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External Coincidence and the Photoinducible Phase in the *Sarcophaga* Photoperiodic Clock

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Summary. 1. Data from single light pulse resetting experiments on the circadian rhythm of pupal eclosion in *Sarcophaga argyrostoma* were used to design and predict the outcome of one- and two-pulse cycles in terms of the induction of pupal diapause. Experiments were interpreted in terms of the "External Coincidence" model of Pittendrigh (1966).

2. Two-pulse asymmetrical "skeleton" photoperiods (night-interruption experiments), in which a short supplementary pulse scans the "night" of a diapause-inductive cycle, show two points (A and B) of short-night effect (the non-diapause or "summer" pathway). The External Coincidence model suggests that the photoinducible phase (ϕ_i) lies at point B.

3. Single short pulses of light (1 h) were used in cycles of different length (so-called T-experiments) in such a way that the light pulses fell on particular circadian phases in each cycle. "Short-night" or non-diapause effects were only produced when the light pulse fell, in each cycle, on that phase (Circadian time, Ct, 21.5) equivalent to point B.

4. In T-experiments comprising 1 h pulses of light in a cycle of *LD 1*:20.5 (T 21.5), with the first pulse in the train starting at different circadian phases, the incidence of diapause was shown to be a function of the number of transient cycles before the circadian pacemaker achieved steady-state entrainment to the light cycle.

5. In two-pulse asymmetric "skeletons" in which the scanning pulse was arranged to fall on point A, it was shown that the diapause-averting effects of the scanning pulse could be "reversed" by a terminal dark period greater than the critical night length. In asymmetrical skeletons in which the pulse fell on point B, however, the diapause-averting effects were irreversible. These experiments demonstrate that the photoinducible phase lies late in the subjective night at a circadian phase (Ct 21.5) marked by point B in night interruption experiments.

6. Although the results do not exclude the possibility of an "Internal Coincidence" type of photoperiodic clock (Pittendrigh, 1972) they remain consistent with the External Coincidence model as adopted in earlier papers for *S. argyrostoma*. The results also underscore the essential similarities between the photoperiodic clocks in *Sarcophaga* and the aphid *Megoura viciae* (Lees, 1973).

Introduction

Photoperiodic induction of diapause or diapause-free development in the flesh-fly *Sarcophaga argyrostoma* is a function of the circadian system (Saunders, 1973, 1976a), and may be described in terms of entrainment (Saunders, 1978a). Of the several models currently proposed to account for this form of time measurement (Pittendrigh, 1972), that of External Coincidence seems to explain these data most effectively; consequently this model, with slight modifications (Saunders, 1975, 1978a), has been adopted as a "working hypothesis". The crux of the model is that the photoperiodic oscillation is reset to a phase (circadian time, Ct) close to Ct 12 at the end of a lengthy (> 10 h) light period, and a particular light-sensitive or photoinducible phase (ϕ_i) occurs about $9^1/_2$ h (the critical night-length) later (Pittendrigh, 1966). In a long-night cycle of *LD 12*:12, therefore, ϕ_i falls in the dark and pupal diapause supervenes in the insect's life cycle, whereas in a short-night cycle of *LD 16*:8 ϕ_i falls in the light and diapause is averted (see Fig. 6A and B). The temporal coincidence between light and ϕ_i is thought to result in a "product" which accumulates over several cycles to induce continuous or non-diapause development, perhaps by stimulating the production or transport of brain hormone.

Substantial circumstantial evidence now exists to suggest that such a mechanism measures night-length

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in *S. argyrostoma* (Saunders, 1978a). Phase response curves based on the phase resetting effects of light pulses on the eclosion rhythm have been used to compute theoretical steady-state phase relationships of the peaks of eclosion (ϕ_r) and of the putative photoinducible phase (ϕ_i) to the light pulses, in a number of simple and complex light regimes. Theoretical and observed phases of ϕ_r were in most cases very close (Saunders, 1978a). More importantly, however, diapause incidence was always low when ϕ_i was illuminated, but high when it fell in the dark. In all regimes, however, diapause or non-diapause development was *also* associated with either (1) an alteration in the duration of the light component, or with (2) an alteration in the Zeitgeber period, T. The results are, therefore, also open to interpretation in terms of an alternative model – Internal Coincidence (Pittendrigh, 1972) – in which induction is seen as a function of the mutual phase relationship between two, or possibly more, theoretical oscillations, independently entrained to "dawn" (=morning) and "dusk" (=evening).

An unequivocal distinction between internal and external coincidence – or any other kind of circadian model for that matter – will probably not be made until the "concrete" physiological events involved in induction are better understood. Nevertheless, there are *some* formal experimental approaches which might help clarify the issue. Some of these were considered in an earlier paper (Saunders, 1978b). Experiments are now described which further underline the probability of external coincidence, at least for the *Sarcophaga* case, and suggest that the photoinducible phase may be a physiological "reality".

Materials and Methods

1. Culture and Experimental Techniques

Adults of *S. argyrostoma* were maintained at 25 °C and in either continuous light (*LL*) or in a long-night regime (*LD 12*:12), the former "programming" the larvae for continuous development the latter for diapause (Denlinger, 1971). The flies were supplied with sugar and water *ad libitum*, and a piece of meat which was replaced daily. First instar larvae deposited on this meat were transferred to plastic culture dishes containing a larval supplement made from yeast, dried milk, and agar. These cultures were then maintained in light-proof wooden cabinets at 18° or 22 °C, in a variety of experimental light regimes controlled by Paragon timers operating Philips 4 W striplights. The irradiance at the level of the larval cultures was about 240 $\mu W \cdot cm^{-2}$. Mature larvae were allowed to disperse and form puparia in a thin layer of sawdust. Puparia were collected daily as they formed and incubated in the dark at 18 to 19 °C for about 10 to 14 days, after which they were opened to ascertain whether the pupae within them were in diapause or not (Saunders, 1971).

