J Comp Physiol B (1984) 154:91–96

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Photoperiodic induction of pupal diapause in the flesh fly, *Sarcophaga crassipalpis:* embryonic sensitivity

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Accepted June 16, 1983

Summary. The last two days of embryonic development are crucial in programming pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Short daylength (greater than $10^1/_2$ h of darkness) during this interval permits expression of diapause while long daylength during this brief sensitive stage eliminates the potential for diapause. Length of scotophase rather than photophase programs the diapause although three hours of light is needed to separate tandem dark periods. Early in the scotophase, photosensitivity is restricted to blue light (less than 540 nm). The scotophase can be divided into 4 phases according to the effect of light breaks on diapause expression. During Phase I (0–6 h after scotophase onset) embryos are highly sensitive to light interruption and diapause is effectively eliminated. A period of insensitivity to light, Phase II, extends from 6–9 h after onset of scotophase. Light breaks at 10–11 h coincide with the critical scotophase length and result in a partial reduction of diapause. In Phase IV, the scotophase reaction is complete and diapause competence is preserved even in the presence of light. Although light breaks result in elimination of diapause throughout Phase I, recovery time from a 1 h light break (length of darkness needed to counter the effect of a light break) differs dramatically depending upon when the light break is presented. Early in Phase I (0–3 h) recovery from light interruption is rapid, while late in Phase I (4–6 h), the effects of light are not readily reversible. The scotophase reaction thus appears to follow a step-wise progression rather than represent a simple linear response. We present a molecular model that could account for the dynamics of the scotophase reaction.

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Introduction

Although the genetic capacity of an individual is fixed at fertilization, alternative development pathways can be programmed at a later time in response to environmental factors. Among many insects, the expression of diapause, a state of developmental arrest used to circumvent inimical conditions, represents an alternative to continuous development. Photoperiod and temperature provide the major environmental cues that determine whether diapause will be expressed (Danilevskii 1965; Beck 1980; Saunders 1982a).

Flesh flies, especially *Sarcophaga argyrostoma*, have been used extensively to investigate the clock mechanism underlying the diapause response. In a series of experiments, Saunders (1971, 1973, 1975, 1978, 1979, 1982b) has convincingly demonstrated a role for circadian rhythms in programming pupal diapause in this species. *S. argyrostoma* is especially amenable to an analysis of circadian rhythmicity since the period of photosensitivity is broad and spans the entire duration of larval life.

In contrast, a closely related species, *S. crassipalpis*, will not enter diapause when only the larval stage is exposed to short days. Two days of embryonic exposure to short day are required (Denlinger 1971). Embryonic development is completeld *in utero*, but it is the embryos themselves that directly perceive the photoperiodic signal through the abdominal wall of the mother. Unless short day is received during this two day interval, pupal diapause cannot be induced in *S. crassipalpis*. This brief period of sensitivity precludes the use of resonance experiments and other approaches that have classically been employed to evaluate the role of circidian rhythms because dark periods greater than 48 h would encompass the entire sensitive period. But, since the photosensitive period is so brief

and decisive in determining the developmental commitment of *S. crassipalpis*, the system has special promise for approaching the molecular and biochemical events that underlie the photoperiodic reaction.

As a basis for such future work, this study examines the photoperiodic response of the embryo during its period of sensitivity and evaluates the relative importance of the scotophase (dark portion) and photophase (light portion). Embryonic sensitivity to various portions of the spectrum is defined with the use of restricted wavelengths of light, and the scotophase is interrupted with light at various intervals to further elucidate the dynamics of the scotophase reaction.

Materials and methods

Experimental animals. The colony of *Sarcophaga crassipalpis* Macquart originated from Champaign County, Illinois and was maintained in the laboratory as described by Denlinger (1972). Adult eclosion is a gated circadian event which takes place in the first few hours of the photophase. To obtain synchronous groups of adults, flies emerging within a few hours of each other were placed in the same cage. Adults were kept at 25 °C with a daily light:dark (L:D) cycle of either 15L/9D or 12L/12D. Ten days after adult eclosion, females were included to deposit larvae by placing a piece of beef liver in the cage. Groups of 80 newly deposited larvae were transferred to aluminum foil packets containing 40 g liver and placed in an environmental chamber at 20 °C with a 12L:12D photoperiod unless otherwise indicated. Under these conditions, pupariation occurs 12–14 days after larviposition.

