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PHYSIOLOGY OF INSECT RHYTHMS

I. CIRCADIAN ORGANIZATION OF THE ENDOCRINE EVENTS UNDERLYING THE MOULTING CYCLE OF LARVAL TOBACCO HORNWORMS

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In insects the development of a rigid exoskeleton requires that for growth and metamorphosis to occur a new cuticle must be periodically formed and the old one cast off. This process (the moult) is initiated with the release of the prothoracicotropic hormone (PTTH) from the brain. Then follows the secretion of a second hormone, ecdysone, by the prothoracic glands, the apolysis of the epidermis from the cuticle, the secretion of a new cuticle, and, finally, the shedding or ecdysis of the old cuticle. The type of cuticle formed is regulated by a third hormone, the juvenile hormone (JH), produced by the corpora allata (see Wigglesworth (1971) and Wyatt (1972) for current reviews of the hormonal control of moulting). Recently, a study of the eclosion of adult silkmoths revealed that the last step in the moulting process is also under endocrine control (Truman & Riddiford, 1970). In this case a neurosecretory hormone from the brain acts on the nervous system to trigger the behaviour involved in emergence (Truman, 1971; Truman & Sokolove, 1972).

An interesting feature of adult eclosion is its association with a circadian clock. This underlying clock is sensitive to photoperiod and restricts emergence to temporal 'gates', which occur during a specific period of the day (Pittendrigh, 1966; Skopik & Pittendrigh, 1967). However, in addition to the act of eclosion, other steps in the moulting process may show a sensitivity to photoperiod cycles. Williams & Adkisson (1964) clearly demonstrated that, in diapausing pupae of *Antheraea pernyi*, long-day photoperiods act directly on the brain to promote the secretion of PTTH. Also, a possible gating of PTTH release has been inferred from histological studies on *Drosophila* (Rensing, 1971).

The events beginning with PTTH release, proceeding through apolysis and cuticle deposition, and ending with ecdysis of the old cuticle are considered here to comprise the moult. This paper examines two aspects of the moulting process in lepidopteran larvae. The first involves the question as to whether the mechanism which controls adult eclosion is also used to regulate the time of larval ecdysis. The second examines the possible involvement of a circadian clock in the control of PTTH release and the temporal relationship of the release of this hormone to that of ecdysone and JH.

MATERIALS AND METHODS

A. *Experimental animals*

Larvae of the silkmoth, *Antheraea pernyi*, were reared in wooden trays which were fitted with screen-covered tops. Routinely, larvae were supplied daily with fresh branches of oak leaves sprayed with a solution of antibiotics (Riddiford, 1967).

The tobacco hornworms, *Manduca sexta*, used in these experiments were derived from animals obtained from Dr R. A. Bell, A.R.S., U.S.D.A., Fargo, N.D. Larvae were grown on a modification of the hornworm diet developed by Yamamoto (1969) (R. A. Bell, unpublished). As recommended by Bell (personal communication), hornworm larvae were reared individually in plastic containers. Upon hatching each larva was placed in a 1 oz plastic cup with approximately 3 g of diet. At 25 °C the larvae attained the fifth instar after about 10 days. Each was then transferred to a 8 oz cup containing about 20 g of diet. Four to five days later, the larvae stopped feeding and initiated the larval–pupal transformation.

B. *Recording of larval ecdysis times*

The recording of the time of larval ecdysis was simplified by the observation that during the shedding of the cuticle the old head capsule is torn from the rest of the exuviae and dropped to the substratum. Thus, the ecdysis time could be recorded simply by collecting the discarded head capsule. For this purpose moulting larvae were suspended over a large funnel which was positioned over a Gilson fraction collector. Collecting tubes were changed at half-hour intervals. In the case of *Antheraea*, the rearing trays were checked daily and twigs bearing moulting larvae were removed and taped to a central branch which overhung the funnel. To record *Manduca* ecdysis the larvae were placed on large-mesh hardware cloth which was fitted over the top of the funnel. The discarded head capsules then fell through the wide holes in the mesh. With both species the ecdysis times were determined by the distribution of head capsules in the collecting tubes.

C. *Photoperiod conditions*

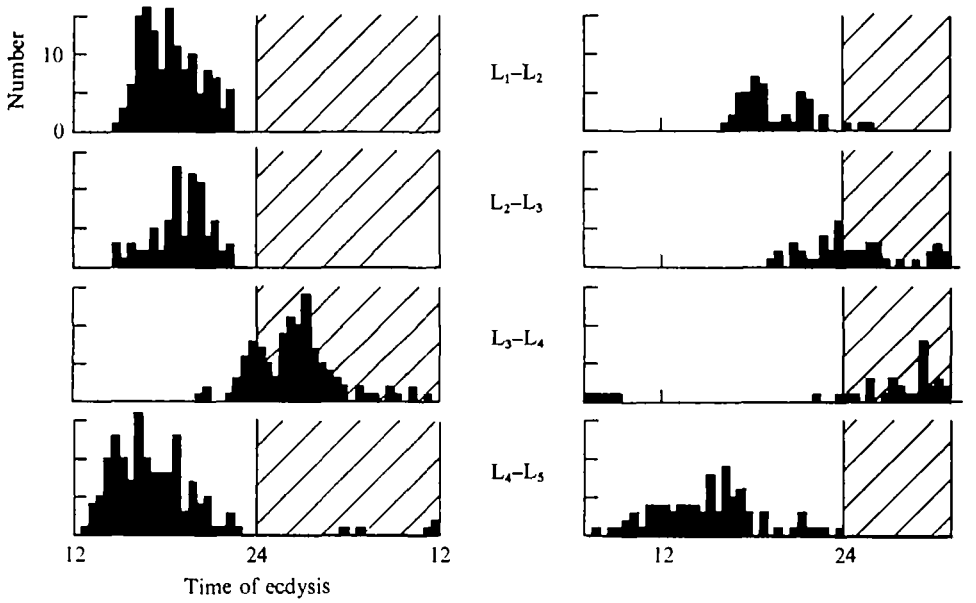
All larvae were reared in constant-temperature rooms with programmed photoperiod cycles. To avoid confusion lights-off has been arbitrarily designated as midnight (24.00) in all cases. According to convention (Pittendrigh, 1965), times are thus given as arbitrary 'zeitgeber' time (A.Z.T.).

