

Investigation of the Interaction between Fat body and Ovary Development during Pupal Transformation in Silkworm, *Bombyx mori*

Esen Poyraz^{1*}, Ebru Göncü², Osman Parlak²

¹Department of Biology, Faculty of Science & Arts, Celal Bayar University, Muradiye, 45140, Manisa, Turkey

²Department of Biology, Faculty of Science & Arts, Ege University, Bornova, 35100, Izmir, Turkey

Abstract

The insect fat body is the major biosynthetic and storage organ involved in lipid, carbohydrate, amino acid and nitrogen metabolism and protein synthesis. It also actively participates in vitellogenesis and ovary development, synthesizing the soluble precursor for yolk, i.e., vitellogenin in stage specific manner. The continual substance exchange among fat body, hemolymph and ovary is controlled by hormones, ecdysteroids and juvenile hormone, during metamorphosis of *Bombyx mori* (L.). The aim of the present study was to clarify the interactions between major proteins of fat body and hemolymph and their effect on ovarian development under hormonal factors during pupal-adult transformation. Detected fat body, hemolymph and ovary proteins are grouped as follows by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): storage proteins (72 kDa and 76 kDa), 30 kDa proteins, ApoLp-I (230-250 kDa), egg specific protein (72 kDa and 64 kDa) and vitellogenins (178 kDa). Our results suggest that changes in the well-defined and characterized protein fractions quality and quantity of the fat body had a direct effect under hormonal factors on the ovary and egg development during metamorphosis.

Keywords: *Bombyx mori*, hemolymph proteins, fat body proteins, yolk proteins, SDS-PAGE.

*Corresponding Author: Esen POYRAZ (e-mail: esen.poyraz@yahoo.com)

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İpekböceği *Bombyx mori*'de Pupal Dönüşümde Yağ Doku ile Ovaryum Gelişimi Arasındaki Etkileşimin Araştırılması

Özet

Böcek yağ dokusu, lipid, karbohidrat, aminoasit ve nitrojen metabolizmalarına katılan ve protein sentezinde rol alan en büyük biyosentetik depo organıdır. Bunun yanı sıra, vitellogenin gibi yumurta depo proteinlerinin öncülerini sentezleyerek aktif olarak vitellogenizde ve ovaryum gelişiminde rol alır. *Bombyx mori*'de (L.)'te metamorfoz süresince, yağ doku, hemolenf ve ovaryum arasında meydana gelen sürekli madde değişimi ekdisteroidler ve juvenile hormonun kontrolü altında gerçekleşir. Elektroforetik olarak belirlenen temel hemolenf proteinleri şu şekilde gruplandırılmıştır: depo proteinleri (72 kDa ve 76 kDa), 30K polipeptidleri, ApoLp-I (230- 250 kDa), yumurta spesifik proteini (72 kDa ve 64 kDa) ve vitellogenin (178 kDa). Çalışmamızın sonucunda metamorfoz süresince yağ doku proteinlerinin nitelik ve niceliğindeki değişimlerin ovaryum ve yumurta gelişimi üzerinde doğrudan etkiye sahip olduğu sonucuna ulaşılmıştır.

Anahtar kelimeler: *Bombyx mori*, hemolenf proteinleri, yağ doku proteinleri, yumurta depo proteinleri, SDS-PAGE.

Introduction

Domesticated silkworm, *Bombyx mori* (L.) has an economic importance for the silk production and it has been referred as the best model organism for biochemical and molecular biology researches (Yang et al. 2010; Nagaraju and Goldsmith 2002).

The insect fat body, which is analogue to vertebrate adipose tissue and liver, is the principle organ that occupies most of the metabolic process, including energy storage, synthesis of carbohydrates, lipids and proteins. It is also responsible for intermediary metabolism and accumulation of toxic materials such as urate (Liu et al. 2009; Oliveira and Cruz-Landim 2004). Insect fat body is also involved in ovary development and maturation by participating in vitellogenesis, the process by which yolk accumulates in the cytoplasm of an ovary, actively by supplying the soluble yolk protein precursors, i.e., vitellogenin (Vg) (Oliverira et al. 2012).

B. mori has a relatively short and non-feeding adult stage. In *Bombyx*, ovarian development occurs during the pupal and pharate adult development. To be ready for fertilization and oviposition of eggs within hours requires a program of ovarian organogenesis before adult ecdysis. *B. mori* has eight ovarioles and each four ovarioles are surrounded by a basal sheet to be ripped by developing follicles in metamorphosis (Kendirgi et al. 2002; Swevers et al. 2005; Mpakou et al. 2008; Telfer 2009). An asynchronous follicle development has seen in the formation of linear arrays (ovarioles) of progressively maturing follicles, with each follicle differing from its immediate neighbor by 2-2, 5 hour of developmental distance. In the merostic polytrophic ovaries of *B. mori*, each follicle includes a single oocyte, seven nurse cells-mitotic siblings of the oocyte- and a surrounding monolayer of approximately 5000 epithelial follicular cells. .

Metabolic functions of fat body and ovarian development are controlled by hormonal factors; especially ecdysone and juvenile hormone (JH) (Liu et al. 2009; Tsuchida et al. 1987). During the metamorphosis, destruction of certain larval tissues and remodeling of various tissues into adult, are depend on synthesis and utiliza-

tion of various macromolecules like proteins which are strictly controlled by the endocrine system of the insect. Therefore, studies on the tissue specific proteins are of paramount significance. The fluctuation in the quality and quantity of tissue specific proteins in *B. mori* have been extensively studied during larval (Uranlı et al. 2011; Hyrsı and Simek 2005) and pupal stages (Janarthanan 1998). In this study, we analyzed specific proteins in female fat body, hemolymph and ovary during pupal stage and demonstrated the relationship between maturation of ovary and fat body-hemolymph protein profiles associated with hormonal factors.

