Archives of Insect Biochemistry and Physiology 21:53–63 (1992)

Initiation of Vitellogenesis in Pharate Adult Females of the Indianmeal Moth, *Plodia interpunctella*

Paul D. Shirk, Grażyna Zimowska, Donald L. Silhacek, and Eli Shaaya

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Gainesville, Florida (P.D.S., G.Z., D.L.S.); The Volcani Center, Agricultural Research Organization, Bet Dagan, Israel (E.S.); Department of Invertebrate Physiology, University of Warsaw, Warsaw, Poland (G.Z.)

Egg maturation in the Indianmeal moth, *Plodia interpunctella* (Hübner), is initiated during adult metamorphosis. The temporal sequence for the initiation of vitellogenin (Vg) synthesis in fat body, the accumulation of Vg in hemolymph, and the accumulation of yolk proteins in terminal follicles during adult development are described. On the basis of quantitation by rocket immunoelectrophoresis, the amount of Vg in hemolymph prior to 80 h after pupation was below 50 ng/μl hemolymph. At 83 h after pupation, Vg was detectable at 0.4 μg/μl hemolymph and increased to over 20 μg/μl within 24 h after adult eclosion. Using immunofluorescent histochemical staining for Vg subunit yolk polypeptide 1 (YP1), the production of Vg was observed to increase rapidly in fat body between 96 and 100 h after pupation. Western blot analysis showed that YP2 was the first YP to accumulate in terminal follicles appearing at 96 h after pupation while the other three major YPs were first observed at 116 h. These findings demonstrate that initiation of vitellogenesis in terminal follicles begins around 96 h after pupation and involves the temporal coordination of fat body and follicle activities that provide Vgs before terminal follicles achieve competency to accumulate yolk proteins.

Key words: yolk proteins, metamorphosis, oocyte development, follicle cell, ecdysteroids

INTRODUCTION

Unlike most insect species, members of the Bombycidae, Pyralidae, and Saturniidae families complete the majority of egg maturation during adult metamorphosis. Inclusion of adult ovarian development and vitellogenesis

Acknowledgments: We thank Curtis Murphy and Karen Ogren for technical assistance, and Drs. A. C. Chen, A. H. Handler, and W. H. Telfer for comments on the manuscript. This work was supported in part by a BARD grant (US-1122-86R) to D.L.S., E.S., and P.D.S. Mention of a proprietary product does not constitute an endorsement by USDA.

Received March 16, 1992; accepted May 26, 1992

Address reprint requests to Paul D. Shirk, P.O. Box 14565, Gainesville, FL 32604.

within the developmental processes of metamorphosis must place constraints on the regulation of egg maturation by the major hormones, ecdysone and juvenile hormone, that typically control this process in the adult stage of other insects.

Changes in the ecdysteroid titer have been implicated as a major temporal regulator of metamorphosis [1]. Early in the pupal stage, an increase in ecdysteroids is necessary to initiate adult development [2]. Measurement of ecdysteroid titers and treatment of isolated pupal abdomens with ecdysteroids showed that initiation of follicular differentiation and ovarian growth is dependent on the early pupal ecdysteroid peak in the silkworm, *Bombyx mori* [3–7]. During late pharate adult development, a decline in ecdysteroid titer is required for the completion of metamorphic processes. Elevated levels of ecdysteroids inhibit late metamorphic events including release of eclosion hormone and eclosion in the tobacco hornworm, *Manduca sexta* [1,8,9].

Similarly, maintenance of high ecdysteroid titers blocks egg maturation and yolk protein synthesis in pharate adult female *Plodia interpunctella* (Hübner) [10]. The oocytes of *P. interpunctella* contain two major yolk proteins: Vg* and follicle cell produced protein [11,12]. Vg (M_r = 475,000) is synthesized and secreted from the fat body and consists of subunits YP1 (M_r = 153,000) and YP3 (M_r = 43,000). The follicle cell produced protein (M_r = 235,000) is produced by the follicular epithelium and consists of subunits YP2 (M_r = 69,000) and YP4 (M_r = 33,000). Repeated treatment of pharate adult females with 20-hydroxyecdysone blocked the accumulation of both proteins in the oocytes and the accumulation of YP2 subunit transcript [10].

