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CONTROL OF MOULTING AND METAMORPHOSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.): GROWTH OF THE LAST-INSTAR LARVA AND THE DECISION TO PUPATE

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SUMMARY

The dynamics of growth and the timing of release of the brain's prothoracicotropic hormone (PTTH) in final instar larvae of *Manduca sexta* are consistent with the following hypothesis. When a 5th-stage larva reaches a critical weight of about 5 g an unidentified process is initiated which requires 24 h to be completed. At the completion of this process the brain is rendered competent to release PTTH. The actual release of PTTH is gated by the photoperiod and occurs when the gate opens during the very next photophase.

INTRODUCTION

Actions and interactions of the prothoracicotropic hormone (PTTH), ecdysone and the juvenile hormone (JH) in directing the moulting and metamorphosis of insects have been the subject of numerous investigations, as is amply evident in the recent reviews presented by Wigglesworth (1970), Wyatt (1972), Doane (1973) and Gilbert & King (1973). Much effort has gone into demonstrating, and in large measure verifying, what Doane (1973) has termed 'the classical scheme' of insect endocrinology. We know that, in most and perhaps all insects, the brain controls the secretion of ecdysone by the action of its tropic hormone on the prothoracic glands. But the questions as to what induces the brain to secrete or not to secrete PTTH, or what causes the corpora allata (CA) to stop secreting JH at the appropriate time, remain for the most part unanswered.

The only definitive study on the intrinsic mechanism for controlling PTTH secretion was carried out by Wigglesworth (1934, 1964) on *Rhodnius prolixus*. In this insect the brain is stimulated to secrete PTTH by nerve impulses arriving via the ventral nerve cord. These impulses originate from stretch receptors in the abdomen which are activated only when the animal engorges a full blood meal. Unfortunately this singular case does not provide a basis for a comprehensive theory because it is highly adapted to the specialized habits of this insect. Temperature and photoperiod are known to be involved in the control of PTTH secretion in many insects with a pupal diapause (Williams & Adkisson, 1964; Williams, 1969; de Wilde, 1970). So

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also, in larval moults as well as the larval–pupal transformation of Lepidoptera, photoperiod is known to provide the 'fine tuning' of PTTH release by determining the time of day at which it will occur (Truman, 1972; Truman & Riddiford, 1974).

Investigations on the control of JH secretion by the CA have centred mainly on the regulation of reproductive cycles and of reproductive diapause in adult insects. In these cases the CA appear to be under the control of either a hypothetical 'allatotropic hormone' (de Wilde & de Boer, 1969; Engelmann, 1970; de Wilde & de Loof, 1973) or under nervous control (Scharrer, 1958). Girardie (1964) has shown that the larval CA of *Locusta* may also be under the control of an allatotropic factor from the brain. No other information is presently available as to mechanisms for controlling JH secretion in larval insects. Wigglesworth (1970) cites persuasive evidence that the CA do not act autonomously. They do not 'count the instars' and cease JH secretion at a predetermined stage, but are controlled by complex internal factors which are yet to be clarified.

The present paper reports on a study of the dynamics of growth of the final larval instar of the tobacco hornworm, *Manduca sexta*. By experiments that altered the normal growth of this lepidopteran, it was possible to affect the timing of the pupal moult in fairly predictable ways. The experiments which we describe here were designed to clarify the physiological interactions that culminate in pupation.

MATERIALS AND METHODS

Our stock of *Manduca sexta* was derived from animals originally received from Dr R.A. Bell, Agricultural Research Service, U.S.D.A., Fargo, N.D. Larvae were fed on an artificial diet described by Dr R. T. Yamamoto (1969) as modified by Dr Bell in a personal communication to Professor Lynn M. Riddiford. As described by Truman (1972), all animals were reared in individual plastic containers at 25 °C under a 12L:12D photoperiod cycle except where noted. Lights-off was at midnight (24.00) and lights-on at 12.00. The day on which each larva ecdysed to the 5th instar is designated as day 0. Weight determinations were carried out on unanaesthetized individuals by means of a top-loading Mettler balance (Model P-120), and unless otherwise specified all weighings were performed during the photophase between 16.00 and 18.00 h.

