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CONTROL OF MOULTING AND METAMORPHOSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.): CESSATION OF JUVENILE HORMONE SECRETION AS A TRIGGER FOR PUPATION

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SUMMARY

During the final larval instar of the tobacco hornworm the presence of juvenile hormone (JH) inhibits the secretion of the brain's prothoracicotropic hormone (PTTH). The corpora allata cease to secrete JH when the larvae attain a weight of approximately 5 g. The JH is cleared from the haemolymph in about 24 h. This process in itself renders the brain competent to release PTTH. The actual release of PTTH occurs at the very first photoperiodic gate after the JH has disappeared from the haemolymph. A functional failure of this normal mechanism is apparently responsible for the developmental standstill of Lepidoptera which diapause as mature larvae.

INTRODUCTION

Tobacco hornworms weigh about 1 g at the outset of the final (5th) larval instar. After 4–5 days of continuous feeding at 25 °C mature larvae attain live weights ranging from 8 to 10 g. Only then does one witness the first indications that pupation is about to begin: these include the cessation of feeding, the purging of the gut, and the exposure of the dorsal vessel (Truman & Riddiford, 1974).

Nijhout & Williams (1974) have shown that the 'decision' to pupate is made when the larva attains a certain critical weight; not the 8–10 g of the mature larva, but a weight of 5 g attained 1–2 days earlier. An unidentified process lasting about 24 h is then initiated which renders the brain competent to release its prothoracicotropic hormone (PTTH). In larvae exposed to a 12L:12D photoperiod the actual release of PTTH takes place during an 8–10 h 'gate' in the middle of the next photophase (Truman & Riddiford, 1974). The hormone entrains the secretory activity of the prothoracic glands and provokes the secretion of ecdysone which within 12–15 h triggers the gut purge and other prodromes of metamorphosis (Nijhout & Williams, 1974).

The objective of the present study was to identify the process that activates the endocrine mechanism for metamorphosis. Three lines of evidence led us to suspect

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that a declining titre of juvenile hormone (JH) was involved. Firstly, there is comprehensive body of evidence that a declining titre of JH is a prerequisite for pupation. Secondly, larval–pupal intermediates were produced when larvae were starved prior to attaining the weight of 4 g, whereas small but otherwise normal pupae were produced when starvation was begun thereafter (Nijhout & Williams, 1974). The third line of evidence was based on experiments described by Truman (1972) in which precocious pupation of 4th-instar hornworms was provoked by head ligation to remove the corpora allata (CA). A few days prior to pupation a white, chalky material was discharged, presumably from the Malpighian tubules, and escaped from the rectum of the ligated animals; since this material is never discharged during larval moults, it was presumed to be in response to the declining titre of JH enforced by head ligation (J. W. Truman, personal communication). It seemed not unlikely that this chalky substance was the same as that which coats the 'frosted frass' of normal 5th-stage larvae shortly after they attain the critical weight of 5 g (Nijhout & Williams, 1974).

In the experiments reported here we sought to determine how CA activity and JH titre relate to the unidentified process that is initiated when a larva attains the critical weight of 5 g.

MATERIALS AND METHODS

1. *Experimental animals*

Manduca sexta larvae were reared as described by Nijhout & Williams (1974). They were maintained under a 12L:12D photoperiod cycle at 25 °C; lights-off was at midnight (24.00) and lights-on at 12.00. Under these conditions the gate for PTTH release occurs between 14.00 and 23.00 h (Truman & Riddiford, 1974). The exact time of PTTH release was determined by extrapolating back from the known time of gut purging, as described by Nijhout & Williams (1974).

2. *Juvenile hormone assay*

To determine the titre of JH in the haemolymph we used the 'black larval assay' described by Truman, Riddiford & Safranek (1973). Haemolymph of three larvae in each weight class was pooled; 0·5 ml was then extracted with ether/ethanol according to the procedure of de Wilde *et al.* (1968). Solvent was evaporated in a nitrogen stream. The dried extract was dissolved in 50 μl acetone and 10 μl applied to each of five test larvae. After 48 h JH activity was assayed and averaged. In all data reported here the five assays of each extract were within 0·5 units of one another. A few 'false positives' were obtained in the control experiment in which acetone alone was applied to test larvae. The mean activity was 0·05 and this was adopted as the base-line for the experiment.

