J Comp Physiol A (1984) 154:789–794

Journal of Comparative Physiology A Sensory, Neural, and Behavioral Physiology

Photoperiodic time measurement in *Sarcophaga argyrostoma*: An attempt to use daily temperature cycles to distinguish external from internal coincidence*

D.S. Saunders
Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.

Accepted March 19, 1984

Summary. 1. Larvae of *Sarcophaga argyrostoma* raised in continuous darkness (DD) and in 'square-wave' temperature cycles or thermoperiods (*H*ot: Cold *4*:20 to *20*:4, where $H = 25$ °C and $C = 15$ °C) entered pupal diapause at a rate equivalent to that in DD and a constant temperature equal to the arithmetic mean of the cycle. Therefore, unlike many other insects so tested, *S. argyrostoma* appears to show no thermoperiodic regulation of diapause induction.

2. A daily low temperature pulse (3 h at 5 °C; = *HC 21*:3), however, had marked phase-dependent effects on diapause incidence when administered with a concomitant light cycle (*LD 14*:10), diapause increasing when the cold pulse fell early in the night, but decreasing when late in the night. This result is interpreted in terms of the phase-resetting effects of the cold pulse on the circadian oscillations making up the clock.

3. These results are considered to be consistent with an 'external coincidence' model for the photoperiodic clock and appear to differentiate between the 'internal' and 'external' alternatives (for *S. argyrostoma*) by demonstrating an inductive requirement for light in addition to that for entrainment.

Introduction

There is now strong experimental evidence to suggest that photoperiodic time measurement in a wide range of plants and animals (insects, mites, birds and mammals) is a function of the circadian system (Pittendrigh 1981; Follett and Follett 1981; Saunders 1982). Theoretical consideration of the nature of the circadian involvement, however, has suggested at least two possible classes of model: internal and external coincidence (Pittendrigh 1972, 1981). In the former, the daily light cycle is thought to entrain or phase-set multiple constituent oscillators in the circadian system in such a way that different phase relationships are set up between them in long or short days. In the latter, light is thought to have a dual role, not only in the entrainment of circadian oscillators within the system, but also a direct inductive role in which a particular light sensitive phase (the photo-inducible phase, Φ_i) is either illuminated (as in long-day cycles) or not illuminated (as in short-day cycles).

The flesh-fly *Sarcophaga argyrostoma* is sensitive to photoperiod during its embryonic and larval stages (Saunders 1971, 1980), exposure to a series of long nights (short days) leading to the production of overwintering diapause pupae, whereas exposure to short nights (long days) to continuous or non-diapause development. The clock which *Sarcophaga* uses to discriminate long nights from short nights is circadian-based and, although probably composed of a number of oscillators, conforms most closely to the external coincidence type (Pittendrigh 1966; Saunders 1973a, 1981). Indeed, adoption of this model as a working hypothesis has facilitated an extensive analysis of photoperiodic time measurement in a wide range of natural and exotic light cycles (Saunders 1975, 1976, 1978, 1979, 1981). Nevertheless, a convincing discrimination between external and internal coincidence is still lacking, for *S. argyrostoma* or any other species.

The daily light cycle is the principal entraining agent for circadian rhythms, as well as the main environmental signal for photoperiodic control. However, temperature steps and pulses can phase-shift circadian rhythms (Zimmerman et al. 1968;

* Dedicated to Professor C.S. Pittendrigh on his 65th birthday

Chandrashekaran 1974) and daily temperature cycles can entrain circadian rhythms in darkness (Roberts 1962). In a number of species, temperature cycles can also substitute for photoperiods in diapause induction (Saunders 1973b; Chippendale et al. 1976; Dumortier and Brunnarius 1977; Masaki and Kikukawa 1981; Beck 1982). This paper now uses thermoperiods to distinguish between the external and internal types of photoperiodic clock for the *Sarcophaga* case, by attempting to separate the entraining and inducing roles of light.

Materials and methods

Stock and experimental cultures. Stock cultures of flies were maintained in constant light (*LL*) at 25 °C. They were supplied with water and granulated sugar *ad libitum*, and with a daily supply of fresh meat. Stock and experimental larval cultures were established by transferring pieces of meat carrying newly-deposited larvae from the 'adult cages' to plastic dishes containing a supplementary diet made from agar, dried milk and yeast. Mature larvae were allowed to disperse into dry sawdust to form puparia.