TIMING OF EVENTS PRIOR TO PUPATION

As a basis for describing the experimental findings, it is necessary to present a brief résumé of the final (5th) larval instar of the tobacco hornworm. In this summary we have included unpublished observations, shared with Truman & Riddiford.

Fig. 1 presents a diagrammatic summary of the 5th-instar and prepupal stage of the tobacco hornworm at 25 °C under a 12L:12D photoperiod regimen. The 5th instar is subdivisible into two phases of behaviour. The first of these, which we define as the 'phagoperiod', begins shortly after the ecdysis of the 5th-stage larva and is terminated 4 or 5 days later by cessation of feeding and a massive purge of material from the gut. That accomplished, the 'feeding larva' is transformed into a 'wandering larva'; the latter shows an abrupt onset of negative phototaxis and would normally dig into the soil if allowed to do so. As shown in Fig. 1, the termination of the 5th

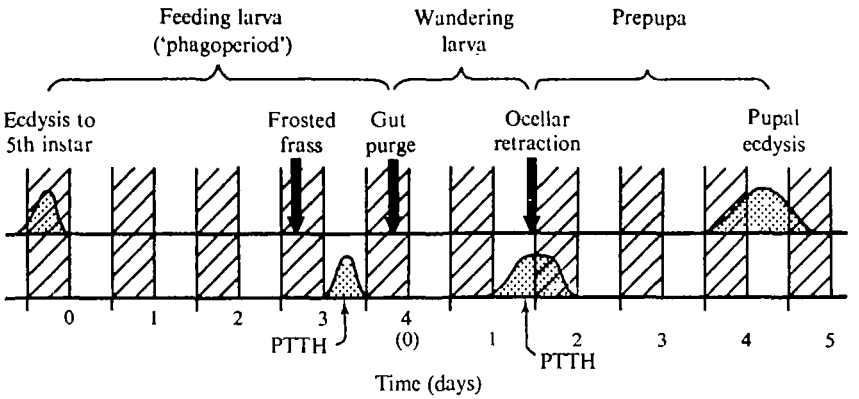

Fig. 1. Timing of morphological events (top panel) and the two periods of PTTH release (bottom panel) during the final (5th) instar and prepupal stage of *Manduca sexta* at 25 °C under a 12L:12D photoperiod. This figure illustrates a population of larvae that purge their gut on day 4. For larvae purging on day 5, all events after ecdysis to the 5th instar are shifted to the right by 24 h. The initial release of PTTH triggers all subsequent endocrine and morphological events. Since this first release can occur on various days, it is convenient to count the insect's age after gut purging on an independent scale. The clear and cross-hatched periods denote photophase and scotophase, respectively. (Modified from Truman & Riddiford, 1974.)

instar and initiation of the prepupal stage are signalled by the apolysis and retraction of the pigment-containing epidermis underlying the larval ocelli.

In addition to the above-mentioned changes, a number of other events occur which are likewise peculiar to the final larval instar. We can find no prior description of the first of these which consists of the excretion of faecal pellets coated with a white chalky substance. Under normal conditions the first appearance of this 'frosted frass' is a sign that the gut purge which terminates the phagoperiod will occur about 24 h later. Two other physiological events accompany the gut purge: the deposition along the dorsum of the caterpillar of a pink ommochrome pigment, and a clearing of the tissues around the dorsal vessel (heart) so that the latter becomes visible along its entire length.

