Archives of Insect Biochemistry and Physiology 15:183–199 (1990)

Ecdysteroids Control Vitellogenesis and Egg Maturation in Pharate Adult Females of the Indianmeal Moth, *Plodia interpunctella*

Paul D. Shirk, Daniel W. Bean, and Victor J. Brookes

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Florida (P.D.S.); Department of Entomology, Oregon State University, Corvallis, Oregon (D.W.B., V.J.B.)

Vitellogenesis occurs during the late pharate adult stage in the Indianmeal moth, *Plodia interpunctella*. Repeated treatment of pharate adult females with doses of 20–hydroxyecdysone (20HE) from 10 to 250 ng per pupa suppressed oocyte growth and inhibited yolk protein accumulation in the oocytes. Treatment of the pharate adults with a biologically inactive ecdysteroid analogue, 22–isoecdysone, had no effect on egg maturation or yolk protein accumulation. The hormonal action of 20HE was not through the inhibition of the corpora allata or juvenile hormone levels, because treatment with a juvenile hormone analogue did not reverse the inhibition by 20HE treatment. Exposure of early vitellogenic ovaries to 20HE in organ cultures in vitro showed that 20HE had a direct effect on the ovarian synthesis of YP2. At 20HE concentrations below 10 nM, YP2 synthesis was minimal, at 10 nM 20HE YP2 synthesis was maximal, and at concentrations higher than 10 nM YP2 synthesis was suppressed to 35% of the maximal level. Synthesis of most other ovarian proteins remained constant with the changing 20HE concentrations. Ovarian RNA from treated females translated in a reticulocyte lysate demonstrated that the hormonal effect of 20HE on the ovarian tissues was on the specific accumulation of translatable YP2 transcript as well as transcripts for a few other polypeptides. This study shows that 20HE controls the rate of egg development during metamorphosis and that declining titers of 20HE regulate the expression of adult genes.

Key words: juvenile hormone, metamorphosis, oocyte development, in vitro translation, yolk proteins

Acknowledgments: We thank K. Ogren and A. Smith for technical assistance. The 22-isoecdysone was a gift from Dr. H. Oberlander. The methoprene was provided by Dr. G. Staal, Zoecon Corp. This work was supported in part by a grant from the Department of Defense (DAAG 29-81-K-0124) to V.J.B. Mention of a proprietary product does not constitute an endorsement by the USDA. These data were presented in part at the UCLA Symposium on Molecular Entomology, 1986 [29].

Received June 15, 1990; accepted August 6, 1990.

Daniel W. Bean is now at the University of North Carolina, Chapel Hill.

INTRODUCTION

The endocrine regulation of vitellogenesis in most insects relies on the stimulation of yolk protein production by JH,* ecdysteroids, and/or neurohormones or combinations of these hormones during the adult stage [1–3]. The coordination of the vitellogenic cycle and the organization of the endocrine regulation employed to control the cycles can be related to the feeding behaviors of the adults [1]. Three models for endocrine regulation of yolk protein production presented by Bownes were organized around the information available for the fruitfly, mosquitoes, locusts, and cockroaches [1]. The fruitfly *Drosophila melanogaster* feeds continuously as an adult and with adequate diet produces eggs continuously. No cycling of egg production occurs under these conditions, and the ecdysteroids and JH appear to perform a permissive role in promoting yolk protein production and are present continuously. In cockroaches and locusts, which also feed continuously, egg production is cyclic, and the maturation of each egg brood is regulated by changing titers of JH; as the titer of JH increases, yolk protein synthesis increases. For anautogenous adult female mosquitoes, the cycle of egg production is tied to the consumption of a blood meal. The regulation of the various physiological components of oocyte maturation and vitellogenesis is dependent on the interactive functions of ecdysteroids, JH, egg development neurohormone, and oostatic hormone. In all these insects, egg production occurs in the adult stage, and yolk protein synthesis is stimulated by ecdysteroids and/or JH [1–3].

In contrast to most insects, several species of Lepidoptera in the families Saturniidae, Bombycidae, and Pyralidae have nonfeeding, short-lived adults. These species achieve sexual competency rapidly as an adult, and at adult eclosion females contain completely mature or nearly mature oocytes, which requires that the majority of oocyte maturation, including vitellogenesis, takes place during the pharate adult stage. Although ovarian differentiation is stimulated by ecdysteroids in these insects [4–8], little is known about the endocrine regulation of yolk protein synthesis in these moths.

The Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), has a short-lived adult and completes most of oocyte maturation during the late pharate adult stage [9]. The mature oocytes contain two major proteins that are each composed of two unique subunits, designated yolk polypeptides [9,10]. Vitellogenin is synthesized and secreted from the fat body and consists of subunits YP1 (153,000 daltons) and YP3 (43,000 daltons). The oocytes also contain a major protein that is synthesized within the ovaries and consists of subunits YP2 (69,000 daltons) and YP4 (33,000 daltons). Since vitellogenesis occurs as a late metamorphic event in this moth, the possibility that the same endocrine mechanisms that control metamorphosis would control the rate of yolk protein synthesis was considered. In the tobacco hornworm, *Manduca sexta*, the rate of metamorphosis, as measured by cuticular darkening, was shown to be regulated by declining levels of ecdysteroids [11]. Although cuticular melanization gave an indirect indication that ecdysteroids

*Abbreviations used: 20HE = 20-hydroxyecdysone; 22IE = 22-isoecdysone; JH = juvenile hormone; JHA = juvenile hormone analogue; NaOAc = sodium acetate; SDS = sodium dodecyl sulfate; TCA = trichloroacetic acid; YP = yolk polypeptide.

were regulating gene activity during metamorphosis, no direct effect of 20HE treatment on gene activity was shown. In this study, we examined the effect of ecdysteroids on the regulation of egg production and yolk protein synthesis in the late pharate adult females of the Indianmeal moth with the intent of determining if ecdysteroids have a direct effect on gene activity during metamorphosis.

MATERIALS AND METHODS

Insect Preparations

The *P. interpunctella* colony was reared according to Silhacek and Miller [12] in a 16 h light:8 h dark cycle at 27°C and 70% relative humidity. Pupae were staged as described by Smithwick and Brady [13]. It was determined by dissection that vitellogenesis began approximately 85 h after pupation, when the pupae showed external morphological characters of tan body color, black eyes, and melanized tarsal claws. The onset of vitellogenesis was marked by the appearance of opaque yolk in the oocytes.

