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THE PHYSIOLOGY OF WANDERING BEHAVIOUR IN *MANDUCA SEXTA*
II. THE ENDOCRINE CONTROL OF WANDERING BEHAVIOUR

BY OLIVER S. DOMINICK* AND JAMES W. TRUMAN
Department of Zoology, University of Washington, Seattle, WA 98195, U.S.A.

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SUMMARY

1. Removal of the prothoracic glands early during the 5th instar of *Manduca sexta* prevented the larvae from wandering and from further development. Infusion of 20-hydroxyecdysone (20-HE) into these larvae induced wandering behaviour.

2. In intact larvae, induction of precocious wandering behaviour required a 20-HE infusion lasting longer than 5 h. Infused 20-HE induced maximal response (90 %) when delivered at a rate of $0{\cdot}06\,\mu g\,g^{-1}$ body weight h^{-1}. At considerably higher concentrations ($0.25\,\mu g\,g^{-1}\,h^{-1}$ larvae performed brief, erratic behaviour or omitted wandering entirely.

3. The latency between appearance of 20-HE and the onset of wandering was dose-dependent with a minimum of 11 h following infusion at $0{\cdot}1\,\mu g\,g^{-1}\,h^{-1}$. Latency was not affected by the duration of 20-HE infusion.

4. The duration of induced wandering behaviour was proportional to the duration of 20-HE infusion. Minimal wandering behaviour lasted 2 h following 20-HE infusions at 5 h, while infusions lasting 11 h induced 9 h of wandering behaviour. Several lines of evidence suggest that the effects of 20-HE accumulate over time and directly determine the duration of wandering behaviour.

5. Many larvae exhibited a series of temporally distinct locomotor periods following various 20-HE infusion protocols, suggesting that a series of separate exposures to 20-HE can result in corresponding serial bouts of locomotion.

6. Responsiveness to 20-HE appeared to be principally modulated by juvenile hormone. Allatectomy of 2nd, 3rd and 4th instar larvae removed juvenile hormone (JH) precociously from these stages and was followed several days later by precocious wandering behaviour. Likewise, application of the JH mimic, EGS, prior to 20-HE exposure or at the start of it, could prevent the behavioural induction. During the 5th instar, 20-HE became increasingly effective in inducing wandering as larvae grew larger than 5 g, the size at which JH normally begins to disappear from the haemolymph. Allatectomized 5th instar larvae responded directly to 20-HE a day sooner than larvae with normal JH titres, but before day 2 the effects of 20-HE on wandering behaviour appear to be indirect, requiring a latency greater than

* Present address: Department of Entomology, Caldwell Hall, Cornell University, Ithaca, New York 14853, U.S.A.

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24 h. Several processes, of which the elimination of JH is the last, appear to be required before 20-HE can induce wandering behaviour.

7. Throughout the entire period when 20-HE was acting to induce wandering, the appearance of JH could prevent further induction of behaviour, but the effects of 20-HE which had already been established up to that time were expressed. Thus, application of EGS progressively later during the interval of 20-HE action was progressively less effective in reducing the length of wandering behaviour. The presence of EGS during wandering had no behavioural effect, indicating that only the induction of behaviour by 20-HE is sensitive to modulation by JH.

INTRODUCTION

In many animals steroid hormones have dual actions on the nervous system, and on non-neural tissues, such that changes in behaviour are coordinated with other physiological changes. This is well documented by numerous studies of modification of vertebrate reproductive behaviour by steroids in conjunction with the regulation of gonadal function (Davidson & Levine, 1972; Pfaff, 1980). Steroids also play a fundamental role in regulating invertebrate development (Highnam & Hill, 1977) but their roles in modulating invertebrate behaviour have not been examined closely.

Previous studies of the endocrine control of insect development suggest that the specialized larval behaviour typically preceding pupation might be released specifically by the hormonal environment which induces pupation. During the day prior to wandering in the tobacco hornworm, *Manduca sexta*, the titre of the steroid moulting hormone, 20-hydroxyecdysone (20-HE), rises (Bollenbacher, Vedeckis & Gilbert, 1975) in the absence of juvenile hormone (JH) (Fain & Riddiford, 1975). This unique endocrine milieu induces the characteristic premetamorphic changes in pigmentation (Nijhout, 1976; Hori & Riddiford, 1982) and cuticular commitment (Riddiford, 1978) of the epidermis. Administration of ecdysteroids to a larva of *Hyalophora cecropia* (Lounibos, 1976) and to larvae of *Ephestia* (Giebultowicz, Zdarek & Chroscikowska, 1980) have induced cocoon spinning, suggesting that ecdysteroids may regulate this behaviour.

In addition, several lines of evidence indicate that JH plays an important role in regulating premetamorphic behaviour. Application of JH causes aberrant cocoon spinning in caterpillars of *Hyalophora cecropia* (Riddiford, 1972), *Galleria* (Piepho, 1950) and *Pieris* (Benz, 1973). Furthermore, removal of the corpora allata (CA) from early instar larvae of the silkmoth, *Bombyx*, or the waxmoth, *Galleria*, causes precocious metamorphosis preceded by the construction of a complete, but miniature cocoon (Bounhiol, 1936; Piepho, 1938, 1939).

In this paper we have used *Manduca sexta* to examine the endocrine control of wandering behaviour, a period of continuous locomotion which accompanies the larva's developmental preparation for pupation. During wandering, the larva abandons its feeding habitat, burrows into the soil and constructs a pupation chamber underground (Dominick & Truman, 1984).

MATERIALS AND METHODS

Experimental animals

Larvae of the tobacco hornworm, *Manduca sexta*, were individually raised in the laboratory on an artificial diet as previously described (Truman 1972; Bel & Joachim, 1976) at 26 °C under a short day photoperiod (12L:12D). Lights off was arbitrarily designated as midnight (00.00). The locomotor activity of wandering behaviour in 5th instar larvae was monitored as previously described (Dominick & Truman, 1984) by placing individual larvae in tilt-sensitive actographs in which locomotion was recorded with an Esterline Angus chart recorder. The wandering behaviour of 3rd and 4th instar larvae was recorded with lightweight tilting actographs constructed from thin acetate sheets.

The temporal interval of the gate in which particular larvae would release prothoracicotropic hormone (PTTH) could be predicted on the basis of their weights during the preceding days of the 5th instar (Nijhout & Williams, 1974*a*). In the experiments reported here, larvae weighing more than 7·0 g at 17.00 on day 2 routinely released PTTH during the gate on day 3 (Gate I larvae), while those weighing 7·5–8·8 g on day 3 released PTTH during the gate on day 4 (Gate II). With animals younger than day 2 the errors in predicting the gate increased, but within a group of larvae which entered the 5th instar synchronously those larvae with weights in the top 20 % of a group were typically Gate I, while the smallest 20 % were usually Gate II.

Surgical procedures

The corpora allata (CA) were removed from 2nd, 3rd and 4th instar larvae which were anaesthetized by immersion in water for about 20 min, according to the method described by Kiguchi & Riddiford (1978). The head was flexed back under a thin piece of plastic and the neck membrane pierced laterally with sharpened no. 5 forceps. Each CA was removed from among the tracheae at the ventral lateral regions of the neck. Following allatectomy, the larvae were returned to their individual cups to feed.

The prothoracic glands were removed on day 1, through an incision slightly dorsal and posterior to the prothoracic spiracle from CO_2-anaesthetized 5th instar larvae which were immersed in saline (Ephrussi & Beadle, 1936). The clustered glandular tissue was found branching over the tracheae, and was carefully separated from its numerous fine tracheal attachments with no. 5 forceps. Particular care was needed to liberate the long branch which projects into the head region without breaking off the terminal lobe of that branch. The incision was subsequently closed with 6–0 gauge braided silk sutures, and the larvae were then returned to their feeding cups.

Ecdysteroid treatments

20-hydroxyecdysone (20-HE) (Rhoto Pharmaceutical, Osaka, Japan) was dissolved in *Manduca* saline (Cherbas, 1973) and concentrations were confirmed spectrophotometrically (E_{240}=12 670; Meltzer, 1971). Larvae were anaesthetized with CO_2 and either received an injection of 20-HE into an abdominal proleg or were infused with the 20-HE solution *via* a long polyethylene catheter (PE-10; Clay Adams)

inserted into the head capsule and stabilized with Tackiwax (Cenco). A 50-μl Hamilton syringe mounted on a syringe pump (Sage Instruments, model 355) was used to deliver the 20-HE solution through the tubing. Solutions were usually infused at a rate of 5 $\mu l\,h^{-1}$, and 20-HE concentration was varied accordingly. Larvae recovered from anaesthesia and remained in their feeding cups during the infusion and were subsequently placed in the actographs without food.

