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Circadian Systems, V. The Driving Oscillation and the Temporal Sequence of Development*

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Abstract. A circadian oscillation (in the brain) of *Drosophila* spp. acts as a gating device restricting the emergence behavior of the adult to a limited fraction of each 24-hour cycle defined by that oscillator, but the oscillation does *not* gate intermediate steps of pupal development. Unlike the emergence act, such intermediate events in development occur at fixed times after prepupa formation and are totally independent of the phase of the ongoing oscillator that gates emergence behavior.

Earlier papers[1–3] from this laboratory have described a circadian oscillation in the larva and pupa of *Drosophila pseudoobscura*. That oscillation serves as a gating device that determines the time of day at which the fully developed adult emerges from the puparium.

In two papers[4, 5] and a general monograph,[6] J. E. Harker has reported some observations on the timing of the developmental sequence in *D. melanogaster* pupae. She relates the timing of easily observed events (head eversion, eye pigmentation, and wing pigmentation) to the time of adult emergence on the one hand and the environmental light cycle on the other. She interprets her observations as evidence against the model developed in this series and in earlier papers, that a circadian oscillation in each individual pupa controls the eclosion time of that pupa. Her own conclusions include the propositions: (1) that the eclosion rhythm is a "population effect" and presumably therefore not a function of an oscillation in the individual; and (2) that a fly simply emerges when its development is concluded—that the act of emergence marks the termination of development, and that the time of emergence is determined not by a gating oscillation but simply as the sum of all intermediate developmental steps. The principal point in the observations Harker reported was that the interval between two successive steps in pupal development such as head eversion (t_h), appearance of yellow eye pigment (t_y), or appearance of wing pigmentation (t_w), etc. is not fixed; specifically, the timing of each event (even at constant temperature) is a function of the phase in the light/dark cycle at which the first event occurs. Thus Harker reports that the interval t_y-t_h is shorter when the earlier event, t_h, occurs during the hours shortly after dawn than at later times in the cycle.

That observation of itself would have led the present writers to a conclusion very different from Harker's: we would have concluded that not only is the terminal event of eclosion coupled to the environmental light cycle by a light-sensitive oscillator, but so also are intermediate developmental events (t_y, t_w, etc.) between t_0 (prepupa formation) and t_e (the eventual act of eclosion). That conclusion from Harker's data was, moreover, very attractive to us insofar as it would have conformed with the speculation published earlier[1, 7] that gene induction may commonly be restricted to a limited phase of the circadian oscillation in cells. We therefore embarked on the present experiments in the hope of confirming Harker's observations in *D. pseudoobscura*.

Materials and Methods. Three species of *Drosophila* (*melanogaster* (Princeton University strain 103), *pseudoobscura* (Princeton University strain 301), and *victoria* (University of Delaware strain 101)) were used. Culture methods and procedures for collecting prepupae have been described previously.[2, 8] All experiments reported here involve populations of pupae (50–75 individuals) that were developmentally synchronous in the sense that they consisted of insects known to have become prepupae within a defined period of 1 hr. The midpoint of that 1-hr "collection window" is taken as time zero (t_0) for further development. The prepupae were mounted, immediately after collection, on temperature-controlled brass platforms. Some collections and subsequent observations were made in white light (about 1000 lux); others were made in dim red light (600 nm and above) which is functionally darkness as far as the light-sensitive circadian oscillation is concerned.[8] In both cases the light was passed through heat-absorbing filters to eliminate (minimize) temperature change in the flies at the time of observation which was made through a dissecting microscope at 2-hr intervals throughout pupal life. The time was recorded for each individual pupa at which the head everted (t_h), yellow pigmentation developed in the eye (t_y), black pigment appeared in the ocellar bristles (t_b), and the adult fly ultimately emerged (t_e). Late in pupal life, when it could be confidently established by pigmentation of the testes, the sex of every pupa was noted. Each population of about 60 developmentally synchronous pupae thus comprises two separate populations of about 30 pupae of each sex. In the discussions that follow, most of our points are made by reference to the males; the female populations behave identically except for their faster absolute rate of development.[9]