Ovarian Transplantation

Newly pupated females and males were washed in 70% ethanol and immersed in O°C Weevers saline [14] until narcotized. The ovaries were then surgically removed from the females and washed three times with fresh sterile saline. A pair of ovaries were implanted into a male by cutting a slit in the cuticle of the fourth abdominal segment just lateral of the middorsal line and inserting the ovaries deep into the hemocoel through the opening. The males were removed from the cold saline and dried, and a 1:1 mixture of crystalline penicillin/streptomycin was then introduced into the wound, and the wound was sealed with beeswax. The host males were allowed to complete metamorphosis in normal culture conditions. After the males eclosed as adults, the ovaries were removed by dissection. The ovaries were homogenized in SDS sample buffer [15] and prepared for SDS-PAGE.

Ecdysteroid And JH Analog Treatments

Newly pupated females were collected and observed until they had reached the tan cuticle-black eye stage prior to melanization of the tarsal claws. The females were injected abdominally with either 20HE (Rohto Pharmaceutical Co. Ltd., Osaka, Japan) or 22IE at the designated concentrations in 0.2 μl of 10% ethanol in Weevers saline every 12 h until killed. As one control group, subsequently referred to as saline injected, females were injected with 10% ethanol in Weevers saline. Methoprene was dissolved in acetone at the designated amounts and administered to the pharate pupae by applying 1 μl of the solution topically once daily for the duration of the treatment period.

Organ Culture of Ovaries

Early vitellogenic female pupae (tarsal claws melanized) were immersed in 70% ethanol for 30 s and then rinsed three times in sterile saline. The pupae were then immersed in sterile saline and dissected. After the reproductive tract was removed intact and cleaned of adhering fat body, the ovaries were

washed three times in sterile saline. The ovaries were finally transferred to Grace's insect medium (Gibco, Grand Island, NY) plus 0.5% fetal calf serum fraction 5 (Gibco) containing varying concentrations of 20HE. Three pairs of ovaries were cocultured in each concentration of hormone. The cultures were placed in a 100% O_2 atmosphere and incubated at 30°C for 22 h. After 22 h, the culture medium was removed, and ovaries were washed once with fresh Grace's insect medium without calf serum at the appropriate concentration of 20HE. The cultures were then transferred to Grace's insect medium without calf serum, with the appropriate concentration of 20HE, plus [^{35}S]methionine (0.5 μCi/μl) and incubated for 2 h under the previous conditions. At the end of 2 h, the medium and tissue from each culture were collected and prepared for SDS-PAGE.

Radio-Labeling, Detection, and Quantification of Yolk Proteins

To label the newly synthesized yolk proteins in vivo, females were injected with 10 μCi of [^{35}S]methionine (1,200 Ci/mmol; NEN, Boston, MA) in 0.2 μl of saline. After 2 h, the moths were chilled to 0°C and then bled and dissected, and the ovaries and abdominal body walls (primarily fat body) were collected separately for homogenization in 100 μl saline. The amount of labeled protein was determined by spotting an aliquot of the sample on a 1 cm^2 of Whatman No. 1 filter paper and precipitating the proteins in cold 10% TCA; boiling in 5% TCA; washing in water, ethanol, and acetone; and counting in a Minaxi β Tri-Carb 4000 liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Protein content was determined on two aliquots of each sample using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Ovarian RNA was isolated by precipitation in the presence of LiCl/urea [16,17]. Ovaries were homogenized in 3 M LiCl, 6 M urea, 10 mM NaOAc, pH 5.0, 0.2 mg/ml heparin, and 0.1% SDS. The homogenate was kept 12 h at 0°C, and then centrifuged 15 min at 14,000*g* and 0°C. The pellet was resuspended in 4 M LiCl and 8 M urea and centrifuged as before. The pellet was then dissolved in 0.1 M NaOAc, pH 5.0, and 0.1% SDS and then precipitated at −20°C in 2 volumes of ethanol. The preparation was centrifuged 15 min at 14,000*g* and −20°C. The supernatant was drained, and the RNA pellet was partially dried before being dissolved in 10 mM Tris HCl, pH 7.5, and 2 mM EDTA. Equal A_{260} units of ovarian RNA were translated in a cell-free rabbit reticulocyte lysate (NEN) in the presence of [^{35}S] methionine.

Proteins were denatured by boiling in SDS sample buffer. Equal quantities of protein or TCA-precipitable radiolabeled protein were resolved on 8–15% gradient SDS-PAGE as described previously [9,18]. The gels either were stained in Coomassie blue R250, dried, and autoradiographed using Kodak X-Omat AR X-ray film or were stained with silver [19]. The autoradiograms were scanned using a 2222 Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ), and the scans were analyzed using 2400 GelScan XL software (Pharmacia LKB Biotechnology). The data were analyzed statistically by a completely randomized, one-way analysis of variance and the means separated with Duncan's multiple range test [20] using STATPAC software (Northwest Analytical, Portland, OR).

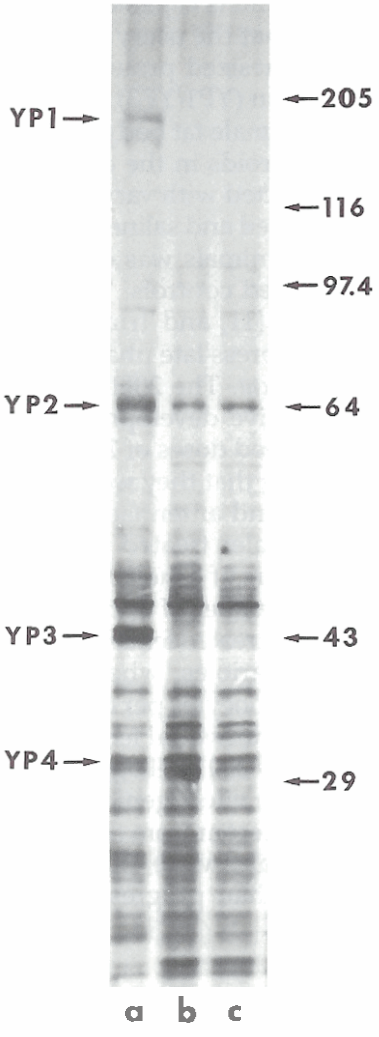

Fig. 1. Accumulation of ovarian yolk proteins in ovaries that metamorphosed in males. Ovarian proteins were resolved by SDS-PAGE and the gel was stained with silver. **Lane a:** Ovaries from normal females. **Lanes b,c:** Ovaries that completed metamorphosis in males. Position of the yolk polypeptides are marked on the left, and the molecular weight markers (×1,000) on the right.

