## JUVENILE HORMONE-INDUCED DELAY OF METAMORPHOSIS OF THE VISCERA OF THE CECROPIA SILKWORM<sup>1</sup>

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Metamorphosis in the Lepidoptera begins when the corpora allata are inactivated in the final larval instar and the juvenile hormone (JH) titer subsequently declines (Williams, 1961; DeWilde, de Kort and de Loof, 1971; Nijhout and Williams, 1974). In response to ecdysone in the presence of this small amount of JH, the integument and viscera progress from the larval to the pupal state. The artificial maintenance of a high JH titer at this stage interferes with this transformation (Piepho, 1942; Sehnal, 1968; Sehnal and Meyer, 1968; Riddiford, 1972; Sehnal and Schneiderman, 1973; Truman, Riddiford and Safranek, 1974).

In the epidermis, the switchover from the commitment to larval differentiation to that for pupal differentiation apparently occurs at the time of gut evacuation (Riddiford, 1972; Truman, Riddiford and Safranek, 1974). After that time, in the giant silkworm, *Hyalophora cecropia*, application of JH does not prevent pupal cuticle synthesis and deposition, but it blocks the pupal differentiation of the viscera when given at any time during the prepupal period (Riddiford, 1972).

In this previous study, the state of differentiation of the viscera was ascertained by their response during the adult development which occurred immediately after pupation. Thus, the possibility that sufficient exogenous JH remained in the animal to act directly on the pupal-adult transformation of the viscera was not ruled out. Accordingly, in this investigation, the gonads and fat body from animals treated with JH as prepupae were transplanted immediately after pupal ecdysis to untreated host pupae. In this manner, their competence for adult differentiation could be assayed by their ability to develop in concert with the host pupa.

## MATERIALS AND METHODS

## Experimental animals

Cecropia larvae were reared on wild cherry trees (Telfer, 1967) or in the laboratory on synthetic medium (Riddiford, 1968). After gut evacuation the animals were maintained at 25–26° C under a 17L:7D photoperiod.

Host pupae were usually in diapause but chilled for varying lengths of time. A few host pupae had had their brains removed to hold them in permanent diapause.

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## Hormonal materials

Cecropia C18-JH (70% all trans) (Eco-Control, Inc.), and its mimics epoxygeranyl sesamole (EGS) (Eco-Control, Inc.), ethyl 3,7,11 trimethyl-dodecadienoate (ZR512) (Zoecon Corporation), and the Williams-Law mixture of chlorinated hydrocarbons (JH-A) prepared according to the method of Vinson and Williams (1967) were used. In the Cecropia pupal assay (Williams, 1961) 0.1  $\mu$ g C18-JH, 2.5  $\mu$ g EGS, 15  $\mu$ g ZR512, or 20  $\mu$ g JH-A was necessary to give a +3 pupal-adult intermediate.

The juvenile hormone materials were freshly prepared in acetone ("Nanograde," Mallinkrodt), and 2 to 5  $\mu$ l were applied along the dorsal midline of the prepupa. Alternatively, the hormonal materials were mixed with light mineral oil (Fisher; Saybolt viscosity 125/135), and 50  $\mu$ l were injected into the prepupa just anterior to the middorsal tubercle on the 8th abdominal segment. 12.5  $\mu$ g of  $\beta$ -ecdysone (either from K. Sláma or from Rohto Co.) in 50  $\mu$ l 10% isopropanol were injected into the mesothoracic tergum of a diapausing host pupa to initiate adult development after a specified period of time.

## Surgical procedures

The surgical techniques employed were as previously described (Williams, 1952; Schneiderman, 1967). The organs to be transplanted were removed from a larva of a known age or from the donor pupa within 12 hours after pupal ecdysis. They were rinsed in Ringer's (Ephrussi and Beadle, 1936) and all extraneous tissue was removed. Each gonad was placed in the tip of the abdomen of a host pupa of the same sex, and the wound covered by a plastic cover slip. The fat body transplants were always made between diet- and leaf-reared animals since the fat body of the former is white whereas that of the latter is yellow. This color difference persisted through metamorphosis; thus, the implant could be readily identified.