RESULTS

A. *Timing of larval ecdysis*

(1) *Untreated larvae*

Groups of *Antheraea* larvae were reared in constant-temperature rooms (26° ± 1 °C) under photoperiod conditions which induce pupal diapause (12L:12D) or which promote continuous development (17L:7D) (Tanaka, 1950). The successive ecdyses were followed through the four moults from the first to the fifth instar. The shorthand notation L_{n-1}–L_n is used to denote the moult to the nth instar; e.g. the L_4–L_5 ecdysis refers to the ecdysis from the fourth- to the fifth-instar larva.

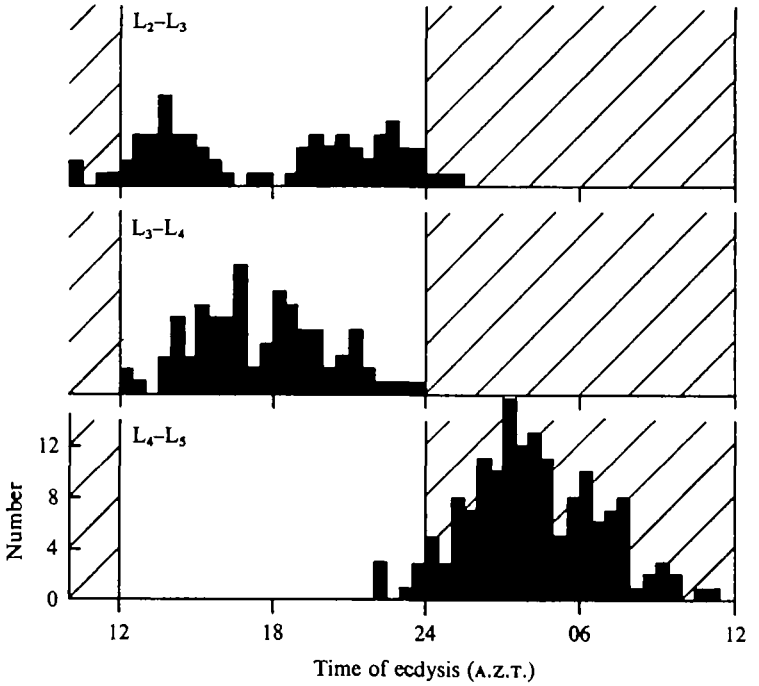


Text-fig. 1. The distributions of the four larval ecdyses of *Antheraea* reared at 26 °C under (left) a 12L:12D photoperiod or (right) a 17L:7D photoperiod.

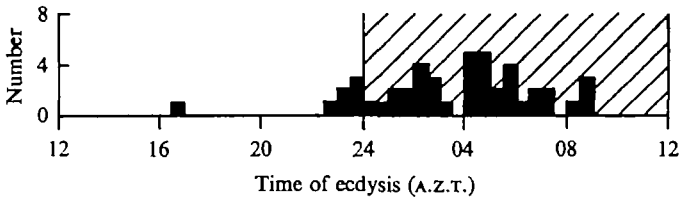
Under both long-day and short-day conditions larval life from hatching to pupation lasted an average of 45 days in *Antheraea*. During this period of growth, the caterpillars underwent four larval ecdyses. The timing of these ecdysial events (Text-fig. 1) shows two features of interest. First, the distributions tended to broaden as larvae progressed from the first to the fifth instar. In every case these distributions were much broader than those seen for adult eclosion under the same photoperiod conditions. Secondly, the time of ecdysis varied with the particular instar. Moreover, each stage underwent ecdysis at a time of day which tended to be later than that seen for the preceding instar. This trend culminated with the L_4–L_5 ecdysis which was delayed into the photophase of the following day.

Text-fig. 2 records the distributions of the L_2–L_3, L_3–L_4 and L_4–L_5 ecdyses of *Manduca* in 12L:12D at 25 ± 1 °C. The last two ecdyses followed the trend seen above for *Antheraea*; the ecdysis to the fifth instar occurred at a later time of day than did the L_3–L_4 ecdysis. However, the ecdysis to the third instar showed a marked bimodality. The significance of this bimodal distribution will be considered in the Discussion.

The occurrence of larval ecdysis at a particular time of day is not, in itself, sufficient grounds for concluding that this particular event is controlled by a biological clock. One alternative explanation is that the first event in the moulting process, PTTH release, is gated and that this event serves to synchronize subsequent development including ecdysis. The following experiments have utilized *Manduca* larvae in an attempt to distinguish between two alternatives.



Text-fig. 2. The distributions of the last three larval ecdyses of *Manduca* reared under a 12L:12D photoperiod at 25 °C.



