

## DURATION OF PUPAL DIAPAUSE IN THE TOBACCO HORNWORM IS DETERMINED BY NUMBER OF SHORT DAYS RECEIVED BY THE LARVA

By DAVID L. DENLINGER AND JAMES Y. BRADFIELD IV

*Department of Entomology, Ohio State University,  
1735 Neil Avenue, Columbus, Ohio 43210 U.S.A.*

(Received 7 July 1980)

### SUMMARY

1. Daylength monitored by the embryos and larvae of the tobacco hornworm, *Manduca sexta*, is used to program both the incidence and duration of pupal diapause.

2. Short daylength throughout embryonic and larval development yields a high diapause incidence, but a diapause of short duration. Hornworms transferred from long- to short-day conditions at later stages of larval development enter diapause at a lower rate, but the resulting diapause is of greater duration.

3. The number of short-day cycles the hornworms receive can be varied by partial starvation and the use of different rearing temperatures. Such manipulations consistently support a model in which diapause duration is inversely related to the number of short-day cycles received.

4. Observations with field-reared hornworms are consistent with the model: September hornworms receive more short-day cycles than August hornworms and thus have a shorter diapause. This mechanism apparently functions in synchronizing the initiation of adult development among individuals that pupate at different times.

5. The diapause duration is programmed by the brain and can readily be transferred to other pupae by brain transplantation.

### INTRODUCTION

Photoperiodic cues are broadly exploited by insects to identify the appropriate time of the year for entering diapause. For most species, daylengths shorter than the 'critical' photoperiod channel the insect toward diapause (Lees, 1955; Danilevskii, 1965; Beck, 1968), but the role of photoperiodism in the developmental stages prior to diapause is usually restricted merely to the decision to enter diapause. In our experiments with the tobacco hornworm, *Manduca sexta*, we find that photoperiodic signals act on the embryos and larvae to determine not only whether the pupae enter diapause (Rabb, 1966; Thurston, 1972; Bell, Rasul & Joachim, 1975) but also how long the diapause will last. We examine the relationship between the number of short-day cycles received and the duration of diapause and investigate the role of the brain in programming this quantitative response to photoperiod.

## MATERIAL AND METHODS

*Experimental animals*

The colony of *Manduca sexta* was of the same origin as described by Truman (1972). Larvae were reared at  $25 \pm 1$  °C on the artificial diet of Bell & Joachim (1976). A daily light:dark cycle of 12L:12D provided 'short'-day conditions and 15L:9D was used as 'long' day. At pupation all pupae were transferred to the same environmental chamber at  $25 \pm 0.5$  °C and 12L:12D. The appearance of black pigmentation in the eyes was used as an easy visual criterion for diapause termination, but since wing apolysis actually occurs 7 days before the appearance of black eyes, the data on diapause duration was corrected to refer to the period between pupation and the onset of wing apolysis.

*Outside rearing*

Natural conditions of photoperiod and temperature were provided by culturing the eggs and larvae on the open window sill of an unheated building, free of artificial lights, in Worthington, Ohio (40° 4' N). At pupation, the hornworms were transferred to an environmental chamber at  $25 \pm 0.5$  °C and 12L:12D.

*Surgical procedures*

Diapausing pupae, aged 4–5 days after pupation, were prepared for surgery with CO<sub>2</sub> anesthesia. The brain, exposed by removing a small flap of cuticle on the dorsal surface of the head, was severed from its connexions with finely sharpened forceps. In 'loose brain' experiments the brain was gently lifted from the head and again returned to the head cavity. In 'brain transplant' experiments a single brain was immediately transferred to the head of a recently debrained pupa. A few crystals of phenylthiourea and streptomycin sulphate were sprinkled on the wound, and the flap of cuticle was repositioned and sealed with molten paraffin.