Results and Discussion. (1) **The time course of development in the absence of a circadian oscillation:** Continuous high-intensity white light damps out the circadian oscillation that gates the act of emergence: populations of developmentally asynchronous insects have an aperiodic distribution of emergence times.[1, 3] Figure 1 shows the time course of development in developmentally synchronous pupae (t_0 range = 1 hr) under continuous light as it is marked by the occurrence of yellow eye pigmentation, ocellar bristle pigmentation, and ultimate adult emergence in *D. pseudoobscura* at 20 and 25°C and in *D. melanogaster* at 25°C. The rate of development is, of course, strongly temperature-dependent and differs between the sexes: females develop faster than males, and *D. melanogaster* develops faster than *D. pseudoobscura*.

There is considerable interindividual variation in the rate of pupal development. In *D. pseudoobscura* males, for example, the initial range of 1 hour within which t_0 occurred was amplified to 14 hours for bristle pigmentation (t_b) and to a range of 28 hours for emergence events (t_e). The distributions of t_y, t_b, and t_e in Figure 1 are frequency distributions that may be treated as probability distributions: they measure the probability with which the event in question (t_y, t_t, t_e) will occur in an individual pupa as a function of time after t_0.

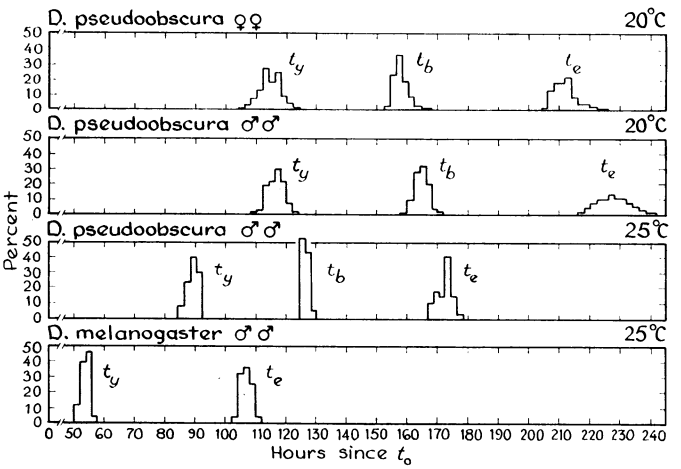

FIG. 1.—The time course (in continuous light) of emergence events, and some prior developmental steps, in populations of *Drosophila* pupae that became prepupae within a known 1-hr interval ($t_0 \pm 0.5$ hr).

(2) **The time course of development in the presence of a circadian oscillation:** Figure 2 summarizes observations on 27 populations of developmentally synchronous male pupae: the individuals in each population were harvested within a one-hour collection window from cultures reared in continuous light. The plotted points in the top panel of the figure are median times—for each population separately—at which development began (t_0), yellow eye pigment appeared (t_y), ocellar bristle pigment appeared (t_b), and adult flies eventually emerged (t_e). The panel also indicates the time (t_i) at which the previously continuous white light was turned off. Earlier papers have established that a circadian oscillation is initiated at the light/dark transition and that it starts from a fixed phase point which is defined as "circadian time" 12 (or ct 12), halfway through the full scale of 24 "hours" of circadian time. The figure also indicates the predicted midpoints (at ct 03) of the gates during which emergence *may* occur. The first gate occurs 15 hours after t_i and subsequent gates recur at 24-hour intervals thereafter.

It is clear from the top panel (*a*) in Figure 2 that emergence activity *does not* occur after a fixed interval from t_0: it occurs only within the gates (~6 hr wide) that recur at ~24-hour intervals after t_i + 15 hours. It is equally clear that the events of eye and bristle pigmentation marking developmental progress in the pupa, *do* occur at fixed intervals after t_0 and that these events (t_y and t_b) are therefore insensitive to the phase of the ongoing oscillation (in the brain) which gates emergence activity. The interval t_y-t_0 is 115.4 hours; t_b-t_0 is 163.4 hours.