RESULTS

To determine if ovarian growth was dependent on specific humoral components, ovaries from newly pupated females were transplanted into male pupae, and the males were allowed to complete metamorphosis. After metamorphosis was complete, the appearance of the ovaries within the males was similar to ovaries from normal females. When the ovarian proteins from male ovaries were resolved by SDS-PAGE and compared with normal ovaries from females, the ovarian polypeptides YP2 and YP4 were observed as the major protein in the oocytes that had completed metamorphosis in males; no vitellin (YP1/YP3) synthesized by the fat body was detectable (Fig. 1). This demon-

strates that the male environment is capable of supporting the differentiation and growth of the ovary and that the ovary remains competent to synthesize and package the ovarian synthesized proteins. In addition, because the oocytes did not contain any vitellin (YP1/YP3), we conclude that the presence of the ovary did not stimulate the male fat body to produce vitellogenin.

To assess the role of ecdysteroids in the control of vitellogenesis, previtellogenic female pupae were injected with varying concentrations of 20HE twice daily for 3 days until the untreated and saline-treated cohorts eclosed as adults. Eclosion of the 20HE-treated animals was delayed past the time of eclosion for untreated and saline-injected controls. This result is consistent with the work of Schwartz and Truman [11] and Truman [21], and Truman et al. [22], showing that ecdysteroids suppress late pharate adult development and eclosion hormone release in *M. sexta*. The 20HE treated *P. interpunctella* females showed a gradient of progressive development correlated with the dose of 20HE. Those females that received doses of 250 ng 20HE showed external morphological characters indicating that they were retarded almost 24 h in development (i.e., black eyes, legs and antennae darkened, and integument over wings light brown). Those females treated with 10 ng 20HE showed normal external morphological characters of a moth just prior to eclosion. The females were dissected, and the total number of oocytes showing any signs of vitellogenesis was counted. Normal untreated females contained 24 ($\pm$2.4) vitellogenic oocytes in each of the eight ovarioles, whereas saline-injected females contained 25.75 ($\pm$1.7) vitellogenic oocytes per ovariole (Fig. 2). As expected from the number of eggs laid by an individual female, normal newly eclosed adult females contained approximately 200 vitellogenic oocytes. Females treated with 10 ng 20HE contained 21 ($\pm$1.9) vitellogenic oocytes per ovariole, which was not statistically significantly different from the controls. However, the oocytes in the 10 ng 20HE-treated females were all in early stages of vitellogenesis, whereas those in the controls had progressed to mature oocytes (data not

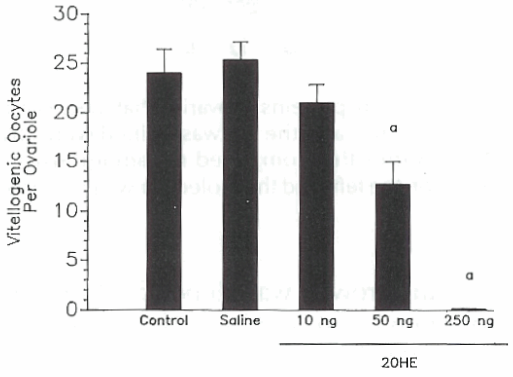

Fig. 2. Number of vitellogenic oocytes per ovariole found in ovaries of females treated with varying concentrations of 20HE. Oocytes were counted as vitellogenic if yolk platelets were observed in the oocyte, but the degree of yolk deposition was not considered. Data were analyzed statistically using ANOVA, and the means were separated using Duncan's multiple range test. Bars represent the mean with standard error (N = 10–16). a: Statistically different from control at 0.01 level of significance.

shown). Females treated with 50 and 250 ng 20HE contained 12.7 (±2.3) and 0.06 (±0.06) vitellogenic oocytes per ovariole, respectively, and thus had significantly fewer vitellogenic oocytes per ovariole from nontreated and saline injected females (Fig. 2).

To determine if the suppression of oocyte development was a specific effect of 20HE, females were treated with 22IE, a hormonally inactive ecdysone analogue [23], following the twice daily dose regime as described for 20HE. Because the number of vitellogenic oocytes per ovariole did not accurately reflect the level of vitellogenesis in the treated females, the total amount of protein accumulated in the ovaries was used as a quantitative measure of vitellogenic activity. As before, the females were treated until the untreated cohorts eclosed as adults, and then the ovaries were removed, and the total protein present in the ovaries was measured. None of the 20HE-treated females eclosed as adults. Ovaries of normal untreated females contained 457 (±21) μg of total protein, and the saline injected females contained 383 (±65) μg of total protein (Table 1). Compared with the untreated and saline-treated controls, treatment with the varying concentrations of 20HE showed a statistically significant decrease in the amount of protein in the ovaries of females at all doses: 230 (±36) μg at 10 ng, 117 (±30) μg at 50 ng, and 46 (±4) μg at 250 ng (Table 1). The amount of protein present in the ovaries of 250 ng 20HE-treated females was tenfold less than the amount of protein present in ovaries of normal newly eclosed adult females. Treatment with 22IE did not produce a statistically significant decrease in the amount of protein in the ovaries at any of the doses except a marginally significant decrease at 250 ng 22IE compared with the untreated controls, but the amount was not significantly different compared with the saline-treated controls. When the 20HE treatments were compared with the 22IE treatments, the two higher doses of 20HE reduced the amount of ovarian protein to levels significantly lower than the 22IE doses (Table 1).

TABLE 1. Comparison of the Effects of 20HE and 22IE Treatments on the Amount of Protein Accumulating in Vitellogenic Oocytes in Ovaries of Female Indianmeal Moths†

	Mean (μg) protein per ovary (S.E.)		20HE			22IE		
		S	10	50	250	10	50	250
UT	457 (21)	–[a]	‡[b]	*[c]	*	–	–	†
S	383 (65)		‡	*	*	–	–	–
20HE 10	230 (36)			–	*	–	‡	–
20HE 50	117 (30)					*	*	‡
20HE 250	46 (4)							
22IE 10	359 (57)						–	–
22IE 50	365 (56)							–
22IE 250	284 (43)							

†Data were analyzed statistically using ANOVA, and the means were separated using Duncan's multiple range test.

N = 10 moths per treatment.

Abbreviations: UT, untreated controls; S, saline-treated; 20HE 10, 10 ng 20HE-treated; 20HE 50, 50 ng 20HE-treated; 20HE 250, 250 ng 20HE-treated; 22IE 10, 10 ng 22IE-treated; 22IE 50, 50 ng 22IE-treated; 22IE 250, 250 ng 22IE-treated.