Abdomens were isolated by slicing through the pupa at the level of the posterior mesothoracic tergum. The midgut was thus left intact and allowed to recede into the abdominal compartment as fat body was removed. The pupa was then transected at the level of the first abdominal segment, and the abdomen sealed with a plastic cover slip. The abdomens were stored in an inverted position at 25° C. Several days later, the gonad was implanted either into the top of the isolated abdomen through the paraffin-plugged hole in the cover slip or into the tip of the abdomen. After varying lengths of time at 25° C, the implant was removed and placed into an intact diapausing host pupa to assay its developmental stage. This further operation was necessary because after  $\beta$ -ecdysone injection, few of the isolated abdomens survived long enough to complete adult development.

#### Results

## Effects of JH application at the initiation of the prepupal stage

I previously reported that when JH-A was administered to a Cecropia prepupa at the time of ocellar retraction, the subsequent pupal diapause was averted and the resultant individual was adult externally but pupal internally (Riddiford,

#### TABLE I

Dosage‡ (µg)	Number treated	Number failing to diapause	Juvenile characters in resulting moths†			
			External*	Internal**		
				Thorax	Fat body	Gonads
		Topical ap	plication in a	cetone		
2.5-5	3	0			_	
10-15	5	3	0.5	3.1	3.3	3.1
25-50	4	4	1.5	4.1	5.0	4.1
100	2	2	3.2	5.0	5.0	5.0
		Injection	in light miner	al oil		
0.1-1	3	0				
2.5-5	5	3	0.5	3.0	4.5	4.0
10-25	8	8	1.0	4.2	4.7	4.2
50	3	3	3.0	4.7	4.9	4.4
100	4	4	4.2	4.9	5.0	4.8

Effects on the incidence of diapause and on adult differentiation of administration of Cecropia C18-JH to Cecropia prepupae at the time of ocellar retraction.

<sup>‡</sup>Doses which produced the same scores were combined; thus, the range is given.

<sup>†</sup>When there is more than one treated individual, an average score is used. This average is based only on the scores for nondiapausing individuals.

\* Scoring of external characteristics according to the 0 to 5 scale of Williams (1961).

\*\* Scoring of internal characteristics according to the 0 to 5 scale of Riddiford (1972).

1972). Since Cecropia C18-JH and more specific JH mimics have become available, a study of their effects at this critical time of pupal development was done in preparation to a study of the effects of JH on the larval-pupal transformation of the viscera.

Table I shows the effects of Cecropia C18-JH on adult differentiation when it is applied to Cecropia prepupae at the time of ocellar retraction. Just as previously found with JH-A (Riddiford, 1972), moderate doses of C18-JH prevented diapause and the metamorphosis of the viscera but had little effect on the integument. With the higher doses of C18-JH, more severe external effects were obtained. Since the resultant pupae had appeared normal externally, these external effects noted in the adult were not due to JH acting at the time of application but rather to the hormone which persisted until the beginning of adult development. As seen in Table I, a given dose of C18-JH was much more effective when injected in mineral oil than when applied topically in acetone. Undoubtedly, this is due to the fact that topically applied hormone is metabolized much faster than that injected in mineral oil (Ajami and Riddiford, 1973; Riddiford and Ajami, 1973).

Similar experiments involving topical application of the JH mimics at the onset on the prepupal period indicated that these compounds were more stable in the animals than was the Cecropia C18-JH since dosages just sufficient to prevent metamorphosis of the viscera also produced external effects in the adult. For



