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Ontogeny of a biological clock in *Drosophila melanogaster*

(circadian rhythms/locomotor activity/entrainment/*per* gene)

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ABSTRACT **_Drosophila melanogaster_ born and reared in constant darkness exhibit circadian locomotor activity rhythms as adults. However, the rhythms of the individual flies composing these populations are not synchronized with one another. This lack of synchrony is evident in populations of flies commencing development at the same time, indicating that a biological clock controlling circadian rhythmicity in *Drosophila* begins to function without a requirement for light and without a developmentally imparted phase. It is possible to synchronize the phases of rhythms produced by dark-reared flies with light treatments ending as early as the developmental transition from embryo to first-instar larva: Light treatments occurring at developmental times preceding hatching of the first-instar larva fail to synchronize adult locomotor activity rhythms, while treatments ending at completion of larval hatching entrain these rhythms. The synchronized rhythmic behavior of adult flies receiving such light treatments suggests that a clock controlling circadian rhythms may function continuously from the time of larval hatching to adulthood.**

Drosophila exhibit locomotor activity rhythms with a circadian (≈24 hr) period. The phases of these rhythms are entrained by light/dark cycles, typically 12 hr of light followed by 12 hr of dark, with flies showing diurnal behavior. Activity rhythms persist when environmental cycles are removed and behavior is monitored in constant darkness, with activity occurring predominantly at times corresponding to subjective day (1, 2). Eclosion, or hatching of the adult fly from the pupa, is also rhythmic and is similarly entrained by light/dark cycles (1, 2).

Several mutations have been identified that affect circadian behavioral rhythms in *Drosophila melanogaster* (3–7). Of particular interest are mutations at the *per* (period) locus. Molecular analysis of *per* has shown that deletion of the gene results in arrhythmicity, while a variety of long- and short-period rhythms can be produced by amino acid substitutions in the *per* protein or by changing the level of *per* expression (8–12). *per* is expressed in many cell types throughout development (13–17). By blot analysis, *per* RNA is first detected in embryos 8–12 hr old when cultured at 25°C (13, 18), and *in situ* hybridization (ref. 13; M. Baylies, S. Kidd, and M.W.Y., unpublished data) and immunocytochemical studies (ref. 16; M. Baylies, S. Kidd, and M.W.Y., unpublished data) show a restricted pattern of *per* expression in the early embryonic central nervous system. Since *per* is essential for the production of adult circadian rhythms, embryonic expression may label cells controlling rhythms in early development. In this report we present behavioral evidence for the existence of a functioning biological clock at the developmental transition from embryo to first-instar larva.

MATERIALS AND METHODS

Staging of Embryos and Light Treatment. *D. melanogaster* (Canton-S strain) were entrained as described in *Results*. All light treatments provided ≈3000 lx of cool fluorescent light. Half-pint "egg collection bottles" were covered with Petri dishes containing grape juice medium for egg deposition (19). Fresh grape plates were added daily, with hourly replacements preceding all 4-hr episodes of egg collection. "Dark" manipulations were carried out under an overhead safelight (15-W bulb with Kodak GBX-2 filter). For embryos receiving light treatment 3C (Fig. 1), ≈6% hatched (producing first-instar larvae) prior to lights-off. For light treatment 3D (Fig. 1), hatching to produce first-instar larvae was essentially complete at lights-off.

Locomotor Activity Assays. Locomotor activity rhythms of individual flies from dark-reared populations and populations that received light during early development were measured, and periodogram analysis was performed as described (ref. 20; Fig. 2 legend). Activity was monitored for 7 days with an Apple IIe data collection program (21). The phase of each fly was defined as the median time of activity offset during the 7-day monitoring period. Degree of synchrony and strength of phasing within populations were calculated as described (ref. 22; Fig. 3 legend).

RESULTS

Dark-Reared *Drosophila* Are Consistently Rhythmic with Varied Phases of Activity. Fig. 1 shows the experimental protocol followed for all studies. Prior to collection of fertilized eggs, mothers were entrained to three 12 hr/12 hr light/dark cycles and then held in constant darkness at 24°C (Fig. 1 *Upper*). Since egg laying is elevated during subjective late afternoon (23), this procedure leads to a high level of egg laying 20–24 hr after transfer to constant darkness. Eggs collected over this 4-hr interval were reared to adulthood in continued total darkness and at constant temperature (24°C) or were subjected to various light treatments and then allowed to develop to adulthood in the dark (Fig. 1 *Lower*). Newly eclosed flies from such cultures were tested for locomotor activity rhythms.

