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Declining Ecdysteroid Levels are Temporally Correlated with the Initiation of Vitellogenesis During Pharate Adult Development in the Indianmeal Moth, *Plodia interpunctella*

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Photo-synchronized pupae were used to determine the titer of ecdysteroids during the pupal and pharate adult stages of the Indianmeal moth, *Plodia interpunctella* (Hübner). For female pupae that were kept in a 16 h light:8 h dark (long-day) photocycle, there was one major peak of ecdysteroids. The ecdysteroids reached a maximum of 2000–2500 pg/mg wet wt between 28–36 h after pupation. The ecdysteroids declined to <500 pg/mg wet wt by 68 h after pupation. For pupae that were kept in continuous darkness, the ecdysteroid peak became broader with a maximum at 24 h (3130 pg/mg wet wt). The composition of ecdysteroids at various times during pharate adult development was determined using gas chromatograph–mass spectrometry with selected ion monitoring [GC–MS (SIM)]. Ecdysone was the major component (93%) in samples taken 28 h after pupation, at the maximum of the pupal peak. Thus, high levels of ecdysone are correlated with the beginning of adult ovarian development. As pharate development progressed, the proportion of 20-hydroxyecdysone increased so that by 100 h after pupation 20-hydroxyecdysone was the only ecdysteroid detected. Previously, the initiation of vitellogenesis was determined to begin between 96 and 100 h after pupation. In conjuction with previous work, these findings demonstrate that the ecdysteroid titers decline before vitellogenesis is initiated and egg maturation can be completed.

Ecdysteroids Vitellogenesis Metamorphosis Radioimmunoassay GC–MS quantification

INTRODUCTION

The hormonal regulation of postembryonic ovarian development is only partially understood in species of the families Bombycidae, Pyralidae, and Saturniidae of Lepidoptera. In these moths which have short-lived adults, ovaries complete all or most of egg maturation before adult eclosion. Placing adult ovarian development within the parameters of metamorphosis requires that the process progress directly to completion, without arrested follicular stages and that regulation of vitellogenesis be controlled by the hormones regulating metamorphosis.

In *Bombyx mori*, the initiation of adult development, including ovarian development and egg maturation, is correlated with the major pupal ecdysteroid peak after pupation (Hanaoka and Ohnishi, 1974; Tsuchida *et al.*, 1987). Additionally, adult ovarian development does not proceed in pupal abdomens of *B. mori* (Sakurai and Hasegawa, 1969; Chatani and Ohnishi, 1976; Ohnishi, 1987) and *Hyalophora cecropia* (Williams, 1952) that were isolated prior to the major ecdysteroid peak. Subsequent treatment of the isolated abdomens with ecdysteroids stimulated development of the ovaries, as well as the other tissues, and mature eggs were found in the fully developed abdomens (Williams, 1952; Sakurai and

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Abbreviations used: 20-HE, 20-hydroxyecdysone; 20,26-HE, 20,26-hydroxyecdysone; wet wt, wet weight.

Hasegawa, 1969; Chatani and Ohnishi, 1976; Ohnishi, 1987; Tsuchida *et al.*, 1987). Although ecdysteroids have been identified as a hormone required for the initiation of adult ovarian development, endocrine controls of vitellogenin synthesis and egg maturation have not been elucidated in these two species. Of the major hormones that have been tested, juvenile hormone has no demonstrable regulatory influence on vitellogenesis in *B. mori* or *H. cecropia* since allatectomy of late larvae or pupae had no effect on egg maturation or viability (Bounhiol, 1942; Fakuda, 1944; Williams, 1959; Pan, 1977). In addition to these two species, egg maturation in *Galleria mellonella* was shown to be independent of control by juvenile hormone through allatectomy as well (Bodenstein, 1938).

Egg maturation and vitellogenesis in late pharate adults of the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) were blocked when the ecdysteroid levels were elevated during this period (Shirk *et al.*, 1990). High levels of ecdysteroids were maintained in late pharate adults by repeated injections of 20-HE. The 20-HE treatments decreased the tissue specific synthesis of the yolk polypeptides in ovaries by inhibiting accumulation of translatable yolk polypeptide transcripts. The effect of the 20-HE treatment was shown to be independent of juvenile hormone activity because concomitant treatment with 20-HE and a juvenile hormone analog did not restore vitellogenesis or egg maturation. From these observations, we have hypothesized that the onset of vitellogenesis occurs following a decline of the ecdysteroid titer in the pharate adult female. To more critically assess the role of ecdysteroids in normal females of the Indianmeal moth, we have measured changes in the ecdysteroid titer during pharate adult development and correlated the changing ecdysteroid titer with the progress of adult ovarian development and the onset of vitellogenin synthesis and vitellogenesis.

