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Research Article/Araştırma Makalesi

Investigation of the Effects of Starvation Stress in the Midgut of the Silkworm *Bombyx mori*

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Abstract: During their evolution, organisms have developed various mechanisms to adapt to changing nutritional conditions such as mobilization of storage molecules and activation of autophagy. In this study, the mechanism of adaptive responses in the midgut of the silkworm *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) larvae, which were starved for different days, was investigated. The study was carried out at the Insect Physiology Research Laboratory and Silkworm Culture Laboratory at Ege University between 2018 and 2020. For this purpose, the histological structure of the midgut was examined using hematoxylin&eosin staining and its protein, sugar, glycogen, and lipid contents were determined. As autophagy markers, lysosomal enzyme activities were measured and expressions of autophagy-related genes (*mTOR, ATG8, and ATG12*) were analyzed by qRT-PCR. The results showed that, depending on the time of onset of starvation stress, autophagy plays no role as an adaptive response under starvation conditions or occurs at a much more moderate level than autophagy which happens as part of cell death during larval-pupal metamorphosis.

Keywords: Lepidoptera, digestive canal, programmed cell death, ATG genes.

İpek Böceği *Bombyx mori*' nin Orta Bağırsağında Açlık Stresinin Etkilerinin Araştırılması

Öz: Organizmalar, evrimleri sırasında değişen beslenme koşullarına uyum sağlamak için depo moleküllerinin mobilizasyonu ve otofajinin aktivasyonu gibi çeşitli mekanizmalar geliştirmiştir. Bu çalışmada, farklı günlerde aç bırakılan ipekböceği *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) larvalarının orta bağırsağındaki adaptif tepkilerin mekanizması araştırılmıştır. Çalışma 2018-2020 yılları arasında Ege Üniversitesi böcek fizyolojisi araştırma laboratuvarı ve ipekböceği kültür laboratuvarında yapılmıştır. Bu amaçla hematoksilen&eozin boyama ile orta bağırsağın histolojik yapısı incelenmiş ve protein, şeker, glikojen ve lipid içerikleri belirlenmiştir. Otofaji belirteçleri olarak lizozomal enzim aktiviteleri ölçülmüş ve otofaji ile ilgili genlerin (*mTOR, ATG8 ve ATG12*) ifadeleri qRT-PCR ile analiz edilmiştir. Sonuçlar, açlık stresinin başlama zamanına bağlı olarak, otofajinin, açlık koşulları altında adaptif bir yanıt olarak hiçbir rolü olmadığını veya larva-pupa metamorfozu sırasında hücre ölümünün bir parçası olarak meydana gelen otofajiden çok daha ılımlı bir seviyede meydana geldiğini göstermiştir.

Anahtar kelimeler: Pul kanatlılar, sindirim kanalı, programlı hücre ölümü, ATG genleri.

1. Introduction

Living beings had to adapt to the constantly changing conditions of obtaining food during their evolution. The reduction in nutrients induces cellular responses to maintain cellular energy levels so that the organism survives in starvation conditions. In the case of limited nutrient availability, mobilization of storage molecules and autophagy can ensure cell survival by mediating cellular ATP production, protein synthesis, and the acquisition of metabolic substrates to sustain the synthesis of fatty acids (Lum et al., 2005).

Autophagy is an evolutionarily well-conserved cellular process, from yeast to mammals, that is effective in maintaining cellular homeostasis by recycling intracellular molecules, especially in the absence of nutrients. Recent studies have revealed that, in addition to its role in cellular metabolism, autophagy is closely connected with cell differentiation, aging, cell death, neurodegenerative diseases, and the functions of the immune system (Xie & Klionsky, 2007). Three basic forms of autophagy, macroautophagy (MA), microautophagy (MI), and chaperone-mediated autophagy (CMA) have been identified in mammals (Klionsky & Codogno, 2013; Li et al., 2012; Kaushik & Cuervo, 2012). Macroautophagy is the most common autophagy type among these, hereafter referred to as autophagy in our study. Autophagy-related genes (ATG) were first identified in the yeast Saccharomyces cerevisiae Meyen ex EC Hansen, 1883 (Saccharomycetales: Saccharomycetaceae) (Xie & Klionsky, 2007) and orthologs of these genes are found in other eukaryotes (Romanelli et al., 2014). TOR (target of rapamycin), an important regulator of autophagy, is a serine/threonine-protein kinase that belongs to the phosphatidylinositol kinase-associated kinase (PIKK) family (Jung et al., 2010) and controls growth, survival, metabolism, development, and protein synthesis (Hietakangas & Cohen, 2009; Wang & Proud, 2009; Zhou et al., 2010). When nutrients are abundant in the cell, TOR is activated and inhibits the initiation of autophagy by phosphorylating the autophagy related-gene, *Atg13*. Under the starvation condition, TOR activity is reduced and dephosphorylation of *Atg13* stimulates vesicle nucleation (Kamada et al., 2000; Chan & Tooze, 2009; Romanelli, 2014). Afterward, autophagosomes are formed with the activation of two ubiquitin-like conjugation systems. The first of these conjugation systems consists of Atg12, Atg5, and Atg16 proteins and provides elongation of the vesicle. The second contains *Atg8* (LC3 in mammals) that binds to phosphatidylethanolamine (PE) to develop an autophagic isolation membrane. In the final step, the

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autophagosomes fuse with the lysosome and their contents are digested by lysosomal enzymes such as acid phosphatase, acid ribonuclease, and cathepsins. (Xie & Klionsky, 2007).

In addition to being a cellular survival mechanism, autophagy plays a role in autophagy-mediated cell death or type II PCD when it occurs excessively in the cell. It has recently been proven that autophagic cell death is an important mechanism in the elimination of large cell populations in insects during metamorphosis and the anomalies that occur in this process have serious effects on insect development and survival (Tettamanti et al., 2007a). The substances obtained from the structures that are degenerated in this process contribute to the necessary energy and acquisition of cellular building blocks during the survival and formation of new tissue in insects that do not feed in the pupal and adult life stages (Rabinowitz & White, 2010).

The silkworm digestive system is a canal composed of epithelial cells and according to its embryonic origin and physiological functions, it is composed of 3 parts foregut, midgut, and hindgut (Klowden, 2007). The functions of the larval midgut are digestion and absorption of nutrients but it undergoes significant changes in the metamorphosis process because of the changing the food habit (Goncu et al., 2016). All of these remodeling events of the midgut are under the control of the insect steroid hormone, 20-hydroxyecdysone (20E). Two events take place in the remodeling process of the midgut during metamorphosis in Lepidoptera. The first is the degeneration of the larval midgut epithelium by PCD and the second is the proliferation and differentiation of stem cells to form the pupa-adult midgut epithelium (Franzetti et al., 2015). Programmed cell death (PCD) of the larval midgut begins in response to an increase in 20E and, in addition to apoptosis, studies have proven that programmed autophagy plays an important role in these developmental processes.

Previous studies have shown the presence of autophagic structures, increased expression levels of autophagy-related genes, and increased lysosomal acid phosphatase enzyme levels in *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) larval midgut cells during larval-pupal metamorphosis. The aim of this study was to investigate the adaptive cellular responses of the midgut under starvation conditions that begin at different developmental times in the final larval stage, the similarities and/or differences between starvation-induced autophagy, and programmed autophagy that occur during metamorphosis in the larval midgut.

2. Material and Methods

2.1. Silkworm rearing and feeding protocol

Silkworm *B. mori* larvae were reared by feeding on fresh mulberry leaves at 25±1 °C, 70-85% relative humidity, 12L:12D photoperiod. For starvation experiments, larvae were starved from day 0 (Group 1) and day 4 (Group 2) of the 5th larval stage. The control group was grown under normal feeding conditions and the midguts of all groups were dissected every 24 hours.

2.2. Histological and histochemical analysis

The dissected midgut samples morphologies were evaluated by staining with Hematoxyline&Eosine. Glycogen granules were detected with Alcian blue (PAS). Photographs of the specimens were taken under a Zeis Axioscope microscope.

2.3. Preparation of samples for lipid, glycogen, and sugar analyses

Midgut tissues were homogenized according to the Van Handel method and separated into phases for glycogen, sugar, and lipid. Glucose was used as a standard for glycogen and sugar determination and corn oil was used as a standard for lipid. Samples and standards were read in a spectrophotometer at 625 nm (adapted from Van Handel, 1985a; Van Handel, 1985b; Van Handel & Day, 1988; Kaufmann & Brown, 2008; Kaufmann & Brown, 2014).

2.4. Total protein concentration

Total protein concentrations in the samples were determined by the Bradford (1976) method. Bovine serum albumin (BSA) was used as a protein standard. Samples and standards were read at 595 nm by using Agilent Cary 60 UV-vis spectrophotometer.

2.5. Acid phosphatase assay

The activity of the acid phosphatase enzyme in tissues was detected according to Bergmeyer's (1974) method. p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) was used as a substrate. The amount of p-nitrophenol, which is the final product of the reaction, was measured at 405 nm spectrophotometrically.

2.6. RNA isolation, cDNA synthesis, and quantitative real-time reverse transcriptase-polymerase chain reaction

The midgut samples were homogenized in Tripure Isolation Reagent (Roche) and RNA isolation was done according to the manufacturer's instructions. The concentration of total RNA and purity were determined with NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fischer) and then evaluated using gel electrophoresis. cDNA synthesis by reverse transcription was performed using 5 µl of RNA and the Evoscript Universal cDNA Master Kit (Roche) according to the manufacturer's instructions.