Panels (*b*), (*c*) and (*d*) of Figure 2 clarify further the fundamentally different relationship to the circadian oscillation that exists between adult emergence on the one hand and intermediate steps in pupal development on the other. The second half of the 27 individual populations (numbers 14 through 27) of panel (*a*) are treated as a single (synthetic) population in panel (*b*). The only event that is synchronous in all 306 pupae of the synthetic population is t_i, the light/dark

transition that initiates a circadian oscillation. In the 16 pupae of population 27, t_0 occurred 64 hours before t_i; in the 22 pupae of population 26, t_0 occurred 62 hours before t_i, and so on; t_0 events in the synthetic population of 306 individual pupae are thus distributed essentially at random through 28 hours as the histogram of panel (*b*) indicates. The observed distributions (within the synthetic population) of t_y, t_b, and t_e for all 306 insects are also given as histograms. The distributions of t_y, t_b, and t_e which are plotted as polygons in panel (*b*) are predictions based on the null hypothesis that the timing of none of these events (t_y, t_b, or t_e) will be affected by the circadian oscillation initiated in all pupae synchronously at t_i. The predicted distributions are obtained by totaling the 306 probability distributions for t_y, t_b, and t_e for each of the 306 pupae whose t_0 is known. The probability distributions used are the frequency distributions of t_y, t_b and t_e in Figure 1.

Figure 2 shows that the observed distributions (histograms) of t_y and t_b are a close fit to the predicted distributions (polygons); but the observed distribution of t_e is radically different from the distribution of t_e predicted on the assumption of no gating. An oscillation does, in fact, split the unimodal distribution of t_e into two entirely discrete peaks, but it has no such effect on the distribution of either t_y or t_b.

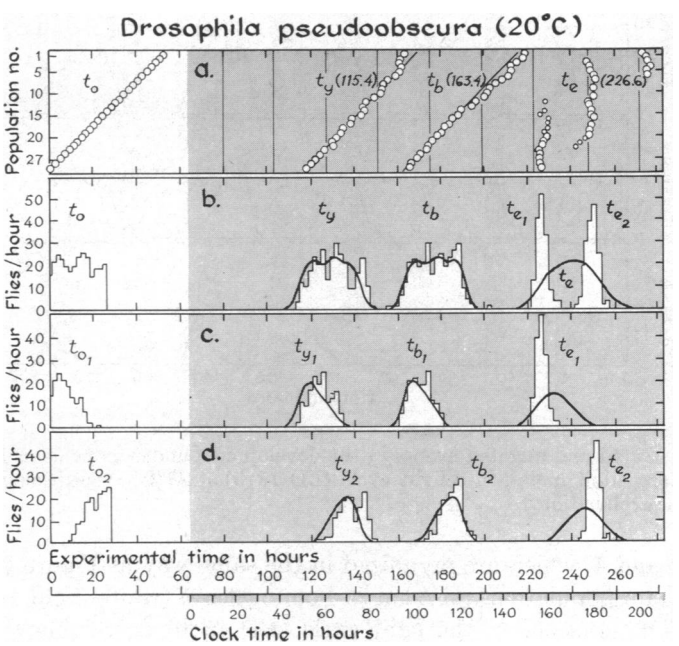

FIG. 2.—Gated and ungated events in the development and emergence of *Drosophila pseudoobscura* adult males after a single step from continuous light to continuous darkness at 20°C. See text for explanation of most of the detail. In panel(*a*) the plotted points are medians for 27 individual populations of developmentally synchronous pupae. In some populations, emergence events (t_e) fall into two discrete peaks as described earlier by Skopik and Pittendrigh.[3] When this happens, the median of the smaller emergence peak is plotted as a smaller circle.

Since the history of each pupa was recorded individually, we can trace the distributions of t_0, t_y, and t_b for all the flies that later happen to emerge in peaks 1 and 2 separately (panels (*c*) and (*d*) of Fig. 2). It is not surprising that the distribution of t_0 is, on average, earlier for flies that emerge in peak 1 than it is for those that emerge in peak 2. The distributions of t_y and t_b can again be predicted on the assumption of no gating and knowledge of the actual distribution of t_0. Such predictions are again closely matched by the observed distributions of those two developmental events. That, however, is not the case for t_e: the oscillation *advances* t_e for the majority of flies that emerge in peak 1, without any comparable advancing effect on the distribution of t_y or t_b; it *delays* the distribution of t_e for the majority of those flies in peak 2 without significantly affecting the distribution of either t_y or t_b.

