# *CIRCADIAN SYSTEMS, II. THE OSCILLATION IN THE INDIVIDUAL DROSOPHILA PUPA; ITS INDEPENDENCE OF DEVELOPMENTAL STAGE**

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The circadian rhythmicity of adult emergences in a population of *Drosophila* pupae has been the object of considerable study.[1–16] The facts that the experimental system is a population and that the act of emergence occurs only once in the life of the individual have been responsible for some confusion in the literature.[8–10] Harker[9] appears to deny the existence of a circadian oscillation in the individual: "the eclosion rhythm is a population effect," and "the final eclosion rhythm is dependent on whatever processes control the developmental rate throughout the pupal stage" (p. 336).

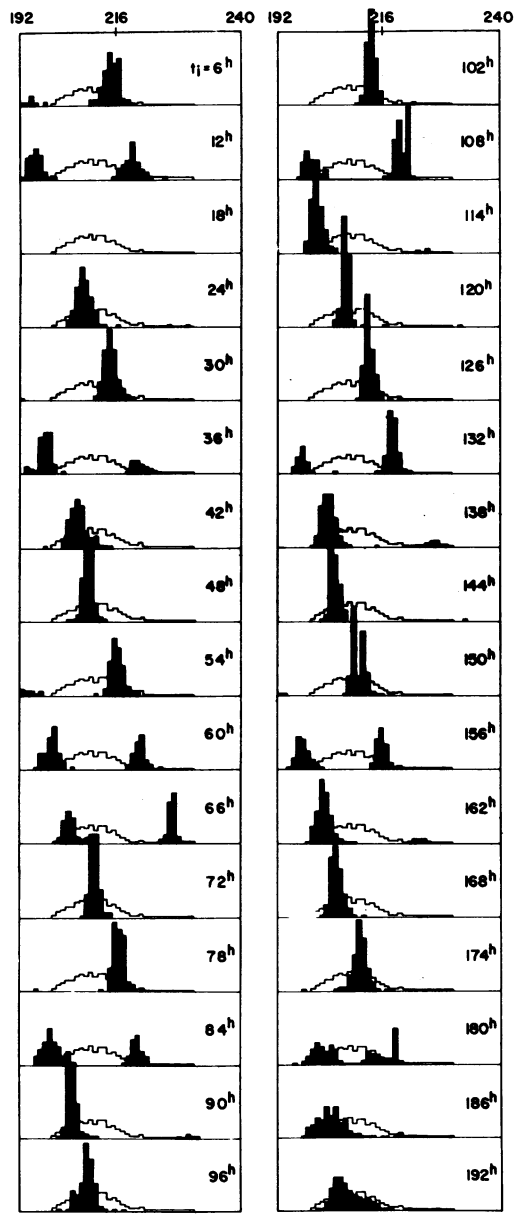
This paper demonstrates the reality of that oscillation without recourse to the use of population rhythmicity. It treats the circadian oscillation as a gating oscillation fully independent of rate and stage of morphogenesis and differentiation;[1, 17] it gates the act of emergence to a restricted phase of the oscillation; and since the oscillation itself locks on to the light cycle in nature, it therefore gates adult emergence to a limited time of day.

Populations of pupae which manifest a rhythm of emergence activity consist of individuals which are *not* synchronous developmentally, but are fully synchronous in their circadian oscillations. While such populations have been the principal, most useful, tool for studying the (individual's) oscillation which underlies and causes the (population's) rhythm, they are by no means necessary for that task. Populations of *developmentally synchronous* pupae can be created by selecting newly formed puparia within a short time interval. These populations do not, of course, display a rhythm of emergence; their eclosion activity is compressed into a single peak (or, at most, two). They do, however, lend themselves well to very explicit demonstrations of the presence of a circadian oscillation throughout pupal life and its control of the eventual time of emergence; and in representing, as they do, an individual pupa merely replicated several hundred times, they minimize the potential conceptual difficulties of utilizing a population to analyze the properties of an individual.

*Materials and Methods.*—All of the experiments were conducted with *Drosophila pseudoobscura* (PU stock 301). About ten pairs of parents were placed in vials (25 × 95 mm) which contained 1 in. of Muller's fly food, and the females were allowed to deposit eggs for a 24-hour period. The parents were then transferred to fresh food vials; for each experiment 2 or 3 transfers were made. The cultures were maintained at 20 ± 1°C and in constant light.