Truman & Riddiford (1974) have shown that pupation is preceded by two periods of PTTH release. Each release of PTTH causes a corresponding surge of ecdysone. The first of these two periods of PTTH secretion is gated by the photoperiod. The gate occurs midway through the photophase, i.e. between 14.00 and 23.00 h. At 25 °C approximately 50% of a synchronous group of final instar larvae release PTTH during the photoperiodic gate on day 3. The remaining 50% do so during the gate on day 4. These two classes of individuals have been designated as 'Gate I' and 'Gate II' larvae, respectively. The surge of ecdysone that follows this first release of PTTH provokes the gut purge (Truman & Riddiford, 1974; Nijhout, unpublished).

In the present communication we shall be concerned only with the causes and consequences of the first period of PTTH secretion. Since the purge ordinarily takes place 12–15 h after PTTH release, the time of PTTH release can be determined by noting the time of the purge.

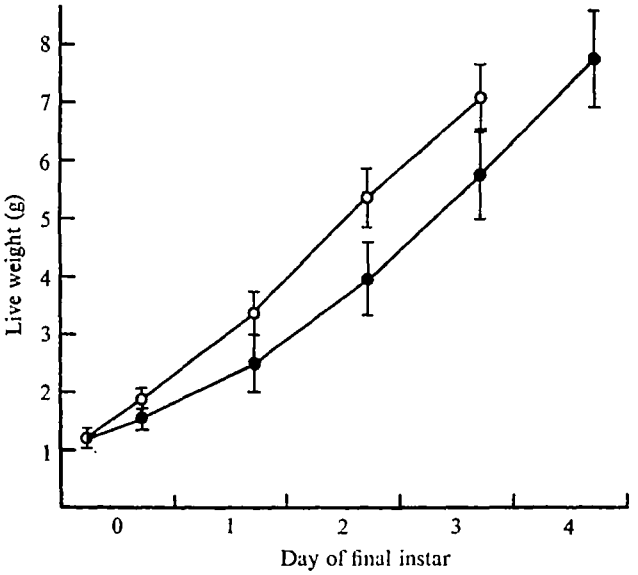

Fig. 2. Growth of *Manduca* larvae during the 5th instar at 25 °C under a 12L:12D photoperiod. The upper curve records the average weight of 18 larvae which purged on day 4; the lower curve represents 16 larvae purging on day 5. The bars indicate standard deviations. The last point on each graph is at the estimated time of PTTH release.

RESULTS

1. *Growth of Gate I and Gate II larvae*

Each of 34 5th-instar larvae was weighed at daily intervals throughout the phagoperiod. At the conclusion of the experiment the data were segregated for Gate I and Gate II larvae.

The average growth curves for these two groups are plotted in Fig. 2. A clear-cut difference between the two curves is evident in that Gate II larvae grew less rapidly than did Gate I larvae during the first 2 days; moreover, they had attained a slightly greater weight at the time of PTTH release.

2. *Duration of the phagoperiod as a function of rate of growth*

Groups of 5th-instar larvae were reared at specific temperatures ranging from 20 to 30 °C. Each individual was weighed at daily intervals and its average rate of growth was calculated throughout the entire phagoperiod. At each temperature we recorded a considerable range of growth rates.

The results summarized in Fig. 3 show that the duration of the phagoperiod was a function of the rate of growth, but that even the fastest growing larvae among over 4000 individuals did not terminate their phagoperiods prior to the 4th day. In the entire group we encountered only five individuals that purged prior to the 4th day; all five were of an experimental group reared under quite abnormal conditions to be considered in a future paper.