Materials and methods

The experiments were performed on female silkworm pupae (*Bombyx mori* L.1758) of the white monovoltine breed: Japan x China poly-hybrid. The larvae were fed on fresh mulberry leaves. Rearing was conducted under $25\pm 1^\circ\text{C}$ temperature and 70-85% relative humidity. The day of pupal ecdysis was designated as day 0 and the pupal stage lasted 11 days. Fat body, ovary and hemolymph samples were obtained from day 0 of pupae to adult ecdysis.

The hemolymph samples were collected into eppendorf tubes by puncturing the wing buds of 8 different pupae with a fine needle. 25 μl phenylthiourea (Sigma, Pierce) solution for every 500 ml of hemolymph sample was added in order to prevent melanization and 25 μl sodium azide was added to prevent bacterial contamination and the samples were kept on ice. Hemolymph samples were centrifugated subsequently at 14000xg for 15 minutes $+4^\circ\text{C}$ to remove the hemocytes and other tissue debris, the supernatants were collected and stored at -80°C until use.

Fat body, ovary and eggs were collected with a curved-tipped pens and samples were washed gently with phosphate buffer saline (PBS, 0,01M phosphate buffer at pH 7,4) in a petri dish.

Collected fat body and ovary samples were homogenized in 300 μl homogenization solution (20mM Tris-HCl, pH 7, 5) containing protease inhibitor (Sigma, Pierce) and Igepal. The homogenates were centrifugated at 16000xg for

10 minutes at +4°C. The supernatants were collected and stored at -80 °C until use.

Total protein concentrations were determined by using Bradford protein assay. Bovine serum albumin (Pierce) was used as a protein standard. Absorbance was measured with a spectrophotometer at 595nm (Unicamhelios α V 1.30).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a discontinuous buffer system. Five micrograms of total proteins were separated by 10% SDS- Polyacrylamide gel electrophoresis system. Gels were stained with silver nitrate. The stained gels were photographed with an Olympus digital camera.

Image J analysis software from the National Institutes of Health, US (<http://rsb.info.nih.gov/nih-image/>) was used for analyzing the protein bands's relative abundance.

After dissection of the animals, tissues were fixed immediately in Bouin's solution (water-saturated picric acid: formalin: acetic acid, 15:5:1 by volume) for 24h at +4°C. Five micrometer-thick sections were cut and dewaxed in xylene, rehydrated through an ascending series of ethanol (%70, %96, and % 100). For morphologic comparison, Hematoxyline-Eosine (H&E) staining was performed. Periodic acid-shiff (PAS) staining was used to demonstrate carbohydrates and carbohydrate compounds in the sample sections which were examined using an Olympus BX-51 microscope and photographed by using an Olympus digital camera.

In order to evaluate the changes on the size of developing follicles, we calculated the range of the last 7-9 follicles each day of larval-pupal metamorphosis and their median value. Kruskal-Wallis one-way analysis of variance was performed in order to analyze whether the follicles sizes changed or not during the pupal stage. If there is a change, the Mann-Whitney Test was implemented to determine the day of the change.

Results and Discussion

Many insect species have a short and non-feeding maturation phase but they have functional sexual glands and fully grown eggs available for fertilization just after adult ecdysis

(Swevers et al. 2005). Regarding holometabolic insects, fat body is the main tissue providing for all the needs of the pupal and adult development, and is the main source of hemolymph and yolk proteins, which are necessary for healthy embryonic development. The maturation of female reproduction system involves the vitellogenesis and oogenesis processes which are regulated by hormones (Nijhout 1994; Belle's, 1998) and take place during pupation (Tsuchida et al. 1987; Ohnishi and Chatani 1977).

Development and maturation of the egg in the *Hyalophora cecropia* (Saturniidae), *Malacosoma alpicornis* (Lasiocampidae), *Lymantria dispar* (Lymantriidae) *Antheraea yamamai* and *Bombyx mori* (Bombycidae) start in the larval stage or early pupal stage and are completed before the pupal-adult ecdysis (Belle's 1998). Ecdysteroids are needed directly or indirectly to begin the vitellogenesis (Ohnishi and Chatani 1977). In *Bombyx*, during pupal-adult development, hemolymph ecdysteroids reach the maximum level on day 2. After the peak value, hemolymph quantity decreases dramatically and continues at low levels during the pupal stage (Hanaoka and Ohnishi 1974). The ovarian follicle cells begin to synthesize oo-ecdysteroids – which are originated from follicular epithelial cells- on day 4 of the pupal period (Ohnishi and Chatani 1977; Ohnishi and Watanabe 1985). Ecdysteroids from the prothoracic gland and from the ovary play a role as gonadotrophic hormones. Besides, it was reported that the development and maturation (vitellogenesis and choriogenesis) of oocytes require low levels of JH in the hemolymph (Chatani and Ohnishi 1976). The JH level in the insects included to the *Lepidoptera* is undetectable in the second half of the pupal stage (Belle's 1995).