If changes in ecdysteroid titers are used by insects to synchronize developmental events, a temporal correlation between changes in the ecdysteroid titers and the initiation of vitellogenesis should occur during late pharate adult development in *P. interpunctella*. The data reported here establish the temporal organization for the initiation of Vg production in fat body and of yolk protein uptake by terminal follicles in late pharate adult *P. interpunctella* females.

MATERIALS AND METHODS

Insect Preparations

The *P. interpunctella* (Hübner) colony was reared according to Silhacek and Miller [13] in a 16 h light : 8 h dark cycle at 30°C and 70% relative humidity. Newly molted white pupae (±1 h) were collected at the beginning of scotophase to obtain synchronous cohorts and then kept 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. [14] and accordingly used to prepare protein extracts or tissue fixations.

*Abbreviations used: BSA = bovine serum albumin; FITC = fluorescein isothiocyanate; PBAT = phosphate buffer, sodium azide, and Triton-X 100; PM = plasma membrane; RER = rough endoplasmic reticulum; SDS = sodium dodecyl sulfate; TBS = tris buffered saline; Vg = vitellogenin; YP = yolk polypeptide.

Quantitation of Vg in Hemolymph

Amounts of Vg (YP1/YP3) in hemolymph were determined by rocket immunoelectrophoresis as described by Weeke [15] using a 2117 Multiphor system (LKB, Piscataway, NJ). Antiserum to purified Vg was diluted 1:500 with 1% SEAKEM LE agarose (FMC, Rockland, ME), which produced the maximal sensitivity, and poured onto Gelbond agarose support medium (FMC) for electrophoresis. Standards were known amounts of purified Vg. Unknowns were quantified by comparison to a linear standard line of Vg peak heights. Hemolymph was collected from 5–20 pupae or young adults and pooled to a total volume of 5 μl. The lowest detectable amount of Vg was 50 pg/μl hemolymph. Quantities of Vg were determined for aliquots of each pooled hemolymph sample. Total protein content of each hemolymph sample was determined for three aliquots of each sample using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Gel Electrophoresis and Immunoblotting

One-dimensional 9% SDS-PAGE was performed according to Laemmli [16]. Lanes were loaded with the designated quantities of total protein. Tissue samples were homogenized in saline [17], centrifuged at 12,000*g* for 2 min at 4°C, and an aliquot of the supernatant was assayed to determine the concentration of protein before addition of SDS-sample buffer [16]. Protein determinations were made using a Bio-Rad protein assay (Bio-Rad) with BSA as a standard. Prestained molecular weight standards (Bio-Rad) were coelectrophoresed for molecular weight estimation.

Monospecific polyclonal antisera were raised to each of the YPs (YP1, YP2, YP3, and YP4) of *P. interpunctella* in New Zealand white rabbits as described by Bean et al. [12]. Immunoblots were prepared according to Towbin et al. [18]. Proteins were electroblotted from the gel to nitrocellulose (BA-85; Schleicher and Schuell, Keene, NH) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol) at 20 V for 12 h using a Transblot cell (Bio-Rad). The electroblot was blocked with 3% gelatin in 20 mM Tris, pH 7.5, and 0.5 M NaCl and then reacted with whole antiserum to each YP diluted 1:500 in 1% gelatin, 0.05% Tween 20, 20 mM Tris, pH 7.5, and 0.5 M NaCl. The immunoreactive bands were visualized with an Immun-Blot color assay (Bio-Rad) using horseradish peroxidase-linked goat anti-rabbit IgG as the second antibody.

