The vertebrate insulin alters the expression profile of steroid hormone ecdysone receptor complex components and autophagy-related genes in the pupal fat body of silkworm, *Bombyx mori*

Sedat Yelkovan[a,b] and Nesrin Ebru Goncu[a*]

[a] Ege University, Faculty of Science, Department of Biology, Izmir, Turkey

[b] Bingol University, Pilot University of Central Coordination Unit, Bingol, Turkey

*Correspondence: ebru.goncu@ege.edu.tr

ORCIDs:

First AUTHOR: https://orcid.org/0000-0002-7209-6350

Second AUTHOR: https://orcid.org/0000-0002-0191-4141

Abstract:

Various studies showed that the insulin-like peptides present in insects and investigation of their possible effects on insect physiology is substantial for clarification the evolutionary developmental period of insulin. Insect fat body is an organ analog to vertebrate adipose tissue and liver. In the insects, which don't show feeding activity during pupal and adult stages such as *Bombyx mori*, substances and energy required for continuation of life and development of tissues and organs are provided by the fat body via autophagy process. In this study, we aim to analyze the action of mammalian insulin in *Bombyx* pupae by studying its effects on physiological parameters, and the transcript levels of both ecdysone receptors and autophagy-related genes- *Atg*8 and *Atg* 12. Our

results showed that biochemical contents of the *Bombyx mori* fat body and the expression of genes related to autophagy and ecdysone receptor complex elements differently responded to insulin treatment depending on the application time.

Keywords: Autophagy, *Bombyx mori*, ecdysone receptor complex, fat body, insulin

1. Introduction

In mammalian, the coordination between insulin and glucagon hormones is important in the control of sugar metabolism. Insulin plays a number of roles in the body's metabolism such as regulation of blood glucose level and triggering the synthesis of glycogen and fatty acids. Insulin-like peptides (ILP) with hypoglycemic effects (Brogiolo et al., 2001; Cao and Brown, 2001) were demonstrated in insects (Steele, 1961; 1963; Bednarova et al., 2013). Bombyxin from *Bombyx mori* brain was the first identified insulin-like molecule with hypoglycemic action (Ishizaki and Suzuki, 1994; Nagasawa et al., 1986). The amino acid sequence of bombyxin-II showed about 50% similarity with mammalian insulin (Nagasawa et al., 1986). Insect steroid hormone, 20 hydroxyecdysone (20E) regulates many physiological events in insect body (Wigglesworth, 1972). Crosstalk between 20E and insulin signaling regulates growth and development and also antagonistic relationship between these two hormones was demonstrated in fat body of *Drosophila melanogaster* (Colombani et al., 2005). 20E regulates the transcription of target genes via the ecdysone receptor complex which consists of the ecdysone receptor (EcR) and its partner ultraspiracle (USP). Two of the three ecdysone receptor isoforms identified in *Drosophila*,- EcR A, and EcR B1-homologs were determined in *Bombyx mori* (Swevers et al., 1996). EcR and USP are orthologs of mammalian farnesoid X receptor (FXR)/liver X receptor (LXR) and

retinoid X receptor (RXR), respectively. These receptors play important roles in metabolism, growth and reproduction of mammalians (Yang et al., 2015). However, it is not clear whether EcR and USP play a role in the regulation of metabolism in *Bombyx mori* other than their role in reproduction and development.

Autophagy is an evolutionarily well-conserved mechanism in eukaryotic cells. In most cells, the autophagy machinery is active at the basal level and is responsible for the recycling of long-lived proteins and damaged structures. Under starvation condition, autophagy allows maintaining of metabolic activities required for life and also plays a role in different diseases such as cancer, neurodegenerative diseases and liver-related disorders (Codogno and Meijer, 2005). The autophagy process begins with the formation of autophagosomes enclosing a part of the cytoplasm and surrounded by a double-layered membrane structure, then it is followed by digestion of the material contained as a result of the association of autophagosomes with lysosomes. The formation of autophagosomes was first shown in the yeast and determined to be mediated by *Atg* proteins. (Tsukada and Ohsumi, 1993). Bioinformatics analyzes have shown that *Bombyx mori* genome contains homologous of more than 20 *Atg* genes. During insect life, endocrine mechanisms strictly control the emergence of intense autophagosomes and autolysosomes in larval tissues such as salivary gland, fat body, midgut and prothoracic glands (Butterworth et al., 1972; Komuves et al., 1985). Steroid hormone 20E stimulates autophagic activity both in vivo and in vitro (Sass and Kovacs, 1975; Lee and Baehrecke, 2007). The 20E-EcR-USP complex in insects has an important role in the induction of autophagy (Berry and Baehrecke, 2007; Jiang and Baehrecke, 1997). This complex and associated transcriptional factors cause starvation-like conditions in the pupal period by terminating food consumption then induce

autophagy (Tian et al., 2010). In mammals, insulin inhibits autophagy via two mechanisms; activation of mTORC1 (Meijer et al., 2015) and protein kinase B which inhibits a transcription factor, FoxO3, for *Atg* gene expression (Mammucari et al., 2007).