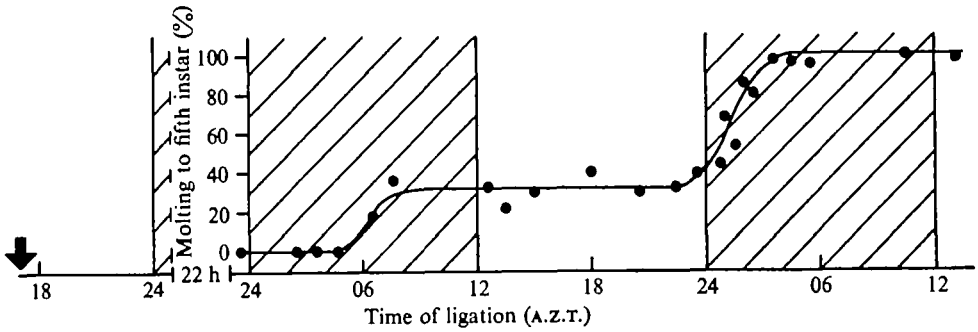
Text-fig. 3. The distributions of L_4–L_5 ecdyses for *Manduca* larvae which had their ocelli cauterized.

(2) *Effect of temperature on the time of larval ecdysis*

Circadian clocks characteristically show a strongly temperature-compensated, free-running period (Sweeney & Hastings, 1960). Similarly, the phase relationship of an entrained rhythm to the photoperiod cycle is relatively stable over a considerable range of temperatures [e.g. adult eclosion of *Drosophila* (Pittendrigh, 1954)]. It was thus of interest that only a 3 °C increase in rearing temperature caused a 7 h shift in the time of the L_4–L_5 ecdysis of *Manduca* larvae. At 25 °C the median time of ecdysis was 03.30, whereas at 28 °C the median ecdysis time advanced to 20.30. For the same changes in temperature the time of eclosion of *Manduca* adults does not change appreciably.

(3) *Effects of cauterization of larval ocelli*

The importance of the lateral ocelli in the timing of the larval ecdyses was determined by cautery of these structures. One day after the L_2–L_3 ecdysis the ocelli were



Text-fig. 4. The effects of applying neck ligatures to fourth-instar *Manduca* larvae at various times after the L_3–L_4 ecdysis. The percentage of the neck-ligatured larvae subsequently developing to the fifth instar is plotted against the time of ligation. Each point represents a sample of about 50 ligatured larvae. The arrow identifies the median time of the L_3–L_4 ecdysis.

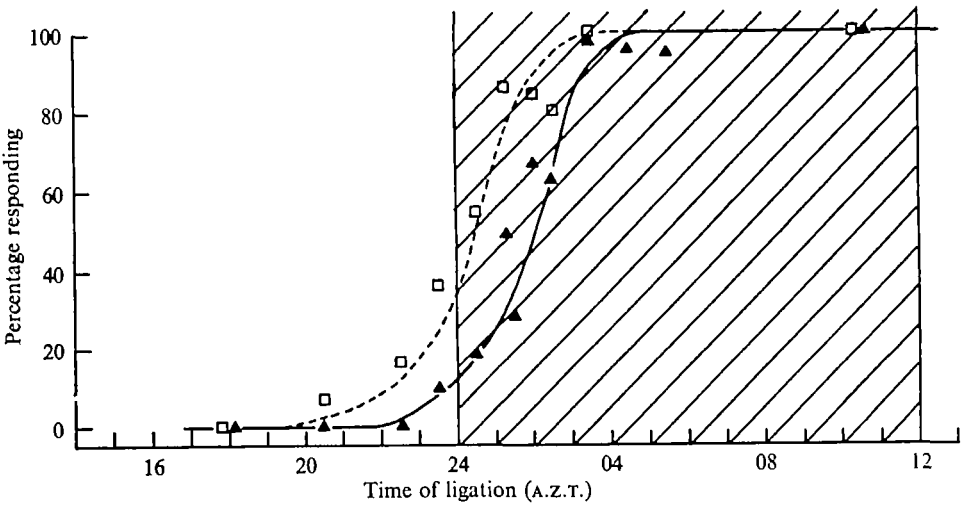
cauterized using a flame-heated needle. After the moult to the fourth instar the burned areas were covered by smooth cuticle which showed no trace of ocellar structures. As seen in Text-fig. 3, cautery of the lateral ocelli did not abolish the ability of the larvae to perceive photoperiod. These larvae underwent ecdysis to the fifth instar at the same time of day as did untreated *Manduca* larvae (Text-fig. 2).

B. *Effects of ligations on the onset of the moulting process*

(1) *Ligations between head and thorax*

(*a*) *Fourth-instar larvae.* If the overt synchrony of larval ecdysis arises from a prior gated event, a likely candidate is the secretion of PTTH. To determine whether the release of this hormone was confined to a specific period of the day, groups of developmentally synchronous *Manduca* were used. These groups were obtained by daily collection of all larvae which had undergone ecdysis to the fourth instar on that day. Since the L_3–L_4 ecdyses are confined to a 12 h period of the day (Text-fig. 2), this procedure yielded larvae which began the fourth instar in relative synchrony (± 6 h). The timing of PTTH release was determined by ligation of over 1200 larvae at various times after the L_3–L_4 ecdysis. A blood-tight ligature was applied between the head and thorax of each larva, thus effectively separating the cephalic endocrine organs – the brain, corpora cardiaca and corpora allata – from the remainder of the body.

