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Effects of Vertebrate Insulin Hormone on Major Hemolymph Proteins and Ovarium Development in The Silkworm Bombyx mori Pupal Stage

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ABSTRACT
Insulin, released from the islets of Langerhans in the pancreas, regulates sugar metabolism and plays a key role in growth, differentiation, metabolism, and apoptosis in both vertebrates and invertebrates. Vertebrate insulin affects anabolic metabolism by activating glucose uptake and conversion while inhibiting glycogen degradation. The first insulin-like peptide (ILP) identified in invertebrates was bombyxin from *Bombyx mori*, which regulates growth, metabolism, and egg development, and is homologous to mammalian insulin. In insects, hemolymph consists of plasma and hemocytes, which are essential for defense against microorganisms and antigens. All events, such as the transportation of hormones and nutrients to target cells and the sending of waste to excretory organs, occur through hemolymph. The key organs that play a role in reproductive physiology in female insects are the fat body and the ovary. Some proteins synthesized by fat body cells are transported to the ovary via hemolymph during the pupal period and play a role in egg development. Due to different physiological processes, the content and amount of hemolymph proteins change in larval, pupal, and adult stages. In this study, vertebrate insulin was injected into *B. mori* female pupae on different days, and its effects on ovarian development and major hemolymph proteins were investigated. For this purpose, histological and electrophoretic methods were used. Findings showed that the vertebrate hormone insulin causes changes in the relative abundance of essential proteins in the ovary and hemolymph of female *B. mori* pupae.

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ÖZET
Omurgalı canlılarda pankreasın Langerhans adacıklarından salınan insülin, vücudun şeker metabolizmasının düzenlenmesinde önemli hormonlardan biridir. Omurgalı ve omurgasız hayvanlarda insülin ve insülin ilişkili peptidlerin büyüme, farklılaşma, metabolizma ve apoptozis gibi hücresel süreçlerin düzenlenmesinde önemli bir rol oynadığı bilinmektedir. Omurgalı insülini, glikojen yıkımını inhibe ederken glikoz alımını ve dönüştürmeyi aktive ederek anabolik metabolizmayı etkiler. Omurgasız hayvanlardaki insülin benzeri peptid olarak belirlenen ilk molekül *Bombyx mori'* de belirlenmiş ve bombyxin adını almıştır. Bombyxinler, böcek büyümesini, karbonhidrat metabolizmasını ve yumurta gelişimini düzenler. Böceklerdeki insülin benzeri peptidler moleküler formda memelilerin insülinlerine homologdurlar, ayrıca işlevsel olarak eşdeğerdirler. Hemolenf, hayvanın vücudunu mikroorganizmalardan ve antijenlerden koruyan hemositlerden ve plazmadan oluşur. Hormonların ve besin maddelerinin hedef hücrelere taşınımı ve atıkların boşaltım organlarına gönderilmesi gibi tüm olaylar hemolenf aracılığıyla olur. Dişi böceklerdeki üreme fizyolojisinde rol oynayan anahtar organlar yağ doku ve ovaryumdur. Yağ doku hücreleri tarafından sentezlenen bazı proteinler pupal dönem

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boyunca hemolenf aracılığıyla ovaryuma taşınır ve yumurta gelişiminde rol alır. Farklı fizyolojik süreçlerden dolayı hemolenf proteinlerin içeriği ve miktarı larval, pupal ve ergin evrelerde değişmektedir. Bu çalışmada pupal evrenin farklı günlerinde *Bombyx mori* dişi pupalarına omurgalı insülini enjekte edilmiş, ovaryum gelişimine ve temel hemolenf proteinleri üzerindeki etkileri araştırılmıştır. Bu amaçla histolojik ve elektroforetik yöntemler kullanılmıştır. Elde edilen bulgular omurgalı hormonu olan insülinin, dişi *Bombyx mori* pupalarının ovaryum ve hemolenfinde bulunan temel proteinlerin nispi yoğunluklarında değişimler meydana getirdiğini göstermiştir.

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INTRODUCTION

In vertebrates, insulin is one of the two hormones essential in the regulation of carbohydrate metabolism. While it lowers the blood glucose level and inhibits glycogen breakdown, it regulates anabolic metabolism by activating glucose uptake and synthesis processes in the cell. Insulin is one of the most important regulators of protein metabolism and, in most cases, has an anabolic effect. It directly stimulates protein synthesis. It has been observed that in insulin deficiency, the destruction of liver and muscle proteins increases, and when it is secreted in excess, protein degradation in the liver decreases (Biolo & Wolfe, 1993; Winther-Sørensen et al., 2020). Also, when the pancreas is unable to produce insulin or use it effectively, blood glucose levels increase (Keleş et al., 2024). As a result, it causes type 2 diabetes, a metabolic disorder associated with insulin resistance (Gültekin, 2024). In vertebrate and invertebrate animals, insulin and insulin-related peptides (IRP) play an important role in the regulation of cellular processes such as growth, differentiation, metabolism, and apoptosis. The first molecule identified as an insulin-like peptide in invertebrate animals was identified in *Bombyx mori* by Nagasawa and colleagues (Nagasawa et al., 1984, 1986) during the process of identifying the prothoracicotropic hormone and was named bombyxin. Bombyxin regulates insect growth (Gu & Lin, 2024), carbohydrate metabolism (Gu et al., 2022), and ovarian development (Yang et al., 2021). Insulin-like peptides in insects are homologous in molecular structure to mammalian insulins and are functionally equivalent (Brogiolo et al., 2001; Semaniuk et al., 2021).

In the silkworm *Bombyx mori*, belonging to the Holometabola superorder, all developmental events, including the development of reproductive organs, as well as the protein content of the hemolymph, are regulated by the endocrine system. Hemolymph in insects mainly contains trehalose, which is the insect blood sugar, various proteins, amino acids, and inorganic substances. The main proteins identified in silkworm hemolymph are lipophorins (200-217 kDa), female-specific vitellogenin (440 kDa), storage proteins (70-80 kDa), and 30 kDa polypeptides (Babila Jasmine et al., 2024).

In silkworm groups such as Bombycidae and Saturniidae, ovary development continues throughout the pupal period, and after the female becomes an adult, she mates and lays eggs immediately (Palli, 2016). It is seen that the ovaries of the silkworm are in a pair in the sixth segment of the abdomen, are meroistic (follicular epithelial cells and trophocytes are present), polytrophic type, and each ovary consists of 4 long ovarioles. Studies conducted with the silkworm *Bombyx mori* reveal three basic developmental stages of ovarian development: previtellogenesis, vitellogenesis, and choriogenesis (Yamauchi & Yoshitake, 1984; Zhang et al., 2017). The previtellogenesis phase includes the structuring of the ovary and the development of the oocyte; The vitellogenesis phase includes the storage of storage material by the oocyte, and the choriogenesis phase includes the formation processes of the eggshell (Swevers et al., 2005). Ovary and oocyte development, which is controlled by the endocrine system, occurs by transporting proteins synthesized in the insect fat body to the oocytes via hemolymph during egg development (Wyatt & Pan, 1978; Liu et al., 2015).