2. Experimental Light Regimes

All experiments were designed and interpreted using a computer program based on the phase resetting effects of light pulses on the *S. argyrostoma* eclosion rhythm (Saunders, 1978a). This program incorporated resetting data for 1, 3, 4, 5, 6, 8, 10, 12, 14, 16 and 20 h of white light, offered in up to 4 pulses per cycle, with the first pulse in the train starting at all circadian times (Ct 01 to Ct 24), in Zeitgeber periods (T) of any length, and for up to 18 consecutive cycles. Particular attention was paid, not only to pulse duration, number of pulses, and Zeitgeber period, but to the *initial conditions* (starting phase) and the rate at which steady-state entrainment was achieved. All of these factors have proved to be important in predicting and interpreting the final incidence of pupal diapause.

Three types of experiment were carried out, the first based on that performed by Pittendrigh and Minis (1964, 1971) on the pink bollworm moth, *Pectinophora gossypiella,* and the second and third on experimental designs used, for another reason, by Lees (1970, 1973) for the photoperiodic clock controlling morph determination in the aphid *Meguora viciae*. These experiments were:

(a) "T-experiments" in which a single short pulse of light (1 h) was used in Zeitgeber cycles from T 21 (*LD 1*:20) to T 30.5 (*LD 1*:29.5), with the first pulse in the train starting at different circadian phases.

(b) "Night interruption" experiments or two-pulse asymmetrical skeletons consisting of a longer "main" photoperiod ($P_1 = 10$ h) and a shorter pulse ($P_2 = 1$ h) which interrupts the night. The supplementary or interrupting pulse was placed 3 h after the end of P_1 and the overall Zeitgeber period varied by systematically altering the *terminal* hours of darkness.

(c) A similar series of asymmetrical skeletons in which the terminal hours of darkness were held at a value greater than the critical night-length ($9^1/_2$ h), but the hours of darkness *between* P_1 and P_2 were systematically varied.

Experiments (b) and (c) were compared with results for "night interruption" experiments in a Zeitgeber cycle where T was *equal to* 24 h. These data are taken from an earlier paper (Saunders, 1975), but receive a fuller analysis here.

In each experiment the computer program was used to calculate whether entrainment took place, how many non-steady-state or transient cycles were necessary before entrainment was accomplished, and the steady-state phase relationship (Ψ) between the photoinducible phase, ϕ_i (at Ct 21.5) and the light pulses.

The following abbreviations will be used throughout this paper:

- DD Continuous darkness.
- *LL* Continuous light.
- *LD* Light-dark cycle (e.g. *LD 12*:12 is a cycle of 12 h of light and 12 h of darkness).
- τ Period of the unentrained (i.e. free-running) circadian oscillation or pacemaker, in h.
- T Period of Zeitgeber (experimental *LD* cycle) in h.
- ϕ_r Phase reference point of the eclosion rhythm (the median of the eclosion peaks).
- ϕ_i The theoretical photoinducible phase of the photoperiodic oscillation.
- Ct Circadian time. A time scale, measured in hours (Ct 00 to 24) covering one full circadian period. Ct 12 is defined arbitrarily as that phase of the oscillation at the end of a 12 h light period when in steady-state entrainment to *LD 12*:12 (Pittendrigh, 1965).
- Ψ The phase relationship or phase angle between the circadian rhythm and the light cycle.
- $+\Delta\phi$ and $-\Delta\phi$ Advance and delay phase shifts.

Results

1. A Single Short Pulse of Light per Cycle: The T-Experiment

When a circadian oscillation (period τ) becomes entrained to an external periodicity (period T) consisting of a single short pulse of light per cycle, the pulse must come to lie, in each cycle, on that part of the phase response curve which generates a phase shift equal to the difference between T and τ (Pittendrigh, 1965). Consequently, when T is greater than τ, the pulse must fall in the early subjective night (Ct 12 to 18) to cause phase delays in each cycle, whereas when T is *less than* τ, the pulse must fall in the *late* subjective night (Ct 18 to 24) to cause phase *advances*. Simply by altering *T*, therefore, a pulse of light can be made to illuminate different circadian phases. This phenomenon was used by Pittendrigh and Minis (1964, 1971) and by Minis (1965) to explore the subjective night of the pink bollworm moth *Pectinophora gossypiella* as a test of the external coincidence model, the theoretical strengths of this so-called "T-experiment" lying in the accuracy with which the pulse of light can be placed at different phases in the absence of any other photoperiodic influence.

In the present experiments, females of *S. argyrostoma* were maintained at 25 °C and *LD* 12:12 until they produced larvae on the meat provided; in this way the larvae were "programmed" for subsequent pupal diapause. Newly-deposited larvae were then transferred, at the end of the last 12 h light period, into experimental cabinets at 18 °C, and in a variety of T cycles all containing 1 h of light per cycle. The phase response curve for 1 h pulses (see Fig. 2) was then used to compute (a) which phase (=circadian time) the first pulse in the train should fall, and (b) which T-value should be used, to illuminate particular phases within the subjective night when in steady-state entrainment. Table 1 shows the various regimes employed. The "amplitude" of the PRC (advances plus delays) provides a guide to the range of entrainment for the *S. argyrostoma* pacemaker exposed to trains of 1 h pulses; in this case the range is from about T 21.5 to 30.5. Outside this range of *T*-values entrainment fails. Entrainment is also doubtful when the pulses fall on phases too close to the point of phase inversion (Ct 17 to 19), a portion of the PRC which is not "available" for normal entrainment (Pittendrigh and Daan, 1976).

