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Expression profiles and possible functions of the ecdysone-related genes in the midgut stem cells of the silkworm, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae)[1]

İpekböceği, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) orta bağırsak kök hücrelerinde ekdizonla ilişkili genlerin ekspresyon profilleri ve olası rolleri

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Abstract

The insect midgut has remarkable similarities with the vertebrate intestine especially concerning controlling cell regulation by the stem cells. While the formation of the pupal midgut from stem cells is regulated by ecdysone, it is inhibited by juvenile hormone via suppression of ecdysone release. This study investigated the possible functions of ecdysone-related genes in the stem cells of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) during larval-pupal metamorphosis. The study was conducted in the Ege University silkworm culture laboratory and insect physiology research laboratory during the years between 2014-2018. Juvenile hormone analogue, fenoxycarb was applied to the fifth instar *Bombyx* larvae to delayed or inhibit the formation of the pupal midgut. Morphologic observations were performed by hematoxylin plus eosin staining; the proliferation rate of stem cells was analyzed by bromodeoxyuridine cell proliferation assay and expression patterns of ecdysone-related genes were detected by quantitative real-time reverse transcriptase-polymerase chain reaction. Expression states of genes and developmental events of the midgut were differently affected by fenoxycarb treatment in an application time-dependent manner. According to results, genes were firstly classified based on their sensitivity of fenoxycarb, then grouped according to their expression profile in connection with morphological evaluations of stem cells.

Keywords: *Bombyx mori*, ecdysone-related genes, midgut, stem cell

Öz

Böcek orta bağırsağı, özellikle kök hücreler tarafından hücre düzenlemesinin kontrol edilmesiyle ilgili olarak omurgalı bağırsağıyla dikkate değer benzerliklere sahiptir. Pupa orta bağırsağı, kök hücreler tarafından oluşturulur ve ekdizon, orta bağırsağın yeniden şekillenme olaylarını düzenlerken, juvenil hormon, ekdizon salınımını baskılayarak engeller. Bu çalışma, larva-pupa metamorfozu sırasında *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae)'nin kök hücrelerinde ekdizonla ilişkili genlerin olası işlevlerini araştırmıştır. Bu çalışma 2014-2018 yılları arasında Ege Üniversitesi ipek böceği kültür laboratuvarı ve böcek fizyolojisi araştırma laboratuvarında yapılmıştır. Juvenil hormon analoğu fenoksikarb, pupa orta bağırsak oluşumunu geciktirmek veya engellemek için 5. dönem *Bombyx* larvalarına uygulanmıştır. Morfolojik gözlemler hematoksilen ve eozin boyama ile gerçekleştirilmiş; kök hücrelerin proliferasyon hızı, bromodeoksiüridin hücre proliferasyon analizi ile analiz edilmiş ve ekdizon ile ilgili genlerin ekspresyon durumları kantitatif gerçek zamanlı ters transkriptaz-polimeraz zincir reaksiyonu ile tespit edilmiştir. Genlerin ekspresyon durumları ve orta bağırsaktaki gelişimsel olaylar, uygulama zamanına bağlı olarak fenoksikarb tedavisinden farklı şekilde etkilenmiştir. Sonuçlara göre, genler önce fenoksikarb duyarlılıklarına göre sınıflandırılmış, daha sonra kök hücrelerin morfolojik değerlendirmeleri ile bağlantılı olarak ekspresyon profillerine göre gruplandırılmıştır.

Anahtar sözcükler: *Bombyx mori*, ekdizon bağlantılı genler, orta bağırsak, kök hücre

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Introduction

In holometabolous insects, midgut stem cells perform different functions throughout the insect life. They proliferate and differentiate before each molt for maintaining the existing larval midgut epithelium. During larval-pupal metamorphosis, the pupal midgut is developed by stem cells suited to the feeding regime of adult insect (Baldwin et al., 1996; Tettamanti et al., 2007) and ecdysone is the main regulator of this process.

