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INDUCTION AND REGULATION OF JUVENILE HORMONE ESTERASES DURING THE LAST LARVAL INSTAR OF THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

THOMAS C. SPARKS and BRUCE D. HAMMOCK*

Department of Entomology, Louisiana State University, Baton Rouge, LA, 70803, U.S.A.

and

*Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA, 92521, U.S.A.

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Abstract—Juvenile hormone esterase titres were monitored in gate I and gate II last instar larvae of *Trichoplusia ni* using JH III as substrate. Two peaks of activity were observed for both gate I and gate II larvae, although the first and second juvenile hormone esterase peaks for the gate II larvae are extended and delayed one day, respectively. Head or thoracic ligations before the prepupal stage lower or block the appearance of both esterase peaks. Juvenile hormone I and II, as well as homo and dihomo juvenoids can induce the second juvenile hormone esterase peak in both normal and ligated larvae, and increase the esterase titre during the first peak in nonligated larvae. Induction of the juvenile hormone esterases is possible in non-ligated larvae as soon as the moult to the last instar has occurred and in ligated larvae as soon as the first esterase peak has started to decline. Distinct mechanisms of regulation are present for the first and second juvenile hormone esterase peaks. Juvenile hormone does not appear to be involved in regulating its own metabolism by directly inducing the first esterase peak; however, evidence is consistent with a brief burst of juvenile hormone which occurs prior to pupation inducing the production of the second peak of juvenile hormone esterase activity.

Key Word Index: Trichoplusia ni, juvenile hormone, juvenoids, anti-juvenile hormone, juvenile hormone esterase, juvenile hormone-regulation, induction-juvenile hormone esterase, ecdysone.

INTRODUCTION

JUVENILE hormone (JH) controls insect metamorphosis (GILBERT *et al.*, 1977). High JH titres maintain the larval state while a decrease in the JH titre initiates the pupation sequence (NIJHOUT and WILLIAMS, 1974) as well as a change in tissue commitment to the pupal stage (RIDDIFORD, 1976). The drop in JH titre at the beginning of the last larval instar in the Lepidoptera appears to be due to a combination of increased metabolism (SANBURG *et al.*, 1975a, b; NOWOCK and GILBERT, 1976) and decreased synthesis (NIJHOUT, 1975). In the Lepidoptera, JH is initially metabolized by ester hydrolysis (SLADE and ZIBITT, 1972; HAMMOCK and QUISTAD, 1976), and esterases capable of hydrolyzing JH are detectable in the haemolymph at times during the last larval instar that appear to coincide with reported drops in the JH titre (WEIRICH *et al.*, 1973; VINCE and GILBERT, 1977; SPARKS *et al.*, 1979).

These JH esterases (JHE's) can be differentiated from α-naphthyl acetate (α-NA) esterases by differential inhibition (SANBURG *et al.*, 1975a, b; KRAMER and DE KORT, 1976; HOOPER, 1976; HAMMOCK *et al.*, 1977; KRAMER and CHILDS, 1977; SPARKS and HAMMOCK, 1979), ability to metabolize JH bound to the binding protein (HAMMOCK *et al.*, 1975; SANBURG *et al.*, 1975a; KRAMER and CHILDS, 1977) and time of occurrence (WEIRICH *et al.*, 1973; SPARKS *et al.*, 1979). The JHE's are also selective for the 2E methyl ester of the naturally occurring JH's (WEIRICH and WREN, 1973, 1976; HAMMOCK *et al.*, 1977). These studies suggest that the JHE's may be important in the regulation of the JH titre and therefore involved in the initiation of and the commitment to the pupal stage. JHE's appear to be produced by the fat body (WHITMORE *et al.*, 1974; HAMMOCK *et al.*, 1975; NOWOCK and GILBERT, 1976) and this production can be stimulated by exogenous JH in *Hyalophora* pupae, a stage devoid of JHE activity (WHITMORE *et al.*, 1972, 1974). Stimulation of JHE activity by JH has also been noted recently in adults of *Leptinotarsa decemlineata* (KRAMER, 1978) and pupae of *Galleria mellonella* (REDDY *et al.*, 1979). However, to date, no reported studies have examined this phenomenon during the last larval instar when these enzymes are thought to be of primary importance. Thus this laboratory undertook an investigation of the haemolymph JHE regulation during the last larval instar of the cabbage looper, *Trichoplusia ni*.

MATERIALS AND METHODS

Insects

Trichoplusia ni larvae were reared on an artificial diet at 27 ± 2°C with a photoperiod of 14 Light (L): 10 Dark (D) (lights on at 5 a.m.) (SHOREY and HALE,

1965). Last instar larvae were reared individually and then separated into gate I (GI: pupate in 4 days) and gate II (GII: pupate in 5 days) larvae on the basis of weight and time of moult to the last instar (SPARKS *et al.*, 1979). GI larvae were used unless otherwise stated. The apparent time of ecdysone release was monitored by ligation, and the T_{50}'s (time for 50% of the insects to respond) for ecdysone release and pupation were determined on a response/no response basis using probit analysis as described earlier (SPARKS *et al.*, 1979).

Esterase assays

Haemolymph from 3–7 larvae was collected from a clipped anal proleg in a culture tube (6 × 50 mm) held at 4°C. Immediately after collection, 25 μl of the pooled haemolymph was withdrawn and diluted with $I = 0.2$ sodium phosphate buffer at pH 7.4 containing 10 mg of phenylthiourea (PTU) per 100 ml to inhibit tyrosinases.