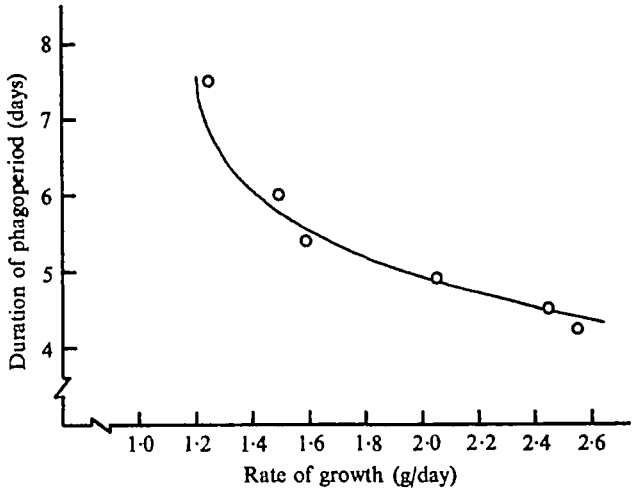

Fig. 3. The relationship between the rate of growth of the final instar and the duration of the phagoperiod. The rate of growth was varied by rearing groups of larvae at specific temperatures from 20 to 30 °C.

Table 1. *Rate of growth and timing of PTTH release in groups of 5th instar* Manduca *fed on normal and diluted diets (25 °C and 12L:12D)*

Dilution of diet (% of normal)	No.	Av. daily consumption (g)	Av. total consumption (g)	Av. weight gain per day (g)	Av. (range) days to PTTH release
100	22	4·5	24	1·6	4·4 (3–6)
66	19	4·8	32	1·4	5·6 (5–7)
33	26	4·9	42	0·9	7·6 (6–10)

3. *Growth on diluted diets*

The preceding experiments show that the timing of PTTH release and the resultant purge are correlated with the rate of growth. We therefore sought to determine which of the various parameters of growth were responsible for triggering the release of PTTH. Among these cues might be the consumption of a critical mass of food, the total time spent in the instar, or the attainment of some critical size or mass.

To examine the first two possibilities, three groups of larvae were reared from the outset of the 5th instar on the normal diet and on diets diluted by 1/3 or 2/3 with non-nutritive material consisting of a mixture of 3 % agar and 8 % powdered cellulose (Alphacel).

The results are summarized in Table 1. It is clear that the diluted diets caused a decrease in the rate of growth and substantially delayed the release of PTTH. Although the daily consumption of diet was little affected, the total amount consumed was greatly increased. On the basis of these results it seems unlikely that either the time spent in the instar, or the total amount of food consumed, serve as cues to determine an individual's readiness to release PTTH. Consequently, we examined in further detail the possibility that PTTH secretion is triggered when the larvae attain a critical size.

The experiment described in Table 1 was repeated a year later, and the two sets of

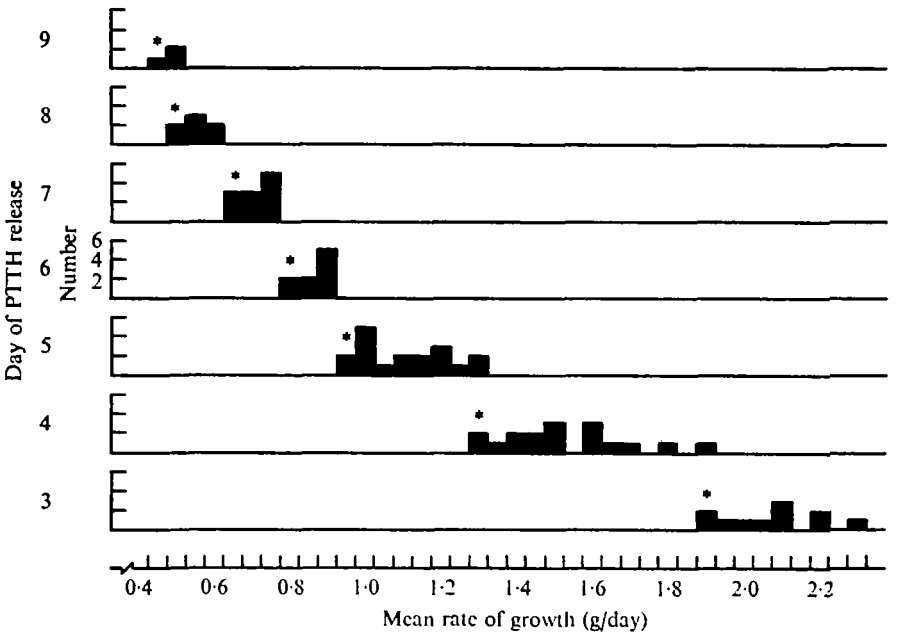

Fig. 4. The distribution of average growth rates for larvae releasing PTTH on various days. The rate of growth was varied by diluting the diet. Individuals from columns labelled with an asterisk were used in the construction of Fig. 5.