Ecdysone-related genes have functions during midgut remodeling in insects (Lee et al., 2002; Nishiura et al., 2005; Goncu et al., 2016). Previous studies have mostly focused on ecdysone-related gene activation involved in programmed cell death events (Goncu & Parlak, 2011; Franzetti et al., 2012), whereas our knowledge of the expression patterns of ecdysone-related genes in insect stem cells is insufficient. Through the few studies on this subject evidence has been obtained regarding the possible functions of ecdysone in the proliferation and differentiation processes of stem cells in different insect species. Inhibition of the ecdysone receptor (EcR) signal in the wing imaginal disc causes a decline in the progression of S phase and mitosis (Cranna & Quinn, 2009) and the ultraspiracle (USP) is involved in the regulation of cell cycle and differentiation in the developing eye imaginal disc of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) (Quinn et al., 2012). Parthasarathy & Palli, (2007) showed that the EcR-A/USP-B heterodimer regulates the development of the pupal/adult midgut in *Aedes aegypti* L., 1762 (Diptera: Culicidae). Lee et al. (2002) reported that mutations in broad impact cell death in the larval midgut but do not affect the development of adult midgut in *D. melanogaster*. Our previous study showed that EcR-A and USP2 mRNAs are produced in the midgut stem cells of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) during remodeling processes but EcR-B1, USP1 and E74B transcripts are detected during the initial phase of metamorphic events (Goncu et al., 2016).

The aim of this study was to investigate possible functions of certain genes in the formation of the pupal midgut. For this purpose, fenoxycarb administration was made to manipulate developmental events in the midgut via suppression of ecdysone release. Fenoxycarb mimics the juvenile hormone action in *B. mori* and delays or inhibits metamorphic events depending on the time of administration. Treatment with fenoxycarb on day 0 causes delay of ecdysone release but day 3 treatment inhibits it completely (Kamimura & Kiuchi, 1998). After the applications, changes in expression profiles of ecdysone-related genes, midgut morphology and proliferation patterns of stem cells were evaluated together, and information was obtained on the critical expression timing of the studied genes and their sensitivity to fenoxycarb.

Materials and Methods

Insect rearing and Fenoxycarb application

This study was conducted in the Ege University silkworm culture laboratory and insect physiology research laboratory during the years between 2014-2018. Hybrid races (Japanese × Chinese) of the silkworm, *B. mori* larvae were reared on fresh mulberry leaves at 25 ± 1ºC, 75-85% RH and 12:12 h L:D photoperiod. Insects were topically treated with 1 ng/10 µL of fenoxycarb dissolved in acetone immediately after the fourth larval ecdysis (day 0) and day 3 of the fifth instar. Throughout the article insects treated with fenoxycarb on day 0 and day 3 are named as group 1 and 2, respectively. Experiments were repeated every 12 h from day 7 larvae of the fifth instar to 24 h after pupation.

Stem cell isolation

Stem cells were isolated from the midgut according to Hakim et al. (2009). Ten insects were used for stem cell isolation every 12 h and midgut cells were classified according to their morphological features (Cermenati et al., 2007; Goncu et al., 2016). Stem cells were observed as clear and round shapes cells and counted by using a hemocytometer slide under an inverted microscope. They were counted in three

arbitrary fields each measuring 1 mm^2. Every calculation was repeated three times and high percentages (>92%) of stem cells were obtained by this method (Hakim et al., 2009). Due to the separation of the larval midgut from the basal lamina into the lumen during pharate pupal stage, the content of the midgut lumen including yellow bodies was discarded, and the remaining tissues were used as stem cell fractions.

Preparation of midgut sections for staining

Midguts were obtained every 12 h from day 7 of fifth instar larvae to the early pupal stage then, they were fixed in Bouin's solution for 5-6 h at 4ºC. Following the fixation, tissue pieces were dehydrated through a graded series of ethanol and processed for embedding in paraffin wax. For morphological evaluation, 5-µm-thick sections were stained with hematoxylin and eosin using the routine protocol. Sections were examined under a Leica DM3000 microscope and photographed with a digital camera.

Bromodeoxyuridine labeling

For in vivo labeling with (bromodeoxyuridine) BrdU, 10 µl of the labeling reagent per 1 g body weight was injected into the hemocoel of staged larvae/pupae. Midguts were dissected 4 h later and then fixed in 10% neutral buffered formalin for 8 h. Paraffin sections of midgut samples were prepared as described above for histological analysis. The sections were processed using In Situ Cell Proliferation Kit, Fluos (Roche, Penzberg, Germany) according to manufacturer’s instructions. The percentage of BrdU positive cells was assessed by Image J analysis (National Institutes of Health, Bethesda, MD, USA). Automated particle counting analyze was performed for all images and percentage of positive cells were calculated according to obtained results.

RNA isolation and cDNA synthesis

Stem cell fractions were pooled and collected in Tripure Isolation Reagent (Roche) for every 12 h. Samples were homogenized in Tripure reagent and total RNA was isolated according to the manufacturer's instructions. Total RNA concentration and purity were evaluated by using Nanodrop UV/VIS spectrophotometer. cDNA synthesis by reverse transcription was performed using 1 µg of RNA and High-Fidelity cDNA Synthesis Kit (Roche) in a 20 µl reaction volume.