About 11 days after egg laying, mature 3rd-instar larvae crawl up out of the food onto the sides of the vials. They form puparia which are white at first but soon tan. Just after puparium formation the prepupae can be removed very easily with a spatula, without injury, from the walls of the vials. In our experiments prepupae (before tanning of the cuticle begins) were so removed during a

FIG. 1.—Distributions of adult emergences from 32 populations of developmentally synchronous pupae (*solid histograms*) that were transferred from constant light to constant darkness ($t_i$) 6 hr, 12 hr, 18 hr, 192 hr after the beginning of their pupal development. The beginning of pupal developmet ($t_0$) is measured from the midpoint of a 5-hr pupal collection period. Each population is plotted against an emergence distribution derived by summing the distributions of emergences in 3 populations of pupae maintained in constant light (*open histograms*). Only the range from 192 to 240 hr. from $t_0$ is presented here, because emergence invariably occurs within these limits when the pupae are maintained at 20°C.




fixed five-hour time interval. Each experimental population contained 250–500 pupae whose development began at a time ($t_0$) defined as the mid-point of the five-hour collection period; and to this extent they were developmentally synchronous.

After collection, the prepupae were attached to brass plates with a nontoxic, water-soluble, casein glue. The brass plates are components of a fraction-collector device which traps newly emerged flies at hourly intervals. This apparatus permits very rigorous control of the lighting and temperature (20.00 ± 0.05°C) conditions under which the pupae proceed to completion of development.

The experimental data discussed here all pertain to females. With respect to the points we develop *in this paper* males give identical results and our entire argument could have been based on them. The sex differences that do exist lead into a discussion that is reserved for a later paper.[18] The terminology of the oscillator model for circadian phenomena is defined in the previous paper of this series.[19]

*Results and Discussion.*—(1) *Emergence time and its variance in the absence of an oscillation:* Circadian rhythmicity in many organisms damps out in sufficiently intense constant light.[4] Figure 1 (*unfilled histograms*) shows the wide aperiodic distribution of emergence times ($t_e$) in a composite of three replicate populations of pupae which were raised and maintained in constant light. Table 1 shows the values of $t_e$ (median emer-

TABLE 1

The history of 32 experimental and 3 control populations. All 35 were "developmentally synchronous" in the sense they comprised individuals which began pupal life within a 5-hr interval, the mid-point of which is taken as zero time ($t_0$). All were reared in LL; the controls (populations 33, 34, and 35) were kept in LL. The others were transferred to DD at times, given as $t_i$ (hours since $t_0$). They all emerged at times given as $t_e$ (hours since $t_0$). The table gives its median to characterize $t_e$ of the population, and gives the variance ($S^2$) on the mean of $t_i$ as a measure of its dispersion.