The development of the ovary was extremely slow in the first three days; however, from the third day a striking increase was detected in the pupal stage (Tsuchida et al. 1987; Chatani and Ohnishi 1976; Parlak et al. 1992). A pair of complete ovarian structure and compact fat body lobules surrounding the organs was observed on 0th, 1st and 2nd days. On day 3, the sheath of the ovarioles was ripped and the

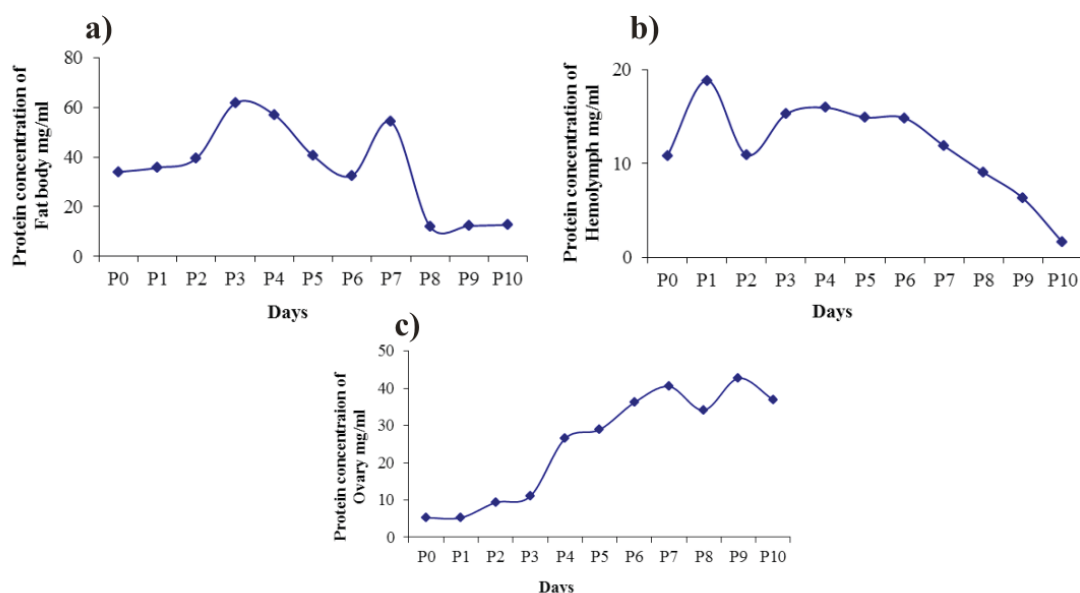


Figure 1. Total protein concentration of a) Fat body, b) Hemolymph and c) Ovary in female *B. mori* pupae. P0, P1, P2, P3, P4, P5, P6, P7, P8, P9 and P10 represented successive days starting from day 0 of pupae.

follicles directly connected with hemolymph and fat body in the abdominal cavity (Fig. 4). According to the results of Kruskal-Wallis one-way analysis of variance and Mann-Whitney test, we found that the increment in the range of follicles began on day 3 and ceased on day 7 of pupae.

Due to the protein accumulation and the expected natural results with the accumulation of hormones which are needed for embryonic development the dimensions of the ovary and follicle increased.

Degeneration in nurse cells starts in the 5th and 6th days of the pupal stage and transferring its contents into the oocytes occurs rapidly (Yamauchi and Yoshitake 1984). In the 7th day, the existent cytoplasmic bridges between the nurse cells and the oocyte is closed and the food substance that flows between these cells is hindered. Thus one of the most important factors in the increase in oocyte volume is eliminated. Based on the statistical data, there was a progress in the follicle dimensions starting the 3rd day and the change in the follicle dimensions a negligible degree after the seventh day. Similarly, constant and rapid rising of ovary protein concentrations from day 3 of the pupa to the end of pupation were reported

earlier (Tojo et al. 1981).

The amount of ovary protein was shown to increase suddenly between the days of 3 and 4 (Fig. 1-c). The transportation and absorption of the proteins are active processes. The transportation of yolk proteins into the oocyte is carried out with synthesis of transporter proteins acting in active transportation and the synthesis of receptor proteins necessary for receptor-mediated endocytosis. When the timetable of these processes and the high protein content of the ovary were considered, they might be stimulated by oo-ecdysteroids.

As a result of the Bradford (1976) assay on day 3, the protein content in the fat body reached its maximum value and decreased until day 7. A sudden drop was detected in total proteins of fat body (Fig. 1-a). The maximum protein content in hemolymph was measured on day 1 and regulated in the following days. The lowest protein content among the three tissues was calculated in the hemolymph just before adult ecdysis (Fig. 1-b). Similarly, in our study the total protein concentration in hemolymph decreases from the 3rd day of the pupal stage (Tojo et al. 1981). The ecdysone hormone secretion immediately before the pupal ecdysis is the triggering factor for the protein absorption