data have been combined in the construction of Fig. 4. The average rate of growth during the phagoperiod was calculated for each individual. The growth rates for all those releasing PTTH on a particular day were grouped together and plotted separately for days 3–9. As shown in Fig. 4, there was little overlap between the growth rates of individuals releasing PTTH on successive days. Consequently, there are definite upper and lower limits to the growth rates which allow a larva to release PTTH on a given day. It also follows that, by measuring the rate of growth throughout the instar, one can predict the day of PTTH release.

Of special interest were those larvae that were on the borderlines between the various groups in the above experiment. The larvae in each group which showed the lowest growth rate (indicated by an asterisk in Fig. 4) presumably were the last individuals which released PTTH on a given day. Consequently, these particular larvae barely attained the presumed critical size at the time the decision to release PTTH was made. Larvae with growth rates that were slightly lower would attain the critical size too late and therefore would have to await the opening of the gate on the succeeding day.

The growth curves of these borderline larvae for the few days preceding PTTH release are plotted in Fig. 5. The weights at the time of PTTH release were not constant but varied with the length of time that the larvae had been in the instar. However, when the growth curves from Fig. 5 were plotted in such a way that PTTH release of all individuals coincided in time (Fig. 6), it was noteworthy that all curves crossed within a remarkably small range of weight and time. These findings indicate that if a larva attains the weight of 5 g about 24 h before the gate for PTTH release, then it is

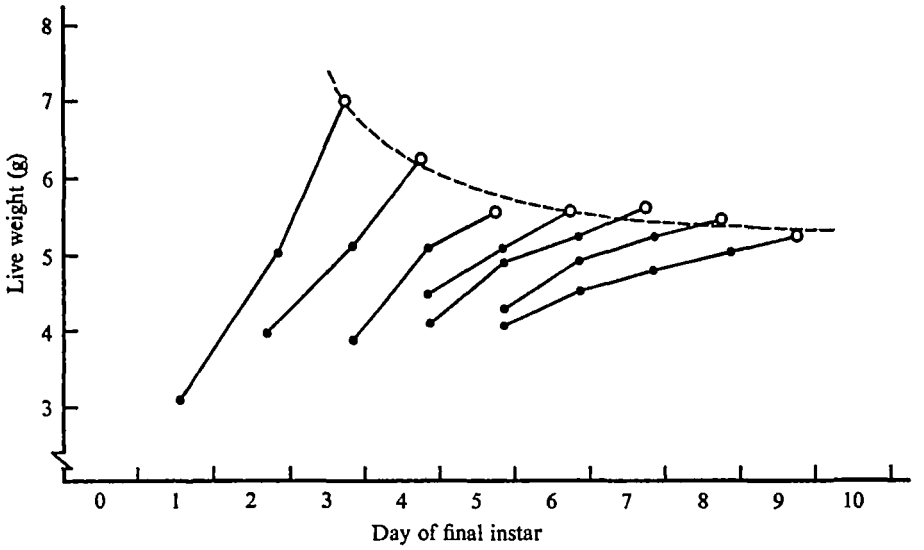

Fig. 5. Daily weights of individual *Manduca* larvae with the minimum rate of growth that allowed PTTH release on successive days. Only the final days of growth are plotted. The average initial weight of the larvae was approximately 1·2 g as in Fig. 2. ○, Weight at the estimated time of PTTH release. ———, Minimum weight at which PTTH release can occur on a given day.