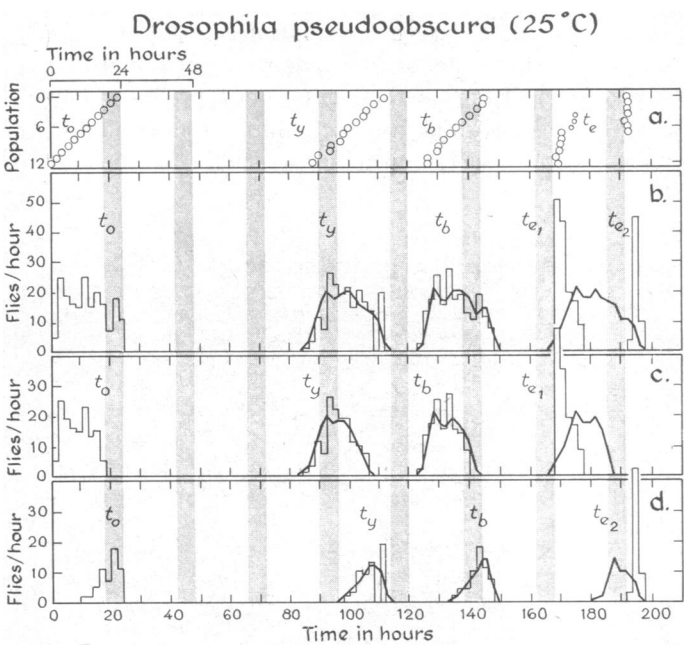

FIG. 3.—Gated and ungated events in the development and emergence of *Drosophila pseudoobscura* adult males, in a light cycle (LD *18*:6) at 25°C. (See legend to Fig. 2 and text for explanation.)

Figures 3 and 4, which are organized in the same way as Figure 2, give comparable data for populations raised in 24-hour cycles of (white) light and darkness at 25°C. The presence of the light cycle (LD *18*:6) entraining the circadian oscillation, has no effect on the basic feature of the observations made in Figure 2: intermediate steps in development are *not coupled* to a circadian oscillation, nor are they coupled in any way to the light cycle directly. Thus the observed distributions of t_y and t_b are a close match to predictions based on the observed distribution of t_0. On the other hand the observed distribution of t_e is again split into two discrete peaks.

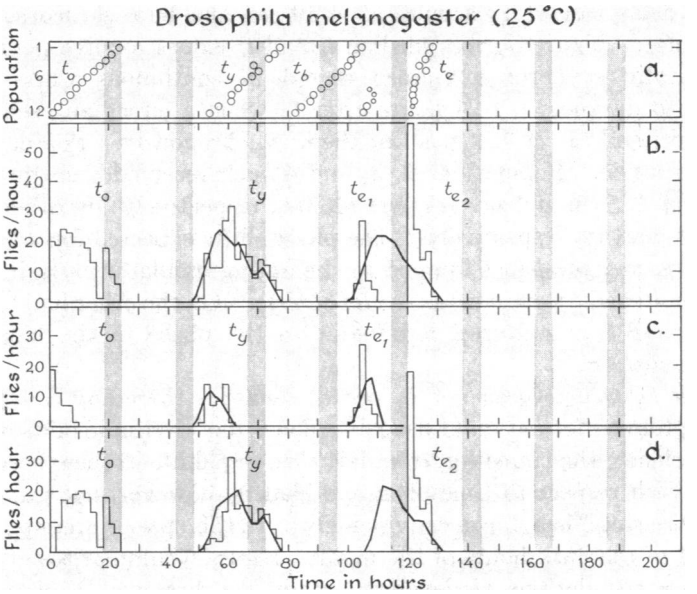

FIG. 4.—Gated and ungated events in the development and emergence of *Drosophila melanogaster* adult males, in a light cycle (LD *18*:6) at 25°C. (See legend to Fig. 2 and text for explanation.) t_b events have been omitted from panels (*b*), (*c*), and (*d*) to avoid overcrowding.