Immunohistochemistry

Immunofluorescent detection of YP1 in whole mounts of fat body was performed as described by Zimowska et al. [14]. Whole fat body preparations were fixed for 12 h at 4°C in 4% (W/V) depolymerized paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The fat body tissues were washed five times for 1 h in 0.1 M phosphate (pH 7.4), 1% sodium azide, and 1% Triton-X 100 (PBAT) at 24°C and placed in PBAT at 4°C overnight. The fat body tissues were blocked with 2% nonreactive goat serum (Jackson Immunoresearch Labs, West Grove, PA) in PBAT at 24°C and then incubated with monospecific rabbit anti-YP1 serum [12] diluted 1:500 in PBAT at 24°C overnight. The monospecific anti-YP1 serum was used for the immunogold labeling because

the anti-Vg serum has low specificity for denatured proteins (Shirk, unreported data). The fat body tissues were washed in PBAT six times for 1 h each and then incubated with anti-rabbit goat IgG-FITC (Sigma, St. Louis, MO) diluted 1:40 in PBAT with 2% nonreactive goat serum at 4°C overnight. The tissues were washed in PBAT five times for 1 h each at 24°C. The tissues were dehydrated and cleared in a graded series of glycerine diluted with 50 mM carbonate buffer (pH 9.4) to 80% glycerine diluted with 50 mM carbonate buffer (pH 9.4) to 80% glycerine. Whole mounts of fat body in 80% glycerine were examined and photographed with an Olympus BHS microscope (Olympus Corp., Marietta, GA) equipped with a BH2-RFL reflected light fluorescence attachment with a blue 490 nm excitation filter, a blue 455 nm supplementary exciter filter, and a G-520 barrier filter.

Immunogold localization of the YPs in ultrathin sections was a modification of Leung et al. [19]. The ovaries were dissected in 0.8% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (0.1 M phosphate [pH 7.5], 0.15 mM $CaCl_2$ and 4% sucrose) and prefixed for 2 h at 4°C. The tissues were fixed overnight in 4% paraformaldehyde in pH 10.4 phosphate buffer at 4°C. The tissues were dehydrated in a graded series of ethanol, and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany). Ultrathin sections were collected on 200 mesh nickel grids coated with formvar (Ernest F. Fulam Inc., Latham, NY). The incubations were performed by floating a grid on a drop of medium and maintaining gentle agitation during each incubation at 24°C. The sections were etched with 3–6% H_2O_2 in double distilled H_2O for 5 min and then blocked with 3% BSA in TBS (0.5 M NaCl, 20 mM Tris, pH 7.5) for 25 min. The sections were incubated with monospecific anti-YP1 serum, a preabsorbed monospecific anti-YP1 serum, or nonreactive serum diluted 1:250 in TBS plus 1% Tween-20 for 60 min. After washing with TBS/Tween three times for 15 min with gentle agitation, the sections were exposed to goat anti-rabbit IgG linked with 20 nm colloidal gold (Polysciences, Inc., Warrington, PA) for 60 min. After washing with 0.3% BSA in TBS, the sections were poststained with 2% uranyl acetate followed by 0.2% Reynolds's lead citrate [20]. Ultrastructural examination was performed on a Hitachi (H-600) transmission electron microscope (Hitachi Instruments, Inc., San Jose, CA) operating at 75 kV.

RESULTS

Hemolymph Titers of Vg

Female pupae and young adults were aged, and hemolymph samples were collected. The total amount of hemolymph protein was determined, and the hemolymph titer of Vg was measured by rocket immunoelectrophoresis (Fig. 1). The amount of total hemolymph protein during most of the pupal stage ranged from 65–70 μg/μl until just before eclosion when the protein levels dropped to 49.8 (±7.1) μg/μl hemolymph at eclosion. The decrease in total protein levels before eclosion was probably due to an increase in hemolymph volume brought about by the resorption of molting fluids [21]. Prior to 83 h after pupation, the amount of Vg in the hemolymph was below 0.05 μg/μl

