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TIMING OF THE ACTION OF PHOTOPERIOD AND TEMPERATURE ON EVENTS LEADING TO DIAPAUSE AND DEVELOPMENT IN PUPAE OF *HELIOTHIS PUNCTIGERA* (LEPIDOPTERA: NOCTUIDAE)

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SUMMARY

1. Diapause in *H. punctigera* occurs in the pupa at some stage between pupation and the pupal-adult apolysis. Its occurrence is induced by 12 L: 12 D photoperiod, which is specifically effective during the last half of larval life, and by low temperature (19 °C).

2. Irrespective of the conditions during larval life, the insects do not diapause if subjected to high temperature (28 °C) after feeding ceases and before the pupal ecdysis. The insects are most sensitive to high temperature during the short perioid between the larval-pupal apolysis and pupal ecdysis.

3. At both 28° and 19 °C the brain and thoracic glands secrete almost simultaneously to trigger the larval-pupal apolysis, which occurs almost immediately after the release of the hormones.

4. High temperature during the prepupal period results in an increased rate of development from pupal ecdysis to emergence of the adult.

INTRODUCTION

Diapause in *Heliothis punctigera* manifests itself by a prolonged period between pupation and the emergence of the adult moth. It is induced by the photoperiodic regime and temperature under which the larva develops. A light:dark regime of 12 L: 12 D is maximally influential in inducing diapause: regimes differing from that by more than about 30 min are much less effective. At 19 °C a high proportion of insects reared at 12 L: 12 D enter diapause, whereas only a few individuals do so if reared at 28 °C, or if they are transferred to 28 °C at pupation (Cullen & Browning, 1978).

Diapause in insects is thought to result from the failure of some part of the endocrine system to stimulate development at a time that would be appropriate in non-diapausing individuals. It is not known what part of the developmental process is blocked in *H. punctigera*, and this knowledge is essential before an analysis can be made of the processes involved in diapause. This study reports observations aimed at defining more precisely the stages in the life-cycle when the influence of both photoperiod and

temperature is exerted and the stage at which diapause occurs, as a background to more detailed study of diapause and development in this insect.

As in many other Lepidoptera, the larva of *H. punctigera*, when it ceases feeding in its last instar, wanders for a while and then burrows under the mass of frass and the remains of food in its rearing vial, forms a cell and spins a cocoon. After some time pupation occurs and finally the adult moth emerges. Hinton (1976) has pointed out the disadvantages of some of the terminology, which has developed from natural history, for describing precisely the stages in development of holometabolous insects. For convenience the period from the beginning of wandering to the larval-pupal ecdysis will be termed the 'prepupal' period except when greater precision is necessary; the larval-pupal ecdysis will be termed 'pupation'; and the period from pupation to the emergence of the adult will be termed the 'pupal' period, even though, for most of this time, the animal is a pharate adult.

MATERIALS AND METHODS

All the insects were derived from stock cultures reared in a room held at 28 °C ± 2 °C and 16 L:8 D. The culture was started from moths caught in the field each spring. Moths were placed in plastic containers lined with paper towelling, on which they laid their eggs. Ten per cent honey solution was provided as food. The paper was removed each morning and eggs were sterilized *in situ* by soaking the paper for several minutes successively in 0·15% solutions of sodium hypochlorite and sodium thiosulphate. They were rinsed in water after each treatment, dried in air and placed in containers in the culture room for incubation. On hatching the larvae were transferred with a fine brush, in groups of 10, to autoclaved glass vials containing food. The diet was similar to that described by Griffith & Smith (1977), except that 1 ml kg^{-1} commercial formalin was added. The vials were then transferred, either to experimental conditions or to stock. After several days, depending on the temperature, larvae in their late second – or early third – instar were transferred individually to clean vials containing enough food for them to complete feeding, begin to 'wander' and burrow. They were then transferred to plastic vials containing enough vermiculite to cover them. There they span a cocoon, and pupated.

The criteria for determining the occurrence of diapause were those used by Cullen & Browning (1978), namely, pupae placed at 19 °C were considered to be in diapause if the moth had not emerged within 50 days of pupation, and at 28 °C, if emergence had not occurred within 20 days. No diapause has been observed in insects in the stock culture.

Experiments were conducted in cabinets in which the temperature was maintained within ± 0·2 °C and 2 × 6 W fluorescent tubes were controlled by time switches.

