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A Lack of Locomotor Activity Rhythms in *Drosophila melanogaster* Larvae (Diptera: Drosophilidae)

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We examined the locomotor activity of Drosophila melanogaster *for the existence of circadian rhythms, using the wild type and two mutants of the* period (per) *gene,* per^{o} *and* per^{s}. *This was accomplished using a newly described apparatus for the recording and measurement of larval path lengths over a 96-h test period. None of the larvae examined exhibited appreciable diel rhythms under cycling conditions of light or temperature. Larvae were also not rhythmic under free-running conditions. Our results suggest that the* per *gene does not influence an observable locomotor behavioral phenotype in the larval stage of development.*

KEY WORDS: circadian rhythms; behavior; light–dark cycles; temperature cycles; *period* mutants.

INTRODUCTION

The daily patterning of animal behavior is presumed to be due to changes in responsiveness to external stimuli, as a function of varying internal states which may be oscillating with a 24-h period (Kyriacou, 1990). The term circadian means "approximately daily" and is used to describe rhythms that have periods of about 24 h (Halberg *et al.*, 1959).

In the fruit fly, *Drosophila melanogaster*, the period (*per*) gene is important

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for the generation of biological rhythms (reviews by Young, 1993). Mutations at the *per* locus disrupt circadian rhythms of adult eclosion from the pupal case and adult locomotor behavior (Konopka and Benzer, 1971). Konopka and Benzer (1971) induced the first single-gene rhythm mutations: the per^{o} allele, a molecularly null mutation (Yu *et al.*, 1987; Baylies *et al.*, 1987), abolishes eclosion rhythmicity and results in essentially undetectable locomotor activity rhythms in adult flies [with hidden, ultradian rhythms (see Dowse and Ringo, 1989a)]; the per^{s} and per^{Clk} alleles shorten the circadian day to about 18–22 h, whereas the per^{L} alleles lengthen it to about 28–30 h (Konopka, 1987; Hall and Kyriacou, 1990; Dushay *et al.*, 1992; Rutila *et al.*, 1992; Baylies *et al.*, 1992). Mutations in *per* have also been observed to affect the cycling of male courtship song (Kyriacou and Hall, 1980; Kyriacou *et al.*, 1990b) and the timing of developmental stages (Kyriacou *et al.*, 1990a).

Given the influence of the *per* gene products on circadian rhythms, it was important to assess the gene's expression during development. Detections *in situ* of *per*-encoded mRNA, protein, and reporter enzyme (in transgenics) have shown that *per* is expressed in a variety of tissues and cell types (review by Hall and Kyriacou, 1990) and that the *per* protein (PER) may be present in different subcellular compartments (Liu *et al.*, 1992). The expression of *per* also occurs at several stages of the life cycle (Hall and Kyriacou, 1990). Expression in larvae, however, has been undetectable in some studies (e.g., James *et al.*, 1986; Siwicki *et al.*, 1988) and observable in others: in Northern blotting experiments (Young *et al.*, 1985) and by *in situ* hybridization or application of an antibody (Bargiello *et al.*, 1987). The latter experiments revealed *per* mRNA and protein only in the larval salivary gland.

The effects of *per* mutations on larval phenotypes have been problematic: heartbeat irregularities in per^{o} did not stand up to further scrutiny (reviewed by Hall, 1990); that this mutation may affect a salivary gland (circadian) rhythm of membrane potential became a moot point when that rhythmicity turned out not to be demonstrable in the wild type (review by Jackson, 1993). A claim that *per* mutations affect intercellular communication in this salivary gland (Bargiello *et al.*, 1987) did not stand the test of time (Siwicki *et al.*, 1992; Saez *et al.*, 1992; Flint *et al.*, 1993). A larval clock is implied by the fact that pupal/adult eclosion and adult behavior can be controlled in part by a "time memory." Pulsed light cues delivered to larvae lead to periodic (hence, synchronous) eclosion and to phased locomotor activity rhythms of adults (Brett, 1955; Sehgal *et al.*, 1992). These rhythmic phenotypes can be manifested many days after the light pulse. It is not known if *per* participates in this larval clock function.