Bombyx mori vitellogenin protein is a protein with a molecular weight of 440kDa and a phospholipoglycoprotein structure consisting of 2 subunits: heavy and light. During the larval-pupal ecdysis, it is synthesized by the fat body cells of females and released into the hemolymph, where it is later absorbed by the developing oocytes (Batcha et al., 2023). Once these proteins enter the oocyte, they are called vitelline. Storage proteins, also known as insect hexamerin proteins, 72-80 kDa in weight, are found in high concentrations in the hemolymph during larval development (Uranlı, 2008). Hexamerins act as amino acids and energy sources during periods when there is no nutrition, such as during the pupal period. Lipids are transported in the hemolymph by binding to lipophorin

proteins (Thangaraj et al., 2017). Lipophorins are synthesized in the fat body and are known to generally transport diacylglycerol to muscle tissue as an energy source and to the ovary for maturation (Ziegler & Van Antwerpen, 2006). Specific plasma proteins called '30K proteins' have a molecular weight of approximately 30,000. These proteins belong to the lipoprotein family (Gamo, 1978; Sun et al., 2007). Although it is seen in both male and female silkworms, it accumulates intensively in the oocytes of females (Shi et al., 2015). It is synthesized in large amounts in the fat body of the larvae and released into the hemolymph. Although their physiological functions are still largely unresolved, there are studies that suggest some of them may inhibit apoptosis (Kim et al., 2003) or play a role in the immune system (Ujita et al., 2005).

This study aimed to analyze the effects of insulin hormone on the major protein content of hemolymph and ovaries during the pupal development process of *Bombyx mori* females. In this context, protein profiles were investigated using the sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) method in hemolymph and ovarian samples obtained after insulin was injected on days 0, 3, 6, and 9 of the pupal periods. Analyses were performed in triplicate, and whether the difference was significant after application was determined by Student's t-test.

MATERIAL and METHOD

Silkworm Rearing

Japanese X Chinese hybrid white monovoltine race silkworm eggs obtained from Bursa Kozabirlik were incubated in the silkworm culture laboratory at 25 ± 1°C temperature and 70-85% humidity, which are optimum conditions for these insects. The silkworm larvae, which hatched in approximately 10 days, were exposed to a photoperiod of 12 hours of light and 12 hours of darkness and began to feed. Feeding was carried out with fresh mulberry leaves at the same times every day: morning (09:30), noon (14:00), and evening (18:30). After 21 days, the silkworms that reached the 5th larval stage were gendered, and the female silkworms continued to feed. Immediately after the larval-pupal ecdysis, bovine insulin (Sigma, I0516) was injected at 50µg in 5µl per individual on days 0, 3, 6, and 9. After the injection, hemolymph and ovarian tissues were taken at the same time every day.

Hemolymph was collected in 1.5 ml eppendorf tubes. To prevent melanization of hemolymph, 5% of N-phenylthiourea (Sigma, P7629) solution dissolved in distilled water was added to eppendorf tubes. Additionally, to prevent contamination, 0.01 g (0.154 M) sodium azide (Sigma, S2002) solution dissolved in 1 ml ultra-pure water was added at a rate of 5%. Hemolymph samples collected in Eppendorfs were centrifuged in a Sigma 2-16 KC refrigerated centrifuge at 7000 g at +4 °C for 15 minutes.

Ovaries were taken from 12 female silkworms every day for 10 days, starting from day 0 immediately after larval-pupal ecdysis. The ovaries were fixed in Bouin fixative for histological analysis and stored at –80°C for protein analysis.

Histological analysis

For histological analyses, Bouin's fixative was used, and the tissues were kept in this fixative at +4°C for 24 hours. Afterwards, the alcohol was changed (70% alcohol) until the yellow color of the fixative disappeared, and these tissues were washed. Then, paraffin-embedded tissues were cut into 5 µm-thick sections by a rotary microtome.

The prepared sections were stained with the Gill's Hematoxylin&Eosin method. The stained sections were examined and photographed by using a Zeiss Axio Scope A1 microscope.

Determination of Total Protein Concentration

Total protein concentration determination in hemolymph and ovaries was performed according to Bradford (1976). 10 mM Tris-HCl (pH: 7.5), to which a protease inhibitor cocktail (Roche) was added, was used as homogenization buffer for the ovaries. The standard chart was prepared using 2 mg/ml stock bovine albumin (BSA, Pierce, Thermo Scientific, Albumin Standard, Prod 23209). 900 µl of Bradford reagent (Sigma) was added to each of 30 µl of samples and standards and vortexed, and then the samples were placed in polystyrene cuvettes. It was read on an Agilent Carry 60 UV-Vis brand spectrophotometer at 595 nm. Standard graphs were drawn, and total protein amounts of each sample were calculated.

Electrophoresis Process

Electrophoretic processes were carried out with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system according to Laemmli (1970). The resulting gels were stained with the silver staining method, and gel photographs were taken on an imaging system (chemidoc mp, Biorad). Based on the calculated molecular weight of the proteins, bands related to the major hemolymph proteins were

determined. Relative abundance of major hemolymph proteins was calculated using the software program of the same imaging system.

Statistical analysis

Significant changes between groups were determined using the PASW SPSS 22 program. Since the sample size was less than 50, the Shapiro-Wilk normality test was performed, and groups showing normal distribution were analyzed using the Independent t-test. Values with $p \leq .05$ were considered significant and were shown with a star on the graphs.

RESULTS AND DISCUSSION

Electrophoretic Findings

Control group and insulin application on day 0 of the pupal stage

Gel images obtained from electrophoresis of the control group hemolymph and ovaries are shown in Figures 1a and 1b, respectively. The lipophorin content of hemolymph shows a fluctuating pattern throughout the pupal stage. The highest lipophorin relative density was determined on the 2nd and 8th days, and approximately 12% of the hemolymph protein content was determined to consist of lipophorin on these days. Towards the adult stage, the relative density of lipophorin started to decrease. Insulin administration on day 0 caused the lipophorin content of hemolymph to be significantly higher than the control group on day 6 of the pupal stage; there was no significant difference on the other days (Figure 2a). In the control group, the relative density of lipophorin in the ovaries increased gradually until the 8th day of the pupal stage; It then started to decline until the adult stage. Insulin administration on day 0 did not show a significant difference in ovarian lipophorin content (Figure 2b).