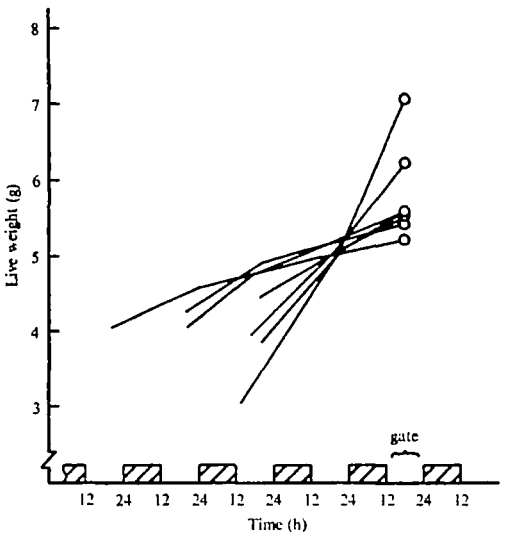

Fig. 6. The curves from Fig. 5 rearranged so that the release of PTTH for all individuals coincide in time (○). The photoperiod is indicated above the abscissa.

'committed' to release PTTH when that gate opens during the following photophase. It will be recalled that the larvae represented in Figs. 5 and 6 are those with the lowest rate of growth that allowed PTTH release to occur on a given day. It follows, therefore, that the weight of these larvae 24 h before PTTH release represents the lowest weight at which these larvae can become committed to release PTTH. This critical weight is about 5 g.

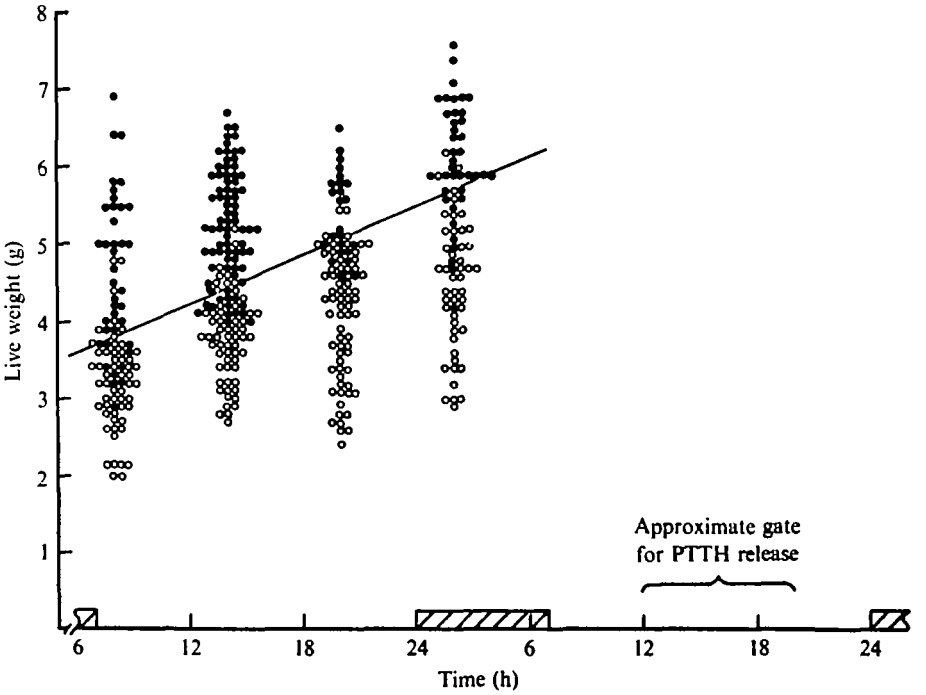

Fig. 7. Weights of feeding larvae are plotted as a function of the time of day during the final days of the 5th instar. ●, Larvae releasing PTTH during the gate on the following day; the position of this gate is indicated. ○, Larvae releasing PTTH on the second day after weighing. The line separating these two groups signifies the minimum weight that allows a larva to utilize the first gate. The photoperiod (17L:7D) is indicated along the abscissa.