Transcript levels of BmATG8, BmATG12, and BmTOR1 were analyzed in a LightCycler 480II real-time PCR cycler (Roche) using a LightCycler® 480 Probes Master (Roche) according to the manufacturer's instructions. The *RP49* gene was used as the housekeeping gene. The primers and probes design was made using the Clustal W align program and Oligo7 software. The specificity of the obtained primers and probes was checked with the blast program. Primer and probe sequences are given in Table 1. qRT-PCR master mix was prepared using 2 µl cDNA, 0,5 µl forward Primer (20mM), 0,5 µl reverse Primer (20mM), 2,7 µl H2O, 0,2 µl Tprobe (20mM) and 5 µl enzyme. PCR conditions were applied as10 minutes for denaturation at 95°C, followed by 45 cycles of the amplification at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second.

2.7. Statistical analysis

The difference between groups was determined using the PASW Statistics 18 program. Since the sample size was less

than 50, the Shapiro-Wilk normality test was performed. The Independent T-test was applied for the normal distributed variable and the Mann-Whitney U test was used for the non-normally distribution variable. When $p \leq 0.05$ in both test results, the difference between the groups was found to be significant and indicated with a star in the columns.

Table 1. Forward and reverse primers use in qRT-PCR

Gene	Forward and reverse primer	Accession no	Taqman probe no
*Bm*ATG8	CAGTTGGCCAGTTCTACTTCTT TGCAGATGTGGGTGGAATG	NM_001046779	Fam- AAACGTATTCACCTGCGGCCTGAA-BBQ
*Bm*ATG12	ATGAGACGCAGAGCCAATC ATTCCCTGTCGCTTTCAGTAG	NM_001142491	Fam- AGTGGCTGGAATGGAAGCATTGAGA -BBQ
*Bm*Tor1	TGGTGGACACGACTCCAAG CATGACTAGACTCCGCACCTG	NM_001184844.1	Fam-CCTCGACCAGTCTCTGCCCGC-BBQ
RP49	GCGGTTCAAGGGTCAATACT GTGAACTAGGACCTTACGGAATC	AB048205.1	Fam- CGGTTCCAACAAGAAGACCCGTCA-BBQ

3. Results

Gut contents were determined until day 7 of the 5th larval stage in the larvae of the control group subjected to normal feeding (Fig. 1). Group 1 larvae, starved from the onset of the fifth larval stage, survived for 7 days. The fluid accumulation in the alimentary canal was detected in this group (Fig. 1). In the control group, gut purge occurred on day 8 and larval-pupal ecdysis happened on day 11 of the 5th larval stage. Group 2 larvae were starved from day 4 of the 5th instar after a small ecdysone release. Gut purge and spinning activity occurred one day before the control group; however, there was no larval-pupal ecdysis (Fig. 2). The total body weight of the starved larvae decreased significantly compared to the control group (Fig. 3).

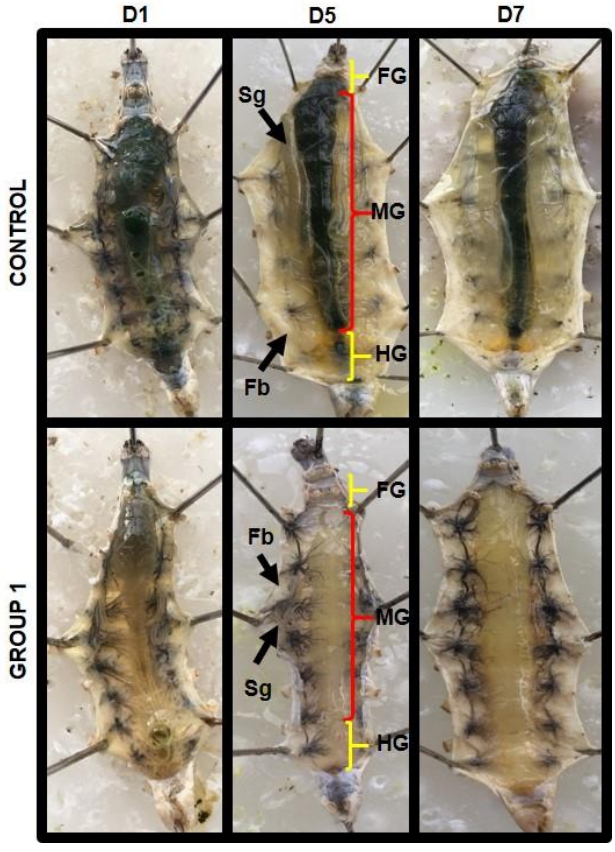

Figure 1. Morphological structure of Control and Group 1 larvae during dissection (FG: Foregut, MG: Midgut, HG: Hindgut, Sg: Silk gland, Fb: Fatbody).

3.1. Histological findings

3.1.1. Hematoxylin & eosin results

In the control group, the single-layered larval epithelium surrounding the lumen, the secretory vesicles, and the brush border membrane were observed until day 7 of the 5th instar.

The midgut histology of the group 1 larvae was similar to the control group until day 7 of the 5th larval stage and no degenerative changes due to starvation were detected. However, on the last day of this group, before the insects died, villi structures were destroyed, the morphology of the cells became irregular, and the brush border membrane was lost. In addition, numerous vesicles on the apical surface of the epithelium were also determined on this day (Fig. 4).

The larval midgut of group 2 appeared as healthy as in the control until day 10 of the 5th instar. In the control group, the degenerated larval epithelium was separated from the basal lamina and stem cells located in the basal region of the epithelium enlarged and proliferated on day 10. In group 2, degenerated larval epithelium was still attached to the proliferating stem cell layer at this time. The formation of pupal midgut epithelium from differentiated stem cells was detected during the early pupal development of the control group. Although larva-pupa ecdysis did not occur, similar developmental events of the pupal midgut were observed in group 2 larvae (Fig. 5).

3.1.2. Alcian blue-periodic acid-schiff (pas) results

While glycogen granules were observed in the midguts of the control group from the early days of the 5th larval stage, glycogen granules were not observed in the group 1 larvae (Fig. 6). Similarly, larval midgut cells of group 2 lacked glycogen granules but staining in the newly formed pupal epithelium indicated the presence of glycogen in these cells. However, the staining intensity was much weaker compared to the control (Fig. 7).

3.2. Glycogen, sugar, lipid, and protein levels

Glycogen levels in midguts from group 1 were significantly lower on days 2 and 3 of the 5th larval stage than in the control group. Midgut glycogen levels detected in group 2 were significantly lower than in the control group throughout the experiment, except on day 1 of starvation (Fig. 8a).

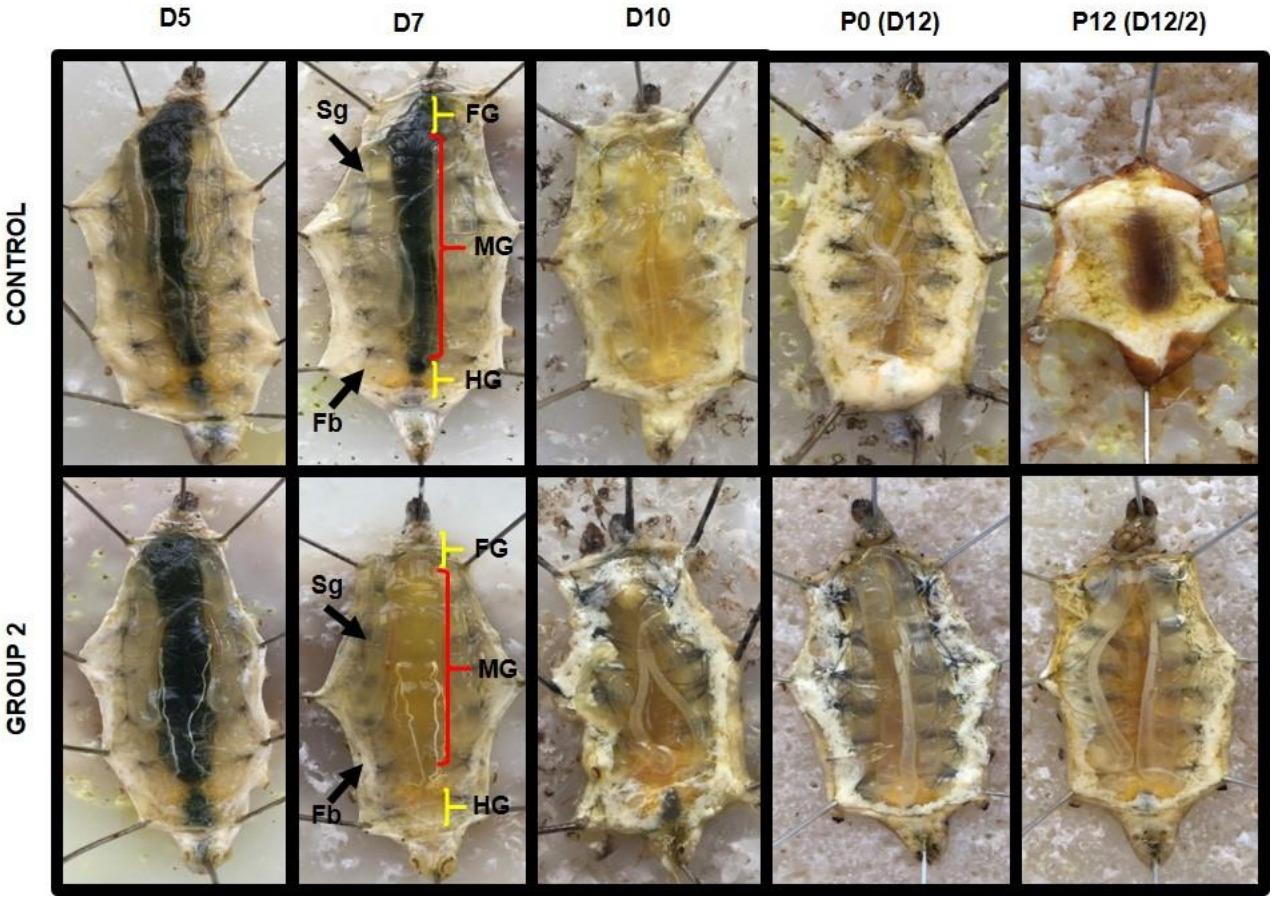

Figure 2. Morphological structure of Control and Group 2 larvae during dissection - developmental days in brackets are shown for group 2 (FG: Foregut, MG: Midgut, HG: Hindgut, Sg: Silk gland, Fb: Fatbody).