MATERIALS AND METHODS

Animal preparations

The *P. interpunctella* colony in the U.S.A. was reared according to Silhacek and Miller (1972) in a 16 h light:8 h dark (long-day) photoperiod at 30°C and 70% r.h.; the colony in Israel was reared on 80% ground wheat, 10% glycerin, 5% brewers yeast, and 5% honey in a 8 h light:16 h dark cycle at 30°C and 70% r.h. As a source of pupae for the photoperiodic condition, newly molted white pupae (±1.5 h) were collected at the beginning of scotophase to obtain synchronous cohorts and then kept in the long-day photoperiod until the appropriate age. The predominant developmental stage at a specific time point was selected on the basis of external morphological characters as described by Zimowska *et al.* (1991) and used to prepare the extracts for that time point. As a source of pupae for the continuous darkness condition, newly molted white pupae (±1.5 h) were collected either at the beginning of scotophase [Fig. 1(A)] or during the photophase [Fig. 1(B)] and placed in total darkness until the appropriate age. Insect age is expressed in hours from the time of pupation.

Ecdysteroid extraction and quantification

For each time point (pupae were ±1.5 h of age), one to six pupae (mean wet wt = 11.0 mg/pupa) were homogenized in 70% methanol at 4°C. The homogenate was centrifuged 10 min at 2600 *g* and 4°C. The supernatant was transferred to another tube, and the pellet was re-extracted in cold 70% methanol. The supernatants were combined and stored for a minimum of 24 h at −20°C. Following cold storage, the samples were centrifuged 10 min at 2600 *g* and 4°C. The supernatants were dried using a stream of nitrogen gas and heating at 22°C. Dried samples were stored at −20°C and were reconstituted with 70% methanol for assaying.

Aliquots of each sample were taken for quantitative ecdysteroid analysis by RIA as described by Shaaya *et al.* (1986). The 20-HE rabbit antiserum, DLII, was a gift of Professor J. Koolman (Marburg/Lahn, Germany), and the rabbit complement serum, HLA-ABC, was purchased from Sigma (St Louis, Mo). Radiolabeled [^{3}H]20-HE (89 Ci/mmol) was purchased from NEN (Boston, Mass.), and 20-HE purchased from Sigma (St Louis, Mo). Quantification of each sample was based on evaluation of triplicates using different concentrations. A standard curve was based on binding with various concentrations of 20-HE.

To determine the molecular composition of the ecdysteroids, methanolic extracts were prepared from females as described above. The methanolic extracts were evaporated to dryness under nitrogen and partitioned between counter-saturated butan-1-ol and water (*ca* 1 ml each); the aqueous layer was re-extracted with butan-1-ol. The combined butan-1-ol phase was back-extracted once with butan-1-ol-saturated water. The butan-1-ol layer was evaporated to dryness under nitrogen.

The butan-1-ol extract was dissolved in 1 ml methanol:water (1:9 v/v) and applied to a C_{18} Sep-Pak cartridge (Waters Assoc.). The cartridge was sequentially eluted with 3 ml methanol:water (1:9 v/v), 4 ml methanol:water (1:4 v/v), 6 ml methanol:water (6:4 v/v), and 4 ml methanol. The 6:4 methanol:water fraction for each sample, which contained the free ecdysteroids, was analyzed by gas chromatograph–mass spectrometry with selected ion monitoring [GC–MS (SIM)] as described by Evershed *et al.* (1987).

RESULTS

Quantification of ecdysteroids in female pupae during development

To establish the ecdysteroid titers during the pupal and pharate adult stages from the U.S.A. culture conditions, females were collected as white pupae at the