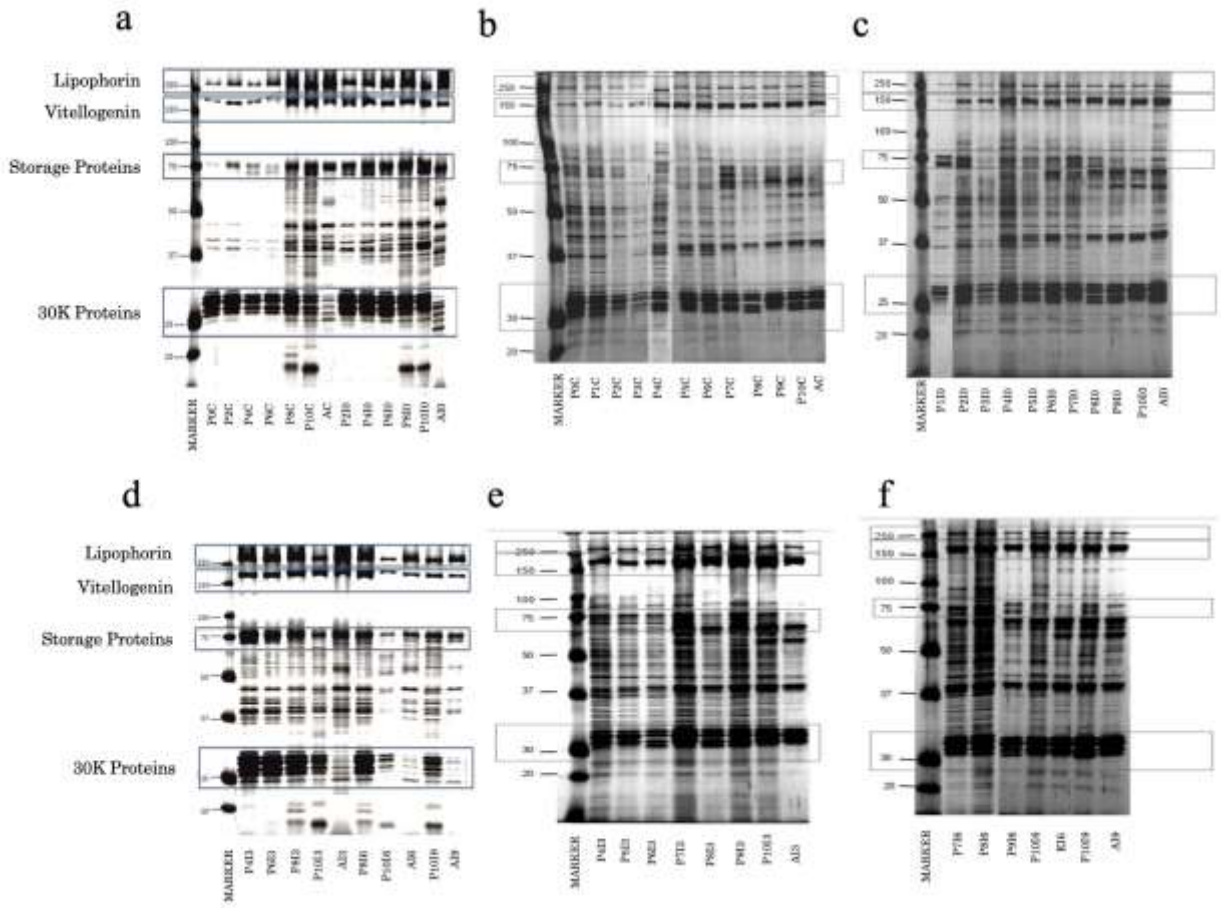

Figure 1. SDS-PAGE image of hemolymph and ovarian protein profiles.
Şekil 1. Hemolenf ve ovaryum protein profillerinin SDS-PAGE görüntüsü.
(a) Gel showing the hemolymph protein profiles of the control group - day 0 insulin application group
Gel showing the protein profiles of the ovaries, control group (b), and day 0 insulin application group (c);
Gel showing the hemolymph protein profiles after the 3rd, 6th, and 9th day insulin applications (d);
Gel showing the ovarian protein profiles in the 3rd day insulin application group (e);
Gel showing the ovarian protein profiles after the 6th and 9th day insulin applications (f).

In the control group, the relative density of vitellogenin, which constituted 3.7% of hemolymph proteins on day 0 of the pupal stage, increased until day 6 and was determined to constitute 7.5% of hemolymph proteins. After the 6th day, it started to decline. Insulin administration on day 0 did not show a significant change in the relative density of hemolymph vitellogenin (Figure 2c). The relative densities of vitelline determined in the control group ovaries increased from the beginning of the pupal stage to the adult stage. Vitelline protein, which has a relative density of 5.9% on the 1st day of the pupal stage, constitutes 15-20% of the ovarian proteins from the second half of the pupal stage. 24 hours after insulin administration, the relative density of vitelline was found to be significantly lower than in the control group. No significant difference was determined in the remaining days of the pupa (Figure 2d).

The relative density of storage proteins in the hemolymph of the control group increased until the 2nd day of the pupal stage, started to decrease from the 4th day, and decreased to the lowest values on the 8th and 10th days of the pupal stage. The relative density of storage protein, which was determined as 10% on the 2nd day of the pupal stage, was calculated as 1.5% on the 10th day. In the adult stage, it increased rapidly and reached a peak, reaching an average relative density of 13% (Figure 2e). The amount of hemolymph storage protein was significantly lower on the 2nd day of insulin administration on day 0; On the contrary, it was found to be significantly higher than the control group on the 8th and 10th days (Figure 2e).