Here we investigated whether *D. melanogaster* exhibits observable circadian rhythmicity in the larval stage by developing a new assay to measure larval locomotor activity during the "foraging phase" of the third instar (Sokolowski, 1980). We looked for both diel rhythms in larvae exposed to entraining condi-

tions (light–dark and warm–cool cycles) and circadian rhythms in larvae held under constant conditions. We used three *per* genotypes to determine whether this gene, whose molecular expression is not detected in larvae, affects larval behavior as it does adult behavior.

MATERIALS AND METHODS

Fly Strains

Experiments were performed on Cantonized strains generated in one of our laboratories (J.C.H.). The strains used had been rendered isogenic as described by Gailey *et al.* (1991). This resulted in four strains—per^+, per^s, per^{LI}, and per^o—that differed only in terms of their alleles at the *per* locus.

Strains were maintained on a medium of dead yeast, sucrose, and agar, supplemented with the mold inhibitor propionic acid. The rearing conditions were 25°C and ~70% relative humidity, with lights on at 0800 h and lights off a 2000 h (LD 12:12).

Larval Path Length

The locomotor component of foraging behavior of third-instar larvae, 96 h post hatching at 25°C (reared under the conditions described above), was examined using a method similar to that described by Sokolowski (1980). Larval path length was determined by placing a single larva on a circular patch, approximately 8.5 cm in diameter, containing a thin, evenly spread, homogeneous layer of aqueous yeast suspension. The visible trail made by the larva during a 5-min test was measured, and the mean path length ±1 SE was calculated for each *per* strain.

Larval Phototaxis

Measurements of the larval phototactic response to white light and red light were made according to Lilly and Carlson (1990). Red light had a wavelength ≥610 nm. The assay plate was a sectioned plastic petri plate (100 × 15 mm; Fischer Scientific) containing four quadrants. Fifteen milliliters of 1% agarose was poured into each of two diametrically opposed quadrants to create clear quadrants; 15 ml of 1% agarose containing 0.5% charcoal powder (BDH Chemicals) was poured into each of the other two quadrants to create dark quadrants. Shortly after the agarose hardened, 12 ml of 1% agarose was distributed so as to provide an even surface over the entire plate. The plates were then allowed to equilibrate to room temperature for a period exceeding 1.5 h. Third-instar larvae, 3.5 days post hatch at 25 ± 1°C and approximately 12 h prior to wandering, were removed using a moist paintbrush from several culture dishes

containing 35 ml of medium and 100 larvae and carefully rinsed with distilled water. Approximately 100 larvae were then randomly selected and placed in the center of the experimental test plate.

The test plate was subsequently placed on a light source (82V ENX 360W; 3M) and the larvae were allowed to migrate. After 5 min the numbers of larvae on the dark and clear (white light or red light) quadrants were counted. There was no significant difference in surface temperature between clear and dark quadrants after 5 min on the light source. Three replicate experiments were conducted in total darkness.

The distribution of larvae on the two assay conditions was observed under illuminated and nonilluminated conditions. The response index (RI) was the number of larvae in clear (C) versus dark (D) sections, RI = (D − C)/(D + C) (Lilly and Carlson, 1990).

Activity Monitoring in Light:Dark Cycles

Preparation for larval observations of activity rhythms commenced with the collection of first-instar larvae 33–34 h following oviposition by adult females on a molasses medium at 19 ± 1°C, ~70% relative humidity, and constant darkness (DD). Larvae were removed and placed on culture medium in an incubator for 72 h, conditions as above with lights on at 0800 h and lights out at 2000 h for L:D 12:12 [dark conditions consisted of ≥610-nm red light; adult Drosophila are "unresponsive" to light of this quality (Dowse and Ringo, 1989b)] necessary for the monitoring of larval locomotor activity. Early third-instar larvae were carefully removed from the medium with a moist paintbrush. Seven larvae were then placed in the experimental dish, each larva in a separate compartment (d = 11 mm, h = 3.0 mm) on a culture medium. Fourteen such compartments were housed in an automatically rotating experimental dish developed for this assay (see Figs. 1 and 2). This dish enabled the simultaneous testing of seven larvae during the 4-day measurement period with each compartment observed consecutively.

