J. Insect Physiol., Vol. 27, No. 11, pp. 779–788, 1981.
Printed in Great Britain.

0022-1910/81/110779-10$02.00/0

GROWTH PARAMETERS ASSOCIATED WITH ENDOCRINE EVENTS IN LARVAL *TRICHOPLUSIA NI* (HÜBNER) AND TIMING OF THESE EVENTS WITH DEVELOPMENTAL MARKERS

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(*Received* 5 *January* 1981; *revised* 8 *May* 1981)

Abstract—Numerous behavioural and morphological markers were found during the last instar of *Trichoplusia ni* which permit selection of highly synchronous groups of larvae for physiological or biochemical experiments. Growth parameters were also examined and it was found that the occurrence of a 6th instar was associated with a head-capsule width below the critical threshold of 1.66 mm. Starvation experiments indicated that the critical body weight triggering the first release of prothoracicotropic hormone (PTTH) was not the same for all larvae and was associated closely with a critical ratio of body size:head-capsule size and/or body size:initial body size at the beginning of the instar. The time of prothoracicotropic hormone–ecdysone release in preultimate instars was also associated with a similarly calculated ratio. The ratio was very similar from instar to instar. Neck or thoracic-abdominal ligation of larvae attaining various markers provided indications of times of release of critical amounts of prothoracicotropic hormone, ecdysone and juvenile hormone. The time of peak juvenile-hormone-esterase (JHE) activity in the haemolymph during the prepupal stage was determined with these markers.

Key Word Index: *Trichoplusia ni*, growth parameters, physiological markers, JH esterase, supernumerary moults, juvenile hormone, ecdysone, PTTH

INTRODUCTION

KNOWLEDGE of the factors initiating release of juvenile hormone (JH), prothoracicotropic hormone (PTTH), ecdysone and juvenile hormone esterase (JHE) and the time of their release is a prerequisite to proper interpretation of events controlling metamorphosis. WIGGLESWORTH (1934) showed that the swelling of the abdomen in *Rhodnius prolixus* after each blood meal triggered release of PTTH, which initiated the endocrine events effecting a moult to the next instar. NIJHOUT and WILLIAMS (1974) pointed out that this system cannot serve as a comprehensive model for insects in general, due to the specialised feeding habits of that species. NIJHOUT (1975) presented strong evidence that the attainment of the last-larval instar in *Manduca sexta* (L.) occurred when the head-capsule size, as indexed by its width, surpassed a critical measurement of 5.1 mm. It also appeared that the release of PTTH during the last instar of this species is triggered by attainment of a critical weight of 5 g (NIJHOUT and WILLIAMS, 1974). However, the mechanism by which the last instar or any of the previous instars establishes a certain instar-specific size as the critical size, or by which they perceive that the critical size has been attained, was not reported.

Recent evidence indicates that the interaction between the three metamorphic hormones in the Lepidoptera is more complex than in the early, classical scheme (NIJHOUT and WILLIAMS, 1974; SAFRANEK *et al.*, 1980), and this complexity underlines the need for precise, reproducible timing of test larvae. During studies on the cabbage looper, *Trichoplusia ni* (Hübner), SMILOWITZ (1971) found considerable variation in protein electrophoretic patterns in the haemolymph when larvae were staged by day and weight. Also, several workers who have reported research recently on *T. ni* physiology reared their insects under different temperature and photoperiodic conditions. These differences in rearing conditions make it difficult to assimilate the published literature on *T. ni* into a comprehensive picture, since the physiological state of, for example, a day-12 larva reared at 24°C in constant light (SMILOWITZ, 1973) is probably not the same as one reared at 28°C 16:8 LD (SPARKS *et al.*, 1979). Indeed, there are differences in the reported time of apparent ecdysone release in last-instar *T. ni* (SMILOWITZ, 1974; SPARKS *et al.*, 1979), and these differences may be a result of different rearing conditions.

Perhaps the best criteria for insuring physiological synchrony of subject animals are growth parameters and behavioural or morphological markers. The use of such markers makes the results from one laboratory much more useful to other laboratories using the same species reared under different conditions. FAIN and RIDDIFORD (1975) used several such markers to synchronise late penultimate and early last-instar *M. sexta* while measuring the juvenile hormone titre. DAHLMAN (1975) used several markers for staging normal and parasitised *M. sexta* prior to haemolymph-biochemical measurements. More recently, REINECKE *et al.* (1980) used time-lapse photography to discern several additional markers in last-instar *M. sexta*. SMILOWITZ (1973) and IWANTSCH and SMILOWITZ (1975) used several markers to stage

parasitised *T. ni* for biochemical studies. In each of the above studies, only several markers were used to stage an entire instar, and apparently there has been no successful attempt to find numerous markers, only several hours apart, for an entire instar. A difference of just several hours can be very important since hormone or enzyme titres can change dramatically within such a short time. Identification of a large number of markers across an entire instar would thus be valuable for precise staging of test larvae.

The purpose of the present study was three-fold: (1) to investigate the mechanism by which PTTH release is stimulated in various instars, (2) to determine whether sufficient markers are available for precise staging of lepidopteran larvae and (3) to use those markers to define the apparent time of release of JH, PTTH, ecdysone, and JHE. Specifically investigated was the possibility of staging several instars, and especially the last instar, of *T. ni* with sufficient growth parameters and markers to enable precise, reproducible, physiological timing of endocrine events. *T. ni* is an especially appropriate test animal since it has been increasing in popularity for use in physiological research recently, and it is of economic importance in vegetables and other crops. Also, all previously published data on *T. ni* cultures has dealt with cultures in which there are normally only 5 larval instars (MCEWEN and HERVEY, 1960; IGNOFFO, 1963; SMILOWITZ and SMITH, 1970; IWANTSCH and SMILOWITZ, 1975). However, we had access to a culture in which both 5 or 6 instars occur normally and we were, therefore, in a unique position to investigate the mechanism effecting different numbers of instars.