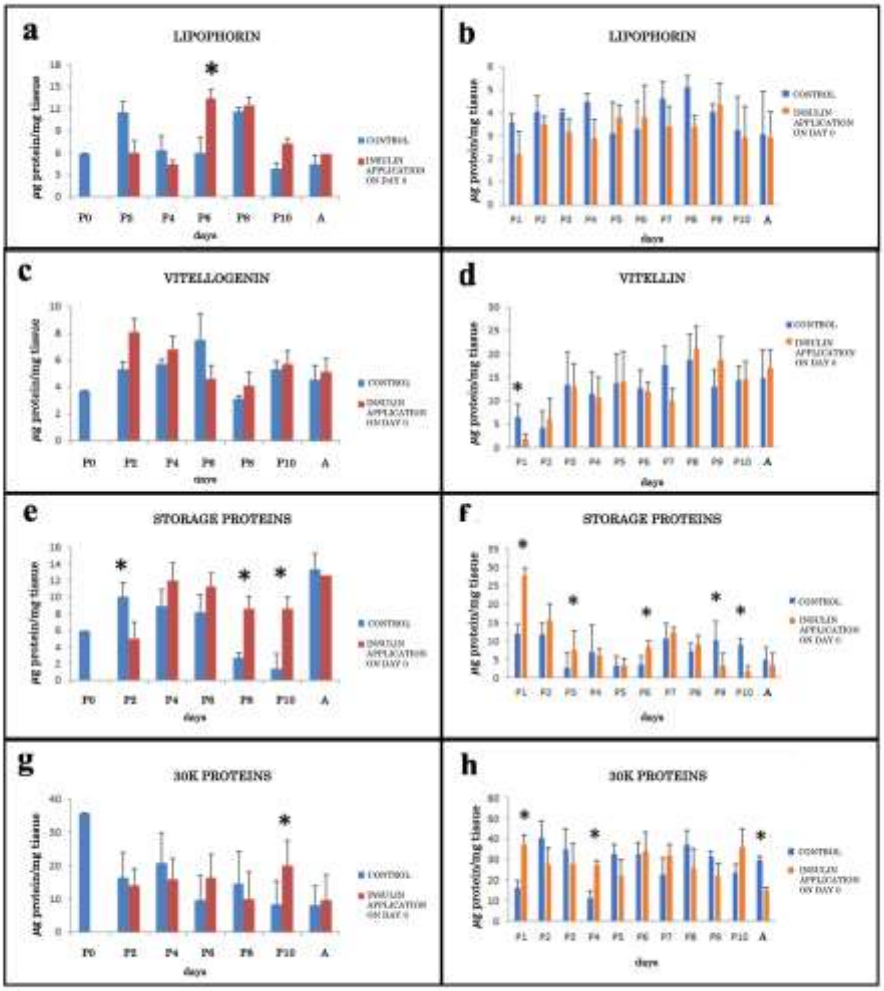

Figure 2. Relative levels of major proteins determined in hemolymph and ovaries of the control group and Day 0 insulin application group (*; t(4)= -26,78, p=.023).

Şekil 2. Kontrol ve 0.gün insulin uygulama grubunun hemolenf ve ovaryumlarında belirlenen temel proteinlerin nispi seviyeleri (; t(4)= -26,78, p=.023).*

Hemolymph lipophorin (a), vitellogenin (c), storage proteins (e), 30K proteins (g); ovaries lipophorin (b), vitellin (d), storage proteins (f), 30K proteins (h).

The relative density of storage proteins, which were determined at average levels on the 1st and 2nd days of the pupal phase in the control group ovaries, remained at relatively low levels until the 7th day. From the 7th day until the end of the pupal stage, it increased to average values again and constituted approximately 10% of the ovarian proteins. Insulin administration on day 0 caused a rapid increase in the relative density of ovarian storage proteins on day 1 of the pupal stage, and the relative density on this day was determined as 27.5%. Similarly, the relative density of storage proteins on days 3 and 6 was significantly higher than in the control group. On the other hand, in the last two days of the pupal stage, it was determined that the levels of storage proteins in the ovaries of the experimental group were significantly lower than those of the control group (Figure 2f).

The relative density of 30K proteins in the hemolymph of the control group was determined at the highest level on day 0 of the pupal stage and constituted 35% of the hemolymph proteins. However, from this day until the adult stage, the relative density of 30K proteins gradually decreased. After insulin administration on day 0 of the pupal stage, a significant change was detected only on the last day of the pupal stage. On this day, the relative density of 30K proteins in the hemolymph of the experimental group is significantly higher than that of the control group (Figure 2g). In the ovaries of the control group, in contrast to hemolymph, the lowest relative density of 30K protein was determined on the 1st and 4th day of the pupal stage, and their relative density varied between 38.5% and 11%. It has been determined that the relative density of 30K proteins in the ovaries is high, especially in the second half of the pupal stage. After insulin administration on day 0, the relative density of 30K proteins in the ovaries increased significantly, and the percentage of relative density in the ovaries on this day was 37.5%. Similarly, the relative density of 30K proteins on the 4th day of the pupal stage was significantly higher than that in the control group. In the adult stage, the situation was reversed, and the relative density of 30K proteins determined in the experimental group was found to be significantly lower than the control group (Figure 2h).

Insulin application group on the 3rd day of the pupal stage

The gel image obtained after the electrophoresis of the hemolymph of the insulin application group on the 3rd day is shown in Figure 1d, and the gel image of the ovary is shown in Figure 1e. The relative density of lipophorin in hemolymph samples after insulin administration was found to be significantly higher than the control group on the last day of the pupal stage and the first day of the adult stage. In the experimental group, the highest relative density of lipophorin protein was determined as 17.5% on day 0 of the adult stage (Figure 3a). The relative densities of lipophorin proteins determined in the ovaries are like the control group, and no significant difference was found (Figure 3b).

After insulin administration on the 3rd day of the pupal stage, there was no significant difference in the relative densities of vitellogenin in the hemolymph compared to the control group (Figure 3c). On the other hand, insulin administration significantly reduced the relative densities of vitelline proteins in the ovaries on the 4th and 7th day of the pupal stage compared to the control group. In the experimental group, the lowest level of vitelline was determined 24 hours after the application, at 2%. The relative densities of vitelline determined in the last 3 days of the pupal stage and the adult stage were found to be high and extremely like the control group. (Figure 3d).

After insulin administration, the relative density of hemolymph storage proteins was significantly higher than the control group on the 8th and 10th days of the pupal stage. On the 8th day, approximately 15% of hemolymph proteins constitute storage proteins (Figure 3e). In the ovaries, the relative density of storage proteins after insulin administration was found to be significantly higher than the control group, especially on the 5th and 6th days. Approximately 10% of ovarian proteins these days constitute storage proteins. On the contrary, the last day of the pupal stage and the first day of the adult stage are significantly lower than the control group (Figure 3f).

After insulin administration, the relative density of hemolymph 30K proteins was found to be significantly higher than the control group on the 6th and 10th days of the pupal stage. The most abundant 30K proteins in the hemolymph is the 6th day of the pupal stage and are approximately 30% (Figure 3g). In the ovaries, after insulin application, there was a significant difference from the control group only on the 9th day of the pupal stage, and it was found to be low. On other days, there was no significant difference (Figure 3h).

Insulin application group on the 6th day of the pupal stage

The gel image obtained after electrophoresis of the hemolymph and ovaries of the 6th day insulin application group is shown in Figure 1d and Figure 1f, respectively. After insulin administration on the 6th day, the relative density of lipophorin in the hemolymph was found to be significantly higher than the control group on the last day of the pupal stage and the first day of the adult stage and constitutes approximately 10% of the hemolymph proteins (Figure 4a). In the ovaries, insulin application caused the relative density of lipophorin on the 8th day of the pupal stage to be significantly lower than the control group; There was no significant difference on the remaining days (Figure 4b). 24 hours after insulin administration, the relative density of vitellogenin in the hemolymph increased

significantly compared to the control group and constituted 9.76% of hemolymph proteins (Figure 4c). In the ovaries, the relative density of vitellogenin determined on all days except the 7th day of the pupal stage after insulin application was significantly lower than the control group. In the control group, an average of 12% of ovarian proteins were vitelline; In the experimental group, this amount is 2% on average (Figure 4d).