As shown in Text-fig. 4, fourth-instar larvae ligated prior to 04.00 A.Z.T. of the second night after ecdysis showed no further larval development. When the ligatures were applied after this time, some larvae subsequently became fifth instars. The percentage beginning the fifth-instar moult depended upon the time of ligation and showed a bimodal distribution. During the period from 04.00 to 08.00 of the second night, approximately one-third of the larvae initiated the moult as manifested by the response of the thorax and abdomen. The remaining two-thirds waited until the onset of the third night. Thus, the head-centred endocrine events which are necessary for the production of the fifth-stage larva are completed during gates occurring in the early to middle portion of the night. The median time for the initiation of fifth-instar



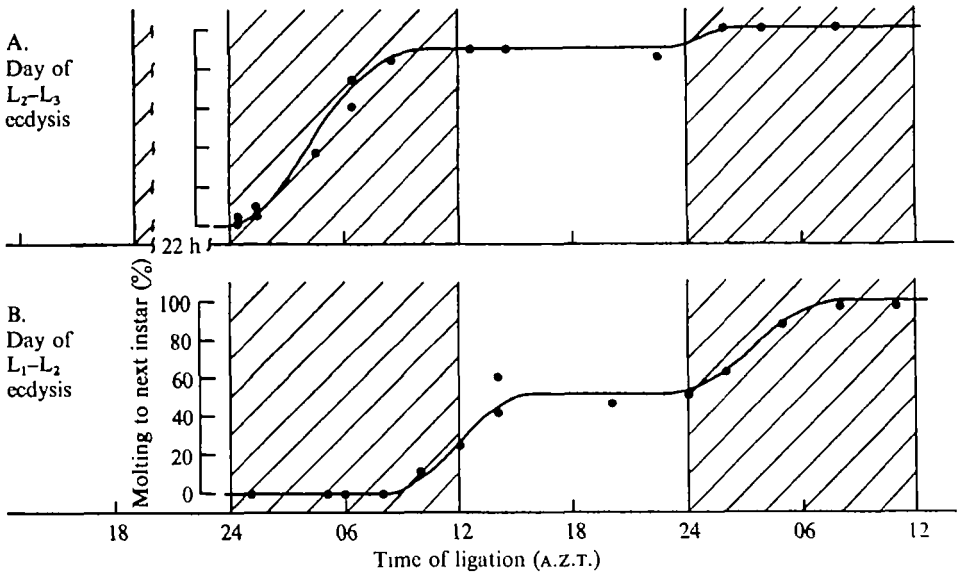
Text-fig. 5. The developmental response of neck-ligatured fourth-instar larvae as a function of the time of ligation; ▲—▲, indicates those becoming fifth-stage larvae; □—□, indicates those becoming fifth-stage larvae, larval–pupal intermediates or miniature pupae. Approximately 50 larvae were ligatured at each time. Only larvae releasing PTTH during the second gate are considered.

moult occurs at approximately 06.00 on the second night (gate I) and at 02.00 on the third night (gate II). This difference in timing between the first and second gates will be discussed below.

The neck-ligatured *Manduca* which subsequently became morphologically perfect fifth-instar larvae showed a curious blackening of their cuticle. This aberrant pigmentation occurred in a definite, reproducible pattern and did not affect the viability of the preparation; such neck-ligatured animals survived for up to 2 weeks. The endocrine basis for this black pigmentation is treated in detail elsewhere (Truman, Riddiford & Safranek, 1972).

The formation of a fifth-stage larva requires not only PTTH but also the secretion of sufficient JH. It was therefore of interest that many of the ligatured larvae did not subsequently moult to the fifth instar, but instead promptly began pupal development. Six to nine days later, these animals became perfect miniature pupae (Pl. 1, fig. 1).

This phenomenon of precocious pupation was studied in detail using larvae which would initiate the moult during the second gate. Neck-ligatured larvae were kept for 9 days after which time the fate of the thorax and abdomen was determined. Nearly 50 miniature pupae were obtained in this manner. Of these only five showed retention of some larval characters. Text-fig. 5 shows the percentage of ligatured larvae which subsequently became fifth-stage larvae, pupae, or larval–pupal intermediates as a function of the time of ligation. This curve is shifted 1·5 h to the left of the curve obtained when only the formation of fifth-stage larvae is considered. This first curve identifies the time at which sufficient PTTH had been released to activate the prothoracic glands; the second curve shows that by 1·5 h later sufficient JH had been secreted to insure the formation of a perfect fifth-stage larva. A smaller series of ligations of first-gate larvae also indicated an approximately 1·5 h delay between PTTH and JH release.



Text-fig. 6. The response of neck-ligatured *Manduca* larvae as a function of the time of ligation. A: ligatured third-instar larvae; B: ligatured second-instar larvae. Each point represents approximately 50 larvae.

(*b*) *Third-instar larvae.* When developmentally synchronous groups of third-instar larvae were ligatured between the head and thorax, the results in Text-fig. 6A were obtained. The first gate for initiation of the moult to the fourth instar occurred on the second night after the L_2–L_3 ecdysis. Approximately 90% of the larvae began fourth-instar development during this gate. The few remaining larvae used the gate on the following night.

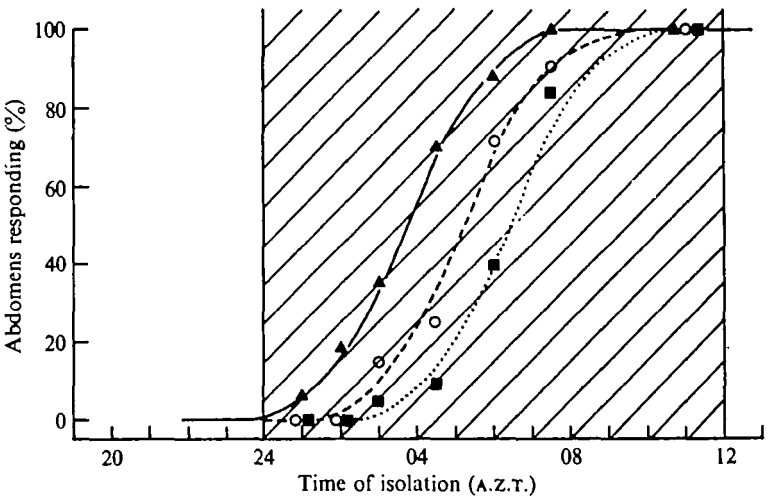
As seen above for fourth-stage larvae, some neck-ligatured third instar larvae became miniature pupae. However, only a few pupae were obtained because of problems in maintaining these small fragments alive for a sufficient length of time.