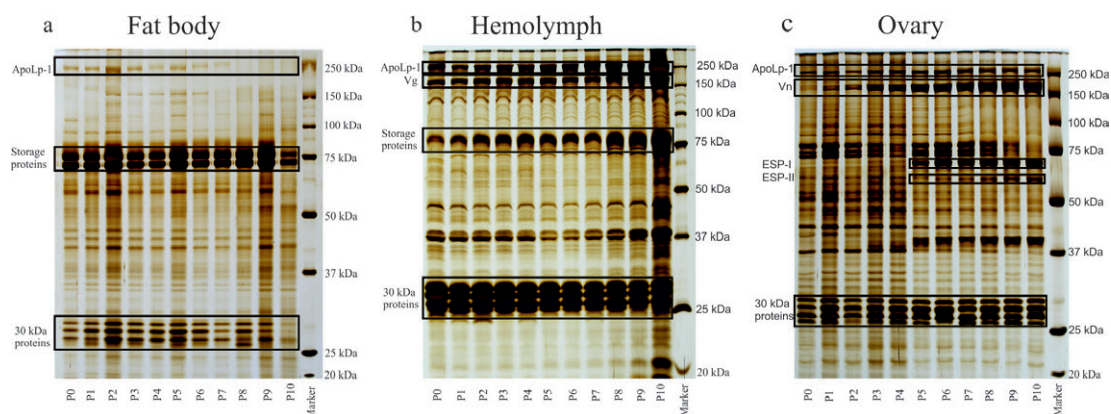


Figure 2. a) Fat body, b) hemolymph and c) ovary protein profiles of female *B. mori* pupae. On the SDS-PAGE gels, according to molecular weights well-defined and characterized protein bands were showed in boxes. apoLp-1 (230 kDa, lipophorin heavy subunit), Vitellogenin (178 kDa, vitellogenin heavy chain), Vitellin (178 kDa, vitellin heavy chain), Storage proteins (SP-1 and SP-2, 76kDa and 72kDa), ESP-I (72 kDa, egg specific protein heavy subunit), ESP-II (64 kDa, egg specific protein light subunit), 30 kDa proteins (25-30 kDa protein groups). P0, P1, P2, P3, P4, P5, P6, P7, P8, P9 and P10 represented successive days starting from day 0 of pupae.

which stored in the hemolymph to the fat body (Tojo et al. 1981). For this reason, the total protein content of hemolymph is expected to show a decrease while the fat body total protein content is expected to show an increase. The maximum protein content among the three tissues was found in the eggs before adult ecdysis. It is thought that along with the increase in the amount of egg total protein and the decrease in the amount of hemolymph protein that this could be explained as the absorption of the hemolymph proteins by oocyte.

To reveal the close relationship between the protein synthesis and metabolic activities in the fat body and ovary development, changes in the protein profiles of the fat body and ovary were carried out considering the changes in the hemolymph protein profiles and hormonal conditions during pupation.

Using SDS-PAGE with silver staining, the fat body, hemolymph and ovary proteins during pupal period of *B. mori* were separated to 35, 29 and 52 protein fractions, respectively. In this study, the most expressive protein groups in tissues during pupal development are grouped as follows: Lipophorins (especially large subunit apoLp-1) with a MW 230-250kDa, Vitellogenin (178 kDa) and its accumulated form in eggs, Vitellin (Vn) (178kDa), storage proteins (SP-1 and SP-2; 76 and 72 kDa), 30kDa proteins

which have 1-5 protein fractions, egg specific proteins in developing eggs (ESP) which have represented by its subunits, with MW 72 kDa (ESP-I) and 64kDa (ESP-II). In addition, lots of undetermined protein fractions were also detected (Fig. 2).

One of the well-defined and characteristic protein fractions during pupal development were storage proteins, SP-1 (female pupa specific protein, methionine-rich storage protein) and SP-2 (arylphorin), respectively 76kDa and 72kDa (Fig. 2 a-b). During the pupal period of *B. mori*, storage proteins (SP-1 and SP-2) provide the biological substances needed to form adult tissues. They are secreted into hemolymph and stored during the larval stage. With the increase of ecdysone level before the larval-pupal ecdysis, storage protein intake is triggered by the fat body cells during the same period; the increase in the juvenile hormone level as well constitutes a pressuring factor on the synthesis of these proteins (Tojo et al. 1981).

Storage proteins were found at all pupal days in female fat body and hemolymph. We evaluated the relative abundance of storage protein bands, SP-1 and SP-2, together. Their density decreased toward the end of the pupal stage in the fat body. The relative abundance of fat body fractions was much higher than in hemolymph. (Fig. 3- a). Storage proteins, destroyed as a re-