The phase response curve was also used to compute the phase relationship between the supposed photoinducible phase (ϕ_i) and the light pulses, assuming theat ϕ_i is "centred" at about Ct 21.5. Figure 1 shows that when T was greater than τ (about 24 h) the pulses came to fall in the early subjective night; consequently ϕ_i fell in the dark. When T was less than 24 h, however, the pulses came to fall in the *late* subjective night and, in two regimes (T 21.5 and T 22) the pulses attained a temporal coincidence with the photoinducible phase itself. The model predicts, therefore, that short night effects (diapause-free devel-

Table 1. T-cycles and phase shift ($\Delta\phi$) required to illuminate particular phases of the *Sarcophaga argyrostoma* circadian pacemaker in steady-state entrainment to light cycles containing a single 1 h pulse of light

First pulse starts (Ct)	T (h)	*LD*	In steady-state entrainment		$\Delta\phi$ (h) in each cycle
			Ct pulse begins	Ct pulse ends	
13	27.5	*1*:26.5	13	10.5	−3.5
14	29.5	*1*:28.5	14	9.5	−5.5
15	30.5	*1*:29.5	15	9.5	−6.5
16	30.5	*1*:29.5	16	10.5	−6.5
17	29.5	*1*:28.5	17	12.5	−5.5
19	21.0	*1*:20	fails to entrain		
20	21.5	*1*:20.5	20	23.5	+2.5
21	22.5	*1*:21.5	22	0.5	+1.5
22	23.0	*1*:22	22	0.0	+1.0
23	23.5	*1*:22.5	23	0.5	+0.5

Ct, Circadian time; *T*, period of driving light cycle; *LD*, ratio of light to dark; $\Delta\phi$, phase shift

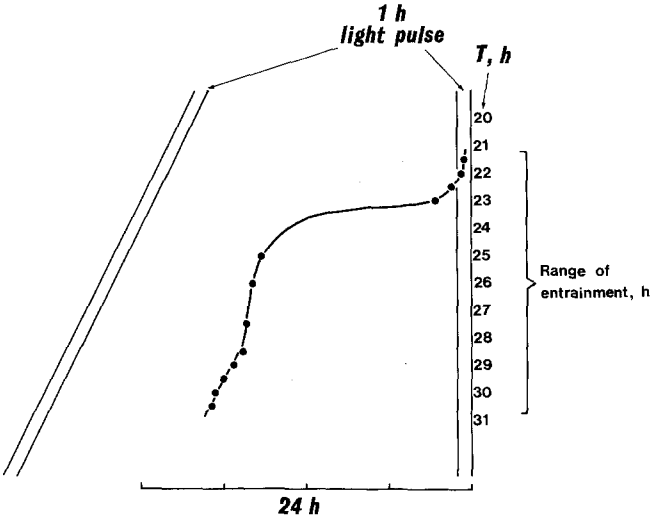

Fig. 1. *Sarcophaga argyrostoma*. Computed steady-state phase relationships of the photoinducible phase (ϕ_i) (at Ct 21.5) in light cycles with periods (T) from 20 to 31 h, each containing a single 1 h pulse of light per cycle. The photoinducible phase coincides with the light pulse only in cycles of T 21.5 (*LD* 1:20.5) and T 22 (*LD* 1:21); in all other regimes it falls in the dark. The range of entrainment of the *Sarcophaga* circadian pacemaker to 1 h pulses of light is from about T 21.5 to T 30.5

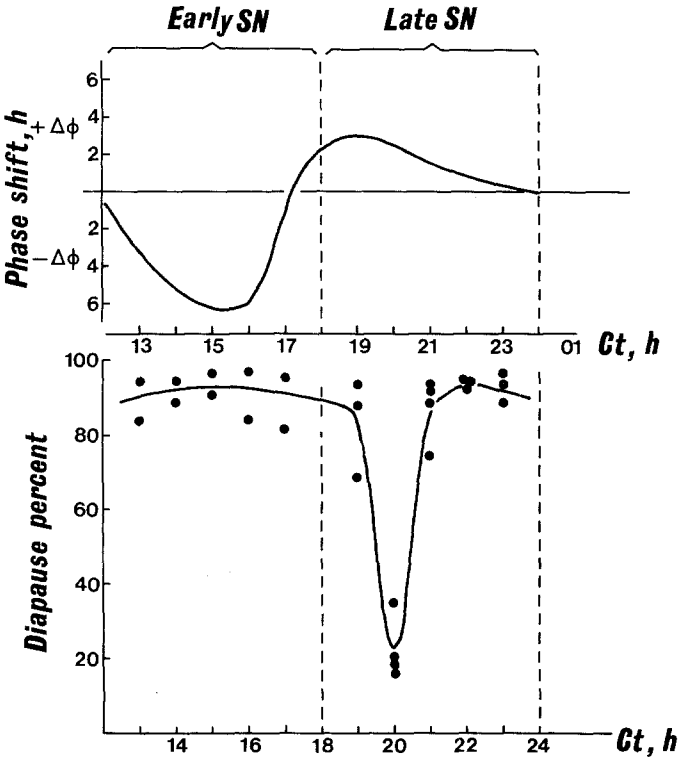

Fig. 2. *Upper panel:* phase response curve for 1 h pulses of white light, showing phase delays ($-\Delta\phi$) in the early subjective night (Ct 12 to 18) and phase advances ($+\Delta\phi$) in the late subjective night (Ct 18 to 24) (Saunders, 1978a). *Lower panel:* the incidence of pupal diapause in *S. argyrostoma* exposed to T cycles (see Table 1) in which the 1 h pulse illuminates a particular phase (circadian time, Ct) of the subjective night *in each cycle* when in steady-state entrainment. Only when the pulse comes on at Ct 20 and is followed by a cycle of T 21.5 (*LD 1*:20.5) are short-night or diapause-free effects obtained

opment) should occur in these cycles *only*; all others should produce a high incidence of pupal diapause.

Figure 2 shows that the experimental results agree closely with this prediction. In the regimes employed (Table 1), a long night effect (high incidence of pupal diapause) was obtained in all *except* that in which the first pulse fell at Ct 20 and subsequent pulses followed in a cycle of T 21.5 (*LD 1*:20.5). In such a regime, the oscillation achieved immediate entrainment and, *in each cycle,* the light pulse came on at Ct 20 and finished at Ct 23.5. It therefore illuminated the supposed photoinducible phase (at about Ct 21.5) and resulted in a high incidence of continuous or diapause-free development typical of the "summer" or long-day response.