The ligation experiments were conducted with the insects placed on a bed of crused ice at about 0–4 °C. When they had become immobile a ligature of strong white cotton thread was applied round the neck and, where necessary, around the abdomen between the first and second or second and third abdominal segments. The insects were left on the ice for a few minutes after ligation and then placed in vials on pa[illegible] tissue.

For histological observations pupae were cut in two at the level of the second abdominal segment while immersed in aqueous Bouin's fixative. After about an hour a small piece was cut from the extreme anterior end to facilitate penetration, and the pieces were left for 24 h. Sections were cut from the thorax. Larvae were decapitated under fixative, and the thorax cut from the abdomen. Sections were cut from the thorax. Specimens were imbedded in wax, sectioned at 7 μm, and stained in iron alum haematoxylin.

RESULTS

The influence of photoperiod at different stages of development

An experiment to determine the most sensitive stage of development of the larvae to the diapause-inducing influence of short days (12 L: 12 D) was designed as follows. Larvae were placed at 19 °C in either 12 L: 12 D or 14 L: 10 D at 6-day intervals throughout their developmental period, as shown in Table 1. The last period, designated '19–' in the Table, was variable because the larvae began 'prepupal' behaviour at variable times, but in all cases they remained in the photoperiod at which they had spent the time since the nineteenth day.

The results (Table 1) show that only in those treatments in which the larvae had spent at least three periods, including the final two periods, at 12 L: 12 D (treatments 1, 8, 12), did the incidence of diapause exceed 90 %. Even in treatment 7, in which the final two periods of 12 L: 12 D were preceded by continuous 14 L: 10 D, the proportion in diapause was considerably reduced. Nevertheless, all treatments resulted in some diapause and even continuous long days resulted in 22 % of the insects diapausing.

The influence of temperature of prepupae and pupae on the expression of the diapause-inducing stimulus

Elevated temperature is known to have the effect of opposing the influence of diapause-inducing photoperiod in many insects (Beck, 1968; Gibbs, 1975; Lees, 1955) and in *Heliothis* spp. in particular (Phillips & Newsom, 1977; Wellso & Adkisson, 1966). Cullen & Browning (1978) showed that the occurrence of diapause was greatly modified if the insects were reared at 19 °C and transferred to 28 °C at the larval-pupal ecdysis. Table 2 shows that diapause was completely prevented when larvae were transferred from 19 °C to 28 °C immediately wandering was observed, irrespective of the photoperiod at which the larvae were reared.

An experiment, whose design is shown in Table 3, was set up to determine the stage in development at which diapause is most effectively prevented by high temperature. Larvae were reared at 19 °C, 12 L: 12 D. One group of controls remained under these conditions throughout (treatment 1), while in another the individuals were transferred to 28 °C on the day they exhibited prepupal behaviour (treatment 2). In other treatments the high temperature was experienced from the day of the larval-pupal ecdysis (treatment 3) or later (treatments 4 and 5), or in short exposures of one day (treatments 4, 7 and 8); the prepupal period only (treatment 9); the prepupal period plus the day of ecdysis (treatment 10); or in 2-day exposures later in pupal life

Table 1. *Occurrence of diapause in pupae when larvae were exposed to long- or short-day photoperiods during development. Larvae and pupae reared at* 19 °C

* (Values followed by the same letter do not differ significantly at the 1 % level of probability.)

Treatment	Days of larval development				No. in diapause	Total	% diapause*
	0–6	7–12	13–18	19–			
1	S	S	S	S	37	40	93 a
2	S	S	S	L	19	43	44 cde
3	S	S	L	L	15	47	32 ef
4	S	L	L	L	18	41	44 cde
5	L	L	L	L	11	49	22 g
6	L	L	L	S	19	45	42 ef
7	L	L	S	S	33	48	68 b
8	L	S	S	S	39	42	93 a
9	L	S	L	L	23	46	50 cd
10	L	L	S	L	17	54	31 fg
11	L	S	S	L	23	46	50 cd
12	S	L	S	S	54	57	95 a
13	S	S	L	S	19	27	70 b
14	S	L	L	S	32	53	60 bc

S = 12 L : 12 D; L = 14 L : 10 D.