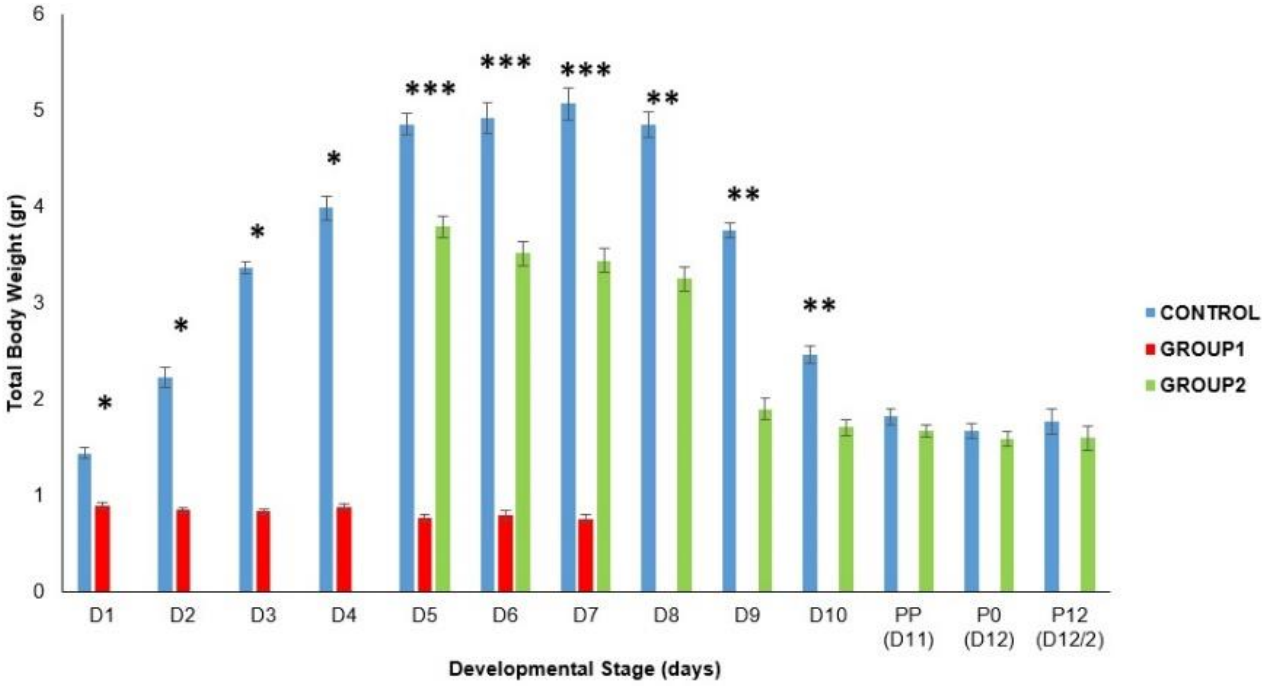

Figure 3. Total body weights of Control, Group 1, and Group 2 larvae - developmental days in brackets are shown for group 2. The bars represent the mean ± standard deviation. Significant changes ($p \leq 0.05$) between * control and group 1; ** control and group 2; *** control and all treatment groups.

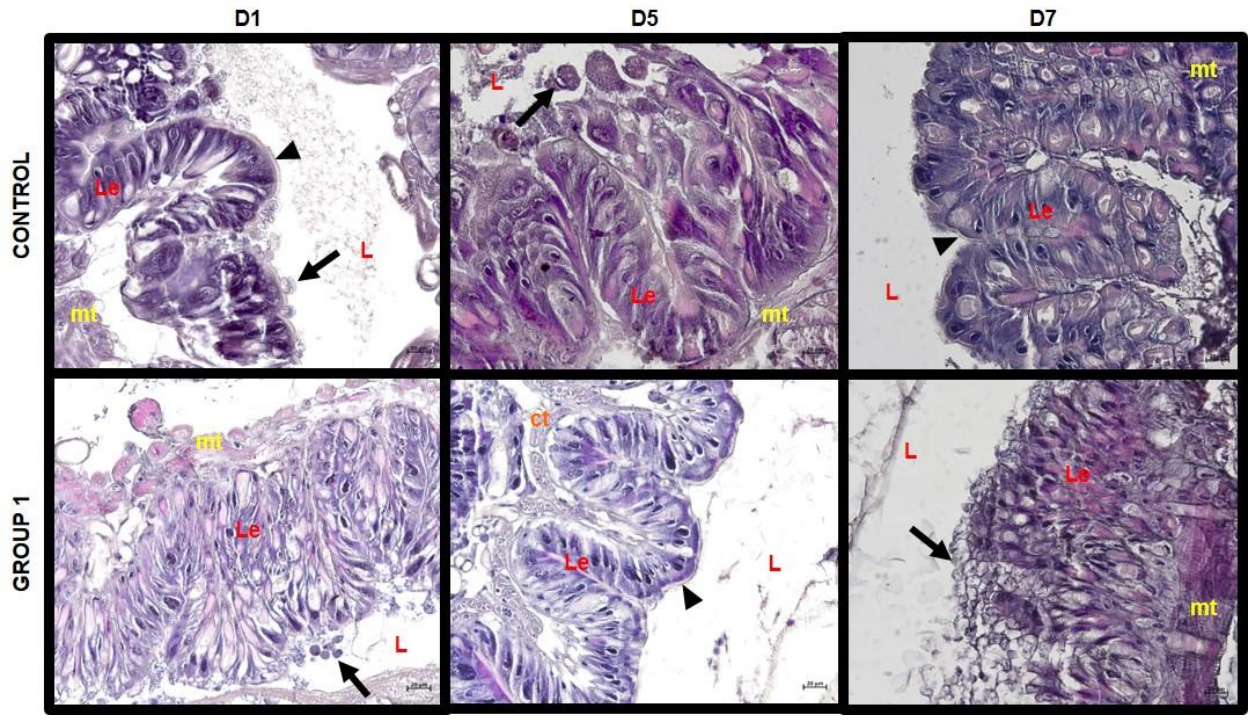

Figure 4. Histological structure of Control and Group 1 larvae with stained H&E (L: Lumen, Le: Larval epithelium, mt: muscle tissue, ct: connective tissue, black arrow mark: secretion vesicles, black triangle: brush border structure).

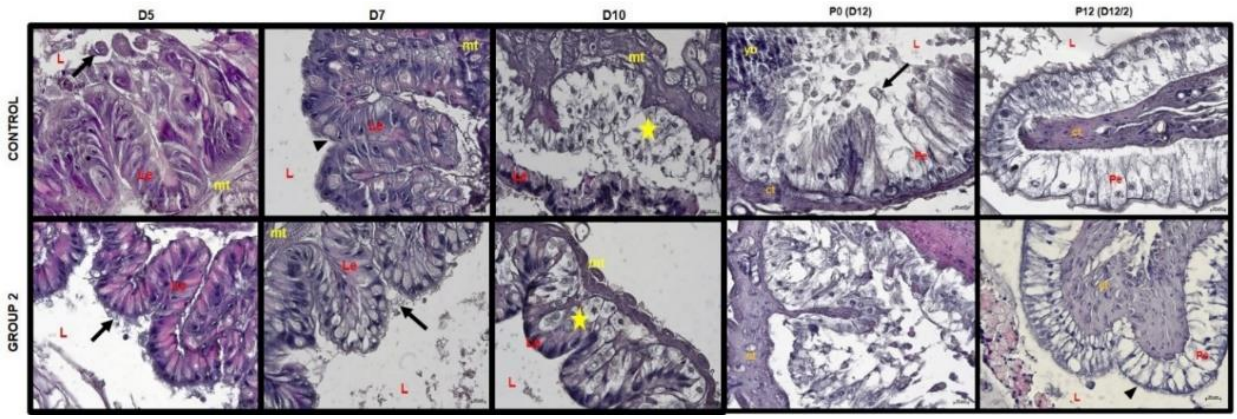

Figure 5. Histological structure of Control and Group 2 larvae with stained H&E (L:Lumen, Le: Larval epithelium, mt: muscle tissue, ct: connective tissue, Pe: Pupal epithelium, yb: yellow body, black arrow mark: secretion vesicles, black triangle: brush border structure, yellow star: stem cells).