On *a priori* grounds one might also have excepted the pulse commencing at Ct 21 to have illuminated ϕ_i and eliminated diapause. Computations for this pulse, followed by the train *LD 1*:21.5 (T 22.5), however, showed that the oscillation only reached steady-state entrainment to the light cycle after 6 cycles, and the pulse came to illuminate Ct 22 to Ct 0.5; this regime, therefore, failed to illuminate ϕ_i. In all other groups the pulse of light also fell outside Ct 21.5. Merely by altering T, therefore, it is possible to operate the photoperiodic "switch" in *Sarcophaga,* apparently by causing the short light pulse to fall on a particular, and quite restricted, phase of the photoperiodic oscillation.

Figure 3 shows that a high incidence of pupal diapause was associated with a greatly protracted larval development. The low incidence of diapause obtained when the light pulse coincided with ϕ_i, on the other hand, was associated with a relatively rapid rate of growth. This result agrees with earlier observations on the effects of T and photoperiod in *S. argyrostoma* (Saunders, 1972), which showed that slow growth and diapause were always associated with long nights, whereas rapid growth and diapause-free development were associated with short nights.

In a second form of the T-experiment, attention was paid to the initial conditions or starting phases. For example, although all cultures exposed to T 21.5 (*LD 1*:20.5) reached a steady-state in which the pulse illuminated Ct 20 to Ct 23.5, regardless of the circadian time at which the first pulse in the train occurred, they did so after a different number of transient or non-steady-state cycles. This is particularly evident in *S. argyrostoma* exposed to 1 h pulses because the PRC for light of this duration is of the "weak" Type 1 and the approach to steady-state is slow (Saunders, 1978a). Cultures in which the first pulse started at Ct 20, for example, achieved "immediate" entrainment, but those starting at Ct 12 passed through 9 transients before the light pulse coincided with ϕ_i (Table 2). Since photoperiodic induction in *S. argyrostoma* involves a "summation" of such coincidences (Saunders, 1971), and the early larval

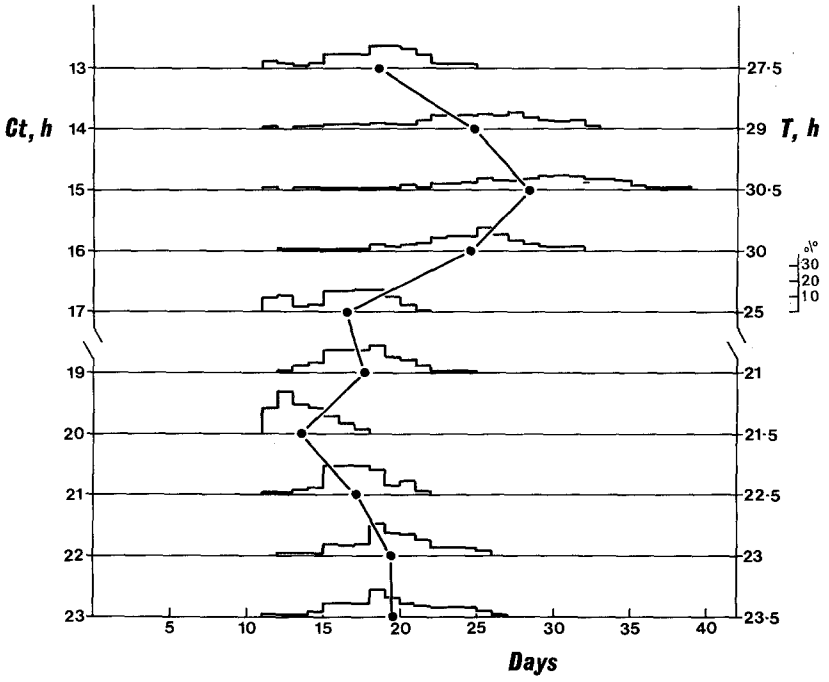

Fig. 3. The effects of Zeitgeber period (T) and phase illuminated (Ct) by the 1 h pulse on the rate of larval development in *S. argyrostoma*. Development is most rapid when the pulses fall in each cycle at Ct 20 in a cycle of T 21.5 (*LD 1*:20.5). Histograms plot the percent pupation per day; zero on the abscissa represents day of larviposition

Table 2. The relationship between the phase (circadian time) of the first light pulse in the train, the number of transient cycles required to bring the circadian pacemaker to steady-state entrainment, and the incidence of pupal diapause. All cultures were maintained in a cycle of *LD 1*:20.5 (T 21.5) at 22 °C with the 1 h light pulse starting at different phases

Phase (Ct) 1 h light pulse begins	Number of transients to steady-state	Number of larvae	Number of diapause pupae	Percentage of pupae in diapause
02	4	223	84	37.7
04	5	219	113	51.6
06	6	300	155	51.7
08	7	359	130	36.2
10	8	605	189	31.2
12	9	268	177	66.0
		301	255	84.7
14	7	286	129	45.1
16	8	715	162	22.7[a]
18	8	692	121	17.5[a]
20	1	427	13	3.0
		722	3	0.4
22	2	587	4	0.7
24	3	364	24	6.6

[a] Groups in which the starting phases were too close to the phase inversion of the phase response curve

instars are more sensitive to photoperiod than mature larvae, the model predicts a positive correlation between the number of transients and the incidence of pupal diapause (or an *inverse* relationship between the number of coincidences and diapause).

This prediction was tested in an experiment in which 14 groups of larvae produced by flies maintained at 25° and *LD 12*:12 were exposed to cycles of *LD 1*:20.5, but with the first pulse beginning at all circadian times. Table 2 shows that the final proportion of the larvae entering pupal diapause was indeed a function of the number of transients, with those cultures starting at Ct 20 (1 transient) producing 0.4 and 3.0%, and those starting at Ct 12 (9 transients) producing 84.7 and 66.0%. Figure 4A illustrates the approaches to steady-state and the diapause incidence for these two initial conditions, and Fig. 4B the linear relationship between the number of transients and diapause. Cultures starting at Ct 16 and 18 produced less diapause than expected: these data have been omitted from Fig. 4B on the grounds that these starting points are too close to the point of phase inversion on the 1 h PRC for "proper" entrainment (Pittendrigh and Daan, 1976). Otherwise, there is a strong evidence that the *number* of coincidences between the light pulses and the supposed photoinducible phase (at Ct 21.5) is an important feature of the photoperiodic response.