Quantitative real-time reverse transcriptase-polymerase chain reaction

Relative expression of selected genes was detected by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using light cycler 480 real-time PCR detection systems (Roche) as described (Gibson et al., 1996). qRT-PCR was performed in a total reaction volume of 10 µl containing 2 µl of cDNA, 0.5 µl each of forward and reverse sequence-specific primers, 2.7 µl H_2O, 0.2 µl tProbe, and 5 µl enzyme. PCR conditions were 95ºC for 10 min followed by 45 cycles of 95ºC for 10 s, 60ºC for the 30 s, 72ºC for 1 s, 40ºC for 30 s. The primers used for PCR that were derived from the sequences of the *Bombyx* genes and references are listed in Table 1. Primer probe design was performed by using Clustal W and Oligo 7 software. The specificity of obtained primers and probes was controlled by using the blast program. *Bombyx mori* Actin 3 was used as an endogenous control. Mean and standard errors were obtained from the averages of three independent sample sets. For each gene, we tested to examine gene expression by using a One-way analysis of variance, SPSS statistics software (IBM, Armonk, NY, USA).

Table 1. Forward and reverse primers used in quantitative real-time reverse transcriptase-polymerase chain reaction

Gene no	Forward primer Reverse primer	Accession no	Upl prob no
Bombyx mori Actin 3	5′- GCTCCCTCGAGAAGTCTTACG-3′ 5′- CTGGGCAACGGAATCTTTC -3′	U49854	9
EcR A	5'-CATCCGGTCAACGGACAC-3' 5'- ACCGTAGCTGCCTGAGGATA-3'	D87118	141
EcR B1	5'- ACTTGGCAGTCGGATGAAG - 3' 5′- CGTCATCTCCGTGATCTGG -3′	L35266	153
USP1	5′- TCAAATAGGCAACAAACAGATAGCCGCTC-3′ 5′- CAGGAACTCCATAGACCG -3	U06073	150
USP2	5′- CAGTGTCACATGTAGAGTGCAAAGA -3′ 5′- CCACTTTCATAGAACAGTTCAGTTGC -3′	AB182582	FAM-GTTCAACGACCTTGTTGCTGACA GGTTC-Tamra- Taqman probe
E74A	5′- CCACTTTCATAGAACAGTTCAGTTGC -3′ 5′- CCACCTATCGAGATAAAGCAAGA -3′	Q1KLS0	141
E74B	5′- ACCCGAGTGACTACGTGAGG -3′ 5′- CGGAGTCTGTGCCTGAGTCT -3′	Q1KLR9	95
E75A	5′- TCGGTCGAGCTTGAGTGAG -3′ 5′- GATGAAGGTCGCTTGTCTCG -3′	AB024904	59
E75B	5′- GGACAGCTCTCAAAGACGTGA -3′ 5′- CGCACCATTCACTACACTCG -3′	AB024905	91
BRC-Z1	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- TACACGCGCTGGCAAATG -3′	AB166725	36
BRC-Z2	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- GTGTATATGTGCGTCATCAGGGA -3′	AB166726	99
BRC-Z4	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- TCTTGTGGTTGTTGAGCGAGTT -3′	AB166727	56
βFTZ-F1	5′- TTCCGCAAGTATCATCATTGAC -3′ 5′- CTTGTCGTGAGTTGGTGGTG -3′	10953	141
BHR3	5′- GGGATGCAAAGGATTCTTCA -3′ 5′-GCGAGGACACTGGTAGTTCAC -3′	AB024902	99

Results

Juvenile hormone analogue, fenoxycarb, delays or inhibits the formation of the pupal midgut epithelium

In the control group, the feeding activity lasted until the end of day 7 of the fifth instar. After the gut purge, larvae were actively spun cocoon on day 8 and 9 of the fifth instar, and these days were considered as the early prepupal stage. Larvae became pharate pupae on day 10 of the fifth instar in which the spinning activity was almost finished and larvae became quiescent. Therefore, this day was determined as the late prepupal stage and larval-pupal ecdysis occurred at the end of day 10. Fenoxycarb treatment in group 1 extended the feeding stage to the end of day 10. Since gut purge was retarded until day 10 of the fifth larval stage, days 11 and 12 were determined as the early pupal stage and day 13 as the pharate pupal stage. Larval-pupal ecdysis occurred at the end of day 13 in this group. Fenoxycarb treatment in group 2 produced dauer larvae with feeding activity until day 13 of the fifth larval stage. Gut purge, spinning activity, and larval-pupal ecdysis did not occur in this group of animals.