MATERIALS AND METHODS

Experimental larvae

Trichoplusia ni larvae were reared at 28°C, 16:8 LD as described elsewhere (SHOREY and HALE, 1965; SPARKS *et al.*, 1979). Larvae exhibiting head-capsule slippage to the 4th or 5th (last) instar were selected from mass-rearing containers between 8:00 and 12:00 hr (day begins at lights on) and reared individually in 30-ml diet cups. The culture used also contains a number of larvae that pupate in the 6th instar and these were isolated during head-capsule slippage to the 6th instar. Isolated larvae were then observed periodically and scored for the presence of possible behavioural or morphological markers.

Growth parameters

For the tests involving head-capsule sizes and body weights, isolated larvae were weighed between 14:00 and 17:00 hr after lights on. Diet cups were searched after each larval moult, and maximum widths of located shed head-capsules were measured to the nearest 0.01 mm. In other tests, last instar larvae were starved at various weights and observed for cocoon spinning 24 hr later.

Haemolymph juvenile hormone esterase activity

Haemolymph JHE activity was monitored using C-10 ^{3}H JH III (New England Nuclear, Boston, MA) as a substrate as described elsewhere (HAMMOCK and SPARKS, 1977). Haemolymph dilutions were such as to insure a linear rate of hydrolysis.

Timing of endocrine events

Ligature experiments were performed to test for the time at which sufficient PTTH or ecdysone had been released to cause ligated larvae to develop simultaneously with the controls. Ligature experiments were also used to determine when sufficient JH had been released to induce a normal amount of prepupal JHE activity. Larvae were ligated, after attaining various developmental markers, behind the head (neck ligation) for PTTH and JHE tests and behind the metathorax (thoracic–abdominal ligation) for ecdysone tests. ALO stands for lights on.

Statistical analyses

Chi-square tests of homogeneity were used to determine whether the percentages of 5th- and 6th-instar larvae were the same or whether the percentages having reached a given marker were the same. The relationship between head-capsule width and larval weight was investigated with the use of parametric least squares regression methods. Comparisons of means were done with the *t*-test and variances were compared with the F_{max} test.

RESULTS

Growth parameters

Attainment of critical weight. Head-capsule widths and weights at head-capsule slippage were recorded beginning with the second instar for a group of larvae, and the resulting data are presented in Table 1. Some larvae pupated in the 5th instar while others pupated in the 6th instar. Head-capsule widths of the two groups diverged to the point of statistical significance by the 4th instar, while the weights at head-capsule slippage were significantly different beginning with the 3rd instar. The weights and head-capsule widths of larvae with 6 instars were less than those with five instars, except that the final head-capsule size of the 6th instar was the largest of all.

NIJHOUT and WILLIAMS (1974) found that in last-instar *M. sexta* there was a critical weight associated with corpora allata inactivation and subsequent PTTH–ecdysone release. It was thought reasonable to also hypothesise an analogous critical weight for *T. ni*. Penultimate instars undergoing head-capsule slippage were placed in groups of similar weight and head-capsules were measured after ecdysis. Using the procedure of NIJHOUT and WILLIAMS (1974), the groups of larvae were starved after reaching various weights. For each group there was a weight range above which starvation did not prevent wandering and cocoon spinning the following day, whereas starvation below that range did. There was no single critical weight (range) associated with wandering, or by deduction, PTTH–ecdysone release; instead, the critical weight (range) increased with the weight (or head-capsule size) of the pharate last instar. For example, the mid-point of the critical weight range of last instars weighing 40–49 mg at the end of the previous instar was 218 mg. The analogous critical weight of those weighing 80–89 mg as late-penultimate

Table 1. Comparison of head-capsule widths and weight at head-capsule slippage of various larval instars of *T. ni* pupating in the 5th vs 6th instar

Pupation instar (sample size)	Mean ± S.D. head-capsule widths (mm) of instars*					
	1	2	3	4	5	6
5	a0.28 ± 0.01	a0.46 ± 0.03	a0.75 ± 0.08	a1.20 ± 0.07	a1.88 ± 0.04	—
(*n*)	10	6	9	7	13	—
6	a0.28 ± 0.01	a0.44 ± 0.02	a0.72 ± 0.10	b1.11 ± 0.14	b1.37 ± 0.24	2.02 ± 0.25
(*n*)	10	7	16	18	22	12
	Mean ± S.D. weight (mg) at head-capsule slippage of instars					
5	nr†	a2.3 ± 0.3	a10.0 ± 1.2	a53.4 ± 4.2	—	
(*n*)		9	9	9	—	
6	nr†	a2.3 ± 0.6	b7.3 ± 3.1	b21.4 ± 8.9	77.3 ± 22.0	
(*n*)		24	28	21	21	

*Means preceded by the same letter are not significantly different in larvae pupating in 5 vs 6 larval instars, *t*-test 0.05.
†nr = not recorded

instars was 315 mg, which is 44% higher than the critical weight for the first group. A correlation between somatic size of sclerotized body parts and critical weight was found for *Oncopeltus fasciatus* (NIJHOUT, 1979).