Insect fat body is the main target of 20E and insulin-like peptides. Hormones (ecdysteroids, insulin-like peptides, adipokinetic hormone and etc.) and nutritional signals regulate the development and function of the fat body. It fulfills critical processes for the survival of insect and development of reproductive cells during the pupal and adult period in which there is no feeding activity. Metabolic and synthetic activities of the fat body mainly regulate organic molecules in the insects (Chapman, 1998). Similar to the mammalian liver, the fat body metabolizes stored products and uses them for the production of new biological molecules. In addition, it transfers organic molecules to other tissues for use as energy sources and biochemical building blocks (Arrese and Soulages, 2010). Autophagy is the main process for mobilization of stored macromolecules in the fat body during the pupal period (Rusten et al., 2004).

Studies on the effects of insulin in insects have increased significantly in recent years. Especially the presence of insulin-like peptides in insects has accelerated this works. Several studies indicated that insulin and insulin growth factor signaling is quite important for fat body function but there is no study on the modulation of the expression of ecdysone receptors and autophagy-related genes by mammalian insulin application to *Bombyx mori* during pupal-adult development. This study aims to understand the action of mammalian insulin in *Bombyx* pupae by studying its effects on physiological parameters, and the transcript levels of both ecdysone receptors and autophagy-related genes- *Atg* 8 and *Atg* 12-. Insulin hormone in mammals affects autophagy activity in

hepatocytes but there is no report about the effects of vertebrate insulin on biochemical contents of fat body and autophagy in *Bombyx* pupae. Our results indicated that the expression of ecdysone receptor complex elements and autophagy-related genes in the fat body of *Bombyx mori* showed the different response to applied insulin depending on the application time.

2. Materials and methods

2.1 Insect rearing

We reared *Bombyx mori* eggs (Chinese x Japanese) from Kozabirlik (Bursa) in *Bombyx mori* culture laboratory at 25 ± 1 °C, 75-80% relative humidity and LD 12:12 photoperiod conditions. The animals were fed 3 times a day with fresh mulberry leaves. After larval-pupal ecdysis, we applied insulin on day 0 and day 6 of the pupal stage. Injection was performed once to each animal. Fat body dissections were performed every 24 hours, following application, until the pupal-adult ecdysis. The reason for the selection of female insects in the study is the amount of ecdysone they have in the pupal period. Studies show that the ecdysone determined during the first 4 days of the pupal period in male insects has decreased to an undetectable level from day 5. In females, because of the hormone secretion activity of the ovarian follicle cells in the second half of the pupal period, ecdysone was found in female's hemolymph until pupal-adult ecdysis. Since the effects of insulin hormone were planned to be investigated in relation to ecdysone, the female silkworms are a more suitable model for this study.

2.2 Hormone applications

Hormone treatments were carried out after larval-pupal ecdysis and day 6 of the pupal stage. Bovine insulin (50 μg of 5 μl) was administered as an abdominal injection with Hamilton syringe. We used 400 animals for the treatment in each group.

2.3 Determination of lipid, sugar and glycogen levels

We detected lipid, glycogen, and total sugar levels using a series of biochemical analysis according to the method as described previously (Van Handel, 1985a; 1985b). Briefly, fat bodies were homogenized using PTFE pestle in a glass tube containing 200 μL of 2% sodium sulfate in distilled water and then placed on ice. The homogenates were extracted with 1,6 ml of chloroform-methanol (1:1), then centrifuged at 3000 rpm for 1 min. The resulting supernatant was transferred to a glass test tube for the sugar and lipid assays and the precipitate was left in the microcentrifuge tube for the glycogen assay. To separate the sugar and lipid reaction, 600 μl of deionizing water was added to supernatant and vortexed, and then centrifuged at 3000 rpm for 1 min. The upper fraction was used for sugar analyze and the bottom fraction was used for lipid assay. All tubes were heated at 90 - 110 °C until all of the solvent had evaporated from the tubes.

2.3.1 Glycogen analysis

For glycogen analysis, 5 ml of anthrone reagent was added to the tube containing the precipitate and heated at 90 - 110 °C for 17 min. The tube was cooled and the absorbance read at 625 nm. Glycogen concentrations were calculated from the prepared standard curve using glucose. Tissue glycogen levels were normalized to wet tissue weight.

2.3.2 Sugar analysis

We added 4.95 ml of anthrone reagent to the sugar fraction, and then heated at 90 - 110 °C for 17 min. after cooling of the tubes, the absorbance was read at 625 nm. As for the glycogen, concentrations were obtained from the prepared standard glucose curve. Tissue sugar levels were normalized to wet tissue weight.

2.3.3 Lipid analysis

To analyze lipids, 200 µL of sulfuric acid was added to the tubes containing the lipid fraction. The tubes were heated again at 90 - 110 °C for 10 min, then 4.9 ml of a vanillin-phosphoric acid reagent was added. The solution in each tube was left at room temperature until the red color developed. The optical density was read at 625 nm using a spectrophotometer. Lipid concentrations were calculated obtained from the prepared standard curve using corn oil. Tissue lipid levels were normalized to wet tissue weight.

2.4 RNA isolation and cDNA synthesis

After homogenization of fat body samples in Tripure isolation reagent (Roche), total RNA was isolated as specified by the manufacturer. The amount and purity of the isolated total RNA were determined by Nanodrop UV / VIS spectrophotometer. cDNA synthesis was performed using the High Fidelity cDNA synthesis kit (Roche) using 1 µg of RNA.