4. *The latent period between the attainment of the critical weight and the actual release of PTTH*

The foregoing results suggest that, after the critical weight of 5 g is attained, there is a latent period of about 1 day before PTTH release actually takes place. Further evidence for this conclusion was obtained in the following experiment. Individual 5th-stage larvae were weighed at a certain time of day and then allowed to continue feeding on a normal diet. They were then grouped into two categories: those which released PTTH during the gate occurring 1 day after weighing and those which released PTTH 2 days after weighing. The results of this experiment are shown in Fig. 7. The line in this figure separates the two groups of larvae and therefore represents the lowest weight at each time of day that will allow a larva to release PTTH on the next day. Unlike all other experiments, these larvae were kept under a 17L:7D photoperiod. We are informed by Dr Truman that the gate under this long-day regimen is advanced 1 or 2 h from its normal time of occurrence under short-day conditions. Fig. 7 shows that larvae must attain the critical weight of 5 g at least 24 h before the closing of the photoperiodic gate. Therefore, in a population of normally growing larvae, those which weigh 5 g or more prior to 20.00 h on day n will release PTTH on day $n+1$. Those which attain this weight after 20.00 h will continue to feed and grow for an additional day and will release PTTH during the gate on day $n+2$.

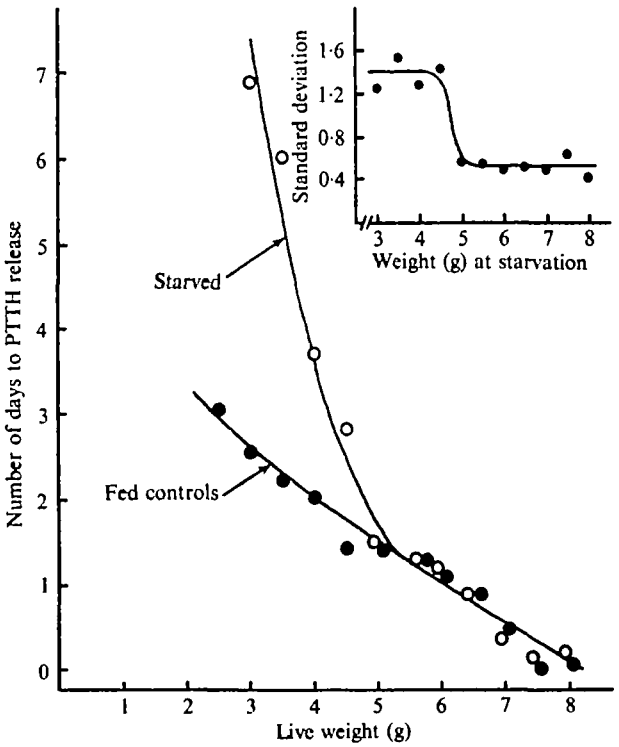

Fig. 8. The effect of starvation of 5th-instar larvae of *Manduca* on the subsequent time to PTTH release. Experimental larvae were weighed and put without food at approximately 17.00 h. Controls were weighed at that time and allowed to continue feeding. Each point represents 30–70 larvae. The inset shows the standard deviations of the mean time to PTTH release of the starved larvae.

5. *Effects of starvation*

The effect of live weight on the timing of PTTH secretion was studied in further detail by starving 5th-stage larvae after they had attained a certain weight and noting the number of days that elapsed until PTTH release. All weighings were made and starvations begun at 17.00 h, i.e. approximately 1 h before the middle of the photophase. Control larvae were weighed and allowed to continue feeding.

The results depicted in Fig. 8 show that when starvation was begun at 5 g or above, the time to PTTH release did not differ from that of fed controls. By contrast, when larvae weighing less than 5 g were starved, PTTH release was substantially delayed.