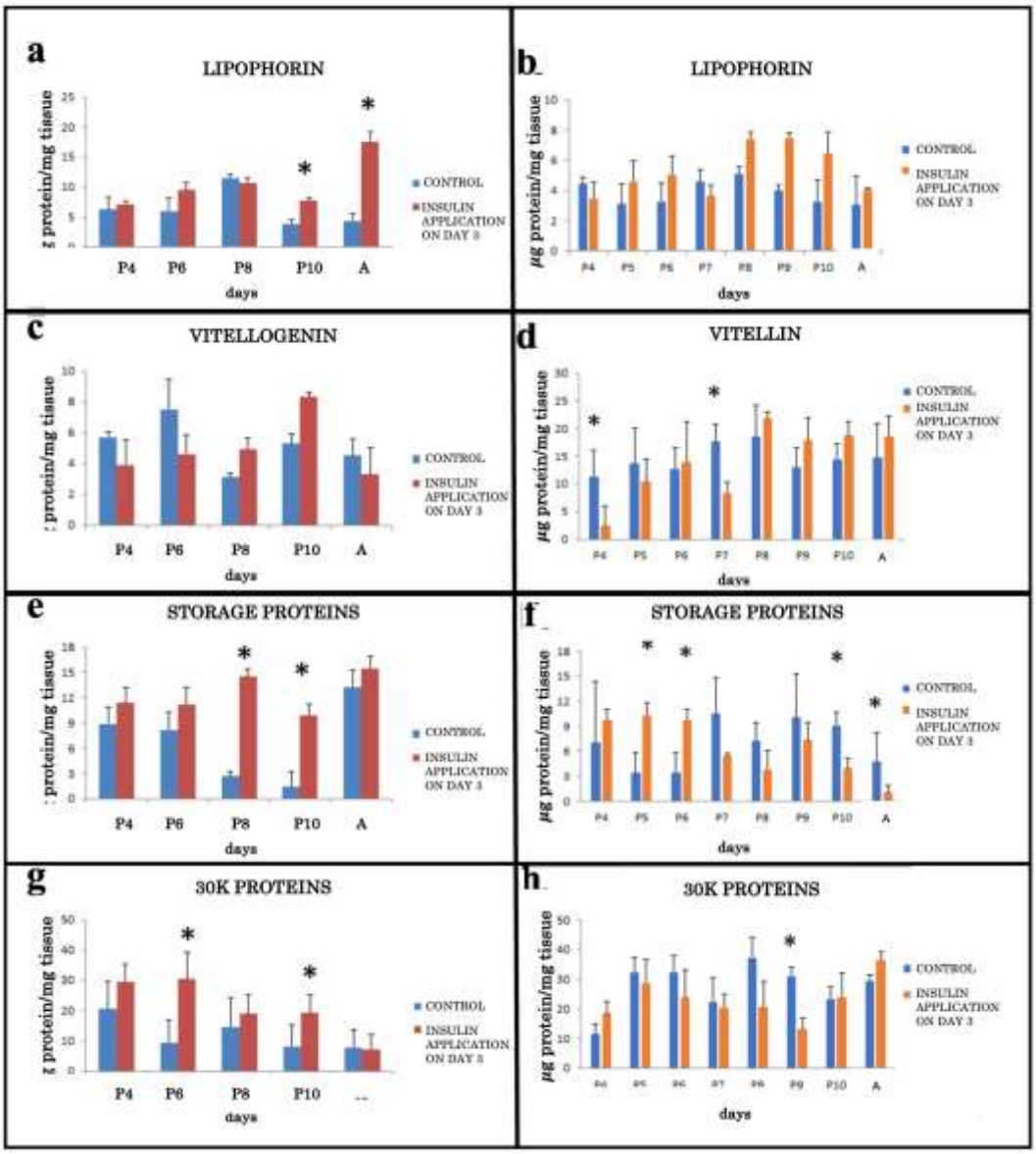

Figure 3. Relative levels of major proteins determined in hemolymph and ovaries of control group and Day 3 insulin application group (*; t(4)= -60,89, p=.030).

Şekil 3. Kontrol ve 3.gün insulin uygulama grubunun hemolenf ve ovaryumlarında belirlenen temel proteinlerin nispi seviyeleri (; t(4)= -60,89, p=.030).*

Hemolymph lipophorin (a), vitellogenin (c), storage proteins (e), 30K proteins (g); ovaries lipophorin (b), vitellin (d), storage proteins (f), 30K proteins (h).

The relative density of storage proteins in hemolymph samples showed a significant increase compared to the control group on the 8th and 10th days of the pupal stage (Figure 4e). In the ovaries, insulin application did not cause a significant change until the adult stage, but the relative density of storage proteins in the adult stage was found to be significantly lower than the control group (Figure 4f).

The relative density of 30K proteins in the hemolymph peaked on the 10th day of the pupal stage and was found to be significantly higher than the control group (Figure 4g). No significant change occurred in the ovary until the adult stage; It was determined that the 30K protein density determined in the adult stage was lower than the control group (Figure 4h).