## RESULTS

*Embryonic and larval determination of diapause duration*

Our development timetable for *M. sexta* at 25 °C was in agreement with the observations of Reinecke, Buckner & Grugel (1980): eggs hatch on day 4, ecdysis to the 5th instar occurs on day 14, the wandering phase begins on day 18 and pupation begins on day 23. When held at short-day conditions (12L:12D) throughout embryonic development and larval life, nearly all the pupae (97.9%,  $N=98$ ) entered diapause (Fig. 1A). The incidence of diapause progressively decreased when larvae were transferred to short-day conditions at later stages of development, thus implying that *M. sexta* has a very broad period of photoperiodic sensitivity. A reciprocal experiment involving transfers from short- to long-day conditions, though not ecologically relevant for *M. sexta*, demonstrated the effectiveness of long days in eliminating the diapause program: only 2.2% ( $N=90$ ) entered diapause when larvae were transferred from short- to long-day conditions on day 17. Earlier transfers completely eliminated diapause.

Although the incidence of diapause was reduced by transferring larvae to short-day conditions at a later age, the duration of pupal diapause increased markedly with age

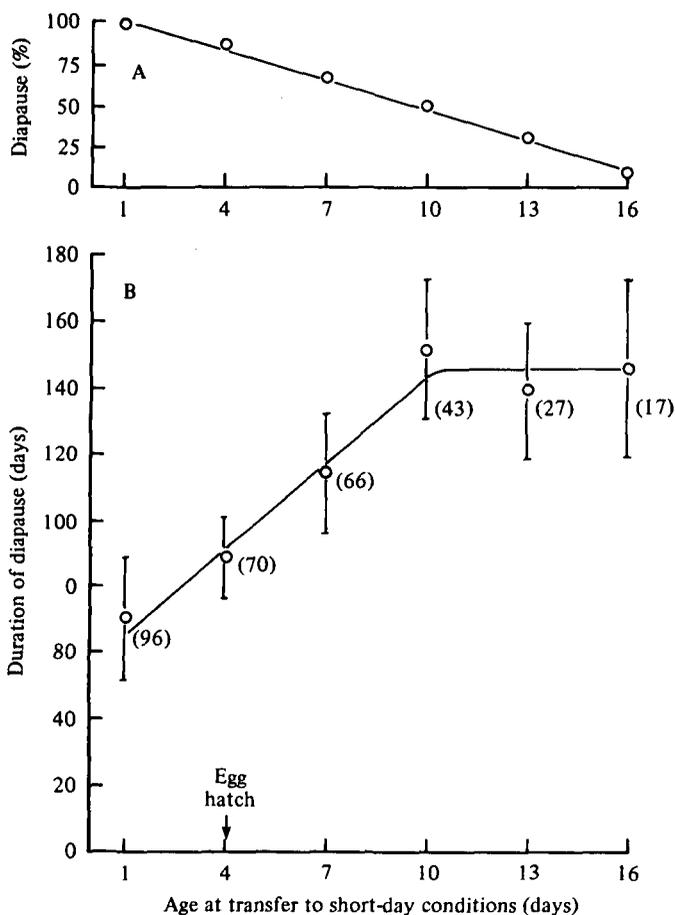


Fig. 1. Incidence (A) and mean  $\pm$ S.D. duration (B) of pupal diapause in *Manduca sexta* at 25 °C when embryos or larvae were transferred from long-day (15L:9D) to short-day (12L:12D) conditions on various days after egg deposition. In (A) each N = 92–98 pupae except on day 16 (N = 196); sample sizes in (B) are indicated in brackets.

of transfer (Fig. 1B). Mean duration of diapause was only 71.9 days for pupae exposed to short days throughout their earlier development, but length of diapause doubled if the larvae were transferred on day 10 or later. An upper limit of about 145 days appears to be reached by day 10.

#### *Manipulation of the number of short-day cycles*

The data in Fig. 1B suggests an inverse relationship between the number of short-day cycles and diapause duration. To test this relationship, several experiments were designed to manipulate the number of short-day cycles received by the larvae.