2.5 Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The relative expressions of the selected genes were determined by using the LightCycler 480 real-time PCR detection system (Roche) according to the the method as described

previously (Gibson et al., 1996). qRT-PCR was performed in 10 µl of reaction volume containing 2 µl of cDNA, 0.5 µl of primers, 2.7 µl of H_2O, 0.2 µl of Tprob and 5 µl of the enzyme. Final concentrations of cDNA, primers and Tprob are 100 ng, 0,5 µmols and 0,2µmols, respectively. PCR conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for the 30 s, 72 °C for 1 s, 40 °C for 30 s. The primers used for PCR that were derived from the sequences of the Bombyx genes and references are listed in Table. Primer probe design was performed by using clustal W align and oligo7 software. Specificity of obtained primers and probes was controlled by using the blast program. In addition, specificity of RT-PCR products was demonstrated in a gradient cycler (Techne TC-400) and carried out in a total volume 45 µl containing 1.8 µl Faststart high fidelity reaction buffer (Roche Diagnostics), 5 µl cDNA, 0.4 µM forward and reverse primers and 2.5 U/µl Faststart High Fidelity Enzyme. Amplification cycles were as follows: 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 50 sec. The 35 amplification cycles were preceded by a primary denaturation step 95 °C for 2 min. RT-PCR products were determined with 4% high-resolution agarose gel electrophoresis containing 4 µg/ml GelRed and analyzed with Chemidoc MP imaging system (Biorad). *Bombyx mori Actin* 3 was used as an endogenous control. Mean and standard errors were obtained from the averages of three independent sample sets.

3. Results

3.1 Effects of insulin on some biochemical parameters of fat body

We detected fat body sugar, glycogen, and lipid levels both in the control and the insulin-treated groups (Figure 1, 2). Insulin treatment on day 0 pupae did not cause any significant effect on sugar content of fat body until day 6 of pupal stage (Figure 1a).

However, on day 6, the amount of sugar determined in the fat body was significantly lower than the control group. Another significant difference in the sugar levels of control and the treated group was determined at the beginning of the adult stage. Insulin treatment caused an increase in sugar level during this developmental period from 0.4 ± 0.003 µg/g to 1.74 ± 0.065 µg/g (Figure 1a). Glycogen contents in pupae treated with insulin on day 0 were quite similar to control until day 6 of pupal stage. However, an increased glycogen accumulation to 34.45 ± 4.38 µg/g was observed in day 6 pupae. After this day, glycogen levels gradually decreased till adult stage and glycogen levels on day 9 was significantly lower than the control (Figure 1b). Insulin treatment on day 0 caused a decrease in lipid level on day 2 of the pupal stage to 1.15 ± 0.58 mg/g, but interestingly, noteworthy increments from day 6 were observed in the fat body lipid contents. The highest lipid levels were detected on the last day of the pupal stage (Figure 1c). In contrast to day 0 application, treatment of insulin on day 6 pupae resulted in a remarkable increase in sugar levels from 0.12 ± 0.0045 µg/g to 23.9 ± 2.76 µg/g (Figure 2a). In contrast to high sugar levels, glycogen was found to be very low which means glycogen appeared to be largely depleted in this group (Figure 2b). In contrast to day 0 application, lipid levels in pupae which treated with insulin on day 6 did not show significant differences from control except day 8. On this day, lipid levels in treated insects decreased from 1,42 ± 0.047 mg/g to 0.76 ± 0,021 mg/g (Figure 2c).

3.2 Expression levels of ecdysone receptor complex and autophagy-related genes in the insulin-treated pupae

The insulin treatment caused different gene expression results depending on the application day of insulin. Insulin application on day 0 caused to decrease in expressions of ecdysone receptor isoforms. In the control group, EcR A mRNA levels

showed three peaks on day 3, 8 and 9 respectively. However, these peaks were not detected after insulin treatment on day 0 (Figure 3A). EcR B1 mRNA levels in the control group gradually increased from day 5 to adult stage. Although a similar expression pattern was observed in the day 0 application group during the same days, it did not reach higher levels which were determined in the control group (Figure 3B). USP 1 mRNA levels in day 0 treated insects did not differ significantly from the control group until day 10 of the pupal stage. On the day 10, however, the USP 1 peak in the control group did not take place in the treated insects, and the transcript amount remained at about one-fifth of the control group (Figure 3C). Similar to ecdysone receptor isoforms, insulin treatment on day 0 mostly suppressed USP 2 expressions during the pupal stage. On the other hand, USP 2 peak occurred on day 8 in treated insects as in the control group (Figure 3D). The insulin application at the beginning of the pupal stage resulted in a decrease in the expression of the autophagy-related genes *Atg* 8 and *Atg* 12 with the ecdysone receptor complex genes especially in the second half of the pupal stage (Figure 3E, F). This suppression in expression is much more severe in the *Atg* 8 gene. The *Atg* 8 mRNA expression profile in the treatment group was similar to the control group until day 6 of the pupal stage and at low levels. A small increase in the expression from day 7 occurred, but until the day 10 of the pupal stage, *Atg* 8 expression was found significantly lower than the control group. However, as in the control, the *Atg* 8 expression rapidly increased to the peak level on day 10 of the pupal stage, and then decreased rapidly by the beginning of the adult stage (Figure 3E). Insulin treatment on day 0 did not cause a significant change in *Atg* 12 mRNA levels until day 6 of the pupal stage but then maintained relatively lower levels until the end of the experiment (Figure 3F).