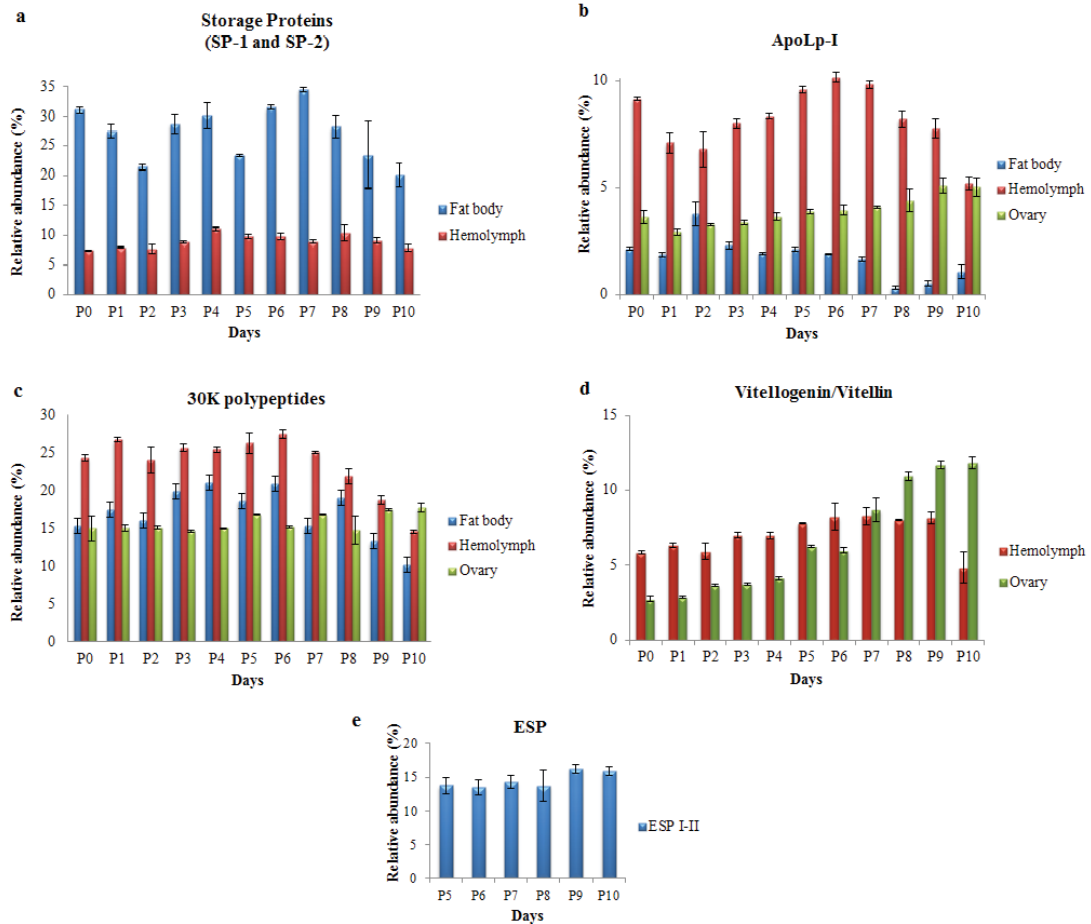


Figure 3. Relative abundance of a) Storage proteins in fat body and hemolymph, b) Apoliphosphorin-I (ApoLp-I), c) 30K proteins in fat body, hemolymph and ovary, d) Vitellogenin in hemolymph and Vitellin in ovary, e) Egg specific proteins ESP-I and ESP-II of female *B. mori* during pupal period. P0, P1, P2, P3, P4, P5, P6, P7, P8, P9 and P10 represented successive days starting from day 0 of pupae.

sult of the metabolic activities which were used in the new tissue and organ generations of the developing pupa. therefore, In the last day of the pupal stage therefore a notable decrease of its amount was noticed in the fat body. Also, storage proteins level in the hemolymph slightly increased during the pupal period. Similarly, according to Ogawa and Tojo (1981) the amount of storage proteins in the hemolymph showed an increase in the middle of the pupal stage. The reason for this could be explained as the programmed cell death occurring in the fat body and the secretion of proteins from the fat body to the hemolymph.

Neutral and polar lipids transporting among the tissues in insects occurs via reusable trans-

port proteins called lipophorins. Lipophorin quantity shows an increase in various metabolic activities such as its usage as an energy source, its need for lipids, the membrane synthesis belonging to different tissues, motion and flying along with the growth, development, diapause and the reproduction of the insect (Nijhout 1994). The presence of the proteins band thought to be the major subunit (ApoLp-I) of the lipophorin whose molecular weight was measured between 230–250kDa showed in gels (Fig. 2). Although the fat body is the main source of lipophorins, there were weak protein bands detected in the pupal fat body (Fig. 2-a). It was thought that these proteins may not be accumulated by the fat body and it is secreted into

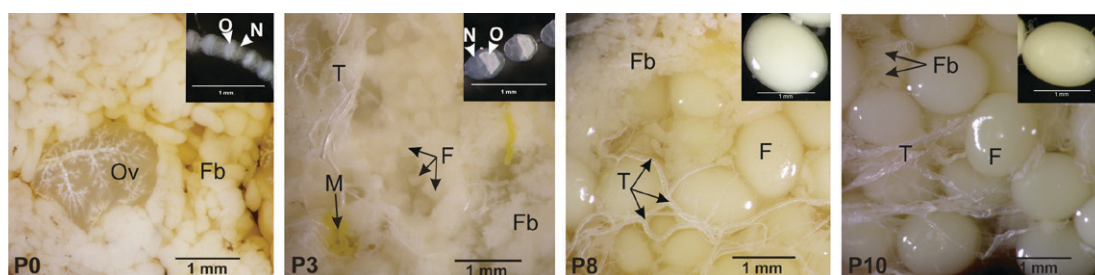


Figure 4 . Stereo microscope images of pupal *B. mori* ovary. Follicle images were shown on the right upper sight of each day. P0, P3, P8 and P10 represented day 0, day 3, day 8 and day 10 of pupae, respectively. O: Oocyte, N: Nurse cell, F: Follicle, Ov: Ovary, Fb: Fat body, M: Malpighi tubules, T: Trache and tracheols.

hemolymph just after its synthesis. The highest level of ApoLp-I was found in the hemolymph, but its ratio was downregulated by at the end of the pupal period (Fig. 3-b). The findings in our study showed parallel results with the reports of Janarthanan et al. (1998), Hyrsl and Simek (2005), Smith et al. (1994) and Pho et al (1996). Rising lipophorin protein level in the insect hemolymph during this period was thought to be explained with the increase in the energy requirement. Lipophorin was observed in the ovary in whole days of the pupal stage (Fig. 2). Its amount showed an increase in the forthcoming days (Fig. 3-b). According to Kawooya and Law (1988) oocytes are the sole structures which the lipophorins might enter into the insect cells. In this case, while the transportation of the lipids which are needed for the embryonic development was performed by the lipophorins which were in a circulation state in the hemolymph. Our results indicated that some amount of lipophorin was stored inside the oocyte.