3. *Ecdysterone* (*β-ecdysone*)

It was difficult to obtain a normal physiological response of 5th-instar larvae to a single injection of ecdysterone. However, a slow infusion of the hormone proved to be effective, apparently because it mimicked the secretory activity of the prothoracic glands. Prior to the initiation of the infusion each larva was paralysed by a single injection of 4 μg/g body weight of tetrodotoxin (TTX) (Williams, 1968). At this low

dosage, TTX has no effect on endocrine secretion in *Manduca*, nor does it interfere with the normal response to ecdysone, as observed in intact control larvae. Fifty μl of a solution of ecdysterone (1 mg/ml in 10% isopropanol) was then infused over a period of 12 h via a 30-gauge needle sealed to a 100 μl gas-tight hypodermic syringe (Hamilton), the latter being mounted on a syringe pump (Sage Instruments, Model 355).

4. *Juvenile hormone injections*

The hormones used in this study were the C18 Cecropia JH and a JH mimic, epoxygeranylsesamole. Both were obtained from Eco-Control Inc., Cambridge, Massachusetts. Epoxygeranylsesamole is an excellent JH mimic in that 1 μg produced a +3 pupal–adult intermediate (Riddiford & Ajami, 1973) when injected into *Manduca* pupae (L. M. Riddiford, personal communication). The C18 Cecropia JH produced a +3 pupal–adult intermediate at a dose of 5 μg. The hormones were dissolved in light mineral oil (Saybolt viscosity 125/135) and injected into the base of a larval proleg.

5. *Surgical techniques*

Allatectomies were performed while the larvae were under CO_2 anaesthesia (Williams, 1946*a*). The mesothorax was constricted with a small hose clamp and the larva was suspended vertically in the anaesthesia funnel. This effectively isolated the abdomen and opposed bleeding. The head capsule was grasped with curved forceps and flexed backwards thereby exposing the ventral neck region. When the neck membrane was fully stretched the forceps were fastened in position with Plasticine. Both CA were removed through a single ventral incision in the neck. They were found embedded among the tracheae on each side of the oesophagus and were attached to the corpora cardiaca and the mandibular gland. When traction was applied to the mandibular gland a slight motion of the CA took place which made them readily detectable. Sterilization precautions proved to be unnecessary. The incision was not sealed after the operation since little bleeding took place. Immediately after the operation each individual was placed in a plastic dish without food.

RESULTS

1. *JH titre in haemolymph of 5th-instar hornworms*

A total of 80 assays was carried out as described under Methods. The results summarized in Fig. 1 show that the titre of JH in the haemolymph begins to decline when larvae attain the weight of approximately 5 g; the titre becomes too low to be determined in larvae weighing 7·5–8·0 g. This result is in accord with that reported for *Philosamia cynthia* by de Wilde, de Kort & de Loof (1971) and Patel & Madhavan (1969). We are informed by Dr Kenneth Judy that he has also observed a sharp drop in JH titre midway through the 5th instar of *Manduca*. Curtailing of CA activity during the final larval instar of *Hyalophora cecropia* has been documented by Williams (1961).