EGS treatment

The JH mimic, 6,7-epoxygeranyl-3′,4′–methylene dioxyphenyl ether (epoxygeranylsesamole, EGS) (Bowers, 1969) (Insert Control, Inc.) was dissolved in cyclohexane (Baker, Inc.) and 10 $\mu g\,\mu l^{-1}$ was applied to the dorsal intersegmental region of unanaesthetized larvae. Mosaic pupae produced by this treatment were scored using the method of Truman, Riddiford & Safranck (1974) in which 0 represents normal pupation and 5 indicates almost completely larval cuticle.

RESULTS

The role of ecdysteroids

Effects of prothoracic gland removal

Prothoracic glands (PTG) were completely removed from four larvae on day 0 of the 5th instar. These larvae grew slowly and continuously to a maximum weight of 15–18 g over the next 12 days, whereas sham-operated larvae wandered by day 6 and pupated by day 10. The PTG– larvae survived for nearly a month without ever showing wandering behaviour, the morphological changes associated with this behaviour, or any sign of pupation.

Sham-operated larvae wandered as usual, although the onset of wandering was often delayed by 1 or 2 days. Of 10 larvae in which removal of the PTG was unsuccessful (as determined by the presence of remnant PTG fragments as small as four cells during subsequent dissection) nine wandered in the gate following attainment of 7–9 g. Most of these wandered by the second gate (day 5) but three larvae grew unusually slowly following surgery and delayed wandering as late as day 8 (Gate V). Therefore, in subsequent experiments requiring PTG– larvae we used only those which reached 7–9 g by day 4 and which failed to wander by Gate III (day 6).

One such larva from which prothoracic glands had been completely removed on day 0 was infused with 50μg of 20-HE (1 $\mu g\,\mu l^{-1}$ at 5 $\mu l\,h^{-1}$) over 10 h on day 6. This treatment resulted in appearance of the dorsal vessel and a 6 h period of wandering beginning 13 h after the start of infusion. This larva never retracted its ocellar pigment or pupated, and, until it died several weeks later, it showed sustained locomotor activity lasting 1–2 h whenever disturbed.

In further experiments, larvae were allatectomized following PTTH release in the 4th instar so that, following removal of their prothoracic glands on day 0 of the 5th instar, there would be no post-surgical, JH-mediated effects on ecdysteroid sensitivity. All unsuccessfully operated larvae wandered by day 6 as described above. Those that showed no wandering were subsequently infused with dosages of 20-HE varying from 5–50 μg over 10 h beginning at 17.00 on day 6 ($N=11$). A wandering response

Table 1. *Effect of 20-HE infusions on wandering behaviour in larvae lacking corpora allata and prothoracic glands*

Dose (μg)	*N*	Latency (h ± S.E.M.)	Wandering duration (h)
5	2	17	6·5
40	5	11 ± 0	5 ± 1
50	2	12·5 ± 0·5	6 ± 0

Infusions were for 10 h at a rate of 5 μl h^{-1} for 50 μl of 20-HE solutions containing 0·1 μg μl^{-1}, 0·8 μg μl^{-1} or 1·0 μg μl^{-1}.
Latency is relative to start of infusion.

was induced in 80 % of the larvae (Table 1). With a relatively small 20-HE dose (5 μg delivered as 0·1 μg μl^{-1} at 5μl h^{-1} over 10 h) the latency between the start of infusion and behavioural onset was longer than that seen after the larger dosages (40–50 μg). Wandering duration in these larvae was 5-6 h regardless of 20-HE dose.

Parameters of ecdysteroid exposure

The effect of prothoracic gland removal clearly implicated ecdysteroids in the induction of wandering behaviour. The role of ecdysteroids was further explored by treating larvae with intact PTG with exogenous 20-HE on the day prior to PTTH and ecdysteroid secretion. Initial experiments involved injecting Gate II larvae with varying amounts of 20-HE (5–100 μg) at 20.00 on day 3. As had been noted earlier by Nijhout (1976), a single injection proved to be a poor mode of delivery for ecdysteroids. Only one larva out of 39 showed early wandering after such treatment. The remainder wandered at their expected time or were delayed for up to 1 day.

To imitate the prolonged exposure to 20-HE which occurs during endogenous hormone secretion (Bollenbacher *et al.* 1975), presumptive Gate II larvae were

Table 2. *The effects of infusing different dosages of 20-HE into presumptive gate II larvae*

Dose (μg)	*N*	% Early	Latency (h ± S.E.)	Duration of induced wandering (h ± S.E.)	% Repeat wandering	Duration of repeat wandering (h ± S.E.)
0*	10	0	(36 ± 2)	(20 ± 4)	0	0
0·30	10	10	14	4	100	16
0·75	8	0	–	0	0	0
1·5	10	70	20 ± 1	6 ± 1	71	7 ± 2
3·5	8	88	15 ± 1	9 ± 2	71	4 ± 1
7·0	8	88	18 ± 2	5 ± 1	89	9 ± 2
15	9	66	14 ± 1	7 ± 1	66	4 ± 1
25	10	90	13 ± 1	7 ± 1	89	9 ± 2
50	21	52	12 ± 1	3 ± 1	40	8 ± 3
100	8	63	11 ± 1	4 ± 1	20	3 ± 1

Solutions were infused over 10 h starting at 19.00 on day 3 using 20-HE concentrations to give the total dose indicated.
* Saline-infused controls with latency and duration for normal wandering in parentheses.

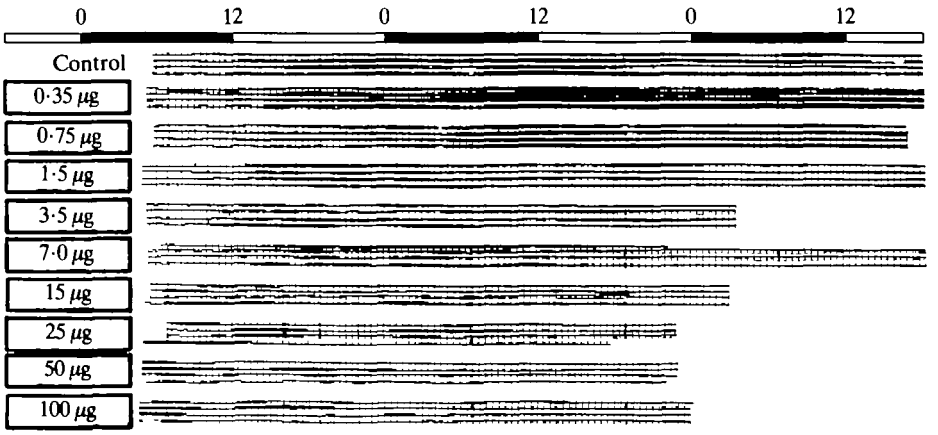

Fig. 1. Activity records of larvae infused with different dosages of 20-HE. Presumptive Gate II larvae were infused with varying dosages of 20-HE (from 0·35–100 μg, as marked) over a 10-h interval (delimited by box surrounding 20-HE dosage) beginning at 19.00 on day 3. For each treatment, the original tilt-dish activity records of four larvae showing typical responses to the hormone are shown. The 12L : 12D photoperiod is shown by the light and dark bars at the top of the figure.

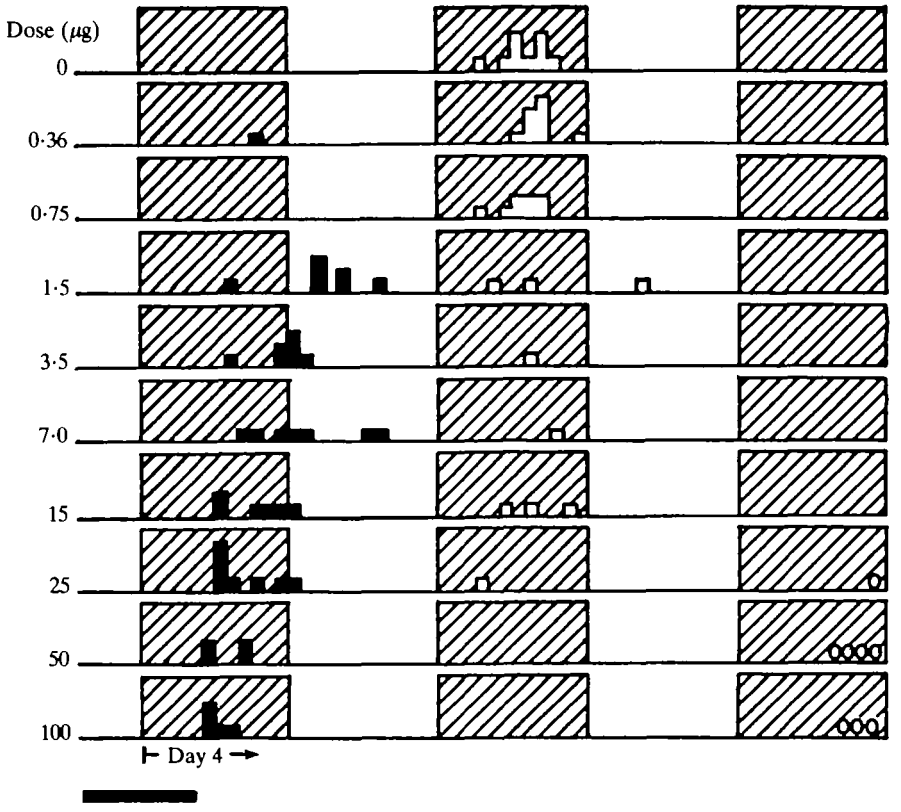

Fig. 2. Onset times of wandering in response to infusions of differing dosages of 20-HE. Data are from the experiment described in Fig. 1. The black bar represents the interval during which 20-HE was infused for each group of larvae. Histograms designate the starting times for wandering behaviour after each treatment. Onset times which are equivalent to Gate II controls are shown as open squares, and advanced onset times are shown as black squares. Larvae which failed to wander are indicated by an open circle to the right. The 12L : 12D photoperiod is represented by the light and cross-hatched background.