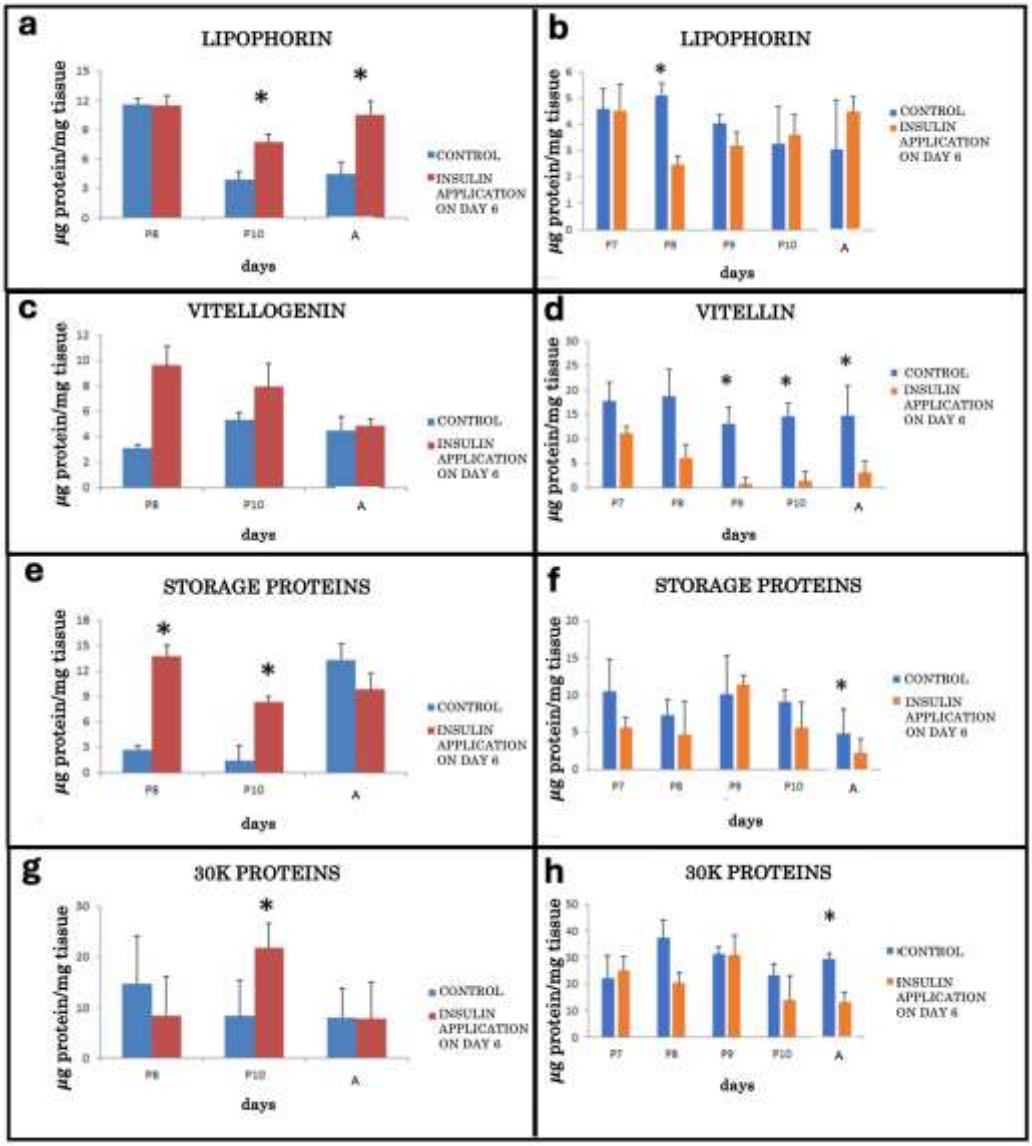

Figure 4. Relative levels of major proteins determined in hemolymph and ovaries of the control group and Day 6 insulin application group (*; t(4)= -39,42, p=.028).

Şekil 4. Kontrol ve 6.gün insulin uygulama grubunun hemolenf ve ovaryumlarında belirlenen temel proteinlerin nispi seviyeleri (; t(4)= -39,42, p=.028).*

Hemolymph lipophorin (a), vitellogenin (c), storage proteins (e), 30K proteins (g); ovaries lipophorin (b), vitellin (d), storage proteins (f), 30K proteins (h).

Insulin application group on the 9th day of the pupal stage

The gel image obtained after electrophoresis of the hemolymph of the 9th day insulin application group is shown in Figure 1d, and the gel image of the ovary is shown in Figure 1f. After insulin administration, the relative density of lipophorin in the hemolymph was found to be significantly higher than in the control group on the last day of the pupal stage and in the adult stage (Figure 5a). In the ovaries, insulin application did not cause a significant difference in the lipophorin content (Figure 5b).

The relative density of hemolymph vitellogenin protein after the experiment was very similar to the control group, and there was no significant difference (Figure 5c). Ovarian vitelline protein relative density was found to be significantly lower than the control group on the last day of the pupal stage and in the adult stage (Figure 5d).

It was determined that the relative density of storage proteins in the hemolymph was higher than in the control group 24 hours after the application. There is no difference in the adult stage (Figure 5e). Insulin administration

had no effect on the relative density of ovarian storage proteins (Figure 5f).

The 30K protein content in the hemolymph samples of the application group is significantly higher than the control group on the last day of the pupal stage (Figure 5g). There was no significant difference in the ovaries (Figure 5h).

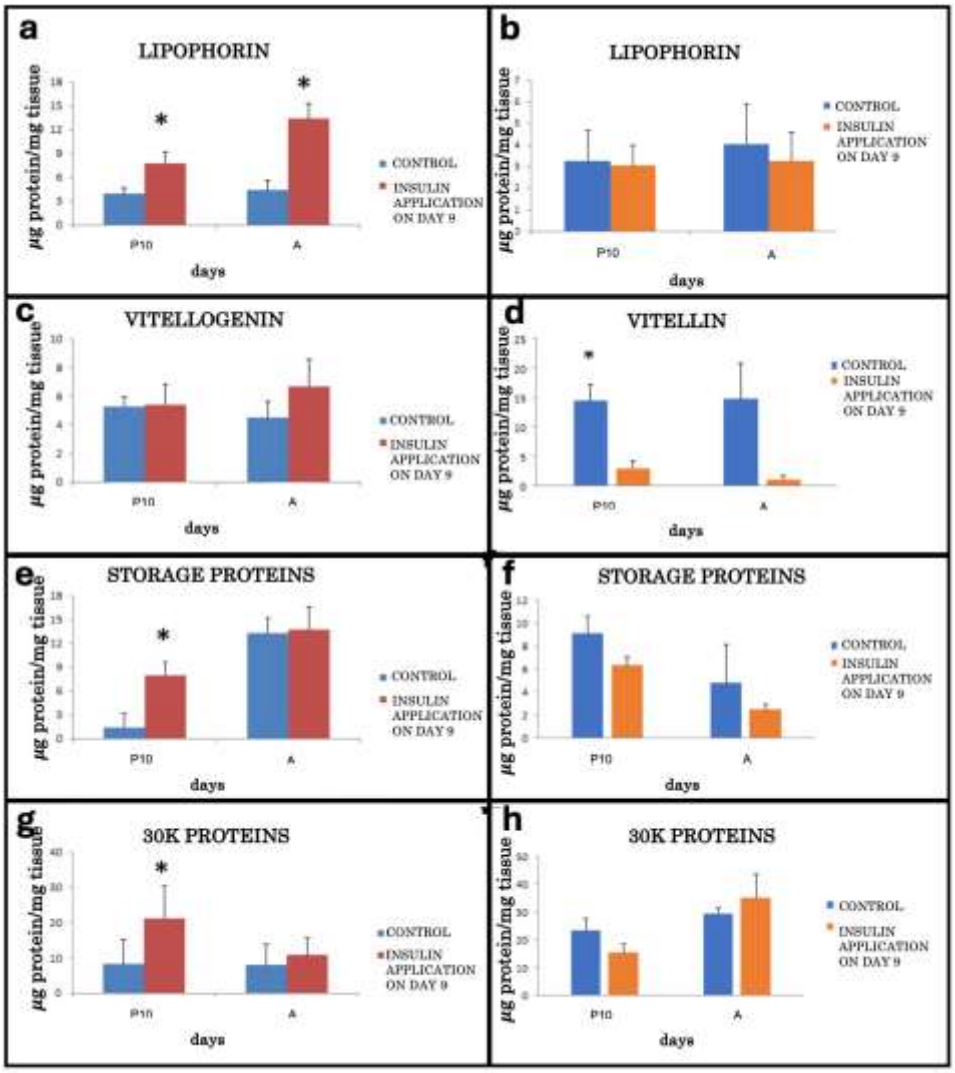

Figure 5. Relative levels of major proteins determined in hemolymph and ovaries of the control group and Day 9 insulin application group (*; t(4)= -95,53, p=.032).