JHE activity was monitored using chain labelled JH I (New England Nuclear Corp.) (HAMMOCK and SPARKS, 1977; SPARKS *et al.*, 1979) and methoxy labelled JH III (SANBURG *et al.*, 1975a; HAMMOCK *et al.*, 1977) as substrates at 5×10^{-6}M. Haemolymph was diluted in buffer at 1:100 and 1:1000 for the JH III and JH I assays respectively. General esterase activity was monitored by using α-naphthyl acetate (α-NA) as substrate at 2.5×10^{-4}M in haemolymph diluted 1:10 in buffer (SPARKS *et al.*, 1979). All esterase assays were performed on enzymes that were diluted so that conditions of substrate saturation were approached and for which the hydrolysis rates were linear throughout the time used for the analysis.

JHE in GI vs GII larvae during development

Larvae were reared as described above, and esterase activity on JH III was monitored in synchronous GI and GII larvae. The assays were carried out every 6 hr; 6 a.m. (1 hr after lights on: ALO), 12 noon (7 hr ALO), 6 p.m. (13 hr ALO), and 12 midnight (19 hr ALO) as soon as the haemolymph was collected. Each pooled haemolymph sample was assayed in triplicate. One and two samples per time point were used for GI and GII larvae respectively, and the whole developmental regime was assayed on three separate occasions for GI larvae and two separate occasions for GII larvae.

Induction and ligation studies

Larvae were ligated using extra fine unwaxed dental floss. Neck ligations were performed by ligating just behind the head capsule, while thoracico-abdominal ligatures were accomplished by ligating between the thorax and abdomen. Ligated larvae were placed in plastic petri dishes on filter paper and kept with the stock culture until assayed. Juvenoids (JH mimics) and JH's were applied topically on the dorsum of abdominal segments 5–8 at 2×10^{-1}M (unless otherwise noted) in 1–2 μl ethanol. Varying doses of ethanol up to 5 μl applied on several occasions led to no detectable effects on JHE activity or time of pupation. 20-hydroxy-ecdysone (Simes) was dissolved in ethanol–water (1:1) and 1 μl was injected dorsally through the first abdominal segment posteriorly along the mid-dorsal line for 3 segments with a Hamilton 10 μl syringe. The larvae were then ligated just posterior to the injection point. Each pooled haemolymph sample from treated and control larvae was assayed in triplicate for JHE activity, using JH III as substrate. Each treatment was composed of at least three (usually four or more) pooled samples taken on (at least) two separate occasions.

RESULTS

Gate I vs Gate II JHE's

For GII fifth (last) instar larvae the T_{50} for apparent ecdysone release based on ligation experiments is 6.5 hr ALO on day 4 (L5D4), and the T_{50} for pupation is 8.1 hr ALO L5D5. In both cases the results were analyzed with probit analysis and within the fiducial limits of the probit lines, these two events are delayed exactly 24 hr from the corresponding events in GI larvae (Fig. 1) (SPARKS *et al.*, 1979).

Esterase activity patterns using JH I and JH III as substrates are identical during the development of GI larvae (SPARKS *et al.*, 1979). Therefore, for the GII larvae only the more rapid JH III esterase assay was used. JHE activity in GII larvae has an overall pattern similar to that of the GI larvae (Fig. 1). Both GI and GII larvae have their first JHE peak early on L5D2 of the last instar (1 hr ALO), and both have a second JHE peak just prior to pupation. In GII larvae, however, this second peak occurs exactly one day later (L5D5: 1 hr ALO) than in the GI larvae (L5D4: 1 hr ALO) (Fig. 1). Although the first esterase peak in the GII larvae reaches maximal activity at the same time as the GI larvae, it takes longer (1 day) for the GII JHE activity to decline to the low levels observed at the time of ecdysone release. Based on these results, it is apparent that there is very little difference in the JHE activity between GI and GII larvae during the first day and a half in last instar larvae; however, JHE levels increase more slowly in L5D1 and D2 GII larvae than in GI larvae and the peak esterase activity is lower. Possibly this depressed activity in GII larvae may reduce the clearance rate of JH in the lighter weight animals so that sufficient JH remains to inhibit the PTTH release needed to initiate metamorphosis (RIDDIFORD,

Table 1. Effects of ligation on apperance of the first (L5D2) and second (L5D4) JHE Peaks in GI *Trichoplusia ni**

Time of ligation day	hr ALO	Point of ligation	JHE Activity† (% control)
L4D2	13	Thorax	1 ± 1
L5D1	4	Head	10 ± 8
	4	Thorax	2 ± 2
	13	Head	6 ± 4
	13	Thorax	5 ± 3
L5D3	4	Head	3 ± 8
	4	Thorax	2 ± 6
	13	Head	67 ± 14
	13	Thorax	43 ± 27

*Larvae were assayed at the times of peak JHE activity in control larvae. L4D2 and L5D1 larvae were assayed on L5D2 3 hr ALO and L5D3 larvae were assayed on L5D4 3 hr ALO. Assays were run 3–4 times.

†Control (non-ligated) JHE activity was 12 nmol JH III acid produced/min-ml for L5D2 and 15 nmol JH III acid produced/min-ml for L5D4 larvae.