FIGURE 1. The average score of the implanted testes, ovaries, and fat body as a function of the mean time ( $\pm 1$  day) required for the initiation of adult development of the host pupa after the implantation. Implants from: closed squares represent untreated fresh (<12-hour old) pupae (15 implants per point until day 8; 6 for day 12; and 3 thereafter); closed circles, fresh pupae given 25 µg C18-JH at onset of prepupal period (at least 15 implants per point except 9 for days 0-3 and 4 for days 3-6); triangles, fresh pupae given JH mimics (150 µg JHA, 50 µg ZR512, or 50 µg EGS) at onset of prepupal period (at least 25 implants per point through 15 days, then 12 for days 17-21, and 4 thereafter); inverted triangles, fresh pupae given 50 µg EGS as white prepupae (at least 10 implants per point); open circles, isolated abdomens 14-36 days after removal from fresh pupae treated with 25 µg C18-JH at onset of prepupal period (6 implants per point except only 1 at 14 days).

instance, 30  $\mu$ g EGS was the minimum dose which prevented diapause, yet treated individuals showed an external score of 3.0 as well as complete inhibition of metamorphosis of the viscera. Similarly, 10  $\mu$ g ZR512 usually prevented diapause with nearly complete inhibition of internal metamorphosis, but it also partially inhibited the pupal-adult transformation of the integument (3.0 score).

## Assessment of the developmental status of viscera from JH-treated pupae

Thus, a low dose of the animal's own juvenile hormone applied at the onset of the prepupal period interfered with metamorphosis of the viscera without affecting the integument. But, as with the previous study with JH-A (Riddiford, 1972), it was possible that the JH was persisting in the animal through the prepupal period and acting primarily at the beginning of adult development. If this

were true, the differences in responses of the viscera and of the integument would only indicate that the internal organs were more sensitive to the hormone. Therefore, the developmental status of the viscera at the time of pupal ecdysis was studied by transplanting various organs into chilled diapausing host pupae. Transplants of any part of the gut, the Malphigian tubules, and the nerve cord of the untreated freshly pupated individuals proved largely unsuccessful. But, as seen in Figure 1, all 45 gonad and fat body implants underwent normal adult development in concert with the host irrespective of the time required for the host to initiate adult development. The number of mature eggs produced by the implanted ovary was small (an average of 22 chorionated ones compared to 125 per ovary in situ). But since the host's ovaries always made their normal complement of eggs, the space and nutrients available to the third implanted ovary was most likely limited. Therefore, in Table II, a score of 0 is assigned to an ovary which makes at least 12 chorionated eggs. These results indicate that in Cecropia by the time of pupal ecdysis the viscera have completed pupal development and are competent to undergo adult development immediately.

To assess the effect of JH on the larval-pupal transformation of the viscera, prepupae were treated at the onset of ocellar retraction with a dose of C18-JH or a JH mimic that prevented the metamorphosis of the viscera (> + 4 score). Five to six days later within 12 hours of pupal ecdysis the gonads and fat body from the treated animals were transplanted into diapausing host pupae. One day after the subsequent adult emergence of the host, the developmental status of the implant was scored as outlined in Table II.

Figure 1 shows the response of the implant as a function of the lag between the time of implantation and the subsequent onset of adult development of the

Score†	Testis*	Ovary	Fat body	
0	Greater than 90% mature sperm.	At least 12 chorionated** and 12 chorionating eggs.	Adult.	
+1	75–90% mature sperm, re- mainder of stages II, III and IV.	5–11 chorionated eggs and some chorionating follicles.	Adult with trace dis- sociated fat body.	
+2	50-75% mature sperm; remainder of stages II to IV.	Up to 4 chorionated eggs; mainly small vitellogenic follicles.	Mixture of adult and granular type.	
+3	Few mature; less than 50% stage IV; remainder stages I-III.	No chorionated eggs; many small vitellogenic follicles.	Granular along trachea, neither pupal nor adult in appearance.	
+4	Less than 10% stage IV; mainly stages I and II.	Ovarioles grown out with distinct follicles but little or no volk deposited.	Friable.	
+5	All stage I: pupal.	Pupal.	Pupal.	

TABLE II

Assessment of developmental status of viscera at time of pupal ecdysis by implantation and subsequent differentiation during adult development of host pupa.

 $\dagger 0$  indicates that viscera were pupal at time of pupal ecdysis and implantation;  $\pm 5$  indicates that viscera were larval at the time.