2. Night Interruption Experiments in T 24

Figure 5 shows the results of a "night interruption" experiment in which the 14 h "night" of an *LD 10*:14 cycle (T 24) was systematically interrupted by a 1 h supplementary light pulse. The lower left panel plots

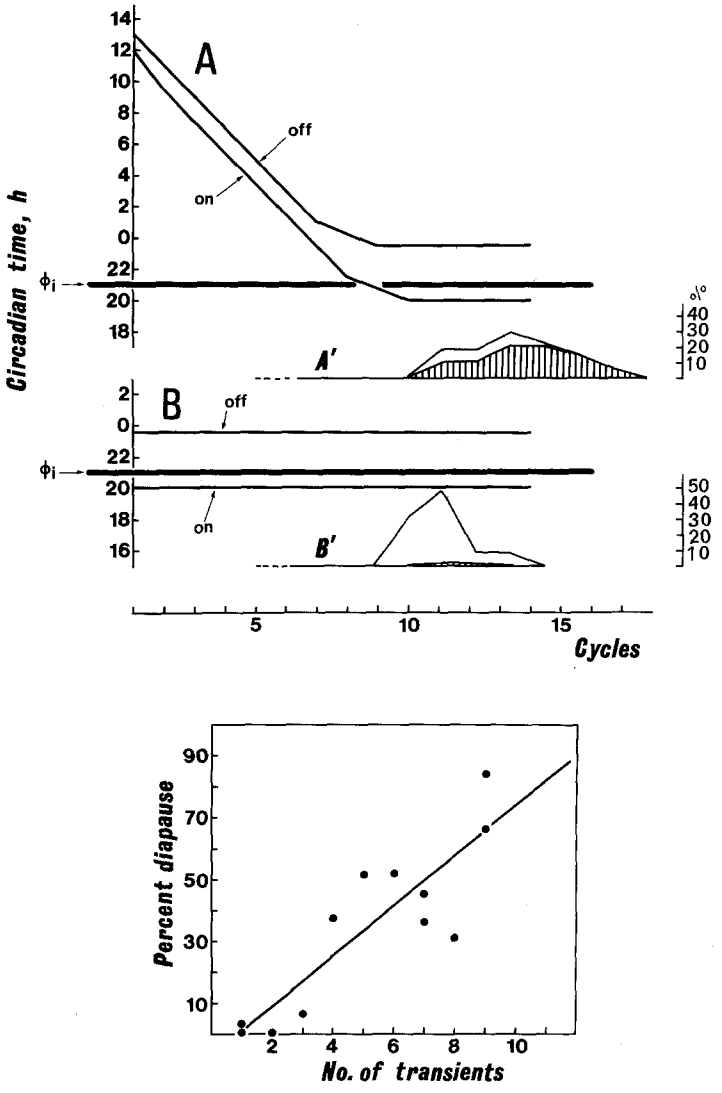

Fig. 4. *Upper panel:* the rate of approach to steady-state entrainment of the *S. argyrostoma* circadian pacemaker to Zeitgeber cycles of *LD 1*:20.5 (T 21.5), and the incidence of pupal diapause. In *A*, the population experienced the first pulse in the train at Ct 12; it took 9 transient cycles to steady-state and coincidence with the photoinducible phase. This resulted in protracted larval development and a high incidence of pupal diapause (inset 'A'). In *B*, the populations experienced its first pulse at Ct 20, underwent immediate entrainment and coincidence with ϕ_i; hence rapid larval development and low incidence of pupal diapause. *Lower panel:* the relationship between diapause incidence and the number of transient cycles before attainment of steady-state

the phase relationships of ϕ_i (Ct 21.5) computed for each regime, and shows first phase delays ($-\Delta\phi$) and then phase advances ($+\Delta\phi$) as the supplementary pulse scans the night. Up to *LD 10*:3:*1*:10 and after *LD 10*:7:*1*:6 there are single, unique, steady-state phase relationships; between *LD 10*:4:*1*:9 and *LD 10*:6:*1*:8, however, there is a "zone of bistability" with *two* possible steady-states, Ψ_x and Ψ_y, and a phase-jump between them. Which of the two modes is adopted by the oscillation is determined by the initial conditions (i.e. the circadian phase illuminated by the first pulse (P_1) in the train). For example, a "population" starting at Ct 07 to Ct 10 would phase jump to Ψ_y after *LD 10*:3:*1*:10, but the phase-jump for a population starting between Ct 16 and Ct 01 would be delayed until after *LD 10*:6:*1*:7. The experimental population whose diapause responses are shown in the right hand panel were transferred straight from *LL* into the first dark period: these show a computed phase-jump between *LD 10*:5:*1*:9 and *LD 10*:6:*1*:8.

The upper panel of Fig. 5 uses the phase response curve as a "measure of the time-course of the oscillation" (Pittendrigh, 1966). It plots, at left, the PRC during the 14 h of darkness following a 10 h light pulse (i.e. in *LD 10*:14) and, at right, in the terminal dark period of *LD 10*:1:*1*:12 in which the supplementary pulse causes a 2 h phase delay. It illustrates the dynamic nature of the entrainment phenomenon in complex regimes such as these asymmetric skeleton photoperiods, and shows that the PRC shifts to the right with phase delays and then, after the phase-jump, to the left with phase advances.