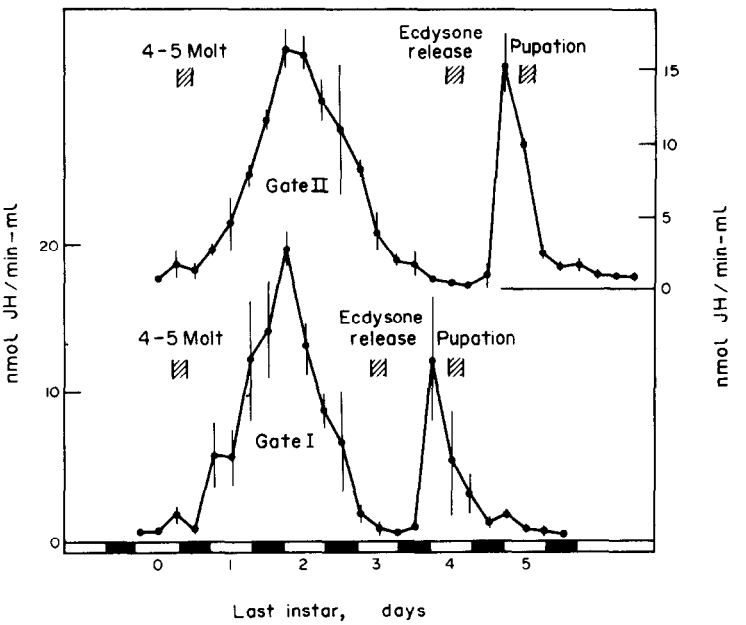


Fig. 1. Haemolymph JH esterase activity during the last instar in gate I (bottom) and gate II (top) larvae. Points are means ± S.D. The JHE activity pattern for GI larvae is from SPARKS *et al.* (1979) The light and dark bars on the abcissa are periods of lights on and off respectively. Each data point is the average of at least 4 assays.

personal communication). It also appears that the second JHE peak must occur just prior to pupation.

Effects of ligations on the first peak of JHE activity

Ligations were performed on L4D2 (13 hr ALO) just before the moult from the 4th to 5th instar and on L5D1 (4 hr ALO and 13 hr ALO), and the haemolymph was assayed for JHE activity at the time of the first JHE peak on L5D2 (3 hr ALO). The JHE activity in haemolymph of larvae following neck or thoracico-abdominal ligations at these times is less than 6% of the JHE activity in haemolymph from non-ligated controls (Table 1). Further ligations were performed to determine the speed of the decline in the JHE activity following ligation. Larvae (L5D1) were ligated (4 hr ALO) at the thorax and then the haemolymph was assayed for JHE activity every few hours (1.5, 3 and 6 hr). JHE activity decreases as the length of time the larvae have been ligated increases (Table 2) and after only 6 hr the JHE activity is reduced to less than 5% of normal. Alternatively, larvae (L5D1) were ligated (14 hr ALO) and the haemolymph assayed for JHE activity (1.5, 3, 6 hr post ligation) and this activity was compared to the JHE level in control larvae at 14 hr ALO and to the JHE level in non-ligated larvae at 15.5, 17 and 20 hr ALO. The JHE level in ligated larvae continued to increase for 1.5 hr, but not so fast as in control larvae. At 3 and 6 hr post ligation the JHE level in ligated insects was lower than the JHE level in larvae at either 0 time or in non-ligated controls of the same age (Table 2). Similar results can be obtained by removing the larvae from the diet (REDDY *et al.*, 1979; LEVINSON and WING, unpublished).

Effects of ligations on the second peak of JHE activity

GI L5D3 larvae were ligated at 4 and 13 hr ALO and assayed for haemolymph JHE activity at the time of the second JHE peak on L5D4 (3 hr ALO) (larvae have ceased feeding at this stage in their development). Ligation of either the head or the thorax at 4 hr ALO results in very low JHE activity compared to non-ligated controls. However, similar ligations performed later in the day (13 hr ALO) only partially block the appearance of the second JHE peak (Table 1). Thus, the stimulus for the production of the second JHE

Table 2. Decreasing JHE activity in L5D1 larvae as the result of increasing the length between thoracico-abdominal ligation and assay

Length of time ligated* before assayed (hr)	JHE Activity (% control)
1.5	69±27
3.0	26±7
6.0	4±2

Length of time after ligation when assayed (hr)	JHE Activity % Control larvae† 10 hr ALO L5D1	% Control larvae‡ of same age
1.5	144±30	83±17
3.0	81±22	56±16
6.0	22±3	15±2

*Larvae were all assayed on L5D1 at 10 hr ALO and compared to the JHE activity in the untreated control larvae (10 nmol JH III acid produced/min-ml). Assays were run 3–4 times.

†Larvae were all ligated on L5D1 at 14 hr ALO and their JHE levels compared to JHE levels in non-ligated controls at the time of ligation or

‡JHE activity in ligated larvae compared to JHE activity in the non-ligated controls of the same age (L5D1 14, 15.5, 17 and 20 hr ALO)

Table 3. Haemolymph JHE activity in 3 hr ALO L5D4 larvae following thorax ligations performed at selected intervals on L5D3

Time of ligation (hr ALO)*	JHE Activity† (% control)
4	2±2
10	10±8
13	40±4
16	102±6

*Thorax.

†Larvae were assayed for JHE activity on L5D4 (4 hr ALO) and the activity was compared to that of the non-ligated control larvae (11 nmol JH III acid produced/min-ml). Assays were run 3 times.

peak appears to have already reached the fat body (FB) by late L5D3 (13 hr ALO). To better determine when this stimulus is occurring, thorax ligations were performed on L5D3 larvae at selected intervals. The larvae were then assayed for JHE activity at the time of the second JHE peak (L5D4, 3 hr ALO). JHE activity increases as the larvae are ligated later in the day, and no decrease in JHE activity is observed in larvae ligated after 16 hr ALO (Table 3). The above observations suggest that the stimulus for the second JHE peak occurs during the late afternoon and early evening of L5D3.