Larval locomotor activity for each compartment was filmed (Sony 700-line-black-and-white compartment I-R spectrum with a CCD videocamera module and 40 mm macro lens system) in conjunction with a videocassette recorder (Panasonic VHS Professional/Industrial video Model AG-1050). One larva was filmed for 1 min every 14 min, hence 4 time point/56 minutes; the filming of each animal was synchronized with the position of the experimental compartments by an electronic timer (Omron digital timer Model H5R series). Each larva was filmed for 96 h. Filming ended prior to the onset of larval wandering behavior, which occurs at the end of the third instar (Sokolowski *et al.*, 1984). All exposures to LD cycles occurred in a 19 ± 1°C incubator (Percival, Boone 1A) in which the light differences were dialed into the incubator settings. Light

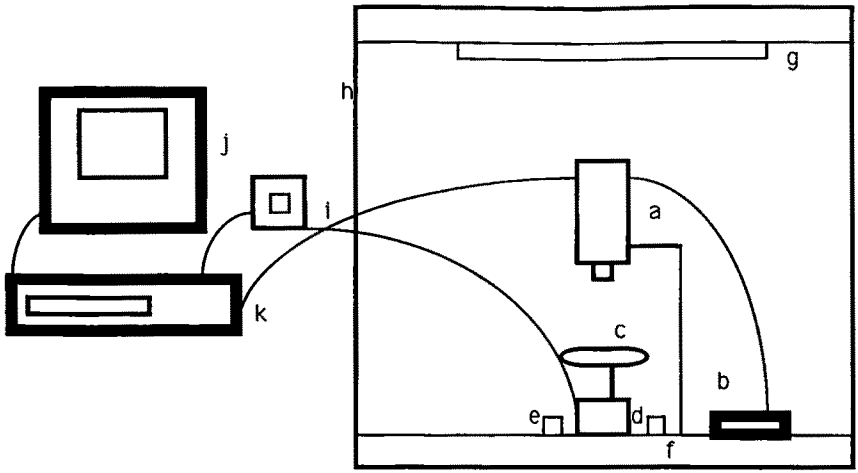

Fig. 1. Apparatus used to test for circadian rhythms in the locomotor activity of *D. Melanogaster* third-instar larvae. (a) Sony 700-line black-and-white camera I-R spectrum with CCD videocamera module and 40-mm macro lens system; (b) DC power supply; (c) experimental plate 95 mm in diameter with 14 circular wells, each 11 mm in diameter and 3 mm deep; (d) low-voltage DC-controlled stepper motor; (e) fluorescent tube with light red and medium red filters that transmits 610 nm and above, 24 h ON; (f) fluorescent tube with light red and medium red filters that transmits 610 nm and above, 12 h ON during night cycle; (g) fluorescent tube white light that emits at ~120 lux, 12 h ON during day cycle; (h) temperature- and light-controlled incubator; (i) Omron digital time Model H5R series; (j) Sony Trinitron color video monitor KV-8AD10, 220 × 213 × 310 mm (w/h/d); (k) videocassette recorder, Panasonic VHS Professional/Industrial video Model AG-1050.

conditions consisted of fluorescent tubes of white light emitting about 120 lux and one fluorescent tube with red filters transmitting ≥ 610 nm.

Activity Monitoring in Temperature Cycles

Larval activity was also measured under dark conditions under a regime of cycling high and low temperatures. Cyclic variations in temperature were programmed automatically by the incubator (Percival, Boone 1A), through dial settings. Larvae were exposed to these temperature cycles in DD for a premonitoring period of 120 h. Filming commenced as above, at early third instar, and continued through to just prior to the larval wandering stage.