[a] –, ≥0.05 level of significance.

[b] ‡, ≤0.05 level of significance.

[c] *, ≤0.01 level of significance.

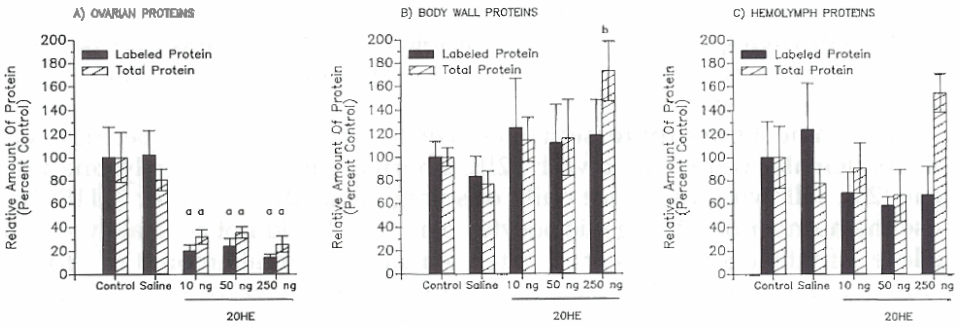

Fig. 3. Protein content and amount of protein synthesis in tissues of females treated with varying concentrations of 20HE. Amounts of protein labeling (closed bars) and total protein (hatched bars) as a percent of the untreated control for ovaries (A), body walls (B), and hemolymph (C). Data analyzed statistically using ANOVA and the means were separated using Duncan's multiple range test. Bars represent the mean with standard error (N = 6). [a]Statistically different from control at 0.01 level of significance. [b]Statistically different from control at 0.05 level of significance.

Tissue-specific effects of 20HE treatment on the amount of protein present in three tissues were examined. Females were injected with 20HE as before until the untreated cohorts had eclosed as adults, and then all were injected with [^{35}S]methionine and allowed to incorporate the labeled amino acid into nascent peptides. The hemolymph, ovaries, and body walls were collected separately, and the total amount of protein and incorporated label were determined. No significant changes in the amount of radiolabeled proteins biosynthesized during the 2 h incubation were observed in the hemolymph or body wall preparations (Fig. 3B,C). The 20HE treatment did not reduce protein synthesis as part of a general suppression of metabolic activity, but affected vitellogenesis specifically. Additionally, YP1/YP3 was not accumulating in the hemolymph to any significant degree, which was confirmed by SDS-PAGE and autoradiography of hemolymph samples (data not shown). This ruled out the possibility that YP1/YP3 was being synthesized at a normal rate by the fat body but was not taken up by the oocytes. No significant changes in the total amount of protein present in the hemolymph and body wall were observed except for a statistically significant increase in the total amount of protein present in the body wall preparations of the 250 ng 20HE-treated females. The greater amount of protein in the fat body of the 250 ng 20HE-treated females was probably due to the maintenance of storage protein granules since the tissue had not progressed in synthetic production of vitellogenin. As before, significantly less total protein was observed in the ovaries of the 20HE-treated females, and in addition significantly less labeled protein was present in the ovaries (Fig. 3A).

In some species, 20HE suppression of oocyte development is effected through the regulation of corpora allata activity [24–28]. Consequently, pharate adult *P. interpunctella* were treated concomitantly with both 20HE and a JHA (methoprene) to determine if JH treatment would rescue vitellogenic activity in 20HE-suppressed females. As a control, the effects of treating pharate adult females with the JHA alone were tested. JHA was administered once daily with vary-

TABLE 2. Combined Effects of Treatment of Pharate Adult Females with 250 ng 20HE and Varying Concentrations of a JHA†

	Mean (μg) protein per ovary (S.E.)		ng 20HE					
			250	250	250	250	250	250
			μg JHA					
			0	0.1	0.5	1.0	5.0	25
Untreated	550	(31)	*[d]	*	*	*	*	
0 μg JHA[a]	65	(5)		–[b]	–		–	
0.1 μg JHA[a]	96	(7)			–	–	–	‡[c]
0.5 μg JHA[a]	106	(11)				–	–	–
1 μg JHA[a]	135	(18)					–	–
5 μg JHA[a]	111	(5)						–
5 μg JHA[a]	148	(20)						

†Data were analyzed statistically using ANOVA, and the means were separated using Duncan's multiple range test. N = 10 moths per treatment.
[a]Injected with 250 ng 20HE.
[b] –, ≥0.05 level of significance.
[c]‡, ≤0.05 level of significance.
[d]*, ≤0.01 level of significance.

ing concentrations ranging from 50 ng to 50 μg beginning during the previtellogenic stage and continuing until the untreated moths had eclosed. No significant change in the amount of protein accumulating in the ovaries was observed compared with untreated females (data not shown). To determine if JHA treatment would rescue the 20HE inhibition of oocyte development, 100 ng to 25 μg JHA was administered concomitantly with twice daily treatments of 250 ng 20HE. All 20HE- and JHA-treated females had statistically significantly less protein accumulating in the ovaries than did the untreated controls (Table 2). None of the JHA-treated females were significantly different from any of the other JHA-treated females except between the 0.1 μg level and the 25 μg level of JHA. The difference between the 20HE treatment alone and the JHA-treated females was not significantly different at the lower JHA concentrations but showed a small, variable response at the highest JHA concentrations.

The hormonal effect of 20HE on a specific tissue was tested by culturing early vitellogenic ovaries in vitro in varying concentrations of 20HE. The amount of YP2 synthesized and secreted into the medium was greatest in 10 nM 20HE (Fig. 4). Ovaries cultured in concentrations of 20HE from 0 M to 0.1 nM 20HE synthesized approximately 14% (significantly less) of the amount of YP2 produced in the 10 nM 20HE cultures (Table 3). The amount of YP2 synthesized in the cultures with 1 μM and 0.1 mM 20HE was significantly reduced, 40% and 35%, respectively, from the amounts in 10 nM 20HE (Fig. 4, Table 3). Whereas the amount of YP2 synthesized varied with the concentration of 20HE present in the culture, the amount of a 107,000 molecular weight protein synthesized in these cultures was unaffected by the changes in 20HE concentration and remained constant between the cultures (data not shown). In these cultures, 20HE had a direct effect on the ovarian tissues without any additional contribution from external hormonal components. In addition, the ovarian tissues were sensitive to the level of 20HE present and required a specific concentration of 20HE for maximal synthesis of YP2.