In the control group, healthy larval midgut epithelium consisted mainly of mature columnar epithelial cells and goblet cells (Figure 1a-b). After the cessation of feeding, several conical or spindle-shaped stem cells located in the basal region of epithelium enlarged and began to proliferate in the early prepupal stage (Figure 1c-d). Proliferating stem cells were observed under the larval epithelium on day 9 (Figure 1e-f). In the late prepupal stage (day 10), when the degenerated larval midgut detached from the basal membrane

stem cells formed a multilayer epithelial structure surrounding the lumen (Figure 1g-h). Stem cells started to differentiate into columnar epithelial cells with brush border membrane to constitute pupal midgut structure in the early pupal stage (Figure 1i-k).

In group 1, pupal midgut formation was temporarily inhibited until the end of feeding on day 11 of the fifth larval stage, and very few stem cells were detected in the basal region of the larval midgut epithelium (Figure 2a-h). Stem cells began to enlarge and proliferate as similar to the control group during the early pupal stage on days 11 and 12 (Figure 2i-l). The mature larval midgut cell layer was detached from the basal membrane at the end of day 13 (Figure 2m-n). The formation of the monolayer columnar epithelium of the pupal midgut continued in the early pupal stage (Figure 2o-q).

Larval midgut structure continued to exist in group 2 until the end of day 13 of the fifth instar. Healthy larval midgut epithelium was observed on days 7, 8 and 9 (Figure 3a-f). Stem cells beneath the larval midgut epithelium begun to enlarge and proliferate at the beginning of day 10 (Figure 3g-h). In addition to the reduction of the connection with the basal lamina in some regions, various morphological changes such as condensation in the cytoplasm and nucleus were indicative of degeneration in the mature larval midgut cells on day 11 (Figure 3i-j). Especially on day 12, degenerated old larval epithelial remnants and undigested mulberry leaves as feeding material were detected in the midgut lumen (Figure 3k-l). On day 13, newly formed larval epithelium from stem cells showed an abnormal multilayered pattern in some regions (Figure 3m-n). The reduced feeding activity observed in these insects was probably related to this abnormal midgut epithelium.

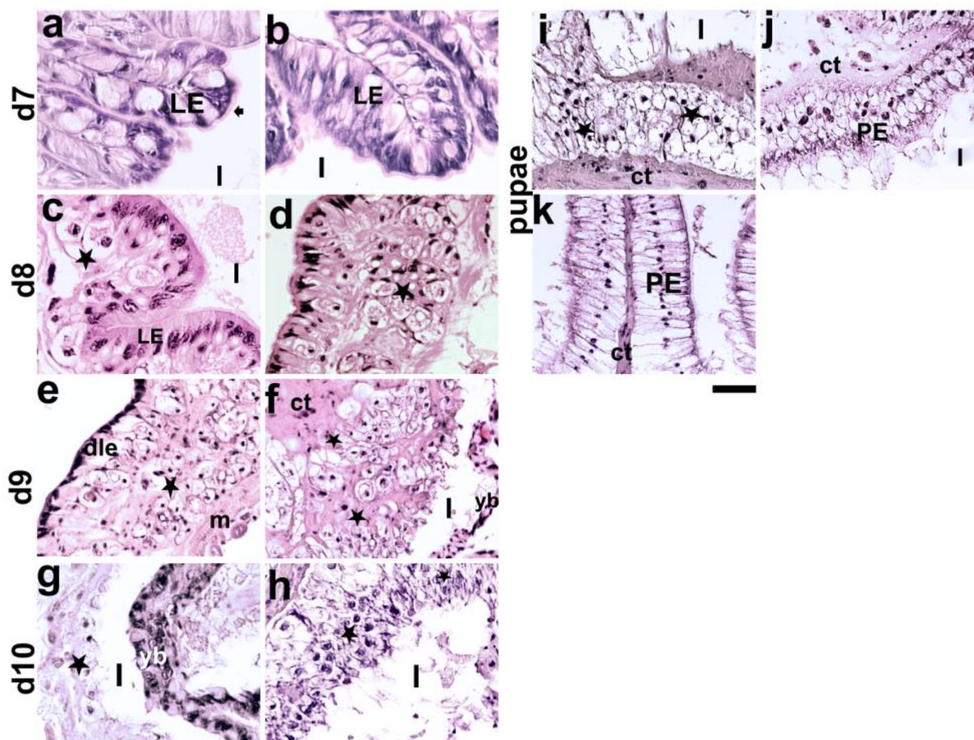

Figure 1. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Control group: a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) pupa, 0 h; j) pupa, 12 h; k) pupa, 24 h (scale bar 50 µm).
Black star, stem cell; ct, connective tissue; dle, degenerated larval epithelium; l, lumen; LE, larval midgut epithelium; m, muscle; PE, pupal epithelium; and yb, yellow body.