Larvae deprived of food 8 h/day from egg hatch until the middle of the fifth instar (weight about 5 g) required 4–7 more days to complete larval development than their non-starved siblings (Fig. 2). Both groups were transferred from long- to short-day

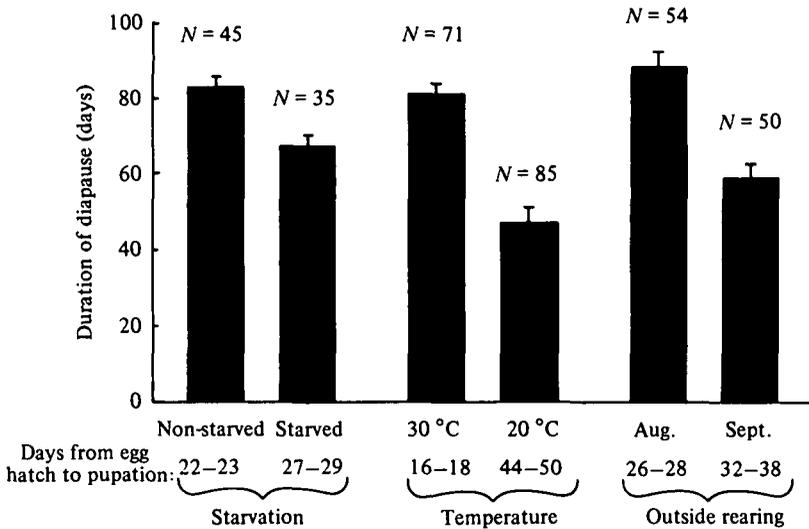


Fig. 2. Manipulation of pupal diapause duration in *M. sexta* at 25 °C by altering the number of short-day cycles (12L:12D) received by the larvae. The interval from egg hatch to pupation was varied by partial starvation, differences in rearing temperatures, and natural outside conditions. In each couplet, means are significantly different (Student's *t* test,  $p < 0.05$ ).

conditions on the day of egg hatch. Although pupae developing from starved larvae were lighter in weight (3.8 g,  $N=35$  v 4.3 g,  $N=45$ ), our unpublished observations on hornworms not subjected to nutritional manipulation show no correlation between pupal weight and diapause duration. We attribute the significant decrease in diapause duration among starved hornworms to their increased exposure to short-day cycles.

The same trend can be noted by using different larval rearing temperatures (Fig. 2). At 30 °C (diapause incidence = 92.2%,  $N=77$ ), larval development was completed in 16–18 days, but lowering the temperature to 20 °C (diapause incidence = 80.9%,  $N=105$ ) more than doubled the time needed for larval development. Although the pupae were all held at 25 °C, the hornworms receiving more short-day cycles as larvae (20 °C rearing) broke diapause earlier.

We have not yet identified the critical photoperiod for our strain of *M. sexta*, but by rearing larvae outside at different times during late summer and early autumn we would expect to obtain groups of hornworms exposed to different numbers of short-day cycles. An experimental group started in July (egg deposition on 19 July 1978, egg hatch on 23 July, mean  $\pm$  s.d. period from egg hatch to pupation was  $28 \pm 1$  days) received an insufficient number of short days to initiate diapause (diapause incidence = 2.4%,  $N=84$ ). A group started in early August (egg deposition on 8 August, egg hatch on 13 August) required nearly the same number of days for larval development (egg hatch to pupation completed in  $27 \pm 1$  days), but a higher portion of the days apparently were interpreted as 'short day', hence the diapause incidence increased to 77.1% ( $N=70$ ). By starting the outside rearing 20 days later (egg deposition on 28 August, egg hatch on 4 September), the period of larval development was extended to  $35 \pm 3$  days and the incidence of diapause increased to 93% ( $N=54$ ).

