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Insect Photoperiodism: An Hourglass Measures Photoperiodic Time in *Ostrinia nubilalis*

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Summary. The European corn borer (*Ostrinia nubilalis*) diapauses as a last instar larva. Both induction and termination of diapause are photoperiodically controlled. Larvae enter diapause (Fig. 1) when raised in light cycles containing 12 or 13 h of light per 24 h (LD 12:12 or LD 13:11). When placed in LD 16:8, diapausing larvae undergo pupation and adult development (Fig. 2, 3).

Long light cycle (T) experiments demonstrate that the timing mechanism involved in terminating diapause has the properties of an interval timer or hourglass mechanism (Fig. 4). Six inductive 8-h dark phases are necessary for optimal termination (Fig. 5), and they must be coupled with at least 4 h of light per cycle (Fig. 6). The circadian system in *Ostrinia* is probably not involved in any way in this response.

Introduction

Seasonally appropriate changes in metabolic strategy are characteristic of many organisms, most conspicuously in those indigenous to temperate latitudes. Garner and Allard (1920) were the first to observe that flowering in plants is often controlled by the relative duration of light and dark in the daily cycle of environmental change. After this initial discovery other photoperiodic phenomena were found in animals, notably insects (Marcovitch, 1924), birds (Rowan, 1926) and mammals (Bissonnette, 1932). Such timed seasonal events are said to be photoperiodically controlled, and the process by which this occurs has been termed photoperiodic induction. That the environmental cue is the daily cycle of light and dark is not surprising since the systematic change in the length of the day and night offers the most precise and reliable seasonal marker. The adaptive significance of such timing is obvious. Processes such as reproduction and development occur only during the time of year which is most favorable for species survival.

The overall mechanism by which organisms respond to photoperiodic cues must involve a clock or timing device. Some process measures the length of the day (or night) and triggers the appropriate response, and over the past several years much work has been directed at trying to elucidate the nature of the photoperiodic clock (see, for example, reviews by Adkission, 1966; Beck, 1968; Hammer and Hoshizaki, 1974; Pittendrigh, 1974; Saunders, 1974).

Bünning (1936) first attempted to relate in a causal way what were then referred to as daily rhythms with photoperiodic time measurement, and in the 1960's Pittendrigh and co-workers extended this line of reasoning in their attempt to formulate a unified theory of the photoperiodic clock. Their model was based on a more complete understanding of the entrainment of circadian oscillations by light and temperature cycles (Minis, 1965; Pittendrigh, 1965, 1966; Pittendrigh and Minis, 1964, 1971). However, despite a substantial body of evidence that now exists which argues for involvement of the circadian system in photoperiodic time measurement in both invertebrates and vertebrates, there are some organisms that appear to use a nonoscillatory hourglass mechanism. This model of the photoperiodic clock states that night length is measured by the accumulation of some metabolic product over a successive number of cycles. Light destroys the product (resets the hourglass) every day. The best example of this is in the aphid *Megoura viciae* (Lees, 1965, 1966, 1971) in which the production of sexual and parthenogenic females is under photoperiodic control.

This paper discusses the nature of the clock mechanism that measures photoperiodic time in the European corn borer, *Ostrinia nubilalis*. *O. nubilalis* diapauses as a late last instar larva.

Materials and Methods

Eggs were obtained from the stock colony of *O. nubilalis* that is maintained by the Department of Entomology and Applied Ecology at the University of Delaware. The eggs for the initiation of the stock colony were originally collected from corn fields in Sussex County, Delaware. Larvae were raised on a lima bean medium (Curl et al., 1975).

Eggs used in the diapause induction experiments were maintained under experimental light cycles from the day of oviposition onward. Each condition contained 4 replicate containers (25 larvae per container) which were examined daily for evidence of pupation.

For the production of diapausing larvae for use in the diapause termination experiments, eggs were maintained in a light cycle of 12 h light alternating with 12 h of darkness (LD 12:12) until a minimum age of 25 days. At this point, the small percentage of larvae that did not enter diapause had pupated (see Fig. 1A). Diapausing larvae were transferred from the food containers to containers lined with filter paper that was continuously saturated with distilled water. Larvae cease all feeding upon entering diapause. All experimental conditions contained four replicate containers (25 diapausing larvae per container) that were examined daily during the first hour of the light (photophase) for evidence of pupation. Containers in constant darkness were examined under a red light.