It was of particular interest to find that when larvae weighing less than 4 g were deprived of food they often transformed, not into pupae, but into intermediates retaining larval characteristics. This was true of 46% of the individuals starved at 3·5 g and 74% of those starved at 3·0 g. The timing of PTTH release could not be scored in the usual way since the intermediates seldom showed the purge that precedes a normal larval-pupal moult. We therefore referenced the PTTH release to the slipping of the head capsule; an event which takes place in a larval–larval moult with the same timing after PTTH release as the purge in a larval–pupal moult (Truman, 1972).

When 5th-stage larvae weighed less than 3·0 g at the outset of starvation, mortality was greatly increased and no reliable data could be obtained. The time interval between ecdysis to the 5th instar and the attainment of 3·0 g thus corresponds to the 'period of indispensable nutrition' as defined by Bounhiol (1938).

In the inset to Fig. 8 it is of interest to observe that the standard deviations of the mean time until PTTH release increased abruptly when starvation began at weights below 5 g. Evidently, the precise control of the secretion of PTTH becomes possible only when a larva attains the critical weight of 5 g.

When larvae were starved at a weight of 5 g or more, the timing of PTTH release was not affected. This finding can be explained by assuming that when a larva reaches the weight of 5 g a process is initiated which culminates in the secretion of PTTH. This process, once initiated, cannot be inhibited by starvation.

The mean time to PTTH release for larvae starved at 5 g is 1·5 days (Fig. 8). Actually, 50% of the larvae (n = 70) released PTTH during the gate on day 1 and 50% did so on day 2 after the beginning of starvation. These larvae were starved shortly before the middle of the photophase (17.00), i.e. 24 h before the first available PTTH gate (Truman & Riddiford, 1974). Thus, when larvae reach 5 g 24 h before a gate, half of them become competent to release PTTH during that gate; the remainder become competent to do so soon after the gate closes, but have to 'wait' almost 24 h for the next gate.

DISCUSSION

Under normal conditions at 25 °C and 12L:12D photoperiod, 5th-instar larvae of the tobacco hornworm terminate the phagoperiod and purge their guts on days 4 or 5, at which time they weigh 8–10 g. As shown in Fig. 1, the purge occurs during the latter half of the scotophase and is in response to the first release of PTTH and the accompanying surge of ecdysone (Truman & Riddiford, 1974). The release of PTTH is gated by photoperiod; it takes place during the photophase some 12 h prior to gut purging.

As shown in Figs. 3 and 4, the timing of PTTH release is correlated with the rate of growth. Fast-growing larvae release PTTH more promptly than do slowly growing ones. Therefore, the total time spent in the instar plays no direct role in the process that renders a larva competent to release PTTH. The experiment summarized in Table 1 strongly argues that the total bulk of food consumed likewise has no effect on the timing of PTTH secretion.

The experiments described in Figs. 5–7 show that a larva must reach a certain weight before PTTH secretion can occur on schedule. Under our experimental conditions the larvae must achieve a critical weight of about 5 g at least 24 h prior to a PTTH gate in order to release the hormone during that gate. If this critical weight is reached less than 24 h before the closing of the gate the latent period is prolonged until the opening of the gate on the succeeding day.

The experiment summarized in Fig. 8 allows us to interpret this phenomenon in the following way. When a last instar larva of *Manduca sexta* attains a weight of 5 g, a hypothetical process is initiated. This process requires approximately 24 h to be completed. When this process has reached its end point the brain has become competent to release PTTH. The actual secretion of this hormone takes place during the

very next photophase. When larvae are starved at weights below 5 g, PTTH secretion may eventually occur (Fig. 8) but only after a long and variable period. The inset in Fig. 8 illustrates this decrease in accuracy of the timing process for PTTH secretion in larvae starved below the critical weight.

The identity of the process that renders the brain competent to secrete PTTH will be explored in the succeeding paper.

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