Insulin treatment on day 6 caused a delay in EcR A peak which was detected on day 8 in the control group and expression levels of EcR A mRNA's in the fat bodies was detected significantly lower than control. On the other hand expression ratio of the gene increased sharply in treated insects on day 9 and reached the almost similar level with the control. At the beginning of adult stage, despite EcR A transcripts decreased almost undetectable level in the control, upregulation in the EcR A expression was detected in the treated insects (Figure 4A). EcR B1 expression profile after insulin treatment on day 6 showed a similar pattern with EcR A. There was a delay occurred in the expression peak on day 9 and quite high EcR B1 mRNA level was detected in the adult (Figure 4B). Insulin treatment on day 6 did not cause significant changes in the USP 1 mRNA levels during the pupal stage, however, it was found significantly high in the adults (Figure 4C). A delay in USP2 peak similar to those occurring in ecdysone receptor isoforms was detected in the treated insects. Besides, the high expression rate of the USP 2 gene was also found at the adult stage (Figure 4D). Rising pattern of *Atg* 8 expression determined in the control group was not observed after insulin treatment. However, its mRNA level in treated insects sharply increased on day 9 and reached the same level those in control. In contrast to the highest *Atg* 8 expression level detected on day 10 in the control group, treated insects showed significantly low transcript level on the same day because of sharp decrease in the expression (Figure 4E). Insulin treatment on day 6 resulted in a decrease in *Atg* 12 mRNA level on day 10 of the pupal stage but then showed a sharp increase after pupal-adult ecdysis (Figure 4F).

4. Discussion

In this study, we showed that the effects of insulin treatment on the fat body contents, expression levels of ecdysone receptor elements and autophagy-related genes changed

depending on the application time. Application of insulin soon after larval-pupal ecdysis suppressed production of ecdysone receptor complex elements and autophagy-related genes in the pupal fat body whereas day 6 treatment did not show an inhibitory effect but caused a delay in the increase of mRNA levels of studied genes. The insect steroid hormone 20-hydroxyecdysone (20E) plays a role in the regulation of metabolism and autophagy as well as many metamorphic processes during insect life. Heterodimer receptor complex, ecdysone receptor (EcR) – ultraspiracle (USP) mediates all of these events (Tian et al., 2010; Chang and Neufels, 2010; Yin and Thummel, 2005). The antagonistic effect of 20E on the insulin/insulin growth factor (IIS) pathway in *Drosophila* has been well studied (Colombani et al., 2005; Rusten et al., 2004). At the same time, however, the insulin signal can also inhibit the ecdysone signal. Therefore, there is a mutual antagonistic interaction between the ecdysone and the insulin signals in the fat body. Previous literature showed that the transcription factor Forkhead box protein subtype (FoxO) is critical between insulin signaling mechanisms and ecdysone (Liu et al., 2010). IIS signaling pathway causes to phosphorylation of FoxO and inhibits its activity, however, 20E inhibited IIS signaling in fat tissue, but increased FoxO activity which leads to upregulation of ecdysone receptors and autophagy-related genes (Colombani et al., 2005; Rusten et al., 2004; Mensch et al., 2008). 20E triggers programmed autophagy of fat bodies and it is important for providing energy and other nutrients for the development of adult insects (McPhee and Baehrecke, 2009). The silencing of genes associated with autophagy by RNAi in *Bombyx mori* led to lethal effects during prepupal and pupal stages (Tian et al, 2010).

Our results suggested that insulin treatment inhibits the ecdysone signaling pathway via blocking the expression of the genes involved in the ecdysone receptor complex and

autophagy. Effects of insulin on the expression of studied genes were permanent when it was applied on day 0 of pupal stage. Low levels of *Atg* 8 and *Atg* 12 mRNAs in the day 0 treatment group indicated that insulin treatment suppresses autophagy in the fat body, as in the liver of mammals. Starvation-induced lipolysis during the pupal period is mostly dependent on the activity of lipase, Brummer (Grönke et al., 2007) and it has been reported that 20E activated EcR suppressed feeding activity and triggers lipolysis by inducing lipase Brummer gene expression in *Drosophila melanogaster* (Kamoshida et al., 2012). When EcR genetically removed, rising lipid accumulation in the fat body was seen in *Drosophila* (Kamoshida et al., 2012; Beckstead et al., 2005). Additionally, autophagy regulates lipid metabolism and intracellular lipid contents of the fat body under starvation condition (Singh et al., 2009). Therefore, a connection between the inhibition the expression of ecdysone receptors and autophagy-related genes due to insulin application on day 0 and suppression of lipid mobilization seems possible.