For experimental purposes, cultures of adult flies were also kept (at 25 °C) in either a cycle of 12 h of light followed by 12 h of darkness (*LD 12*:12) or in continuous darkness (DD), the former to provide a stock of larvae 'pre-programmed' for pupal diapause (Denlinger 1971; Saunders 1980), the latter to provide a stock kept in the dark throughout their development.

Experimental cultures of 300 to 700 larvae derived from these sources were placed in light-proof wooden boxes or incubators. The wooden boxes were held in a constant temperature room, each box being fitted with a fluorescent light source (Philips 4W), water-jacketted to counteract excessive temperature fluctuation. Light cycles, when used, were regulated by Venner time switches. The incubators were Gallenkamp cooled models some of which could be programmed to provide a daily temperature cycle. At the end of each experiment the puparia were collected from the sawdust and maintained in the dark at 19 to 20 °C for a further 10 to 14 days before dissection to ascertain the diapause or non-diapause status of the pupae within them (Fraenkel and Hsiao 1968).

Temperature cycles. The daily temperature cycles (thermoperiods) applied to larval cultures in continuous darkness comprised different number of hours at 25 or 15 °C in each 24 h period: these cycles are described as a ratio *H* (hot, 25°): C (cold, 15°), e.g. *HC 4*:20 or *HC 12*:12. The daily low-temperature pulses (LTPs) applied to cultures in a concomitant light cycle (at 17 °C) were 3 h periods at 5 °C, applied at different times of the light cycle.

These temperature regimes were provided by Gallenkamp cooled incubators programmed for 'square-wave' cycles between the two previously-set temperatures (25 and 15 °C), or by manual transfers from one incubator (at 25°) to another (at 15°), or from wooden boxes in a constant temperature room (at 17°) to cooled incubators (at 5°). All movements of cultures between cabinets and incubators were carried out rapidly, and the temperature changes, both within the body of the incubator or cabinet, and within the larval medium, were monitored with Rustrak temperature recorders. Examples of such recordings are shown in Fig. 1. In the temperature-cycling incubators, and with a temperature probe lying above the larval medium, 80% of the heating or cooling (between 15 and 25°) was achieved within 15 to 30 min of the switch (Fig. 1A and B). With the probe placed within the larval medium, however, such changes took about 60 min (Fig. 1C). Temperature changes resulting from manual shifts between 17 and 5° were completed within about 5 min (Fig. 1D).

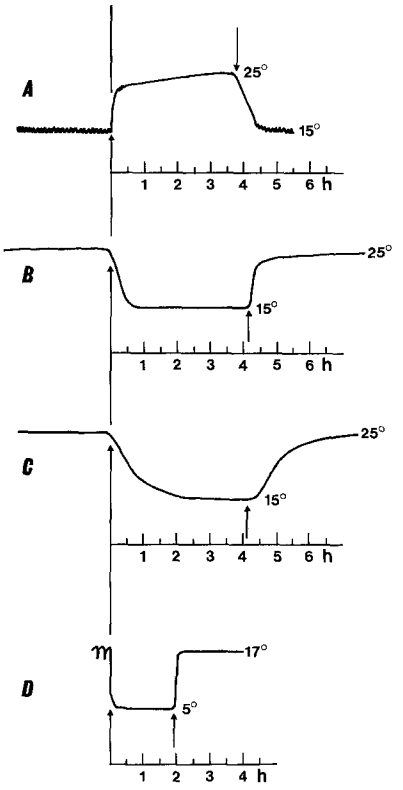

Fig. 1 A–D. Recordings of temperature cycles applied to cultures of *Sarcophaga argyrostoma* larvae. **A** 4 h temperature rise from 15 to 25° in a 'programmed' temperature-cycling incubator, with the temperature probe just above the group of feeding larvae. **B** ditto, 4 h temperature fall from 25 to 15 °C. **C** ditto, with the temperature probe within the larval medium. **D** 2 h low temperature pulse obtained by moving culture manually from 17° to 5 °C and back. Probe lying just above the feeding larvae

Results

The effect of daily thermoperiods in darkness

Larval cultures derived from adult flies kept at 25 °C, *LD 12*:12 or *LL*, were set up in daily thermoperiods (15/25 °C) of *HC 8*:16, *12*:12, *16*:8, and *18*:6, all in continuous darkness (DD). Other cultures were established as controls at 15 and 25 °C, and in constant temperatures of 18.3, 20, 21.7 and 22.5 °C, again in DD, these latter four temperatures being equivalent to the arithmetic means of the thermoperiods cycling between 15 and 25 °C. All temperatures were maintained within about 0.5 °C.