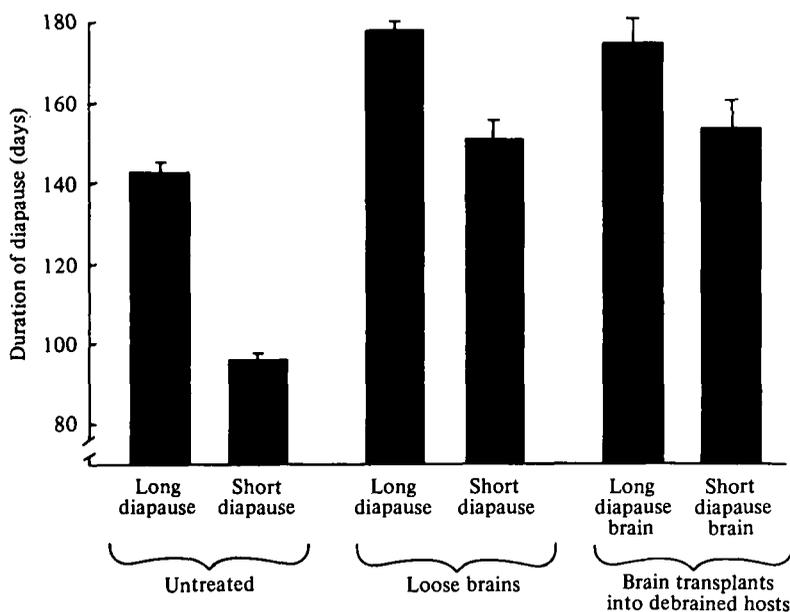


Fig. 3. Surgical manipulation of pupal diapause duration (mean days  $\pm$  S.E.) in *M. sexta* using 'loose' brain operations and brain transplantation. Pupae were programmed for short diapause by transfer from long- to short-day conditions at egg hatch; long diapause was programmed by transferring larvae on day 6 of larval life. Each  $N = 28-32$ . Means in each couplet are significantly different (Student's  $t$  test,  $p < 0.05$ ). Differences between loose brains and corresponding brain transplants are not significant.

As shown in Fig. 2, duration of pupal diapause at 25 °C was significantly shorter for the hornworms reared in September than in hornworms reared during August.

### Brain transplants

Brain transplants were used to evaluate the role of the pupal brain in determining duration of diapause. In this experiment, pupae with a long diapause were produced by transferring larvae from long- to short-day conditions 6 days after egg hatch, while pupae with a short diapause were produced by immediately transferring hornworms to short-day conditions on the day of egg hatch. As shown in Fig. 3, severing the brain from its nervous connexions in the 'loose' brain operation greatly extended the duration of diapause, but the significant difference between long and short diapause persisted. The program for diapause duration could be transferred with the brain: short-diapause pupae that were debrained and received an implanted brain from long-diapause pupae acquired a long-diapause program, while the transplant of a short-diapause brain conferred a short diapause on debrained, long-diapause pupae.

### DISCUSSION

In *M. sexta* the stage sensitive to photoperiodic stimulation is very broad. Our observations and data from Rabb (1966) and Bell *et al.* (1975) indicate that sensitivity begins in embryonic stage and persists until the larva stops feeding near the end of the fifth instar. The sensitive period thus persists for several weeks and, as the horn-

worm develops under field conditions, it is exposed to a significant shift in natural daylength. Like many insects, *M. sexta* has a very precise 'critical photoperiod' (around 13.5 h for a North Carolina strain examined by Bell *et al.* 1975) marking the transition from long-day (non-diapause inducing) to short-day (diapause inducing) conditions. Field populations in late summer are thus exposed to a transition from long days to short days as well as progressive daily decrease in daylength. The tobacco hornworm apparently utilizes this photoperiodic information not only to trigger induction of diapause but also to program the duration of diapause.

A simple model can account for the observed pattern: duration of diapause is inversely related to the number of short-day cycles received. Thus, pupae that received short-day cycles throughout their embryonic and larval development have a much shorter diapause than pupae receiving only a few short-day cycles late in larval life. The model is readily testable, not only by transferring larvae from long- to short-day conditions at different intervals, but also by varying the number of short-day cycles using partial starvation and different rearing temperatures. Consistently, an increase in the number of larval short-day cycles decreased duration of pupal diapause at 25 °C. Under outside conditions, larval development was slower in September than in August and again the shorter diapause in September pupae could be attributed to a longer exposure to short-day cycles during larval life. In several other species, the number of short-day cycles received influences the decision to enter diapause (Saunders, 1976; Beach, 1978), but in *M. sexta* this information garnered during larval life is stored for a much longer period and exerts its effect on the decision to terminate diapause.

As in the classic experiments with silk moths (Williams, 1946, 1952), the brain of *M. sexta* presides over the regulatory decision to enter and break diapause (Bradfield & Denlinger, 1980; Safranek & Williams, 1980). In our present experiments we find that the program for diapause duration also is stored within the brain and can be transferred successfully to another individual by brain transplantation.