(*c*) *Second-instar larvae.* As shown in Text-fig. 6B, about half of the second-instar larvae initiate the moult to the third instar during the first night after the L_1–L_2 ecdysis. The remainder exploit the gate on the second night. No miniature pupae were obtained from this group since most ligatured larvae lived only for a few days.

(2) *Ligations between the thorax and the abdomen*

The timing of ecdysone secretion was estimated for fourth-instar larvae which had released PTTH during the second gate. Transverse ligatures were placed between the first and second abdominal segments of developmentally synchronous groups of larvae. The thorax and head were then cut away. Since this procedure removed the prothoracic glands, the subsequent fate of the abdomen reflected the action of the ecdysone which had been secreted up to the time of ligation (Williams, 1952).

Abdomens isolated from second-gate larvae prior to 01.00 showed no further development and remained permanent fourth-instar abdomens. When ligatures were placed after this time, various degrees of ecdysone effects were subsequently observed. The least affected abdomens showed only the formation of fifth-instar crochets on the



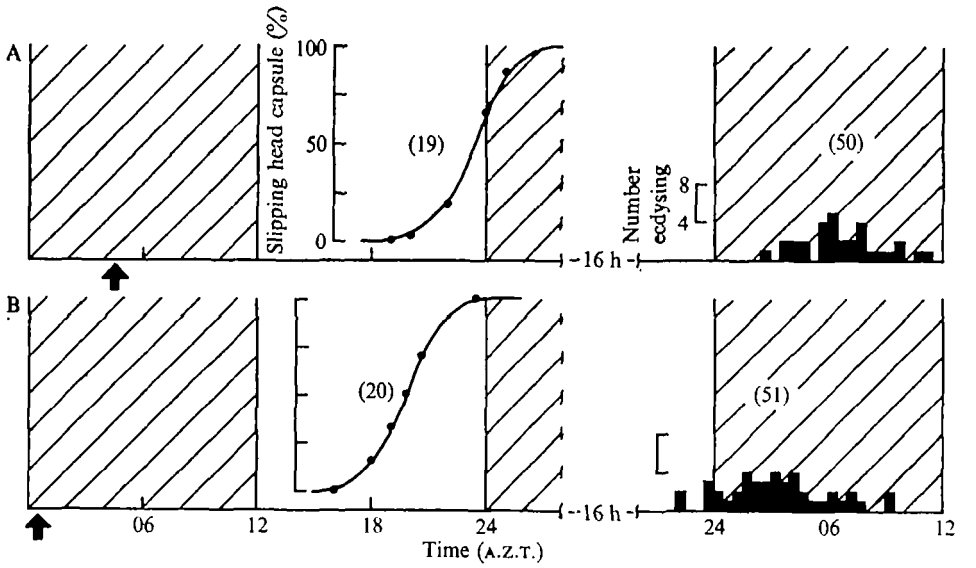
Text-fig. 7. The developmental response of isolated fourth-instar abdomens as a function of the time of isolation; ▲—▲, abdomens which showed at least a grade 1 effect; O----O, abdomens which showed at least a grade 2 effect; ■.....■, abdomens which showed grade 3 effects (for explanation of scoring of ecdysone effects, see text). Thirty to forty abdomens were isolated at each time. Only second-gate larvae are considered.

Table 1. *Ecdysone effects observed in abdomens which were isolated from fourth-instar hornworm larvae during the moult to the fifth instar*

Grade of effect	Description
1	Abdomen shows formation of fifth-instar crochets; remainder of abdomen remains as fourth instar
2	Abdomen shows localized patches of epidermis which undergo retraction, moulting-fluid secretion, and formation of fifth instar cuticle
3	Abdomen forms a perfect fifth-instar abdomen

tip of each proleg. These rows of hooks appeared as a darkened area underlying the fourth-instar crochets. Dissection revealed that these structures were perfectly formed and completely tanned; there was no sign of endocuticle digestion in the overlying fourth-instar cuticle. With one exception, in affected abdomens all prolegs were equally affected. More pronounced ecdysone effects were manifest by abdomens which showed localized areas of retraction, secretion of moulting fluid, and formation of fifth-instar cuticule. From a series of 20 mosaic abdomens a clear hierarchy in response was observed. The structures which most frequently underwent the fifth-instar moult were the prolegs. These were followed in frequency of occurrence by the ventral mid-line, the intersegmental areas, and finally the dorsal and lateral portions of each segment. For the purpose of this paper the ecdysone effects have been separated into three grades as summarized in Table 1.

The effects of isolating fourth-instar abdomens at various times during the second gate are illustrated in Text-fig. 7. Of abdomens isolated at 04.00 A.Z.T., approximately 50% subsequently showed at least a grade 1 response. If ligatures were applied at 05.30, half of the abdomens produced at least a grade 2 effect. By 06.30 ligation yielded a majority of perfect fifth-instar abdomens.



Text-fig. 8. The timing of head-capsule slippage (●—●) and of the L_4–L_5 ecdysis (histograms) for groups of larvae which moulted to the fifth instar in a 12L:12D photoperiod at 25 °C. A: larvae releasing PTTH during the first gate; B: second-gate larvae. The arrows identify the median time of PTTH secretion. The median times for head-capsule slippage and for ecdysis relative to PTTH release are given in parentheses.