When previously entrained wild-type flies are monitored in constant darkness, they show 24-hr rhythms with activity predominantly confined to subjective day. The phase of the rhythm (or distribution of activity) is the same in all flies that have seen the same entrainment regime. Fig. 2*a* shows a representative activity record (*Left*) and periodogram (*Right*) for a fly from a control population entrained by two 12 hr/12 hr light/dark cycles (see Fig. 1, treatment 2A). The first 12-hr light treatment was initiated during embryogenesis (from 12 to 24 hr after egg collection). The second began during the first larval instar, 12 hr after completion of the first exposure (from 36 to 48 hr after egg collection). Flies from populations entrained in this manner produced circadian locomotor activity rhythms that were synchronized to the phase of the entraining light regime (for Fig. 2*a*, time 0 corresponds to lights on and time 12 corresponds to lights off during entrainment).

Fig. 2 *b–f* show five representative periodograms and corresponding activity records for flies reared throughout

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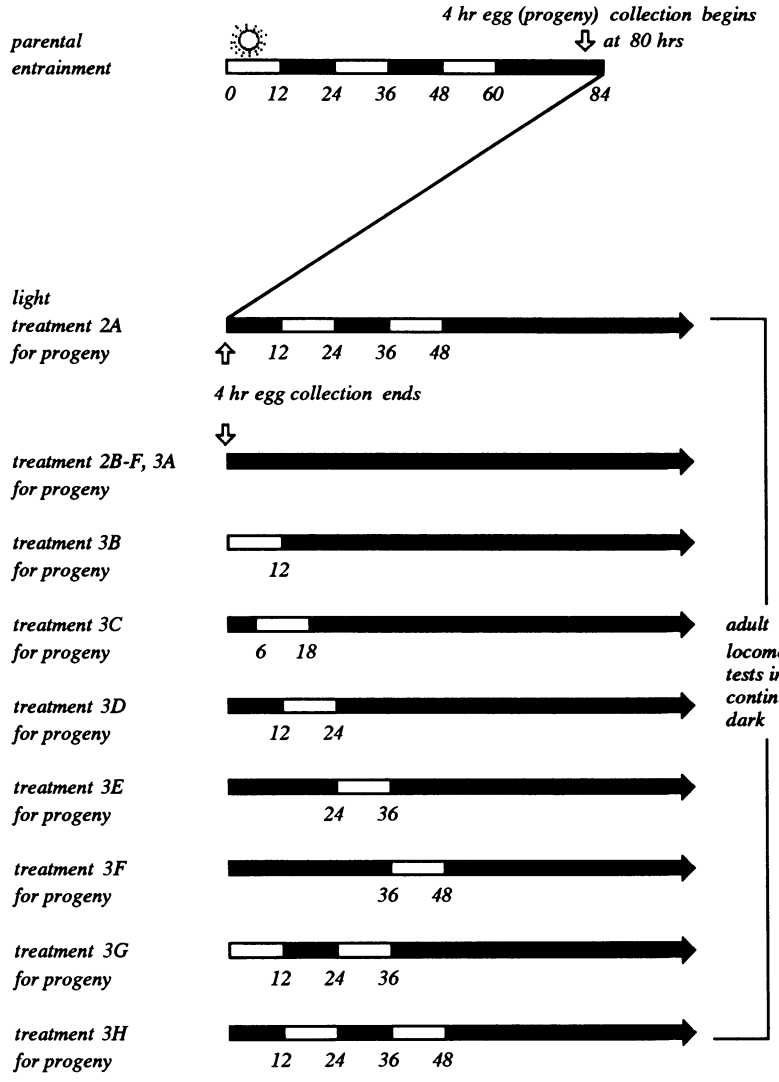