In both diapause induction and termination experiments, all containers were maintained in light-tight black boxes containing ceiling-mounted 7-watt cool-white fluorescent tubes (Sylvania F4T5/D) which were water-jacketed to avoid temperature cycles. The floor level illumination was approximately 220 lux. The boxes were also equipped with a system of baffles which were continuous with a small ventilator fan, and they were housed in a temperature controlled walk-in growth chamber (30 ± 1.0 °C). The light cycles were controlled by standard electric timers.

Results

Induction of Diapause

The photoperiodic response curve for Delaware-collected borers is presented in Figure 1B (broken line). LD 12:12 and LD 13:11 induce a high amount of diapause, but larvae raised in cycles containing less than 12 or more than 13 h of light per 24 h undergo pupation and adult development (see Fig. 1A for the time course of pupation in LD 16:8 vs. LD 12:12).

Experiments designed to elucidate the nature of the photoperiodic clock often involve protocols that call for light cycles with relatively long periods (e.g., LD36:12; period=T=L+D=48 h). Larvae raised under long T's behave as if they are in constant light and no, or very little, induction of diapause occurs (Skopik, unpublished data). One interpretation of these experiments is that the incidence of diapause depends on the number of inductive cycles the larvae experience, and the relatively short length of larval development (approximately 16 days at 30 °C) precludes many cycles of long period length. For this reason we turned to a study of the clock mechanism involved in the termination of diapause.

Termination of Diapause

Diapause can be terminated in *O. nubilalis* by transferring diapausing larvae to a light cycle containing a 16 h photophase (McLeod and Beck, 1963). In

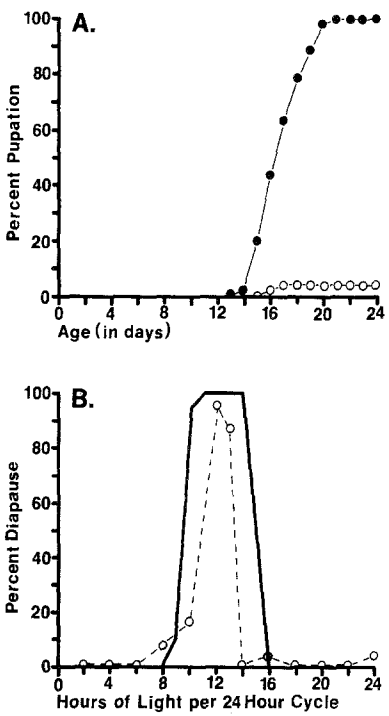

Fig. 1A and B. Induction of larval diapause. **A** Time course of pupation in LD 16:8 (solid circles) and LD 12:12 (open circles). Time 0 is the day of oviposition. Data from four replicates (25 larvae/replicate) in each light cycle are combined. The sample size in this, and all subsequent figures, is 100. **B** Photoperiodic response curves for two races of *Ostrinia nubilalis* (broken line, Delaware-collected borers; solid line, Wisconsin-collected borers [from Beck, 1962])