Activity Monitoring Under Free-Running Conditions

Larvae were harvested and experiments were conducted as described earlier for activity monitoring in light : dark cycles, except that premonitoring and monitoring were done under "dark" conditions (no light below 610 nm).

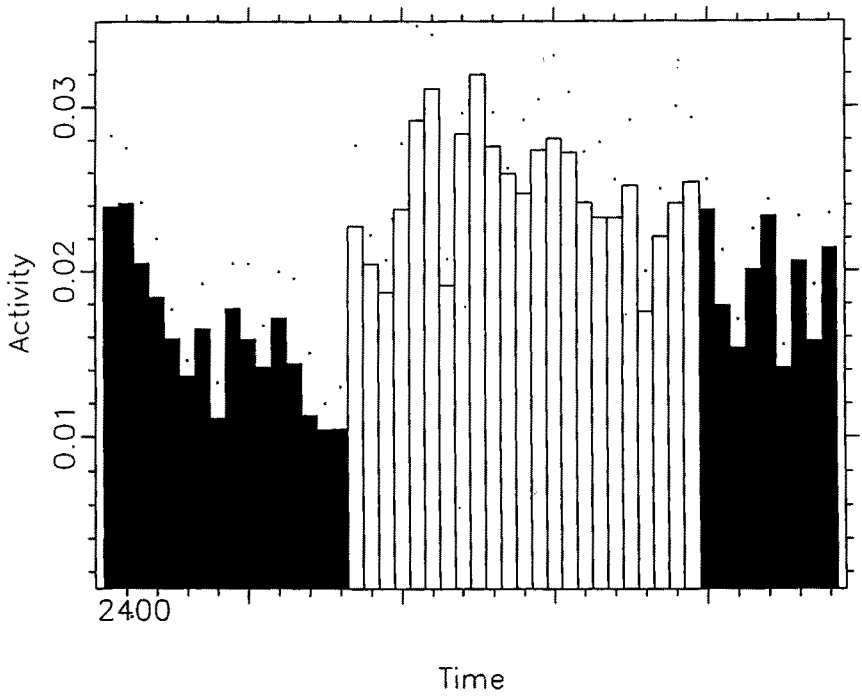

Fig. 2. Diel locomotor activity of *D. melanogaster* wild-type *per*$^+$ larvae. Fifteen larvae were monitored in LD 12:12; four such larvae were significantly periodic (Table I). Phase analysis superimposed and plotted the behavioral records. The filled bars (corresponding to 0.5 h) represent relative activity levels of the four larvae during darkness (Hamblen-Coyle *et al.*, 1989). The open bars show activity levels in the light. Dots above the bars indicate variations in the relative activity levels for a given bin (SE).

Data Collection and Computational Methods

The movement of a foraging larva across a nutritive agar–yeast substrate was traced to give path lengths of the recorded locomotor activity. Path lengths were viewed on a monitor (Sony Trinitron color video monitor Model KV-8A010) and subsequently digitized (Bauer and Sokolowski, 1984) to give a quantitative measure of larval locomotion as centimeters per hour. The ordering of path lengths over time created an activity profile for each larvae of approximately 412 min. The data from larvae that were later observed to pupate and emerge were used in subsequent analyses to ensure that the larvae observed were healthy.

Analyses of digitized activity data were performed by applying chi-square periodograms (Hamblen *et al.*, 1986), maximum entropy spectral analysis

(MESA), and autocorrelations (Dowse and Ringo, 1989a) to determine if periodic components were present in the data sets.

RESULTS

Larval Locomotion

No significant path length differences were observed (Student–Newman–Keuls, $\alpha = 0.05$) among the wild-type larvae and the three *per* types. The behavior of 50 larvae was measured for each strain. The mean larval path length (cm $\pm$ SE) for each of the strains was as follows: per^+, 7.0 $\pm$ 0.4; per^s, 6.7 $\pm$ 0.3; per^{LI}, 6.8 $\pm$ 0.3; and per^o, 7.5 $\pm$ 0.4. Thus the strains did not differ in their larval locomotion on an aqueous yeast suspension.