Effects of ecdysteroid and a juvenoid on JHE activity

The results of the ligation studies suggested that ecdysones may be involved in the release and/or production of the JHE's. To test this hypothesis, 20-hydroxy-ecdysone (1 μg) was injected into ligated 4 hr ALO L5D1 and L5D3 larvae which were then assayed for JHE activity 3 hr ALO the next day on L5D2 and L5D4, respectively. L5D1 haemolymph contained little JHE activity in either the ethanol–water controls or in those treated with ecdysteroid compared to untreated control larvae (Table 4). In contrast to the L5D1 larvae, L5D3 larvae treated with 20-hydroxy-ecdysone contained some JHE activity compared to the ligated and ligated + solvent controls (Table 4). However, the FB and other tissues appeared to be histolyzing which may lead to the release of (FB) esterases and thus may account for the low JHE activity observed. Thorax ligated L5D1 and L5D3 larvae were topically treated with JH I to determine if JH could cause the appearance of the JHE's. Haemolymph from these JH I treated larvae contained very little JHE activity when assayed 24 hr later compared to the JHE activity of the untreated non-ligated control larvae (Table 4). Thorax ligated L5D1 and L5D3 larvae were also treated with a juvenoid Ro-10-3108, (1-(4′-ethylphenoxy)-6, 7-epoxy-3-ethyl-7-methylnonane). Ro-10-3108, which is effective on the Lepidoptera (ZURFLUEH, 1976), lacks an ester functionality and therefore is not subject to metabolism by esterases. L5D1 larvae treated with Ro-10-3108 or Ro-10-3108 + 20-hydroxy-ecdysone show no increase in JHE activity compared to ligated and ligated + solvent controls. Surprisingly, treatment with Ro-10-3108 on L5D3 results in the normal (non-ligated) JHE activity being restored (Table 4).

Table 4. Induction of JHE activity by 20-hydroxy-ecdysone and Ro-10-3108 in ligated L5D2 and L5D4 larvae*

	Treatment‡	JHE Activity† (% control)
L5D1	Ligated (thorax)	3±3
	Ligated + ethanol (2 μl)	4±2
	Ligated + JH I (60 μg)	4±3
	Ligated + Ro–10–3108 (60 μg)	3±3
	Ligated + ethanol–H_2O	5±4
	Ligated + ecdysteroid (1 μg)	8±6
	Ligated + ecdysteroid (1 μg) + Ro–10–3108 (30 μg)	6±2
L5D3	Ligated (thorax)	2±3
	Ligated + ethanol (2 μl)	1±2
	Ligated + JH I (60 μg)	2±2§
	Ligated + Ro–10–3108 (60 μg)	100±6
	Ligated + ethanol–H_2O	3±3
	Ligated + ecdysteroid (1 μg)	33±11

*Larvae were ligated and treated on early (4 hr ALO) L5D1 and L5D3 and assayed 24 hr later on L5D2 (JHE peak 1) and L5D4 (JHE peak 2) respectively. Assays were run at least 3 times.

†Control (non-ligated) JHE activity is 13 nmol JH III acid produced/min-ml on L5D2 and 14 nmol JH III acid produced/min-ml on L5D4.

‡Ethanol and Ro–10–3108 were applied topically while the 20–hydroxy-ecdysone and ethanol–H_2O were injected.

§When JHE levels are assayed 12 hr after the application of JH I (17% t,t,c) high induction is observed (50% Ro–10–3108). See also Fig. 2.

JHE induction in day 3 larvae

Because L5D3 GI larvae have low JHE activity (Fig. 1), they were used to examine further the increase in JHE activity resulting from JH treatment. This phenomenon will be referred to as induction. L5D3 GI (2 or 5 hr ALO) larvae were treated topically with Ro-10-3108 (60 μg) and assayed for JHE activity at 3 hr intervals. JHE activity rose to detectable levels above background within 6 hr after treatement. Although JHE activity continued to increase in treated larvae for at least 15 hr and appears to remain high for over 24 hr in ligated larvae (Table 4), the greatest

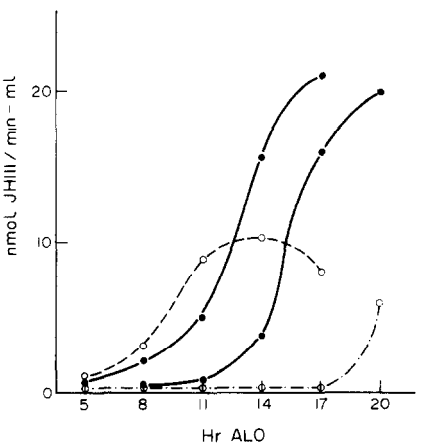


Fig. 2. JH esterase activity in L5D3 GI last instar *T. ni* larvae at selected intervals after treatment with JH I (17% t, t, c) or Ro–10–3108 at 5 or 8 hr ALO at 60 μg/larva. Ro–10–3108 (●——●); JH I (○——○); Control (untreated or ethanol: ○——○). Assays were run 3–4 times.

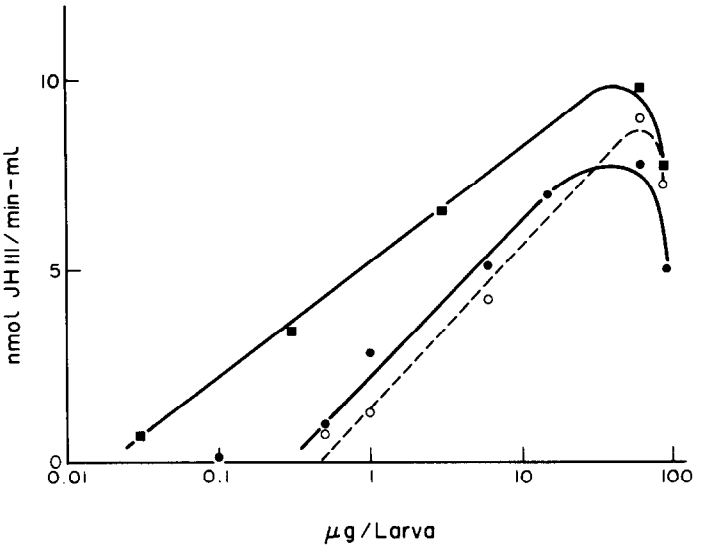


Fig. 3. JH esterase activity in L5D3 GI larvae in response to varying concentrations of Ro–10–3108, normal (●—●) and ligated (○—○) and JH I (95% t, t, c) (■—■). Larvae were assayed 12 hr after treatment and all assays were run at least 4 times.