| Culture no. | $t_i$ | $t_{e(1)}$ | $S^2$ | $t_{e(1)}-t_i$ | Gate no. | $t_{e(2)}$ | $S^2$ | $t_{e(2)}-t_i$ | Gate no. | Interval between $t_{e(1)}$ and $t_{e(2)}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 | 211.8 | 3.1 | 205.8 | 9 | — | — | — | — | — |
| 2 | 12 | 195.3 | 2.6 | 183.3 | 8 | 216.4 | 3.6 | 204.4 | 9 | 21.1 |
| 3 | 18 | — | — | — | — | — | — | — | — | — |
| 4 | 24 | 205.8 | 3.5 | 181.8 | 8 | — | — | — | — | — |
| 5 | 30 | 211.6 | 2.2 | 181.6 | 8 | — | — | — | — | — |
| 6 | 36 | 198.1 | 2.8 | 162.1 | 7 | 217.8 | 1.9 | 181.8 | 8 | 19.7 |
| 7 | 42 | 204.6 | 3.8 | 162.6 | 7 | — | — | — | — | — |
| 8 | 48 | 206.8 | 1.6 | 158.8 | 7 | — | — | — | — | — |
| 9 | 54 | 213.0 | 2.8 | 159.0 | 7 | — | — | — | — | — |
| 10 | 60 | 199.0 | 2.3 | 139.0 | 6 | 218.1 | 2.4 | 158.1 | 7 | 19.1 |
| 11 | 66 | 202.9 | 3.1 | 136.9 | 6 | 225.1 | 1.1 | 159.1 | 7 | 22.2 |
| 12 | 72 | 208.1 | 1.2 | 136.1 | 6 | — | — | — | — | — |
| 13 | 78 | 213.3 | 1.7 | 135.3 | 6 | — | — | — | — | — |
| 14 | 84 | 198.5 | 3.0 | 114.5 | 5 | 218.3 | 2.0 | 134.3 | 6 | 19.6 |
| 15 | 90 | 203.1 | 2.6 | 113.1 | 5 | — | — | — | — | — |
| 16 | 96 | 206.7 | 3.5 | 110.7 | 5 | — | — | — | — | — |
| 17 | 102 | 212.4 | 1.5 | 110.4 | 5 | — | — | — | — | — |
| 18 | 108 | 199.0 | 1.7 | 91.0 | 4 | 218.4 | 1.8 | 110.4 | 5 | 19.4 |
| 19 | 114 | 201.6 | 2.5 | 87.6 | 4 | — | — | — | — | — |
| 20 | 120 | 206.8 | 1.8 | 87.8 | 4 | — | — | — | — | — |
| 21 | 126 | 211.7 | 1.4 | 85.7 | 4 | — | — | — | — | — |
| 22 | 132 | 198.6 | 1.7 | 65.6 | 3 | 217.0 | 1.1 | 85.0 | 4 | 19.4 |
| 23 | 138 | 203.0 | 2.2 | 65.0 | 3 | — | — | — | — | — |
| 24 | 144 | 204.3 | 1.4 | 60.3 | 3 | — | — | — | — | — |
| 25 | 150 | 209.3 | 2.6 | 59.3 | 3 | — | — | — | — | — |
| 26 | 156 | 197.3 | 2.5 | 41.3 | 2 | 214.5 | 1.7 | 58.5 | 3 | 17.2 |
| 27 | 162 | 201.9 | 2.7 | 39.9 | 2 | — | — | — | — | — |
| 28 | 168 | 204.6 | 2.2 | 36.6 | 2 | — | — | — | — | — |
| 29 | 174 | 211.0 | 2.7 | 36.9 | 2 | — | — | — | — | — |
| 30 | 180 | 201.7 | 5.8 | 21.7 | 1 | 215.5 | 7.6 | 35.5 | 2 | 13.8 |
| 31 | 186 | 203.4 | 27.5 | 17.4 | 1 | — | — | — | — | — |
| 32 | 192 | 206.3 | 19.4 | 14.3 | 1 | — | — | — | — | — |
| 33 | Control | 206.7 | 24.5 | | | | | | | |
| 34 | Control | 209.7 | 36.4 | Mean $t_i$ = 208.1 ± 0.9 hr | | | | | | |
| 35 | Control | 207.8 | 26.2 | | | | | | | |

gence time) for each of the three populations and also the variances on the mean emergence times. The mean value of $t_e$ for the three populations is 208.1 ± 0.9 hours. The differences between the three medians are trivial; but the variance for each individual population is substantial—individual $t_e$ values vary over a range in excess of 24 hours.

(2) *Emergence time and its variance in the presence of a circadian oscillation:* The transfer of populations of developmentally asynchronous pupae from constant light (LL) to constant dark (DD) initiates a circadian rhythm of emergence activity.[4] The phase of the rhythm is determined by the light/dark transition. Bursts of activity occur at times after the transition equal to 15 hours + $n\ \tau$, where $\tau$ is close to 24 hours. The *inference*[4] is that an oscillation is initiated at the LL/DD transition in all individuals irrespective of the developmental stage; subsequent emergence times are restricted to a limited phase of the oscillation so initiated.

Thirty-two populations of developmentally synchronous pupae were created from cultures raised in LL. They were then transferred to DD to initiate an

oscillation which, on hypothesis, would dictate the subsequent time of adult emergence. The LL/DD transfer, $t_t$, was made at different times of development in each population; it occurs 6 hours after $t_0$ in population No. 1, 12 hours after $t_0$ in population No. 2, etc. Table 1 lists $t_t$ and $t_e$ for all 32 populations; it also gives the variance on the mean emergence time and the value of the interval $t_e$–$t_t$. The raw data are summarized graphically in Figure 1. Several points are immediately clear.