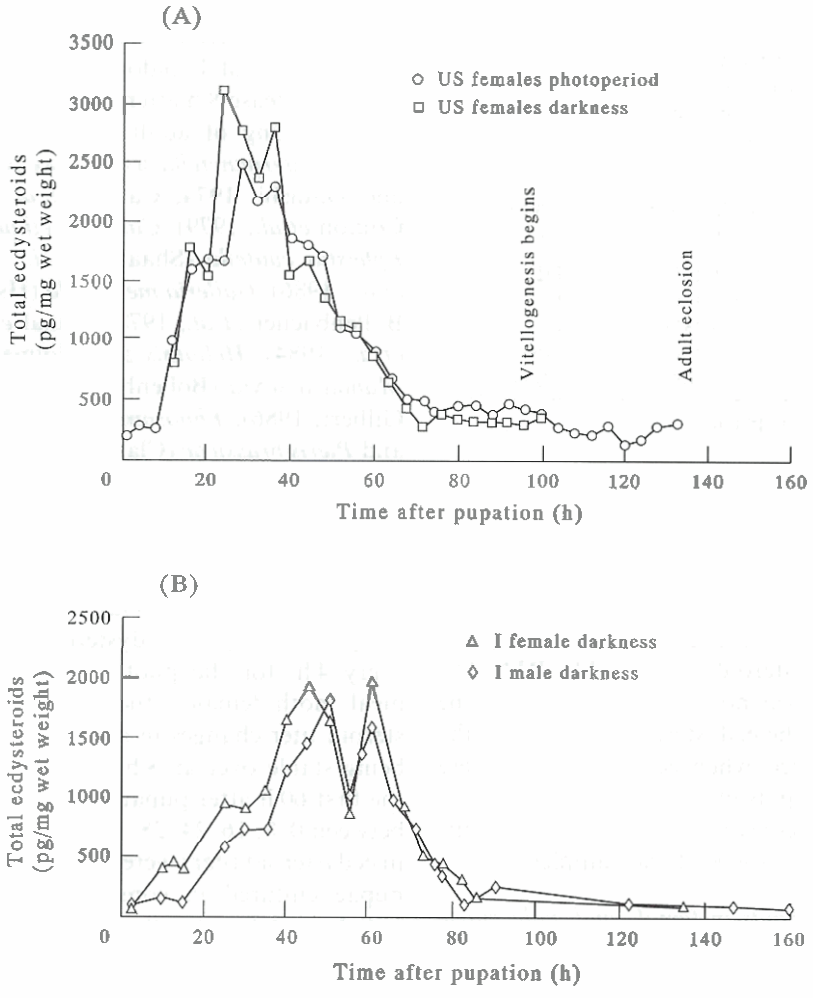

FIGURE 1. Changes in ecdysteroid titers (as 20-HE equivalents) during pupal and pharate adult development. (A) Female pupae from the U.S.A. culture were selected at the beginning of scotophase and kept either in the same photoperiod (○) or in constant darkness (□). Each point represents the mean of at least three replicates of five animals. The maximum standard error for the photoperiod samples was 246 and for the constant darkness samples was 277. (B) Female (△) or male (◇) pupae from the Israel culture were selected during the photophase and kept in constant darkness. Each point represents the mean of at least two replicates of six animals. The maximum standard error for the female samples was 99 and for the male samples was 166.

beginning of a scotophase, kept in a long-day photoperiod, and collected every 4 h until adult eclosion (136 h after pupation). Ecdysteroids were determined for each time point [Fig. 1(A)]. For the first 8 h after pupation, the titers were <300 pg/mg wet wt and then increased first to a plateau of 1600–1700 pg/mg wet wt between 16–24 h and then attained a maximum of 2200–2500 pg/mg wet wt between 28–36 h after pupation. The titer then began to decline with plateaus at 40–48 h (1700–1870 pg/mg wet wt) and at 52–60 h (935–1200 pg/mg wet wt). The ecdysteroid titers fell below 500 pg/mg wet wt by 68 h and remained below this level for the remainder of pharate adult development.

In the U.S.A. pupae were maintained in continuous darkness instead of in a long-day photoperiod, the profile was essentially the same except that the major ecdysteroid peak between 12 and 68 h reached a maximum earlier at 24 h (3130 pg/mg) and was broader than the peak in females kept in photoperiodic conditions.

To determine which ecdysteroids contribute qualitatively to the total titers prior to the onset of vitellogenesis, methanol extracts were made from female eye class 1 (Piepho, 1938) pharate pupae [late 5th (last) instar larvae] and from photo-synchronized female pupae at 28, 52, 76 and 100 h after pupation (mid-scotophase for each time point). The free ecdysteroid fractions were analyzed by GC–MS (SIM). In eye class 1 pharate pupae, 20-HE comprised 81% of the ecdysteroids measured (Table 1). At 28 h after pupation, the maximum of the pharate adult peak, the distribution had switched and ecdysone comprised the greater proportion of the ecdysteroids (93%). As pharate adult development progressed, the proportion of ecdysone decreased as the proportion of 20-HE and 20,26-HE increased. By 100 h after pupation, at the time of initiation of vitellogenesis (Zimowska *et al.*, 1991), no ecdysone was detected; 20-HE and 20,26-HE comprised all of the ecdysteroids measured. No detectable amounts of 26-HE were found in any of the samples.