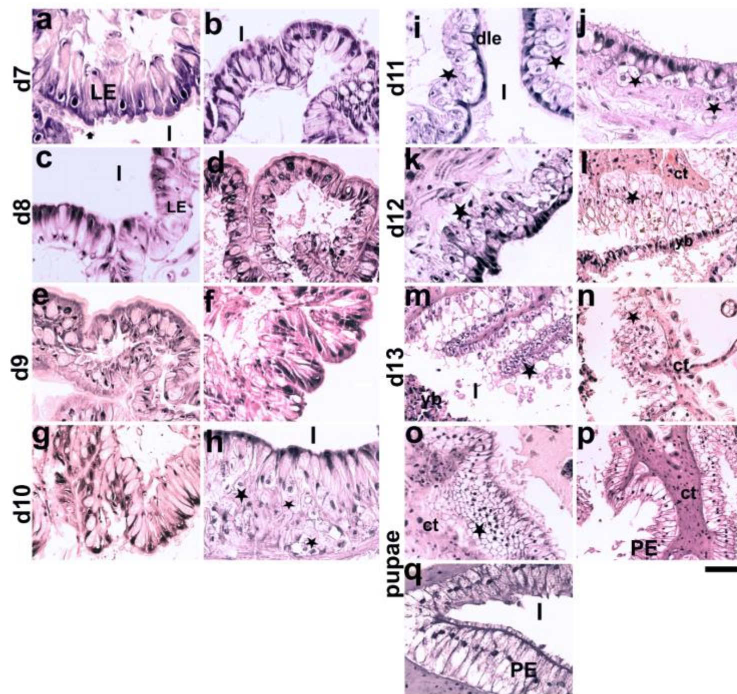

Figure 2. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Group 1 (day 0 fenoxycarb treated insects): a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) day 11; j) day 11, 12 h; k) day 12; l) day 12, 12 h; m) day 13; n) day 13, 12 h; o) pupa, 0 h; p) pupa, 12 h; q) pupa, 24 h (scale bar 50 µm).

Black star, stem cell; ct, connective tissue; dle, degenerated larval epithelium; l, lumen; LE, larval midgut epithelium; PE, pupal epithelium; and yb, yellow body.

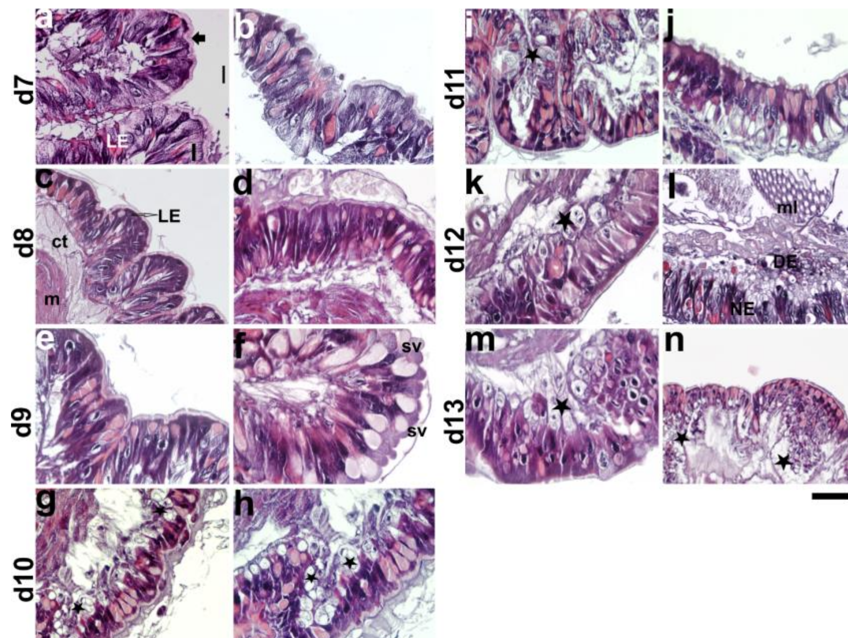

Figure 3. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Group 2 (day 3 fenoxycarb treated insects): a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) day 11; j) day 11, 12 h; k) day 12; l) day 12, 12 h; m) day 13; n) day 13, 12 h (scale bar = 50 µm).