First, $t_e$ is no longer fixed at the 208.1 hours characteristic of the LL controls; in some populations it is as low as 195 hours or as high as 225. There is an obvious periodicity in the value of $t_e$ in the series beginning with population 1 and ending with population 32.

Second, the significance of that periodicity is clear from inspection of the values of $t_e$–$t_t$: they all fall into a series defined by 15 hours + $n\ \tau$ ($\tau \sim 24$ hr). The time to emergence after $t_t$ is thus modulo $\tau$—it is controlled by a (periodic) process that measures intervals equal to $\tau$. The interpopulation differences in $t_e$ cannot be due to any light-growth effect—that is, to any action of light which accelerates or decelerates developmental rates. The value of $t_e$ is periodic in the series: populations with the same $t_e$ have experienced major differences in total illumination.

Third, the variance on emergence time is greatly reduced in all populations, compared to the variance in the LL populations unaffected by an oscillation. This reduction of variance is what is expected of the gating-oscillation model. The initial range of 5 hours in $t_0$ is enlarged to over 24 hours in the LL controls because (1) there is an interindividual variation in developmental rates, even in rigorously constant temperature; and (2) because the behavioral process of emergence itself is subject to its own very high variance.[20] The oscillation initiated by the LL/DD transition defines recurrent gates during which emergence can occur; it is *because* the gates are only about six-hours wide that the large variance characteristic of LL is compressed into a narrow range; and *because* the gates recur at intervals of $\tau$, the values of $t_e$ fall into values that are modulo $\tau$.

Fourth, the small differences in the value of $t_e$–$t_t$ among the several cultures exploiting a given gate (see Table 1) are explained by the fact the gate is about 6-hours wide. Thus, for gate 5, $t_e$–$t_t$ is 114.5 in population 14 and 110.4 in population 17. The oscillation in population 17 was initiated 18 hours later in its development than the oscillation in population 14. The flies in culture 17 were thus ready to exploit gate 5 (development was completed) sooner than even the fastest individuals in culture 14, and consequently their emergence activity falls earlier within the six-hour opportunity which gate 5 offers for emergence.

Fifth, the phasing of the gating oscillation relative to the ungated variance has other spectacular effects; it can be such (e.g., cultures 2, 6, 10, 11, 14, 18, 22, 26, and 30) that only the fastest-developing pupae in the population can exploit a given gate; slower pupae completing development in the "forbidden" phase are forced to await the next gate. Separation of the population into two discrete peaks recurs in every fourth culture. Since in our series successive $t_t$'s are separated by six-hour intervals, the bimodal distributions recur in every population with identically phased oscillations: they recur when the oscillation assumes a particular phase relative to the LL variance; that phase recurs every 24 hours ($\tau$); and the values of $t_e$ obtained for the two peaks so created are about 18–19 hours apart because the

first peak lies to the extreme right of (late in) the first gate exploited and to the left (early in) the second gate exploited.

(3) *The phase-response curve: Assay of the oscillation:* Evidence for the existence of an oscillation in the individual pupa and the independence of its period and phase from developmental stage is thus far an inference (albeit inescapable) from the values of $t_e$–$t_i$ which are modulo $\tau$. More direct demonstration that an oscillation is present throughout pupal life exploits its phase-response curve which summarizes the oscillation's responses—as phase-shifts—to single 15-minute flashes of white fluorescent light (100 ft-c) applied to phase-points throughout its cycle. All earlier studies, including the previous paper in the series,[19] derived the response curve from populations of developmentally asynchronous pupae, and used it (the curve) for *inferences* about the oscillation in the individual. Pittendrigh[4] inferred that: (1) on transfer from LL to DD the oscillation entered its steady-state motion from the phase-point circadian time (ct) 12; and (2) that its wave form in the first cycle of a DD free run was the same no matter at what developmental stage the free run was initiated. Pittendrigh[19] similarly inferred from its response curve that the oscillation remained unchanged through three full cycles of its free run in constant darkness. All of these *inferences* from the behavior of *population rhythmicity* are tested directly here in experiments which examine the effect of single light pulses (15 min, 100 ft-c white fluorescent) on $t_e$ of populations of developmentally synchronous pupae.