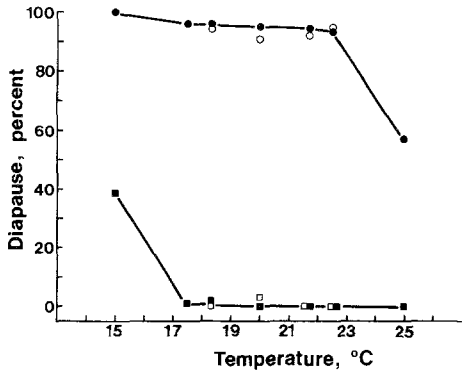

Fig. 2. *S. argyrostoma.* Incidence of pupal diapause in cultures maintained as larvae in either constant temperature and darkness (solid circles and squares) or in a daily thermoperiod and darkness (open circles and squares). Circles (solid and open) show data for larvae deposited by flies maintained at *LD 12*:12, 25 °C until larviposition; squares (solid and open) data for larvae deposited by flies maintained in *LL*, 25 °C. Data for thermoperiods (*HC 8*:16, *HC 12*:12, *HC 16*:8, and *HC 18*:6) are plotted as their arithmetic mean temperatures (18.3, 20, 21.7, and 22.5 °C)

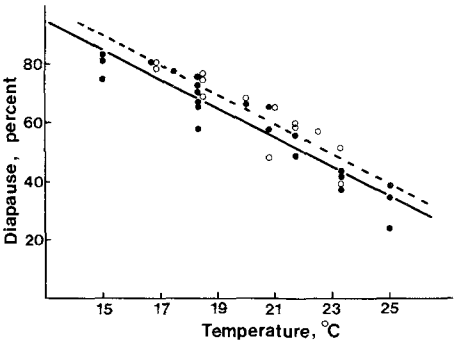

Fig. 3. *S. argyrostoma.* Incidence of pupal diapause in cultures maintained as larvae in either constant temperature and darkness (solid circles) or in a daily thermoperiod and darkness (open circles). The adult flies which deposited these larvae were kept in continuous darkness at 25 °C, so that both larvae *and* intra-uterine embryos were never exposed to light. Data for thermoperiods (*HC 4*:20, *HC 8*:16, *HC 12*:12, *HC 14*:10, *HC 16*:8, *HC 18*:6, and *HC 20*:4) are plotted as their arithmetic mean temperatures (16.7, 18.3, 20, 20.8, 21.7, 22.5 and 23.3 °C)

Figure 2 shows that cultures derived from *LD* females produced a high incidence of pupal diapause at all temperatures up to 22.5°, but a lower incidence at 25°. Conversely, all cultures derived from *LL* females produced a low incidence of diapause except at the lowest temperature (15°). This difference underlines the observaton that the embryos within the maternal uterus are maximally sensitive to photoperiod (Denlinger 1971; Saunders 1980), and that larvae are to some extent 'pre-programmed' for diapause or non-diapause development by the time they are deposited on the meat. The results also show that the incidence of pupal diapause in daily thermoperiods is very close to that in a constant temperature when that in the former is plotted against the arithmetic mean temperature of the cycle.

Since the embryonic stage is the most sensitive to photoperiod, and the incidence of pupal diapause in *LD*-derived and *LL*-derived cultures was close to 100% and 0% respectively, the experiment was repeated with larvae derived from adult flies maintained at 25° and in continuous darkness since the pupal stage. These larvae, therefore, experienced no illumination at any point in their embryonic or post-embryonic development. The second experiment was extended to include thermoperiods of *HC 4*:20, *14*:10 and *20*:4, and the corresponding mean temperatures of 16.7, 20.8 and 23.3 °C.