infused with varying amounts of 20-HE over a 9-h period beginning at 19.00 on day 3 (Table 2) to ensure that exogenous 20-HE appeared well before the time of endogenous ecdysteroid secretion. Animals infused with less than 1·5 μg of 20-HE were rarely observed to wander earlier than their expected gate (Figs 1, 2). With infusions of 1·5 μg or more, wandering began at progressively earlier times as the hormone dose increased, but even with extremely large doses (up to 100 μg), the minimal latency between the beginning of the infusion and behavioural onset was 11 h (Fig. 2). Indeed, at the larger doses, wandering was often delayed by 1 day or omitted. The duration of induced wandering varied from 3–9 h, independent of dose (Fig. 1; Table 2).

As seen in Fig. 3 there was a clear dosage optimum for the induction of wandering locomotion by 20-HE. In Gate II larvae the incidence of early wandering rose from 10 % to about 90 % as the infused 20-HE dose increased from 0 to 5 μg. Presumptive Gate I larvae showed essentially the same dose-response relationship but the proportion responding at each dose was 20–30 % lower (Fig. 3). At higher doses a gradually increasing number of animals omitted the behaviour, in spite of the appearance of the dorsal vessel, such that at 50 μg and above, between 40 % and 50 % of the infused larvae failed ever to show wandering locomotion.

In the ecdysteroid-infused larvae the dorsal vessel appeared prematurely (Fig. 4) as previously reported by Nijhout (1976). Interestingly, the induction of wandering

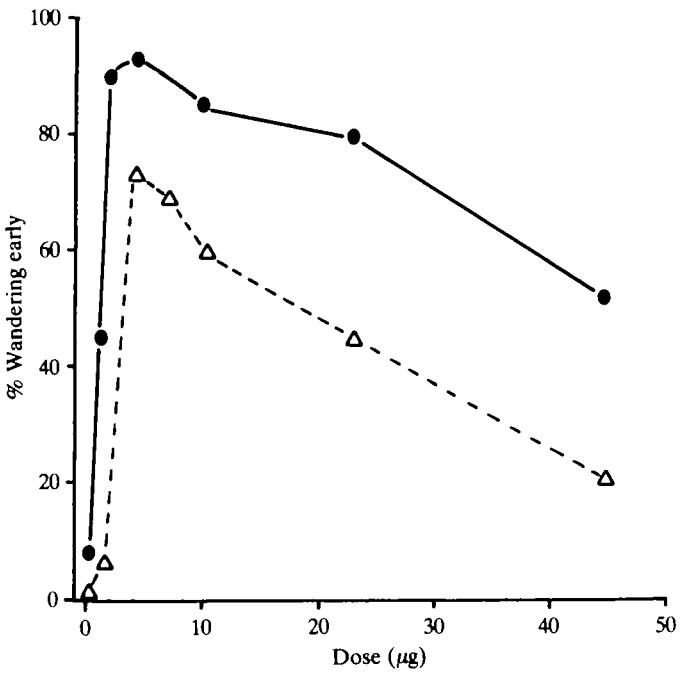

Fig. 3. The effectiveness of 20-HE infusions for inducing wandering behaviour as a function of hormone dose. After infusion with different dosages of 20-HE as described for Fig. 1, the proportion of Gate II larvae which began wandering earlier than the normal gate was noted (filled circles). In a parallel experiment, presumptive Gate I larvae were infused with 20-HE on day 2, and wandering response was observed (open triangles). The value of each point is based on 10–40 larvae.

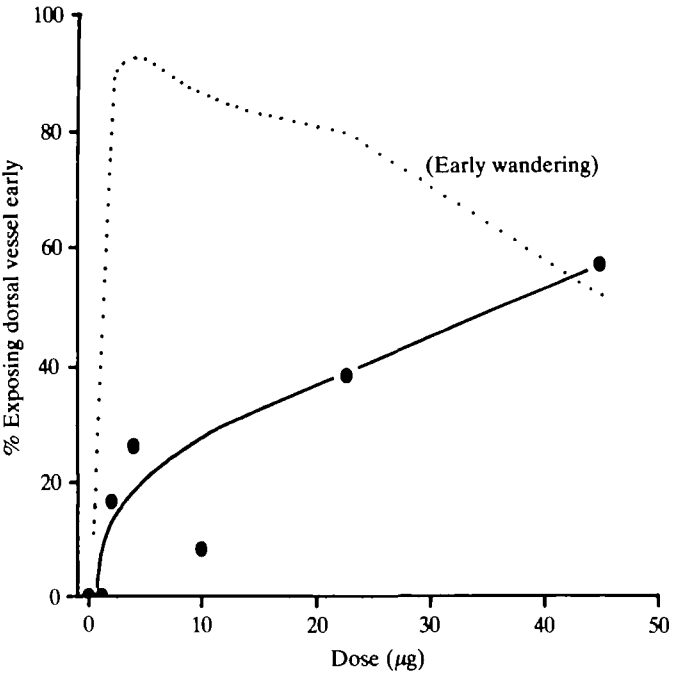

Fig. 4. The effect of infused 20-HE on heart exposure. The time when the heart was exposed following 20-HE infusion was noted for the Gate II larvae described in Fig. 1 ($N = 10$–40 per point). For each dose, the solid circles indicate the proportion of 20-HE infused larvae exposing their hearts earlier than usual. For comparison, the dotted line reiterates the behavioural response (Fig. 3) of these larvae at these dosages.

behaviour was more sensitive to the infused 20-HE than were the epidermal changes. Thus, the behavioural responses to 20-HE can occur in the absence of obvious peripheral changes that normally occur at the same time.

The relationship between dose and the apparent requirement for prolonged exposure to the hormone was examined by varying dosage and duration of 20-HE infusion over a wide range. Fig. 5 summarizes the results of an experiment in which Gate II larvae received a total dosage of 5 μg of 20-HE (as a 0·1 mg ml^{-1} solution) infused over time periods which varied from a single instantaneous injection to 10 h. The effectiveness of the 5 μg dosage in inducing early wandering increased abruptly as the length of exposure to hormone exceeded 4 h with 54 % of the larvae wandering in response to a 5-h infusion, and the maximal response of 80 % occurring after 8–10 h of infusion. The results (Figs 3–5; Table 3) indicate that the maximal behavioural effectiveness of 20-HE occurred under relatively limited conditions of low hormone dose (2–15 μg) and long infusion time (8–10 h).

Effects of 20-hydroxyecdysone on wandering duration

Possible relationships between the amount of ecdysteroid infused and the duration of wandering behaviour are complicated by the existence of overdosage effects which result in the omission of wandering behaviour. Consequently we examined temporal aspects of ecdysteroid exposure on the behaviour of the animal by infusing low dosages of 20-HE (0·5 μg h^{-1}) for varying amounts of time beginning at 19.00 on day 3.

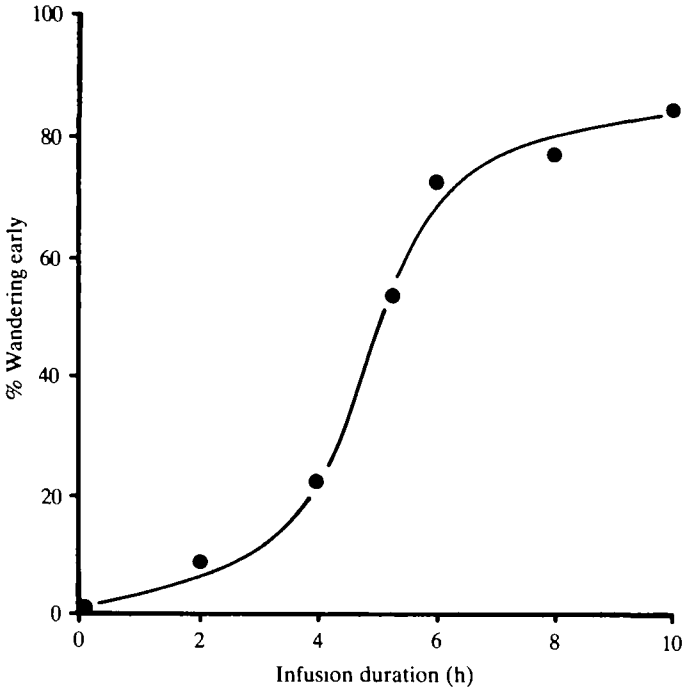

Fig. 5. The length of 20-HE infusion needed to induce wandering behaviour. A total dose of 5 μg of 20-HE was infused into presumptive Gate II larvae over a 0-(single injection) to 10-h period, beginning at 20.00 on day 3. The proportion of larvae wandering earlier than saline-infused controls is plotted for each infusion duration (N = 10–40 animals per point).