FIG. 1. Experimental protocol. (*Upper*) Parental entrainment. Before collection of fertilized eggs, parents were entrained to three 12 hr/12 hr light/dark cycles and then held in constant darkness at 24°C. Lights on, open blocks; dark intervals, filled blocks. Numbers below the time line represent hours after first lights on during parental entrainment. Beginning 80 hr after this first lights on, eggs were collected for 4 hr. (*Lower*) Treatments for progeny. Collected eggs were allowed to develop to adulthood in complete darkness (treatment 2B–F, 3A) or in darkness following one or two exposures to light during embryogenesis or development of the first larval instar (treatments 2A, 3B–3H). Numbers below each time line represent hours after completion of egg collection. Locomotor activity tests were performed on newly eclosed flies from such cultures as described in *Materials and Methods*. Results of adult locomotor activity tests for flies exposed to treatments 2A–F and 3A–H during development are found in corresponding panels of Figs. 2 and 3 and in Table 1.

development in constant darkness and at constant temperature. No measurable differences in the periods of the rhythms are observed in comparing flies from the experimental (constant dark) and control (entrained) populations (compare also populations A and H in Table 1). Moreover, the fraction of flies showing rhythmic activity was not diminished in the experimental group as compared with entrained controls (Table 1).

Activity records for dark-reared flies differed from those of entrained flies when the phases of the activity rhythms were examined (Fig. 2 *b–f Left*). For the control population, phases of locomotor activity rhythms were synchronized by the lighting regime provided during embryonic and larval development, whereas rhythmic bouts of activity occurred at different times of day for the different dark-reared flies. For example, a comparison of activity records in Fig. 2 *b–f* illustrates the behavior of several flies developing from the same egg collection, but what is subjective day for one fly clearly can be subjective night for another (compare Fig. 2*b Left* with Fig. 2*c Left*). A total of 117 dark-reared flies (including the 83 listed in Table 1, population A) were analyzed in locomotor activity assays. Of these flies, 93% were rhythmic but showed no evidence for similar phasing (Table 1, population A, and data not shown). The randomness of the phases of rhythmic activity for flies reared in constant darkness is demonstrated by plotting phase data for flies from one such experimental group as depicted in Fig. 3*A*. When the average phase (time of activity offset) is plotted for each fly around the circumference of a 24-hr clock face, there is no significant bias in the distribution that would indicate coordination of the individual fly activity rhythms (see further discussion below). Since no statistically significant phase could be assigned to the population of constant-dark-reared flies (Table 1, population A, and Fig. 3*A*), the phases of the progeny also did not bear any measurable relation to the time of egg collection or to the entrainment regime of the parents. Thus, no evidence was seen for maternal inheritance of phase information or for developmentally imparted phase.

Consistent with previous observations (24, 25), the eclosion profile of flies raised in constant darkness was found to be arrhythmic (data not shown). The eclosion data can now be explained, given that dark-reared flies are individually rhythmic whereas their phases are not synchronized within the population. In fact, eclosion, which is a population rhythm, is expected to be arrhythmic from our analysis of the behavior of individual dark-reared flies.

Light Treatments Ending Early in Larval Development Set the Phase of the Adult Locomotor Activity Rhythm. We examined the effects of light treatments provided at different stages of development (listed in Table 1) in an attempt to synchronize the phases of otherwise dark-reared flies. Each population produced a high percentage of rhythmic individuals, all of which had periods close to 24 hr (Table 1). In agreement with results described for constant-dark-reared flies, no correlation was seen between duration of light received by the variously entrained populations and relative numbers of flies showing rhythmicity in the populations. Nor were there differential effects on the period lengths of activity