Larval Light Responses

Larvae of all strains distributed themselves randomly under red light conditions [$\chi^2_{11} = 10.4$, NS (Stalker, 1942)]. This condition consisted of an assay plate with four quadrants, two having no illumination and two with red light (see Materials and Methods). The mean $\pm$ standard error (sample size) of the response indices were $per^+ = 0.03 \pm 0.09$ (3), $per^s = 0.05 \pm 0.07$ (3), $per^{LI} = 0.00 \pm 0.04$ (3), and $per^o = 0.15 \pm 0.07$ (3). These results indicated that we could use these red light conditions in our larval circadian locomotor monitoring experiments. A one-way ANOVA showed that there was no significant strain differences in the larval phototactic response to white light [$F_{(3,8)} = 0.89$, NS]. Therefore mutations at the *per* locus did not affect larval photobehavior and all larvae responded to light by showing a pattern indistinguishable from that of the wild type.

Diel Behavior in Light:Dark Cycles

Four of 15 per^+ larvae under L:D 12:12 and constant temperature exhibited periodic behavior which could be construed as rhythmicity in light:dark cycles (Table I). However, subsequent phase analysis showed that these larvae exhibited no anticipation of lights-on. Their locomotor activity increased only after the lights went on (Fig. 2 shows the larval response after lights-on graph). This increase in activity subsided and remained fairly constant until lights-off, which triggered another increase in locomotion (Fig. 2). This indicates that these rhythms were not mediated by a clock; rather, they were a response to changing light conditions (see Hamblen-Coyle *et al.*, 1992; Wheeler *et al.*, 1993).

We examined the short *period* mutant (per^s) in an attempt to increase the probability [for instance, per^s has been reported to exhibit especially strong adult rhythms (Dowse and Ringo, 1987)] of observing any behavioral manifes-

Table I. Summary of Diel Behavior in Third-Instar *D. melanogaster* Larvae Under Varied Light and Temperature Regimes

Genotype	Number rhythmic[a]	Number circadian[b]	Total larvae tested	Condition	
				Light	Temperature
per^+	4	0	15	L:D 12:12	19°C
	1	0	15	.DD	20°C:15°C, 12:12
per^o	0	0	6	L:D 12:12	19°C
	0	0	10	DD	20°C:15°C, 12:12
per^s	1	0	16	L:D 12:12	19°C

[a]Data files were analyzed by autocorrelation and MESA ($\alpha = 0.05$) (Dowse *et al.*, 1987; Dowse and Ringo, 1987, 1989, 1993), to ask if a larva showed significant rhythmicity in its behavioural record. Autocorrelation was used to determine if a rhythm was significant ($\alpha = 0.05$). Periodicity was determined using MESA.

[b]The determination of circadian rhythmicity was based upon the anticipation of the 24-h period. Anticipation of periodic cycle changes was assessed in larvae which exhibited periodicity that resembled circadian cycling using phase analysis (Hamblen-Coyle *et al.*, 1992; Wheeler *et al.*, 1993) which determined larval locomotor peak positions related to the onset of environmental cycle changes.

tation of *per* in larvae under a circadian light environment. One of 16 per^s larvae had a period resembling circadian behavior (Table I). However, once again, phase analysis (see Hamblen-Coyle *et al.*, 1992) showed no anticipation of light transitions. Therefore the periodicity in this per^s larva was a response to light cycles, and not circadian.

None of the six per^o mutants, tested under light:dark cycles, exhibited periodic behavior resembling circadian rhythmicity. In addition, all of the per^o mutants failed to anticipate light transitions and did not increase locomotor activity in response to ambient light changes (Table I).