Table 2. *Incidence of diapause in pupae when larvae were reared at different photoperiods at* 19 °C *and transferred to* 19 °C *or* 28 °C *when wandering began*

	Photo-regime	Pupal temp. (°C)	Diapause	Total pupae
1	12 L : 12 D	19	52	62
2	12 L : 12 D	28	0	25
3	14 L : 10 D	19	11	68
4	14 L : 10 D	28	0	21

Table 3. *Occurrence of diapause when individuals were treated at* 19 °C *or* 28 °C *at intervals from beginning of wandering to emergence of adult. Larvae reared at* 19 °C, 12 L : 12 D

No.	Pp	P	P+1	P+2	P+3	P+4	E	% diapause	n*
1	19	19	19	19	19	19	19	19	25 a
2	28	28	28	28	28	28	28	0	25 c
3	19	28	28	28	28	28	28	12	25 c
4	19	19	19	28	28	28	28	28	25 bc
5	19	19	19	19	19	28	28	32	25 b
6	19	28	19	19	19	19	19	24	25 bc
7	19	19	19	28	19	19	19	54	24 ab
8	19	19	19	19	19	19	19	79	24 ab
9	28	19	19	19	19	19	19	4	25 c
10	28	28	19	19	19	19	19	0	25 c
11	19	19	28	28	19	19	19	25	24 bc
12	19	19	19	19	28	28	19	50	24 ab

Pp, period from cessation of feeding to larval-pupal ecdysis. P, day on which ecdysis occurred. P + 1 etc., one (etc.) day(s) after ecdysis. E, period from P + 4 to emergence or until diapause was manifest.

Table 4. *Number of individuals on which dark brown pupal cuticle occurred after ligation:* (A) *round the neck and* (B) *round both the neck and abdomen. Neck ligatures applied at intervals after wandering began and abdominal ligatures at intervals after the neck ligature. In* (A) *brown cuticle scored anywhere on body; in* (*B*) *on abdomen*

(A)

28 °C							
Hours after wandering	18	21	24	27	30	33	36
No. larvae	57	67	69	73	59	56	36
No. pupal cuticle	4	10	21	39	53	40	29

19 °C					
Days after wandering	1·5	2·0	2·5	3·0	3·5
No. larvae	11	22	26	24	36
No. pupal cuticle	0	0	1	3	17

(B)

28 °C	Neck ligature 30 h after wandering				
Hours after first ligature	0	3	6	9	12
No.	20	20	20	20	18
No. pupal cuticle	17	14	15	15	16

19 °C	Neck ligature 3·5 days after wandering			
Hours after first ligature	0	4	8	12
No.	23	25	24	21
No. pupal cuticle	11	12	5	7

(treatments 11 and 12). The results show clearly that exposing the insect to 28 °C during the prepupal period and on the day of ecdysis (treatment 10) was as effective in preventing diapause as continuous high temperature throughout the prepupal and pupal periods (treatment 2). Furthermore, treating only the prepupa (treatment 9) was little less effective. Exposing the pupae to high temperature progressively later in their development was progressively less effective, whether the treatment was continuous (treatments 3, 4 and 5) or in short exposures (treatments 6, 7, 8, 11 and 12).

During the 'prepupal' period the secretion of the endocrine organs controlling development and the apolysis from larva to pupa occurs, and it was thought that there may be a differential effect of high temperature on the expression of diapause depending upon whether it was experienced by larval or pupal structures. A series of experiments was conducted to determine the timing of these events.

To establish the timing of the secretions from the brain-corpus allatum, ligatures were tied round the necks of larvae at intervals after wandering began and the animals were observed for signs of pupation. Any browning of the cuticle was taken as an indication of some endocrine activity mediated by the brain. Larvae were reared at 28° and 19 °C and maintained at those temperatures after ligation. Table 4(A) shows that at 28 °C the brain had secreted in about half the individuals 27 h after wandering had begun and at 19 °C after 3·5 days. In a second experiment larvae were left at 28 °C for 30 h after wandering began and at 19 °C for 3·5 days. A ligature was then applied round the neck of all animals and a second ligature round the abdomen at intervals up to 12 h. The results in Table 4(B) show that there was no difference at either temperature in the number showing pupal development between those in which the second

Table 5. *Temperature at certain stages of development and resulting incidence of diapause Larvae reared at 19 °C and 12 L:12 D*

Treatment	*n*	Wand-apol I	Apol I-ecdysis	Ecdysis-apol II	Apol II-emergence	% diapause
1	22	19	19	19	19	86
2	22	28	28	28	28	0
3	23	19	28	28	28	0
4	25	19	19	28	28	0
5	21	28	19	19	19	24
6	23	28	28	19	19	0
8	24	19	28	19	19	0
9	20	19	19	28	19	15
10	21	19	28	28	19	0

n, number of individuals per treatment. Apol I, larval-pupal apolysis. Apol II, pupal adult apolysis. Ecdysis, larval-pupal ecdysis. Wand, beginning of wandering.

ligature had been applied immediately after the first and others in which the second ligature was applied later, indicating that the thoracic glands secrete immediately after the brain-corpus allatum; so soon that these experiments were unable to distinguish between the two events.