Infusions lasting 3 h or less were ineffective in inducing early wandering (Figs 6, 7).

As the length of the infusion increased to 5 h or more, precocious wandering appeared in increasing numbers of larvae. Importantly, the interval between the start of the infusion and behavioural onset was constant regardless of infusion duration, averaging 14±1 h (N=53), indicating that the initiation of wandering was not accelerated by increasing the duration of 20-HE exposure.

The systematic lengthening of the time of 20-HE infusion caused a proportional increase in the average duration of induced wandering behaviour, from a minimum

Table 3. *The effect of infusion of 20-HE for varying lengths of time*

Infusion duration (h)	Total dose (μg)	N	% Early	Latency (h ± S.E.M.)	Induced duration (h ± S.E.M.)	% Repeat	Repeat duration (h)
0(control)	0·0	25	0	(36 ± 2)	(20 ± 2)	0	0
1	0·5	7	0	–	–	–	–
3	1·5	19	0	–	–	–	–
5	2·5	16	50	16 ± 2	2 ± 0·5	100	9 ± 1
7	3·5	23	61	13 ± 1	5 ± 1	57	4 ± 1
9	4·5	26	73	13 ± 1	7 ± 1	63	9 ± 2
11	5·5	17	71	15 ± 1	9 ± 1	67	8 ± 2

Presumptive Gate II larvae were infused with 0·5 μg 20-HE h^{-1} beginning at 19.00 on day 3.

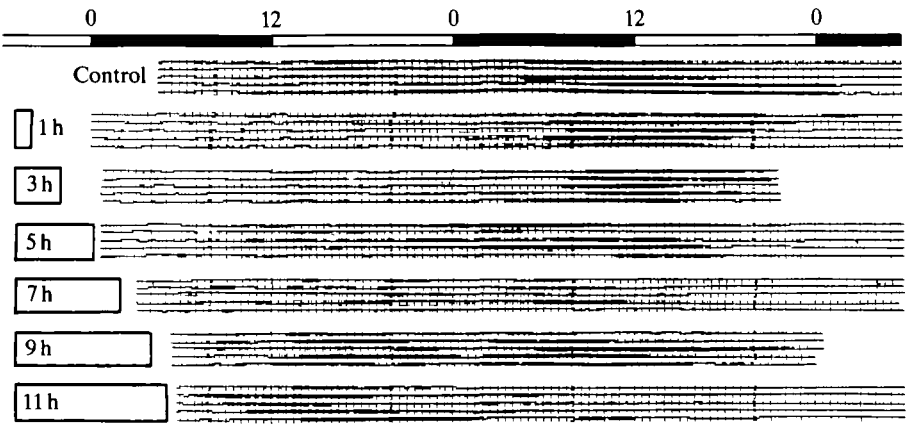

Fig. 6. Activity records of wandering behaviour induced by varying the length of infusion of a constant 20-HE concentration. Predicted Gate II larvae were infused with 20-HE at a rate of 0·5 μg h^{-1} for varying lengths of time from 1–11 h starting at 19.00 on day 3 (as indicated by the numbers and the white bars at the left side of the figure). Typical records from five larvae responding in each experimental group are shown. Records of uninfused control Gate II larvae are also shown. The 12L : 12D photoperiod is shown as light and dark bars at the top of the figure.

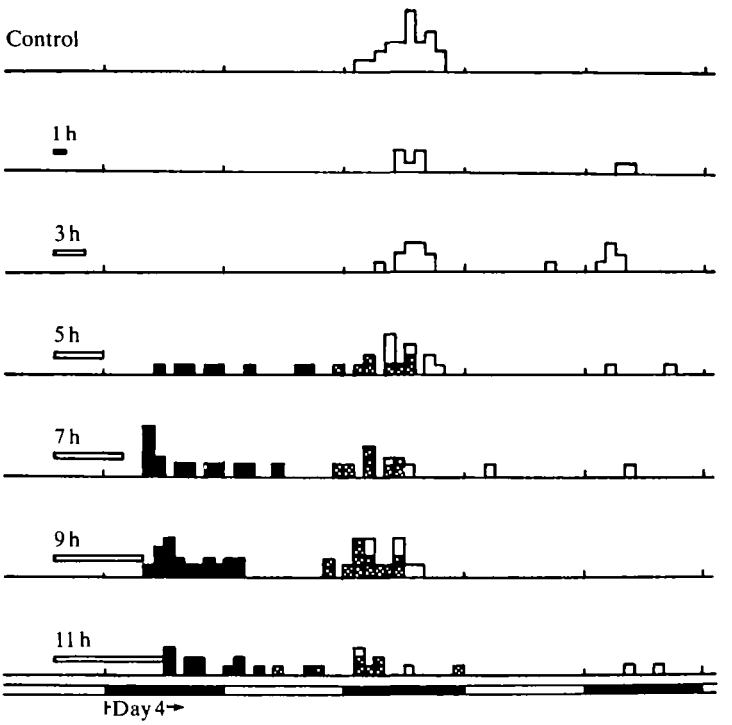

Fig. 7. Onset of wandering behaviour induced by 20-HE infusions of varying duration. Data represent the behaviour of all larvae infused in the experiment for which representative activity records were shown in Fig. 6. Each group of 20-HE infusions is illustrated as a thin white bar on the left side of the figure, and is labelled with the duration in hours. Black histograms represent onset times of wandering which were earlier than control Gate II times (which are shown as white histograms). The appearance of a second bout of wandering is shown as a cross-hatched histogram. The 12L : 12D photoperiod is shown below the figure.

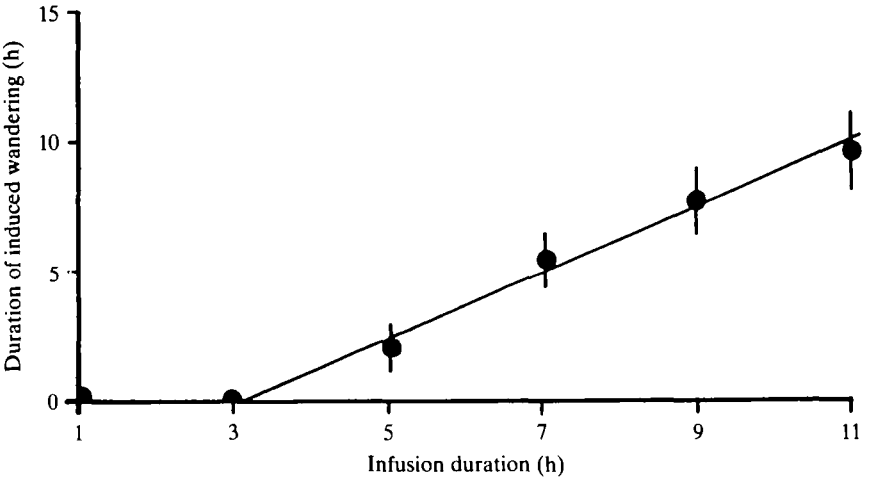

Fig. 8. The control of wandering duration by length of 20-HE infusion. The durations of early bouts of wandering behaviour induced by the 20-HE infusions of varying length (as described in Figs 6 and 7) were measured and plotted (mean ± S.E.) with reference to the length of the 20-HE infusion ($N = 7$–26 per point; see Table 3). The line calculated by linear regression ($y = 1{\cdot}2x + 3{\cdot}2$) represents a significant correlation ($r = 0{\cdot}9$; $P < 0{\cdot}01$).