30kDa polypeptides are synthesized by the fat body cells during the 5th larval stage and accumulated in the hemolymph. During the pupal stage, they are absorbed and stored as one of the yolk proteins in the oocytes for embryonic development (Hyrsl and Simek 2005; Zhu et al. 1986). All of the 30kDa proteins could not be absorbed by developing follicles. They were also used as an amino acid source in the maturation of other tissues.

The presence of 30kDa polypeptides was detected in all pupal tissues (Fig. 2).The highest amount was found in the hemolymph, then in the fat body and a lesser amount in the ovary

(Fig. 3-c). 30K polypeptides became the most common proteins in the pupal hemolymph (Kishimoto et al. 1999; Sun et al. 2007). Maximum relative abundance of 30K polypeptides was detected in hemolymph (Fig. 3-c). According to Janarthanan et al. (1998) and Hyrsl and Simek (2005), 30K polypeptides decline to nearly undetectable levels in the hemolymph in the last days of the pupa. However in our study, a noticeable decrease was not detected. The reasons for these differences were thought to be based on the methods applied and procedures followed in the studies.

It was determined that the amount of 30K polypeptides in the ovary increased unlike fat body and hemolymph (Fig. 3-c). These result suggested that its accumulation ese proteins within the eggs (Fig. 2-c).

The relative abundance of 30K polypeptides in the fat body -the main source of 30kDa polypeptides- decreased up to adult ecdysis (Fig. 3-c). As a result of the cell death in the fat body, metabolic activities would decline and protein levels would decline as well.

ESP is the yolk protein produced by the follicular epithelium of pupae. It is also deposited in yolk bodies and used as a marker of vitellogenesis (Raikhel and Dhadialla 1992). Kawaguchi et al. (1996) found ESP protein on day 5 of the pupal stage in the ovary. According to Ohno et al. (1975), ESP protein was not observed in the larval, pupal or pharate adult hemolymph, it was found only in the eggs. Similarly, predicted protein bands which belong to the subunits of ESP, 72kDa and 64kDa, were detected in developing oocytes beginning from the 5th day of pupae (Fig. 2-c). They were only found in the egg on day 5 of pupa.

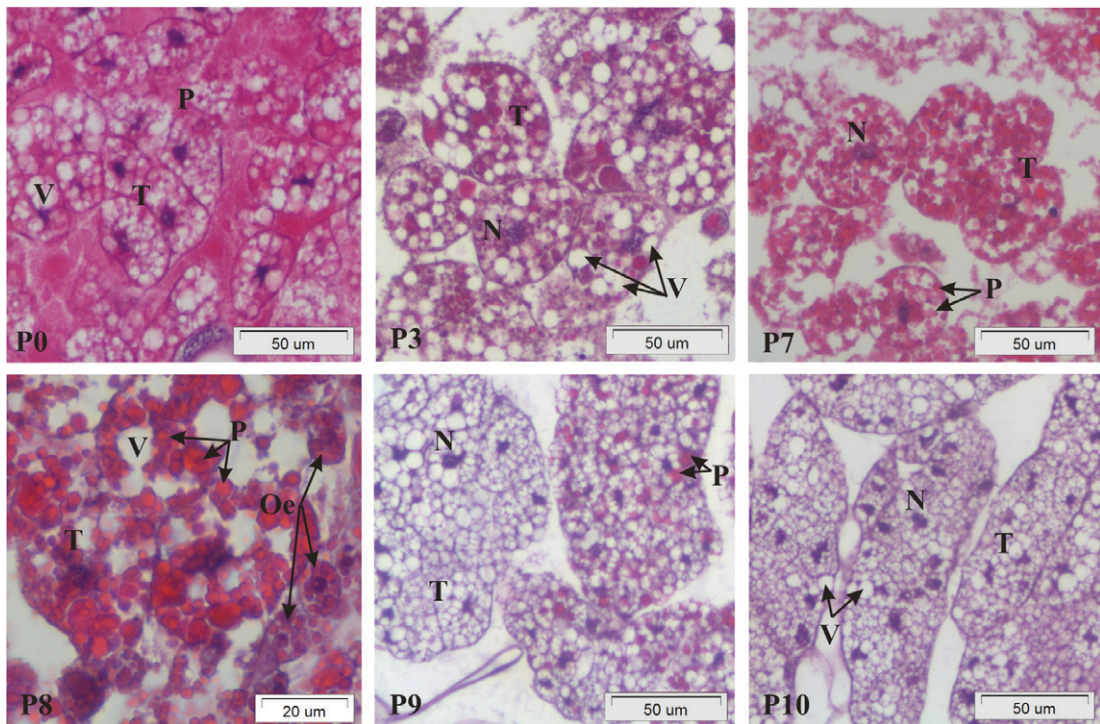


Figure 5. The histology of the fat body of female *Bombyx mori* pupae (Lepidoptera) stained with H&E. Oenocytes (Oe) were interspersed among trophocytes (T). They had several protein granules (P), eosinophil granules which distributed throughout the cytoplasm. Also lots of lipid droplets, vacuoles (V) and lucid vesicles were found. Trophocytes exhibited heterochromatic structure in their nucleus (N). P0, P3, P7, P8, P9 and P10 represented days of pupae starting from day 0.

The relative abundance of ESP subunits showed no significance protein expression fluctuations up to adult ecdysis. They maintained constant levels (Fig. 3-e).