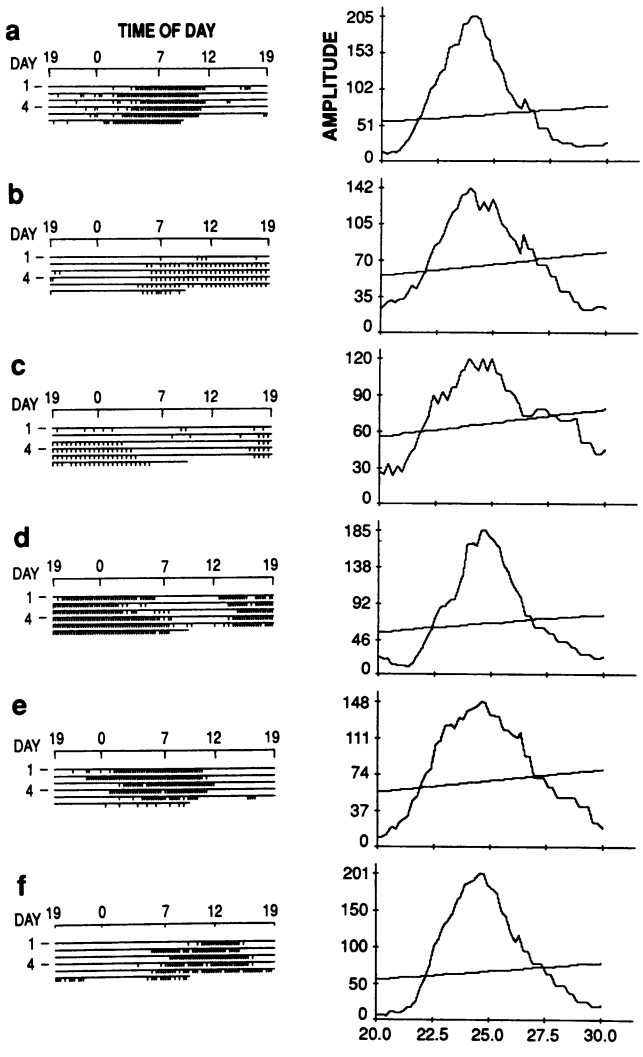

FIG. 2. Locomotor activity rhythms of dark-reared flies. (*a*) Activity record (*Left*) and periodogram (*Right*) from a control fly that was exposed to two 12 hr/12 hr light/dark cycles (Fig. 1, treatment 2A, and *Materials and Methods*). (*b–f*) Activity records and corresponding periodograms of dark-reared files (Fig. 1, treatment 2B–F, 3A). Time 0 shown in activity record *a* is lights on for the entrained fly and time 12 is lights off, whereas 0 and 12 in activity records *b–f* indicate lights on and lights off, respectively, for entrained parents of dark-reared flies (see Fig. 1). A 0.05 level of significance (indicated by the sloping line) was used for periodogram analysis (20). Activity records display consecutive days (top to bottom). Each inflection on the activity record represents >50 signals (>5 in case of *b*) per half-hour. Numbers at bottom of periodogram *f* refer to tested range of periodicities (in hours) for all periodograms.

rhythms (Table 1). As described for constant-dark-reared flies, the phases of the circadian locomotor activity rhythms for all *Drosophila* tested in a particular entrainment regime were plotted on a circular clock face. For Fig. 3*B–H*, 0 on the clock face indicates lights on and 12 indicates lights off during entrainment. For Fig. 3*D*, lights on (0 time) occurred during embryogenesis, and lights off (12 hr) occurred at the time of hatching of the first larval instar (*Materials and Methods*; light treatments illustrated in Fig. 1 *Lower*). An average locomotor activity phase for each population of flies reared with a given lighting regime was calculated by vector addition (22) and is indicated by the direction of the arrow inscribed on each clock face. The strength of this phasing (*r* value; see Fig. 3 legend) is indicated by the length of the arrow within each clock face, and the statistical significance of phasing was determined by applying the Rayleigh test (ref. 22; calculations in Fig. 3 legend).

A significant result to emerge from these tests involved the behavior of adult *Drosophila* receiving light during the developmental transition from embryo to first-instar larva. In contrast to the behavior of constant-dark-reared flies (Fig. 3*A*) and flies reared with light treatments ending during embryogenesis (Fig. 3 *B* and *C*), whose activity rhythms show phases scattered around the clock face, a single 12-hr light treatment beginning at mid-embryogenesis and ending at completion of larval hatching produces clear phasing (Fig. 3*D*). Comparison of Fig. 3 *C* and *D* indicates a special role for development around the time of larval hatching; a light treatment ending just prior to hatching had no measurable effect on phase of the adult rhythm (Fig. 3*C*), whereas extension of the light treatment to cover the period of larval hatching resulted in unambiguous phasing (Fig. 3*D*). While the data presented in Fig. 3*B* indicated possible phasing of a population receiving light treatments only during early embryonic stages of development, related experiments involving 4- and 8-hr light treatments ending 12 hr after egg collection failed to confirm phasing (37 flies receiving an 8- to 12-hr light treatment and 27 flies receiving a 4- to 12-hr light treatment showed no significant phasing, $P > 0.10$, and produced *r* values of 0.18 and 0.035, respectively; clock faces not shown). Note also the low *r* value calculated for Fig. 3*B* (representing strength of phasing; see Fig. 3 legend), and the poor correlation of the estimated phase with phase of entrainment (see also Table 1 legend).