The sugar levels in the midguts obtained from group 1 were found to be significantly lower than the control group throughout the experiment. In group 2, sugar levels are low on days 6 and 8 of the last larval stage and the last two days of the experiment. On days 9 and 11 of the 5th larval stage, the sugar levels detected were significantly higher than in the control group (Fig. 8b).

Lipid levels in the midguts obtained from group 1 did not differ from the control group until day 4 of the 5th larval stage. However, the lipid levels determined from day 5 were found to be significantly lower than the control group. The lipid levels in the midgut obtained from group 2 were found to be lower than the control group on day 6 of the 5th larval stage and the last 2 days of the experiment (Fig. 8c).

The total protein concentration of midguts obtained from the group 1 was found to be significantly higher than the control group larvae on the 2nd day of starvation. However, especially after day 4 of the 5th larval stage, protein levels remained at lower levels compared to the control group. Total protein amounts in the midguts from group 2 decreased in the first two days of the starvation period. Following this, the increasing total protein amount showed a graph that fluctuated until the end of the experiment (Fig. 8d).

3.3. Acid phosphatase assay findings

Acid phosphatase is a lysosomal enzyme and its activity has been determined to increase in autophagy-mediated cell death (Selek et al., 2016). In the midguts obtained from group 1, there was a significant increase in the acid phosphatase enzyme on days 3 and 5 compared to the control group. It was determined that acid phosphatase activity in the midguts obtained from group 2 was higher on days 6 and 10 of the 5th larval stage compared to the control group. However, the acid phosphatase levels determined during the prepupal period of the control group were significantly higher than the starved group 2

larvae (Fig. 9).

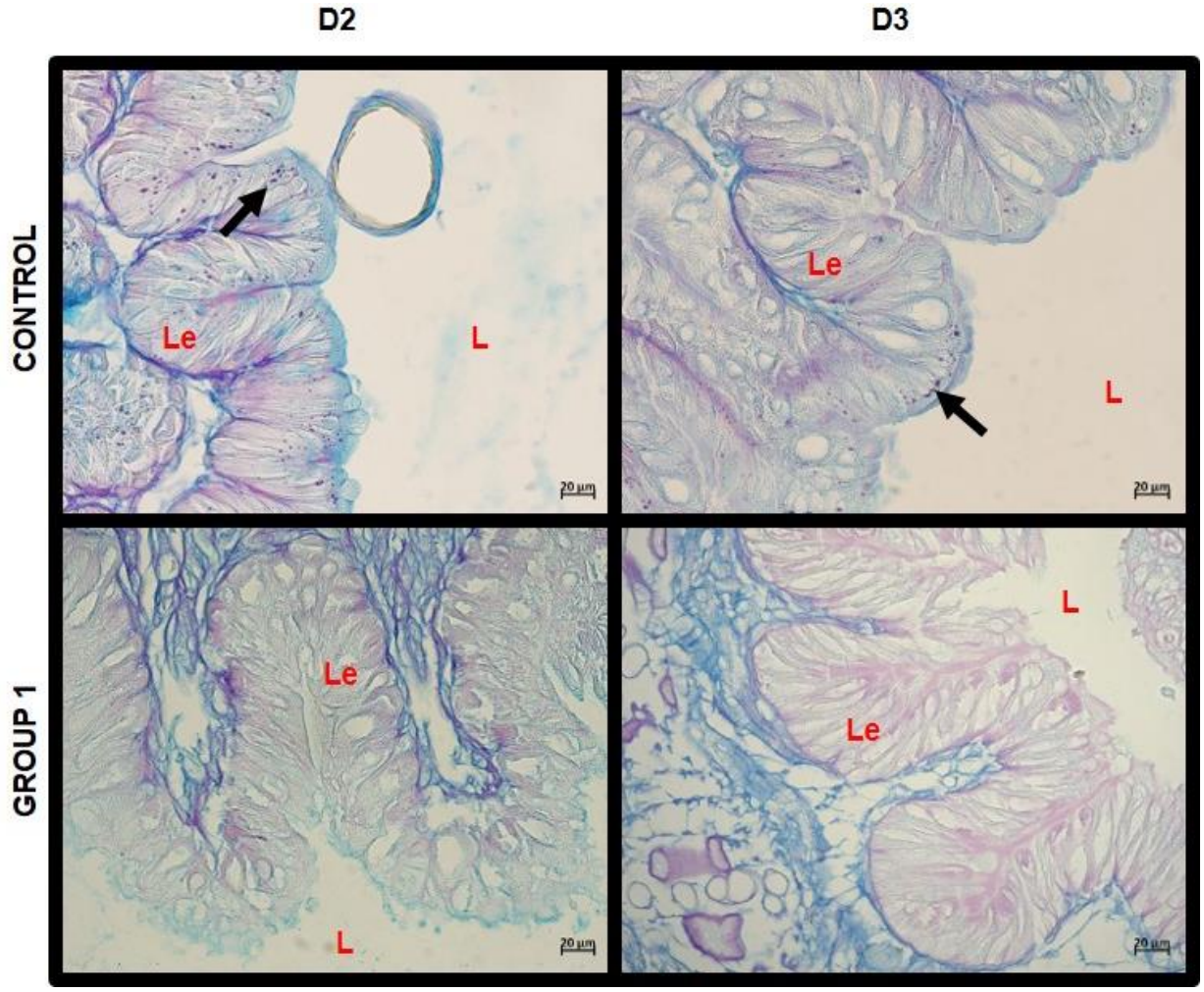

Figure 6. Histological structure of Control and Group 1 larvae with stained Alcian blue-PAS (L:Lumen, Le: Larval epithelium, black arrow mark: glycogen granules).

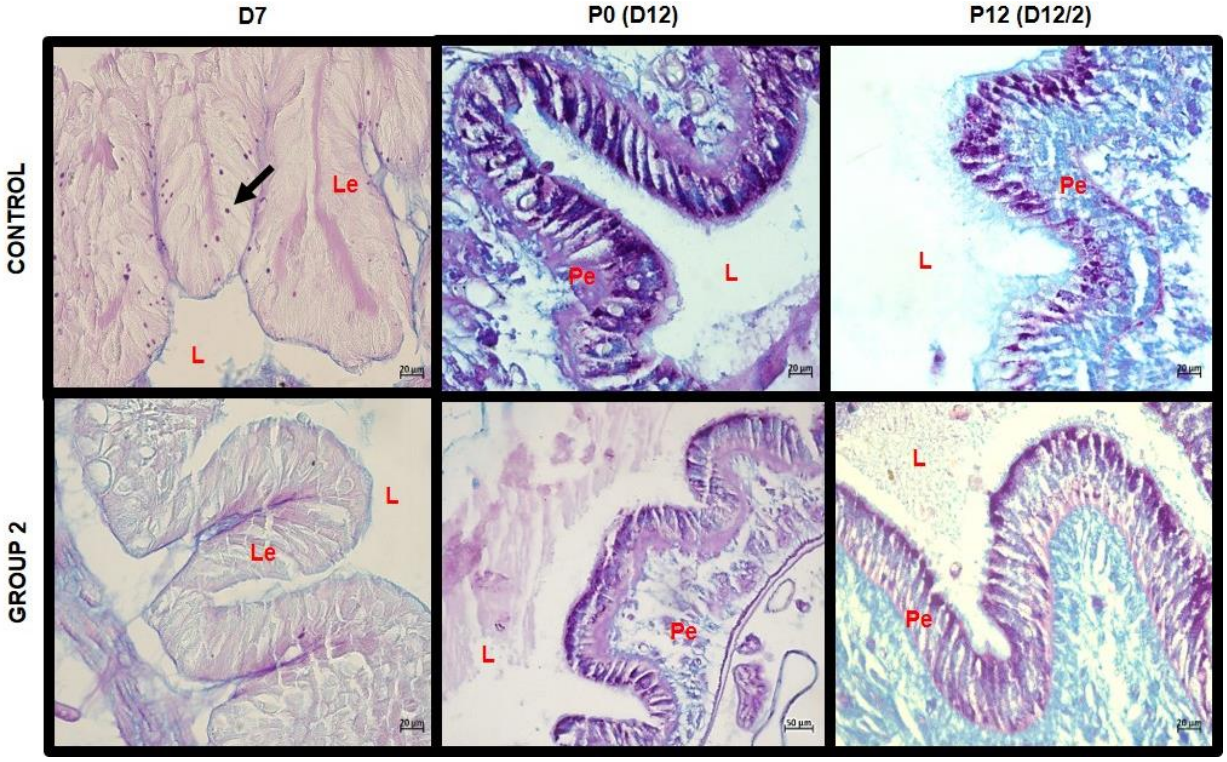

Figure 7. Histological structure of Control and Group 2 larvae with stained Alcian blue-PAS - developmental days in brackets are shown for group 2 (L:Lumen, Le: Larval epithelium, black arrow mark: glycogen granules).