The critical weight of penultimate and earlier instars which may stimulate PTTH–ecdysone release is not known, but since larvae stop feeding upon ecdysone release, their final weight at head-capsule slippage to the next instar is an index of the critical weight. From the weight gain data in Table 1, it is evident that, as with the last instar, there is no one critical weight associated with PTTH–ecdysone release in the 3rd and 4th instars since the weight at head-capsule slippage during these instars was significantly different in larvae pupating as 5th rather than 6th instars. Rather, there is a trend for the apparent critical weight of an instar to vary with the initial weight of the instar or head-capsule size.

Since the critical weight associated with PTTH–ecdysone release apparently varies with change in head-capsule size, a logical hypothesis is that a critical ratio between these two parameters is involved. For example, by sensing the allometric relationship between a constant parameter (head-capsule size) and a changing parameter (body size) the insect could monitor its relative growth during the instar. To test this hypothesis, the ratio of body weight (mg):head-capsule width (mm) was calculated for each last-instar larva. The critical ratio range (Fig. 1) was not constant for each group, although it did not change as much as did critical body size alone. Although the difference between the critical ratios for the two extreme groups in Fig. 1 was 29%, as opposed to the 44% found by merely comparing critical body weights, this variation is still too large for this ratio to be an acceptable tool for predicting the critical weight signalling PTTH–ecdysone release.

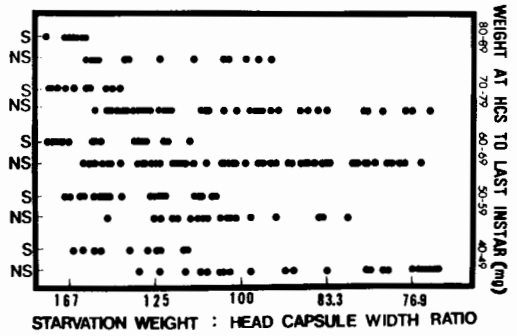

Fig. 1. Reduction in the ability of last-instar *T. ni* larvae to spin cocoons as the ratio of body weight at starvation to head-capsule width increases. Each point represents data from a single insect, and observations (S = spinning, NS = not spinning) were made the day after initiation of starvation. The weight at head-capsule slippage is shown on the right axis.

The index of head-capsule size being used is its width, which is a linear measurement. Assuming that the head and remaining body are both cubical and that the specific gravity of the insect is close to 1, a corresponding linear index of body size then would not be its weight, which is a function of volume. Rather, an appropriate linear index would be the cube root of its weight (i.e. cube root of 1 gram = 1 cm). The ratios of cube root of critical weight:head-capsule width for last instars are presented in Table 2. It is evident that putting measurements of the two parameters into hypothetically similar dimensional units by taking the cube root of the last-instar critical weight remarkably stabilised the calculated critical ratio. The difference between the ratios of the lightest- and heaviest-pupating groups is only 4%. The necessary assumptions are actually quite reasonable. For instance, the specific gravity of large, feeding last-instar *T. ni* larvae is 1.1. Obviously, mathematical relationships of increasing complexity can be derived which assume a more natural shape for the insect. One such relationship is based upon the assumption that the head is spherical and the body cylindrical. Additional measurements and/or assumptions are required for such a treatment, and in this study this more sophisticated analysis did not result in substantial improvement over the cube root approach.

Since head-capsule width of an instar is correlated closely with size of the larva at ecdysis to that instar, it could be that head-capsule width is not the key factor

Table 2. Relationship between head-capsule width (mm) and weight (mg) at which head-capsule slippage or stimulus to pupate occurs

Instar	*n*	Mean ± S.D. head-capsule width	Mean ± S.D. weight at head-capsule slippage or critical pupation weight	(weight)$^{1/3}$	Mean ± S.D. ratio weight: head-capsule width	Mean ± S.D. ratio (weight)$^{1/3}$: head-capsule width	Weight at head-capsule slippage to last instar
				5-instar larvae			
2	6	0.46 ± 0.03	2.26 ± 0.15	1.32 ± 0.03	4.91 ± 0.23	2.85 ± 0.10	
3	9	0.75 ± 0.08	11.55 ± 2.50	2.22 ± 0.15	15.61 ± 3.16	3.02 ± 0.20	
4	7	1.20 ± 0.74	53.25 ± 4.83	3.75 ± 0.14	46.40 ± 8.40	3.12 ± 0.23	
5*		1.80	218	6.02	121	3.34	40–49
		1.88	227	6.10	121	3.24	50–59
		1.93	257	6.36	133	3.29	60–69
				6-instar larvae			
2	4	0.45 ± 0.02	2.08 ± 0.23	1.27 ± 0.05	4.54 ± 0.37	2.79 ± 0.05	
3	10	0.67 ± 0.06	6.63 ± 1.77	1.86 ± 0.18	9.91 ± 2.34	2.81 ± 0.24	
4	10	0.99 ± 0.13	19.68 ± 7.39	2.66 ± 0.32	19.66 ± 5.63	2.72 ± 0.30	
5	14	1.38 ± 0.14	77.01 ± 25.24	4.17 ± 0.43	54.57 ± 15.41	3.00 ± 0.27	
6*		1.97	288	6.61	146	3.35	70–79
		2.01	315	6.80	156	3.38	80–89

* Data is derived from the relationship between weight at head-capsule slippage to last instar and head-capsule width (Fig. 3) or critical pupation weight (Fig. 1) of last instars.

in determining the body size that triggers PTTH-ecdysone release, but rather body size at ecdysis is the key factor and head-capsule width appears important because it is a correlate of the key factor. Indeed, computation of critical ratios as in Table 2 using body weight at ecdysis rather than head-capsule width yields similar results to those presented in that table. Finally, there is, of course, the possibility that neither head-capsule width nor body size at ecdysis is involved, but both appear to be so because they are both correlated with the true key factor.