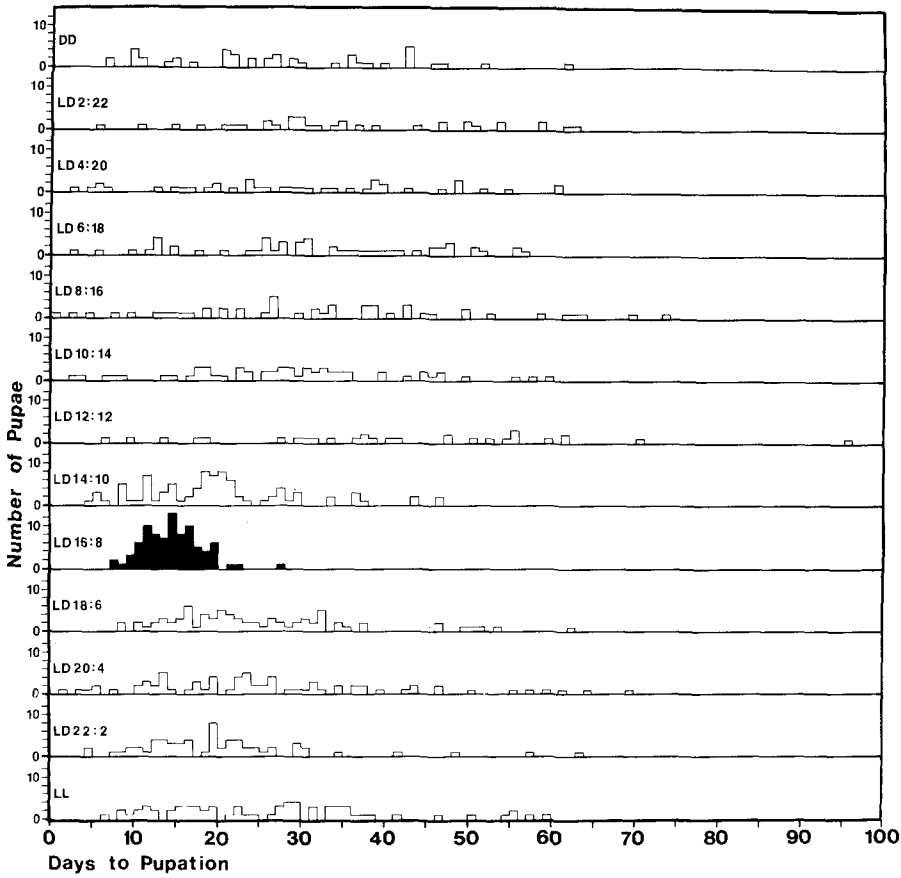

Fig. 2. Photoperiodic termination of larval diapause. Frequency distributions of pupation for light cycles ranging from constant darkness (DD) to constant light (LL) after transfer from LD 12:12 are presented. See text for discussion

addition, *O. nubilalis,* unlike many insect species (see, for example, Truman, 1971a) does not require a prolonged period of chilling before diapause can be terminated.

Frequency distributions of pupation for populations of diapausing larvae that were transferred from LD 12:12 to DD, LD 2:22, LD 4:20 and so on in two hour increments up to constant light are shown in Figure 2. These data demonstrate several points: (1) The LD 12:12 control population is characterized by high larval mortality (71%), a mean time to pupation of 42.0 days for the survivors, and a high standard error (see Table 1 for the summary statistics of the distributions). The sporadic pupation observed in LD 12:12 is probably brought about by the high temperature employed in these experiments, and the high mortality is probably due to a depletion of metabolic reserves. (2) Populations of larvae placed in cycles with photophases shorter than 12 h are also characterized by sporadic pupation, mean pupation times that range from 26.9 to 35.5 days, and high standard errors. (3) Sporadic

Table 1. Summary statistics for frequency distributions of pupation plotted in Figure 2

Light cycle	# Pupae	# Dead larvae	$\bar{x}$ time (in days) to pupation (±SEM)
DD	45	55	26.9 (±2.0)
LD 2:22	35	65	35.5 (±2.5)
LD 4:20	40	60	29.9 (±2.5)
LD 6:18	53	47	32.6 (±1.9)
LD 8:16	50	50	31.3 (±2.5)
LD 10:14	58	42	28.9 (±1.7)
LD 12:12	29	71	42.0 (±3.6)
LD 14:10	88	12	20.1 (±1.0)
LD 16:8	86	14	14.6 (±0.4)
LD 18:6	79	21	24.9 (±1.3)
LD 20:4	72	28	26.8 (±1.8)
LD 22:2	62	38	20.6 (±1.4)
LL	68	32	26.5 (±1.6)

pupation is not characteristic of larvae transferred to LD 14:10. In this light cycle a peak of pupation is observed, and the mean time to pupation is 20.1 days with a standard error that is lower than in all of the light cycles with shorter photophases. (4) LD 16:8 is clearly the optimal light cycle for terminating diapause. The reproducibility of this result is shown in Figure 3, where LD 16:8 populations from 5 different experiments are compared. The mean time to pupation in these experiments is 16.8 days with a range from 14.6 to 18.1 days. Finally, (5) In cycles with photophases longer than 16 h the synchrony of pupation that is observed in LD 16:8 breaks down. These populations also exhibit higher larval mortality than LD 14:10 and LD 16:8.

Evidence for an Hourglass Mechanism

Although it is clear from the data in Figure 2 that LD 16:8 must set in motion the neuroendocrine events that control pupation and adult development, it is impossible to say whether or not the circadian system is involved in any way in this response. Or, if an hourglass or interval timer is involved, whether the light or dark phase of the cycle is being measured.