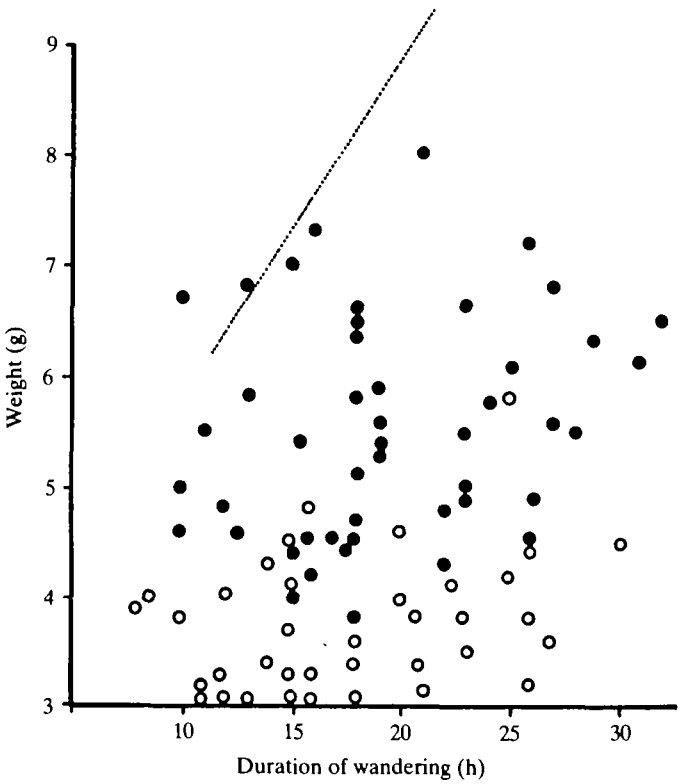

Fig. 9. Relationship between weight and wandering duration in allatectomized larvae. Weights were taken at 17.00 on day 1 and day 2 for Gate I (open circles) and Gate II (filled circles) larvae respectively. The correlation coefficient ($r = 0{\cdot}09$) is not significant. The dotted line indicates the regression line for Gate I and II larvae with intact CA (Dominick & Truman, 1984).

of 2 h in response to a 5-h infusion, to 9 h of locomotion after 11 h of 20-HE infusion (Fig. 8). Infusions longer than 11 h were impractical because wandering often started before the infusion was completed.

Following the period of early wandering induced by 20-HE, many of the larvae appeared to wander a second time, early in their normal wandering gate (Figs 1, 6; Table 2). The length of this second behavioural period was shorter than that of control animals, lasting less than 10 h, and bearing no relationship to the dose of hormone infused.

The role of juvenile hormone

Effects of removal of the corpora allata

A number of previous experiments indicate that the induction of cocoon spinning behaviour of various caterpillars is inhibited by JH secreted from the CA (Bounhiol, 1938; Piepho, 1939, 1950, 1960; Riddiford, 1972; Akai & Kobayashi, 1970; Benz, 1973). We initially examined the role of the CA in wandering of *Manduca* by removing these glands from 4th instar larvae on the day prior to ecdysis to the 5th instar (14–18 h after PTTH and ecdysone release). Under this protocol successful allatectomy was clearly indicated by melanization of the new 5th instar cuticle (Kiguchi & Riddiford, 1978). These larvae began wandering during gates on the third and fourth nights following ecdysis, 1 day earlier than the wandering gates for sham-operated larvae (Nijhout, 1975*b*; Kiguchi & Riddiford, 1978). As with the onset of wandering in sham-operated controls, the wandering gates for allatectomized larvae were about 8–9 h wide during the scotophase and showed a 'gating bias' (Pittendrigh & Skopik, 1970) such that larvae began wandering later during the first gate than they did during the second.

In contrast to the correlation between larval size and average wandering duration in normal larvae (Dominick & Truman, 1984) there was no correlation between larval size and the duration of wandering (average, 19·5 h) in allatectomized 5th instar larvae (Fig. 9). The observation supports the earlier suggestion (Dominick & Truman, 1984) that the correlation between larval size and wandering duration is based on JH effects.

Larvae were also allatectomized early in the 2nd, 3rd and 4th instars. These larvae wandered during a broad gate on the third scotophase following surgery for an average of 10 h (3rd instar) to 19 h (4th instar). Detailed data were not collected from the tiny 2nd instar larvae. These results indicate that wandering can be induced by the appearance of ecdysteroids in any larval instar following the elimination of JH.

Effects of exogenous juvenile hormone

The above results suggest that JH may inhibit the performance of wandering behaviour when early instars are exposed to ecdysteroids and it may modulate the expression of wandering behaviour by the last instar larvae. When the JH mimic, EGS, was applied to Gate II, 5th instar larvae at progressively later times up until about 05.00 during the scotophase of day 4, the wandering gate was delayed by 1–3 days (Fig. 10). Throughout this period, progressively later EGS applications became increasingly effective both in causing the retention of larval characteristics at pupation

Fig. 10. Effect of EGS on onset of wandering. EGS (10 μg) was applied topically to Gate II larvae at various times (arrows) during the day prior to wandering. Following EGS treatment, the onset times of wandering were recorded in tilt-dish actographs and are shown as black histograms. The starting times of a distinct second wandering period which occurred in many of these larvae are also shown (open histograms). The distribution of wandering onset times in untreated Gate II larvae is shown in the top panel. The cross-hatched areas represent scotophases, beginning with day 4.

(Truman *et al.* 1974; personal observations) and in reducing the duration of wandering in the delayed gates to as little as 1 h.

Between 05.00 and 06.30 there was an abrupt increase in the proportion of EGS-treated larvae which wandered in their expected gate, although the onset time within the gate was influenced by the treatment. Thus, the ability of EGS applications to postpone wandering declined sharply during this period, as did its effectiveness in causing the retention of larval features at pupation (Truman *et al.* 1974).

Fig. 10 also shows that in response to EGS applications over the 9-h period beginning at about 06.30, wandering in the normal gate was followed by a second gated period of wandering in about 25 % of the larvae. The onset times of the repeated behaviour usually coincided with the start of wandering for animals which had been delayed by 1 day. Furthermore, wandering behaviour in this gate was extremely short: 2 ± 1 h for repeats; 5 ± 2 h for delayed larvae.

Although treatment with EGS after 06.30 could no longer delay the onset of wandering, it still had a marked effect on the duration of the behaviour (Fig. 11). With application of EGS at 06.30, 63 % of the treated larvae wandered in their usual gate, but the average duration of locomotion was only 2 ± 1 h. As EGS was applied at progressively later times, the duration of wandering increased proportionately until 15–20 h later, at which time behavioural duration could no longer be affected by EGS. Thus, it appears that the duration of wandering behaviour is determined by the gradual accumulation of effects of a process sensitive to interference by JH over an approximately 20-h period on the day prior to wandering.

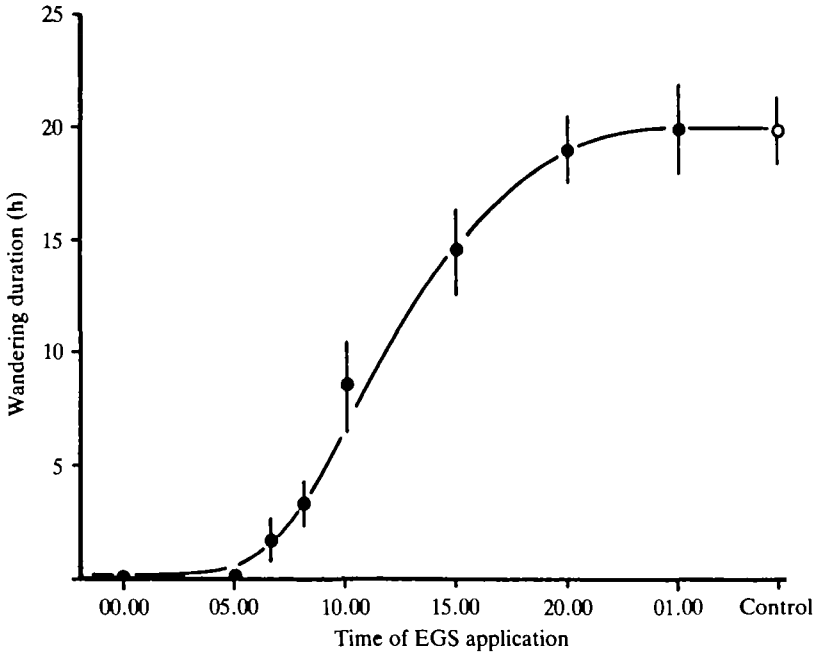

Fig. 11. Effect of EGS application on Gate II wandering duration. The duration (shown as mean ± S.E.) of wandering behaviour during the second gate (day 5) was measured in tilt-dish actographs following the EGS applications described in Fig. 10 ($N = 11–19$ per point).