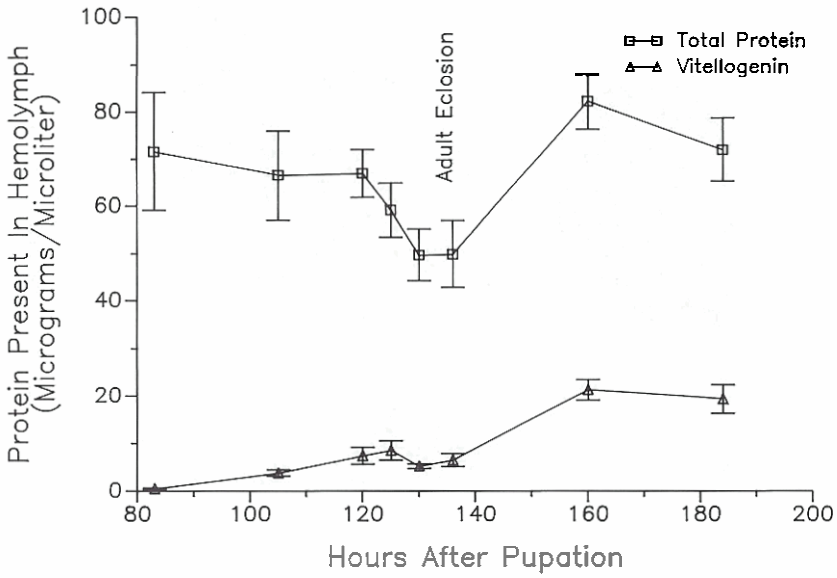

Fig. 1. Changes in the amount of total protein and Vg during vitellogenesis. Each point represents the mean (± S.E.) of three to eight pooled hemolymph samples from pupae or adults.

(data not shown). At 83 h the amount of Vg was 0.4 (±0.1) μg/μl of hemolymph and rose to 8.5 (±2) μg/μl by 125 h. Vitellogenic oocytes can be observed in ovaries of females at 98 h. The amount of Vg decreased slightly just prior to eclosion as did the amount of total hemolymph protein, and then increased to 21.2 (±2.2) μg/μl at 160 h (24 h after adult eclosion). By 184 h (48 h after adult eclosion), the amount of Vg comprised 27% of the total hemolymph protein.

Increased YP1 Production in Fat Body During Adult Development

Within 24 h after pupation the fat body dissociated and dispersed throughout the hemocoel [14]. The fat body did not begin to reassociate into the adult form until the 4th scotophase (approximately 80 h) and by 83 h the fat body had reassociated. To assess the vitellogenic activity of the reassociated fat body, ultrathin sections and whole mounts of fat body from females were examined after using anti-YP1 primary serum in conjunction with immunogold and immunofluorescence labeling, respectively. Immunogold labeling of ultrathin sections of fat body from 92 h and 96 h females showed no significant amounts of Vg accumulated in secretory granules or cytoplasm of fat body cells (Fig. 2A,B). Most of the YP1 labeling was observed in association with PM and occasionally with RER. Whole mounted fat body from 94 h old females (developmentally staged to just prior to melanization of the tarsal claws) showed only background reactivity with the anti-YP1 serum (Fig. 3A). Whole mounted fat body from 100 h females (showing melanization of the tarsal claws) had considerable reactivity with the anti-YP1 serum (Fig. 3B); fat body from females of the same age exposed to control nonreactive serum or preabsorbed serum showed no reactivity (data not shown). The immuno-