To determine whether or not the circadian system is involved in diapause termination a T experiment of the type first used by Nanda and Hamner (1958) was conducted. Pittendrigh (1972), in a review of the problems that exist in the study of photoperiodism, has suggested that this technique would be most useful in clarifying the diverse roles that circadian organization can play in photoperiodic induction. It involves coupling a fixed photophase (e.g., 16 h) with extended periods of darkness to produce various T's. When induction rises and falls as a function of T (e.g., peaks of induction at T24, T48, and T72) the circadian system is probably involved in some way as the photoperiodic clock. The T's used were T24 (LD 16:8), T36 (LD 16:20), T48 (LD 16:32) and T60 (LD 16:44). Figure 4A shows a more complete account of the results

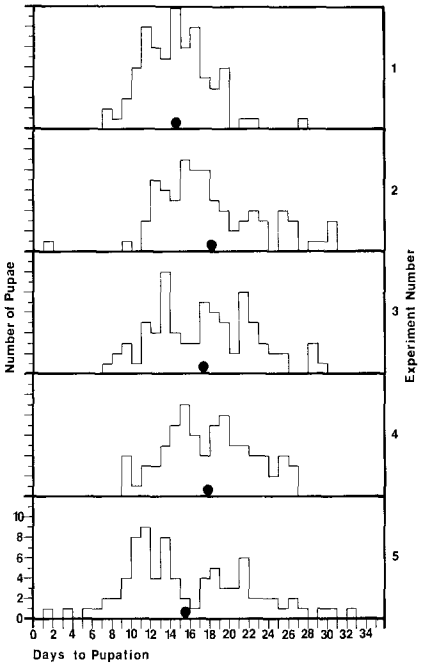

Fig. 3. Reproduciblity of the system. The five frequency distributions of pupation are from different experiments involving LD 16:8. The means of pupation, indicated by the solid circles, range from 14.6 to 18.1 days

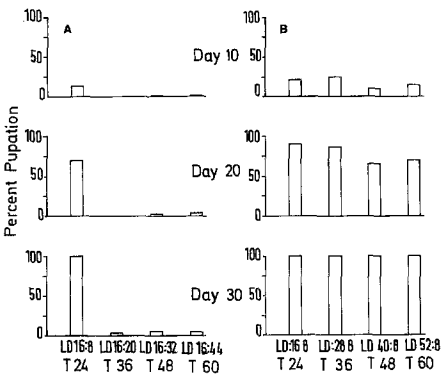

Fig. 4A and B. Percentage of pupation of survivors 10, 20, and 30 days after transfer from LD 12:12 to various T's.
In **A**, L was held constant at 16 h and T was varied by extending D.
In **B**, D was held constant at 8 h and T was varied by extending L

from this experiment that have been summarized elsewhere (Bowen and Skopik, 1976). The percentage of pupation after 10, 20 and 30 days in the various T's show that 100% of the survivors had pupated, as expected, by day 30 in the control group (LD 16:8). However, pupation was negligible in the remaining T's. These data suggest that circadian rhythmicity is probably not involved in the time measurement nor is 16 h of light the component of the cycle important in diapause termination. Moreover, the data suggest that an hourglass mechanism is involved, and that it measures the length of the dark phase (scotophase) with 8 h being optimal. In order to test this, the standard protocol for the T experiment was reversed. The scotophase was held constant at 8 h while the duration of the photophase was lengthened to produce T's of 36 (LD

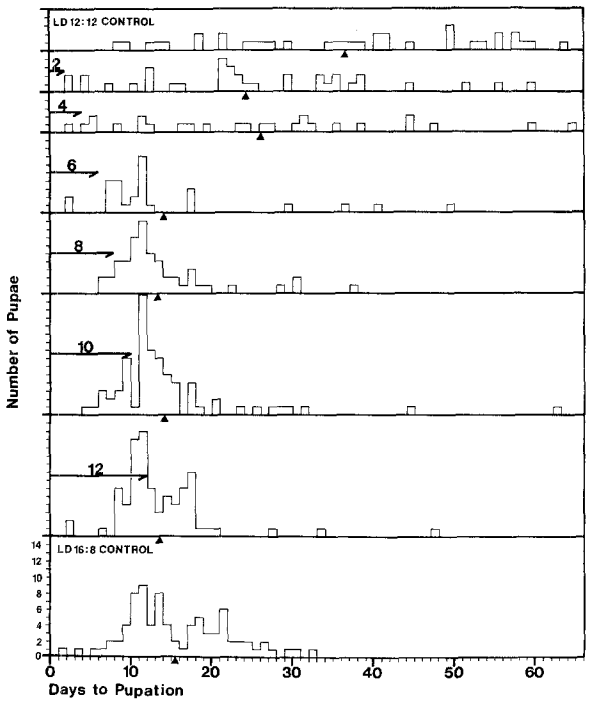

Fig. 5. Number of 8-h scotophases necessary for termination of diapause. Populations of diapausing larvae were transferred to LD 16:8 for 2, 4, 6, 8, 10 and 12 days (indicated by the heavy lines) and then returned to LD 12:12 and examined daily for pupation. Triangles indicate the mean pupation time for each condition

28:8), 48 (LD 40:8) and 60 (LD 52:8) hours. The results (Fig. 4B) support this hypothesis. Pupation of the survivors reached 100% after 30 days in all T's indicating that 8 h of darkness was being measured and that the measurement can take place regardless of the period of the light cycle.