difference in JHE activity between control and treated insects occurred 12 hr after L5D3 larvae were treated at 5 hr ALO (Fig. 2). Therefore, subsequent experiments were performed by treating L5D3 larvae at 5 hr ALO and assaying haemolymph JHE at 17 hr ALO. Similar experiments with JH I indicated that maximal JHE activity appeared 12 hr after treatment (Fig. 2). In contrast to JHE activity in the haemolymph of larvae treated with Ro-10-3108, JHE activity dropped rapidly in larvae treated over 12 hr previously with JH I, and JHE activity was not detectable in ligated larvae treated 24 hr previously (Table 4).

Early (5 hr ALO) ligated and non-ligated L5D3 GI larvae were treated with different doses of Ro-10-3108 and assayed 12 hr later for JHE activity. The induction of the JHE's acts in a dose dependent manner, with 60 μg/insect (~20 μg/100 mg live wt) yielding the highest response (Fig. 3). At doses above 60 μg, the ability of Ro-10-3108 to induce the JHE's decreases and below 0.5 μg/larva no detectable induction occurs. Similar results are obtained when JH I is used as the JHE substrate. Thus, within the limits of the assay, the induction response to Ro-10-3108 appears to be identical for both ligated and non-ligated larvae. JH I (95% t,t,c) was examined for its ability to induce the JHE's in non-ligated larvae, at several doses, compared to Ro-10-3108. As with Ro-10-3108, JH I induces the JHE's in a dose-dependent manner (Fig. 3). However, the induction process is much more sensitive to JH I than it is to Ro-10-3108 because the dose of JH I necessary to induce 50% of the maximal JHE activity (effective dose: ED_{50}) as induced by JH I is 1 μg/larva compared to 5 μg/larva for Ro-10-3108. The maximal response to JH I also occurs at 60 μg/larva and like Ro-10-3108 JHE activity decreases at higher doses. The decrease in JHE activity observed at doses greater than 60 μg/larva may be the result of JH directly interfering with the JHE or acting elsewhere to inhibit the JHE induction. The inability of Ro-10-3108 to elicit as high a response as t,t,c JH I may indicate that it is a partial agonist. This interpretation of the data predicts that some juvenoids with even very high JH mimicking activity may have anti-JH properties.

Ro-10-3108 induces JHE activity with both JH I and JH III as substrate; however, when α-NA is substrate, no increase in esterase activity is observed (Table 5). Thus, the JHE induction does not appear to be due to an increase in general (α-NA) esterase activity.

Several compounds were assayed for their ability to induce JHE activity relative to Ro-10-3108 (Table 6). Among the geranyl phenyl ether juvenoids, several trends are apparent. Optimal induction was obtained with the 3,7-dihomo juvenoids Ro-7-9767 and Ro-10-3108. Apparently both the methylene dioxyphenyl and *p*-ethylphenyl substituents confer high activity with unsaturation at Δ2 causing little change. Hydration of Ro-10-3108 to its diol led to a total loss of activity. R-20458 is a close analogue of Ro-10-3108 differing in Δ2 unsaturation and the lack of ethyl branches at Δ3 and 7; however, it shows negligible activity. PMM (9-(4′-ethylphenoxymethyl)-1,2-epoxymenthane, isomer mixture) (SPARKS and HAMMOCK, unpublished data)

Table 5. Induction of esterase activity by Ro–10–3108 in L5D3 larvae

Treatment†	Substrate (nmol/min-ml)* α-NA	JH I	JH III
Control	79±13	9±2	0
Ro–10–3108 (60 μg)	80±18	84±13	12±3

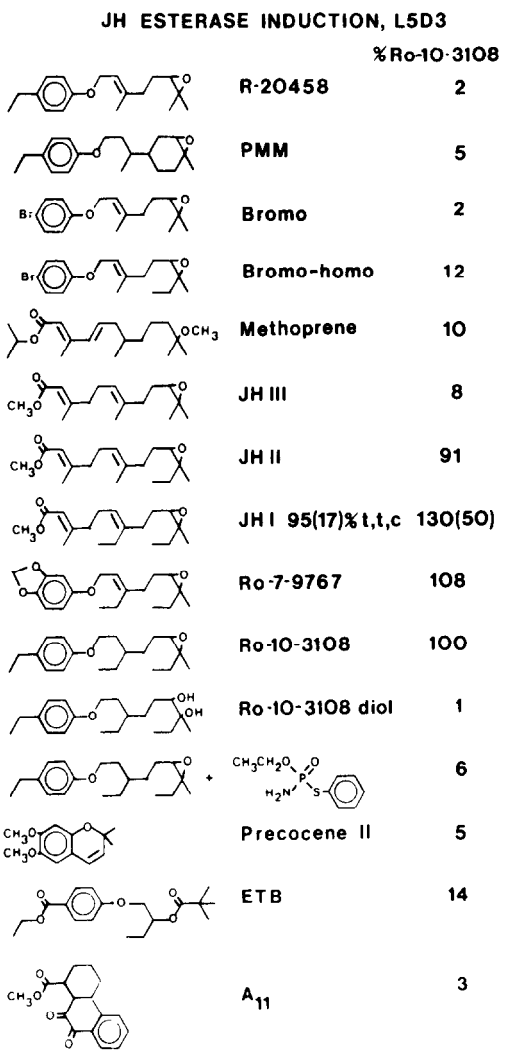
*Substrate concentrations: 2.5×10^{-4}M (α-NA) and 5×10^{-6}M (JH I and JH III).