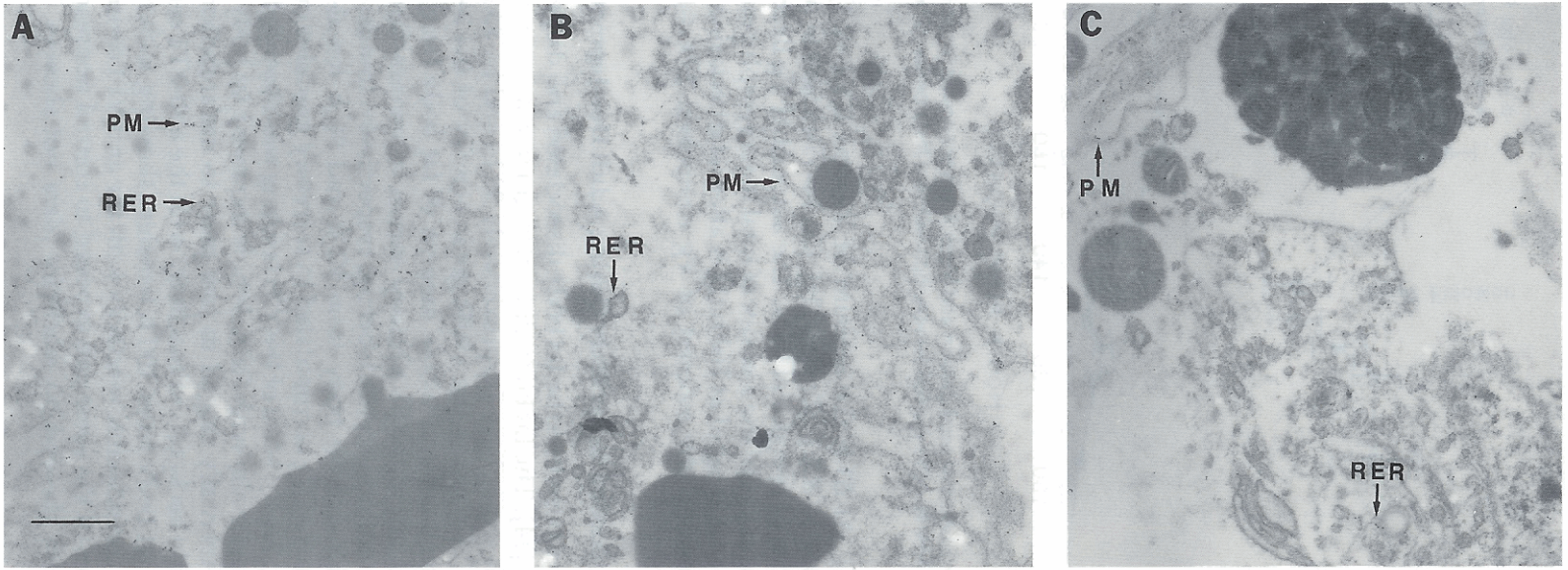

Fig. 2. Presence of YP1 in fat body of pharate adult females. Ultrathin sections of fat body from (**A**) 92 h, (**B**) 96 h, and (**C**) 112 h pupae were immunogold labeled with anti-YP1 serum. Arrows point to areas of immunogold labeling. PM, plasma membrane; RER, rough endoplasmic reticulum. Magnification bar = 10 μm.

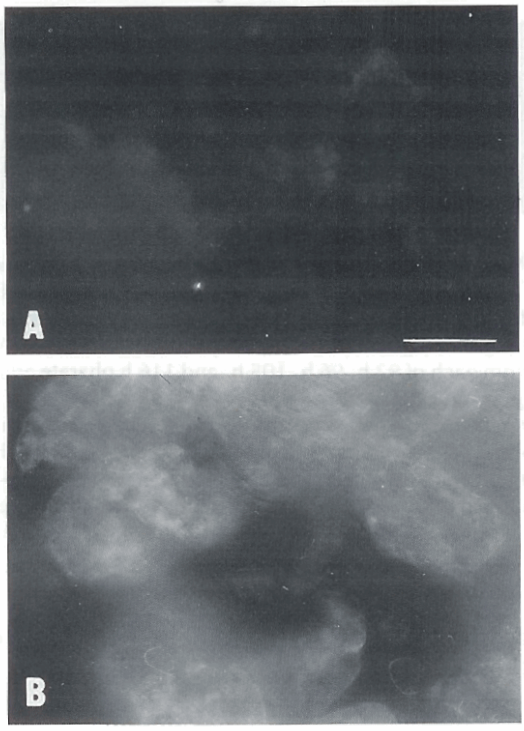

Fig. 3. Temporal increase in YP1 associated with previtellogenic and vitellogenic fat body cells. Whole mounted fat body from (A) 94 h female pupae and (B) 100 h female pupae were stained with YP1 antiserum. Magnification bar = 25 μm.