Diel Behavior in High:Low-Temperature Cycles

Cyclic variations in temperature have been shown to have an effect on rhythmic activity in adult *Drosophila* (Wheeler *et al.*, 1993). To determine the effect of temperature upon larvae, we examined *per* normal and null mutant larvae for "forced" rhythmicity under the conditions of circadian cycling high and low temperatures (H:L 12:12) in constant darkness.

Fifteen wild-type larvae were placed under cyclical temperature conditions (H:L 12:12) throughout development and subsequently observed. One of the 15 larvae tested did exhibit what appeared to be diel locomotor rhythms (Table I). The activity plot for this larva is shown in Fig. 3. Phase analysis determined that this larva was indeed responding to the periodic temperature changes and was thus "forced" into rhythmicity (Table I). This was also the case for separate rhythmic larvae (of this genotype) tested under L:D 12:12. Rhythmic larval

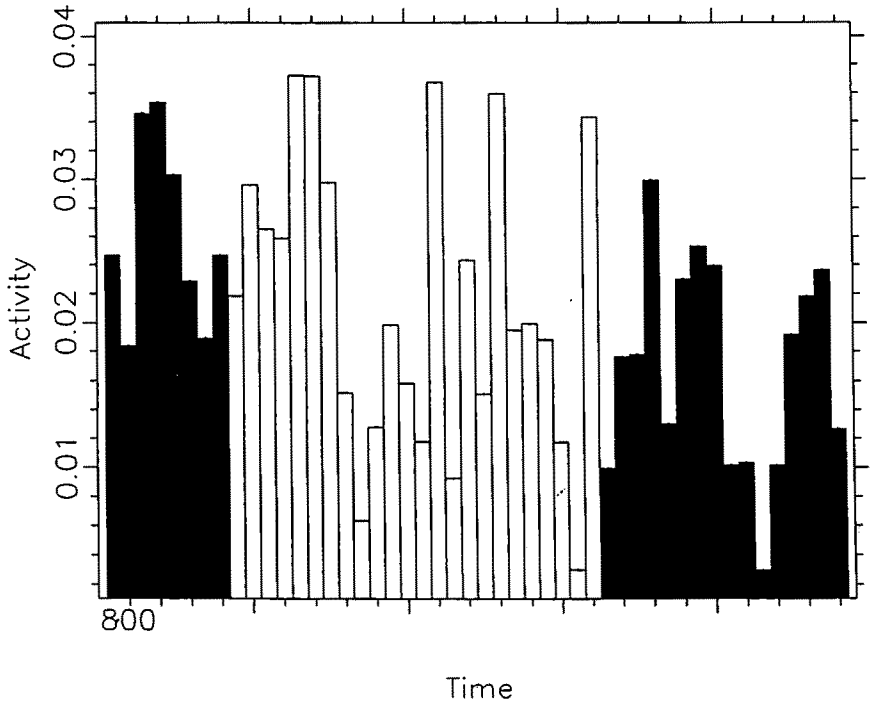

Fig. 3. Diel activity average for the one *D. melanogaster* wild-type per^+ larva that was significantly periodic in daily temperature cycles of H:L 12:12. This average activity plot was generated as described in the legend to Fig. 1, with filled bars corresponding to activity levels at low temperatures and open bars representing behavior at high temperatures. In this single-larva plot, 4 day's worth of diel activity is displayed by superposition of separate days of activity.

behavior was a response to environmental changes, and not the behavioral manifestation of *per*.

Larvae expressing the per^o mutation failed to express circadian rhythmically under constant-dark conditions and H:L 12:12.