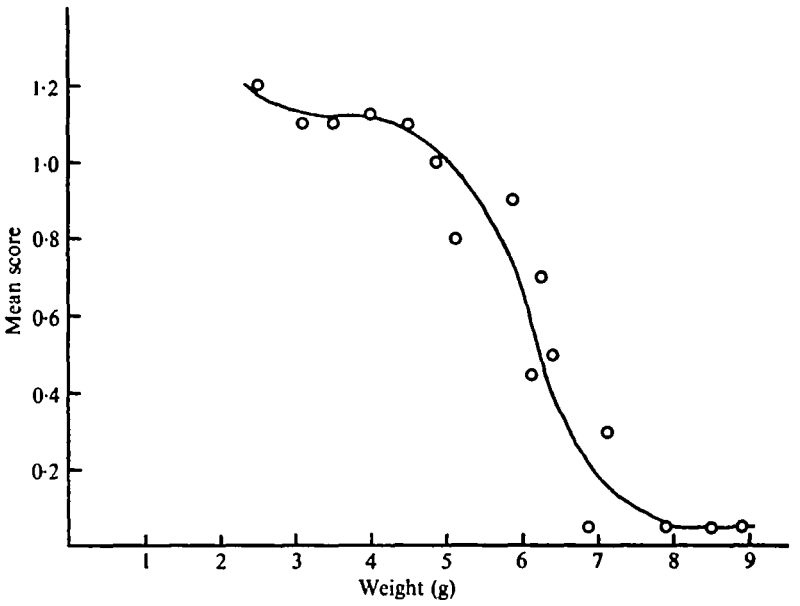

Fig. 1. The titre of juvenile hormone in the haemolymph of *Manduca* larvae at various weights in the 5th instar. Each point is the average of five assays.

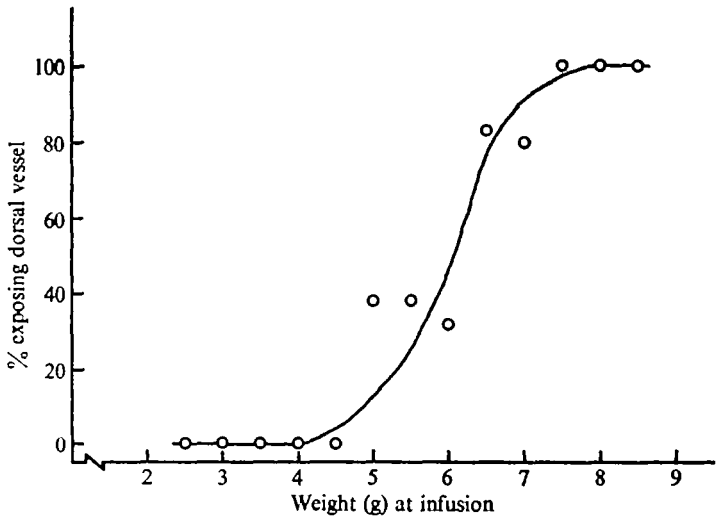

Fig. 2. The percentage of 5th-instar larvae at various weights that expose their dorsal vessel upon perfusion with ecdysterone. Each point represents 8–12 animals.

2. *Effects of ecdysterone infusion*

Homogeneous groups of 5th-instar larvae were paralysed by the injection of tetrodotoxin (TTX) and then infused for 12 h with ecdysterone, as described under Methods. Each individual was scored as to whether it exposed its dorsal vessel – the only prodrome of pupation that can be induced in larvae paralysed by TTX. A 'larval moulting response' was scored upon failure of the dorsal vessel to become exposed

Table 1. *Effects of JH injections on the timing of the purge.*

(All larvae weighed 7·5–8·0 g.)

Expt no.	Hormone and dosage	Time of injection	No. of larvae	No. of animals that purge on day*					
				1	2	3	4	5	6
1	Control	13.00	34	23	11	0	0	0	0
2	25 μg EGS†	13.00	27	0	6	16	4	1	0
3	5 μg C18	13.00	11	0	6	5	0	0	0
4	20 μg EGS on day 0, 1 and 2	13.00	14	0	1	0	2	8	3
5	25 μg EGS	1.00	29	13	2	11	3	0	0

* Day of injection = day 0.
† Epoxygeranylsesamole.

and, moreover, by the formation of a double row of black spots along the dorsum. These spots appear when larvae are infused with a subthreshold dose of ecdysone in the presence of JH (Nijhout, unpublished). They consist of a melanization of the larval cuticle and are probably analogous to those reported by Sehnal (1972) when larvae of *Galleria mellonella* were injected with ecdysterone.