For Fig. 3 *D–H*, generally, the same 12-hr light treatment produces somewhat stronger effects on phasing when administered later in development, with maximum phasing probably achieved in first-instar larvae (J.P., unpublished observation; see also *r*-value calculations of Fig. 3 legend). The progressive improvement in synchronization could be due to ongoing development of tissues composing a biological clock, to incomplete development of tissues essential for entrainment, or to incomplete development of photoreception at earlier developmental stages.

As shown in the circular plots of Fig. 3 *D–H*, not only was each lighting condition capable of synchronizing the locomotor activity rhythms of flies composing each experimental population, but also the phase of average activity offset at adulthood was predicted by the time of lights off during entrainment (compare phases of Fig. 3 with light treatments of Fig. 1 *Lower*; see also Table 1 legend). A strong correlation is also observed by *inter se* comparison of the plots of phase shown in Fig. 3 (similar orientations of phase arrows in clock faces *D–H*; Table 1 legend). There is apparently little damping or change in the phase of the synchronized rhythms, even after at least 10 days of development in constant darkness. Together, the results indicate that by initiation of the first larval instar, *Drosophila* are able to perceive light and, without further stimulation, can store this phase information for later manifestation in the adult. This strongly implies the action of a biological clock at the developmental boundary separating embryogenesis and the first larval instar.

DISCUSSION

The results demonstrate that a clock controlling circadian behavioral rhythms can assemble in *Drosophila* in the absence of light. A similar result also was observed in *Drosophila* carrying the per^s mutation. per^s flies reared in constant darkness produced endogenous locomotor activity rhythms with 19-hr periods (data not shown), consistent with behavior previously observed for entrained per^s flies (3). Development of circadian rhythms in the absence of light, or light cycles, has already been reported for several distantly related organisms (26–28). For example, hamsters born to mothers with