Determination of diapause incidence. Diapause incidence was determined by removing the anterior portion of the puparium and examining the pupa for signs of antennal formation and the pigmentation characteristic of pharate adult development (Fraenkel and Hsiao 1968). Diapausing pupae remain white and undifferentiated. Pupae held at 20 °C were scored 30 days after larviposition; pupae at 15 °C were scored after 60 days.

Spectral study. Corning (Corning Glass Works, Corning, New York) and Kodak (Eastman Kodak Company, Rochester, New York) short and long pass filters were used in combination to obtain desired wavelengths. A 100 W lamp (General Electric CDS/CDX) projected through a lucite rod (Circon Corporation, Santa Barbara, California) served as the light source and provided an intensity of 275–365 lux.

Results

Embryonic sensitivity

In this species, the egg is ovulated and fertilized on day 5 of adult life, and embryonic development is completed inside the uterus by day 10. The period of photoperiodic sensitivity was previously described for this fly colony (Denlinger 1971), but since the preliminary work was carried out more than 10 years ago, experiments were designed to determine whether the embryonic period still retained its high sensitivity. To examine the duration of the sensitive period, synchronous groups of adult flies were exposed to the four different photoperiods shown in Table 1 during the first seven days after eclosion. On day 7, each group was subdivided into two groups (15L:9D or 12L:12D) until larviposition. Progeny were then transferred to 12L:12D, 20 °C. A high incidence of pupal diapause is restricted to flies receiving embryonic exposure to short daylength. The photoperiod received by females from eclosion to day 7 had no effect on the diapause incidence of her progeny. Lowering the larval rearing temperature to 15 °C was likewise inadequate for diapause induction in flies receiving long daylength during the sensitive embryonic period. While embryos exposed to 12L:12D and reared as larvae at 12L:12D and 15 °C entered diapause at a high rate (99.4%, $N=400$), embryos exposed to 15L:9D and then reared as larvae at 12L:12D and 15 °C entered diapause at a very low rate (2.8%, $N=400$). The ability of *S. crassipalpis* to enter diapause is thus irreversibly repressed if the embryos are exposed to long daylengths. Expression of diapause in individuals reared under short day conditions as embryos can be eliminated if larvae are held at 25 °C and exposed to 15L:9D (diapause incidence = 0%, $N=480$).

Table 1. Effect of photoperiod received by adult females and embryos of *Sarcophaga crassipalpis* on the incidence of pupal diapause

Adult photoperiod (day 1 to 7)	Photoperiod of embryonic stage (day 7–10)			
	15L:9D		12L:12D	
	N	Diapause (%)	*N*	Diapause (%)
15L: 9D	425	0	203	97.5
12L:12D	618	0.2	480	99.2
0L:24D	312	0	234	97.4
24L: 0D	240	0	165	97.5

Critical daylength

The critical daylength for this population of *S. crassipalpis* is between 13.5 and 14 h of light per day (Fig. 1). Embryonic exposure to fewer hours of light or continuous darkness induced a high incidence of diapause, while longer daylength or continuous light produced no diapausing individuals.

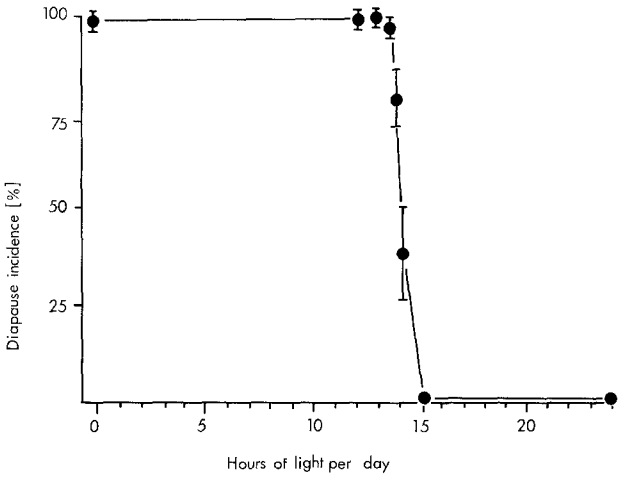

Fig. 1. Critical daylength for pupal diapause induction in *Sarcophaga crassipalpis*

Action spectrum

During the period of embryonic sensitivity a 12 h photophase was extended by 3 h with light of restricted wavelength but similar intensity. If the embryo perceives the additional 3 h as light, the effect should be the same as a 15 h photophase; that is, no diapausing pupae. If the embryo cannot distinguish the supplemental restricted spectrum from darkness, a high diapause incidence should be observed. The results in Table 2 indicate that wavelengths less than 540 nm are involved in the diapause response.