fluorescence of whole mounted fat body from females older than 100 h was similar to that of the 100 h females (data not shown). Immunogold labeling of ultrathin sections of fat body from 112 h females showed the YP1 distribution to be similar to that observed in 92 h and 96 h fat body, i.e., most of the YP1 label associated with PM, occasionally with RER, and no major accumulations within the cytoplasm (Fig. 2C). The immunogold labeling of the fat body tissue was not sufficiently quantitative to make any accurate assessment of biosynthetic activity. Immunolabeling of whole mounted or ultrathin sections with anti-YP3 serum gave similar results as observed for YP1 (data not shown). Together, these data indicate that the fat body undergoes a major increase in Vg production over the 6 h period between 94 h and 100 h after pupation.

Initiation of Yolk Protein Uptake by the Oocytes

From our previous studies [14], yolk bodies first appeared in terminal follicles of pharate adult females between 96 and 105 h after pupation (during the 5th scotophase). To determine the time period when accumulation of the YPs could first be detected in the terminal oocytes, ovaries of females timed at 92 h, 96 h, 105 h, and 116 h after pupation were removed and homogenized in SDS-buffer. The proteins were analyzed for the presence of YPs on electroblots using monospecific antisera for each of the YPs. Whereas YP2 was observable first at the lowest detectable level in ovaries of 96 h pharate adult

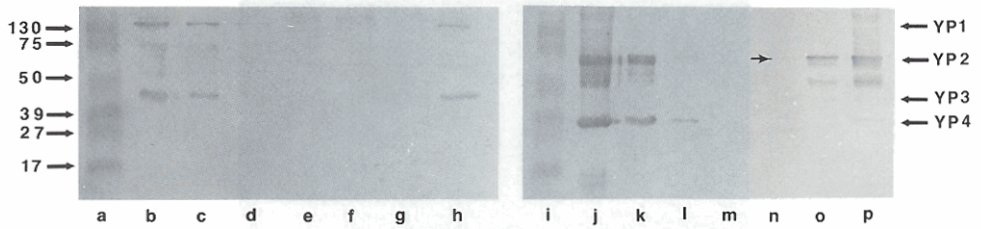

Fig. 4. Initiation of YP accumulation in ovaries of pharate adults. Immunoblots of ovarian protein extracts from pharate adults were reacted with the combined monospecific antisera for YP1 and YP3 (**lanes a–h**) and for YP2 and YP4 (**lanes i–p**). Lanes a and i are prestained molecular weight markers. **Lanes b–d:** 1 μg, 0.1 μg, and 0.01 μg, respectively, of purified Vg; **lanes e–h:** 10 μg of total ovarian proteins from each of 92 h, 96 h, 105 h, and 116 h pharate adult females, respectively; **lanes j–l:** 1 μg, 0.1 μg, and 0.01 μg, respectively, of purified YP2/YP4 protein; **lanes m–p:** 10 μg of total ovarian proteins from each of 92 h, 96 h, 105 h, and 116 h pharate adult females, respectively. The arrow in lane n points to a weakly staining band of YP2. The multiple bands appearing between the molecular weights of 50,000 and 69,000 in lanes j–p have previously been shown to be degradation products of YP2 [12]. Positions of the molecular weight markers are designated on the left borders of the gels, and the positions of the YPs are designated on the right borders of the gels.

females, the remaining three YPs were initially detectable only in the ovaries of 116 h females (Fig. 4).

DISCUSSION

The initiation of vitellogenesis in pharate adult female *P. interpunctella* was shown to be a coordinated process between fat body and ovarian activities. Prior to 80 h after pupation, neither synthesis of yolk proteins nor vitellogenic processes were detected in either fat body or follicles. Coincident with the reassociation of the fat body at 83 h after pupation, Vg became detectable in the hemolymph. The production of Vg by the fat body prior to 94 h was low based on a lack of the presence of any immunogold labeling material accumulating in the fat body cells and on an undetectable amount of staining in whole mounted tissues. Subsequently there was an increase in the fat body production of Vg between 94 and 100 h after pupation that was coincident with the first accumulation of YP2 in terminal follicles. Although the amount of YP1 or YP3 staining in whole mounted fat body tissue increased during this period, no accumulation of immunogold labeled YPs was detected in the cytoplasm of the fat body cells. This suggests that the synthesis and secretion of the Vg into the hemolymph from the fat body cells is rapid.