The results of this experiment (Fig. 3) showed that diapause incidence dropped from about 97% at 15° to about 30% at 25° and that responses to daily thermoperiods were almost identical to constant temperatures when the former were calculated as the arithmetic mean of the number of hours at 15 or 25°. Calculated regressions for diapause on temperature for constant temperatures and thermoperiods (Fig. 3) show that the slopes were almost identical (−4.97 and −5.05; F = 0.0097 for 1, 31 df, *P* n.s.), but the elevations were significantly different at the 5% level (F = 5.2873 for 1, 32 df). Both slopes were significantly different from zero.

The most important aspect of these results is that there is a negative *linear* relationship between temperature and diapause incidence in the absence of a light cycle. There is no evidence for a 'critical thermoperiod' which would suggest that daily temperature cycles may substitute for light cycles in diapause regulation.

The effect of daily low temperature pulses with a concomitant light cycle

Cultures of larvae deposited by flies kept in continuous light at 25° were raised in a daily light cycle (*LD* 14:10, 17 °C) just short of the critical photoperiod (about 14.5 h/24). This combination of adult and larval temperatures and photoperiod was chosen to give an 'unsaturated' diapause response (44 to 61%) in the control groups. Experimental cultures were then exposed to the same light cycle, but additionally received a daily pulse of low temperature (5°) starting, in different cultures, at progressively later times of the night (Fig. 4). This experiment was repeated three times.

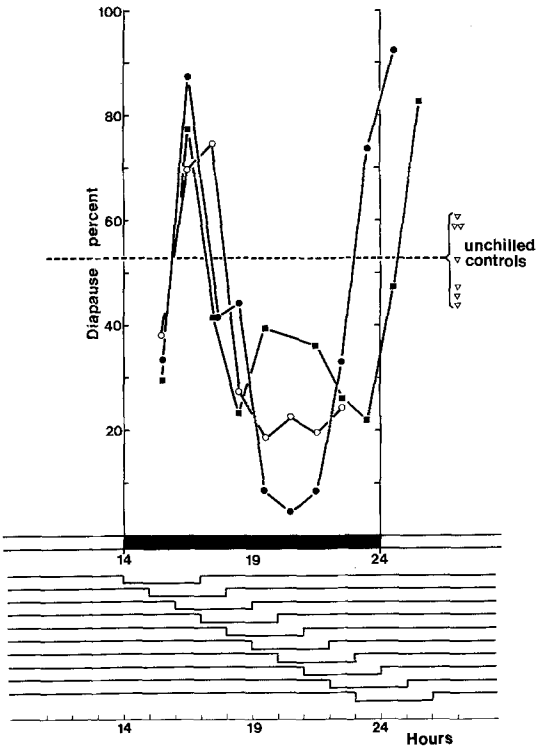

Fig. 4. *S. argyrostoma.* Incidence of pupal diapause in 3 replicate cultures (● ■ ○) of larvae exposed to a daily light cycle (*LD 14*:10) and a concomitant temperature cycle (*HC 21*:3; 17/5 °C) with the 3 h low temperature pulse starting daily at sequentially later times in the 10 h 'night'. Open triangles show 7 replicate controls for unchilled larvae in the light cycle, and the horizontal dotted line their mean value. Experimental design is shown at the bottom

The addition of the daily low temperature pulse (LTP) was found to have marked effects on the incidence of pupal diapause. For example, when the pulse commenced 2 to 3 h after light-off, diapause incidence rose to 70 to 87%. When the LTP commenced at hour 6, on the other hand, diapause fell sharply to a minimum of 5%. With still later pulses the incidence of diapause climbed back to about 80%. The results of the three replicate experiments were essentially the same.

An interpretation of this result in terms of resetting the circadian photoperiodic clock is offered in the Discussion. Here, however, it is pointed out that temperature cycles (*HC 21*:3, where H = 17° and C = 5°) in conjunction with *LD 14*:10 have marked (and phase-dependent) effects on diapause induction, whereas daily thermoperiods (25°:15°) offered in darkness (Fig. 3) do not.