The addition of a known quantity of makisterone A to each sample permitted quantification of the ecdysteroids present in each of the samples (Table 1).

TABLE 1. Qualitative analysis of ecdysteroids from female *Plodia interpunctella* during metamorphosis as determined by GC–MS (SIM). Quantitative determinations were based on comparison with a known amount of makisterone A added to each sample

Stage	Ecdysone pg/mg wet wt (%)	20-HE pg/mg wet wt (%)	20,26-HE pg/mg wet wt (%)
EC1PP*	18 (19)	76 (81)	ND‡
28 h P‡	1640 (93)	115 (7)	ND
52 h P	16 (19)	68 (81)	ND
76 h P	4 (3)	62 (51)	55 (46)
100 h P	nd (0)	19 (68)	9 (32)

*Eye class 1 pharate female pupae.
†ND = none detected.
‡P = timed females after pupation.

Although the amounts chemically determined followed the general trend of RIA determinations, the absolute quantities of ecdysone, 20-HE, and 20,26-HE, as determined by chemical quantification, were consistently less than the amounts of ecdysteroids measured by RIA. The source of this difference was not determined but may be due in part to assessing the ecdysteroids by RIA on the basis of a 20-HE standard when ecdysone levels were high, to interference by lipids in the methanolic extracts, and, perhaps, to other ecdysone metabolites contributing significant material to some of the samples.

Quantification of ecdysteroids in female and male pupae during development

When white pupae were selected from the mid-photophase from the short-day Israel culture and then kept in continuous darkness until collection, the overall changes in ecdysteroid titers were similar to those of long-day pupae collected at the beginning of a scotophase. However, the major peak of ecdysteroids occurred later than the major peak in the long-day pupae and was followed by a second peak before the titers fell to a low level during the later part of pharate adult development [Fig. 1(B)]. The peaks in the short-day pupae had maxima occurring at 45 h (1932 pg/mg wet wt) and 60 h (1970 pg/mg wet wt) for females and at 50 h (1832 pg/mg wet wt) and 60 h (1600 pg/mg wet wt) for males. The presence of two ecdysteroid peaks in the first half of pharate adult development in the short-day pupae may suggest that the shoulder at 36 h (2800 pg/mg wet wt) in the long-day pupae kept in constant darkness represents a bimodal character for this peak in the long-day pupae as well. From the maxima at 60 h, the ecdysteroid titers declined rapidly to 166 pg/mg wet wt at 82 h in males and 170 pg/mg wet wt at 85 h in females. After 85 h, the ecdysteroid titers remained below 400 pg/mg wet wt for both females and males until adult eclosion.

DISCUSSION

The developmental profile of ecdysteroids during pupal and pharate adult development in *Plodia interpunctella* is generally similar to the profiles reported for other species of Lepidoptera. The ecdysteroid titer begins to increase 8 h after pupation and is correlated with the beginning of adult development (Shirk, unpubl.). For *P. interpunctella* as well as for *B. mori* (Hanaoka and Ohnishi, 1974; Calvez *et al.*, 1976; Böhm, 1979; Coulon *et al.*, 1979), *Calpodes ethius* (Dean *et al.*, 1980), *Ephestia cautella* (Shaaya *et al.*, 1986; Spindler-Barth *et al.*, 1986), *Galleria mellonella* (Hsiao and Hsiao, 1977; Bollenbacher *et al.*, 1978; Sehnal *et al.*, 1981; Plantevin *et al.*, 1984), *Heliothis zea* (Holman and Meola, 1978), *Manduca sexta* (Bollenbacher *et al.*, 1981; Warren and Gilbert, 1986), *Philosamia cynthia* (Calvez *et al.*, 1976), and *Pieris brassicae* (Claret *et al.*, 1977; Böhm, 1979), the ecdysteroids increase to a single major peak during the first half of pharate adult development. The only exception to this pattern has been *H. virescens* where the major peak of ecdysteroids occurs during the last half of adult development (Loeb, 1982).