Principle yolk protein precursor vitellogenin (Vg) has 420-440 kDa molecular weights (MW) a native and two subunits as 180 and 42 kDa, in *B. mori* (Izumi et al. 1980; Izumi and Tomino, 1983; Zhu et al. 1986; Vale, 1993). After being synthesised by fat body cells, they are immediately secreting to the hemolymph (Telfer 1965; Engelmann 1979) and transported into developing oocytes, stored as vitellin (Vn) for use in embryonic development (Oliveira and Cruz-Landim 2004). Similarly, in our study, Vg was seen only in hemolymph and ovary (Fig. 2 a-b-c). This finding showed that the fat body cells did not store but released Vg immediately after synthesizing.

Ecdysteroids triggered the hemolymph protein synthesis involving Vg (Ono et al. 1975). Vg syntheses started immediately after the pupation (Izumi and Tomino 1983; Tsuchida et al. 1987)

and it is found in the pupal hemolymph (Kawaguchi and Doira 1973) According to Uranlı (2011) the day before pupal ecdysis, Vg was already found in the larval hemolymph. We determined that the Vg -estimated molecular weights of 178kDa- found all pupal days in hemolymph (Fig. 2). Its relative abundance decreased in hemolymph by the end of pupal stage and probably depends on absorption from the ovary (Fig. 3-d). Early reports suggest that its amount in hemolymph remained stable (Ogawa and Tojo 1981). Furthermore, Janarthanan et al (1998) stated that between day 1 and day 6 of the pupa, Vg was accumulated in the hemolymph. Different from the study of Janarthanan et al (1998), the hemolymph level was thought to maintain almost steady quantities in whole days. Ogawa and Tojo (1981) found the Vg only in hemolymph in the first half of the pupal stage while its presence was detected in the ovarioles and hemolymph in the second half of the stage. According to Tsuchida et al (1987), on day 2 of the pupal stage when

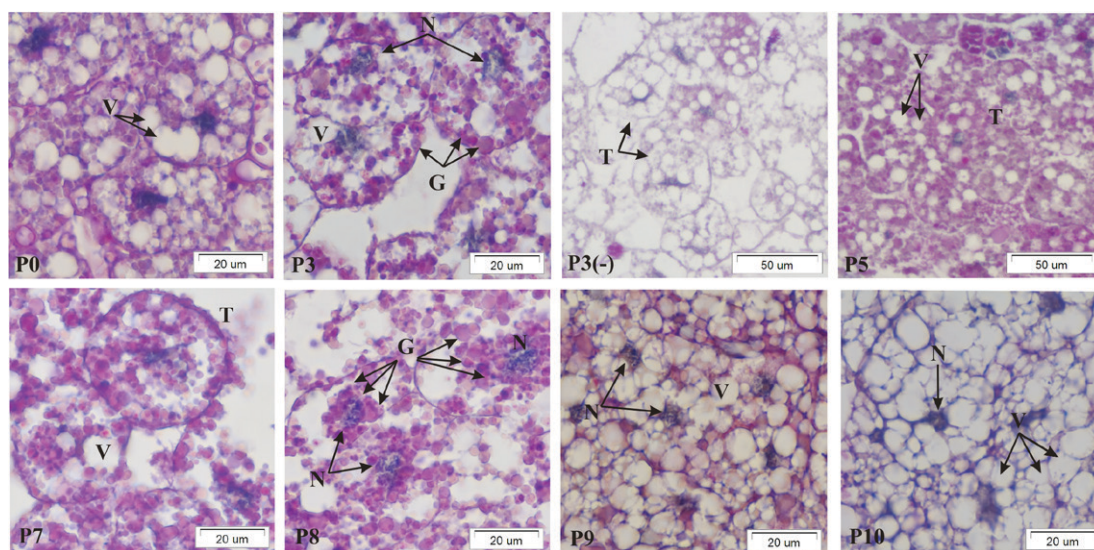


Figure 6. Histological sections of the female pupal fat body of *Bombyx mori*, stained with Periodic acid-Schiff reagent (PAS). Trophocytes (T) showed vacuolization in their cytoplasm. It was detected that glycogen containing vacuoles (G), staining with PAS, rested upon nucleus (N) on day 8 of pupae. sudden decreasing on the glycogen deposits (G) was detected. P0, P3, P5, P7, P8, P9 and P10 represent pupal days starting from day 0, P3 (-) showed PAS negative staining belonging to day 3 sections.

the ecdysone level reached its highest level in the hemolymph, Vn protein was found in the ovary immunologically. In our study it was found that the bands having the same molecular weight as the Vn were observed in ovary and eggs in whole days of the pupal stage and the amount in the ovary increased throughout the pupal stage (Fig. 2 b-c). Vn reached its maximum level just before adult ecdysis. The presence of bands on day 0 and day 1 was thought to be the result of a different protein expression having the same molecular weight.

Early reports in the pupal period of *B. mori* suggest that Vg was detected in the hemolymph and ovary, it was not found in the fat body in females (Ogawa and Tojo 1981).

Histologically, the fat body of *B. mori* showed three different cell types: Trophocyte, oenocyte and urocyte (Oliveira and Cruz-Landim, 2003) (Fig. 5, 6, 7 and 8). Trophocytes are polygonal cells with centrally round nuclei containing intensely chromatic content and a cytoplasm with plenty of lipid and protein containing vacuoles (Fig. 5). Oenocytes are large; spherical cells. They have single rounded nucleus and eosinophilic cytoplasm with variable numbers of inclusions (Fig. 5, 8). They are intercalated between the other fat body cells and

intimately associated with them. Urate cells or urocytes are specialized in accumulation of uric acid for excretion and storage as urate granules which are generated from protein degradation and do not show organic material accumulation or protein synthesis (Wigglesworth 1942; Arrese and Soulages 2010). They had a central nucleus with a large amount of heterochromatin and several small and lucid vesicles in their cytoplasm (Fig. 7). Their numbers were lesser than trophocytes or oenocytes.