Discussion

External and internal coincidence represent two broad classes of model for the photoperiodic clock which differ mainly in that light is seen to have a circadian entraining role in both, but an additional inductive role in the former (Pittendrigh 1972). Bearing in mind that alternative explanations for photoperiodic time measurement exist, this difference invites tests to distinguish external from internal coincidence by separating these two roles for light. Following suggestions made by C.S. Pittendrigh, for example, it was found that larval diapause in the parasitic wasp *Nasonia vitripennis* could be regulated by the use of daily thermoperiods in continuous darkness (Saunders 1973b); this result led to the conclusion that internal rather than external coincidence was the most likely explanation. Similar thermoperiodic control of diapause has since been demonstrated in *Diatraea grandiosella* (Chippendale et al. 1976), *Pieris brassicae* (Dumortier and Brunnarius 1977), *Plodia interpunctella* (Masaki and Kikukawa 1981) and *Ostrinia nubilalis* (Beck 1982). External coincidence was clearly excluded in the case of *P. interpunctella* since thermoperiodic regulation was equally effective in DD or *LL*, but in the other species (and in *N. vitripennis*) a modified form of external coincidence involving a temperature-sensitive phase, rather than a light-sensitive phase, remained a possibility. Such approaches, therefore, failed to make an unequivocal distinction between the two types of clock.

The present results with *S. argyrostoma*, however, clearly demonstrate (1) the absence of a thermoperiodic effect on diapause induction in DD, and (2) a strong and phase-dependent effect of low temperature pulses with a concurrent light cycle, although it should be borne in mind that experiments (1) and (2) used different temperature cycles, 25:15° and 17:5°, respectively. Although it is possible that a low temperature pulse (in 2) acts directly on the diapause inducing mechanism rather than as an entraining agent, low temperature normally enhances diapause induction whereas, in these experiments, it almost abolished it when delivered late at night. In addition, although a few insect species appear not to use *light* as a Zeitgeber (Tweedy and Stephen 1970; Riba 1976), the temperature cycle, in poikilotherms, may be a universal entraining agent. Therefore, assuming that temperature *is* an effective Zeitgeber, observation (1) would seem to exclude a form of internal coincidence in which constituent ('dawn' and 'dusk') oscillators were entrained by the thermoperiod (as well as by light); the second observation (2) would strongly suggest a requirement for light in addition to entrainment; and the two observations together would constitute evidence consistent with an external type of clock.