†Control larvae were untreated or treated only with ethanol (2 μl).

Larvae (L5D3; 4 hr ALO) were treated topically and assayed 12 hr later. Each assay was run 5 times.

Table 6. Induction of JHE activity in L5D3 GI larvae by selected compounds as compared to Ro–10–3108. Larvae (L5D3; 4 hr ALO) were treated topically (2 μl at 10^{-1} M) and then assayed for JHE activity 12 hr later. Ro–10–3108 treated larvae typically metabolize JH III (5×10^{-6}M) at 7 nmol/min-ml. No JHE activity is present in untreated or ethanol treated larvae. Each compound was assayed at least 6 times.

Sources of the compounds are as follows: Methoprene, ETB, A_{11}, G. Quistad and G. B. Staal (Zoecon Corp); Ro–10–3108, Ro–7–9767, U. Schwieter (Hoffman-LaRoche); precocene II, W. S. Bowers (Cornell University); EPPAT, T. R. Fukuto (University of California, Riverside); JH I (17% t, t, c), A. J. Manson (Ayerst Labs); JH I (95% t, t, c or E, E, c), JH II Calbiochem; and JH III, Ro–10–3108 diol, PMM, Bromo-epoxide, Bromo-homo epoxide, were synthesized in this laboratory. For the 95% and 17% t, t, c JH I's, the other 5% and 83% are largely a mixture of the other 7 JH I isomers with a trace of the unepoxidized compound as verified by normal and reversed phase high resolution liquid chromatography. All potent inducers of JHE are also good JH mimics in *T. ni* and related Lepidoptera (SPARKS and HAMMOCK; BOONE and HAMMOND, unpublished).



restricts the possible confirmations of the juvenoid by bonding the ethyl branch to the terpene chain, and it has very low activity. Neither of the *p*-bromophenyl juvenoids have high activity, but the Δ7-homo derivative is much more active than the corresponding methyl compound. Similar trends also exist for the homologues of JH and the dienoate juvenoid, methopreme. JH III and methoprene are weak inducers of JHE activity, while the homo and dihomo compounds (JH II and I) are very active. The anti-juvenile hormones, precocene II, A_{11} (methyl-6,7-dioxo-5α,10α-podocarpa-8,11,13-triene-15-oate) and ETB (ethyl-4-(2-(*t*-butylcarbonyloxy)-butoxy)-benzoate) ZR-2646) are all poor JHE inducers. Also, the addition of EPPAT (*O*-ethyl-*S*-phenyl phosphoramidothiolate), a potent JHE inhibitor (HAMMOCK *et al.*, unpublished data), at the time Ro-10-3108 is applied prevents the detection of the induced JHE's probably by directly inhibiting the JHE. The JHE induction is also somewhat selective for the natural isomer of JH as 95% t,t,c JH I is a much better inducer than 17% t,t,c JH I (Table 6). Furthermore, based on the dose–response curve for 95% t,t,c JH I (Fig. 3), it appears that some of the other isomers of JH I may, in fact, be inhibiting the induction process because 60 μg of 17% JH I gives much lower induction than would be anticipated based on its content of t,t,c JH I.

Induction of JHE's during development

Ligated and non-ligated larvae were treated with Ro-10-3108 (20 μg/100 mg live wt) and compared to normal and ligated controls at selected intervals during development. The JHE's cannot be induced by Ro-10-3108 in either normal or ligated larvae before the moult to the last instar has occurred (Fig. 4). JHE induction is possible in non-ligated individuals once the larvae have moulted. However, it is not possible to induce the JHE's in ligated larvae until early day 2 (Fig. 4).

DISCUSSION

During the last larval instar of the Lepidoptera examined there are two peaks of JHE activity (Fig. 1) (WEIRICH *et al.*, 1973; VINCE and GILBERT, 1977; SPARKS *et al.*, 1979). Based on time of occurrence, substrate selectivity and ability to metabolize JH bound to the binding protein, the JHE's appear to be specifically produced when the insect needs to remove JH from its system. However, the exact timing of the decline in the JH titre relative to the appearance of the JHE's is uncertain and, as indicated by NIJHOUT (1975), the first JHE peak may appear after the JH titre has already reached low levels.

If the JHE's are indeed necessary for the removal of JH, then they should occur at about the same relative physiological time during the last larval instar, regardless of its length. In fact, such a pattern does occur in *T. ni* (Fig. 1). The overall pattern is the same for GI and GII larvae; however, in the GII insects the second JHE peak is delayed exactly 1 day. This delay implies that the second JHE peak must occur just prior to pupation. During the early last instar the JH titre has to be reduced to sufficiently low levels to allow the release of the first bursts of prothoracicotropic hormone (PTTH) and ecdysone which initiate metamorphosis (NIJHOUT and WILLIAMS, 1974) and the change in commitment to pupal programming