It is also very important to note that when a calculation was made of the critical ratio of, for example, the cube root of body weight:head-capsule width for the earlier instars, the resulting ratios for all the instars were very similar (Table 2). The clear implication is that a similar critical ratio of body size to head-capsule size (or something of which it is a correlate) is a consistent signal in all instars that sufficient growth has been achieved to permit a moult to the next instar.

Critical head-capsule width. Although the data in Table 1 show a significant difference between 5th instar head-capsule widths of larvae pupating in the 5th vs 6th instar, they do not indicate what is the critical head-capsule width associated with attainment of the pupation instar. However, when the 5th instar head-capsule size was plotted against the occurrence of 5th instar pupation, it was very clear that a critical head-capsule width of about 1.66 mm was associated with the pupation instar (Fig. 2). Fifth instars with a head capsule larger than 1.66 mm in width pupated while those with smaller head capsules moulted to a 6th instar. Of several hundred larvae observed, few had head-capsule widths between 1.60 and 1.70 mm.

Although the pupation instar can thus be predicted on the basis of its head-capsule width, such measurements are tedious and a more convenient predictive tool was needed. NIJHOUT (1975) found that the mass of a larva at the time of the moult is instrumental in determining the size of the new head capsule of the next instar. Therefore, weights of 4th and 5th instars at head-capsule slippage were plotted against next instar head-capsule sizes (Fig. 3). The next problem lies with selecting the most appropriate regression equation to be used. A linear regression is

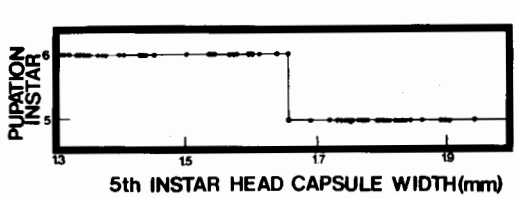

Fig. 2. Relationship between head-capsule width and attainment of the pupation instar in larvae of *T. ni*. Each data point represents a single insect.

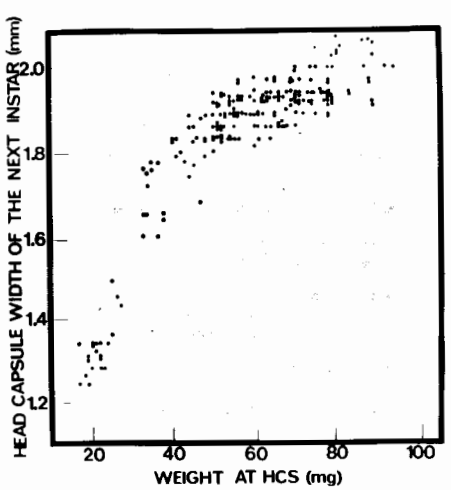

Fig. 3. Relationship between weight at head-capsule slippage (HCS) and head-capsule width of next instar in *T. ni*. Each data point represents a single insect.

not appropriate since the relationship seems curvilinear. Numerous polynomial, log or double-log equations are available and have been used by other workers, but it is desirable to select a model which might be more meaningful biologically. Since the mass (volume) of the larva is being used to predict the size of the head capsule of the next instar, the model most descriptive of the biological event occurring would be one which puts the measurements of both parameters into analogous dimensional units for analysis, i.e. cube the head-capsule width to provide a volume measurement for both or take the cube root of the weight to provide a linear measurement for both. We chose the alternative in which Y (head-capsule width) is regressed on the cube root of X (weight at head-capsule slippage). Such an approach resulted in a significant ($r^2 = 0.90$, $P < 0.001$, $Y = 0.28 + 0.40\ \sqrt[3]{X}$) regression. From the regression it can be found that larvae at the stage of head-capsule slippage with weights less than 33 mg were associated with 5th instars possessing head-capsule widths less than the critical threshold. These larvae later underwent head-capsule slippage to the 6th instar and most weighed 70–120 mg at that time. Fourth instars weighing 35–70 mg were usually associated with 5th instars possessing head-capsule widths greater than the critical threshold. Thus, on the basis of weight at head-capsule slippage of the 4th instar, it was possible to select larvae as 4th instars that would pupate in the desired subsequent instar. In a test of the apparent heritability of occurrence of a supernumerary (6th) instar the F_1 progeny of a group of gate-I pupating 5th instars was composed of 57% 6-instar larvae ($n = 42$). The F_1 progeny from a group of gate-I pupating 6th instars exhibited 79% supernumerary instars ($n = 47$). The difference in % supernumerary larvae between the two groups was significant ($\chi^2_{0.05}$).

Behavioural and morphological markers

With the above information, it was then possible to search for markers associated with various stages of the last (pupation) instar in larvae known to be pupating in the 5th vs 6th instar. Fourth instars showing the first signs of imminent head-capsule slippage (migration to the top of the rearing container, pale green colour) were weighed and, on the basis of Fig. 3, separated into 5th-instar vs 6th-instar pupation groups. Those destined to pupate in the 5th instar were observed subsequently and individually every few hours, while those to pupate as 6th instars were so observed, beginning at the first sign of head-capsule slippage in the 5th instar. Fig. 4 illustrates the temporal sequence of markers observed in last instar larvae. Since the temporal sequence was the same ($\chi^2_{0.05}$) in 5th and 6th instars for nearly every marker, the data for two groups were combined for analysis.