Since the larvae in the longer T's in Figure 4B, i.e., those with the fewest number of 8 h scotophases, also exhibited 100% pupation by day 30, the number of scotophases needed to effect optimal induction is small.

Number of Cycles Necessary for Termination of Diapause and the Light Requirement

The number of cycles (8 h scotophases) necessary for termination of diapause was determined by allowing populations of larvae to measure time for a given number of cycles (2, 4, 6, 8, 10 and 12) in LD 16:8. They were then placed back in LD 12:12 and examined daily for pupation (see Fig. 5). Larvae receiving either 2 or 4 cycles of LD 16:8 do not terminate diapause synchronously. The distributions of pupation more closely resemble the LD 12:12 control (upper panel) than they do the LD 16:8 control (lower panel). On the other hand,

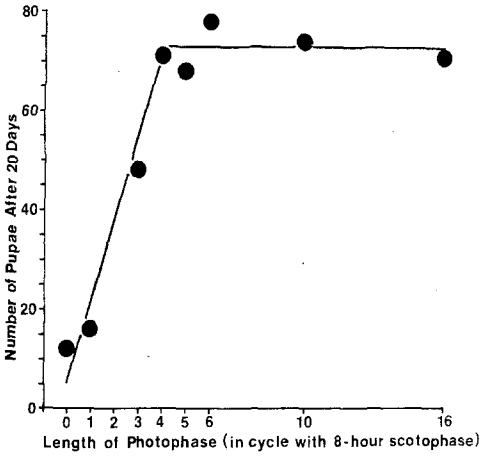

Fig. 6. Number of hours of light required for optimal termination of diapause. Eight-hour scotophases were coupled with 1, 3, 4, 5, 6, 10 and 16 h of light. Filled circles represent the number of pupae after 20 days in each condition

populations receiving 6, 8, 10 or 12 cycles of LD 16:8 show synchrony of pupation and mean pupation times that are almost identical with the LD 16:8 control. From these data it is clear that at least six 8-h scotophases are necessary for optimal termination of diapause.

In addition to an 8-h scotophase, there is also a light requirement for terminating diapause. It is 16 h or less. To determine the length of this light requirement 8-h scotophases were coupled with 1, 3, 4, 5, 6, 10 and 16 h of light, thus creating T's of 9, 11, 12, 13, 14, 18 and 24 h. The data in Figure 6 indicate that 4 h of light are sufficient, when coupled with 8 h of darkness, for optimal termination. LD 4:8 is as effective as LD 16:8. An intermediate response was obtained with LD 3:8 and LD 1:8 was identical with the DD control.

Discussion

Induction of Diapause

Evidence from two kinds of experiments led Beck (1962) to originally conclude that diapause in *O. nubilalis* is probably controlled by an hourglass mechanism or interval timer that measures the length of the scotophase. Induction is maximal when 12 h of darkness are measured. The first involved a study of the effect of different photophase:scotophase ratios on the incidence of diapause induction. Ratios of 1:1 yielded 100% induction only in LD 12:12. When the ratio was 2:1, induction was maximal in LD 24:12 but negligible in LD 12:6. Finally, 1:2 ratios effected maximum induction in LD 6:12. The second experiment utilized a protocol common in studies of photoperiodism. A long scotophase that was part of a non-inductive cycle (LD 7:17) was systematically broken up with 1 h pulses of light. Beck found two regions in the scotophase (one early and one late) where a one hour pulse of light would result in a peak of induction. In both cases it was creating a 12 h uninterrupted dark phase.