\* The stages of spermatogenesis are those given by Kambysellis and Williams (1971).

\*\* Average of 22 chorionated and 12 chorionating eggs among the 14 control implants.

host. When the host pupa initiated adult development within three days of implantation, the implants showed only traces of adult characters and responded quite similarly to organs left *in situ* in JH-treated animals. In contrast to the control implants, then, these JH-treated viscera had not completed pupal differentiation by the time of pupal ecdysis so were unable to undergo adult development. But if development of the host pupae was delayed, the implant rapidly became competent to undergo adult development; and by three to four weeks all implants were able to differentiate into adult structures in concert with the host. As seen in Figure 1 the loss of these effects and the acquisition of competence for adult differentiation occurred more rapidly after treatment of prepupae with C18-JH than with the more stable JH mimics. Thus, when treated with JH at the onset of the prepupal period, the viscera are unable to complete the larvalpupal transformation by the time of pupal ecdysis but can complete it in the JHfree environment of the diapausing pupal host.

My previous studies (Riddiford, 1972) had indicated that JH treatment at the white prepupal stage (just before pupal ecdysis) had a definite but less pronounced effect on the metamorphosis of the viscera. In this instance, the effect might not be on the larval-pupal transformation of viscera which had been in progress for 3 to 4 days at the time of application but rather only on their pupaladult transformation which began about 2 to 3 days after application. In order to differentiate between these two possibilities, white prepupae were treated with 50  $\mu$ g EGS, and the gonads and fat body subsequently removed about 26 hours later within 12 hours of pupal ecdysis. As seen in Figure 1, when the host pupae immediately initiated adult development, the implants showed little adult differentiation, similar to those from animals treated with EGS at the outset of pupal development. But the time necessary for recovery was much faster as would be expected since the larval-pupal transformation was nearly complete at the time of JH application.

## Role of brain and prothoracic glands in pupal development of JH-treated viscera

The above results clearly indicated that the JH-blocked viscera could gain competence to undergo adult development when they were implanted into diapausing pupae. To determine if this recovery was influenced by the endocrine environment of the host, the effects of brain and prothoracic gland removal were examined.

For assay of the role of the brain, brains were removed from the diapausing pupae. The organ from JH-treated prepupae was removed immediately after pupal ecdysis and implanted into the brainless host; then  $\beta$ -ecdysone was injected at specific times thereafter. The time of recovery of the implant in a debrained pupa was found to be the same as that for an intact host. Therefore, the data in Figure 1 are a composite of implants into hosts with and without brains.

To assess the effects of the prothoracic glands on the recovery of the viscera, gonads were removed from the JH-treated individuals and placed into isolated diapausing pupal abdomens. After 14, 21, or 36 days the implants were removed and implanted into chilled diapausing host pupae to assay their developmental status. In no instance did the gonad undergo adult differentiation when adult development of the host began immediately. In fact, the developmental status of

Stage of donor	Time for host to initiate development	Number implants	Developmental capacity of implant average score $\pm$ s.d.
4th instar	1-3 days	4	$4.8 \pm 0.5$
(1-2 g)	$3\frac{1}{2}$ months*	3	$2.7 \pm 1.5$
5th instar	1-3 days	5	$5.0 \pm 0.0$
(2-8 g)	5-8 days	4	$4.5 \pm 0.6$
	10-14 days	6	$3.2 \pm 0.9$
	$3\frac{1}{2}$ months*	7	$1.2 \pm 1.4$
Gut evacuation	5-8 days	1	4.0
	$3\frac{1}{2}$ months*	2	$0.5 \pm 0.7$

## TABLE III Developmental capacity of larval gonads after varying lengths of time in a diapausing pupa.

\* Brainless host pupae injected with 12.5  $\mu$ g  $\beta$ -ecdysone  $3\frac{1}{2}$  months after implantation.

the implant had not changed during the 14 to 36 days in the isolated abdomens. In Figure 1 all implants from isolated abdomens are grouped together as if they had been transplanted into the chilled host after 21 days. The data show that the treated gonads did not become competent to undergo adult development until after they were placed into the intact pupa. Then the kinetics of recovery was essentially the same as that seen after implantation of the treated gonad immediately after pupation. The average score of 1.6 at 12 days is for 6 implants, 4 of which formed normal adult structures whereas both ovaries from one treated individual remained pupal. Apparently then the presence of prothoracic glands was essential for the completion of the pupal transformation after the JH effects had decayed.