Interaction between juvenile hormone and ecdysteroids

Effects of EGS application during 20-HE infusion

The above results show that appropriately timed applications of the JH analogue, EGS, during the period of endogenous 20-HE secretion, caused systematic changes in the duration of wandering. We examined whether this was due to the progressive interference with the 20-HE effects which normally lead to the 20 h long wandering behaviour. Gate II larvae were infused with 5 μg of 20-HE over 10 h starting at 00.00 on day 4, and 10 μg of EGS was administered either at the start of the infusion or 6 h later. As seen in Table 4, both EGS-treated groups of the larvae which wandered exhibited a duration of locomotion which was shorter than that seen when the 20-HE infusion was given alone. Those receiving EGS at the onset of ecdysteroid infusion

Table 4. *Effects of ECG treatment during 20-HE infusion into Gate II larvae*

EGS application time	*N*	% Early (*N*)	Induced duration (h ± S.E.M.)	Latency* (h ± S.E.M.)
00.00	29	38(11)	1·5 ± 0·5	15 ± 1
06.00	25	84(21)	5·5 ± 0·5	14 ± 0·5
none	20	85(17)	8·1 ± 1	14 ± 1

Gate II larvae were treated topically with 10 μg of EGS (1 μg EGS μl^{-1} cyclohexane) during 20-HE infusion (5 μg 20-HE over 10 h beginning at 00.00 on day 4).

* Latency is relative to start of 20-HE infusion.

only wandered for 2 h whereas those receiving EGS 6 h later spent 6 h wandering. Importantly, the time of EGS application did not alter the interval between the start of the infusion and the onset of induced wandering, which remained at 14–15 h.

In larvae receiving EGS during the period of endogenous or infused ecdysteroid action, wandering ended abruptly at about 16 h following the time of EGS application. The animals treated with EGS at the start of infusion showed a duration of wandering that was similar to larvae that had received 6 h of uninterrupted ecdysteroids. This relationship is consistent with the proposed 5-h equilibration period for topically applied EGS (Riddiford & Ajami, 1973).

The development of sensitivity to 20-HE

The effects of both allatectomy and EGS application on wandering behaviour are consistent with the idea that ecdysteroids must act in the absence of JH to induce wandering (Nijhout & Williams, 1974*b*; Truman *et al.* 1974). It was therefore important to determine when the 5th instar larvae acquired competence to wander in response to 20-HE, and to relate this to the normal fluctuation of the JH titre in larvae. When larvae were infused with 5 μg 20-HE over 10 h prior to 16.00 on day 2, wandering was not directly induced by 20-HE (Fig. 12). Many of these larvae (57 %) delayed

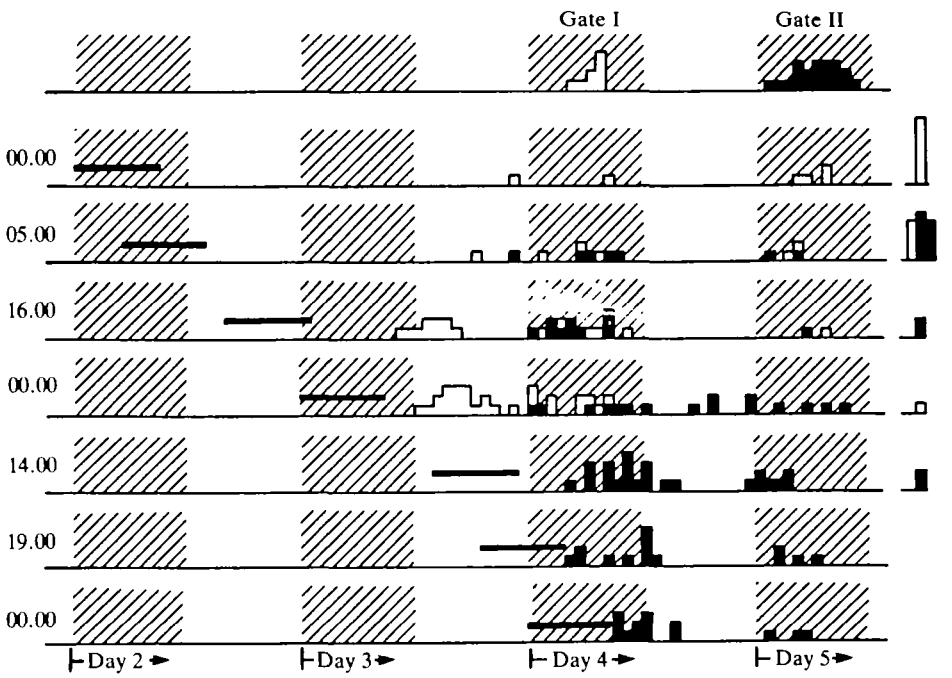

Fig. 12. The development of behavioural sensitivity to 20-HE during the 5th instar. Larvae were predicted to be either Gate I or Gate II by weight at different times during the 5th instar and were then infused with 20-HE (5 μg over 10 h) as indicated by the black bar to the left of each group. The subsequent wandering onset time was recorded in a tilt-dish actograph for each larva and is shown for predicted Gate I (white histograms) and Gate II (black histograms) larvae. The normal onset times for wandering in Gate I and Gate II larvae are shown at the top of the figure. Larvae wandering later than their normal gate are grouped to the right of each treatment.

wandering past the second gate as a result of starvation following the hormone treatment. By 16.00 on day 2, about 36 h before the first wandering gate was expected, infused 20-HE induced a clear wandering response in 67 % of the larger larvae with a mean latency of 25 ± 1 h. Most of the smaller, presumptive Gate II larvae were shifted to the first gate with a latency of more than 36 h from the start of the infusion. When 20-HE was infused into presumptive Gate I larvae starting at 00.00 on day 3 the response latency was further reduced to 16 ± 1 h. A similar pattern of declining latency associated with later infusions also occurred in the Gate II larvae. Therefore, once behavioural responsiveness to 20-HE had clearly appeared in one size class of larvae, later infusions decreased the interval between the start of infusion and the onset of behaviour from 20–25 h during the early stages of sensitivity, to 11–14 h when full sensitivity had been acquired.

The behavioural response to 20-HE was analysed in terms of larval size at the start of the infusion (Fig. 13). Prior to attaining a weight of 5·5 g, the onset time of wandering could not be advanced by 20-HE and was delayed by starvation following

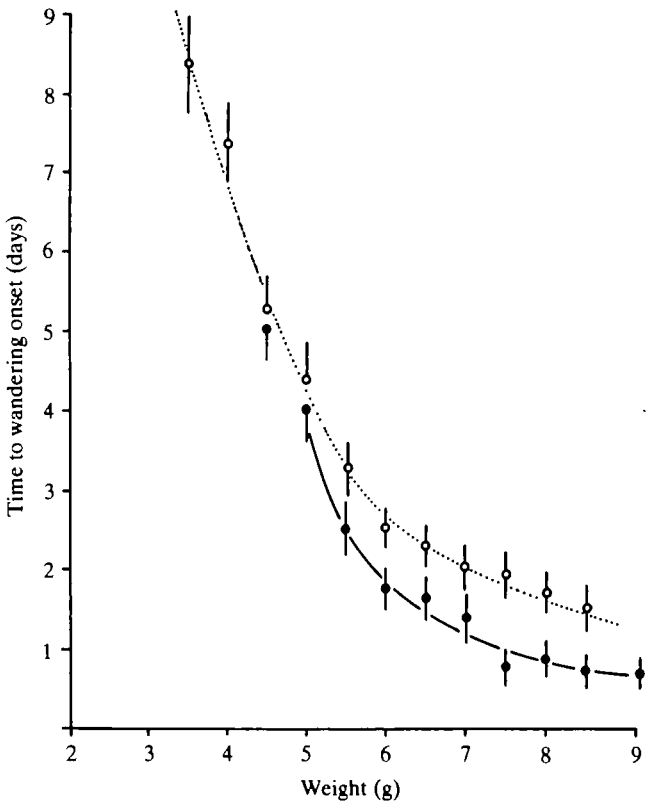

Fig. 13. Development of sensitivity to 20-HE as a function of larval weight during the 5th instar. Latencies to wandering were obtained from the results in Fig. 12 and were grouped according to weight at the time of 20-HE infusion. Open circles represent the latency (mean ± S.E., $N = 8–15$) to wandering of uninfused, starved control larvae; filled circles show the latency for larvae of each weight class ($N = 9–27$) following infusion with 20–HE (5 μg over 10 h).

20-HE infusion, as expected from the results of Nijhout & Williams (1974*a*). As larvae grew beyond 5·5 g, 20-HE infusion caused increasingly pronounced advancement of the behaviour relative to control larvae.

The appearance of sensitivity to 20-HE when the larvae attained a weight of 5·5 g supports the idea that the size-dependent decline of JH which begins at a critical weight of about 5 g (Nijhout & Williams, 1974*b*; Nijhout, 1975*a*) initiates competence for wandering in response to 20-HE. When allatectomized 5th instar larvae were infused with the standard dosage of 5 μg of 20-HE over 10 h, a slow but pronounced wandering response was evoked in 82% of the larvae by infused 20-HE as early as 14.00 on day 1, about 56 h prior to the expected wandering gate (Fig. 14). In these cases the latencies were exceptionally long, being 36 ± 4 h. Infusions before this time had a weaker, (but still significant) effect, in that larvae wandered in the normal gate 1 day earlier than fed, allatectomized controls. Such long latencies for response again suggest that very early 20-HE infusions had only an indirect effect on wandering even in allatectomized larvae. Later infusions required a shorter (13 h) latency, indicating direct induction of wandering by 20-HE.