The experiments summarized by Figure 5 extend the demonstration of this point, using *D. pseudoobscura* in which the effect of single light pulses on the phase of the oscillation controlling emergence time is well known.[1, 2] Single pulses cause phase shifts ($\Delta\phi$) whose sign and magnitude are a function of the phase (ϕ) at which the light is seen. Pulses of 15-minute duration of high intensity white light (400<lux) at circadian times (ct) 16, 17, 18, and 19 will cause, for example, phase delays of 6, 8, 11, and 14 hours, respectively. The 14-

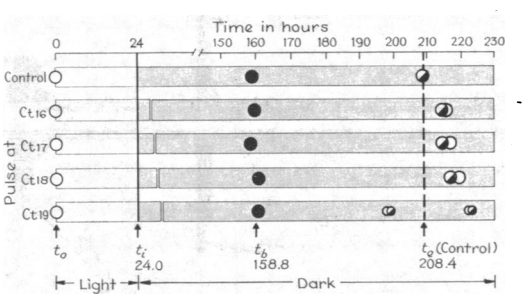

FIG. 5.—Insensitivity of t_b to phase shifts of gating oscillator that controls t_e. *Drosophila pseudoobscura* ♂♂ at 20°C (see text).

Light pulses of 15 min falling at ct 16, 17, 18, and 19 are indicated in histories of the 2nd, 3rd, 4th, and 5th populations of developmentally synchronous pupae. In the control population the midpoint of the 8th gate after t_i occurs at 207 hr [15 + 8(24)]; observed t_e in the control was 208.4 hr. In the 2nd, 3rd, and 4th populations all the flies emerge in the delayed 8th gate. Phase shift caused by pulse at ct 19 is such as to permit fastest developing flies to emerge in gate 7; the remainder emerge in gate 8. None of the phase shifts disturbs t_b relative to the control.

hour phase delay caused by a pulse at ct 19 can also be regarded as a 10-hour phase advance. Figure 5 gives median t_b and t_e values for five populations of developmentally synchronous pupae reared in continuous white light and transferred to darkness (t_i) 24 hours after t_0, when a circadian oscillation was therefore initiated (at ct 12) in all of them synchronously. Population 1 was left as the control. Populations 2, 3, and 4 each received a single 15-minute pulse of light 4, 5, 6, and seven hours after t_i, respectively; in other words, at ct 16, 17, 18, and 19, respectively. The phase shifts expected (*open circles*) as a result of these perturbations applied to the gating oscillator early in pupal life are closely matched by observation (*solid circles*). On the other hand, t_b remains the same in all four cultures, indifferent to the phase of the light-sensitive circadian oscillation.

In neither *D. melanogaster* nor *D. pseudoobscura* is there any convincing evidence that prepupa formation or head eversion is day rhythmic in its occurrence. In our experience, the transition from larval to pupal life in these species occurs at random with respect to time of day. That is, however, not the case in *D. victoria*, as Rensing[10] has shown convincingly. In this species prepupa formation is restricted to the dark hours of the night. Figure 6 summarizes observations made on two populations of developmentally synchronous *D. victoria* pupae. Both populations consist of insects that formed prepupae in the same one-hour interval that was the modal hour (plotted in solid black) in a total distribution which is fully reported in that figure as an otherwise open histogram. In the upper panel it is seen that in those insects, head eversion (t_h) occurred at 21.9 hours, t_y occurred at 125.2 hours, t_b at 179.4 hours, and t_e at 226.0 hours after t_0.