Şekil 4. Kontrol ve 9.gün insulin uygulama grubunun hemolenf ve ovaryumlarında belirlenen temel proteinlerin nispi seviyeleri (; t(4)= -95,53, p=.032).*

Hemolymph lipophorin (a), vitellogenin (c), storage proteins (e), 30K proteins (g); ovaries lipophorin (b), vitellin (d), storage proteins (f), 30K proteins (h).

Histological Examination

In research, the changes in the histological structure and morphology of the ovaries and follicles of the silkworm *Bombyx mori* during the pupal and adult periods were examined using the Hematoxylin-Eosin staining method.

The ovary is defined as the organ surrounded by a sheath after the larval-pupal ecdysis. The ovarioles, which are surrounded by the ovarian sheath on day 0 of the pupal stage, are released into the abdominal cavity on the 1st day, with the rupture of the ovarian sheath, and the oocytes are surrounded by the follicular epithelium, except for the intercellular connections (Figure 6a-d). On day 2, the oocyte nucleus was observed anterior to the oocyte. From this day on, the oocyte is surrounded by 7 nurse cells and follicle epithelial cells (Figure 6e-f). On the 3rd day of the pupal stage, the oocyte is seen to occupy half of the follicle volume (Figure 6 g-h). On day 4, the oocyte is completely covered by follicular epithelium. At this stage, nurse cells are observed in their largest size (Figure 6 i-i). The oocyte growth rate peaks on the 5th day of the pupa (Figure 6j-k). Starting from the 6th day of the pupa, a

decrease in the size of the nurse cells begins to be observed (Figure 6 l-m). On the 7th day of the pupa, the vitelline membrane formed by the follicular epithelium is observed. There is an accumulation of egg yolk proteins in the oocyte (Figure 6n-o). Chorion accumulation is observed on the 8th day of the pupa. On the 9th and 10th day of the pupal stage, follicle cells extend towards the outer layer of the chorion, and at the end of choriogenesis, the follicular epithelial layer of the mature egg is peeled off (Figure 6 u-v). In morphological examinations, it was observed that follicular epithelial cells began to separate from the oocyte around the 10th day of the pupal stage (Figure 6s-t). In the adult stage, the formation of the chorion is completely completed (Figure 6 y-z).

In the morphological examinations of the experimental group, no difference was detected from the control group (Photos not shown).

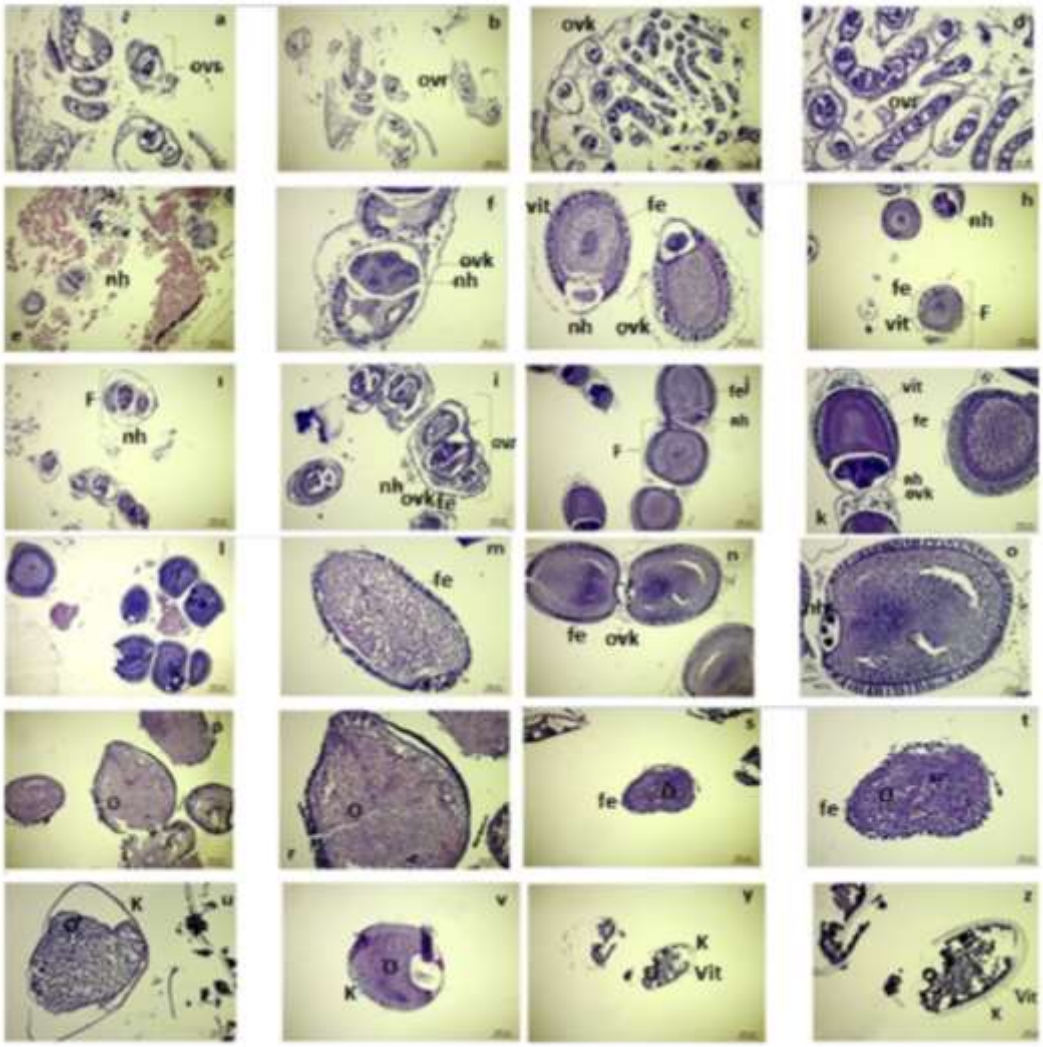

Figure 6. Development of ovary and follicles during pupal period in the silkworm Bombyx mori, control group. H&E staining.

Şekil 6. İpek böceği Bombyx mori kontrol grubunda pupal dönem boyunca ovaryumun ve foliküllerin gelişimi. H&E boyama.