The panel on the right shows the experimentally determined diapause responses for populations of

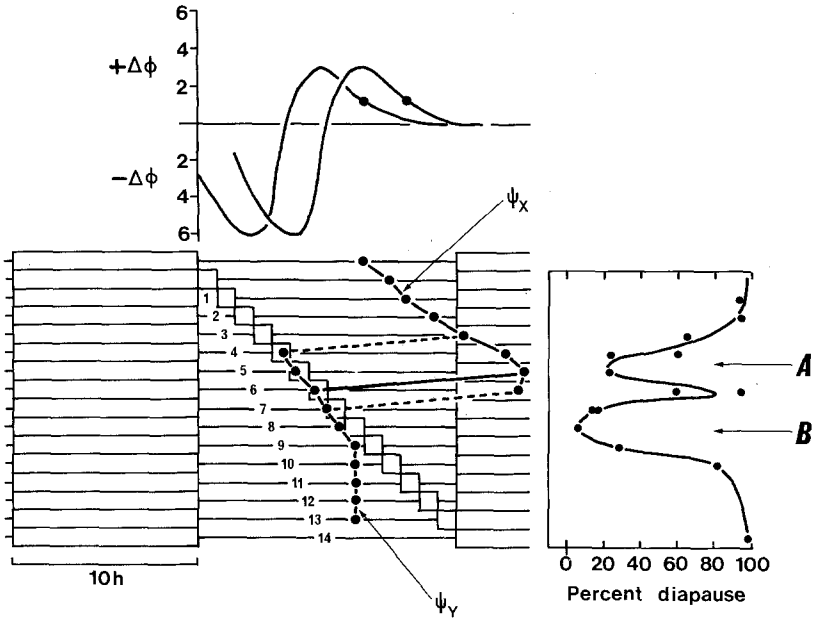

Fig. 5. Phase relationships of the photoinducible phase (ϕ_i) and diapause incidence in asymmetric "skeleton" photoperiods. *Upper panel:* the 1 h phase response curve for *S. argyrostoma* plotted, at left, for *LD 10*:14 and, at right, for *LD 10*:1:*1*:12 in which the "scanning" pulse causes a 2 h phase delay. Solid points – photoinducible phase (ϕ_i). *Lower panel, left:* computed phase relationships of ϕ_i (solid points) showing the two phase relationships (Ψ_x and Ψ_y) and the zone of bistability between them. Dotted connecting lines show the "earliest" and "latest" phase-jumps; the solid connecting line shows the phase-jump for the experimental population on the right. Open "boxes" represent light periods; connecting lines the intervening dark hours. *Lower panel, right:* incidence of pupal diapause in these asymmetrical skeleton photoperiods, showing the two points of short-night or diapause-free development, A and B

larvae which entered the first and variable dark period directly from *LL*. It can be seen that diapause incidence is high when ϕ_i (Ct 21.5) falls in the dark, but diapause is averted when ϕ_i falls in the light, or when the light comes on immediately after Ct 21.5. The characteristic bimodal response with two "peaks" of short-night effect (labelled points A and B), so frequent in the insects (Saunders, 1976b), is brought about by the higher incidence of diapause at the point of maximum-phase-jump between Ψ_x and Ψ_y (at *LD 10*:6:*1*:7). Analysis of diapause induction in terms of entrainment and in terms of the illumination or non-illumination of Ct 21.5, thus provides a close match between prediction and observation. The results are therefore clearly interpretable in terms of the "External Coincidence" model.

It is perhaps timely, at this point, to restate the External Coincidence model as it behaves in night interruption experiments (Fig. 6). A pulse of light placed in the early subjective night (Fig. 6C) causes a phase delay in the circadian pacemaker until ϕ_i (at Ct 21.5) passes into the light and diapause is eliminated; this gives rise to "point A" in night-interruption experiments. Pulses of light placed *late* in the subjective night (Fig. 6D) cause phase *advances* until, when the pulse comes on at about Ct 20, it coincides *directly* with ϕ_i and again eliminates diapause; this produces "point B" in night-interruption experiments. The apparently "insensitive" point between A and B occurs, as stated above, when the oscillation undergoes its phase-jump between delays and advances.

3. Night Interruption Experiments in Cycles Where T Is Not Equal to 24 h

Two types of night interruption experiment in which T was *not* equal to 24 h were carried out, both of them based on designs used by Lees (1970, 1973) for *Megoura viciae*. In the first, the supplementary light pulse was placed 3 h after the end of the 10 h "main" photoperiod, in a position where it begins to reverse diapause incidence at "point A" (Fig. 5). The subsequent hours of darkness were then systematically increased from 7 to 11 h to provide Zeitgeber cycles from T 21 to T 25. In the second, using a "main" photoperiod of 8 h, the hours of darkness following the supplementary light pulse were held constant at 12 h (greater than the critical night length,

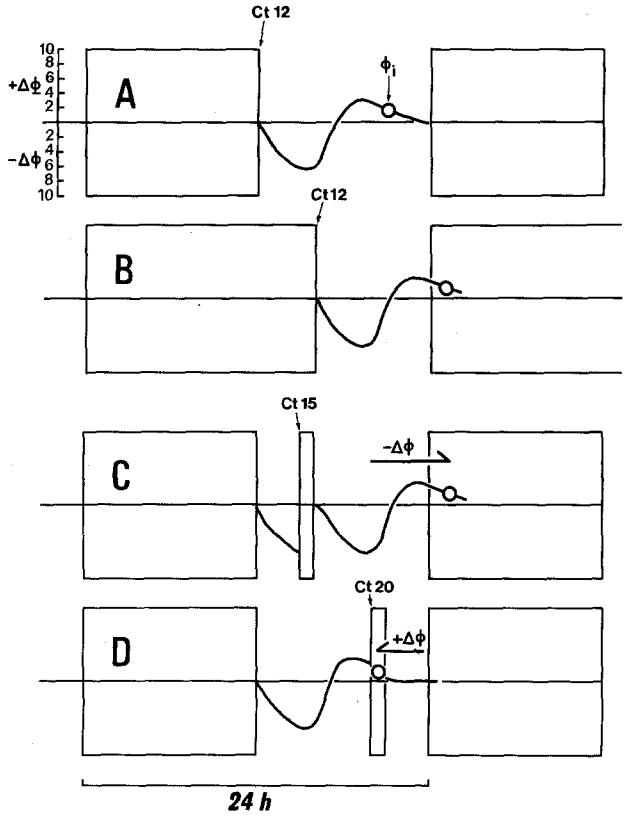

Fig. 6. The "External Coincidence" model of Pittendrigh (1966) as applied to *S. argyrostoma*. *A* in *LD 12*:12: ϕ_i falls in the dark inducing high incidence of pupal diapause. *B* in *LD 16*:8: ϕ_i is illuminated, resulting in a low incidence of pupal diapause. *C* in *LD 12*:3:*1*:8: the "scanning" pulse causes a delay phase shift, pushing ϕ_i into the light; hence the diapause-averting effects of a pulse of light at point A. *D* in *LD 12*:8:*1*:3: the "scanning" pulse causes a phase advance and coincides *directly* with ϕ_i; hence point B

$9^1/_2$ h), but the hours of darkness *before* the pulse were systematically increased from 3 to 11 h to give Zeitgeber cycles from T 24 to T 32. These experimental designs are illustrated in Figs. 7 and 8.