Pittendrigh (1966, 1972) in a reanalysis of Beck's data emphasized that irrespective of the way time measurement is effected in *Ostrinia,* the quantitative expression of the response it underlies (in terms of percentage of the population entering diapause) is influenced by the state of the circadian system. The circadian system, which has a period, τ, of approximately 24 h is in resonance with the light cycle of the environment when $T = n\tau$. Under these conditions the circadian organization is most nearly normal and phenomena like time measurement are most effectively executed. On the other hand, the system is least normal when driven by $T = n\tau + \tau/2$. Beck's data support this concept. Diapause induction is 100% in LD 12:12 (T24) but it drops to 65% in LD 24:12 (T36). Circadian organization could not be completely ruled out in Beck's data because his experimental light cycles did not extend beyond T40. If it is involved in some way as the photoperiodic clock another peak of induction would be predicted around T48 (LD 36:12) and T72 (LD 60:12). We have tried, and failed, however, to induce diapause with these long T's. Time measurement is probably effected by an hourglass, and the larvae raised in long T's are unable to measure the required number of 12-h scotophases for induction to be triggered. Nevertheless, the fact still remains that in light cycles with period lengths close to τ, the state of the circadian system does modify the response.

Danilevskii and co-workers (see Danilevskii, 1965, for a review) have discussed the implications of photoperiodic control in insects inhabiting wide geographical ranges. They have shown that in general the critical daylength, that daylength at which there is a switch from development to diapause in anticipation of winter, increases with increasing latitude. This means that the photoperiodic response is adaptively correlated with local conditions. In regions like Delaware, where winters are milder than at the northern end of the distribution range, multivoltine races or ecotypes can evolve (see Brindley et al., 1975). It is, therefore, not surprising that the photoperiodic response curve for diapause induction in Delaware-collected borers (Fig. 1B; broken line) shows a shorter critical daylength (approximately 13.5 h) than borers adapted to Wisconsin conditions (approximately 15 h). Not only is the critical daylength shorter, the range over which maximum induction can be effected is significantly narrower. Although the meaning of the almost all-or-none response around LD 12:12 is unclear, it has also been observed in *Pieris brassicae* from different latitudes (Danilevskii, 1965).

Termination of Diapause

Diapausing *Ostrinia* larvae represent an ideal system in which one can study the properties of the photoperiodic clock. They have completed the complex sequence of events involved in larval growth and development. All that they require, to undergo pupation and adult development, is moisture and the appropriate (inductive) light cycle. The larvae can easily be subjected to the long-period light cycles that have proven to be so useful in studies of photoperiodism (Pittendrigh, 1974; Saunders, 1974).

In light cycles with periods of 24 h, either LD 14:10 or LD 16:8 will terminate larval diapause, with the latter result being optimal and reproducible (Fig. 2, 3). On the other hand, light cycles with scotophases outside the range of 8–10 h lose their effectiveness. This is unlike the situation in *Megoura* (Lees, 1965) where all scotophases longer than the critical length promote the development of egg-laying oviparae (vs. parthenogenetic virginoparae).

Light cycles with periods longer than 24 h have helped clarify the issue of involvement of the circadian system in photoperiodic time measurement in *Ostrinia*. The results from long T experiments (see Fig. 4) demonstrate that neither 16 h of light nor the period of the light cycle are important in termination. What is important is whether or not the light cycle contains an inductive 8-h scotophase. LD 52:8 (T60) terminates diapause as effectively as LD 16:8 (T24), and six of these 8-h scotophases are required for optimal termination (Fig. 5).

One might argue that the circadian system itself has hourglass properties. For example, it has been known for some time that the circadian oscillation controlling adult emergence in *Drosophila* damps out at a fixed point in the cycle (circadian time 12) in photophases longer than 12 h. It resumes its motion from this point when darkness occurs (Pittendrigh, 1966; Skopik and Pittendrigh, 1967). Truman (1971b) has observed a similar phenomenon in the silkworm, *Antheraea pernyi*. If the circadian system in *Ostrinia* behaves like that of *Drosophila*, then in all of the T's discussed in Figure 4 the oscillation damps out during the photophase. It resumes its motion at the beginning of the scotophase and this might be the basis for time measurement. The results in Figure 6 do not support this view. Diapause is terminated in larvae raised in short-period light cycles (e.g., LD 4:8 T12 or LD 6:8 T14). Based on studies of the entrainment mechanism in other insects, one would infer that the photophases in these cycles are probably not of sufficient length for damping of the circadian system to occur. More importantly, since induction is being effected with short T's that are outside the normal range of entrainment (see, for example, Pittendrigh, 1965), and since there is little possibility that entrainment is occurring via frequency demultiplication at least in T14 and T18, the circadian system most likely is not involved at all in this response in *Ostrinia*.

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