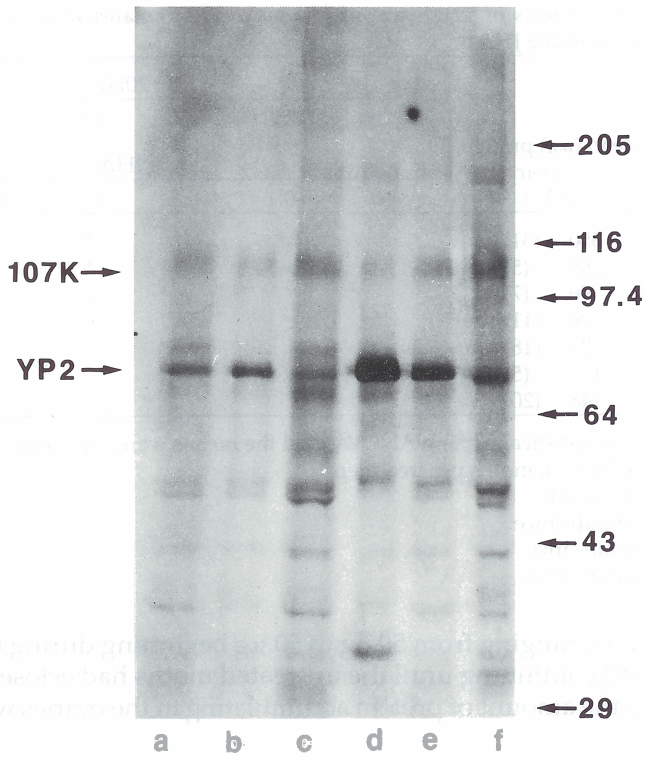

Fig. 4. Proteins secreted into culture medium by early vitellogenic ovaries cultured in the presence of varying concentrations of 20HE. Equal amounts of TCA-precipitable radiolabeled protein were layered per lane. **Lane a:** No 20HE. **Lane b:** 1 pM 20HE. **Lane c:** 0.1 nM 20HE. **Lane d:** 10 nM 20HE. **Lane e:** 10 μM 20HE. **Lane f:** 0.1 mM 20HE. Position of YP2 and a 107 K molecular weight polypeptide are marked on the left, and the molecular weight markers (×1,000) are marked on the right.

TABLE 3. 20HE Regulation of YP2 Synthesis in Early Vitellogenic Ovarioles Cultured In Vitro†

Concentration of 20HE	Mean (SE)	1 pM	0.1 nM	10 nM	1 μM	0.1 mM
0	1.1 (0.3)	–[a]	–	*[c]	–	–
1 pM	0.7 (0.3)		–	*	‡[b]	–
0.1 nM	0.8 (0.3)			*	‡	–
10 nM	4.9 (0.5)				*	*
1 μM	2.0 (0.4)					–
0.1 nM	1.7 (0.2)					

†Table shows the cross comparisons of the means in Figure 4. The units of the means are in AU_{633}/mm^2. Data were analyzed statistically using ANOVA, and the means were separated using Duncan's multiple range test.
N = 3 organ cultures containing three pairs of ovaries.
[a] –, ≥0.05 level of significance.
[b] ‡, ≤0.05 level of significance.
[c] *, ≤0.01 level of significance.

To assess the hormonal effect of 20HE on YP synthesis in a specific tissue, the regulation of the ovarian synthesis of YP2 was examined because 20HE was found to have a direct effect on the rate of YP2 synthesis in cultured ovarioles. In addition, the YP2 translate constituted a high percentage of the material synthesized by ovarian RNA translates, and the primary translate of YP2 was sufficiently resolved from other translation products which facilitated quantitation. Females were treated with 20HE as before until the untreated cohorts had eclosed. Total RNAs were extracted from the ovaries and translated in vitro. The polypeptides produced in the translates were resolved by SDS-PAGE and autoradiographed. The amount of YP2 polypeptide translate decreased with the increasing dose of 20HE (Fig. 5), and the decrease was statistically significant between the doses demonstrating that 20HE influenced the accumulation of translatable YP2 transcript in the ovarian tissues. The identity of the YP2 translate was previously confirmed by peptide mapping [29] and by immunoprecipitation (Shirk, unpublished). The amount of YP2 transcript synthesized in the 250 ng 20HE-treated females was 40-fold less than the saline treated controls. In addition to YP2, there were other translated polypeptides that decreased significantly with increasing doses as well, i.e., a 109,000 molecular weight polypeptide (Fig. 5). Most importantly, not all the translated polypeptides decreased with the increasing concentration of 20HE, i.e., 43,000 molecular weight polypeptide (Fig. 5), indicating that the response to 20HE was a specific hormonal effect on the accumulation of individual gene products.

DISCUSSION

In those moths that complete vitellogenesis during the pharate adult stage, inclusion of egg maturation in metamorphosis places constraints on the endocrine mechanisms available for regulating the production and uptake of yolk proteins. Because the events of adult metamorphosis require adherence to specific hormonal regimens in order to achieve correct temporal sequencing of developmental events [30], the hormonal mechanisms that have evolved for regulation of vitellogenesis in the adult stage of other insects would be inappropriate for regulating vitellogenesis during the pharate adult stage.

The differentiation and maturation of ovaries in insects appears to require two major points of regulation: the initiation of adult differentiation and the initiation of vitellogenesis. The separation of these two events is clearly demonstrated in anautogenous mosquitoes in which the ovaries differentiate during the pupal stage and the follicles progress to a previtellogenic stage when development is arrested until the consumption of a blood meal [reviewed in 1,2]; vitellogenesis is then stimulated by an endocrine cascade initiated by a blood meal. In *Drosophila* the separation of differentiation and vitellogenesis can be experimentally demonstrated by isolating the abdomens from the anterior endocrine organs at adult eclosion, which causes the oocytes to arrest at a previtellogenic stage [reviewed in 1]. Treatment of the isolated abdomens with ecdysteroids or juvenoids then stimulates some or all of the vitellogenic processes.