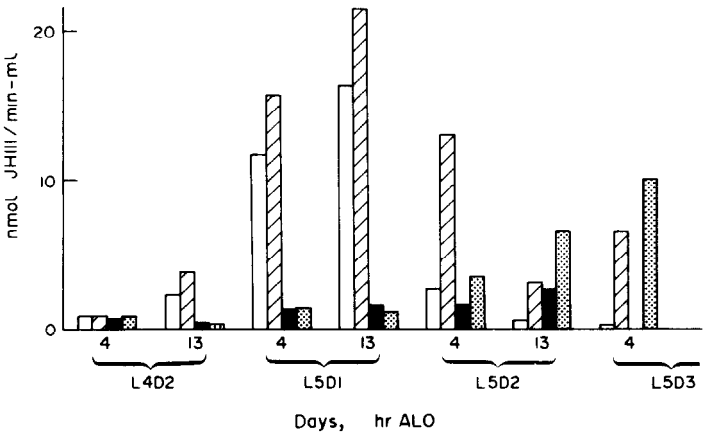


Fig. 4. JH esterase activity in normal and ligated GI larvae, with and without Ro–10–3108 (20 μg/100 mg live wt) during the last instar. Larvae were treated at either 4 hr ALO (8–9 a.m.) or 13 hr ALO (8–9 p.m. and assayed 12 hr later. Control (□); normal + Ro–10–3108 (▨); ligated (■); and ligated + Ro–10–3108 (▩). All assays were run 4–5 times.

(RIDDIFORD, 1976; MITSUI and RIDDIFORD, 1978) respectively. The first JHE peak must also disappear before the second burst of JH can be released. This second JH peak has been shown to be necessary to prevent the precocious development of the wing patches, eyes and mouth parts in the pupae of *Hyalophora cecropia* (WILLIAMS, 1961) and *M. sexta* (KIGUCHI and RIDDIFORD, 1978). *In vivo* studies indicate that rapid metabolism of the second JH peak is necessary. Partial inhibition of the first and second JHE activity peaks by EPPAT results in a delay in the time of pupation and malformed larvae that resemble larval–pupal intermediates, respectively (SPARKS and HAMMOCK, unpublished). Thus, immediately before pupation the insect must both release JH and then swiftly degrade it in order for normal development to occur.

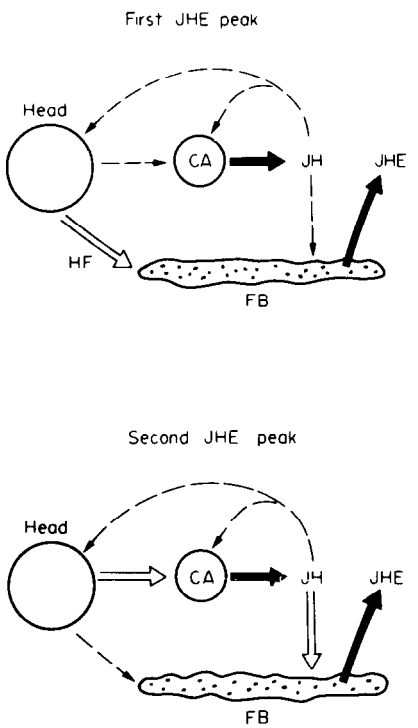


Fig. 5. Possible mechanisms of JH esterase regulation in last instar larvae of *T. ni*. Established pathways of production and/or release of JH and JHE (➡); major route of regulation of JHE (⇒); possible routes of regulation of JHE (⇢). HF. head factor regulating JHE in early last instar larvae; CA: corpora allata; FB: fat body; JHE: JH esterase. The transition from the top scheme to the bottom one seems to occur during the night of L5D1.

Induction of esterases active on JH was initially demonstrated by WHITMORE *et al.* (1972) in the pupal stage of *Hyalophora gloveri*. Treatment with JH induced enzymes capable of hydrolyzing α-NA, *p*-nitrophenyl acetate and JH, but no physiological role was shown for the induction phenomenon. KRAMER (1978) has reported that JH induces esterases metabolizing JH but not α-NA in adult *L. decemlineata*. These JHE's may be partially responsible for lowering the JH titre in short day adults in preparation for diapause. In contrast, JH I (17% t,t,c) has recently been found to lower both JHE and α-NA esterase activity in last instar larvae of *G. mellonella*, while it induces only JHE activity in the pupa (REDDY *et al.*, 1979). These results clearly indicate a difference in the biology of JHE regulation in *G. mellonella* and *T. ni*, but they are not necessarily contradictory to the model presented in this study (see below). For instance, the change in control of JH esterase production from the head factor to JH may occur after D5 in *G. mellonella* which is the oldest larval age examined for the induction of JHE with JH (REDDY *et al.*, 1979). Also, as shown in Fig. 3, very high doses of topically applied JH I will reduce the amount of JHE in the haemolymph in *T. ni*, and possibly injected doses of JH I will result in a similar phenomenon in *G. mellonella*.

In last instar *T. ni*, JHE but not α-NA esterase activity can be induced by JH I and II and homoisoprene containing juvenoids. Based on the compounds so far assayed, an epoxide and ethyl branches appear necessary for proper receptor binding and induction. Such functionalities are also generally important for juvenilizing effects in *T. ni* and related Lepidoptera. The JHE's resulting from this induction also metabolize JH I faster than JH III (SPARKS and HAMMOCK, 1979). Assuming that the larval JH of *T. ni*

is JH I and/or II as in *M. sexta* (DAHM *et al.*, 1976; SCHOOLEY *et al.*, 1976), then the observed specificity for the induction phenomenon is appropriate. KRAMER (1978) found that besides JH I; methoprene and hydroprene (ethyl, 3,7,11-trimethyl-2,4-dodecadienoate), both lacking homoisoprene units, could also induce the JHE's in adults of *Leptinotarsa*. However, induction by these compounds is reasonable since JH III is thought to be the JH present in the adult beetle (TRAUTMANN *et al.*, 1976).