Insulin treatment on day 6 showed completely different effects both in gene expression levels and fat body contents. In contrast to day 0 application, mRNA levels of studied genes suppressed transiently thereafter increased as in the control. We speculated that production of ecdysone from the ovary by the beginning of day 6 of pupal stage (Ohnishi and Chatani, 1977) may break down the inhibitory effect of insulin. Interestingly mRNA levels of all studied genes were found significantly high in adult insects after insulin treatment on day 6. These results may relate to the interaction between insulin and bombyxin, because, bombyxin is produced in high levels during second half of the pupal stage especially in female insects (Saegusa et al., 1992). These results may relate to the interaction between insulin and bombyxin which is found high in the pupal hemolymph during the second half of the pupal stage (Saegusa et al., 1992).

It has been reported that both bombyxin and mammalian insulin trigger ecdysone synthesis from the ovary of blowfly, *Phormina regina* (Manière et al., 2004). Therefore, the presence of insulin and bombyxin at the same time could increase ecdysone production capacity of the ovary which upregulates ecdysone response genes like ecdysone receptor elements and autophagy-related genes. Determination of 20E levels after insulin treatment will help to clarify this possibility. In contrast to day 0 treatment, sugar metabolism in the fat body completely altered after insulin application on day 6 which means a factor which is present on day 6 was responsible for that effect. According to previous literature, one possible factor is thought to be bombyxin, because it has been reported that bombyxin decreases glycogen content of fat body (Satake et al., 1997). Therefore, in this study, the presence of bombyxin at the same time with insulin may enhance catabolic effects of insulin on glycogen.

4. Conclusions

According to results from day 0 treatment, permanent inhibitory effects of insulin on the expression of studied genes suggested that endocrine factors and other physiological conditions at the beginning of the pupal stage are critical for ecdysone signaling during the second half of the pupal stage. Results obtained from day 6 treatment group also prove the importance of the early pupal day's conditions for the upregulation of ecdysone response genes during the late pupal stage.

Insulin is released when mammals take food into their body and then anabolic metabolic reactions such as glycogen production and lipid storage are triggered. However, in this study, we applied insulin when the insects were under a kind of starvation conditions due to pupal stage. In addition, insect steroid hormone ecdysone controls both

metamorphic changes and metabolic processes during pupal stage and previous studies showed interaction between insulin and ecdysone signaling pathway. These are physiological differences between insects and mammals. The effects of insulin in lipid metabolism of *Bombyx* pupae is quite similar to those in mammalian, on the other hand, its role on sugar metabolism should be clarified. When metabolic disturbances such as abnormal lipid accumulation and glycogen catabolism are taken into consideration, we propose that mammalian insulin act a role on insect metabolism by interacting to the ecdysone signaling pathway and bombyxin, respectively.

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Table Forward and reverse primers used in qRT-PCR

Gene	Forward primer Reverse primer	Accession no	upl prob no
B. mori actin 3	5′- GCTCCCTCGAGAAGTCTTACG-3′	U49854	9
	5′- CTGGGCAACGGAATCTTTC -3′		
EcR-A	5'-CATCCGGTCAACGGACAC-3'	D87118	141
	5'- ACCGTAGCTGCCTGAGGATA-3'		
EcR-B1	5'- ACTTGGCAGTCGGATGAAG - 3'	L35266	153
	5′- CGTCATCTCCGTGATCTGG -3′		
USP-1	5′- TCAAATAGGCAACAAACAGATAGCCGCTC-3′	U06073	150
	5′- CAGGAACTCCATAGACCG -3′		
USP-2	5′- CAGTGTCACATGTAGAGTGCAAAGA -3′	AB182582	
	FAM-GTTCAACGACCTTGTTGCTGACAGGTTC-Tamra		
	5′- CCACTTTCATAGAACAGTTCAGTTGC -3′	Taqman probe	
BmATG8	5′- TCCGGAAACGTATTCACCTG -3′	FJ416330	124
	5′- GGGTGGAATGACATTGTTCAC -3′		
BmATG12	5′- CCTGTATGTGAATCAGACTTTTGC -3′	FJ416329	9
	5′- CCGAAGCACTCATAAAGATTCC -3′		