For some of the moths, the endocrine regulation of ovarian differentiation has been determined, but little is known about the control of the regulation of

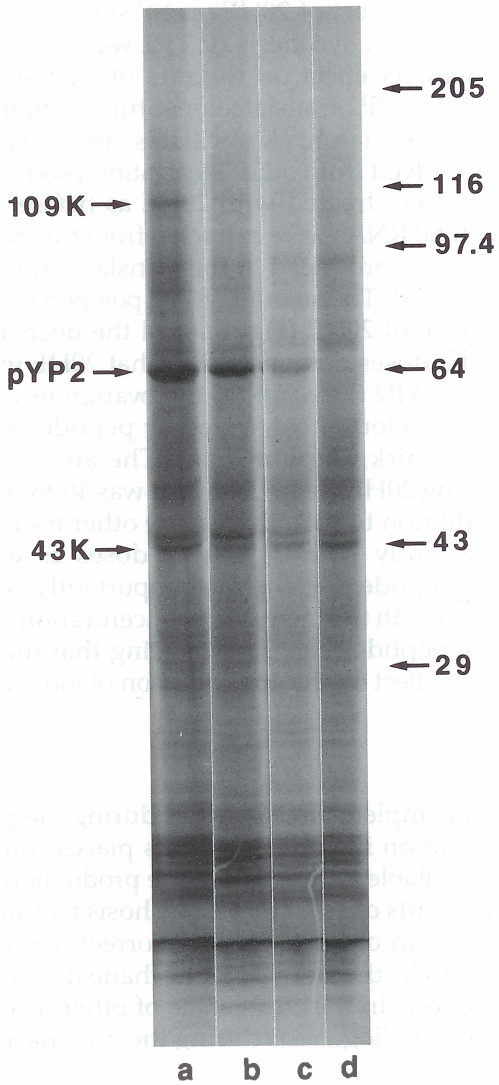

Fig. 5. Translation products from RNAs isolated from ovaries of females treated with varying concentrations of 20HE. Equal amounts of A_{260} units of RNA were translated, and equal amounts of TCA-precipitable radiolabeled protein were layered per lane. **Lane a:** Saline treated. **Lane b:** 10 ng 20HE. **Lane c:** 50 ng 20HE. **Lane d:** 250 ng 20HE. Position of the translates of YP2 (pYP2), 109,000 molecular weight polypeptide (109 K), and 43,000 molecular weight polypeptide (43 K) are marked on the left, and the molecular weight markers (×1,000) are on the right.

vitellogenesis. Initiation of ovarian differentiation is correlated with the early ecdysteroid peak in the pupal stage of *Bombyx mori* [8,31]. By isolating the abdomens of pupae prior to the initiation of adult development, ovarian differentiation is blocked in both *B. mori* [5–8] and *Hyalophora cecropia* [4] but can be stimulated by injection of ecdysteroids. Ovarian differentiation appears to be regulated in a similar manner in the pyralid moths as well. Ovarian differentiation is blocked in isolated abdomens of *P. interpunctella* and initiated only

after injection of the isolated abdomens with 20HE (Bean and Shirk, unpublished). Additionally, the ovaries of last-instar larvae of *Galleria mellonella* cultured in vitro in the presence of 20HE were found to differentiate, which led to the presence of identifiable oocytes, nurse cells, and follicular cells in the ovarioles [32].

Maturation of the ovaries and oocytes in either *B. mori* [33] or *P. interpunctella* does not depend on completing development in females. In both moths, ovaries that were transplanted into male pupae to complete metamorphosis became vitellogenic and produced chorionated oocytes even though they lacked vitellin. For *B. mori*, the mature oocytes were even shown to be viable by partheogenically activating the oocytes after removal from the males [33]. The maturation of ovaries in males suggests that there are no female-specific factors regulating ovarian development and oogenesis in these moths, i.e. the hormonal milieu regulating pupal-adult metamorphosis is similar if not identical in females and males of these moths.

By treating *P. interpunctella* female pupae with high levels of ecdysteroids from a point prior to the beginning of vitellogenesis, we found that vitellogenesis and oocyte maturation were arrested. Analysis of the ecdysteroid titers during the pupal stage has confirmed that the 20HE treatments were begun when the ecdysteroids were at the highest level (Shaaya, Shirk, and Silhacek, unpublished), and the 20HE injections probably maintained the titers above the normal levels. The effect of the 20HE treatment in *P. interpunctella* was similar to that in *M. sexta* where declining 20HE titers were shown to regulate the rate of developmental processes during metamorphosis [11]. This action of 20HE can also account for the observation that normal development in *B. mori* was delayed following injection of a single large dose of 20HE (100 μg/pupa) into 2-day-old pupae [34]. The 20HE treatment had the additional effect of initially delaying the normal increase in ovarian weight that would be expected if vitellogenesis was also delayed. Thus the 20HE suppression of egg development in moths that complete oogenesis during pharate adult development is in agreement with the initial hypothesis that vitellogenesis is under the general regulation of metamorphosis and requires a decline in ecdysteroid titers for the process to progress.

The suppression of oocyte maturation by 20HE in *P. interpunctella* is a direct hormonal effect and does not depend on a secondary endocrine modulator. This is unlike the suppression of ovarian growth by 20HE in the monarch butterfly, *Danaus plexippus* [24], the milkweed bug, *Oncopeltus fasciatus* [25,26], and the cockroach, *Diploptera punctata* [27,28] in which 20HE-suppressed ovarian growth is rescuable by treatment with JH. In both *O. fasciatus* [25,26] and *D. punctata* [27,28], the effect of 20HE was due to an inhibition of corpora allata activity. The 20HE suppression of ovarian growth in *P. interpunctella* could not be rescued by concomitant treatment with a JHA and is therefore probably not mediated through inhibition of the corpora allata activity. Although there was a marginal stimulation of protein accumulation in moths treated with the highest doses of JHA, the nature of the JHA action was not identified in these experiments and may have been the result of a pharmacological effect of the hormone analogue. The lack of an endocrine role for JH in regulating oocyte maturation in *P. interpunctella* is similar to that demonstrated in

the cecropia silkworm [35] and *Bombyx* [36–38], in which allatectomy of pupae had no observable effect on the subsequent egg production and viability.

The suppression of oocyte development by 20HE was the result of an endocrine action and not the consequence of a general pharmacological effect from steroid treatment, since equivalent treatments with 22IE, a biologically inactive ecdysteroid, did not have any effect on either oocyte development or the progress of metamorphosis. The action of 20HE on the suppression of yolk protein synthesis in *P. interpunctella* was at the cellular level on the accumulation of translatable YP transcript. Culturing early vitellogenic ovaries in the presence of varying concentrations of 20HE showed that YP2 synthesis in the tissues was directly regulated by the molar concentration of 20HE. The amount of YP2 synthesized was maximal at 10 nM 20HE and was either synthesized at a minimal rate at concentrations below 10 nM 20HE or decreased at concentrations greater than 10 nM. This suggests that 20HE must be present at a critical titer to promote YP2 synthesis and that the ovarian tissues monitor the titer of 20HE very accurately and respond differentially to the changing concentrations.