Importance of scotophase

The requirement for less than 13.5 h of light per day to induce diapause could also be interpreted as a necessity for at least 10.5 h of darkness per day. To evaluate the relative importance of the photophase and scotophase, the photophase of long day (15 h) was combined with the scotophase of short day (12 h), and the photophase of short day (12 h) was combined with the scotophase of long day (9 h). As shown in Table 3, scotophase length governs diapause expression.

Minimum photophase

Nine hours of darkness is not adequate for diapause induction (Table 3), but continuous darkness will produce nearly 100% diapause (Fig. 1). To determine the minimum amount of light effective in separating successive scotophases, 9 h-periods of darkness were punctuated by various intervals of light indicated in Table 4. Based on the differences in diapause incidence resulting from these treatments, a light interruption of greater than two hours is required to effectively separate tandem scotophases

Table 2. Diapause incidence in embryos exposed to a 12 h photophase extended by 3 h with light of restricted wavelength but similar intensity. % diapause expressed as a weighted mean ±SD of several unequal replications

Wavelength (nm)	*N*	Diapause (%)
390–440	347	6.1± 4.8
490–535	921	1.2± 0.3
500–540	436	1.4± 1.0
550–595	89	97.7± 1.6
550–620	362	90.0±13.3
550–650	545	98.0± 0.7
580–660	413	95.2± 2.8
650–750	550	95.2± 3.9

Table 3. Pupal diapause incidence in *Sarcophaga crassipalpis* when embryos are exposed to various combinations of 'long' or 'short' photophase or scotophase yielding cycles of 21, 24 and 27 h

Embryonic photoperiod	*N*	Diapause (%)
15L: 9D	309	0
12L:12D	645	98.2
12L: 9D	393	0
15L:12D	479	94.3

Table 4. Pupal diapause incidence when successive 9 h-periods of darkness are separated by light interruptions of various durations. Adult females were placed in the regimes 7 days after emergence, and progeny were collected on day 10 and maintained thereafter at 12L:12D, 20 °C

Length of light interruption	*N*	Diapause (%)
1 h	486	43.2
2 h	562	42.4
3 h	489	0
4 h	693	0

Night interruption

The relative importance of scotophase in contrast to photophase suggests that a dark dependent reaction must be allowed to go to completion if diapause is to be induced. To begin characterizing this reaction, females with photosensitive embryos in utero (7–10 days after adult eclosion) were exposed to a 12 h daily scotophase which was interrupted by 1 h of light. The first interruption began one hour after scotophase onset with subsequent light breaks at progressvely later times (Fig. 2).

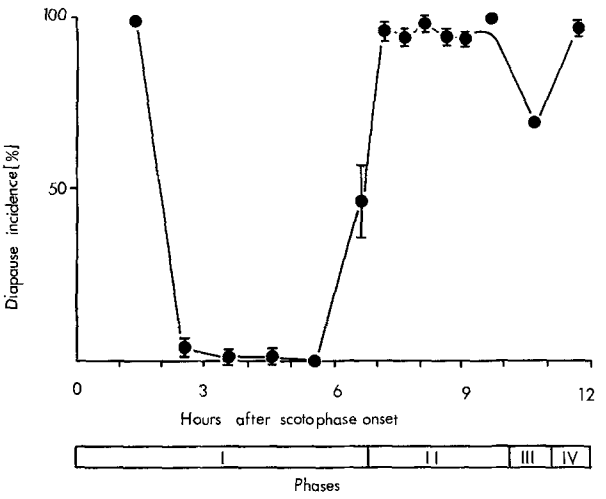

Fig. 2. Effect of one hour light interruptions on the incidence of pupal diapause in *Sarcophaga crassipalpis*. The scotophase was preceded by an eleven hour photophase. Diapause incidence expressed as a weighted average ± SD of several unequal replications with sample sizes ranging from 309 to 449

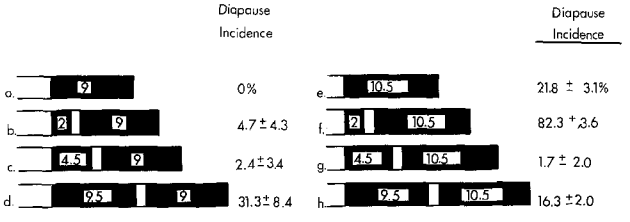

Fig. 3a–h. Pupal diapause incidence resulting from night interruptions followed by either 10.5 or 9 h of darkness. Interruptions in **b, c, f** and **g** occur during Phase I, and interruptions in **d** and **h** occur during Phase III. Diapause incidence is expressed as weighted average ± SD of several unequal replications with sample sizes ranging from 400 to 600