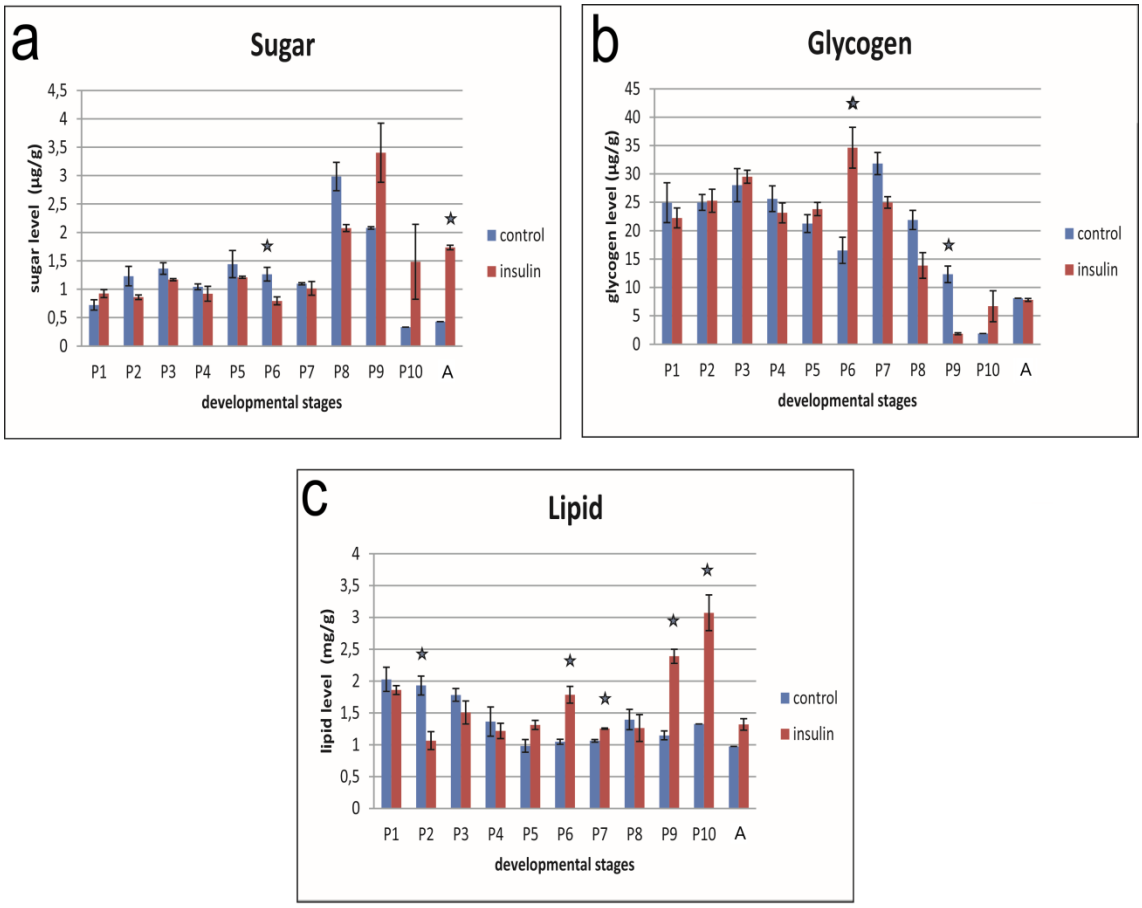

Figure 1. Some biochemical parameters of female fat body after insulin treatment on day 0 of the pupal stage. Sugar levels (a), glycogen levels (b), lipid levels (c) . The X-axis shows the developmental days from day 0 (P0) of the pupal stage to the day 0 (A) of the adult stage. Mean ± S.D. are for the three independent experiments. Asterisks indicate significant differences in the expression ($P \leq 0.05$) using t-test.