The relationship between larval size and induction of wandering in these infused, allatectomized larvae is shown in Fig. 15. In the absence of JH, even 1·5-g larvae showed a behavioural response to 20-HE. Although the response had a very long latency (3·5 ± 0·5 days), these larvae wandered several days before the equivalent saline-infused controls. These exceptionally long latencies suggest that early 20-HE treatment might be acting indirectly by altering the normal timing mechanism for wandering rather than directly inducing the behaviour. Infusion of a much larger dose

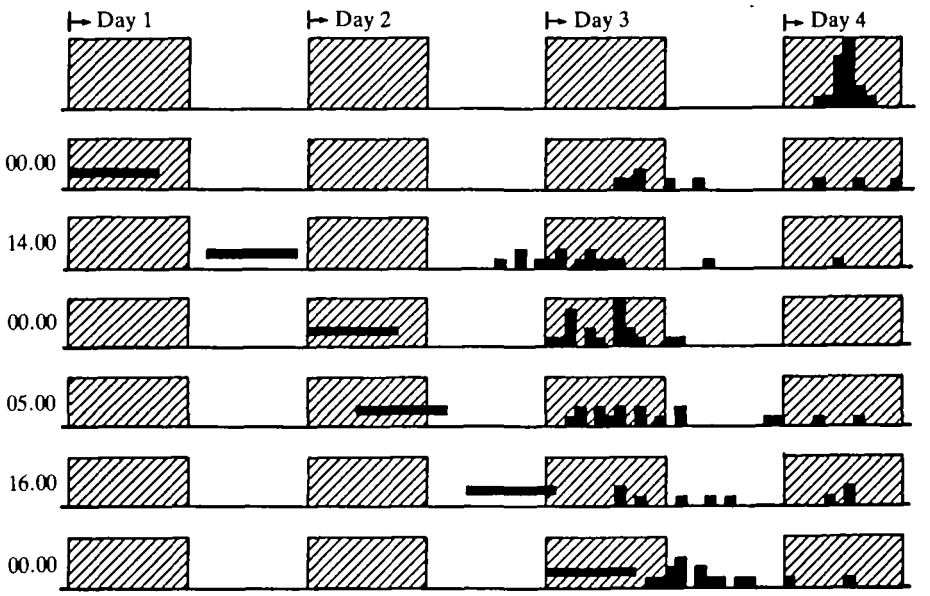

Fig. 14. Development of behavioural sensitivity to 20-HE in allatectomized larvae during the 5th instar. The time of wandering onset was recorded in tilt-dish actographs following 20-HE infusion (5 μg over 10 h) (black bars) into allatectomized larvae at various times during the 5th instar. The onset time for uninfused, allatectomized larvae is shown as a histogram at the top of the figure.

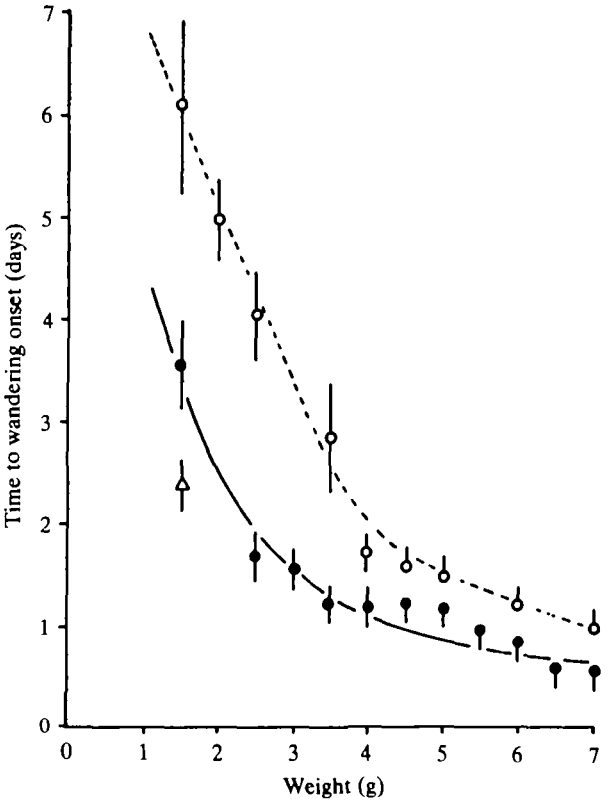

Fig. 15. Development of sensitivity to 20-HE as a function of weight during the 5th instar in allatectomized larvae. The latencies to wandering behaviour were obtained with respect to weight from the results shown in Fig. 15. Open circles show latency (mean ± S.E.) for uninfused, allatectomized controls ($N = 6$–12). Filled circles show the latency to wandering in each weight class following 20-HE infusion ($N = 9$–17). The open triangle represents the latency to wandering in response to a 20-HE infusion of 40 μg over 10 h ($N = 6$).

of 20-HE (40 μg) at this time resulted in a substantial reduction of the latency to 2·5 ± 0·2 days. As larvae grew past 1·5 g the latency following infusion of 5 μg over 10 h decreased rapidly, but it remained in excess of 24 h until, in 5- to 6-g larvae, the latency decreased to a minimum of about 15 h.

DISCUSSION

The role of ecdysteroids

Ecdysteroids have been implicated in the premetamorphic behaviour of the giant silkworm, *Samia cynthia* (Fujishita, Ohnishi & Ishizaki, 1982), *Hyalophora cecropia* (Lounibos, 1976) and *Ephestia kuhniella* (Giebultwicz *et al.* 1980). The results reported in this paper show that the wandering behaviour of *Manduca* larvae is induced by 20–HE. The requirement for ecdysteroids was shown by removing the prothoracic glands, the source of ecdysone, from larvae. This prevented the appearance of wandering behaviour as well as all other developmental changes known to require ecdysteroids. When these larvae were infused with 20-HE, wandering was induced,

followed by a state of quiescence from which bouts of locomotion could be briefly re-evoked by disturbance. This post-wandering excitability represented a distinct behavioural state of the larvae, persisting until the larvae died several weeks later without undergoing any further development.

The effectiveness of 20-HE in activating wandering was a function of the dose and duration of the hormone treatment as well as of the JH environment. Prolonging the exposure to 20-HE by multiple injections or continuous infusion has previously been shown to enhance the effect of this hormone compared with single injections (Ohtaki, Milkman & Williams, 1968; Zdarek & Fraenkel, 1970; Nijhout, 1976). The observation is particularly pertinent to the induction of wandering behaviour, where even large amounts (up to 100 μg) of injected 20-HE were unsuccessful in causing wandering. Prolonged exposure to 20-HE was necessary to induce a behavioural response. The maximal effectiveness of this treatment was reached with 9- to 10-h infusions of the steroid. The most effective infusion rate was 0.5 $\mu g\,h^{-1}$ or 0·06 $\mu g\,g^{-1}$ body weight h^{-1}. This rate of 20-HE delivery is consistent with the levels of ecdysteroid that are normally seen preceding wandering, ie. 0·07 $\mu g\,ml^{-1}$ (Wielgus, Bollenbacher & Gilbert, 1979).

Dosages greater than the optimal amount resulted in increasingly disrupted behaviour and at 5 $\mu g\,h^{-1}$ about 50 % of the larvae omitted wandering entirely (Fig. 3). In the silkmoth, *Samia cynthia*, large amounts of 20-HE caused an acceleration and compression of the early events of adult development (Williams, 1968). A similar effect of large infused dosages of 20-HE may accelerate the response of *Manduca* larvae such that they compress part or all of the spontaneous wandering period. Curiously, pupation was not noticeably advanced in such larvae. Thus, in order to activate the physiological process leading to wandering behaviour, an adequate, but not excessive, 20-HE concentration must be maintained in the larva for a sustained period of time.

Within the range of 20-HE infusions which induced wandering, the latency between the beginning of the infusion and behavioural onset varied with the rate of the infusion. A 20-h latency was seen when the 20-HE concentration was at the lower limit of effectiveness (0·15 $\mu g\,h^{-1}$) (Fig. 2; Table 2). This latency gradually shortened as dose increased, until at very high rates (5 and 10 $\mu g\,h^{-1}$) the minimal latency of about 11 h was observed. Infusions in the range of 0·2–0·5 $\mu g\,h^{-1}$ are thought to approximate the *in vivo* secretion of ecdysteroids. That these infusion rates resulted in an approximately 15-h latency suggests that the behaviourally relevant secretion of ecdysone is under way in normal animals by about 15 h prior to the onset of wandering behaviour. Photoperiod shift experiments (Dominick & Truman, 1984) also indicate an interval of about 15 h between a photoperiod-sensitive event and wandering behaviour. This time would coincide with the second pulse of PTTH proposed by Gilbert *et al.* (1981) which apparently elevates the ecdysteroid titre to a level leading to the induction of the behaviour.