Similar criteria for ecdysteroid regulation of metamorphic events have been described for other tissues in several insects. The puffing patterns of glue protein gene loci in the polytene chromosomes of salivary glands in *D. melanogaster* responded in a concentration-dependent manner, and individual loci puffed or regressed at specific concentrations of 20HE [39]. Imaginal wing discs of *P. interpunctella* cultured in vitro began synthesis of cuticle only after a pulse and withdrawal of 20HE; continuous exposure of the wing discs to 20HE prevented chitin synthesis [40]. Similarly, cultured imaginal discs from *D. melanogaster* produce epicuticle and deposit procuticle only when exposed to a high level of 20HE and then moved to reduced levels [41]. In addition, tanning of epidermal structures in cultures of pupal cuticle [42] and larval crochets [43] was prevented by continuous exposure to 20HE.

Direct ecdysteroid suppression of gene activity has been demonstrated for only a few genes in insects. Chromosomal locus 25B, which contains the structural gene for the glue protein *Sgs.1*, is normally puffed in salivary gland polytene chromosomes of last instar *D. melanogaster*, but the locus regresses when the ecdysteroid titers are increased either in vivo or in vitro [44]. In *M. sexta* the appearance of dopa decarboxylase activity was shown to depend on an increase and subsequent decline in the titers of ecdysteroids during the last larval molt [45–47]. The increase in ecdysteroid titers at the last larval molt determined the subsequent appearance of dopa decarboxylase, and the declining titers regulated the temporal appearance of the transcript and enzyme. The control of YP2 synthesis appears to be similar to that of dopa decarboxylase because the differentiation of the ovaries requires an increase in ecdysteroids, and the synthesis of YP2 can begin only after the ecdysteroid titers decline.

Steroid hormone suppression of gene activity has also been observed in vertebrates. Glucocorticoids generally have a stimulatory effect on gene activity but have a negative regulation on the transcriptional activity of proopiomelanocortin and prolactin genes [48,49] and a negative response element has been delineated in the 5′ regulatory sequences for the prolactin gene [49]. Although the molecular action of the glucocorticoids is through direct regula-

tion of transcriptional activity, the estrogen-induced suppression of the 74 kDa albumin gene transcript in *Xenopus* was found to be a combination of deinduction of transcription and destabilization of the mRNA [50]. Whether the ecdysteroid suppression of yolk protein synthesis in *P. interpunctella* is directly on the transcriptional activity of the YP genes or is through the destabilization of the mRNAs has not been determined and requires further analysis.

Ecdysteroids exert two actions on tissues during metamorphosis; initially they provide the impetus for differentiation [4–8,32] and subsequently provide the meter for tissues to assess and coordinate the progress of metamorphosis [11]. In this work, we examined the role of ecdysteroids following the onset of adult differentiation and showed that declining ecdysteroid titers regulate the onset and progress of vitellogenesis in *P. interpunctella*. Since the ovarian tissues responded directly to the concentration of 20HE and not through secondary endocrine regulators, the analysis of YP2 synthesis in ovaries permits a direct quantitation of the 20HE effects during metamorphosis. Thus, the analysis of yolk polypeptide regulation in pharate adult *P. interpunctella* should provide an excellent model for determining the nature of the regulatory mechanisms that are cued by the ecdysteroid titers and operate to coordinate developmental events during metamorphosis.

LITERATURE CITED

1. Bownes M: Expression of the genes coding for vitellogenin (yolk protein). Annu Rev Entomol *31*, 507 (1986).
2. Hagedorn HH: The role of ecdysteroids in reproduction. In Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Vol. 8. Endocrinology II. Kerkut GA, Gilbert LI, eds. Pergamon Press, New York, pp 205–262 (1985).
3. Koeppe JK, Fuch M, Chen TT, Hunt L-M, Kovalick GE, Briers T: The role of juvenile hormone in reproduction. In Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Vol. 8. Endocrinology II. Kerkut GA, Gilbert LI, eds. Pergamon Press, New York, pp 165–204 (1985).
4. Williams CM: Physiology of insect diapause. IV. The brain and prothoracic gland as an endocrine system in the cecropia silkworm. Biol Bull *103*, 120 (1952).
5. Sakurai H, Hasegawa K: Response of isolated pupal abdomens of silkworms, *Bombyx mori* L., to injected ponasterone A (Lepidoptera: Bombycidae). Appl Entomol Zool *4*, 59 (1969).
6. Chatani R, Ohnishi E: Effect of ecdysone on the ovarian development of *Bombyx* silkworm. Dev Growth Differ *18*, 481 (1976).
7. Ohnishi E: Growth and maturation of ovaries in isolated abdomens of *Bombyx mori*: Response to ecdysteroids and other steroids. Zool Sci *4*, 315 (1987).
8. Tsuchida K, Nagata M, Suzuki A: Hormonal control of ovarian development in the silkworm, *Bombyx mori*. Arch Insect Biochem Physiol *5*, 167 (1987).
9. Shirk PD, Bean D, Millemann AM, Brookes VJ: Identification, synthesis, and characterization of the yolk polypeptides of *Plodia interpunctella*. J Exp Zool *232*, 87 (1984).
10. Bean DW, Shirk PD, Brookes VJ: Characterization of yolk proteins from the eggs of the Indian meal moth, *Plodia interpunctella*. Insect Biochem *18*, 199 (1988).
11. Schwartz LM, Truman JW: Hormonal control of rates of metamorphic development in the tobacco hornworm, *Manduca sexta*. Dev Biol *90*, 103 (1983).
12. Silhacek DL, Miller GL: Growth and development of the Indian meal moth *Plodia interpunctella* (Lepidoptera: Phycitidae), under laboratory mass-rearing conditions. Ann Entomol Soc Am *65*, 1084 (1972).
13. Smithwick EB, Brady UE: Integumentary pigmentation as an age index in Indian-meal moth pupae. J Georgia Entomol Soc *6*, 137 (1971).
14. Weevers RdG: A lepidopteran saline: Effects of inorganic cation concentrations on sensory, reflex, and motor responses in a herbivorous insect. J Exp Zool *44*, 163 (1966).