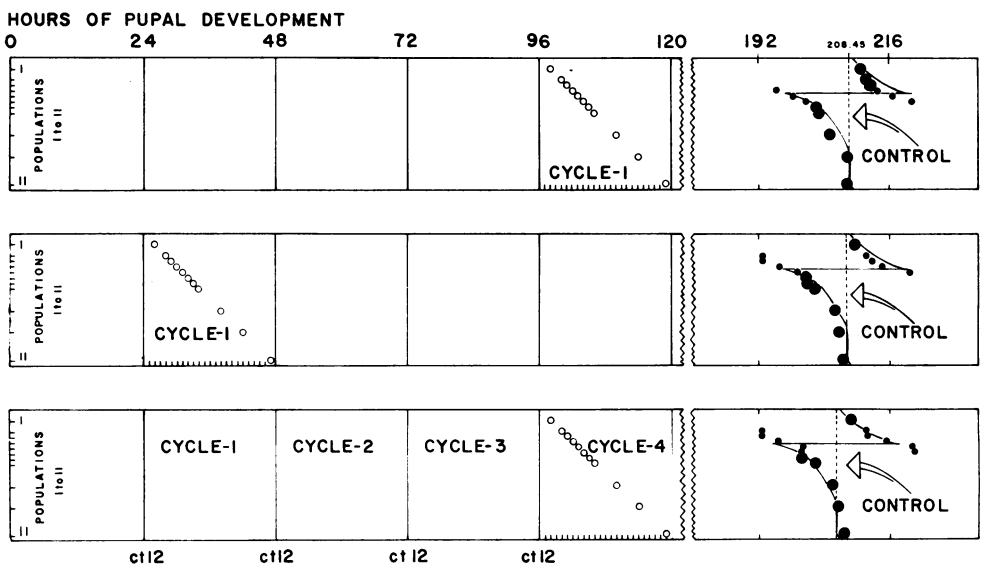



FIG. 2.—The effect of light (15 min, 100 ft-c, white fluorescent) pulses (*open circles*) on emergence times in populations of developmentally synchronous pupae (*filled circles*). Populations emerging entirely in one gate are plotted as large circles, those exploiting two gates as small circles. *Top panel:* $t_i$ = 96 hr; and light pulses were applied to 11 populations during the 1st cycle of the oscillation's free run, i.e., at 96 + 2$^h$, 96 + 4$^h$, 96 + 5$^h$, etc. *Middle panel:* $t_i$ = 24$^h$ and again light pulses given during the 1st cycle of the free run (at 24 + 2$^h$, 24 + 4$^h$, 24 + 5$^h$, etc.). *Bottom panel:* $t_i$ = 24$^h$ but light pulses given on the fourth cycle of the oscillation's free run (at 24 + 74$^h$, 24 + 76$^h$, 24 + 77$^h$, etc.). The dashed line is $t_e$ of the control culture in each experiment and the standard response curve (*solid line*) gives the predicted phase shifts that the light pulses should effect.

The top panel of Figure 2 shows the history of 12 populations of synchronous pupae transferred from LL to DD after 96 hours of development. Fifteen-minute light pulses (open circles in the figure) were applied to 11 of the 12 cultures (one left to free run as a control) at various times (2, 4, 5, 6, 7 hr, etc.) after $t_i$. We predict from previous studies of population rhythmicity that the pulse two hours after the LL/DD transfer will hit the phase-point ct 14 of the oscillation; that the pulse four hours after $t_i$ will hit ct 16, and so on. These lead to the collective prediction at the right-hand end of the panel given by the solid curve: viz. the phase-response curve rotated 90° from the usual axis of presentation. Thus the pulse at $t_i$ + 4 hours (= ct 16) should phase delay 5.8 hours. The plotted points are the medians ($t_e$) of the observed eclosion distributions and they closely conform to prediction. The slight discrepancies from the curve based on developmentally asynchronous populations are real and important, and are discussed in a later paper.[20] It is especially noteworthy that the pulses (at ct 18–20) which shift the phase of the oscillation about 12 hours, split the total population, part of which advances to the gate now coming earlier than the control and part of which delays to the gate now coming later than the control. This is the same phenomenon we saw earlier for populations 2, 6, 10, etc., in Figure 1. The splitting of the population into two discrete distributions by the same light pulse excludes (as did cultures 2, 6, 10, etc., in Fig. 1) any alternative explanation of the present facts in terms of light accelerating or decelerating developmental rates.