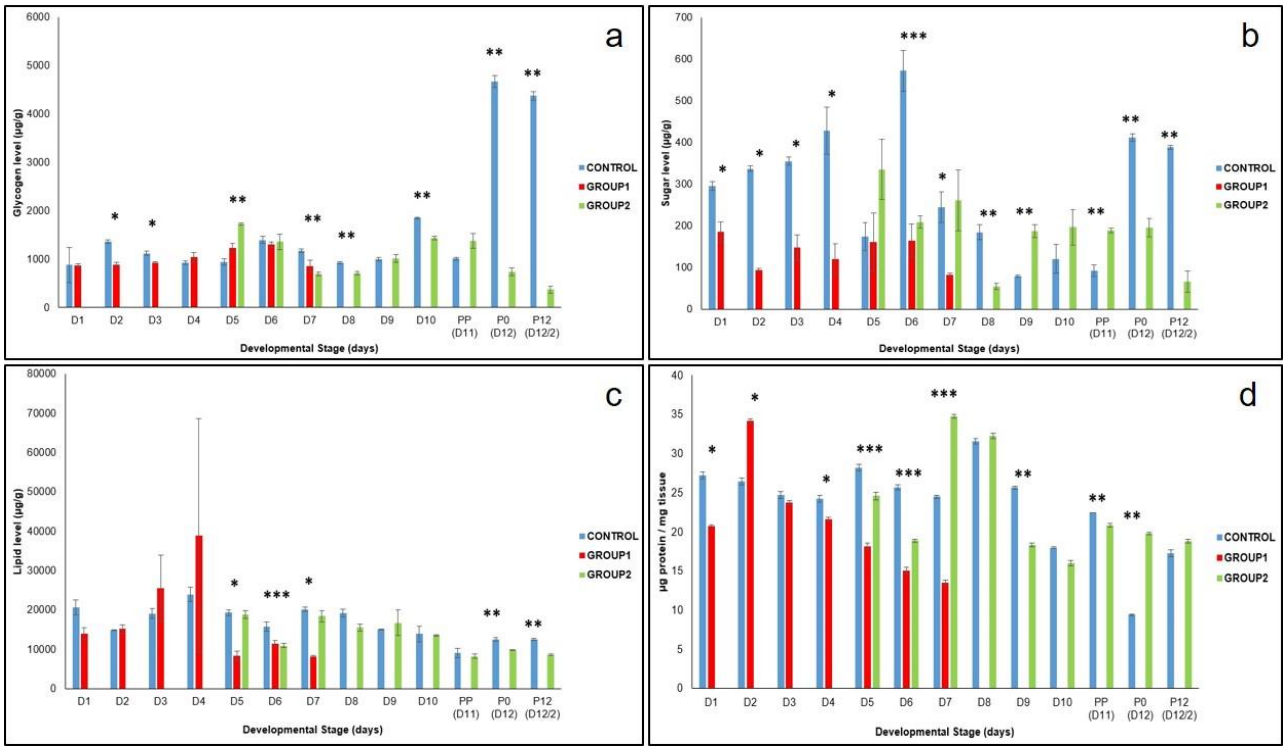

Figure 8. a) Glycogen, b) sugar, c) lipid, d) protein levels of Control, Group 1 and Group 2 larvae - developmental days in brackets are shown for group 2. The bars represent the mean ± standart deviation. Significant changes (p≤ 0.05) between * control and group 1; ** control and group 2; *** control and all treatment groups.

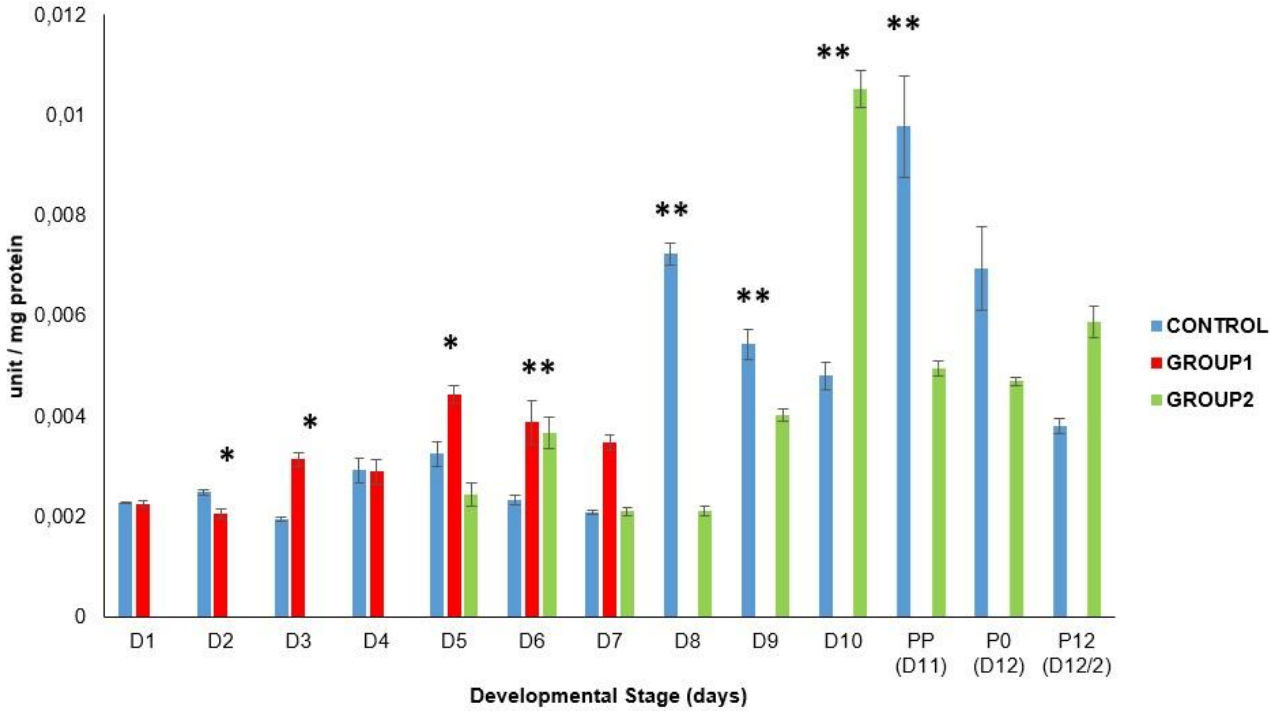

Figure 9. Specific acid phosphatase enzyme activities of Control, Group 1, and Group 2 larvae - developmental days in brackets are shown for group 2. The bars represent the mean ± standard deviation. Significant changes (p≤ 0.05) between * control and group 1; ** control and group 2; *** control and all treatment groups.

3.4. Expressions levels of *Bm*Tor1, ATG12, and ATG8

*Bm*Tor 1 expression in the midguts of the control group was determined to be at low levels until day 7 of the 5th larval stage and then peaked rapidly on this day. *Bm*Tor1 mRNA levels detected from the midguts of group 1 larvae were found to be significantly higher than the control group on days 1, 4, 5, and 6 of the 5th instar. The transcript levels determined on the other experimental days were lower than those in the control group.

Moderate *Bm*Tor 1 mRNA levels in the midguts of the control group were maintained until 12th hour pupation. In the midguts obtained from group 2, *Bm*Tor 1 transcript levels were higher than those in control group, except on day 8 of the 5th instar (Fig. 10a).

The ATG12 expression levels in the midguts of group 1 were significantly lower than the control group throughout the experiment. ATG12 mRNA levels determined in midguts from group 2 were significantly higher than those in determined in the control group at days 5, 9, and 10 of the 5th larval stage (Fig. 10b).

The ATG8 mRNA levels in the midguts of the control group showed a gradual increase until day 7 of the 5th larval stage. On the other hand, a similar increase did not occur in the midguts obtained from group 1 and remained at lower levels than the control group throughout the experiment. In the midguts obtained from group 2, ATG8 mRNA levels were higher than the control group on days 5, 6, and 10 and remained at low levels on the other days of the experiment (Fig. 10c)