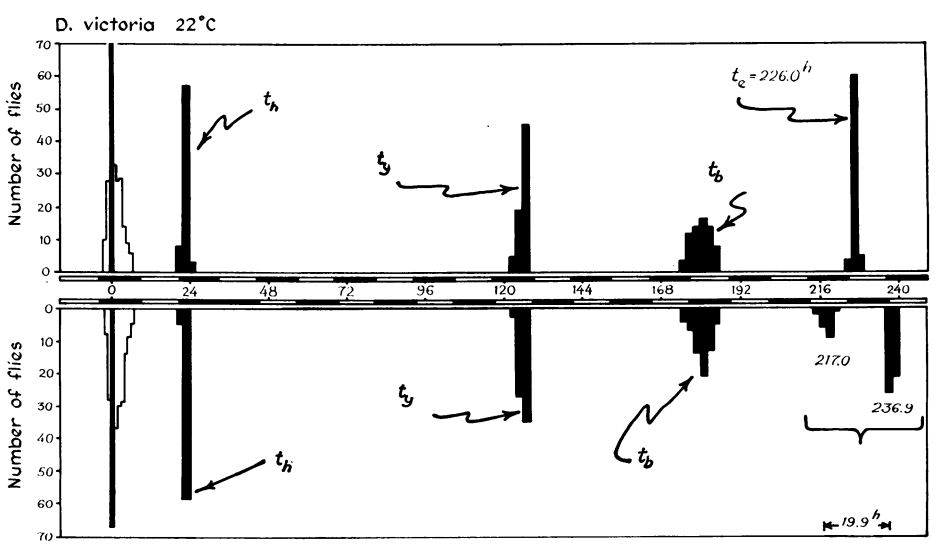

FIG. 6.—Gated and ungated elements in developmental history of *Drosophila victoria* pupae at 22°C.

The value of t_h in the upper panel is 21.9 hr; it is also 21.9 hr in the lower panel.
The value of t_y in the upper panel is 125.2 hr; it is 125.0 hr in the lower panel.
The value of t_b in the upper panel is 179.4 hr; it is 179.6 hr in the lower panel.

Emergence activity, unlike prepupa formation, occurs at the beginning of the light period. In the lower panel of the figure the phase of the light/dark cycle is shifted 180°, after head eversion, by extending the second dark period by an additional 12 hours. In spite of the resulting phase shift in the light cycle, t_y and t_b occur at the same time as before but emergence activity is split into two totally discrete peaks which occur at the beginning of two successive light periods. Thus in spite of the fact that both the beginning and the end of pupal life are clock controlled in *D. victoria*, the timing of intermediate developmental steps is not.

Discussion. The data presented here show that the situation Harker reported for her strains of *D. melanogaster* does not obtain in *D. pseudoobscura*, it does not obtain in *D. victoria*, nor is it found in the strain of *D. melanogaster* that we used. In these three strains, involving three different species, the temporal sequence of developmental steps is *not* coupled to the circadian oscillation that is demonstrably present throughout pupal development and whose phase so clearly controls the time of emergence. Nor is the rate of intermediate steps in development affected directly by the light/dark cycle.

We cannot explain the observations Harker reported; but we can proceed with our analysis of how the *behavioral* act of emergence is timed, confident that, as such, it is not just the terminal step of the developmental sequence in the strict sense; and that it is phased by a circadian oscillator which on the one hand we can manipulate predictably with light pulses, and on the other has no role in regulating the rate at which the developmental sequence, as such, proceeds.

We have had unpublished data for many years showing that the phase of the gating oscillator can be established by light seen at least as far back as the third larval instar. This fact and Kalmus' old evidence[11] that the *Drosophila* clock is in the anterior end of the pupa strongly suggest that the brain is the locus of the driving oscillation; the brain is about the only larval structure that survives the upheaval of metamorphosis.

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[1] Pittendrigh, C. S., *Z. Pflanzenphysiol.*, **54**, 275 (1966).

[2] Pittendrigh, C. S., these PROCEEDINGS, **58**, 1762 (1967).

[3] Skopik, S. D., and C. S. Pittendrigh, these PROCEEDINGS, **58**, 1862 (1967).

[4] Harker, J. E., *J. Exptl. Biol.*, **42**, 323 (1965).

[5] Ibid., **43**, 411 (1965).

[6] Harker, J. E., *The Physiology of Diurnal Rhythms* (London: Cambridge University Press, 1964), 114 pp.

[7] Pittendrigh, C. S., in *Science and the Sixties* (U.S. Air Force Office of Scientific Research, Cloudcroft Symposium, 1966), p. 96.

[8] Frank, K. D., and W. F. Zimmerman, *Science*, **163**, 688 (1969).

[9] We thank Mr. Arthur Winfree for the program used for the machine computation of means and variances from our raw data on developmental steps and eclosion events.

[10] Rensing, L., and R. Hardeland, *J. Insect Physiol.*, **13**, 1547 (1967).

[11] Kalmus, H., *Z. Vergl. Physiol.*, **16**, 362 (1938).