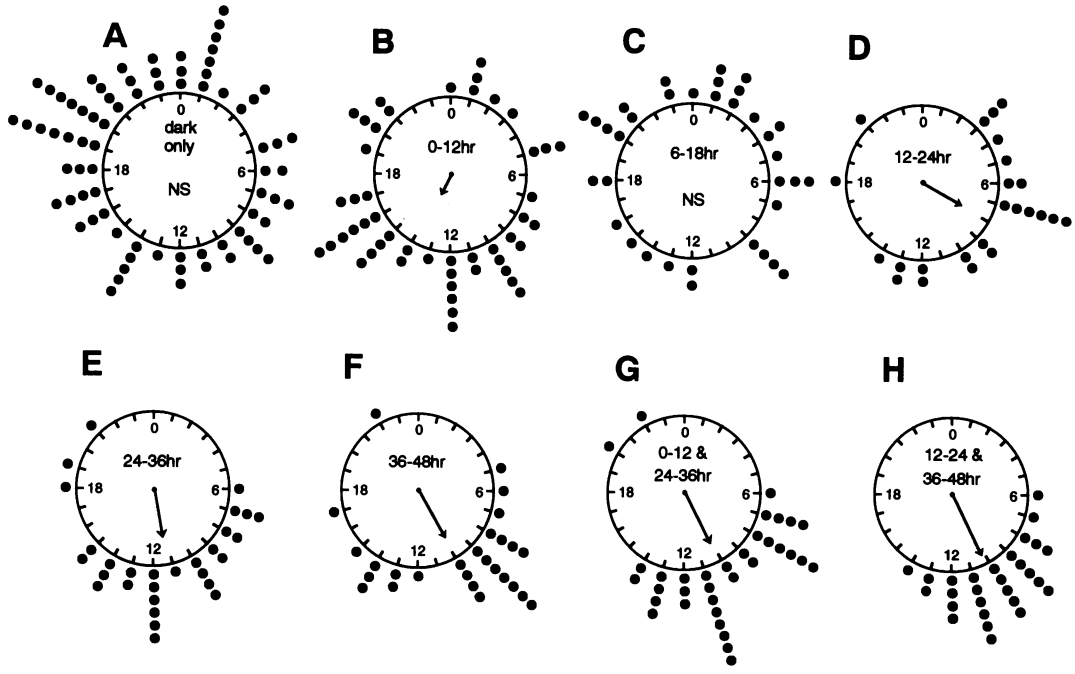

FIG. 3. Distribution of phases for dark-reared flies and flies exposed to light in early development. Each open circle represents a 24-hr clock face. Small filled circles show locomotor activity phases of individual flies. Light treatments received by populations A–H are indicated in Fig. 1 and Table 1 and inside each clock face. For *B–H*, time 0 corresponds to lights on and 12 to lights off during entrainment. For *A*, numbering on the clock face reflects entrainment of the parents of these dark-reared flies (see also Fig. 1 and *Materials and Methods*). For all clock faces except *A* and *C* (see below), the average phase (daily activity offset) of each population is indicated by the orientation of the arrow at the center of the face. The length of the arrow reflects the degree of synchrony in the population and corresponds to the *r* value as described (22). The *r* values were calculated as 0.19 (*A*), 0.32 (*B*), 0.18 (*C*), 0.54 (*D*), 0.65 (*E*), 0.68 (*F*), 0.73 (*G*), and 0.89 (*H*). Probabilities for *A–H* [probability that the distribution of observed results from sampling a population of randomly phased individuals as calculated by the Rayleigh test (22)] were $P > 0.05$ (*A*), $P < 0.01$ (*B*), $P > 0.10$ (*C*), $P < 0.001$ (*D*), $P < 0.001$ (*E*), $P < 0.001$ (*F*), $P < 0.001$ (*G*), and $P < 0.001$ (*H*). For *A* and *C*, NS indicates no significant phase ($P > 0.05$).

ablated suprachiasmatic nuclei and subsequently raised in constant dim light are rhythmic with scattered phases (29). However, earlier studies of the ontogeny of *Drosophila* rhythms have been inconclusive (25, 30, 31). In particular, Dowse and Ringo (32) suggested that dark-rearing may produce a high frequency of locomotor arrhythmia; only 7 of 31 flies studied produced rhythms with periods close to 24 hr, while the rest showed locomotor arrhythmia or produced rhythms with a variety of long and short periodicities. Analysis of a larger population of dark-reared flies in the present study indicates no difference in the frequency of circadian rhythmicity in dark-reared flies compared with flies exposed to light/dark cycles, but there are several differences in experimental protocol that may account for the quantitative differences between this and the former study. In particular, flies in the former study were reared for several generations in constant darkness. Nevertheless, the results from both studies are qualitatively similar. In both cases, circadian rhythms were found but without consistent phase.

A second conclusion from this work is that a biological clock is active very early in *D. melanogaster* development. Earlier work in this and other *Drosophila* species indicated function of a clock controlling the adult eclosion rhythm in larvae and pupae (2, 25, 30, 33). However, no attempt was made in those studies to map the early ontogeny of such a clock(s), particularly in reference to embryogenesis. Previous reports have demonstrated that older embryos of cockroach and moth can be entrained, but embryogenesis for both

Table 1. Effects of different light treatments on rhythmicity, period length, and phase of dark-reared flies

Population	Light treatment, hr after egg collection	No. of flies in sample	% rhythmic	Average period, hr (mean ± SD)	Average phase
A	None	83	92	24.22 ± 0.52	NS
B	0–12	53	92	23.86 ± 0.63	13:20
C	6–18	38	92	24.13 ± 0.57	NS
D	12–24	29	83	23.96 ± 0.74	7:50
E	24–36	31	94	24.11 ± 0.46	11:20
F	36–48	29	93	24.05 ± 0.66	10:00
G	0–12, 24–36	36	94	24.12 ± 0.71	10:10
H	12–24, 36–48	33	85	24.05 ± 0.53	10:10