The data in Figure 2 (*top panel*) document the oscillation's wave form for cycle 1 of a free run beginning at $t_0$ + 96 hours of development. The middle panel shows that the same wave form is found for cycle 1 of a free run begun three days earlier ($t_0$ +24 hours) in development; and the bottom panel shows that the oscillation has not measurably damped by cycle 4 of a free run begun at $t_0$ + 24 hours.

Figure 3 further documents this important point. In all 11 populations involved, $t_i$ occurred 24 hours after $t_0$. Population 1 was left as control. Populations 2

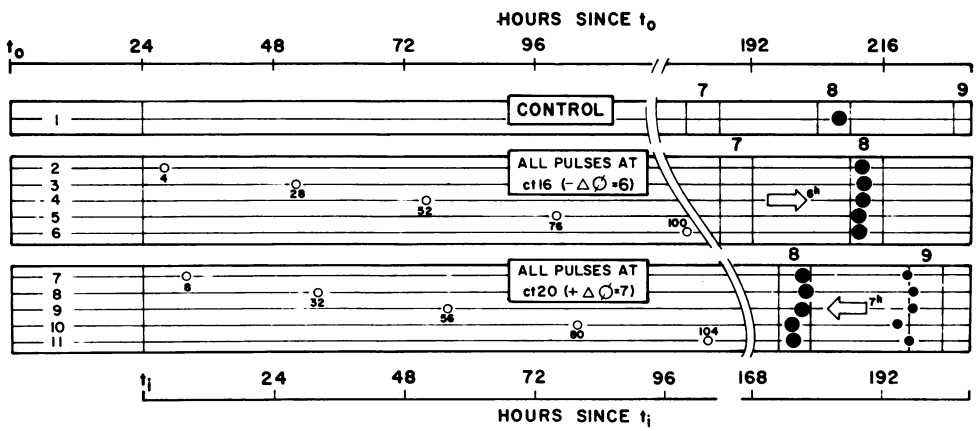



FIG. 3.—The effect on 10 experimental populations of developmentally synchronous pupae of single 15-min light pulses (*small open circles*) applied at predicted ct 16 (populations 2–6) and ct 20 (populations 7–11), but in a different cycle of the free run of each population. Population 1 was the control. After the break in the time scale, the sixth, eighth, and ninth gates are shown. Solid circles indicate observed eclosion peaks (medians); populations illuminated at ct 20 exploit mainly the advanced eighth gate, but a few flies (*small circles*) await the ninth gate.

through 6 each received a single 15-minute light pulse; at $t_i$ + 4 hours; $t_i$ + 28 hours (= 4 + 1$\tau$); $t_i$ + 52 hours (= 4 + 2$\tau$); etc. Thus, populations 2 through 6 are all expected to be at ct 16 when the light pulse falls; it falls in the first cycle of the free run in population 2, in its second cycle in population 3, and in the fifth cycle of the free run in population 6. The right-hand end of the figure shows the positions of the seventh, eighth, and ninth gates defined by the oscillation; it shows, too (*solid circles*), the medians of the observed eclosion peaks. Cultures 2, 3, 4, 5, and 6 all are delayed, as expected, into the delayed eighth gate. Emergence in cultures 7, 8, 9, 10, and 11 is bimodal; the bulk of each population is able to exploit the phase-advanced eighth gate but a small fraction of each is forced to await the very beginning of the succeeding ninth gate.