## Capacity of larval viscera for adult differentiation

Since the hormonal environment of an intact or brainless diapausing pupa was sufficient to allow organs from JH-treated prepupae to attain competence for adult differentiation, it was of interest to determine whether organs from feeding larvae could also undergo the pupal transformation in a diapausing pupal host.

The gonads of 4th and 5th instar larvae of known age were transplanted into diapausing host pupae. Table III shows that when the host pupa initiated adult development within 8 days after implantation, the larval gonad was not capable of undergoing adult development. When development of the host was delayed for 10 to 14 days, then the implant showed some adult development forming a few mature sperm or oocytes but remained less than half normal adult size. After  $3\frac{1}{2}$ months in a brainless diapausing host pupa, the testis was able to mature fully in spite of its small size. The ovary was able to form some chorionated eggs and at least an equal number of vitellogenic follicles but many fewer than implants from freshly ecdysed pupae. The lack of the period of growth of the ovary which normally occurs during the larval-pupal transformation may account for the fewer developing follicles and could explain the disparity between the developmental capacities of the ovary and the testis. These experiments however clearly indicate that when placed in a IH-free environment under the influence of the prothoracic glands, the larval gonad can undergo pupal development without the concomitant growth that normally occurs during the larval-pupal transformation.

## DISCUSSION

During larval life the gonads grow slowly. Then when the JH titer declines and metamorphosis begins, they show an increased rate of growth and an initial differentiation prerequisite for subsequent adult development (Sehnal, 1968). The results obtained here show that gonads from early final instar larvae can be induced to make adult structures in response to ecdysone after being held in a diapausing host pupa for several weeks. During this holding period, conditions in the host pupa were apparently suitable for these organs to undergo their initial pupal differentiation. Since these larval implants never attained normal adult size, it is obvious that the period of enhanced growth is not necessary for their larval-pupal transformation.

This initial differentiation of the viscera to the pupal condition requires ecdysone in the presence of little or no JH (Williams, 1961). Juvenile hormone treatment of the prepupa prevented pupal differentiation, and treated viscera showed no recovery after 2 to 5 weeks in isolated abdomens. But after reimplantation into intact pupae, pupal differentiation occurred during the ensuing two weeks before adult development was initiated, and the implants completed adult differentiation in concert with their hosts. Since recovery of treated implants occurred at the same rate, irrespective of whether or not the host pupa had a brain, this process was undoubtedly due to ecdysone release from the prothoracic glands.

Normally, the prothoracic glands of Cecropia secrete sufficient ecdysone to initiate molting only after activation by the prothoracicotropic hormone from the brain (Williams, 1952). But wounding can subliminally activate these glands in Cecropia, and in other saturniid species can fully activate them (McDaniel and Berry, 1967). Furthermore, debrained tobacco hornworm (*Manduca sexta*) pupae can nevertheless eventually develop (Judy, 1972). Therefore, it seems likely that a small amount of ecdysone may "leak out" of the prothoracic glands in the wounded diapausing pupal hosts. While this ecdysone is insufficient to trigger adult development, it is sufficient for the completion of pupal differentiation of the implant. This amount of ecdysone also allows pupal differentiation of the larval gonad but appears inadequate to induce the increased growth rate normally seen at the outset of metamorphosis.

The recovery of the JH-treated viscera in host pupae can be divided into three phases: 1) the decay of the exogenous hormone; 2) the decay of the covert effects of the hormone; and 3) the subsequent completion of the larval-pupal transformation. When the third phase is completed, the pupal viscera are able to respond to ecdysone in the absence of JH and become adult. Similar recovery after JH treatment was noted by Sehnal and Schneiderman (1973) in the waxmoth, *Galleria mellonella*.