In the first set of experiments (Fig. 7) the initiating 10 h pulse in the train (P_1) started at Ct 22. The left hand panel plots the theoretical phase relationships of ϕ_i (Ct 21.5) to the various light regimes, and shows that ϕ_i falls in the light when the terminal hours of darkness are less than 10 h, but in the dark when the terminal dark period exceeds this value; all starting conditions (i.e. P_1 commencing at all circadian times, Ct 01 to 24) attain the same phase relationship. The right hand panel shows that diapause incidences in the experimental populations were low or at zero when ϕ_i fell in the light, but high when the terminal dark hours exceeded the critical value ($9^1/_2$ h) and ϕ_i fell in the dark.

For the second set of experiments (Fig. 8), the left hand panel plots the phase relationships of ϕ_i to the skeleton regimes, and the right hand panel the diapause incidence, as before. Computed phase relationships show a "zone of bistability" with two possible steady-states, Ψ_x and Ψ_y, as in the asymmetrical skeleton shown earlier (Fig. 5). Data from two experimental populations are included in this Figure. In one, in which the first 8 h pulse in the train started at Ct 09, the phase jump to Ψ_y occurred between *LD 8*:5:*1*:12 and *LD 8*:6:*1*:12; in the second, starting at Ct 22, the phase jump occurred between *LD 8*:8:*1*:12 and *LD 8*:9:*1*:12. In both experiments diapause incidence was low when ϕ_i either fell in the light, or when the "main" photoperiod came on shortly after Ct 21.5. On the other hand, diapause incidence was high when ϕ_i fell in the dark. The supplementary pulse (P_2) can thus be seen to be diapause-averting as soon as it lands on that part of the night (6 to 9 h after "dusk") where it acts as point B. Once again the experimental results provide a close match for those predicted by computer simulations, and are generally consistent with the "External Coincidence" model.

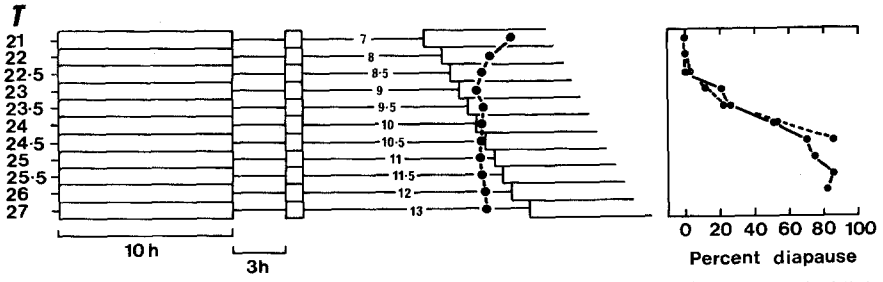

Fig. 7. *Left-hand panel:* computed phase relationships of ϕ_i (solid points) in asymmetrical "skeleton" photoperiods in which a "scanning" pulse placed at point A is followed by an increasing terminal dark period (7 to 13 h). *Right-hand panel:* results of two experiments showing that diapause incidence is low when ϕ_i falls in the light, but high when it falls in the dark: short-night effects of a pulse at A are therefore reversible by a subsequent long night

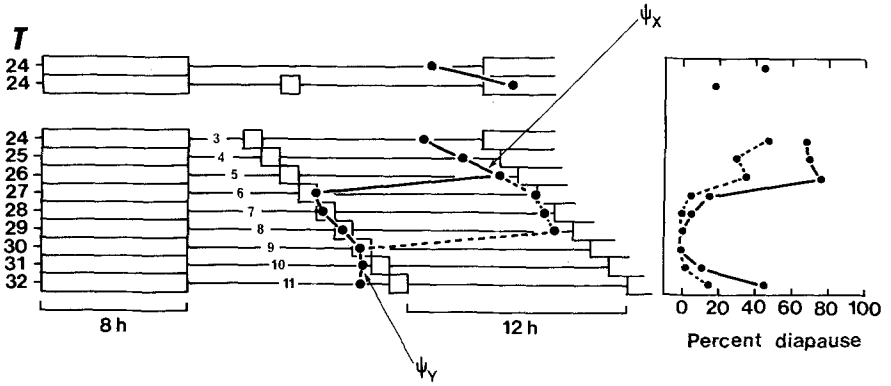

Fig. 8. *Left-hand panel:* computed phase relationships of ϕ_i (solid points) in asymmetrical "skeleton" photoperiods in which the "scanning" pulse is in all cases followed by a dark period greater than the critical night (i.e. 12 h), but the hours of darkness before the pulse are systematically varied from 3 to 11 h, Ψ_x and Ψ_y are the two phase relationships, the connecting lines (solid and dotted) the "earliest" and "latest" phase jumps. *Right-hand panel:* diapause incidence in two experiments in which the first 8 h pulse in the train started at Ct 22 (dotted line) and at Ct 09 (solid line). Note that diapause incidence is high when ϕ_i falls in the dark, but is low when it falls in the light. The trough in diapause incidence is equivalent to point B in Fig. 5, and is clearly *irreversible* by a subsequent long-night

Discussion

The experiments described in this paper provide a further demonstration that the photoperiodic mechanism in *S. argyrostoma* can be accounted for in terms of entrainment within the circadian system, using data derived from a study of the pupal eclosion rhythm. This suggests that the circadian pacemakers involved in eclosion and photoperiodism are extremely similar in their entrainment properties – or even "identical". It also suggests that the "External Coincidence" model (Pittendrigh, 1966, 1972) is relevant to the *Sarcophaga* case, although the experiments do not unequivocally exclude the alternative view of "Internal Coincidence", for reasons outlined in the Introduction.