Having determined the time at which the thoracic glands secreted, a search was made for external signs of the occurrence of apolysis, such as have been described, for example, in *Manduca sexta* (Nijhout & Williams, 1974). No such signs could be found. Two groups of larvae, one reared at 28 °C and the other at 19 °C, were taken and samples were fixed at intervals of 12 h, spanning the time when the thoracic gland was known to have been active. Sections were cut of at least three individuals at each time interval. Apolysis was oberved to have occurred between 24 and 36 h at 28 °C and between 3·5 and 4 days at 19 °C. Thus the major developmental events, secretion of the brain-corpus allatum, secretion of the thoracic glands, and apolysis, were found to occur in very close sequence. Pupae held at 28 °C were also sectioned at 12 h intervals after pupation and it was found that the pupal-adult apolysis occurred between 3 and 3·5 days after pupation.

An experiment was designed to investigate the effect on the incidence of diapause of treatment at high temperature administered to prepupae before or after the larval-pupal apolysis. Some treatments were also included in which the high temperature was administered from pupation to the pupal-adult apolysis.

The design of the experiment is shown in Table 5. The larvae were reared at 19 °C and 12 L:12 D, so a high proportion were expected to diapause. The controls (treatment 1) showed that to be so. The results in Table 5 show that no diapause occurred in any treatment that experienced 28 °C during the period from the larval-pupal apolysis to pupation (treatments 2, 3, 6, 7, 8 and 10). High temperature was rather less effective before the larval-pupal apolysis (treatment 5) and from pupation to the pupal-adult apolysis (treatment 9).

The duration of the pupal period

The mean period that elapsed at 28 °C from the beginning of wandering to pupation was 2·5–3 days. Histological observations showed that apolysis began 24–36 h after wandering began. Similarly, sections of pupae fixed at intervals after pupation showed apolysis beginning between 60 and 72 h after ecdysis. The pupation period (in the strict sense) thus occupied only between 84 and 109 h in a total mean period of 13 days. It was noted that the stigmata of the compound eyes had started to move posteriorly (a sure sign that diapause is complete) (Cullen & Browning, 1978; Phillips & Newsom, 1966) before apolysis occurred. Diapause is thus truly a pupal phenomenon.

DISCUSSION

Diapause in *H. punctigera* occurs at some stage between the pupal ecdysis and the pupal-adult apolysis, a period occupying only 2·5–3 days at 28 °C in non-diapausing individuals. The effect of the environmental stimuli that induce a high proportion of pupae to enter diapause – low temperature and a photoperiod of 12:12 D during larval development – can be completely negated if the pupa spends only the one to 1·5 days between the larval-pupal apolysis and ecdysis at 28 °C. The endocrine system, that has been programmed during the later stages of larval development to become inoperative once the pupal ecdysis has occurred, is switched on by the short exposure to high temperature. It was also fully operational, just before the high temperature treatment, to organize the larval-pupal apolysis. Meola & Adkisson (1977) have recently provided evidence that in *Heliothis zea* the pupal brain secretes its prothoracicotropic hormone very soon after the pupal ecdysis, irrespective of whether the insect is to enter diapause or not. This contrasts with the situation in many other insects (Williams, 1969; Wilson & Larsen, 1974). It is not yet known whether *H. punctigera* behaves like *H. zea* in this respect, but if it does, it would seem that the effect of high temperature is to be sought in some systems other than the neurosecretory cells of the brain. During the pharate pupal period rapid reorganization of the insect's tissues occurs and the nature of the results of such reorganization may well depend on the temperature at which it takes place. In particular the constitution of certain critical membranes may differ, depending upon the temperature at which they are formed. Further work is aimed at determining which aspect of the endocrine system in *H. punctigera*, production, secretion or reception of the hormones, is responsible for diapause, and how it is influenced by temperature.