Diel Behavior in Free Run

Tests of two per^+ and five per^o larvae were conducted under free-running conditions. As expected (given the LD and HL results) these per^+ and per^o larvae failed to show circadian rhythmicity under constant darkness, DD, and constant temperature. Figure 4 shows an example of an actogram, a correlogram, and a MESA plot from a per^+ larva which did not exhibit circadian rhythmicity but did show ultradian periodicities characteristic of many larvae tested. Therefore, in DD, all larvae tested proved to be arrhythmic. The only periodic components extractable were of the ultradian variety. Ultradian rhythms were found

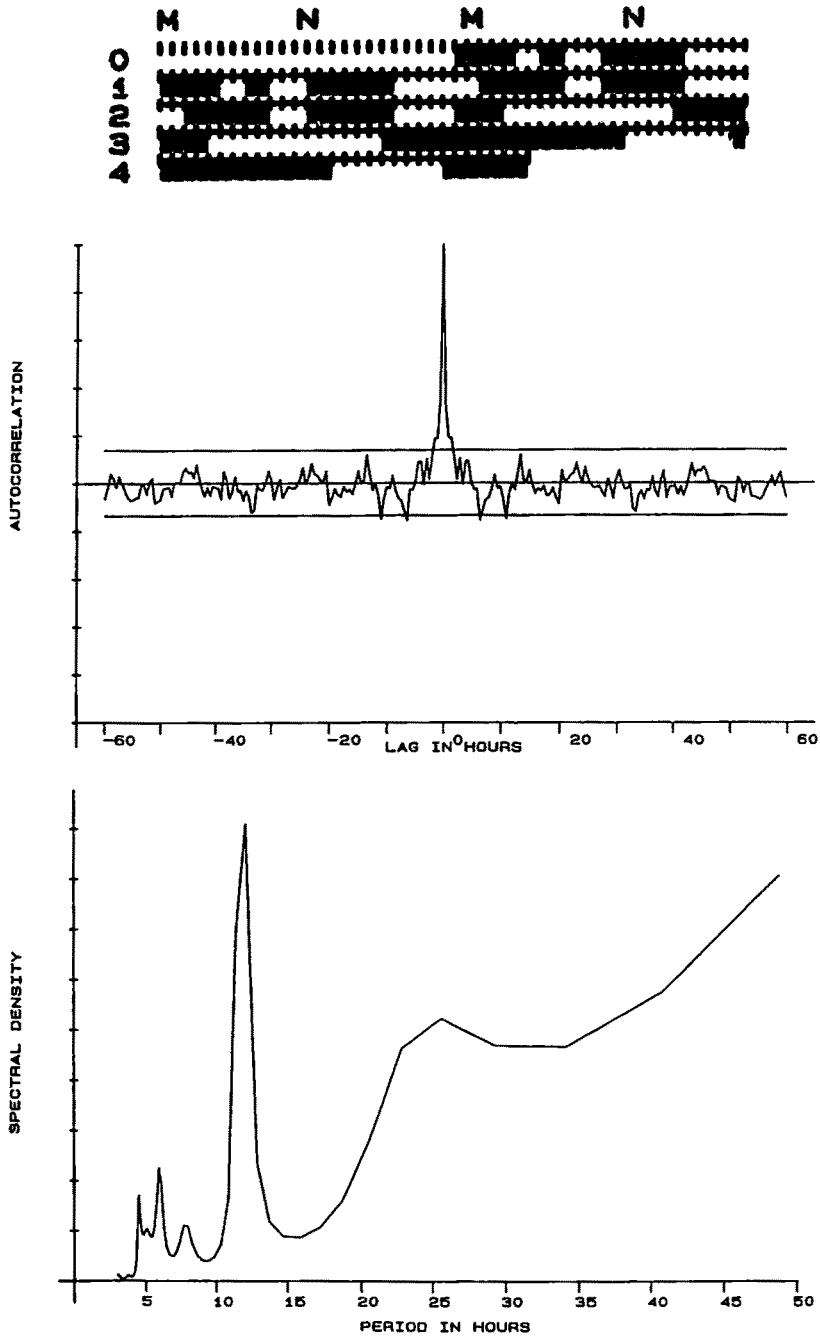

Fig. 4. Analysis of locomotor activity in a DD-reared and -tested *D. melanogaster* wild-type larva. The activity profile shows ultradian activity; circadian periodicities are absent. Top, actogram; middle, correlogram; bottom, MESA plot showing a 12.0-h periodicity.

in 54% of all larvae tested in all strains. Ultradian rhythms have periods about 5–15 h long and have been reported previously to accompany the adult locomotor activity of *per*-null mutants (e.g., Dowse *et al.*, 1987; Hamblen-Coyle *et al.*, 1989).