An important feature of the wandering behaviour is that the duration of the spontaneous locomotor activity appears not to be a predetermined characteristic but instead is a function of the duration of ecdysteroid action which precedes the behaviour. Application of the JH mimic, EGS, at various times during exposure to endogenous or infused ecdysteroids resulted in behavioural durations proportional to the interval

of ecdysteroid action preceding the appearance of JH (Fig. 11). More directly, varying the duration of 20-HE infusion at 0·5 $\mu g\,h^{-1}$ resulted in a linear proportionality between the length of the infusion and the duration of the subsequently induced behaviour (Fig. 8). Thus, the duration of wandering behaviour is established as a direct consequence of the duration of ecdysteroid presence, and not as a preprogrammed repertoire which is simply triggered by a phasic hormonal signal.

In studies of vertebrate reproductive behaviour, relatively long but dose-dependent latencies similar to those reported here exist before the onset of lordosis behaviour induced in rabbits (30 h, McDonald, Vidal & Beyer, 1970) and in rats (16–22 h, Green, Luttge & Whalen, 1970). The long latency typically required for the induction of behavioural effects by steroids probably represents the time required for a sequence of hormonally stimulated RNA and protein synthetic steps which are thought to constitute the mechanism by which steroid mediated behavioural changes occur (McEwan, Davis, Parsons & Pfaff, 1979).

The duration or intensity of steroid-induced behaviour can be graded in vertebrates in response to varying the parameters of hormone exposure, as has been demonstrated with oestrogen-dependent mating behaviour in the rat (Aren-Engelbrektsson, Larsson, Sodersten & Wilhelmsson, 1970; Demassa, Smith, Tennent & Davidson, 1977), rabbit (McDonald *et al.* 1970), cat (Peretz, 1968) and guinea pig (Goy & Young, 1957). Therefore, the programming of the duration or intensity of induced behaviour as a cumulative steroid effect may be a phylogenetically widespread phenomenon.

Wandering latency and duration are controlled independently by different parameters of 20-HE exposure. The minimal effective 20-HE infusion lasts 4 h during which time the steroid probably acts by inducing primary biochemical changes such as mRNA and protein synthesis. Since the behavioural duration induced by such a short 20-HE infusion is also short (Fig. 11), the relevant biochemical changes would appear to have a relatively short half-life and quantitatively to support the behaviour. Thus, by increasing the duration of 20-HE exposure, the induced product might be synthesized over a longer period of time, thereby enabling the behaviour for a correspondingly extended period. Induction of new neurotransmitter or postsynaptic receptor species in the 20-HE target neurones are possible examples which would conform to this interpretation.

Although 4 h infusion with 20-HE was sufficient to induce wandering behaviour, a minimum latency of 11 h must intervene before the induced behaviour appears. Since this latency varies strictly as a function of 20-HE dose (Table 2) and is not influenced by infusion duration (Table 3) the rate of accumulation of the presumed induction product would be dose dependent as is typical with steroid hormones. In the simplest case the product might accumulate to a critical level (the threshold for wandering) at a rate proportional to the 20-HE concentration, thereby advancing the start of behaviour as 20-HE concentration increases. This accumulation would require at least 7 h in addition to the 4 h infusion of 20-HE (a total latency of 11 h at the highest effective hormone concentration. Alternatively, the dose-dependent accumulation of product to the threshold level may be more rapid but additional time could be required for a secondary, hormone-independent process which contributes significantly to the latency. Transport of induced neurotransmitter or receptor

molecules to appropriate pre- or postsynaptic regions of the putative 20-HE target neurones could provide such a delay.

Wandering behaviour induced by 20-HE infusion was often followed by a distinct second period of wandering which began early in the usual wandering gate (Figs 1, 7) and which was uniformly shorter than normal, averaging 7 h in length (Tables 2, 3). This reappearance of wandering was apparently due to the endogenous gated secretion of ecdysone, since second bouts of wandering behaviour were not observed in 20-HE-infused larvae lacking prothoracic glands. Evidently, the behavioural action of 20-HE does not necessarily include a programmed loss of behavioural sensitivity to subsequent re-induction as is true for eclosion hormone (Truman, 1978). Furthermore, varying the starting time and duration of 20-HE infusion into unoperated larvae greatly affected the onset time and duration of the induced behaviour, but had no effect on the initiation or duration of the repeat wandering. Indeed, when larvae were restrained and infused with 20-HE at the start of wandering about half of them entered a distinct second period of wandering extending well past the usual time when control larvae become inactive.

Juvenile hormone effects on wandering induction by 20-HE

The disappearance of JH from the tissues of the last instar larva of *Manduca* is a prerequisite for PTTH release (Nijhout & Williams, 1974*b*) and for ecdysteroids to induce premetamorphic changes in the epidermis (Riddiford, 1978). Allatectomy resulted in precocious wandering behaviour in earlier instars of *Manduca* as has been found for *Bombyx* (Bounhiol, 1938) and *Galleria* (Piepho, 1938). Thus, the requisite behavioural circuitry appears to be both present and responsive to its endocrine trigger from a time very early in post-embryonic development.

The release of PTTH and ecdysteroid necessary for wandering behaviour can be delayed if JH reappears before these secretory processes begin. Application of the JH mimic, EGS, to larvae during ecdysteroid infusion or during their normal time of release of ecdysteroid showed that JH can antagonize the action of 20-HE apparently at any time during the 24-h ecdysteroid release. Interestingly, any effects of ecdysteroid that have occurred prior to the application of EGS are subsequently manifest as the normal initiation and progression of the behaviour. Since applications of EGS just prior to the start of wandering had no effect, only the induction and not the expression of the behaviour is sensitive to JH. The spinning of very thin or flat cocoons by *H. cecropia* larvae which were treated with JH during the period when epidermal commitment could be disrupted (Riddiford, 1972) suggests that the endocrine activation of cocoon spinning is analogous to that of *Manduca* wandering in that the behavioural programme accumulates as a function of ecdysteroid action over time.

The ability of JH to modify the duration of wandering is probably the basis for the size-related variation of behavioural duration seen in *Manduca* larvae (Dominick & Truman, 1984). Wandering duration lengthens as larval weight increases in larvae with intact CA, but allatectomized 5th instar larvae show no such relationship. Therefore, it appears that following the elimination of measureable JH which starts when the larva reaches a critical size of about 5 g (Nijhout & Williams, 1974*b*), JH or its effects gradually decrease with time as the larva continues to grow. The residual JH effects may influence the behavioural action of 20-HE in proportion to their

magnitude. Presumably, in smaller larvae the level of covert JH effects is somewhat greater at the time of ecdysteroid release than in larger larvae, resulting in a slightly diminished ecdysteroid effect, and consequently in a shorter behaviour. Another interpretation is that the level of residual JH effects regulates the dynamics of ecdysteroid secretion from the prothoracic glands (Safranek, Cymborowski & Williams, 1980) and the altered ecdysteroid titre results in a modified behaviour.

Infusions of 20-HE into larvae with intact CA at various times during the 5th instar showed that responsiveness to infused hormone appears by the time larvae have reached 5·5 g (Fig. 13), a weight close to that described for 20-HE sensitivity of heart exposure (Nijhout, 1976). At this time the JH titre of the animals is rapidly declining as the CA become inactive (Granger *et al.* 1979) and JH-specific esterase appears (Vince & Gilbert, 1977; Beckage & Riddiford, 1982). As the effects of JH are erased during the period of growth beyond 5 g, the response to 20-HE becomes more rapid. The acquisition of a minimal latency at 7·5 probably represents maximal ecdysteroid sensitivity as a consequence of the virtual elimination of JH. This correlation of sensitivity with weight suggests that elimination of JH during the feeding stage of the 5th instar is the primary factor determining the ability of the larva to wander in response to 20-HE.

In larvae which had been allatectomized just prior to the 5th instar, wandering was induced to start only about 12 h earlier than the earliest 20-HE infused larvae with intact CA (Fig. 14 *vs* 12). Here, too, the infused 20-HE became increasingly effective with time, demonstrating that responsiveness to 20-HE improves over several days, even in the absence of measurable JH. Thus, endocrine events at the beginning of the instar, as well as at the 5 g size, are probably important to developing sensitivity to 20-HE.

In summary, wandering behaviour in *Manduca*, and the probably analogous prepupal behaviour patterns of many other Lepidoptera, are quantitatively determined by effects of ecdysteroids. These effects are prevented by the relatively high JH titres accompanying ecdysteroid action prior to the last instar, and may be subtly modified by the persistence of slight JH effects in the larval tissues during the period of ecdysteroid action which induces the behaviour.

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