In each case eggs were collected over 4 hr, so that if a 12-hr light treatment starts immediately after egg collection, it ends when embryos are 12–16 hr of age, etc. (see also Fig. 1). Populations A–H correspond to data plotted in Fig. 3 *A–H*. Average phases shown were calculated from circular plots of Fig. 3, as described in the figure legend, and represent average activity offsets. For B–H if activity offset and lights off during entrainment were coincident, predicted phase would be 12:00 (rightmost column). However, activity offsets tend to occur ≈2 hr prior to lights off for these entrained populations (e.g., Fig. 2*a* and Fig. 3*H*; unpublished observations). Average phases provided in the rightmost column of the table indicate hours and minutes. NS, no significant phase at 0.05 level (see also Fig. 3 legend).

lasts many days (26, 34). In contrast, a *D. melanogaster* clock emerges within the first few hours of development. As eggs are always collected over a 4-hr period prior to application of any of our lighting schedules, a 12- to 24-hr light treatment would have been complete when *Drosophila* ranged in age from 24 to 28 hr. Single light pulses lasting only minutes will synchronize eclosion rhythms when provided during later larval and pupal stages of *D. melanogaster* development (25). This suggests that a *Drosophila* clock may begin to function in the relatively short developmental interval encompassing hatching of the first larval instar. Alternatively, a clock regulating circadian rhythms could form during embryogenesis, followed at larval hatching by development of tissues supporting photic entrainment.

The data suggest that *Drosophila* must develop a system for linking photoreception and circadian behavioral rhythms by the onset of the first larval instar. The photoreceptor organ (Bolwig's organ) that mediates negative phototactic behavior of the *D. melanogaster* larva is present in the embryo and expresses a photoreceptor-specific antigen by 16 hr of development (35, 36), but it is not known to function in light reception prior to larval development. Although the development of this photosensory organ is well correlated with first light-dependent entrainment of circadian rhythms, reception and processing of light for adult entrainment of these rhythms do not appear to depend upon ocular photoreceptors, which are derived, in part, from tissue associated with Bolwig's organ in the larva (33, 37). Several eye mutants exhibit circadian rhythms that can be entrained, indicating that extraocular elements for light reception are sufficient or required (6, 39). This also has been suggested by surgical ablation and implantation studies in flies and moths (38, 40). It may also be important that carotenoid-based photopigments do not appear to play a role in photic entrainment of *D. melanogaster* eclosion rhythms (33, 41).

It is intriguing that *per*, a gene central to the control of adult circadian rhythms, is first expressed in the embryo at a time somewhat earlier than the onset of clock activity measured by our behavioral studies. *per* mRNA is detected in developing embryos by *in situ* hybridization with *per* DNA probes about 5 hr after egg laying (M. Baylies, S. Kidd, and M.W.Y., unpublished data). Until the 11th hour of development at 25°C, *per* mRNA is expressed in <200 cells composing the developing embryonic central nervous system (M. Baylies, S. Kidd, and M.W.Y., unpublished data). The number of *per*-expressing cells increases severalfold by the end of embryogenesis, but *per* expression falls dramatically upon hatching of the first-instar larva (refs. 13–18; M. Baylies, S. Kidd, and M.W.Y., unpublished data). Possibly, cessation of *per* expression marks completion and initial function of a *Drosophila* clock that runs continuously from larval hatching through adulthood.