DISCUSSION

Experiments in the present study applied newly developed analysis of larval locomotor behavior to extend our knowledge of temporal expression of the *per* gene in *D. melanogaster*. Specifically, the experiments we performed were designed to analyze larval locomotor activity patterns for circadian rhythmicity.

The detection of behavioral rhythms at the larval stage has not been previously attempted in *Drosophila*. In this study, the locomotor activity in light : dark cycles of *D. melanogaster* larvae expressing both the wild-type (per^+) and the short period (per^s) genotypes at the *period* locus were found to be devoid of any kind of obvious rhythmicity. Behaviorally, this was characterized by either an arrhythmic phenotype or periodic fluctuations induced by environmental changes, providing no evidence of clock function. Such forced periodicities consisted of one locomotor maximum following a given light-to-dark transition and a second maximum very soon after a dark-to-light transition. For a locomotor rhythm detected under cyclically fluctuating conditions to be considered clock-controlled, anticipation of the cyclic changes must be observed (e.g., Wheeler *et al.*, 1993). Since *D. melanogaster* larvae did not anticipate these changes, (and in any case only 5 of 31 of the animals tested showed forced rhythms), our results indicate that these animals' circadian clock (which is running, given the aforementioned studies of time-memory) is weakly coupled to, or does not interact at all with, output pathways that mediate larval behavior.

Cyclical fluctuations in larval locomotion were observed in our temperature fluctuation experiments. Temperature cyclings alone (i.e., in constant darkness) are relatively effective Zeitgebers for poikilothermic insects in postembryonic stages (Zimmerman *et al.*, 1968; Winfree, 1972; Saunders, 1982; Wheeler *et al.*, 1993). Such cycles could, however, be expected to entrain larval locomotor behavior more effectively than light cycles, since larvae live within their food medium. Therefore temperature may be a more pertinent phase cue than light. Yet the current study revealed that the 24-h periodicities exhibited by larvae subjected to temperature cycles of HL 12 : 12 (1 of 15 larvae tested) reflected activity-level increases following the environmental transitions; again, we infer these to be mere responses to temperature changes.

Analysis of larval activity rhythms revealed strong periodicities in the ultradian range ($\tau < 18$ h; $\alpha = 0.05$) in 54% of the animals tested, with individuals usually having multiple ultradian rhythms. Such ultradian components are also observed in conjunction with locomotor activity (monitored in DD) of various

kinds of aperiodic adult *Drosophila* [*per*^{o}-expressing, *disco* mutant, constant dark-reared (Dowse *et al.*, 1987, 1989; Dowse and Ringo, 1989b)]. That the larva's behavioral records also can have ultradian components extracted analytically (such high-frequency rhythms were not readily observable in the behavioral records) implies that this anomalous rhythmicity may routinely accompany *Drosophila* behavior that is devoid of circadian components. This is an additional argument against the larva's ability to mediate a circadian behavioral rhythm.

The lack of observable circadian locomotor activity found in the present study can be interpreted as consistent with the absence of detectable *per* expression in the larval nervous system—if one also assumes that all circadian behavioral rhythms in this species are controlled by this gene's action. It could have been that embryonic *per* expression (see references above) would have influenced behavioral rhythmicity at a later stage (i.e., pupae); but this does not fit with studies of adult rhythms, which have shown that ongoing action of the gene is necessary for the clock to run, or for fluctuating behavior to be manifested, or both (Ewer *et al.*, 1988, 1990).

Finally, the thoroughgoing absence of any known influence of the *period* gene on larval phenotypes—to which conclusion the current study contributes—suggests that the clock running in *Drosophila* developing through this stage of the life cycle is controlled by factors other than *per*.

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