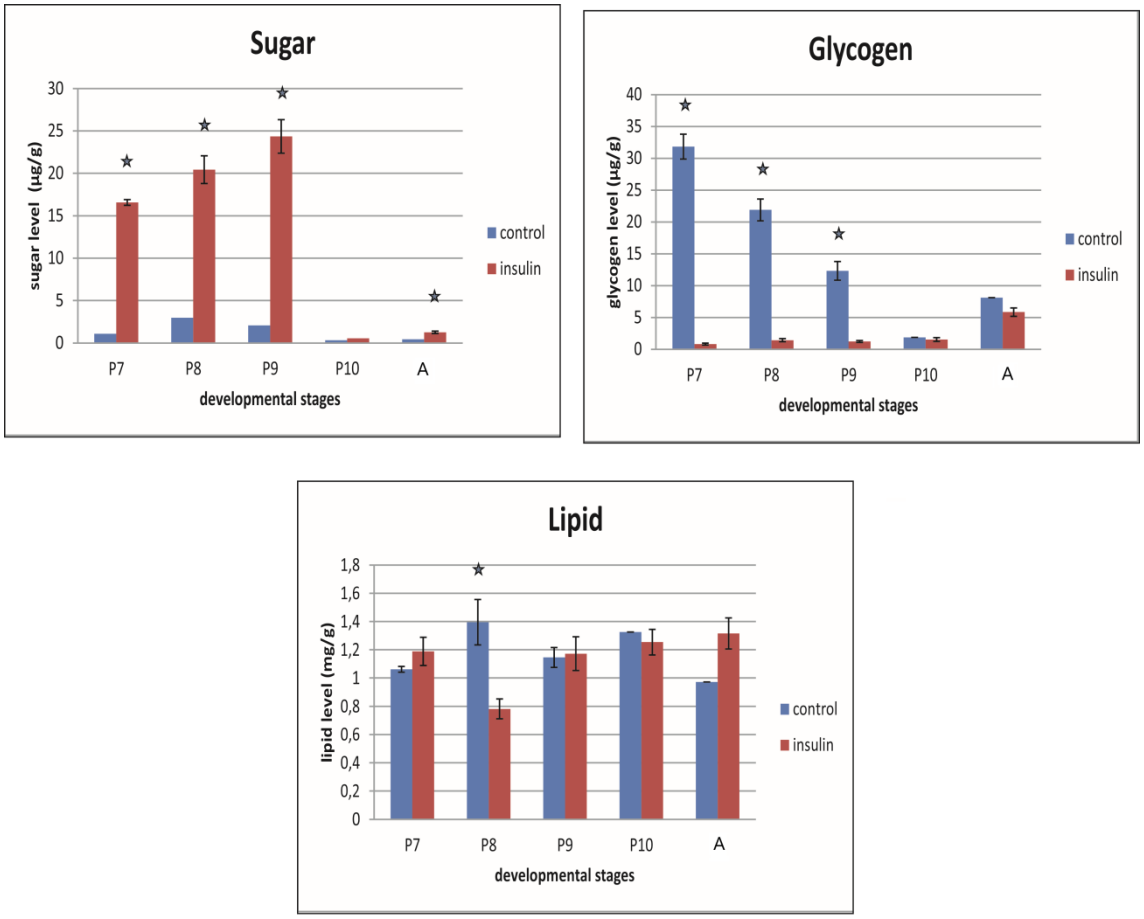

Figure 2. Some physiological parameters of female fat body after insulin treatment on day 6 of pupal stage. Sugar levels (a), glycogen levels (b), lipid levels (c) . The X-axis shows the developmental days from day 0 (P0) of the pupal stage to day 0 (A) of the adult stage. Mean ± S.D. are for the three independent experiments. Asterisks indicate significant differences in the expression ($P \leq 0.05$) using t-test.

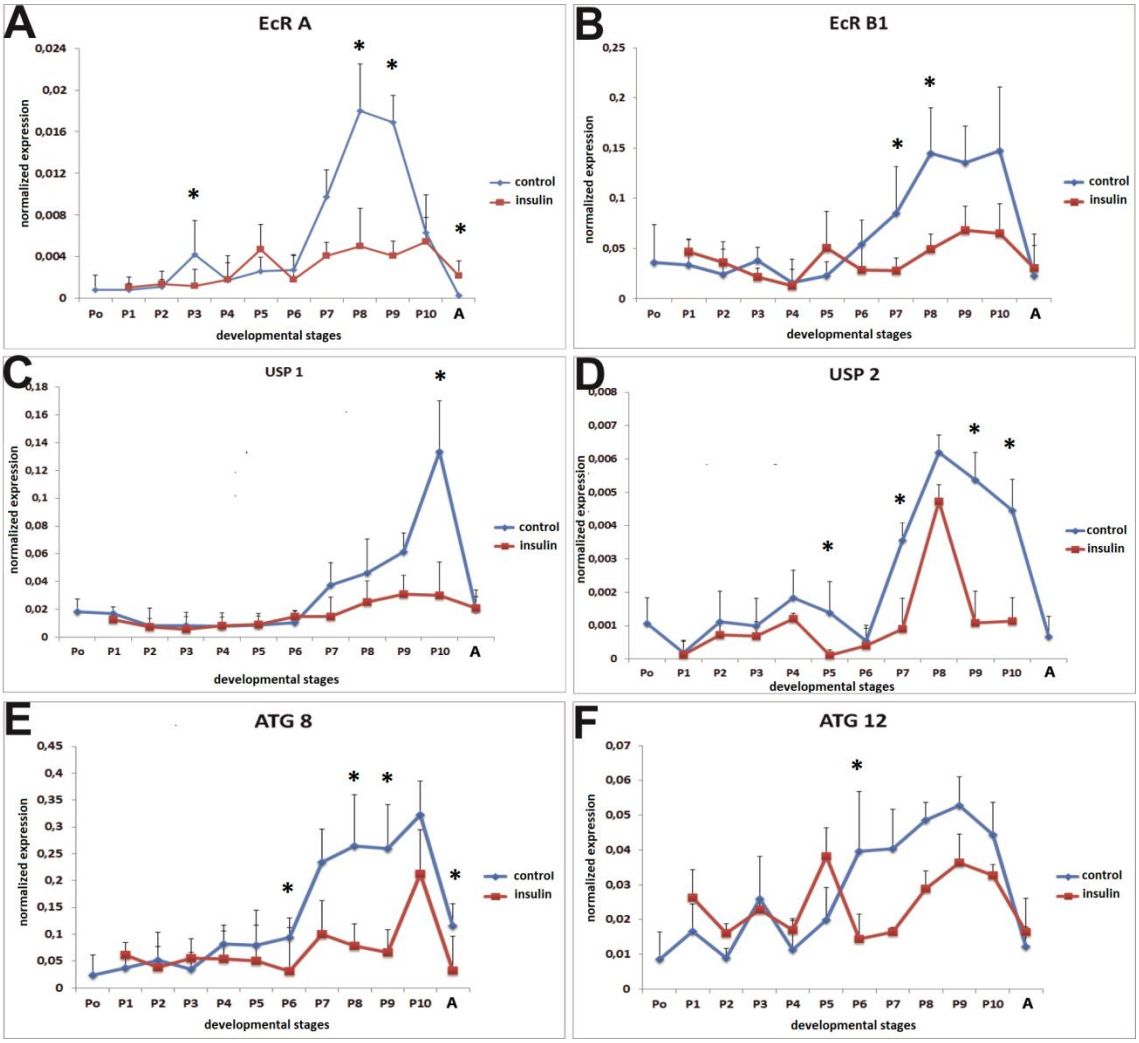

Figure 3. Expression profiles of studied genes in the fat body of *Bombyx mori* after insulin treatment on day 0 of pupal stage. Ecdysone receptor A (EcR A) **(A)**; ecdysone receptor B1 (EcR B1) **(B)**; ultraspiracle 1 (USP 1) **(C)**; ultraspiracle 2 (USP 2) **(D)**; ATG 8 **(E)**; ATG 12 **(F)**. The X-axis shows the developmental days from day 0 (P0) of the pupal stage to day 0 (A) of the adult stage. Expression levels of the genes were normalized using *Bombyx mori Actin* 3 RNA. Mean ± S.D. are for the three independent experiments. Asterisks indicate significant differences in the expression ($P \leq 0.05$) using one-way ANOVA.