The JHE's apparently cannot be induced by the topical application of Ro-10-3108 before the moult to the last instar has occurred. During the penultimate instar, the JH titres are high (FAIN and RIDDIFORD, 1975; VARJAS *et al.*, 1976) and the JHE's are low (WEIRICH *et al.*, 1973; SANBURG *et al.*, 1975a; SPARKS *et al.*, 1979). Thus, this lack of JHE induction in L4D2 *T. ni* lends further support to the hypothesis that the JHE's are not produced until the last larval instar.

The inability of Ro-10-3108 to induce the JHE's in early ligated last instar (i.e. L5D1) larvae, suggests that some head factor other than JH is directly responsible for stimulating the fat body to produce and/or release the JHE's. Ecdysteroids were other candidates for this stimulus; however, ecdysteroids with or without Ro-10-3108 also fail to induce the JHE's in ligated L5D1 larvae (Table 3). During L5D1 the JHE production and/or release seems to be regulated via some process from the anterior region of the insect. Ligation of the larvae on L5D1 results in the JHE levels dropping to less than 6% of normal within 6 hr (Table 2). Thus, it also appears that the stimulus from the head region is necessary on a continuing basis for the JHE's to be released. Because the application of Ro-10-3108 causes increased JHE levels in nonligated L5D1 larvae, it appears that the JH titre could indirectly influence the amount of JHE produced and/or released. The JH's or juvenoids may be acting directly on the FB or via some organ in the head region, to either modify the FB's response to the stimulus, or to directly modify the amount of the stimulus from the head. This stimulus could be in the form of a neurosecretion released into the haemolymph or via direct neural stimulation. In view of this information, it is interesting that the destruction of the A and B neurosecretory cells in *Locusta migratoria* during the early last instar results in a drop in the JHE titre (RETNAKARAN and JOLY, 1976).

Induction of the JHE's during an early instar could cause the JH titre to be lowered prematurely which could then initiate precocious development. Unfortunately, none of the anti-JH's tested are potent inducers of the JHE's. For precocene II this lack of JHE induction is not surprising since it does not appear to be effective in lepidopterous insects and its site of activity appears to be the corpora allata (PRATT and BOWERS, 1977; UNNITHAN *et al.*, 1977). ETB and A_{11} are effective on the lepidopterans *M. sexta* (STAAL, personal communication; ROSELAND and RIDDIFORD, 1978) and *Bombyx mori* (MURAKOSHI *et al.*, 1975; SLAMA, 1978), respectively, and their modes of action are currently unknown. It is therefore of interest that ETB can induce JHE activity to higher levels than Ro-10-3108 in L5D1 *T. ni*, although it induces JHE's only at very high doses and actually inhibits JHE induction resulting from Ro-10-3108 application to L5D3 larvae (SPARKS and HAMMOCK, unpublished). After the first day the JHE's can be increasingly induced by Ro-10-3108 in ligated GI larvae as the endogenous JHE titre drops (Fig. 4). This ability to induce the JHE's in ligated larvae also appears to coincide with the apparent release of PTTH during the night of L5D1 (SMILOWITZ *et al.*, 1974). After this critical period the FB is apparently physiologically reprogrammed to produce JHE in response to the presence of JH I and II or closely related compounds. The JHE's resulting from this induction appear biochemically identical to those of both the first and second JHE peaks (SPARKS and HAMMOCK, 1979). Because the FB is a likely source of the haemolymph JHE's (WHITMORE *et al.*, 1974; HAMMOCK *et al.*, 1975; NOWOCK and GILBERT, 1976), the induction of the JHE's appears to be the result of JH acting directly on the FB and not via some intermediate messenger from the head region. Based on the above information, the second JHE peak is probably the result of the JHE's being induced by the short burst of JH that occurs just prior to pupation. In effect, JH is inducing the enzymes that are responsible for its own removal.

Assuming that JH does induce its own catabolism and that the length of time required for the JHE induction is the same for exogenous and endogenous JH (12 hr), then ligation of L5D3 larvae 12 hr (or less) before the second JHE peak should not prevent the second JHE peak from appearing. Metabolism and distribution studies in *T. ni* following the topical application of 60 μg of radiolabelled JH I or Ro-10-3108 indicate that haemolymph JH titres reach a maximum of 1 μg/ml of haemolymph at 6 hr and rapidly decline, while Ro-10-3108 levels continue to increase reaching 3.6 μg/ml at 24 hr (SPARKS and HAMMOCK, unpublished information). Extrapolating from these haemolymph levels following 60 μg JH I (50% 10R,11S) applications to 0.03 μg applications, it is conceivable that physiological levels of JH can induce JHE (DAHM *et al.*, 1976; SCHOOLEY *et al.*, 1976; JUDY and GILBERT, unpublished information). Based on the above assumptions and information, the data in Table 3 predicts that in *T. ni* the second burst of JH occurs during the evening (~13 ALO) of L5D3 in GI larvae.

The results of the ligation and induction studies indicate that there are distinct phases in the control of the JHE's in *T. ni* (Fig. 5). Before the moult to the last instar occurs, the JHE's cannot be induced by exogenous JH in either normal or ligated larvae. However, at the time of the moult some process occurs that initiates JHE production and allows exogenous JH to increase the JHE activity in nonligated larvae. Because the JHE's appear to have a relatively short life in ligated larvae, some constant stimulus from the head region is necessary for their continued production. The stimulus for the JHE production and/or release in L5D1 larvae does not seem to be due to JH or ecdysone acting directly on the FB, although they may be involved indirectly via some process in the head region. After early L5D2 the amount of induction possible in ligated larvae increases as the insect's own endogenous JHE levels decline. Thus during the night of L5D1 the control of the production

and/or release of the same enzyme responsible for the two peaks of JHE activity appears to pass from some unidentified head factor (possibly via neural or neurosecretory mediation) to the epithelial endocrine glands, the corpora allata.

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