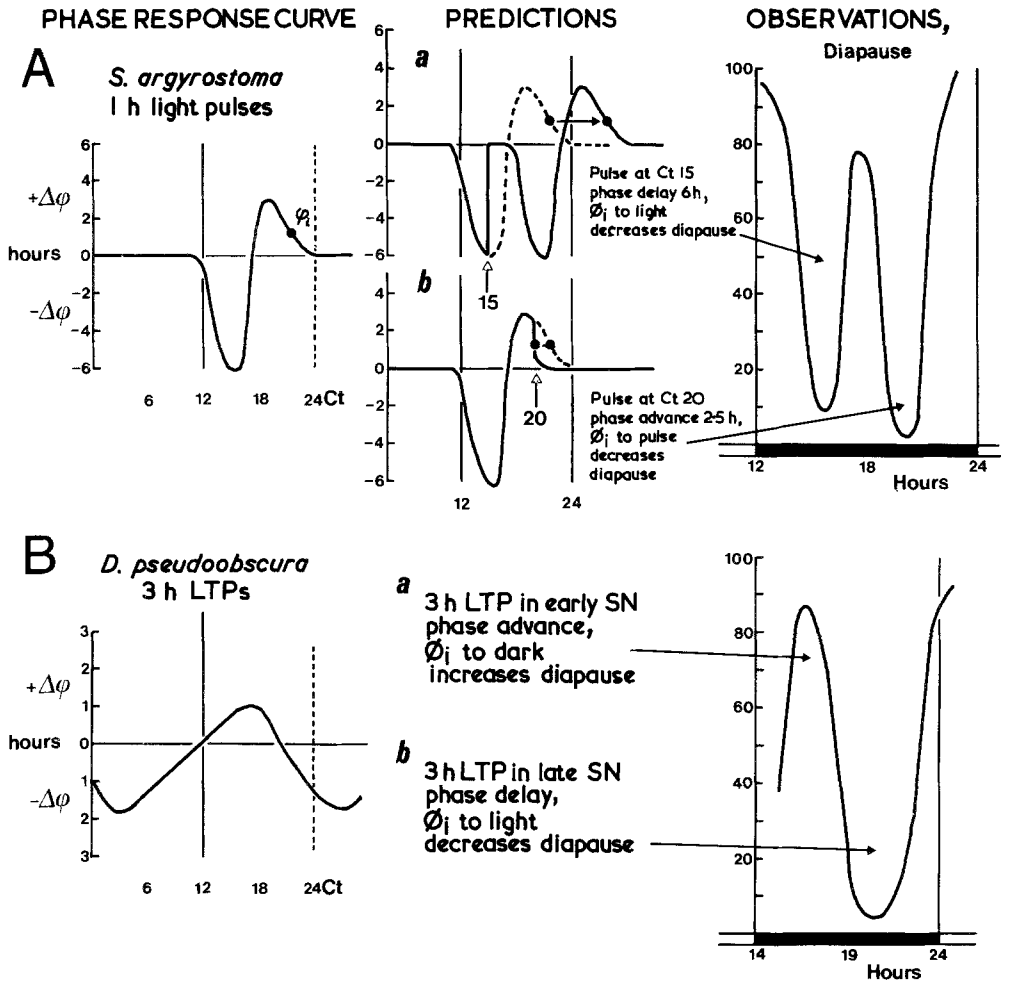

Fig. 5A, B. An interpretation of the experimental data in Fig, 4 in terms of the resetting behaviour of the circadian oscillation(s) within the external coincidence photoperiodic clock. **A** *Responses to 1 h light pulses. Left:* Phase response curve for 1 h light pulses (from Saunders 1978) showing phase delays ($-\Delta\Phi$) when pulses fall in the early subjective night, and phase advances ($+\Delta\Phi$) when they fall in the late subjective night. Φ_i – photoinducible phase. *Centre:* (a) predicted phase delays of Φ_i into next light fraction when pulse falls at Ct 15. (b) predicted phase advance and coincidence of Φ_i with pulse when the pulse falls at Ct 20. *Right:* Observed bimodality in diapause incidence in asymmetrical skeleton photoperiods (night interruption experiments) (from Saunders 1978). **B** *Response to 3 h low temperature pulses. Left:* Phase response curve (*Drosophila pseudoobscura,* Chandrashekaran 1974) for 3 h LTPs, showing phase advances ($+\Delta\Phi$) when pulses fall in the early subjective night, and phase delays ($-\Delta\Phi$) when they fall in the late subjective night. *Centre:* (a) and (b) predictions for phase-shifting the photoinducible phase (Φ_i). *Right:* Observed incidence of pupal diapause in combined light and temperature cycles (see Fig. 4 for comparison, and text)

Although unequivocal evidence for external coincidence is still lacking, the low temperature pulse experiment (Fig. 4) may be interpreted in terms of this model. In Fig. 5 the top panel (A) interprets the effect of a 1 h light pulse falling early in the subjective night (at circadian time, Ct 15). At this phase it causes a phase delay in the oscillation, 'pushes' the photoinducible phase (Φ_i, at Ct 21.5) into the next light component of the cycle, and therefore causes a low incidence of diapause (Pittendrigh 1966; Saunders 1981). A pulse falling late in the night (at Ct 20), on the other hand, causes phase advances until Φ_i coincides directly with the light pulse, again eliminating diapause. Systematic pulsing of the night with a 1 h light pulse thus gives the characteristic bimodality associated with 'night interruption' experiments, which is a result of the dual roles of light, entrainment and induction. The bottom panel (B) shows a similar interpretation for the current low temperature pulse experiments. In the absence of an LTP phase response curve for *S. argyrostoma,* a PRC for 3 h LTPs in the *Drosophila pseudoobscura* eclosion rhythm (Chandrashekaran 1974) is used; this curve shows phase advances early in the subjective night (Ct 12 to 18) and phase delays in the late subjective night (Ct 18 to 24), and is assumed to be qualitatively representative of the unknown PRC for *S.*

argyrostoma. Using this PRC, an LTP commencing early in the night would be expected to phase advance Φ_i to earlier times of the night and thus enhance the light cycle's diapause-inducing effect, whilst an LTP commencing late in the night would phase delay Φ_i into the light fraction of the cycle so producing a diapause-averting response. Such an interpretation predicts a 'wave-shaped' curve (Fig. 5B; lower right) comparable to the experimental results shown in Fig. 4. The obvious similarities between prediction and observation encourage interpretation in terms of external coincidence, and suggest that although both light and temperature cycles can entrain the circadian oscillation(s) comprising the clock, only light falling on Φ_i can effect the photoperiodic switch. This phase occurs about $9^1/_2$ h (the critical night length) after the end of the light component (='day') in excess of about 10 h (Saunders 1978), and the success of the model in explaining a wide array of experimental data strongly suggests that attention should now be directed at the photochemical events which must occur at that time.