(a ; b, Pupa day 0), (c ; d, Pupa day 1), (e ; f, Pupa day 2), (g ; h, Pupa day 3), (ı ; i, Pupa day 4), (j ; k, Pupa day 5), (l ; m, Pupa day 6), (n ; o, Pupa day 7), (p ; r, Pupa day 8), (s ; t, Pupa day 9), (u ; v, Pupa day 10), (y ; z, Adult). (F: follicle, Fe: follicular epithelial layer, Fh: follicle cell, K: chorion, O: oocyte, nh: nurse cell, Ovr: ovariole, ovk: ovariole sheath, vit: vitelline)

Insulin is an extremely well-preserved hormone. The presence of insulin-like peptides in insects reveals the importance of this hormone in insect physiology. In the silkworm *Bombyx mori*, the development of female reproductive organs occurs in the pupal stage. During this development process, proteins synthesized in the fat body and important for egg development are transported to the maturing oocyte via hemolymph. The effects of insulin and insulin-like peptides on ovarian development have been studied in different insects. Manière et al.

(2004) reported that there are insulin receptors in the ovaries of *Phormia regina*. Riehle and Brown (1999) demonstrated the protein synthesis-stimulating effects of bovine insulin in *Aedes aegypti* in vitro.

In this study, insulin administration increased the hemolymph lipophorin content. Lipophorins are responsible for the transport of fat molecules and hormones in the hemolymph. The increase in the relative densities of lipophorin in all groups after insulin administration on different days may have occurred due to the increase in the fat content of hemolymph and the transport of these fat molecules to target organs such as the ovary. In contrast to hemolymph, insulin administration did not cause a significant change in ovarian lipophorin content. Studies have shown that lipophorin transports lipids to different tissues through different mechanisms (Jouni et al., 2003). It is thought that this result between the tissues in our study may be related to different mechanisms.

In the silkworm *Bombyx mori*, vitellogenin is synthesized in the female fat body during larval-pupal molting (Batcha et al., 2023). In *Bombyx mori*, vitellogenin consists of 2 heavy and 2 light chains. (Izumi et al., 1980; Yang et al., 2014). The onset of vitellogenin synthesis is closely related to ovarian maturation in many insect species (Engelmann, 2013). The vitellogenin content in the newly pupated female is 5% of the total protein. It gradually increases in the early pupal stage and then reaches several times the initial amount during pharate adult development. In the newly molted adult, it has been observed to reach 40% of the total protein. In the first half of the pupal stage, vitellogenin was detected only in the hemolymph, but in the second half it increased rapidly in the ovary while maintaining its level in the hemolymph (Ogawa & Tojo, 1981). Hyršl and Šimek (2005) performed electrophoresis of silkworm hemolymph proteins and showed lipophorins, storage proteins, sex-specific vitellogenin protein, 30K proteins, and some unidentified protein fractions by electrophoresis. They stated that there was no difference in hemolymph proteins between male and female silkworms until the end of the 5th larval stage. However, they showed that vitellogenin synthesis occurred in females at the end of the larval stage. Our study of the effect of insulin application on the hemolymph vitellogenin content did not cause a significant change, except for the 6th day. After insulin application on the 6th day, the significant increase in the amount of vitellogenin the next day may be related to Bombyxin, which is an insulin-like hormone in insects and is found in high amounts in hemolymph on this day. The presence of both Bombyxin and insulin in the body may have caused the release of more vitellogenin from the fat body into the hemolymph. The effect of the insulin hormone on ovarian vitelline protein was realized in a decreasing direction. 24 hours after insulin administration on day 0; In the 3rd day application, on the 4th day and the 7th day; In the 6th day application, on all days except the 7th day; On the 9th day of application, ovarian vitellin contents were significantly lower than the control group two days after application. Although vitellogenin is present in the hemolymph, its low determination in the ovaries indicates that insulin administration largely inhibits the uptake of vitellogenins into the oocyte.

During times when there is no feeding, such as during the pupal period, hexamerins serve as amino acid and energy sources. Storage proteins, which have two forms, SP-1 and SP-2, are synthesized in large amounts in the fat body of actively feeding larvae and released into the hemolymph. During the larval-pupal transformation period, these proteins are gradually taken up by fat body cells and stored as protein granules (Tojo et al., 1980; Yan et al., 2022). Storage proteins are very important proteins in the formation of new tissues and organs, and the development of reproductive organs in insects. It is observed that insulin applications cause significant increases in the hemolymph storage protein content, especially in the last days of the pupal stage. The only exception was that after the application on day 0, the relative density of storage protein in the control group was found to be significantly higher than the experimental group on the 2nd day of the pupa. In contrast to hemolymph, the content of ovarian storage proteins increased compared to the control group in the first half of the pupal stage, especially after insulin applications on day 0 and day 3; It decreased in the second half. This contrasting result between hemolymph and ovaries is since insulin administration initially stimulates the uptake of storage proteins into the ovaries and thus reduces their content in the hemolymph; On the other hand, it may be due to the reversal of this effect in the second half of the pupal stage.

30K proteins are included in the lipoprotein family (Ye et al., 2021). The majority of protein components in the hemolymph of fifth instar larvae are 30K proteins (Izumi & Tomino, 1976). They are synthesized in large amounts in the fat body during the larval period and secreted into the hemolymph. It has been shown that the periods when 30K proteins are most abundant in *Bombyx mori* hemolymph are the late larval stage and the early pupal stage (Izumi & Tomino, 1976). Many studies are being carried out on its functions. Insulin applications have an increasing effect on hemolymph 30K protein content. A similar result occurred in the ovaries after day 0 insulin administration. These results show that insulin increases the release of 30K proteins from fat body into hemolymph and stimulates their transport to target tissues such as the ovary.

CONCLUSION

The results obtained from this study show that insulin application has effects on hemolymph and ovarian protein

content in the pupal period of the silkworm *Bombyx mori* females. By analyzing the effects using more advanced molecular methods and considering other hormones present in the insect's physiology, more detailed information can be obtained regarding the physiological mechanisms of insulin hormone in insects. In addition, the effects of insulin applications on ovarian development did not cause any significant differences in light microscopy examinations, and ovarian development was the same as in the control group. This result reveals that the insulin hormone does not have an inhibitory or stimulating effect on ovarian development.

Determination of fat body protein profiles after insulin application may clarify the results determined in this study. Investigation of the presence of insulin-like receptors in the ovary and changes that may occur in the receptor after insulin applications may reveal the receptor-mediated effect of insulin. Determination of the effects of insulin on endocytosis in the transport of proteins in the hemolymph to the ovary via endocytosis should be the next stage of the study. Finally, determining the productivity of eggs obtained after insulin administration may provide new insights into the reproductive biology of insulin in *Bombyx mori*.

Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

Conflict of Interest

The authors declare that there is no conflict of interest.

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