The effect of light interruption varies markedly during the scotophase. One hour after scotophase onset the diapause incidence remains high since the 11 h of darkness that follow light interruption still exceed the critical scotophase of 10.5 h. When light interruption is followed by only 10 h of darkness, diapause incidence drops sharply and remains low until 6 h into the scotophase (Phase I). From 6–9 h after scotophase onset, light interruptions have no effect on diapause induction (Phase II). A light interruption between 10 and 11 h coincides with the critical scotophase and results in a partial reduction in diapause incidence (Phase III). After 11 h of darkness the scotophase reaction is complete and the diapause program remains intact (Phase IV).

A light break of 15 min presented 4 h after scotophase onset is as effective in eliminating diapause (0.2% diapause, $N = 524$) as a one hour break. A 5 min break was also effective (52.4% diapause, $N = 540$) although to a lesser extent.

To test the hypothesis that the scotophase reaction involves a linear accumulation of a substance, three points during scotophase were selected: early (2 h after onset of darkness) and late (4.5 h after onset of darkness) in Phase I and 9.5 h after onset of darkness (Phase III). As shown in Fig. 3, the 1 h light breaks were followed by either 9 h (insufficient for diapause, Fig. 3a) or 10.5 h (critical scotophase length, 3e) of darkness. Photophase was shortened to 10 h to ensure that embryos would be exposed to at least two complete cycles before being transferred as larvae to 12L:12D.

A light break 2 h after the onset of darkness followed by 9 h of darkness produced a low level of diapause (Fig. 3b) whereas 10.5 h of darkness resulted in a high diapause incidence. Neither the 9 h (Fig. 3c) nor the 10.5 h (Fig. 3g) scotophase were effective in diapause induction when the light break occurred late in Phase I. Light interruption in Phase III produced a low diapause incidence for both the 9 h (Fig. 3d) and 10.5 h (Fig. 3h) scotophase extensions.

Discussion

Although our colony of *S. crassipalpis* has been maintained in the laboratory over 10 years, a photosensitive period late in embryonic development (Denlinger 1971) remains crucial for the programming of pupal diapause. Short daylength and cool temperatures during larval life are inadequate for pupal diapause induction in this species. The embryos, rather than the mother, perceive the photoperiodic signals directly from the environment (Denlinger 1971). Diapause is thus not influenced by a circadian rhythm entrained within the adult, but is programmed by the embryo at an early stage coincident with the ontogeny of the light-sensing mechanism. A brief period of short daylength (12L:12D) during late embryonic life preserves diapause competence while long daylength (15L:9D) irreversibly disrupts it.

As in many other species with similar photoperiodic responses (Danilevskii 1965; Beck 1980; Saunders 1982a) the critical daylength is sharply defined. The critical daylength for *S. crassipalpis* is very similar to that observed in a population of *S. bullata* collected from the same locality (Denlinger 1972).

During early scotophase, the spectral sensitivity for diapause induction in *S. crassipalpis* falls into the category of blue-light mediated photomorphogenic responses. There are a large number of these types of responses, many of which alter growth and differentiation (Shropshire 1977). Both carot-