Acknowledgements. This work was supported by a grant from the Nuffield Foundation. Kathleen Rothwell is thanked for her expert technical assistance.

References

Beck SD (1982) Thermoperiodic induction of larval diapause in the European corn borer, *Ostrinia nubilalis*. J Insect Physiol 28:273–277

Chandrashekaran MK (1974) Phase shifts in the *Drosophila pseudoobscura* circadian rhythm evoked by temperature pulses of varying length. J Interdiscipl Cycle Res 5:371–380

Chippendale GM, Reddy AS, Catt CL (1976) Photoperiodic and thermoperiodic interactions in the regulation of the larval diapause of *Diatraea grandiosella*. J Insect Physiol 22:823–828

Denlinger DL (1971) Embryonic determination of pupal diapause in the flesh fly *Sarcophaga crassipalpis*. J Insect Physiol 17:1815–1822

Dumortier B, Brunnarius J (1977) L'information thermopériodique et l'induction de la diapause chez *Pieris brassicae*. C R Acad Sci Paris D 284:957–960

Follett BK, Follett DE (1981) Biological clocks in seasonal reproductive cycles. John Wright & Sons, Bristol

Fraenkel G, Hsiao C (1968) Manifestations of a pupal diapause in two species of flies, *Sarcophaga argyrostoma* and *S. bullata*. J Insect Physiol 14:689–705

Masaki S, Kikukawa S (1981) The diapause clock in a moth: response to temperature signals. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright & Sons, Bristol, pp 101–112

Pittendrigh CS (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. Z Pflanzenphysiol 54:275–307

Pittendrigh CS (1972) Circadian surfaces and the diversity of possible roles of circadian organisation in photoperiodic induction. Proc Natl Acad Sci USA 69:2734–2737

Pittendrigh CS (1981) Circadian organisation and the photoperiodic phenomena. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright & Sons, Bristol, pp 1–35

Riba G (1976) Alimentation des larves de *Rhagium inquisitor* L. (Coleoptera, Cerambycidae) en conditions expérimentales, rythme prandial, transit intestinal, indices de consommation et de croissance. Ann Zool Ecol Anim 21:499–511

Roberts SK de F (1962) Circadian activity in cockroaches. II. Entraiment and phase-shifting. J Cell Comp Physiol 59:175–186

Saunders DS (1971) The temperature-compensated photoperiodic clock 'programming' development and pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. J Insect Physiol 17:801–812

Saunders DS (1973a) The photoperiodic clock in the flesh-fly, *Sarcophaga argyrostoma*. J Insect Physiol 19:1941–1954

Saunders DS (1973b) Thermoperiodic control of diapause in an insect: theory of internal coincidence. Science 181:358–360

Saunders DS (1975) 'Skeleton' photoperiods and the control of diapause and development in the flesh-fly, *Sarcophaga argyrostoma*. J Comp Physiol 97:97–112

Saunders DS (1976) The circadian eclosion rhythm in *Sarcophaga argyrostoma:* some comparisons with the photoperiodic clock. J Comp Physiol 110:111–133

Saunders DS (1978) An experimental and theoretical analysis of photoperiodic induction in the flesh-fly *Sarcophaga argyrostoma*. J Comp Physiol 124:75–95

Saunders DS (1979) External coincidence and the photoinducible phase in the *Sarcophaga* photoperiodic clock. J Comp Physiol 132:179–189

Saunders DS (1980) Some effects of constant temperature and photoperiod on the diapause response of the flesh-fly, *Sarcophaga argyrostoma*. Physiol Entomol 5:191–198

Saunders DS (1981) Insect photoperiodism: entrainment within the circadian system as a basis for time measurement. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright & Sons, Bristol, pp 67–81

Saunders DS (1982) Insect Clocks, Second edition. Pergamon Press, Oxford

Tweedy DG, Stephen WP (1970) Light-refractive emergence rhythm in the leaf-cutter bee, *Megachile rotundata* (F.) (Hy., Apoidea). Experientia 26:377–379

Zimmerman WF, Pittendrigh CS, Pavlidis T (1968) Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. J Insect Physiol 14:669–684