Numerous markers were found for the penultimate–ultimate instar intramoult period and for the time from wandering to the pupal moult. Markers observed, and their sequence, for the intramoult period were: (1) pale green colour, (2) migration to the top of the container, (3) slight clearing behind the head capsule, (4) head capsule slipped forward but not past any ocelli, (5) the old head-capsule slipped forward until the posterior margin has passed 1–3 ocelli, (6) the old head capsule slipped forward past all ocelli, (7)

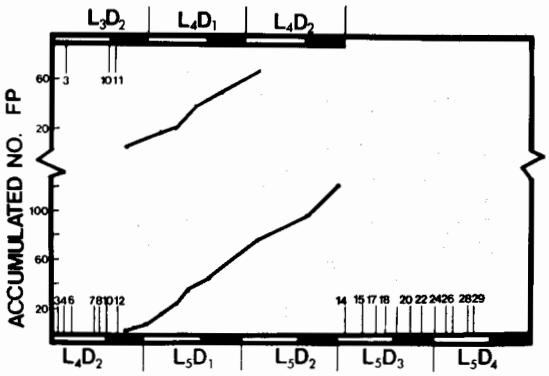

Fig. 4. Temporal sequence of behavioural and morphological markers and faecal pellet (FP) accumulation observed during Gate-I 4th (upper) and 5th (lower) instar larvae of *T. ni*. For markers associated with each code number see text. (L = larval instar, D = day of specified instar).

setae of the pharate instar are crossed along the dorsum of the abdomen, (8) the mandibles of the pharate instar becoming brown pigmented, (9) act of moulting, (10) newly moulted last instar larva positioned by old cuticle, (11) old cuticle consumed and observed as a black mass through the translucent last-instar integument, (12) consumed old cuticle voided as the first faecal pellet (black pellet). Markers (7) and (11) were first used by SMILOWITZ (1973) and were found valuable in the present study as well.

Markers observed, and their sequence, for the period of wandering to the pupal moult were: (13) cessation of feeding, (14) uniform green colour, (15) migration to the top of the container, (16) voiding of the last chalky, white faeces (white pellet), (17) spinning of the first few strands of silk, (18) larva completely enclosed by a thin fragile bubble of silk, (19) enclosure of the larva in a strong, dense silk cocoon, (20) larva positioned ventral side down, (21) initial ocellar pigment retraction, (22) crochet retraction, (23) loss of ability to curl the body, (24) migration of a large black eye-pigment spot posteriorly to where it straddles the hind margin of the head capsule, (25) larva, when prodded, unable to turn itself upright, (26) one of 6 posteriorly migrating white ocellar pigment spots at the head capsule hind margin, (27) several white pigment spots at or posterior to the head-capsule hind margin, (28) prolegs wither and shrivel, (29) act of moult, (30) newly moulted pupa entirely green, (31), dorsum of abdomen light brown, rest of body green, (32) entire body light brown except wings, (33) wings light brown.

It was very difficult to find well-defined markers for the feeding stage of the last instar. The larvae began with several distinct longitudinal white stripes and became almost entirely grey-white, but this was a gradual change. Weight would not be a good index for staging individuals from mass culture since the weight of pupating 5th instars was significantly lower (t, $P < 0.05$) than that of pupating 6th instars, and weights of pupating 5th or 6th instars also showed great variation within each group. Faecal pellet accumulation followed a similar pattern in last-instar 5th and 6th stages and could be measured objectively (Fig. 4). After a while, however, the number of accumulated faecal pellets was enough to make counting them very tedious, so this marker was satisfactory only for the

Table 3. Effect of neck or thoracic–abdominal ligation at various behavioural and morphological markers on metamorphic and biochemical events in post-feeding last instar larvae of *Trichoplusia ni*

Developmental marker at ligation or bleeding (code)*	% Neck-ligated larvae				% Thoracic–abdominal-ligated larvae				JHE activity (nmole/min-ml)		
	n	Moulting at 50% control moult	*n*	Moulting or tanning by 48 hr post control moult	*n*	Moulting at 50% control moult	*n*	Moulting or tanning by 48 hr post control moult	*n*	At each marker	*n*	In larvae head-ligated at marker, bled at time of control maximum
Pale green with white stripes	14	0	3	66	7	0	4	25				
Uniform green migrates upward (14)	39	0	13	87	12	0	13	23	10	1.8 ± 1.1	8	1.9 ± 2.0
First silk (17)	27	4	9	100	18	0	7	100	6	0.4 ± 0.2	7	1.2 ± 0.7
Thin silk bubble (18)	46	2	5	100	30	0	5	100	5	0.8 ± 0.8	8	9.1 ± 3.5
Dense silk cocoon (19)	21	5	23	100	17	0	8	100	4	0.4 ± 0.1	7	9.9 ± 7.3
Larva ventral side down (20)	17	6	14	100	27	0	10	100	3	3.3 ± 1.4	4	14.7 ± 5.8
Initial ocellar pigment retraction (21)	18	33			32	3	18	100	5	1.7 ± 0.5	5	27.6 ± 6.0
Crochet retraction (22)	10	50			11	0	5	100	7	6.6 ± 3.7		
Black eye spot (24)					33	12	17	100	19	20.2 ± 7.3		
One white spot (26)					67	15	41	100	11	31.7 ± 12.3		
Several white spots (27)					38	37	34	100	4	14.1 ± 8.2		
Prolegs withered (28)					11	50	9	100	2	10.6 ± 0.1		

*Numbers in parentheses refer to code number for the marker used in text and Fig. 4.

early part of the feeding stage. An alternative would be to weigh a larva at head-capsule slippage to the last instar and again at the time of test use to, in conjunction with the critical ratio data in Table 2, stage the larva by its developmental distance from the critical weight associated PTTH–ecdysone release.