The ability to program a variable diapause duration confers a conspicuous ecological advantage. One of the major requirements for a successful pattern of seasonal development is synchronous maturation within the population after the inimical season has passed. Since different individuals enter diapause over a period of several months, a fixed period of dormancy could lead to asynchronous development. Although several options have been exploited successfully by other insects to achieve synchronous post-diapause development (Beck, 1968; Denlinger, 1972; Tauber & Tauber, 1976), *M. sexta* compensates for its broad period of entering diapause by adjusting the duration according to the date of entry into diapause. Early in the season it encounters only a few short-day cycles during larval development, hence the resulting diapause is long. Larvae developing later in the season are exposed to many more short-day cycles and thus remain in pupal diapause for a much shorter period. The cooler temperatures in early autumn, while retarding the rate of larval development, further shorten the duration of diapause by increasing the number of short-day cycles the hornworms receive. The seasonal response is further amplified by the hornworm's ability to discriminate among different short daylengths: within a range of 12–13.25 h of light/day, diapause is shortest at the shortest daylength (Bell *et al.* 1975). The tobacco hornworm is thus equipped with an extremely sophisticated

Photoperiodic mechanism that can precisely monitor the seasons and generate a long-term development program finely tuned to the calendar year. The early determination of diapause duration in *M. sexta* is especially appropriate in this species since the overwintering pupa is buried underground in a site inaccessible to direct photoperiodic cues.

W. A. Foster kindly provided a unheated building for our outside experiments. We also thank M. P. Murtaugh, A. L. Gnagey and V. C. Henrich for their comments on the manuscript. This research was supported in part by the Science and Education Administration of the U.S. Department of Agriculture under grant no. 7800595 from the Competitive Research Grants Office.

## REFERENCES

- BEACH, R. (1978). The required day number and timely induction of diapause in geographic strains of the mosquito *Aedes atropalpus*. *J. Insect. Physiol.* **24**, 449-455.
- BECK, S. D. (1968). *Insect Photoperiodism*. New York: Academic Press.
- BELL, R. A. & JOACHIM, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. ent. Soc. Am.* **69**, 365-373.
- BELL, R. A., RASUL, C. G. & JOACHIM, F. G. (1975). Photoperiodic induction of the pupal diapause in the tobacco hornworm, *Manduca sexta*. *J. Insect. Physiol.* **21**, 1471-1480.
- BRADFIELD, J. Y. IV, & DENLINGER, D. L. (1980). Diapause development in the tobacco hornworm: a role for ecdysone or juvenile hormone? *Gen. comp. Endocrin.* **41**, 101-107.
- DANILEVSKII, A. S. (1965). *Photoperiodism and Seasonal Development of Insects*. Edinburgh: Oliver & Boyd.
- DENLINGER, D. L. (1972). Seasonal phenology of diapause in the flesh fly *Sarcophaga bullata*. *Ann. ent. Soc. Am.* **65**, 410-414.
- LEES, A. D. (1955). *The Physiology of Diapause in Arthropods*. Cambridge University Press.
- RABB, R. L. (1966). Diapause in *Protoparce sexta* (Lepidoptera: Sphingidae). *Ann. ent. Soc. Am.* **59**, 160-165.
- REINECKE, J. P., BUCKNER, J. S. & GRUGEL, S. R. (1980). Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. *Biol. Bull. mar. Biol. Lab., Woods Hole* **158**, 129-140.
- SAFRANEK, L. & WILLIAMS, C. M. (1980). Studies on the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. mar. Biol. Lab., Woods Hole* **158**, 141-153.
- SAUNDERS, D. S. (1976). *Insect Clocks*. Oxford: Pergamon Press.
- TAUBER, M. J. & TAUBER, C. A. (1976). Insect seasonality: diapause maintenance, termination, and postdiapause development. *A. Rev. Ent.* **21**, 81-107.
- THURSTON, R. (1972). Induction of diapause in tobacco hornworms from Kentucky and California by temperature and photoperiod. *Envir. Ent.* **1**, 638-640.
- TRUMAN, J. W. (1972). Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworms. *J. exp. Biol.* **57**, 805-820.
- WILLIAMS, C. M. (1946). Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull. mar. Biol. Lab., Woods Hole* **90**, 234-243.
- WILLIAMS, C. M. (1952). Physiology of insect diapause. IV. The brain and thoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull. mar. Biol. Lab., Woods Hole* **103**, 120-138.