In the larger larvae it was necessary to discriminate between the effects of the infusion and a possible release of ecdysone by the insect's own prothoracic glands. When endogenous secretion takes place, it occurs during the photophase and provokes exposure of the dorsal vessel during the ensuing scotophase (Truman & Riddiford, 1974). All infusions were therefore carried out during the scotophase so that the reaction could be scored towards the end of the next photophase, thereby avoiding the above-mentioned complication.

The results summarized in Fig. 2 show that, up to a weight of 4·5 g, the infusion of ecdysterone was unable to provoke the exposure of the dorsal vessel. Instead, all these individuals developed the distinctive double row of black spots along their dorsum. When the infused larvae weighed over 7·5 g, these spots failed to appear and all individuals exposed their dorsal vessel. What these results imply is that the rapid decline in JH titre (Fig. 1) is accompanied by a change in the response to ecdysterone so that the latter provokes a typical prodrome of pupation rather than of a larval moult.

3. *JH injections*

When JH was injected into 5th-instar larvae a few hours before the projected release of PTTH, the phagoperiod was routinely prolonged for 1–4 days. The purging of the gut then took place followed by the normal train of events culminating in pupation. Normal pupae were formed with no evidence of larval characters.

As seen in Table 1, when JH was injected at the onset of the photophase (13.00) into larvae weighing 7·5–8·0 g, none of the larvae purged on the next day, whereas 70% of the control larvae did so. The phagoperiod was prolonged an average of 1–2 days by a single injection of 5 μg of C18 hormone or 25 μg of epoxygeranylsesamole. As illustrated in Expt 4 in Table 1, the phagoperiod was further prolonged in individuals injected with JH on successive days. Therefore the injected JH inhibited

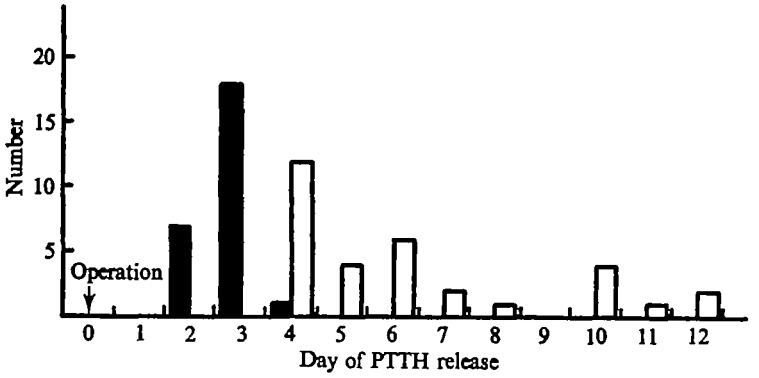

Fig. 3. The timing of PTTH release in 5th-instar larvae that were allatectomized (black, $n = 26$) or sham operated (white, $n = 32$). All larvae weighed between 3·5 and 4·0 g and were starved after the operation. The day of the operation is designated as day zero.

either the release of PTTH or the ability of the larvae to respond to it. When the injection was postponed 12 h until the beginning of the scotophase (1.00), then, as shown in Expt 5 in Table 1, many larvae purged during the same night. Those that failed to do so delayed the purge for 1–3 days.

4. *Effects of allatectomy*

The preceding findings suggested that the release of PTTH might be accelerated by provoking a premature decline in JH titre. The effect of removing the CA upon the time of PTTH release was therefore studied (Fig. 3).

As previously described for larvae starved at weights less than 5 g (Nijhout & Williams, 1974), the sham-operated controls showed a long and variable delay in their release of PTTH. Here again, a number of individuals weighing less than 4 g underwent apolysis instead of purging and proceeded to form larval–pupal intermediates. By contrast, the allatectomized individuals released PTTH mainly on the 2nd and 3rd days and showed no retention of larval characters.