The External Coincidence model in its original form (Pittendrigh and Minis, 1964) developed from the observations of Adkisson (1964) on the pink boll worm moth *Pectinophora gossypiella* – and now known to be widespread in the insects (Saunders, 1976b) – that "night interruption" experiments produce *two* points of diapause reversal (or short-night effects) in an otherwise diapause-inductive long-night. The two points have been called "A" and "B", and their interpretation, first propounded by Pittendrigh and Minis (1964), is essentially that developed in this paper.

Pittendrigh and Minis (1964) pointed out that the photoinducible phase (ϕ_i), if it were a reality, might be at either point A *or* B. In subsequent refinements of the model (Pittendrigh, 1966), however, it was observed that since the phase of the oscillation at the end of the "main" photoperiod was always close to Ct 12, dawn must, in effect, "move backwards" in relation to the circadian time scale as night-length shortened; consequently ϕ_i *must* be at point B, and not A (see Fig. 6A and B). Although this deduction was, and is, undoubtedly correct, the present experiments provide stronger and more direct evidence that this is so.

In the T-experiment (Fig. 2), itself powerful evidence for the circadian basis of photoperiodic induction (Pittendrigh and Minis, 1971), the subjective night is systematically probed by a short light pulse in the absence of any other photoperiodic influence, particularly a "main" photoperiod. The results show that only when the light pulse comes on at Ct 20 and off at Ct 23.5 (and hence illuminates the putative photoinducible phase at Ct 21.5) are short-night or non-diapause effects observed. Consequently, ϕ_i must lie at about Ct 21.5 which, in "complete" photoperiods or in asymmetrical skeletons, lies about $9^1/_2$ h (the critical night-length) after dusk, or at point B (Saunders, 1975).

The two experimental designs applying the night interruption technique to non-24 h Zeitgeber cycles were introduced by Lees (1970, 1973) to analyse the photoperiodic clock in the aphid *Megoura viciae*, which also shows the two points A and B. The present results for *S. argyrostoma* are essentially the same as those for *M. viciae*, but their interpretation is strikingly different.

In a series of papers, Lees (1966, 1968, 1973) has developed the concept that the photoperiodic clock in *M. viciae* is a non-circadian "hour-glass", set in motion *in each cycle* by the dusk transition, and measuring night-length. In this model the two points A and B are seen as components of a linear biochemical sequence characterised by its responses to light. Thus: (1) for the first few hours of darkness the measurement of a long-night can be easily reversed by a light pulse (i.e. at point A). If, however, a reversing pulse of light at A is followed by a terminal dark period in excess of the critical night-length (9.75 h), the "photoperiodic reversal" can *itself* be reversed. (2) Between the 3rd and the 4th hour of the night (i.e. between points A and B) the system is insensitive to light, because in Lees' model a necessary substrate disappears. (3) Between the 5th hour and the end of the critical night (9.75 h) (i.e. the position of point B) the system is again photosensitive, but if a pulse given at B is followed by a terminal dark period of *greater* than the critical value, it is found to be *irreversible*. Lastly, (4) light pulses applied during the hours of darkness in excess of the critical night-length (9.75 h) are seen as ineffective because they leave an uninterrupted residue greater than 9.75 h. Lees stresses the differences between a *reversible* effect of light at A and an *irreversible* effect at B; in *Megoura* the spectral sensitivity at B also extends further into the red end of the spectrum (Lees, 1971).

The present data with *S. argyrostoma* also show that the short-night effects of light falling on A are reversible (Fig. 7) whilst those falling on B are not (Fig. 8). However, the explanation given here, which is based on circadian rhythmicity and the "external coincidence" model, is as follows. In Fig. 7 the supplementary light pulse falling 3 h after the end of the "main" photoperiod causes a phase delay so that ϕ_i (Ct 21.5) moves into the light and short-night effects (non-diapause development) occur. Once the terminal dark hours exceed $9^1/_2$ to 10 h, however, ϕ_i falls in the dark and the larvae enter pupae diapause. The short-night effects of light falling at A are therefore *reversed* by a terminal long-night, as in *Megoura*. In Fig. 8, the supplementary pulse causes first phase delays and then phase advances until it falls in a position in the night (6 to 9 h after dusk) where it operates as point B and eliminates diapause. Since all regimes include a terminal dark period in excess of 9.5 h, the short-night effects brought about by this *direct* interaction with ϕ_i are clearly *irreversible*. Essentially identical results in *Megoura* and *Sarcophaga* can thus be interpreted in radically different ways: as a linear biochemical sequence in a dark-period "hour-glass", *or* in terms of entrainment within the circadian system and calculations of the phase relationships of a particular light-sensitive phase to the light cycle. The results for *Sarcophaga* clearly remain consistent with this "External Coincidence" model, and demonstrate that ϕ_i *must* be at B.

In earlier papers (Saunders, 1978a, b) the close similarities between the photoperiodic clocks in *Megoura* and *Sarcophaga* were stressed. The present results provide yet another close similarity. Indeed, the two clocks are practically identical in every respect except one. In *Sarcophaga* the clock "resets itself" in very long periods of darkness, thereby producing the cyclic (~24 h) incidence of diapause and non-diapause development seen in "resonance" experiments (Saunders, 1973). In *Megoura,* on the other hand, the clock apparently measures only one night-length before it stops. The former therefore exhibits the generally-accepted signs of a circadian-based timing system, whereas the latter is best interpreted as a "pure" hour-glass. However, this seemingly fundamental difference can be resolved (Saunders, 1978b) if, following the suggestion of Bünning (1969), the clock in *Megoura* is regarded as a redundant oscillation, one which is so rapidly "damped out" in DD that it requires to be reset by the next "main" photoperiod after accomplishing only one night-measuring event. This interpretation is particularly attractive if one seeks simplicity in biological theory: it suggests that organisms measuring "photoperiodic time" use *already existing* circadian pacemakers.

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