Time of PTTH release

Neck ligation of feeding larvae (which weighed 50–59 mg at 4th-instar head-capsule slippage) on day 2 prior to about 21 hr ALO prevented them from turning a pale green colour the following day at the same time as the controls. Thus, prior to the time the average larva reaches this time probably not enough PTTH has been released to cause larvae to initiate wandering behaviour on schedule. This PTTH release is apparently gated photoperiodically, as in *M. sexta* (TRUMAN, 1972), since gate-I larvae usually stopped feeding and began turning pale green late on the night of day 2. Slower-developing larvae did not cease feeding or change colour until the following night on day 3.

Neck ligation of larvae prior to the pale green colour marker prevented both tanning and successful pupation, both events being associated with a second period of PTTH release during the last instar (TRUMAN and RIDDIFORD, 1974). Less ecdysone, and thus less PTTH, is needed for tanning than for ecdysis. Apparently, release of enough PTTH to stimulate ecdysone release to cause tanning in 50% of the larvae occurs just before the time of pale green colour (Table 3). Neck ligation as late as ocellar-pigment retraction did prevent ecdysis. The markers of pale green colour and ocellar-pigment retraction define the minimum bounds for the second period of PTTH release.

Time of ecdysone release

Thoracic–abdominal ligation of feeding larvae (which weighed 50–59 mg at head-capsule slippage in the 4th instar) on day 3 prior to about 1 hr ALO prevented them from turning a pale green colour the following day at the same time as the controls. Thus, prior to 1 hr ALO the average larva has probably released insufficient ecdysone to cause larvae to initiate wandering behaviour on schedule.

Thoracic-abdominal ligation of larvae prior to the marker of first-silk production prevented tanning from occurring within 48 hr after control pupation (Table 3). Ligation prior to withering of the prolegs prevented pupation but did not prevent tanning. The markers of first-silk production and proleg withering define the minimum bounds for the second, or prepupal, period of ecdysone release.

Time of prepupal JHE peak

The haemolymph JHE activity was low during the phases of wandering and cocoon spinning. Beginning with the marker of crochet retraction, the activity began to rise, reaching a peak at the time several white ocellar spots had reached the hind margin of the head capsule. After this time, the activity decreased as the pupal moult approached.

Time of JH release

Since the prepupal peak of haemolymph JHE activity in final instar *T. ni* is known to be induced by JH (SPARKS and HAMMOCK, 1979), the appearance of JHE activity in neck-ligated larvae is one index of JH release. The data in Table 3 show that head ligation prior to the formation of a thin bubble of silk during cocoon formation will prevent a rise in JHE activity above the baseline occurring during wandering. Ligation at subsequent times will still prevent a normal amount of activity from occurring until the marker of ocellar-pigment retraction. Apparently, JH is being released during the prepupal stage at least between the markers of thin bubble and pigment withdrawal.

Pattern of tanning

In normal prepupae of *T. ni* which have been in the marker of 'several white spots' for some time, a pair of small tanned stripes or spots appear close to the prothoracic spiracles. Soon after, the caudal tip of the abdomen bearing the hooks for anchorage in the cocoon also tans. In larvae ligated around the thorax soon enough to prevent pupal ecdysis and complete tanning, the first tanning appeared on the 5th-abdominal segment. This tanning was in the form of a thin dorsal transverse band. Larvae ligated at later intervals showed dorsal tanning bands on sequentially more-anterior segments. Finally, the ventral surface showed tanning. It was observed also that often in a group of ligated larvae, those that did not ecdyse often tanned, while those that reached ecdysis (as evidenced by trachael withdrawal) had not tanned.

DISCUSSION

Growth parameters

The head-capsule widths of various larval instars of *T. ni* have been reported for several different cultures (MCEWEN and HERVEY, 1960; IGNOFFO, 1963; SMILOWITZ and SMITH, 1970; IWANTSCH and SMILOWITZ, 1975), but all of these reports dealt only with 5-instar cultures. The present study provides the first comparison of growth parameters of 5th vs 6th instars of *T. ni* larvae, and also an explanation of why there were only 5 instars in other *T. ni* cultures. The reported increase in head-capsule width during the first 4 moults is, for the most part, the same for all 5-instar cultures of *T. ni* (Table 4). The average increase in usually 1.57–1.69-fold, except for the moult from 4th to 5th instar, where the fold increase in all 5-instar larvae is a more uniform 1.54–1.58-fold. The 5-instar larvae in these cultures all had average 5th-instar head-capsule widths greater than 1.66 mm, which is the critical width associated with attainment of the last instar (Fig. 2). Comparison of 5- and 6-instar larvae in the present study shows that increases in head-capsule width from instar to instar in the 6-instar larvae were generally lower than those for 5-instar larvae. Associated with this observation is the trend for smaller cube root of body weight:head-capsule width ratios for 6-instar larvae (Table 2). In fact, the ratios for 3rd and 4th instars were slightly, but significantly, smaller (t, $P < 0.05$) in 6-instar larvae as compared with 5-instar larvae. One interpretation of this event is that there is a genetically based smaller critical ratio in 6-instar larva which causes them to experience PTTH–ecdysone release sooner, thus causing them to stop feeding and begin ecdysis at a lower weight. An

Table 4. Fold increase in head-capsule widths at specified instar–instar moults in various cultures of *T. ni*