The major accumulation of the other major YPs (YP1, YP3, and YP4) in the terminal follicles was not detectable until 116 h after pupation. The delayed accumulation of the three YPs relative to YP2 was due to at least two factors, one of which must be the development of patency by the follicular epithelium. The synthesis and secretion of YP2 by follicular epithelium cells towards the brush border allows YP2 to be taken up by the oocyte as soon as synthesis begins and does not require the development of patency by the follicular epithelium cells [22]. However, the inability of terminal follicles to take up Vg until 116 h even though the protein is found in the hemolymph prior to this

time indicates that the terminal follicles only become patent during this period.

The most difficult to explain was the difference in the initiation of the uptake of YP2 and YP4 from the follicular epithelium cells. Although YP4 is present in follicular epithelium cells of previtellogenic follicles even before YP2 is observed (Zimowska et al., unreported data), the accumulation of YP4 in terminal follicles was found to begin 8–16 h after the initiation of YP2 accumulation. The difference in the time of initiation of YP2 and YP4 accumulation in the terminal follicles remains to be resolved.

The timing of the first appearance of Vg in the hemolymph is most likely species specific. The first appearance of Vg in the hemolymph of *P. interpunctella* occurred with two thirds of the time of metamorphosis completed and just at the time when the fat body reassociated into the adult form. This is similar to the temporal sequence for Vg appearance in *M. sexta* where Vg was first detected in the hemolymph 4 days prior to adult eclosion [22]. However, these two species contrast with both *B. mori* [23–25] and *Hyalophora cecropia* [26,27] where Vg was first detected in hemolymph of pharate pupae, and the major increase in Vg synthesis occurred midway through pharate adult development.

The titers of Vg in the hemolymph during the pupal period also appeared to be species specific. In *H. cecropia* the relative concentration of Vg in the hemolymph initially increased to the maximal level during the pharate pupal stage [26,27] and accounted for 50% of the newly synthesized protein in the hemolymph [28]. After pupal ecdysis, the amount of newly synthesized Vg decreased to less than 10% of hemolymph protein [28] and remained at this level until day 13 of adult development (two thirds of the time of adult development) when synthesis and accumulation of Vg in the hemolymph rapidly increased [27,28]. By day 18 of adult development, the relative amount of Vg began to decrease [26] even though the proportion of newly synthesized protein increased [27]. Although Vg began to increase in the hemolymph at the same developmental period during metamorphosis in *P. interpunctella*, the titers did not decrease during late pharate adult development but continued to increase through 24 h after adult eclosion. Both of these species had Vg titers that were completely different from those reported for *B. mori* which maintained a constant level of Vg in the hemolymph throughout adult development [25].

The initiation of the vitellogenic processes in the fat body and the ovaries of *P. interpunctella* was shown to be inhibited by maintaining an elevated ecdysteroid titer during late pharate adult development [10]. Measurement of the ecdysteroid titers during the pupal period showed that the ecdysteroids rose to a peak of 2,500 pg/mg wet weight at 28 h and then had declined to a minimal level of 200 pg/mg wet weight by 60–70 h after pupation (Shaaya et al., unreported data). The completion of the decline is prior to the initiation of the first events in competency for vitellogenesis, i.e., the reassociation of the fat body and the initiation of Vg synthesis. These data suggest that the overall regulation of vitellogenesis is dependent on ecdysteroid titers declining to a lowered level. However, the sequential initiation of individual components of vitellogenesis, i.e., fat body reassociation, increased Vg synthesis, follicular epithelium cell

synthesis of YPs, and the accumulation of yolk proteins by the terminal follicles, indicates the presence of additional regulators besides ecdysteroids that contribute to the initiation and control of vitellogenesis and oocyte maturation in the pharate adult females of *P. interpunctella.*

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