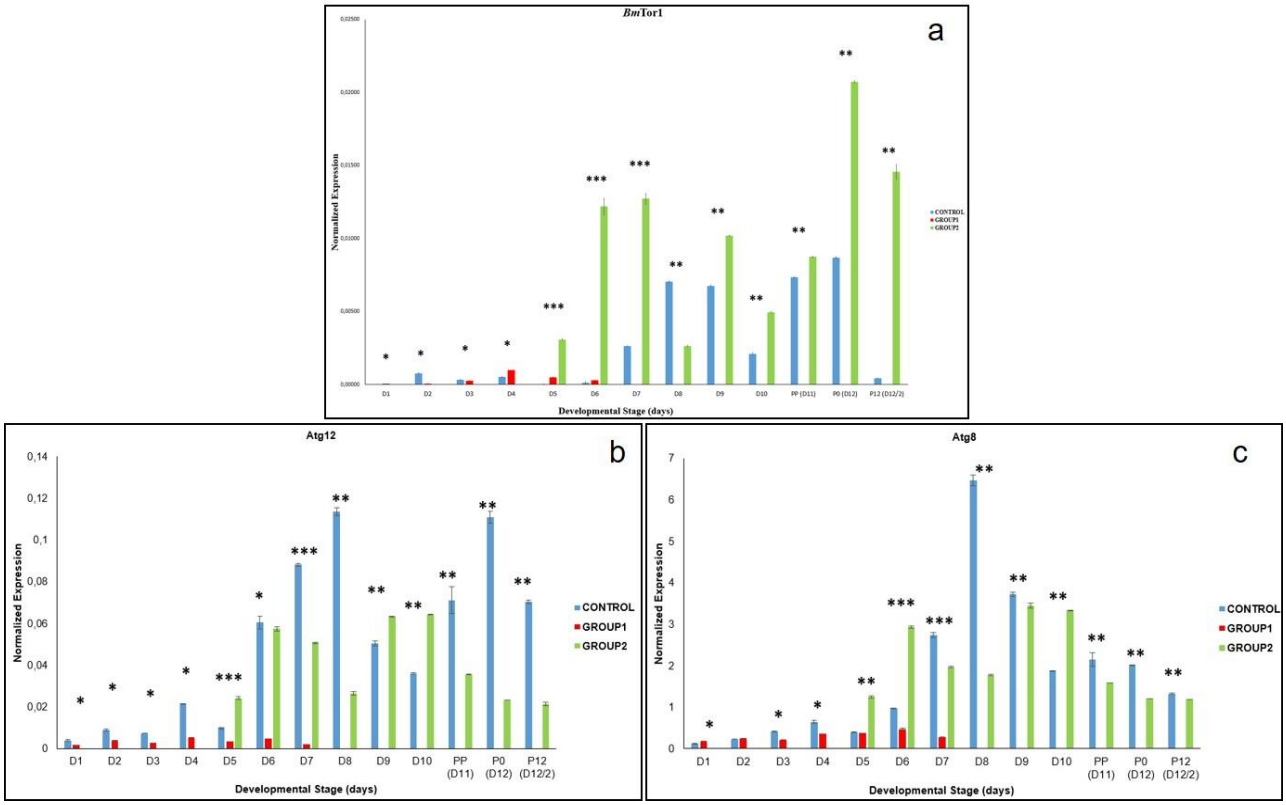

Figure 10. a) BmTor1 b) ATG12 c) ATG8 mRNA expression levels of Control, Group 1 and Group 2 larvae - developmental days in brackets are shown for group 2. The bars represent the mean ± standart deviation. Significant changes ($p \leq 0.05$) between * control and group 1; ** control and group 2; *** control and all treatment groups.

4. Discussion

Under starvation conditions, the cell tries to adapt to this stress condition by reducing its needs, mobilizing energy-rich storage molecules, and activating the autophagy pathway. Autophagy, as a cellular response to starvation, has been studied in detail in many different species including yeast, nematodes, flies, and mice. Loss of autophagy resulted in death in yeast, developmental defects in *Caenorhabditis elegans* Maupas, 1900 (Rhabditida: Rhabditidae), hypersensitivity to starvation in *Drosophila melonagaster* Meigen, 1830 (Diptera: Drosophilidae), and postnatal death in mice (Tsukada & Ohsumi 1993; Melendez et al., 2003; Kuma et al., 2004; Scott et al. 2004). In addition, important functions of autophagy have been demonstrated in insects during the metamorphosis process in which the organism is remodeled but there is no nutrient entry into the body (Malagoli et al., 2010; Romanelli et al., 2014). Lepidopteran insects, including the silkworm *Bombyx mori*, are widely used as model organisms in studies on the function of autophagy in insect metamorphosis. However, it is unclear whether autophagy plays a role in the midgut of *B. mori* under starvation conditions. Furthermore, no information is available on the similarities or differences between autophagy in the remodeling process in the midgut of *B. mori* and autophagy in the starvation state.

If cells cannot adapt to starvation stress, this can lead to apoptosis or necrosis (Caro-Maldonado & Muñoz-Pinedo, 2011). There have been several previous reports showing that starvation causes cell death in various insect cells. Wu et al. (2011) reported that *B. mori* ovarian cells die in a non-apoptotic way when starvation lasts longer than 48 hours. When *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae) ovarian cells were exposed to amino acid starvation, an increase in autophagic cell death activity was observed and it was determined that the cells died by apoptosis after 48 hours of starvation (Wu et al., 2011). Park et al. (2009) showed that starvation triggers apoptosis in midgut tissue in their study on *Periplaneta americana* L., 1758 (Blattodea: Blattidae). In contrast to these studies, starvation did not cause cell death in larval midgut cells until the last day of the experiment. The preservation of the normal histological

structure of the midguts obtained from starved insects showed that starvation did not cause any abnormal or degenerative changes until the death of the insect. However, the significant reduction in the fat body, which is the main storage organ of insects, and the consequent decrease in body weight of starved insects suggested that death was related to the depletion of energy reserves.

In a study (Xie et al., 2016) in the *Bombyx mori* cell line, apoptosis was shown to occur following autophagy after 20E treatment and starvation. In the same study, it was reported that, the rate of cells undergoing both autophagy and apoptosis decreased after 20E and starvation experiments as a result of decreasing the expression of Atg5 and Atg6 by RNAi. Our results show that autophagy does not occur in the midgut in vivo after starvation.

Lysosomes play a central role in cellular catabolic processes by being at the junction between endocytic and autophagic pathways. The lysosomal acid phosphatase enzyme hydrolyzes organic phosphates at acidic pH and an increase in acid phosphatase activity has been used as an indicator of autophagy in different studies (Goncu & Parlak, 2008; Klionsky et al., 2016). Relatively high acid phosphatase activity in the midguts during the mid-starvation period of group 1 indicated rising lysosomal activity in this group. However, the low expression levels of autophagy-related genes, ATG8 and ATG12, in the midguts obtained from group 1 suggested that starvation did not cause an increase in autophagy in this group. On the first day of the experiment, the increase in the expression of TOR kinase, the main regulator of autophagy, in the midguts obtained from group 1 was the first response to starvation. Zhou et al. (2010) also reported that starvation induces TOR expression especially in the brain and fat body of *B. mori*. However, this increase in TOR expression in group 1 was not associated with autophagy. If autophagy is not stimulated in the midgut when the starvation condition is established from the beginning of the 5th larval stage, how was the viability and normal morphological structure of the cell preserved until day 7 of 5th instar?

Endosomal microautophagy (eMI) has been recently described in mammals as a process by which endosomes engulf cytosolic material through the formation of multivesicular bodies (MVBs) that is subsequently degraded in late endosomes or upon their fusion with lysosomes. Mukherjee et al. (2016) reported a starvation-inducible catabolic process resembling endosomal microautophagy in the *Drosophila* fat body. Significantly, eMI in the *Drosophila* fat body is a transient physiological mechanism that occurs after more than 12 hours of starvation. It has also been noted that it is genetically different from macroautophagy as it is independent of Atg5, Atg7, and Atg12. Although there was no increase in the expression of the ATG genes studied, the increased lysosomal activity suggested that endosomal microautophagy may have occurred during the prolonged starvation period as demonstrated in the *Drosophila* fat body and this may be one of the answers to the question above.

Mobilization of energy substrates is another response that occurs during starvation. The decrease in sugar levels together with the amount of midgut glycogen in starved larvae, determined by Alcian blue-PAS staining and spectrophotometric analysis of glycogen, showed that the first response to starvation was the mobilization of glycogen to be used as an energy source. In a study (Franzetti et al., 2016) conducted in the midgut of *B. mori*, it was also reported that autophagy was not activated during the starvation process during larval-larval molt instead, intracellular energy sources such as glycogen and lipid were used. It was also shown that even in this process, the midgut is metabolically active and the brush border structure is not affected by this starvation process. The decrease in TOR expression in group 1 over the next two days of onset the starvation may be the result of intracellular glycogen breakdown and/or uptake of trehalose from hemolymph as an energy substrate. This can temporarily eliminate the starvation stress that the cell was exposed to. The recurrent increase in TOR expression during the later days of starvation was an indicator that the cells were again under starvation stress. The decrease in the lipid and protein levels occurring these days also supported this finding. Lipids are another energy-rich storage molecule. In a study (Arrese & Soulages, 2010) conducted in *Manduca sexta* L., 1763 (Lepidoptera: Sphingidae), it was reported that starvation doubled the hemolymph lipid concentration but the presence of high trehalose levels in the hemolymph inhibited lipid mobilization. This result supports that lipid mobilization occurs after the decrease in hemolymph trehalose levels. The decrease in the glycogen levels in the early stages of starvation and lipid levels in the late stages of starvation in group 1 larvae in our study indicates that a similar regulation mechanism also exists in the midgut. In addition to carbohydrates and lipids, proteins can be catabolized during starvation. Our results showed that starvation stimulated protein degradation in the midgut of *B. mori*. The reduction in protein amounts in the midgut of group 1 larvae during the last days of starvation indicated that the final response to prolonged starvation stress was increased protein degradation. Because proteins are also structural organic molecules, their use as energy substrates in vertebrates occurs in the late stages of starvation. The decrease in the amount of protein in the midguts of group 1 larvae in the pre-mortem period indicated an increase in the protein breakdown and thus a depletion of energy reserves.

As a result of the proteomic analysis performed in *B. mori* midgut, it was reported that a 10kDa small heat shock protein (sHSP) and a diapause hormone precursor were produced under starvation stress. Studies have found that sHSPs have a protective role in cells and their expression is particularly prominent where cells are exposed to harmful conditions such as hyperthermia (hence the name heat shock), hypoxia, and starvation. They are also known to be involved in protein repair and maintaining cell survival by keeping proteins from fatally aggregating in stressed cells (Soti et al., 2003). Although sHSPs can bind a wide variety of cellular proteins in prokaryotes, it is determined that they preferentially protect certain classes of functional proteins such as translational proteins and metabolic enzymes (Fu, 2014). A similar function may apply in eukaryotes. In a study performed in the heart, it was reported that sHSP stimulates glucose uptake and glycolysis by cells and

regulates fatty acid metabolism (Sun et al., 2021). Thus, sHSP produced under starvation conditions in the midgut of *B. mori* may play a role in metabolic enzymes and may contribute to the obtaining of the energy required for life from carbohydrates, lipids, and proteins and thus to the inhibition of autophagy. In addition to sHSP, the production of the diapause hormone precursor after starvation suggests that the larva delayed its development in order to survive.