Trophocytes underwent a distinct rise in size by accumulation of cellular inclusions and enlarging vacuoles until day 8 of the pupal period then the size of the cells showed an abrupt decrease on day 9 and day 10 (Fig. 5). Various sizes eosinophilic granules and vacuoles, probably lipid and/or autophagic, within the cells were observed, especially on day 7 and other day samples (Fig. 5). The ecdysone level, secreting on day 2, probably act as the triggering factor for the absorption proteins from hemolymph to the fat body cells. Especially on day 3, when the total protein concentration of fat body reached peak value, the sizes of the trophocytes were enlarged and plentiful everywhere (Fig. 5-6). Glycogen and protein reserves are sharply consumed on day 9. Degrading of biomaterials be-



Figure 7. Urate cell (U) dispersed among the trophocytes (T) stained with H&E (a,b) and PAS(c) on day 0 of pupae. Central nucleus (N) showed heterochromatin structure and the cytoplasm had electron lucid material in small vesicles (v). There were some small eosinophil granules, proteinic structure, could be found in urate cell cytoplasm and the size of the cell was much higher than the other fat body cells.

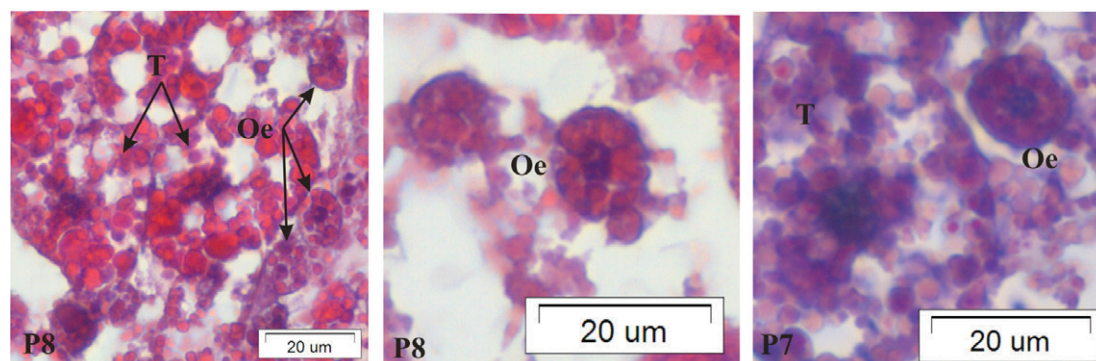


Figure 8. Histological section of the fat body cells stained with H&E on day 7 and 8 of *Bombyx*. Oenocytes (Oe) distributed among trophocytes (T) these cells showed eosinophilic vacuolization in their cytoplasm. Oenocytes and trophocytes showed PAS positive reaction.

cause of increasing energy supply and ongoing programmed cell death process may deeply affect the content of materials inside the cells.

In mosquito larvae (Wigglesworth 1942) and in *Pachycondyla villosa* larvae (Zara and Caetano 2004) glycogen is absent from the oenocytes. In our study, the oenocytes histochemically reacted positively to the PAS test for carbohydrates. Glycogen is widely dispersed and it is plentiful in the fat body cells, both trophocytes and oenocytes. It is found that the number of oenocytes increased with age. Oenocytes are the cells which actively join cuticular lipid synthesis in arthropod groups. As far as the adult ecdysis is concerned, the requirement of newly cuticle, is expected to be rising in the number of these cells, as in our study.

Fat body cells appear as looser aggregated small nodules suspended in the hemocoel and in contact with the hemolymph, making pos-

sible the metabolic changes between cells and hemolymph. Sumithra et al. (2010) reported that after cessation of feeding the larval fat body loses its structural integrity and moreover disintegrates completely before the larval-pupal ecdysis. Similarly, the lobular structure of the fat body was also observed at the beginning of pupal period but at the end of pupal period it lost its compact structure (Fig. 4). The combination of cell death mechanisms together degraded the fat body during metamorphosis (Sumithra et al. 2010). Programmed cell death, apoptosis and autophagy, is involved in the degradation of the larval tissues during metamorphosis in Lepidoptera (Goncu and Parlak 2008). Autophagy is characterized by the formation of autophagic vacuoles used for cytoplasmic destruction (Goncu and Parlak, 2008; Sumithra et al. 2010). Also the disintegration of pupal fat body tissues is typical morpholo-

gy observed in tissues undergoing apoptosis. In our study, there were lots of vacuoles detected especially towards the end of the pupal stage. Hence, the vacuolization in trophocytes can be interpreted as auto-phagocytic cell vacuoles and the disintegrity of fat body can be interpreted as a programmed cell marker during transformation of *B. mori*.

We refer to the fact that the protein quantities and contents of the tissues show fluctuations and changes which are controlled by the hormonal factors. All of these biochemical changes were associated with follicle size depending on the pupal days. As a result, total protein amount in the ovary and eggs increased from day 0 to the 10th day of the pupal stage in contrast to hemolymph and fat body. It was observed that the secretion and the synthesis of proteins in insect tissues and their absorption by the other tissues or organs were organized under the hormonal control of the metamorphosis. According to our results, changes in the protein contents of the fat body and hemolymph had a direct effect on the ovary and egg development.

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