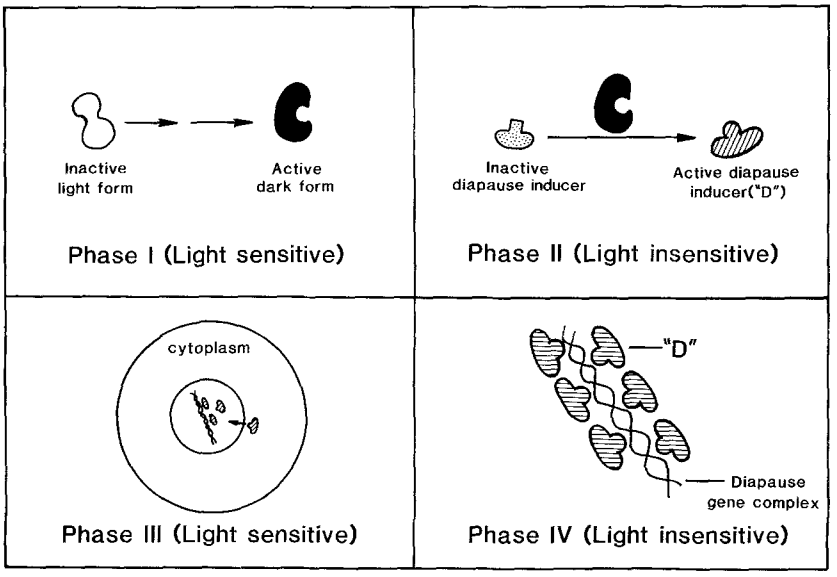

Fig. 4. Molecular model to account for results obtained in Fig. 3. *Phase I:* activation of a protein which is inactive in the presence of light. *Phase II:* the active dark form of the protein produced in Phase I is light insensitive and activates the diapause inducer, "D". *Phase III:* "D" enters the nucleus where it binds to the DNA of the diapause gene complex, a process which is sensitive to light. *Phase IV:* Once complexed with the diapause programming genes, the diapause dark reaction is complete. The potential for diapause expression in the pupal stage is maintained

enoids and flavins absorb in this region and could be the photoreceptor pigments involved in the diapause response. The aphid *Megoura viciae* is also sensitive to a narrow band of the spectrum in the blue range (450–470 nm), and Lees (1981) suggests the photoreceptor may be a caroteno-protein. Experiments with spider mites (Veerman 1980; Van Zon et al. 1981) provide strong evidence that diapause induction is mediated by carotenoids.

In *S. crassipalpis* light is not essential for diapause induction since constant darkness produces a high diapause response. Light apparently functions as part of a resetting mechanism that allows the duration of each new scotophase to be measured. Greater than 2 h of light effectively completes the separation of two successive scotophases. Diapause is determined, not by the photophase, but by a scotophase reaction that requires at least 10.5 h of uninterrupted darkness. The central importance of the scotophase is well documented for a number of insect examples (Beck 1980; Saunders 1982a) and several authors (Truman 1971; Beck 1980) have speculated on a crucial biochemical reaction occurring during darkness that leads to accumulation of a dark induced substance. Subsequent exposure to light degrades this hypothetical substance.

Light pulses during the scotophase can be utilized to further analyze the scotophase reaction. The precise shape of such response curves varies greatly among different species (Lees 1973; Bünning and Joerrens 1959; Beck 1962) and can vary according to the duration of the light pulse (Ankersmit and Adkisson 1968), temperature (Saunders 1975), the photoperiodic regime (Saunders 1975), and the number of cycles interrupted (Bell et al. 1975). Using a 1 h light pulse to interrupt a 12 h daily scotophase in *S. crassipalpis*, the scotophase was separated into 4 distinct phases: Phase I (6 h of photosensitivity), Phase II (3 h period of light insensitivity), Phase III (1 h of partial photosensitivity) and Phase IV (final phase of insensitivity to light).

The scotophase reaction is highly dependent upon when the light break is presented. Although light interruption throughout Phase I eliminates diapause when the total dark period is 12 h, the effect of light on the scotophase reaction differs greatly between early and late Phase I.

The model proposed in Fig. 4 could account for the results obtained from the above experiments. During Phase I a protein which is inactive during photophase is dark-activated through several steps. Although this whole process is sensitive to light, the latter portion requires a longer recovery period (Fig. 3c and 3g). In Phase II, the active dark form of the protein activates a substance that is capable of entering the nucleus. This reaction is insensitive to light (Fig. 2). In Phase III, substance D enters the nucleus where it binds to the DNA involved in diapause programming. This process is also sensitive to light, but recovery time is less than the latter portion of Phase I (compare Fig. 3b and f with d and h). Presence of substance D is necessary to allow this gene complex to be expressed later in the pupal stage. Upon entry into Phase IV, the diapause dark reaction is complete and the diapause gene's potential for expression in the pupal stage is maintained.

It is clear that the scotophase reaction is a step-

wise progression that is not uniform in its response to light interruption. The reaction must be allowed to go to completion during the sensitive embryonic period. If not, the program is irreversibly repressed and cannot be restored by subsequent rearing in cold temperatures and short days.

Since the major developmental decision for or against diapause is determined in this species during such a brief period of embryonic sensitivity, the search for the biochemical basis of the clock can thus focus on the last two days of embryonic development and attempt to identify a crucial reaction sequence that occurs during the first $10^1/_2$ h of scotophase.

Acknowledgements. We thank Dr. Elizabeth Gross for kindly providing the light filters used in this study and Mr. Paul Brunsman for his technical assistance. The research was supported in part by the Science and Education Administration of the U.S. Department of Agriculture under grant no. 8000233 from the Competitive Research Grants Office.

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