The main inducer of autophagy is starvation stress but autophagy also plays a special role in the degeneration of larval-specific tissues and organs of Holometabolous insects during metamorphosis (Tettamanti & Casartelli, 2019). During the larval-pupal metamorphosis in *B. mori*, larval-specific tissues such as the prothoracic gland, fat body, silk glands, intersegmental muscles, and larval midgut are degenerated by programmed cell death including autophagy (Kakei et al., 2005). Since insects don't feed during pupal and adult stages, substances obtained from degenerated tissues provide necessary energy and basic building blocks during the formation of a new tissue (Rabinowitz & White, 2010). For this reason, autophagy, which plays a role in the degeneration of larval organs, plays a very important role in gaining the necessary energy and molecules for the later life periods of the insect where there is no food intake.

With further studies, it was shown that autophagy in the midgut of *B. mori* is activated at the onset of metamorphosis (Goncu et al., 2017). In *D. melanogaster*, it was shown that autophagy rather than apoptosis is required for cell death in the midgut during metamorphosis (Franzetti et al., 2012). It was been reported that autophagy begins immediately after termination of feeding and gradually increases until the larval epithelium is digested (Tettamanti et al., 2007b).

In group 2, increased ATG gene expression and higher acid phosphatase activity in the first days of starvation compared to the control group indicated that autophagy was stimulated at the onset of starvation. Histological analyses of the midguts of this group of insects revealed that remodeling processes in the midgut occurred despite starvation. According to the previous studies in *B. mori*, autophagy plays a very important role in the remodeling process of the midgut resulting in increased autophagic structures in cells, high expression levels of autophagy-related genes, and increased lysosomal acid phosphatase enzyme levels during prepupal development (Franzetti et al., 2012; Romanelli et al., 2016; Goncu et al., 2016). Although remodeling occurred in the midgut of group 2 insects during development, the absence of an increase in the expression of ATG genes and acid phosphatase activity as in the control group indicated that starvation stress reduced autophagy during the remodeling of the midgut. Romanelli et al. (2016) reported that autophagy in the midgut of *B. mori* is stimulated by 20E during metamorphosis. Although the prothoracic gland gains its secretory activity in the first days of the 5th larval stage, the development of the gland takes place in the later days of the 5th larval stage (Keshan et al., 2015), and the hemolymph ecdysone levels begin to increase gradually until pupal ecdysis (Sakurai et al., 1998; Mizoguchi et al., 2002). However, inhibition of gland growth due to starvation in group 2 may have reduced the amount of 20E produced. Since the expression of autophagy genes is induced by 20E in larval-pupal metamorphosis (Tian et al., 2013), low expression levels of ATG genes may be associated with insufficient ecdysone production. Despite cocoon spinning, the absence of larval-pupal ecdysis supports this possibility. In addition, high TOR1 expression in this group throughout the experiment was also an indicaticator of continued starvation stress.

TOR expression was reported to increase not only during starvation but also during metamorphosis (Zhou et al. 2010); therefore, the large increase in gene expression in the control group on day 7 may be associated with the onset of the prepupal period. The increase in glycogen content in the midgut of the control group in the early pupal stage and its localization in the newly formed pupal midgut epithelium suggested that it was a result of the digestion of the degenerated larval midgut due to apoptosis and autophagy by the pupal midgut epithelium in this period. According to a previous study in *B. mori*, glycogen granules were found in the cytoplasm of larval midgut cells during the 5th larval stage, while after pupal midgut formation, the positivity was mainly localized in the new epithelial layer (Franzetti et al., 2015). Extremely low glycogen levels in pupal midgut epithelial cells of group 2 were related to depleted resources during the larval period due to starvation and this may result in insufficient energy molecules to be supplied to the newly formed pupal midgut cells from the degenerated larval midgut. Although a reduction in lipid levels occurred in group 2 larvae before larval epithelial degeneration as a response to starvation, determination of similar lipid levels to the control group during larval-pupal metamorphosis indicated inhibition of lipid mobilization in the midgut. It was determined that the triacylglycerol levels in the adipose tissue decrease due to ecdysone during each molt and the hemolymph diacylglycerol levels increase to be used by the tissues during the metamorphosis process where there is no nutrient entry into the body (Liu et al. 2009). The reason why lipid levels did not decrease in the midgut during metamorphosis in group 2 larvae might be due to the uptake of lipids by midgut cells released into the hemolymph due to increased ecdysone levels in this process. The decrease in protein levels determined at the beginning of starvation in group 2 larvae occurred because of protein degradation due to autophagy or physiological changes that occur with the onset of metamorphosis. Energy consumption increases during larval-pupae metamorphosis; thus, energy stores during larval feeding must support pupal development and meet the reproductive and survival needs of adults. In holometabolous insects, energy expenditure at metamorphosis forms a U-shaped curve and energy expenditure is high in the early stages of metamorphosis (Nestel et al., 2003). Protein degradation in the early stages of starvation in group 2 larvae occurred in order to obtain amino acids to be used in the synthesis of new proteins for larval-pupa metamorphosis and/or to meet the increased energy requirements under starvation conditions.

In conclusion, autophagy either plays no role or occurs at a moderate level during starvation depending

on the time of the onset of starvation. While starvation from the onset of the fifth larval stage causes gradual mobilization of energy storage molecules rather than stimulating macroautophagy, increased lysosomal activity may be associated with the newly described endosomal microautophagy. Future work will focus on detailed analyzes of this case. Autophagy, which takes place in the midgut during larval-pupal metamorphosis, occurs intensely as a part of programmed cell death given the expression levels of genes and lysosomal activity involved in this process. From day 4 of the 5th larval stage, starvation caused an increase in autophagy as the first response while it caused a decrease in the intensity of the autophagy process that occurs during metamorphosis. Considering the absence of larval-pupa ecdysis in this group, it can be thought that decreased autophagy may be due to insufficient ecdysone production during the metamorphosis.

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References

Arrese, E.L., & Soulages, J.L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual review of entomology*, 55, 207. https://doi.org/10.1146/annurev-ento-112408-085356

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Bergmeyer, H.U. (1974). Enzymes as biochemical reagents. "*Methods of Enzymatic Analysis*", Academic Press, New York, 428 pp.

Caro-Maldonado, A., & Muñoz-Pinedo, C. (2011). Dying for something to eat: how cells respond to starvation. *The Open Cell Signalling Journal*, 3(1), 42-51. https://doi.org/10.2174/1876390101103010042

Chan, E.Y., & Tooze, S.A. (2009). Evolution of Atg1 function and regulation. *Autophagy*, 5(6), 758-765. https://doi.org/10.4161/auto.8709

Franzetti, E., Huang, Z.J., Shi, Y.X., Xie, K., Deng, X.J., Li, J.P., ... & Feng, Q. (2012). Autophagy precedes apoptosis during the remodelling of silkworm larval midgut. *Apoptosis*, 17(3), 305-324. https://doi.org/10.1007/s10495-011-0675-0

Franzetti, E., Romanelli, D., Caccia, S., Cappellozza, S., Congiu, T., Rajagopalan, M., ... & Tettamanti, G. (2015). The midgut of the silkmoth *Bombyx mori* is able to recycle molecules derived from degeneration of the larval midgut epithelium. *Cell and tissue research*, 361(2), 509-528. https://doi.org/10.1007/s00441-014-2081-8

Franzetti, E., Casartelli, M., D'Antona, P., Montali, A., Romanelli, D., Cappellozza, S., ... & Tettamanti, G. (2016). Midgut epithelium in molting silkworm: a fine balance among cell growth, differentiation, and survival. *Arthropod Structure & Development*, 45(4), 368-379. https://doi.org/10.1016/j.asd.2016.06.002

Fu, X. (2014). Chaperone function and mechanism of small heat-shock proteins. *Acta Biochimica et Biophysica Sinica*, 46(5), 347-356. https://doi.org/10.1093/abbs/gmt152

Goncu, E., & Parlak, O. (2008). Some autophagic and apoptotic features of programmed cell death in the anterior silk glands of the silkworm, *Bombyx mori*. *Autophagy*, 4(8), 1069-1072. https://doi.org/10.4161/auto.6953

Goncu, E., Uranlı, R., Selek, G., & Parlak, O. (2016). Developmental expression of ecdysone-related genes associated with metamorphic changes during midgut remodeling of silkworm *Bombyx mori* (Lepidoptera: Bombycidae). *Journal of Insect Science*, 16(1), 86. https://doi.org/10.1093/jisesa/iew061

Goncu, E., Uranli, R., & Parlak, O. (2017). Juvenile hormone analogue, fenoxycarb, modulates ecdysone-triggered transcriptional hierarchy during programmed cell death of midgut in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *European Journal of Entomology*, 114, 235. https://doi.org/10.14411/eje.2017.029

Hietakangas, V., & Cohen, S.M. (2009). Regulation of tissue growth through nutrient sensing. *Annual review of genetics*, 43, 389-410. https://doi.org/10.1146/annurev-genet-102108-134815

Jung, C.H., Ro, S.H., Cao, J., Otto, N.M., & Kim, D.H. (2010). mTOR regulation of autophagy. *FEBS letters*, 584(7), 1287-1295. https://doi.org/10.1016/j.febslet.2010.01.017

Kakei, M., Iwami, M., & Sakurai, S. (2005). Death commitment in the anterior silk gland of the silkworm, *Bombyx mori*. *Journal of insect physiology*, 51(1), 17-25. https://doi.org/10.1016/j.jinsphys.2004.10.012

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., & Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *The Journal of cell biology*, 150(6), 1507-1513. https://doi.org/10.1083/jcb.150.6.1507

Kaufmann, C., & Brown, M.R. (2008). Regulation of carbohydrate metabolism and flight performance by a hypertrehalosaemic hormone in the mosquito Anopheles gambiae. *Journal of insect physiology*, 54(2), 367-377. https://doi.org/10.1016/j.jinsphys.2007.10.007

Kaufmann, C., & Brown, M. (2014). Determination of lipid, glycogen and sugars in mosquitoes. *MR4 methods in Anopheles research, 4th ed. BEI Resources, Manassas, VA*.