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1. Pittendrigh, C. S. (1974) in *The Neurosciences Third Study Program*, eds. Schmitt, F. O. & Worden, F. G. (Massachusetts Institute of Technology Press, Cambridge, MA), pp. 437–458.
2. Konopka, R. J. (1981) *Handb. Behav. Neurobiol.* **4**, 173–181.
3. Konopka, R. J. & Benzer, S. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2112–2116.
4. Jackson, F. R. (1983) *J. Neurogenet.* **1**, 3–15.
5. Newby, L. M., White, L., DiBartolomeis, S. M., Walker, B. J., Dowse, H. B., Ringo, J. M., Khuda, N. & Jackson, F. R. (1991) *Genetics* **128**, 571–582.
6. Dushay, M. S., Rosbash, M. & Hall, J. C. (1989) *J. Biol. Rhythms* **4**, 1–27.
7. Dushay, M. S., Konopka, R. J., Orr, D., Greenacre, M. L., Kyriacou, C. P., Rosbash, M. & Hall, J. C. (1990) *Genetics* **125**, 557–578.
8. Bargiello, T. A. & Young, M. W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2142–2146.
9. Jackson, F. R., Bargiello, T. A., Yun, S. H. & Young, M. W. (1986) *Nature (London)* **320**, 185–187.
10. Citri, Y., Colot, H. V., Jacquier, A. C., Yu, Q., Hall, J. C., Baltimore, D. & Rosbash, M. (1987) *Nature (London)* **326**, 42–47.
11. Baylies, M. K., Bargiello, T. A., Jackson, F. R. & Young, M. W. (1987) *Nature (London)* **326**, 390–392.
12. Yu, Q., Jacquier, A. C., Citri, Y., Hamblen, M., Hall, J. C. & Rosbash, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 784–788.
13. James, A. A., Ewer, J., Reddy, P., Hall, J. C. & Rosbash, M. (1986) *EMBO J.* **5**, 2313–2320.
14. Bargiello, T. A., Saez, L., Baylies, M. K., Gasic, G. & Young, M. W. (1987) *Nature (London)* **328**, 686–691.
15. Liu, X., Lorenz, L., Yu, Q., Hall, J. C. & Rosbash, M. (1988) *Genes Dev.* **2**, 228–238.
16. Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M. & Hall, J. C. (1988) *Neuron* **1**, 141–150.
17. Saez, L. & Young, M. W. (1988) *Mol. Cell. Biol.* **8**, 5378–5385.
18. Young, M. W., Jackson, F. R., Shin, H. S. & Bargiello, T. A. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **50**, 865–875.
19. Ashburner, M. & Wright, T. R. F. eds. (1978) *Genetics and Biology of Drosophila* (Academic, New York), Vol. 2a.
20. Hamblen, M., Zehring, W. A., Kyriacou, C. P., Reddy, P., Yu, Q., Wheeler, D. A., Zweibel, L. J., Konopka, R. J., Rosbash, M. & Hall, J. C. (1986) *J. Neurogenet.* **3**, 249–291.
21. Sulzman, F. M. (1982) *Comput. Biol. Med.* **12**, 253–261.
22. Batschelet, E. (1972) in *Animal Orientation and Navigation*, eds. Galler, S. R., Schmidt-Koenig, K., Jacobs, G. J. & Belleville, R. E. (National Aeronautics and Space Administration, Washington), pp. 61–91.
23. Allemand, R. (1977) *C. R. Acad. Sci. Ser. D* **284**, 1553–1556.
24. Kalmus, H. (1940) *Nature (London)* **145**, 72–73.
25. Brett, W. J. (1955) *Ann. Ent. Soc. Am.* **48**, 119–131.
26. Page, T. L. (1990) *J. Biol. Rhythms* **5**, 273–289.
27. Aschoff, J. (1960) *Cold Spring Harbor Symp. Quant. Biol.* **25**, 11–28.
28. Browman, L. G. (1952) *Am. J. Physiol.* **168**, 694–697.
29. Davis, F. C. & Gorski, R. A. (1988) *J. Comp. Physiol. A* **162**, 601–610.
30. Zimmerman, W. F. (1969) *Biol. Bull. (Woods Hole, Mass)* **136**, 494–500.
31. Winfree, A. T. (1973) *Biological & Biochemical Oscillators*, eds. Chance, B., Kendall Pye, E., Ghosh, A. K. & Hess, B. (Academic, New York), pp. 461–501.
32. Dowse, H. B. & Ringo, J. M. (1989) *Physiol. Zool.* **62**, 785–803.
33. Zimmerman, W. F. & Ives, D. (1971) in *Biochronometry*, (Natl. Res. Council, Washington), pp. 381–391.
34. Minis, D. H. & Pittendrigh, C. S. (1968) *Science* **159**, 534–536.
35. Pollock, J. A. & Benzer, S. (1988) *Nature (London)* **333**, 779–782.
36. Steller, H., Fischbach, K. & Rubin, G. (1987) *Cell* **50**, 1139–1153.
37. Page, T. L. (1982) *Experentia* **38**, 1007–1013.
38. Truman, J. W. (1974) *J. Comp. Physiol.* **95**, 281–296.
39. Helfrich, C. (1986) *J. Neurogenet.* **3**, 321–343.
40. Helfrich, C., Cymborowski, B. & Engelmann, W. (1985) *Chronobiol. Internatl.* **2**, 19–32.
41. Zimmerman, W. F. & Goldsmith, T. H. (1971) *Science* **171**, 1167–1169.