference, which plays a key role in an organism's survival remains unchanged.

Most laboratory strains used for genetic analyses are maintained at rather small population sizes (often for many years) and, consequently, are likely to be highly inbred. One problem with generalising from the results of any phenotypic manipulation of inbred populations is that one is never sure to what extent fortuitous fixation of particular alleles at some locus in that particular strain contributes to any observed response. Similarly it is also not clear to what extent responses to phenotypic manipulations observed in inbred lines are representative of the kinds of response one is likely to see in larger outbred populations, especially if the species is a normally outbreeding one (Rose et al., 1996; Mueller, 1997; Reznick and Ghalambor, 1999; Harshman and Hoffmann, 2000; Mueller and Joshi, 2000), a problem that is relatively unappreciated in the literature on chronobiology (Sheeba et al., 2000). Given, moreover, that *Drosophila* is a commonly used system in chronobiology, it is of obvious interest to have some knowledge of the effects that pre-adult rearing conditions have on 'wild type' individuals from large outbred populations with a large amount of standing genetic variation. Unlike experiments of Konopka et al. (1989) and Tomioka et al. (1997), which were done on laboratory populations of wild type and mutant Canton S strains of *D. melanogaster*, and therefore are likely to be highly inbred, our studies were carried out using four independent outbred populations. These four populations share a common ancestry but have been maintained as separate populations for over 600 generations, i.e., without any gene flow among them. The fact that all four populations show a similar trend in the pattern of larval light regime effects on the τ of locomotor activity rhythm further strengthens the argument that the developmental modifications in the circadian phenotype

are not merely because of fortuitous fixation of alleles at certain loci that influence the overt circadian rhythm.

The *Drosophila* circadian pacemaker is believed to be composed of at least two interlocked feedback loops: one consisting of the cycling of mRNA production of a gene *dclk* and another that involves the cycling of the mRNA of two other genes, *per* and *tim*. Under LD cycles, the transcripts of *per*, *tim* and *dclk*, and their protein products PER, TIM and dCLK, show robust oscillation within the cells of *D. melanogaster* (Sehgal et al., 1994; Bae et al., 1998; Darlington et al., 1998; Dunlap, 1999). Entrainment to LD cycles is achieved by light-dependent degradation of the protein TIM. The level of TIM falls rapidly at 'lights-on', thus preventing the formation of PER-TIM heterodimers required for the negative feedback loop to function. Instead, PER gets phosphorylated by another protein DBT thus causing a fall in the level of PER-TIM heterodimers. This allows another heterodimer dCLK-CYC to bind to the E-box of the promoter region of *per* and *tim* genes and transcription is initiated once again. Thus light is involved in resetting the phase of the circadian oscillator (Zeng et al., 1996).

In LL, the levels of PER abundance and phosphorylation are suppressed and low levels of PER are constitutively expressed (Price et al., 1995). It has also been observed that a minimum length of 6–8 h of darkness is required for the feedback loop to function (Qui and Hardin, 1996). Hence, in the *Drosophila* circadian clock the lengthening of τ in LL can be due to degradation of TIM by light, which would result in longer time being required to build up sufficient amount of PER-TIM heterodimer. In DD, in the absence of light induced degradation of TIM, the PER-TIM heterodimer formation and its subsequent nuclear entry occurs at shorter intervals than that in LD or LL regimes. Thus, the current understanding of the *Drosophila* circadian oscillator seems to imply that lengthening or shortening of the overt rhythm assayed under different light regimes is a reflection of changes in the molecular mechanisms underlying overt circadian rhythms.

The overall effects of light regimes on the τ of locomotor activity rhythm in *Drosophila* may be mediated by interactions between the light regimes experienced during the pre-adult and early adult stages. If so, the fact that the pre-adult light regimes in the study by Konopka et al. (1989) were LD 12:12 h, 25°C and LL, 22°C and that the study by Tomioka et al. (1997) confounds light regime effects at different life stages, may together perhaps provide a resolution of the discrepancy between these two studies. In both our experiments the flies were reared in three different light regimes (LL, LD 12:12 h and DD) only during the pre-adult stage and therefore the modification in the free-running period can only be ascribed to the influence of light regimes during pre-adult stages. However, factorial experiments with different combinations of pre-adult and early adult light regimes may yield the answer.

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