Study	Fold increase head-capsule width 1–2	2–3	3–4	4–5	5th instar head-capsule width (mm)	5–6	6th instar head-capsule width (mm)
IGNOFFO (1963)	1.39	1.63	1.61	1.56	1.76	—	—
MCEWEN and HERVEY (1960)	1.62	1.57	1.58	1.55	1.79	—	—
SMILOWITZ and SMITH (1970)	1.57	1.61	1.60	1.54	1.77	—	—
IWANTSCH and SMILOWITZ (1975)	—	—	1.69	1.58	1.71	—	—
5-instar larvae Present study	1.68	1.63	1.60	1.57	1.88	—	—
6-instar larvae Present study	1.57	1.63	1.54	1.23	1.37	1.47	2.02

alternative explanation that we favour is that in fast-growing 5-instar larvae, the critical weights of each instar were reached in advance of the gate period of PTTH-ecdysone release. These larvae thus continued feeding and gaining 'extra' weight until the gate opened. The 6-instar larvae did not reach the critical weights until much closer to the gate of PTTH–ecdysone release and thus did not gain much additional weight after achieving the critical weight. The latter would then appear to have had a lower critical cube root of body weight:head-capsule width ratio. The result of a 6-instar larva having a lighter weight at ecdysone release is that the subsequent instar would be smaller than its 5-instar counterpart. After several instars, the cumulative effect of 6-instar larvae moulting at sub-maximal weights is that their 5th instar is stunted and possesses a head-capsule width below that associated with attainment of the pupation instar. The below-threshold head-capsule width is apparently responsible for the occurrence of an extra larval moult. This second interpretation would explain why the occurrence of 6th instars in *T. ni* is not constant but will vary with temperature ($\chi^2_{0.05}$, FYE and MCADA, 1972).

This interpretation would also explain the results of ZENNER-POLANIA and HELGESEN (1973) who report a supernumerary 6th instar in (apparently only females of) the omnivorous leaf roller *Platynota stultana* (Walsingham). Close examination of their data shows that female 6-instar larvae grew less in size at each moult (as evidenced by comparative increases in head-capsule widths) than did their female 5-instar counterparts. The average 5th-instar head-capsule width of 6-instar larvae was never greater than 0.83 mm while that for 5-instar females was never less than 0.97 mm. If the critical head-capsule width associated with pupation in *P. stultana* is, for instance, 0.87 mm, then using our model one would predict the results they observed. One would also predict that no male 6th instars would be observed under their rearing conditions since the average head-capsule width of 5th instar males was never less than 0.90 mm. However, since the head-capsule width of 5th instar males was shown to decrease with higher rearing temperatures, it is possible that had temperatures above 35°C been used the head-capsule width of 5th-instar males would have dipped below the critical threshold and a supernumerary male instar observed.

It was noted that the critical cube root of body weight:head-capsule width ratios of preultimate *T. ni* larvae were very similar. When similar methods of analysis were applied to the head-capsule and body weights reported in the literature for preultimate-instar *M. sexta* (NIJHOUT, 1975) critical ratios were again found which are remarkably similar from instar to instar (2.44–2.99).

Behavioural and morphological markers

The numerous markers and critical ratios provided in Fig. 4 and Table 2 should be very useful in selecting highly synchronous larvae for experiments. For example, the standard deviation about the average JHE activity during a transient JHE peak in 4th-instar larvae at head-capsule slippage (SPARKS *et al.*, 1979) is significantly less when derived by staging procedures of this paper (4.5) than when derived from larvae staged by time of day (15.1) (WING *et al.*, 1981) (F_{max} test, $P < 0.05$). Also, D. JONES *et al.* (1981), found that the biology and markers defined in this study greatly facilitated elucidation of the nature of the developmental interaction between *T. ni* and a parasitic *Chelonus* sp. The times of occurrence of endocrine events found in the present study will be applicable to other laboratories using *T. ni* reared under different conditions since the larvae in those laboratories will still pass through the set of markers reported here. The growth parameters and markers investigated here will provide leads toward locating useful staging tools in other lepidopteran species as demonstrated by our results with *M. sexta*.

Timing of endocrine events in T. ni

Data presented in the present study provide an explanation for inconsistent results in studies of *T. ni* endocrinology performed by different laboratories. SMILOWITZ (1974) investigated the time of PTTH release and found that neck ligations of *T. ni* after about the middle of day 2, when the still-feeding larvae had reached a little over half of their maximum weight, did not prevent pupation. The present study shows that neck ligation prior to markers occurring on late day 3 prevented the pupal moult at the time of the unligated controls. One explanation for the apparent contradiction between the two studies lies with the different endpoints being used. Procedures in the present study used pupation of the ligated larvae simultaneously with the controls, whereas SMILOWITZ (1974) apparently observed whether the larvae

pupated at all. It is now known that there are two major periods of PTTH and ecdysone release in Lepidoptera (TRUMAN and RIDDIFORD, 1974; RIDDIFORD, 1980). It has also been shown that moulting can eventually occur after a long delay in larvae incapable of further PTTH secretion (due to neck ligation) once the first PTTH release has already occurred, because of leakage of ecdysone from the prothoracic glands (TRUMAN, 1972). If the neck-ligated larvae in the study of SMILOWITZ (1974) were actually scored only by whether or not they eventually moulted, and not by whether they moulted simultaneously with the controls, then the time of PTTH release found in that study may have been the first PTTH release period instead of the second one.