*Summary and Comment on the Meaning of Population Rhythmicity.*—We have demonstrated here the presence throughout pupal life of an oscillation whose period (at 20°C) is not significantly different from 24 hours, and is independent of the pupa's developmental stage. The most compelling evidence is the circadian periodicity of the effect of light signals applied to the pupa in darkness. Those effects cannot be explained by any effect of light accelerating or decelerating developmental rates. All the effects here described are, however, explained by the gating oscillation model Pittendrigh advanced in 1954, using the terminology of "allowed" and "forbidden" phases. And none of our conclusions on the circadian oscillation in the individual pupa is dependent on the rhythmicity of populations. Figure 4, however, makes the meaning of population rhythmicity clear. It reorders the

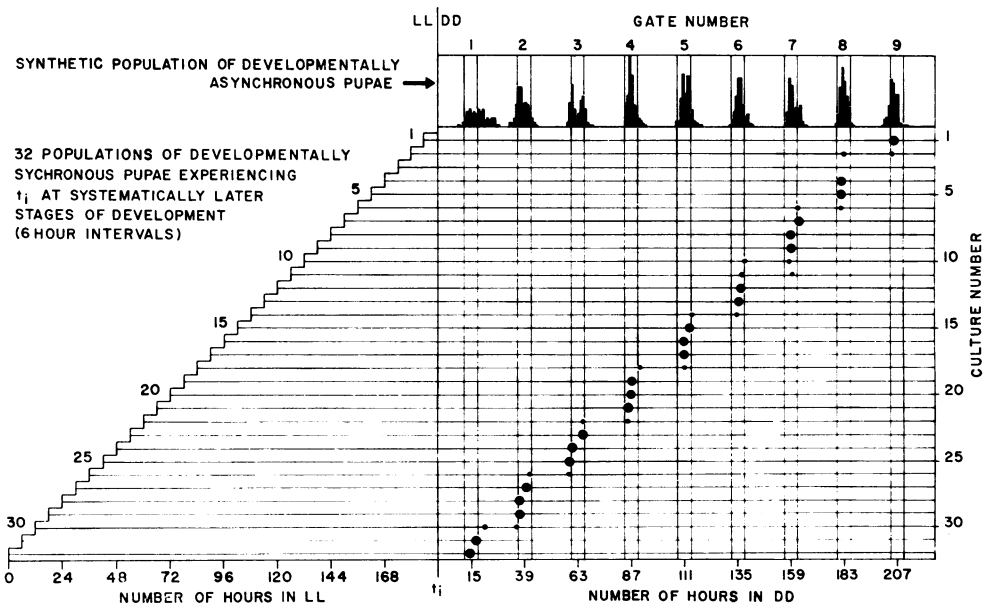



FIG. 4.—The medians of the emergence distributions plotted in Fig. 1. The data have been rearranged graphically so that the light/dark transition ($t_i$), rather than $t_0$, is synchronous. After the light/dark transition, eclosion gates (6 hr wide) occur at times, 15 hr + $n\ \tau$, where $\tau$ is equal to about 24 hr. The larger circles are cases where 100% of the flies in the population emerged in a single gate and the smaller circles are cases where the population was partitioned into two gates. The synthetic peaks were derived by summing, from raw data, the incidences of emergence in all the populations occurring in each hour after $t_i$. The peaks so derived for each gate were normalized to equal areas.

TABLE 2

Predicted times of eclosion (peaks at 15 hr + $n$ $\tau$ after $t_i$) and observed times in the 32 cultures of developmentally synchronous pupae. "Observed $\tau$" is the interval between two of the observed peaks.

| Gate | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| $t_e$–$t_i$ | | | | | | | |
| (Predicted at 15 + $n\tau$) | 39.0 | 63.0 | 87.0 | 111.0 | 135.0 | 159.0 | 183.0 |
| (Observed at) | 37.8 | 61.5 | 86.7 | 111.4 | 135.7 | 160.1 | 181.4 |
| Observed $\tau$ | | 23.7 | 25.2 | 24.7 | 24.3 | 24.6 | 21.3 |

data of Figure 1 so as to synchronize $t_i$. The series of 32 populations then constitutes a model population of developmentally asynchronous pupae in which an ocsillation is initiated synchronously in all 8000 individuals. By summing the incidence of emergence events every hour after $t_i$ we reconstitute the observed *population rhythmicity* in which peaks occur at times 15 hours + $n$ $\tau$ after the LL/DD transition (also see Table 2).

The fact which Harker surprisingly stresses, that the individual emerges only once, has no bearing on the presence or absence of an oscillation throughout the whole of pupal—and for that matter larval—life which functions as a gating system restricting the ultimate emergence act to a limited ($\sim$90°) fraction of its cycle.

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