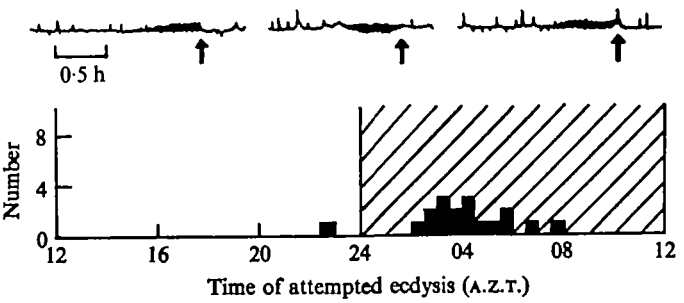
Table 2. *Time table of endocrine and developmental events which occur during the moult to the fifth-instar larva at 25 °C*

Time (h)	Event
0	Sufficient PTTH released; brain no longer necessary for moulting
1·5	Sufficient JH secreted; corpora allata no longer needed for fifth-instar larval development
3	The first epidermal tissue, the crochets, becomes independent of the prothoracic glands
6	Prothoracic glands no longer necessary for the complete moulting of the abdomen
19	Head capsule slipped by the developing fifth-stage larva
39	Mandibles of fifth-stage larva begin to darken
44	Initiation of moulting-fluid resorption; air present in old fourth-instar head capsule
50	Fifth-instar ecdysis

Thus we see that at the onset of the moult to the fifth-instar larva ecdysone is actively secreted during at least a 2·5 h period. Moreover, the prothoracic glands are no longer required by 6 h after PTTH release.

C. *Relation of larval ecdysis times to PTTH release time*

The data presented in the preceding section clearly show that the release of PTTH is confined to gates which occur during a specific period of the day. It was therefore possible that the synchrony observed in larval ecdysis was derived from the gating of this primary endocrine act. To explore this possibility further, I analysed the timing of certain developmental events with respect to the time of PTTH secretion. This



Text-fig. 9. 'Ecdysis' of neck ligatured fourth-instar larvae. Top: tracings of kymograph records showing the characteristic record produced during 'ecdysis'. The arrows show the points selected as the time of ecdysis. Bottom: the distribution of L_4–L_5 ecdyses shown by neck-ligatured larvae.

analysis was simplified by the fact that PTTH release during the first gate occurs at a mean time of 04.30 A.Z.T., whereas the mean time for secretion is 00.30 A.Z.T. for gate II. Thus, relative to time of day, first-gate and second-gate larvae show a 4 h difference in the time of initiation of the moult to the fifth instar. Groups of gate I and gate II larvae were therefore followed through development with special attention being given to the times of head-capsule slippage and of ecdysis. The former event was signalled by the withdrawal of the occipital region of the developing head from the old head capsule. At this stage the old capsule formed a 'muzzle' which covered the mouthparts and most of the genal and frontal area of the developing head. Slippage was considered complete after the posterior margin of the head capsule moved past the developing ocelli.

Text-fig. 8 shows the timing of head-capsule slippage and of ecdysis for both gate I and gate II larvae. It is of importance that the initial time difference seen in the onset of the moult for the two groups is maintained through the formation of the fifth instar. The median time for head-capsule slippage occurs at 23.30 A.Z.T. for gate I larvae and at 20.00 A.Z.T. for gate II. Similarly, median ecdysis times of 06.30 A.Z.T. and of 03.00 A.Z.T. are observed for larvae from gate I and gate II, respectively. Thus, the L_4–L_5 ecdysis occurs at a fixed time (50 h) after PTTH release, irrespective of which gate the larva uses. Table 2 provides a schedule of selected endocrine and developmental events which occur during the moult to the fifth instar larva. Events occurring during this span are referred to PTTH release at hour zero.

D. *Effect of neck ligation on the time of ecdysis*

The controlling mechanism for the photoperiodic termination of pupal diapause (Williams & Adkisson, 1964) and for the gating of eclosion (Truman & Riddiford, 1970) are brain-centred. If there were a clock controlling larval ecdysis, it probably would also be located in the head. Therefore, *Manduca* larvae were neck-ligatured at approximately hour 12 of the moult. To provide support and to prevent wandering of the caterpillars, their prolegs were fixed to a substrate with wax. Movements were then recorded by attaching the restrained larvae to a lever by means of a thread tied to the caudal 'horn'. The lever then wrote on a revolving smoked drum. These neck-

ligatured animals did not show sufficient coordination to shed the old cuticle, but at the normal ecdysis time they initiated a series of weak peristaltic waves which moved along the abdomen in a manner similar to that seen in intact larvae during ecdysis. These subtle movements produced a distinct widening of the kymograph trace which lasted about 0·5 h (Text-fig. 9). Of 20 larvae which were treated in this fashion, 18 left unambiguous records as to when 'ecdysis' occurred. Text-fig. 9 shows that even though these larvae were ligatured about 1·5 days before, they nevertheless underwent 'ecdysis' synchronously during the normal period in the night.

DISCUSSION

A. *Comparison of larval ecdysis with adult eclosion*

As mentioned above, the time of adult eclosion is controlled by a circadian clock and has been classified as a 'gated event' (Pittendrigh & Skopik, 1970). These authors have shown that in *Drosophila* the act of eclosion is not simply the last step in the developmental sequence which transforms the maggot into the fly. Events during adult development occur without respect to time of day, but the eclosion event occurs only during specific temporal gates as dictated by an underlying circadian clock. Thus, flies which complete development after one gate has closed wait until the opening of the gate on the following day.

In the silkmoths the gating of adult eclosion is controlled by a brain-centred mechanism (Truman & Riddiford, 1970). At the proper time of day the brain liberates the eclosion hormone which then provokes the emergence of the moth (Truman & Riddiford, 1970; Truman, 1971). The results presented above strongly argue that the larval ecdyses are *not* controlled by the same or similar mechanisms. A direct correlation between the time of PTTH release and the time of subsequent ecdysis is not compatible with the control of the ecdysial act by an independent, circadian timing mechanism. Similarly, the striking temperature sensitivity of the time of larval ecdysis is consistent with the hypothesis that this event is developmentally triggered. It is also of importance that the eclosion hormone cannot be recovered in significant amounts from the brain or corpora cardiaca of *Manduca* larvae at any stage of the moult or intermoult period (J. W. Truman, unpublished). Finally, the maintenance of 'ecdysis' synchrony after neck ligation shows that the head is not needed to trigger this event.