15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature *227*, 680 (1970).
16. Auffray C, Rougeon F: Purification of mouse immunoglobin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur J Biochem *107*, 303 (1980).
17. LeMeur M, Glanville N, Mandel JL, Gerlinger P, Palmiter R, Chambon P: The ovalbumin gene family: Hormonal control of X and Y gene transcription and mRNA accumulation. Cell *23*, 561 (1980).
18. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem *250*, 4007 (1975).
19. Wray W, Boulikas T, Wray VP, Hancock R: Silver staining of proteins in polyacrylamide gels. Anal Biochem *118*, 197 (1981).
20. Duncan, DB: A significance test for differences between ranked treatments in an analysis of variance. Virginia J Sci *2*, 171 (1951).
21. Truman JW: Interaction between ecdysteroids, eclosion hormone and bursicon titers in *Manduca sexta*. Am Zool *21*, 655 (1981).
22. Truman JW, Roundtree DB, Reiss SR, Schwartz LM: Ecdysteroids regulate the release of eclosion hormone in moths. J Insect Physiol *29*, 895 (1983).
23. Oberlander H: α-Ecdysone induced DNA synthesis in cultured wing disks of *Galleria mellonella*: Inhibition by 20-hydroxyecdysone and 22-isoecdysone. J Insect Physiol *18*, 223 (1972).
24. Herman WS, Barker JF: Ecdysterone antagonism, mimicry, and synergism of juvenile hormone action on the monarch butterfly reproductive tract. J Insect Physiol *22*, 643 (1976).
25. Aldrich JR: Inhibition of juvenile hormone synthesis by β-ecdysone in adult female milkweed bugs. Am Zool *20*, 900 (abstract) (1980).
26. Aldrich JR, Svoboda JA, Thompson MJ: Cuticle synthesis and inhibition of vitellogenesis: Makisterone A is more active than 20-hydroxyecdysone in female milkweed bugs. J Exp Zool *218*, 133 (1981).
27. Friedel T, Feyereisen R, Mundall EC, Tobe SS: The allatostatic effect of 20-hydroxyecdysone on the adult viviparous cockroach, *Diploptera punctata*. J Insect Physiol *26*, 665 (1980).
28. Stay B, Friedel T, Tobe SS, Mundall EC: Feedback control of juvenile hormone synthesis in cockroaches: Possible role for ecdysterone. Science *207*, 898 (1980).
29. Shirk PD, Brookes VJ: 20–Hydroxyecdysone suppresses yolk production in the Indianmeal moth. In: Molecular Entomology. Law JH, ed. Alan R. Liss, Inc., New York, pp 415–424 (1987).
30. Riddiford LM: Hormone action at the cellular level. In Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Vol. 8. Endocrinology II. Kerkut GA, Gilbert LI, eds. Pergamon Press, New York, pp 37–84 (1985).
31. Hanaoka K, Ohnishi E: Changes in ecdysone titre during pupal-adult development in the silkworm, *Bombyx mori*. J Insect Physiol *20*, 2375 (1974).
32. Shibuya I, Yagi S: Effects of ecdysteroids on cultivated ovaries of the greater wax moth larvae (Lepidoptera:Pyralidae). Appl Entomol Zool *7*, 97 (1972).
33. Yamashita O, Irie K: Larval hatching from vitellogenin-deficient eggs developed in male hosts of the silkworm. Nature *283*, 385 (1980).
34. Hamada N, Koga K, Hayashi K: Effects of a large dose of ecdysteroid on development of ovary in *Bombyx* pupae. J Faculty Agricult Kyushu Univ *28*, 23 (1983).
35. Williams CM: The juvenile hormone. I. Endocrine activity of the corpora allata of the adult cecropia silkworm. Biol Bull *116*, 323 (1959).
36. Bounhiol JJ: L'ablation des corps allates au dernier âge larvaire n'affecte pas, ultérieurement, la reproduction chez *Bombyx mori*. CR Acad Sci *215*, 334 (1942).
37. Fakuda S: The hormonal mechanism of larval molting and metamorphosis in the silkworm. J Faculty Sci Imperial Univ Tokyo, Sect IV, *6*, 477 (1944).
38. Bodenstein D: Untersuchungen zum Metamorphose-problem, III. Über die Entwicklung der Ovarien im thoraxen losen Puppenabdomen. Biol Z. *58*, 329 (1938).
39. Ashburner M, Richards G: Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. III. Consequences of ecdysone withdrawal. Dev Biol *54*, 241 (1976).
40. Oberlander H, Ferkovich SM, Van Essen F, Leach CE: Chitin biosynthesis in imaginal discs cultured *in vitro*. Wilhelm Roux Arch *185*, 95 (1978).
41. Fristrom JW, Yund MA: A comparative analysis of ecdysteroid action in larval and imaginal tissues of *Drosophila melanogaster*. In Progress In Ecdysteroid Research. Hoffman JA, ed. Elsevier, New York, p 349 (1980).

42. Mitsui T, Riddiford LM: Hormonal requirements for the larval-pupal transformation of the epidermis of *Manduca sexta in vitro*. Dev Biol *62*, 193 (1978).
43. Fain MJ, Riddiford LM: Requirements for molting of the crochet epidermis of the tobacco hornworm larva *in vivo* and *in vitro*. Wilhelm Roux Arch *181*, 285 (1977).
44. Velissariou V, Ashburner M: The secretory proteins of the larval salivary gland of *Drosophila melanogaster*. Chromosoma *77*, 13 (1980).
45. Hiruma K, Riddiford LM: Hormonal regulation of Dopa decarboxylase during a larval molt. Dev Biol *110*, 509 (1985).
46. Hiruma K, Riddiford LM: Inhibition of DOPA decarboxylase synthesis by 20-hydroxyecdysone during the last larval moult of *Manduca sexta*. Insect Biochem *16*, 225 (1986).
47. Hiruma K, Riddiford LM: Regulation of DOPA decarboxylase gene expression in the larval epidermis of the tobacco hornworm by 20-hydroxyecdysone and juvenile hormone. Dev Biol *138*, 214 (1990).
48. Gagner J-P, Drouin J: Opposite regulation of pro-opiomelanocortin gene transcription by glucocorticoids and CRH. Mol Cell Endocrinol *40*, 25 (1985).
49. Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson J-A, Rottman FM, Yamamoto KR: Hormone-mediated repression: A negative glucocorticoid response element form the bovine prolactin gene. Genes Dev *2*, 1144 (1988).
50. Wolffe AP, Glover JF, Martin SC, Tenniswood MPR, Williams JL, Tata JR: Deinduction of transcription of *Xenopus* 74-kDa albumin genes and destabilization of mRNA by estrogen *in vivo* and in hepatocyte cultures. Eur J Biochem *146*, 489 (1985).