The decay of exogenous hormone is quite rapid—the half-life of Cecropia C18-JH in the blood is at most two hours (Ajami and Riddiford, 1973). The half-life of the JH mimics is somewhat longer (Staal, 1975) and could be seen from their effects on the integument when adult development began about 10 days after application. Thus, the organs from JH-mimic treated prepupae were exposed to a higher level of JH at the time of their removal and consequently, as seen in Figure 1, required a longer time to attain competence for adult differentiation. It is interesting in this context that only a rare host with an implant formed pupal

cuticle at the wound site indicating that the implant *per se* did not contain significant amounts of unbound hormone.

The separation of the last two phases of recovery-the decay of covert effects and the completion of larval-pupal differentiation-is more difficult. One difference between the two is that the latter requires the presence of ecdysone whereas the former apparently does not. Kimura (1974) and Fain and Riddiford (1973) have shown that the covert effects of JH that are prerequisite for a larval molt have completely decayed by 48 hours and 72 hours in isolated abdomens of Bombyx mori and Manduca sexta respectively. Thus, after 2 to 5 weeks in an isolated abdomen the Cecropia implants were presumably free of exogenous JH and its covert effects. The subsequent time then required for these organs to recover after implantation into diapausing hosts must reflect the time needed to complete differentiation to the pupal condition. As seen in Figure 1, this time is only slightly shorter than that required by implants taken directly from <sup>18</sup>C-JH-treated individuals. Thus, the recovery rates indicated by the curves in Figure 1 reflect primarily the time required for the completion of pupal differentiation after the effects of juvenile hormone have disappeared. Although normally pupal differentiation in Cecropia requires 4 to 5 days (Williams, 1952), the increased time found necessary in these experiments likely is due to the very low level of ecdysone in the diapausing hosts.

Presumably in Cecropia as in *Manduca* (Bollenbacher, Vedeckis, Gilbert and O'Connor, 1975) there is a high titer of ecdysone at the onset of the prepupal period. This large amount apparently is necessary to initiate pupal cuticle synthesis and also probably accounts for the growth of the gonads. The experiments reported here, however, indicate that pupal differentiation of the viscera can occur in the presence of much lower amounts of ecdysone. But this differentiation of the viscera requires the virtual absence of JH throughout the prepupal period. In the epidermis, only the commitment to pupal differentiation which occurs in response to the first ecdysone release that initiates gut evacuation (Truman and Riddiford, 1974; Bollenbacher *et al.*, 1975) can be prevented by JH (Riddiford, 1972; Truman, Riddiford and Safranek, 1974). After this time epidermal differentiation becomes aloof to JH. Thus, the epidermis and viscera differ not only in the timing of their irrevocable switchover from larval to pupal commitment, but also in the amount of ecdysone necessary for the expression of that commitment.

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## Summary

1. Cecropia C18-juvenile hormone (C18-JH) when given to Cecropia larvae at the onset of pupal development prevented metamorphosis of the viscera but had

little effect on the integument as had been previously reported for a mixture of JH mimics (Riddiford, 1972).

2. The developmental status of the viscera of freshly ecdysed pupae which had been treated with juvenile hormone (C18-JH or JH mimics) as prepupae was ascertained by transplantation into normal host pupae.

3. Recovery as signaled by the completion of the larval-pupal transformation of these implanted viscera occurred in diapausing pupae with or without brains but not in isolated pupal abdomens. Thus, ecdysone is necessary for the resumption of differentiation after JH and its effects have decayed.

4. Similarly, pupal differentiation of larval gonads occurred during  $3\frac{1}{2}$  months in a diapausing host pupa. Thus, for pupal differentiation of the viscera all that is required is a low level of ecdysone in the absence of JH.

5. The epidermis and the viscera thus differ in their hormonal requirements for pupal differentiation. The epidermis requires only an absence of JH during the time of commitment but not during its expression; furthermore, this expression requires a high level of ecdysone.

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