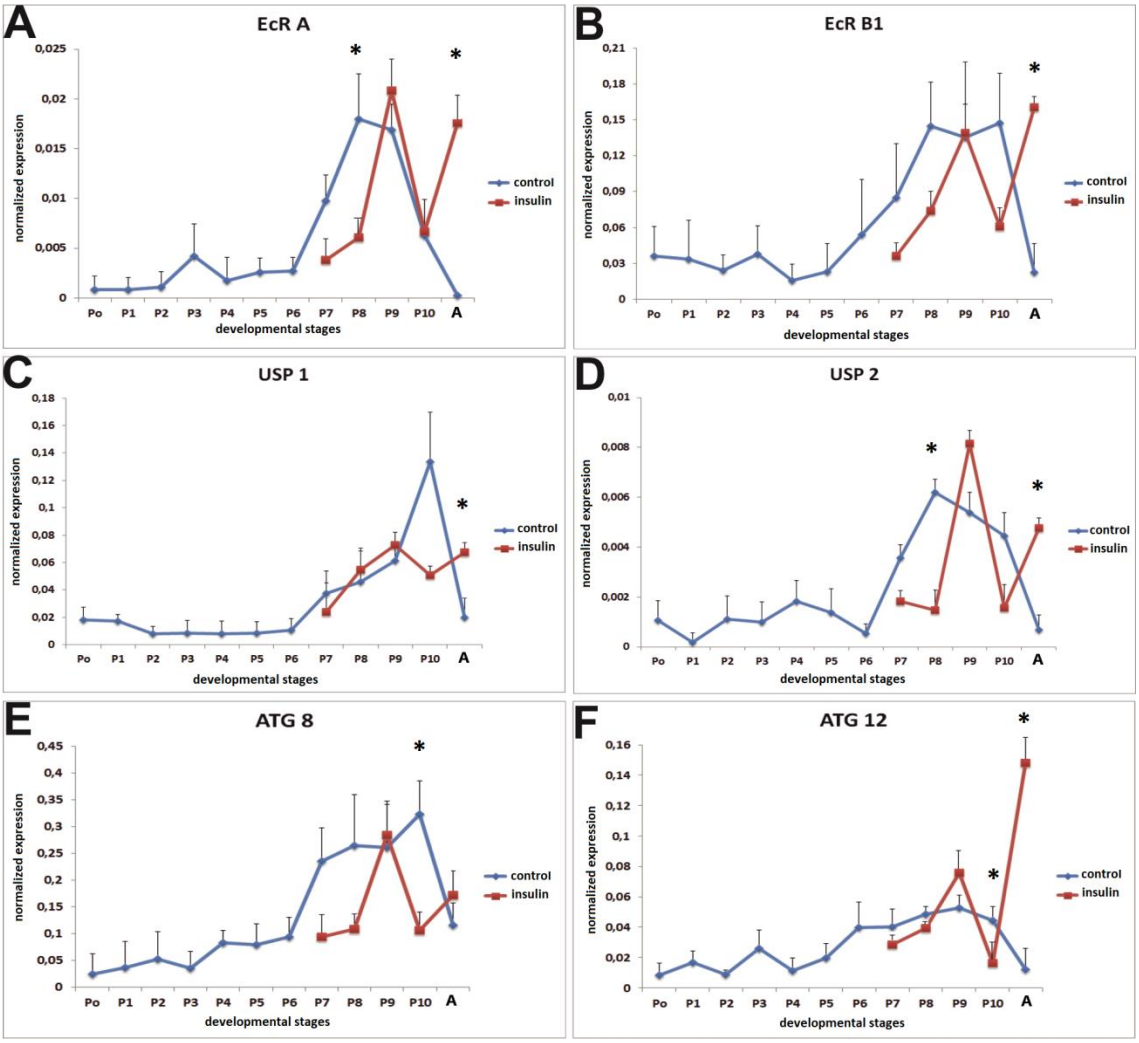

Figure 4. Expression profiles of studied genes in the fat body of *Bombyx mori* after insulin treatment on day 6 of pupal stage. Ecdysone receptor A (EcR A) **(A)**; ecdysone receptor B1 (EcR B1) **(B)**; ultraspiracle 1 (USP 1) **(C)**; ultraspiracle 2 (USP 2) **(D)**; ATG 8 **(E)**; ATG 12 **(F)**. The X-axis shows the developmental days from day 0 (P0) of the pupal stage to day 0 (A) of the adult stage. Expression levels of the genes were normalized using *Bombyx mori Actin* 3 RNA. Mean ± S.D. are for the three independent experiments. Asterisks indicate significant differences in the expression ($P \leq 0.05$) using one-way ANOVA.