In the experiments reported in Tables 3 and 5 there were several treatments in which the animals developed at either 19° or 28 °C after ecdysis but where the conditions prior to ecdysis differed. These are shown in Table 6, which also shows the mean time that elapsed from pupal ecdysis to the emergence of the adult in non-diapausing individuals. It is clear that in those treatments where the period from larval-pupal apolysis to ecdysis was spent at 28 °C the insects developed more rapidly after ecdysis. The differences are considerable and statistically significant. High temperature during the pharate pupal period not only eliminated the propensity for diapause if it were present, but in some way potentiated the development of the insect following pupation.

Table 6. *The influence of temperature during prepupal life on the rate of development from pupation to emergence. Data from various treatments shown in Tables* 3 *and* 4

(Values followed by the same letter do not differ significantly at the 1 % level.)

Treatment and	Wand-apol I	Apol I-ecdysis	Ecdysis-emergence	Mean time (days)
2*(3)†	28	28	28	10·3 a
2 (4)	28	28	28	10·9 a
3 (4)	19	28	28	11·0 a
3 (3)	19	19	28	12·2 b
4 (4)	19	19	28	12·4 b
6 (4)	28	28	19	26·6 c
8 (4)	19	28	19	28·9 d
5 (4)	28	19	19	31·1 e

* Treatment no. † Table no.

In order to try to assess whether the temperature of the pharate pupa was influencing the development of the pupa after ecdysis or of the pharate adult, two groups of larvae, reared at 19 °C and 14 L: 10 D and thus unlikely to diapause, were taken at the wandering stage and placed at either 19° or 28 °C until pupation and then allowed to complete their development and emerge at 28 °C. Samples of four were taken at 12 h intervals, fixed and sectioned to observe apolysis. No difference could be found between the two groups; apolysis occurred between 2·5 and 3 days in each. The developmental periods of the controls were 10·2 days for those that had spent the prepupal period at 28 °C and 11·3 days for those at 19 °C; a significant difference. The potentiation in the rate of development cannot then be attributed to the later pupal period alone, but must also involve pharate adult development.

During the experiments in which two ligatures were applied to larvae, apolysis was signalled by the appearance of brown patches of new pupal cuticle below the larval cuticle. Frequently the region posterior to the ligature underwent ecdysis, with the larval cuticle splitting and contracting to expose the pupal abdomen, whereas this did not occur on the thorax nor that part of the abdomen anterior to the ligature. In almost all cases where apolysis occurred the abdomen showed the first signs of browning. Often the posterior end would metamorphose into a complete pupal abdomen and only one or two days later would brown pupal cuticle appear on the anterior part. This delay occurred even in cases where the only signs of apolysis on the posterior part were small dorsal brown strips, and the anterior part later became pupal. In those cases where the whole abdomen did not become brown there was a consistent pattern of browning. Strips of brown appeared dorsolaterally and these spread posteriorly and ventrally. Patches of brown cuticle thus occurred. Truman (1972) described a similar phenomenon in *Manduca sexta* which he ascribed to epidermal structures becoming competent to respond at different times, but it may equally result from certain parts of the epidermis responding to lower titres of ecdysone.

In a very few cases (eight out of 129), double-ligatured larvae produced pupal cuticle anterior to the second ligature only, the posterior part remaining with no trace of pupal cuticle. In all cases where this was observed it occurred only after a prolonged period, 8–10 days after the ligatures had been applied instead of 3–5 in other cases.

Truman (1972) reports a rather similar phenomenon in *M. sexta* and ascribes it to the prothoracic glands 'leaking' ecdysone in the absence of a stimulus from the brain.

In their detailed analysis of the endocrine events leading from the cessation of feeding to pupal ecdysis in *Manduca sexta*, Truman & Riddiford (1974) found that insects ligatured round the neck just before the cessation of feeding did not show the pink coloration nor the exposure of the heart that ordinarily accompany the onset of the wandering period, whereas if the ligature was applied later both these events occurred. They concluded that these changes are controlled by a pulse of prothoracicotropic hormone and ecdysone, which do not trigger the larval-pupal apolysis but which do control the release of a second pulse of hormones that are responsible for the apolysis. In *H. punctigera* it has not proved possible to find morphological characters that occur after the cessation of feeding and before ecdysis. Even the purging of the gut, described by Nijhout & Williams (1974) is not conspicuous in *H. punctigera*. It is possible that prepupal behaviour in *H. punctigera* is triggered by a pulse of hormones different from that which triggers apolysis, but if so it will be necessary to assay for it by a method other than ligation.

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