Kaushik, S., & Cuervo, A.M. (2012). Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends in cell biology*, 22(8), 407-417. https://doi.org/10.1016/j.tcb.2012.05.006

Keshan, B., Thounaojam, B., & Kh, S.D. (2015). A comprehensive study of the changes in ecdysteroid levels during the feeding phase of fifth instar larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *European Journal of Entomology*, 112(4), 632. https://doi.org/10.14411/eje.2015.088

Klionsky, D.J., & Codogno, P. (2013). The mechanism and physiological function of macroautophagy. *Journal of innate immunity*, 5(5), 427-433. https://doi.org/10.1159/000351979

Klowden, M.J., (2007), Physiological Systems in Insects, 298-395 pp.

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., ... & Mizushima, N. (2004). The role of autophagy during the early neonatal starvation period. *Nature*, 432(7020), 1032-1036. https://doi.org/10.1038/nature03029

Li, W.W., Li, J., & Bao, J.K. (2012). Microautophagy: lesser-known self-eating. *Cellular and molecular life sciences*, 69(7), 1125-1136. https://doi.org/10.1007/s00018-011-0865-5

Liu, Y., Liu, H., Liu, S., Wang, S., Jiang, R. J., & Li, S. (2009). Hormonal and nutritional regulation of insect fat body development and function. *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, 71(1), 16-30. https://doi.org/10.1002/arch.20290

Lum, J.J., Bauer, D.E., Kong, M., Harris, M.H., Li, C., Lindsten, T., & Thompson, C.B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, 120(2), 237-248. https://doi.org/10.1016/j.cell.2004.11.046

Malagoli, D., Abdalla, F.C., Cao, Y., Feng, Q., Fujisaki, K., Gregorc, A., ... & Umemiya-Shirafuji, R. (2010). Autophagy and its physiological relevance in arthropods: current knowledge and perspectives. *Autophagy*, 6(5), 575-588. https://doi.org/10.4161/auto.6.5.11962

Meléndez, A., Tallóczy, Z., Seaman, M., Eskelinen, E.L., Hall, D.H., & Levine, B. (2003). Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science*, 301(5638), 1387-1391. https://doi.org/10.1126/science.1087782

Mizoguchi, A., Dedos, S.G., Fugo, H., & Kataoka, H. (2002). Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*. *General and comparative endocrinology*, 127(2), 181-189. https://doi.org/10.1016/S0016-6480(02)00043-6

Mukherjee, A., Patel, B., Koga, H., Cuervo, A.M., & Jenny, A. (2016). Selective endosomal microautophagy is starvation-inducible in

Drosophila. *Autophagy*, 12(11), 1984-1999. https://doi.org/10.1080/15548627.2016.1208887

Nestel, D., Tolmasky, D., Rabossi, A., & Quesada-Allué, L.A. (2003). Lipid, carbohydrates and protein patterns during metamorphosis of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Annals of the Entomological Society of America*, 96(3), 237-244. https://doi.org/10.1603/0013-8746(2003)096[0237:LCAPPD]2.0.CO;2

Park, M.S., Park, P., & Takeda, M. (2009). Starvation induces apoptosis in the midgut nidi of *Periplaneta americana*: a histochemical and ultrastructural study. *Cell and tissue research*, 335(3), 631-638. https://doi.org/10.1007/s00441-008-0737-y

Rabinowitz, J.D., & White, E. (2010). Autophagy and metabolism. *Science*, 330(6009), 1344-1348. https://doi.org/10.1126/science.1193497

Romanelli, D., Casati, B., Franzetti, E., & Tettamanti, G. (2014). A molecular view of autophagy in Lepidoptera. *BioMed research international*, 2014. https://doi.org/10.1155/2014/902315

Romanelli, D., Casartelli, M., Cappellozza, S., de Eguileor, M., & Tettamanti, G. (2016). Roles and regulation of autophagy and apoptosis in the remodelling of the lepidopteran midgut epithelium during metamorphosis. *Scientific reports*, 6(1), 1-15. https://doi.org/10.1038/srep32939

Sakurai, S., & Kaya, M. (1998). Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval–pupal metamorphosis and a reappraisal of the head critical period. *Journal of Insect Physiology*, 44(10), 867-881. https://doi.org/10.1016/S0022-1910(98)00075-4

Scott, R.C., Schuldiner, O., & Neufeld, T.P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Developmental cell*, 7(2), 167-178. https://doi.org/10.1016/j.devcel.2004.07.009

Selek, G., Göncü, E., & Parlak, O. (2016). Programmed Cell Death in the Digestive Canal of *Bombyx mori* (Lepidoptera: Bombycidae) during Prepupal Period. *European Journal of Biology*, 73(2), 1-29.

Sõti, C., Sreedhar, A.S., & Csermely, P. (2003). Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. *Aging cell*, 2(1), 39-45. https://doi.org/10.1046/j.1474-9728.2003.00031.x

Sun, X., Siri, S., Hurst, A., & Qiu, H. (2021). Heat Shock Protein 22 in Physiological and Pathological Hearts: Small Molecule, Large Potentials. *Cells*, 11(1), 114. https://doi.org/10.3390/cells11010114

Tettamanti, G., Grimaldi, A., Pennacchio, F., & de Eguileor, M. (2007a). Lepidopteran larval midgut during prepupal instar: digestion or self-digestion?. *Autophagy*, 3(6), 630-631. https://doi.org/10.4161/auto.4908

Tettamanti, G., Grimaldi, A., Casartelli, M., Ambrosetti, E., Ponti, B., Congiu, T., ... & Eguileor, M.D. (2007b). Programmed cell death and stem cell differentiation are responsible for midgut replacement in Heliothis virescens during prepupal instar. *Cell and tissue research*, 330(2), 345-359. https://doi.org/10.1007/s00441-007-0449-8

Tettamanti, G., & Casartelli, M. (2019). Cell death during complete metamorphosis. *Philosophical Transactions of the Royal Society B*, 374(1783), 20190065. https://doi.org/10.1098/rstb.2019.0065

Tian, L., Ma, L., Guo, E., Deng, X., Ma, S., Xia, Q., ... & Li, S. (2013). 20-Hydroxyecdysone upregulates Atg genes to induce autophagy in the *Bombyx* fat body. *Autophagy*, 9(8), 1172-1187. https://doi.org/10.4161/auto.24731

Tsukada, M., & Ohsumi, Y. (1993). Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS letters*, 333(1-2), 169-174. https://doi.org/10.1016/0014-5793(93)80398-E

Van Handel, E. (1985a). Rapid determination of glycogen and sugars in mosquitoes. *Journal of the American Mosquito Control Association*, 1(3), 299-304.

Van Handel, E. (1985b). Rapid determination of total lipids in mosquitoes. *Journal of the American Mosquito Control Association*, 1(3), 302-304.

Van Handel, E., & Day, J. (1988). Assay of lipids, glycogen and sugars in individual mosquitoes: correlations with wing length in field-collected Aedes vexans. *Journal of the American Mosquito Control Association*, 4(4), 549-550.

Wang, X., & Proud, C.G. (2009). Nutrient control of TORC1, a cell-cycle regulator. *Trends in cell biology*, 19(6), 260-267. https://doi.org/10.1016/j.tcb.2009.03.005

Wu, W., Wei, W., Ablimit, M., Ma, Y., Fu, T., Liu, K., ... & Hong, H. (2011). Responses of two insect cell lines to starvation: autophagy prevents them from undergoing apoptosis and necrosis, respectively. *Journal of insect physiology*, 57(6), 723-734. https://doi.org/10.1016/j.jinsphys.2011.02.008

Xie, Z., & Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. *Nature cell biology*, 9(10), 1102-1109. https://doi.org/10.1038/ncb1007-1102

Xie, K., Tian, L., Guo, X., Li, K., Li, J., Deng, X., ... & Cao, Y. (2016). BmATG5 and BmATG6 mediate apoptosis following autophagy induced by 20-hydroxyecdysone or starvation. *Autophagy*, 12(2), 381-396. https://doi.org/10.1080/15548627.2015.1134079

Zhou, S., Zhou, Q., Liu, Y., Wang, S., Wen, D., He, Q., ... & Li, S. (2010). Two Tor genes in the silkworm *Bombyx mori*. *Insect molecular biology*, 19(6), 727-735. https://doi.org/10.1111/j.1365-2583.2010.01026.x