By measuring the ecdysteroids at short time intervals, every 4 h, for the photo-synchronized U.S.A. Indianmeal moth females, the profile shows that the ecdysteroid titer changes in a stepwise fashion with the titer being stable over an 8 h period of a step or plateau. In the first 60 h after pupation, 8 h plateaus were observed between 0–8, 16–24, 28–36, 40–48 and 52–60 h. Plateaus in ecdysteroid titers were observed also in the profiles for pupae cultured in constant darkness from both the U.S.A. and Israel cultures. Similar stepwise or plateau changes in the ecdysteroid titer have been observed in *Drosophila melanogaster* when short intervals were used between sample collection (Handler, 1982). The significance of these 8 h plateaus was not determined.

By the beginning (68 h after pupation) of the last half of pharate adult development in *P. interpunctella*, the ecdysteroid titer had declined to below 500 pg/mg wet wt and was maintained below this level through the remainder of pharate adult development. Similar observations have been made in *C. ethius* (Dean *et al.*, 1980), *E. cautella* (Shaaya *et al.*, 1986; Spindler-Barth *et al.*, 1986), *M. sexta* (Bollenbacher *et al.*, 1981; Warren and Gilbert, 1986), *Pieris brassicae* (Claret *et al.*, 1977; Böhm, 1979) and the males of *B. mori* (Hanaoka and Ohnishi, 1974; Coulon *et al.*, 1979) and *G. mellonella* (Bollenbacher *et al.*, 1978; Sehnal *et al.*, 1981; Plantevin *et al.*, 1984) in which the ecdysteroid titers declined and remained low during the last half of adult development. However, in the females of *B. mori* (Hanaoka and Ohnishi, 1974; Calvez *et al.*, 1976; Böhm, 1979) and *G. mellonella* (Hsiao and Hsiao, 1977; Bollenbacher *et al.*, 1978; Sehnal *et al.*, 1981; Plantevin *et al.*, 1984), the ecdysteroid titers increased just prior to adult eclosion.

Ecdysone was the major component of the early pharate adult ecdysteroid peak. Our data show that high levels of ecdysone, with only minor amounts of other metabolites, are correlated with the initiation of adult ovarian development. As development progressed, the amount of ecdysone progressively decreased as well as the proportion of ecdysone to 20-HE. By 100 h after

pupation, when ecdysteroid titers had declined to <500 pg/mg wet wt, no ecdysone was detected in the samples and 20-HE was the primary ecdysteroid present in the pharate adult females. This switch from ecdysone to 20-HE as the primary ecdysteroid as pharate adult development progresses has also been reported for *H. zea* (Holman and Meola, 1978), and *M. sexta* (Warren and Gilbert, 1986).

The decline in the ecdysteroid titer in *P. interpunctella* appears to be regulated for completion before the 5th scotophase. When placed in a short-day photoperiod (8 h light:16 h dark), the major pupal ecdysteroid peak was delayed by 10 h from when the peak occurred in the long-day photoperiod (16 h light:8 h dark). However, even though the peak was delayed in the short-day photoperiod, the level of ecdysteroids was reduced prior to 90 h after pupation, i.e. before the 5th scotophase for those insects kept in a photoperiod. In addition, the decline of ecdysteroid titers took only 20 h in the short-day photoperiod while it was nearly 40 h in the long-day photoperiod. Because vitellogenesis began at approximately the same time in both the short-day photoperiod and long-day photoperiod females, i.e. 96–100 h after pupation, the data suggest that the ecdysteroid decline is significant to the initiation of vitellogenesis.

Vitellogenesis in *P. interpunctella* begins between 96 and 100 h after pupation (Zimowska *et al.*, 1991). Previous experiments demonstrated that increasing the ecdysteroid titers by treatment with exogenous 20-HE during pharate adult development blocked yolk protein synthesis and egg maturation (Shirk *et al.*, 1990). Our data show that the ecdysteroid titer has declined to a basal level by the time of the initiation of vitellogenesis. These data support the hypothesis that the ecdysteroid titers must decline before vitellogenesis can proceed (Shirk *et al.*, 1990). Taken together, the data suggest that ecdysteroids play a central role in the regulation of vitellogenesis during pharate adult development. However, the mechanism that leads directly to the initiation of vitellogenesis appears to involve additional regulators because the initiation of vitellogenesis does not occur until 24 h after the ecdysteroids have completed the decline.

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