It is therefore likely that the gating mechanism controlling adult eclosion does not have a counterpart in larval ecdysis. The activation of the behaviour involved in shedding the larval cuticle is presumably a developmentally triggered process which occurs a fixed number of hours (depending upon temperature) after the gated release of PTTH. Due to the increase in size during larval growth, more time is required to form each successive larval stage. Thus, as seen in *Antheraea* (Text-fig. 1), each successive ecdysis occurs at a progressively later time of day and the distribution tends to broaden. In the case of second-instar *Manduca*, the PTTH release gate is broader than that seen in later instars (Text-fig. 6B). As a result, the median time of secretion during the first gate occurs at a time of day which is almost 9 h later than that seen during the second gate. This extreme difference in PTTH release times for the first versus the second gate is reflected in the marked bimodality which is seen in the

subsequent L_2–L_3 ecdysis (Text-fig. 2). Primarily second-gate larvae are found in the early peak, whereas the late peak is composed almost exclusively of first-gate larvae.

B. *The gating of PTTH release*

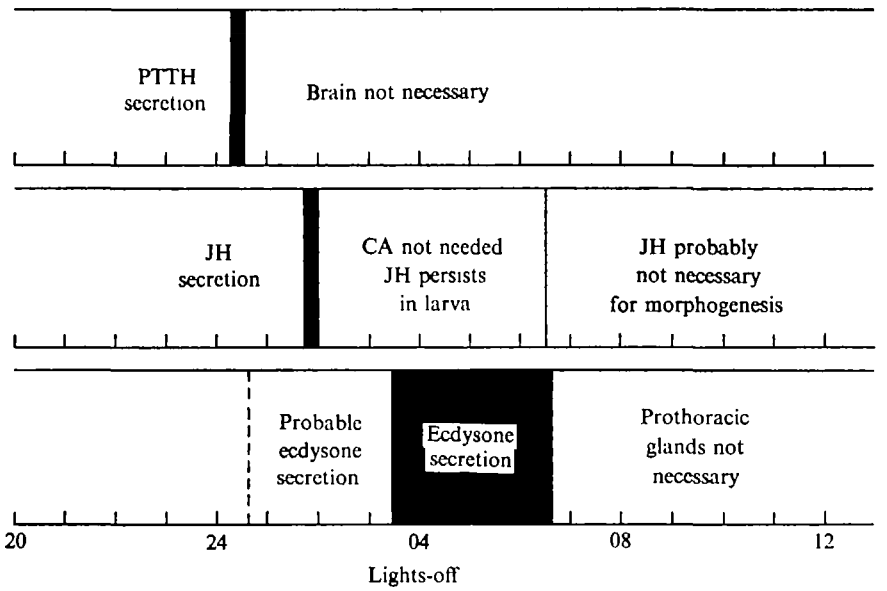
The ultimate stimuli which trigger PTTH release in growing larvae are well understood only in the case of *Rhodnius* (Wigglesworth, 1934). In this blood-sucking bug the state of nutrition is not the crucial stimulus, but rather the mechanical stretching of the abdomen. This information is then relayed to the brain through abdominal stretch receptors (van der Kloot, 1961). In *Locusta* the onset of a moult is presumably dependent upon information coming from the frontal ganglion (Clarke & Langley, 1963). But, as pointed out by Wigglesworth (1971), the arrest of moulting after removal of this ganglion may be the result of starvation since this structure is necessary for swallowing. The present study in no way identifies the nature of the stimuli which cause the secretion of PTTH in *Manduca*. Rather, it demonstrates that once these proper stimuli have been received, the brain then releases hormone only during specific times of the day.

The difference in mean hormone-release times for the first against the second gate in the fourth instar (Pl. 1) is a case of 'gating bias' as first described for *Drosophila* eclosion (Skopik & Pittendrigh, 1967). In *Manduca* this difference suggests that when the first gate opens, none of the larvae have attained a competence to release PTTH. But while the gate is open some of the population reach the appropriate condition and by the time the gate shuts, a number have released hormone and thus started the moult to the fifth instar. During the following day the remainder of the larvae become competent and upon the opening of the next gate they rapidly initiate the moult. Thus, as noted for *Drosophila* eclosion (Skopik & Pittendrigh, 1967), the distribution of releases in the first gate indicates when the gate closes and those in the second gate indicate when the second gate opens. Therefore, in the fourth instar the total duration of the PTTH gate is 10 h from 22.30 to 08.30 A.Z.T. in a 12L:12D photoperiod. Assuming that the 1·5 h lag between PTTH and JH secretion also holds for earlier instars, then the gate for the third instar occurs at essentially the same time of day. As indicated above, the PTTH gate for the second stage also opens at the same time, but it closes later. Thus, the gate width in the second instar is about 14 h.

Although the position and width of the gate are relatively temperature independent, the percentage of the population which exploit a given gate is temperature dependent. At 25 °C the percentages initiating the moult on the second and third nights after the L_3–L_4 ecdysis are approximately 30 and 70%, respectively. At 28 °C the percentages have changed to 85 and 15%. Indeed, one would expect that any variable which affects the rate of larval growth such as temperature, food quality, etc., would influence which gate was used by the larva.