DISCUSSION

The results of the infusion experiment (Fig. 2) together with the sharp decline in the titre of JH during the second half of the 5th instar (Fig. 1) led us to formulate the following hypothesis. We suggest that when a 5th-instar larva attains a weight of approximately 5 g, the CA are turned off and the secretion of JH stops. Consequently, the titre of JH in the haemolymph drops to an undetectable level by the time the larva comes to weigh 7·5 g. Since the larvae at this stage are growing at a rate of about 2 g per day (Nijhout & Williams, 1974), the period of JH decline requires slightly more than 24 h. We suspect that these events comprise the 'unidentified process' (Nijhout & Williams, 1974) which restores the competence of the brain to secrete PTTH.

Further support for the foregoing hypothesis is provided by the experiments in which JH or one of its mimics was injected into larvae prior to the gate for PTTH release. As shown in Table 1, the phagoperiod was substantially prolonged. Thi

bility of JH to prolong the final instar is in accord with previous observations on the lepidopterans *Cerura vinula* (Hintze-Podufal & Fricke, 1971), *Bombyx mori* (Akai & Kobayashi, 1971; Akai, Kiguchi & Mori, 1971, 1973; Nihmura *et al.* 1972), *Hyalophora cecropia* (Riddiford, 1972) and *Diatraea grandiosella* (Chippendale & Yin, 1973).

Expt 5 in Table 1 is especially informative. JH was here injected at 1, i.e. shortly after the closing of the gate for PTTH release. Nearly 50% of the larvae purged during the very next day (day 1). Evidently these larvae had already released PTTH a few hours before the injection. The other larvae which would have released PTTH during the next photoperiod gate were unable to do so and the purge was therefore delayed. These findings strongly support the argument that JH blocks the release of PTTH rather than the ability of the larvae to respond to PTTH.

Conversely, one can cause a premature decline in the titre of JH by allatectomizing larvae before they attain the weight of 5 g. When this was done, the resulting larvae did indeed release PTTH considerably earlier than sham-operated controls (Fig. 3). It must be noted in this connection that the mean time from allatectomy to PTTH secretion was substantially longer than the 1·5 days that would be predicted if the JH is eliminated from the blood in 24 h (Nijhout & Williams, 1974). This discrepancy will be examined on a subsequent occasion.

We conclude from these experiments that when the JH has been eliminated from the haemolymph, the larval brain becomes competent to release PTTH and will do so at the very next photoperiodic gate. When larvae were starved (Nijhout & Williams, 1974), the activity of the CA was dependent on the weight that the individual had attained. When larvae were starved at weights below 5 g, the CA remained active for a much longer period than when larvae were allowed to feed and grow normally. The CA can eventually be 'turned off' in certain larvae starved below 5 g, but only after a long and unpredictable delay. Moreover, when larvae were starved at weights below 4 g, a considerable percentage of larval–pupal intermediates was formed (Fig. 3 and Nijhout & Williams, 1974). This implies that in many of these small starved larvae the CA were never fully shut off. The moult that ultimately took place was probably provoked by autonomous activity of the prothoracic glands (Judy, 1972; Truman, 1972) without any secretion of PTTH. By contrast, when larvae were starved at weights greater than 5 g, the CA had already been turned off and JH breakdown proceeded on schedule. This can explain why starvation at 5 or more grams can no longer delay the purge (see Fig. 8 in Nijhout & Williams, 1974).

Fukaya & Mitsuhashi (1957, 1961) have presented persuasive evidence that, in the rice stem borer, the CA remain active in the diapausing larvae and that the persistence of a high titre of JH is causally related to the developmental standstill. The same has been reported for diapausing larvae of the southwestern corn borer, *Diatraea grandiosella* (Chippendale & Yin, 1973; Yin & Chippendale, 1973).

We are now in a position to interpret these puzzling findings. Thus, as in the case of pupal diapause (Williams, 1946*b*, 1952), the larval dormancy is once again due to a failure of the brain to release PTTH; the brain's inactivity being enforced in this case by a high titre of JH. The onset and persistence of the diapause of mature larvae can be accounted for by a functional failure of the normal mechanism which inactivates the CA of non-diapausing larvae.

The turning-off of the CA is, in more ways than one, the ultimate cause of the

formation of pupal characters when ecdysone provokes the succeeding moult. It now appears that the inactivation of the CA sets in motion the endocrine events that lead to the moult itself.

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