Both SMILOWITZ (1974) and SPARKS *et al.* (1979) used thoracic–abdominal ligations to locate the time of sufficient ecdysone release for successful pupation. SMILOWITZ (1974) used ecdysis to the pupa as the measured endpoint while SPARKS *et al.* (1979) used the formation of tanned pupal cuticle beneath the larval cuticle and scored the larvae 1 day after the controls pupated. SMILOWITZ (1964) placed the time of sufficient ecdysone release as the mid-prepupal stage, while SPARKS *et al.* (1979) placed it earlier at a time associated with cocoon spinning. Since less ecdysone is needed to cause tanning than for the actual moult, SPARKS *et al.* (1979) placed the time of ecdysone release earlier than SMILOWITZ (1974). The present study used the time of the moult of the controls as the endpoint, and placed the time of sufficient ecdysone release late in the prepupal stage, later than that in the study of SMILOWITZ (1974). This second discrepancy would be anticipated if ligated larvae pupating later than the controls were also scored positively by SMILOWITZ (1974), since less ecdysone is needed to cause eventual pupation than to cause pupation at the same time as the controls.

The first period of ecdysone release in the last-instar gate-I larvae used in this study was indicated to occur on the night of day 2. Data from other studies indicate that a decline in the JH titre must have occurred by this time to enable PTTH-ecdysone release or ecdysone effects (CYMBOROWSKI and STOLARZ, 1979; SAFRANEK *et al.*, 1980; WILLIAMS, 1976). It has been hypothesised that the role of JHE in feeding larvae is clearance of JH, which then permits initiation of metamorphosis (GILBERT *et al.*, 1978; RIDDIFORD, 1980), and the results of *in vivo* inhibition of JHE activity substantiates this hypothesis (SPARKS and HAMMOCK, 1981). However, this hypothesis does not explain why in gate-II *T. ni*, in which JHE peaks on day 2 as in gate-I larvae, apparent ecdysone release occurs 24 hr later than it does in gate-I individuals (SPARKS and HAMMOCK, 1979).

As a result of the present study, it is now possible to compile the most detailed outline yet available of the endocrinological events leading to metamorphosis in last-instar *T. ni*, and this outline is provided in Fig. 5. G. JONES *et al.* (1981) found that a factor from the brain and suboesophageal ganglion induced the first but not the second peak of JHE activity during the last instar. The second peak is induced directly by JH (SPARKS and HAMMOCK, 1979). G. JONES *et al.* (1981) hypothesised that the switchover in sensitivity of the source of JHE to JH may be caused by a release of ecdysone and

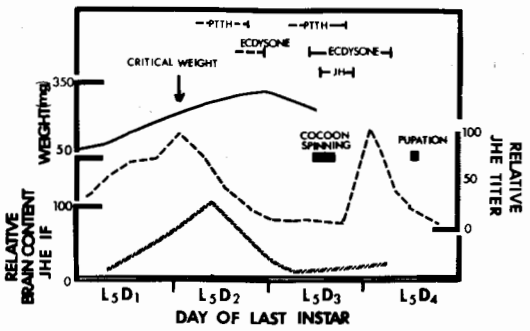

Fig. 5. Time of occurrence of endocrine events associated with metamorphosis in gate-I, last-instar *T. ni* which weighed 50–59 mg at head-capsule slippage in the 4th instar. Solid PTTH, JH and ecdysone bars closed at both ends indicate minimal periods of release of these hormones. Dotted PTTH and ecdysone bars are closed at one end, and the time of the closure indicates the time sufficient PTTH and ecdysone have been released to cause normal cocoon spinning. The solid curve represents weight, the dotted line the relative JHE titre (adapted from SPARKS *et al.*, 1979) and the slashed line the relative brain content of JHE inducing factor (IF) (adapted from G. JONES *et al.*, 1981).

indeed it can be seen (Fig. 5) that the first release of ecdysone has apparently at least begun during the decline of the JHE inducing factor from the brain-suboesophageal ganglion complex. Also, the prepupal releases of JH and ecdysone appear to be concommitant, which is consistent with the hypothesis that in post-wandering lepidopteran larvae JH promotes the release or effects of ecdysone (RIDDIFORD, 1980; SAFRANEK and WILLIAMS, 1980).

Pattern of tanning

Both the present study and that of VINCE and GILBERT (1977) report tanning to occur on the thorax of normal, unecdysed prepupae. However, the two events are probably not homologous since the tanned areas occur on the prothorax in *T. ni* and metathorax in *M. sexta*. The pattern of tanning of pupal cuticle in *T. ni* was also different from *M. sexta* in that the more-caudal segments tanned first in thoracic–abdominal ligated *T. ni* and just the opposite was true in *M. sexta* (TRUMAN *et al.*, 1974). The differences in time and place of tanning of the thoracic and abdominal epidermis between *T. ni* and *M. sexta* indicate that the pattern of attainment of competence to secrete pupal cuticle is not the same in the two species.

To conclude, the behavioural and morphological markers here described have proven valuable in this laboratory for comparing the developmental state of normal, parasitised and experimental *T. ni* involved in endocrinological and ecological studies. As illustrated by this report, such markers also facilitate the comparison of data generated in different laboratories. Such techniques can certainly be applied to a variety of leipidopterous larvae, and we have found that many of these markers were useful in comparing different species. The ratio of cube root of weight:head-capsule width correlates with developmental events in each larval instar of *T. ni* and apparently other species as well. Certainly relationships such as this one will be useful in investigating insect developmental biology, and it may

additionally relate to how the insect discerns its critical size.

Acknowledgement—The research was supported, in part, by the National Institutes of Health Grant 7 RO1 ES02710-01 and PHS/NIEHS Research Career Development Award 1 K04 ES00046-03 (B. D. HAMMOCK).

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