C. *Temporal organization of the release of hormones by the brain, corpora allata and prothoracic glands*

The prothoracicotropic hormone acts on the prothoracic glands to promote the secretion of the insect growth hormone, ecdysone (Williams, 1952). In *Manduca* larvae this tropic action of PTTH enforces a timed release of ecdysone from the prothoracic glands. But unlike the case of *Hyalophora cecropia* (Williams, 1952), the



Text-fig. 10. A schematic summary of the timing of the endocrine events which are required for the moult to the fifth instar. Based on median times obtained from second-gate larvae.

larval prothoracic glands of *Manduca* are not completely inactive in the absence of the brain because brainless fifth-instar larvae can undergo a pupal moult (K. Judy, personal communication). Also, a ligature applied to the neck of a mid-fifth-stage larva usually does not prevent pupation (J. W. Truman, unpublished; H. F. Nijhout, personal communication). But it was noted that at least 2 weeks were required by these preparations to undergo pupal 'ecdysis' as compared to the one week normally needed for pupation. Similarly, some fourth-instar larvae which were ligatured before the time of the PTTH gate also pupated, but again only after 12–14 days. This delayed pupation due to 'leaky prothoracic glands' is therefore readily distinguishable by its time course from those precocious pupations observed when the prothoracic glands are driven by the tropic action of the brain.

As summarized in Text-fig. 10, the function of the prothoracic glands is completed within 6 h after the tropic influence of the brain is no longer necessary. During at least the last 2·5 h of this period the prothoracic glands are actively secreting ecdysone. As in *Bombyx* (Fukuda, 1944), isolated larval abdomens of *Manduca* sometimes show a mosaic moult, and thus are covered with a patchwork of old and new cuticle. Apparently the epidermal structures become competent to respond to ecdysone at different times after the beginning of ecdysone secretion. In *Manduca* this competence begins with the crochets and then spreads to the prolegs, the ventral midline, the intersegmental areas and, finally, the remainder of the abdomen.

The studies of Wigglesworth (1934) on *Rhodnius* and of Fukuda (1944) on *Bombyx* clearly showed that PTTH release must precede JH secretion. However, Fukuda also reported that when fourth-stage *Bombyx* larvae were ligatured posterior to the prothorax, pupal characters were occasionally found on the abdomen. This would indicate that significant titres of ecdysone are present before the appearance of JH. In a total

of more than 200 isolated abdomens prepared from fourth-stage *Manduca* larvae no trace of pupal characters was observed.

At the time of PTTH release the circulating titre of JH is too low to oppose pupal development. By 1·5 h later the blood concentration is high enough to assure complete larval development. An animal showing a mixture of larval and pupal characters presumably results from the application of a neck ligature during the time when the blood titre is at an intermediate concentration. The percentage of larval–pupal intermediates produced after such treatment thus provides a quantitative estimate of the rate of JH secretion. The rarity of these animals (approximately 10% of the pupae showed larval characters) indicates that this transition occurs very rapidly within a span of less than 15 min. Thus, JH is apparently released in a rapid, pulsed fashion.

An indication of the length of time that the hormone released during the JH 'surge' persists in the animal is obtained from the larval–pupal intermediates. For this consideration it is important to note that the frequency of retention of certain larval structures in the intermediates paralleled the order in which these structures became competent to produce fifth-instar cuticle (as shown in the isolated abdomens discussed above). Thus, all of the intermediates had crochets, most retained prolegs, some had larval cuticle in the intersegmental areas, and one had pupal cuticle only in small patches lying in the dorsal and lateral area of each segment.

This similarity is most simply explained by assuming that epidermal structures become insensitive to JH after they have achieved competence to moult to the next instar. Indeed, in *Galleria* the larval epidermis becomes unresponsive to JH once the cells have replicated their DNA (Sehnal, 1969; Schneiderman *et al.* 1969), a manifestation of ecdysone action. This reasoning would then suggest that in order for a *Manduca* larva ligatured at hour 1·5 to form a perfect fifth-stage caterpillar, the JH liberated prior to the time of ligation must persist until the last epidermal structure has attained competence (hour 6). Therefore, the hormone liberated during the JH surge must be present in physiological concentrations through at least the next 4·5 h.

SUMMARY

1. The larval ecdyses of the tobacco hornworm, *Manduca*, and of the oak silkmoth, *Antheraea*, occur during specific times of the day. These times are instar-specific and species-specific and depend upon the photoperiod and temperature conditions.

2. The larval ocelli are not required for the synchronization of larval ecdysis with the photoperiod.

3. The synchrony observed in larval ecdysis does not arise from a gating of the ecdysial event *per se* by a biological clock. Rather, it is an outcome of the gating of the endocrine events which initiate the moulting process.

4. Application of neck ligatures to fourth-instar hornworm larvae at various times of day showed that prothoracicotropic hormone (PTTH) secretion occurred only during a gate in the early to middle portion of the night. The times of the gates for second- and third-instar larvae were similar.

5. When neck ligatures were applied to fourth-instar larvae within the first 1·5 h after PTTH release, the larvae underwent precocious metamorphosis rather than moulting to the fifth instar. A consideration of the number of larval–pupal interme-

diates produced indicated that within a span of 15 min sufficient JH is liberated from the corpora allata to ensure full larval differentiation.

6. Larval abdomens which were isolated during the period from 3 to 6 h after PTTH release underwent a mosaic larval moult – i.e. they became a patchwork of new and old cuticle. Thus, the epidermal structures of the abdomen vary as to the time that each becomes independent of the prothoracic glands. The first structures which continue fifth-instar differentiation in the absence of the prothoracic glands are the crochets. These are then followed in order by the prolegs, the ventral midline, the intersegmental areas, and, by 6 h after PTTH release, the remainder of the abdomen. During this 3 h period the prothoracic glands are presumably secreting ecdysone.

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EXPLANATION OF PLATE 1

Fig. 1. Right: a normal pupa of *Manduca*; left: a miniature pupa which was produced by application of a neck ligature to a fourth instar larva during the second gate of PTTH secretion.

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(*Facing p.* 820)