Black star, stem cell; ct, connective tissue; DE, degenerated epithelium; l, lumen; LE, larval midgut epithelium; m, muscle; ml, mulberry leaf; NE, newly formed epithelium; sv, secretory vesicle.

Bromodeoxyuridine (BrdU) incorporation assay was used to evaluate the stem cell proliferation rate (Figure 4-6), and the percentage of positive cells was assessed by Image J analysis (Figure 7). In the control group, a few numbers of BrdU positive cells were detected in the basal layer of the larval epithelium on day 7 of the fifth instar (Figure 4a). The number of stem cells with a positive signal increased on day 10 (Figure 4b). Image J analysis of midgut sections from control group insects revealed that the percentage of maximum BrdU incorporated cells were 26 ± 2.9% just before pupal ecdysis (Figure 7). After pupation, the number of positive cells gradually decreased (Figure 4c, d). BrdU positive cells in the midguts of group 1 were determined as 4.7 ± 0.5% on day 7 (Figures 5a and 7) then decreased to very low levels until day 12 of the fifth instar (Figures 5b and 7). The significant increase in BrdU positive cells in group 1 was detected just before pupation (Figure 5c) and 20 ± 2.4% of the cells were BrdU positive (Figure 7). As detected in the control insects, the number of cells including BrdU gradually decreased after pupation (Figure 5d, e). In the midgut of group 2, the amount of BrdU positive cells did not exhibit significant changes during the experiment (Figure 6) and the maximum BrdU positive cells were determined to be 6 ± 1.0% on day 10 of the fifth instar (Figure 7).

Expression patterns of ecdysone receptors and ecdysone-related transcription factors in the midgut stem cells

Since the application of fenoxycarb caused prolongation of the feeding stage, x-axis diagrams were prepared separately for the control and treated insects. In stem cells isolated from the control group, moderate amounts of EcR A transcripts during feeding and in the early prepupal stage increased sharply on day 10 and then remained moderate levels up to 24 h after pupation (Figure 8a). However, EcR A expression was temporarily suppressed in group 1 until day 12 of the fifth instar. The highest EcR A mRNAs were detected in the first half of day 13 and followed by a gradual decrease up to 24 h after pupation (Figure 8a). EcR A expression in the stem cells of group 2 was completely inhibited until day 13 of the fifth instar (Figure 8a).

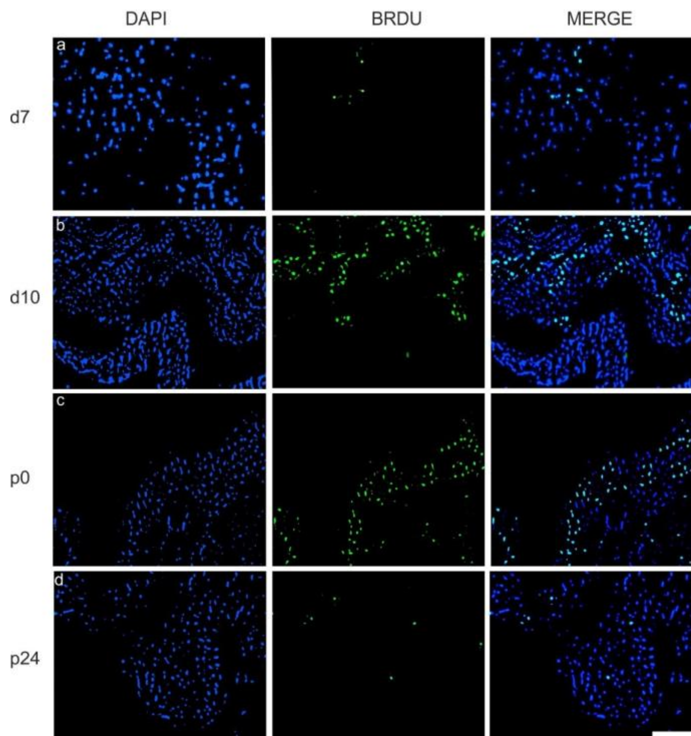

Figure 4. The proliferation rate of stem cells in the control group visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 10 (d10); c) at pupation (p0); d) pupa 24 h (p24) (scale bar 150 µm).

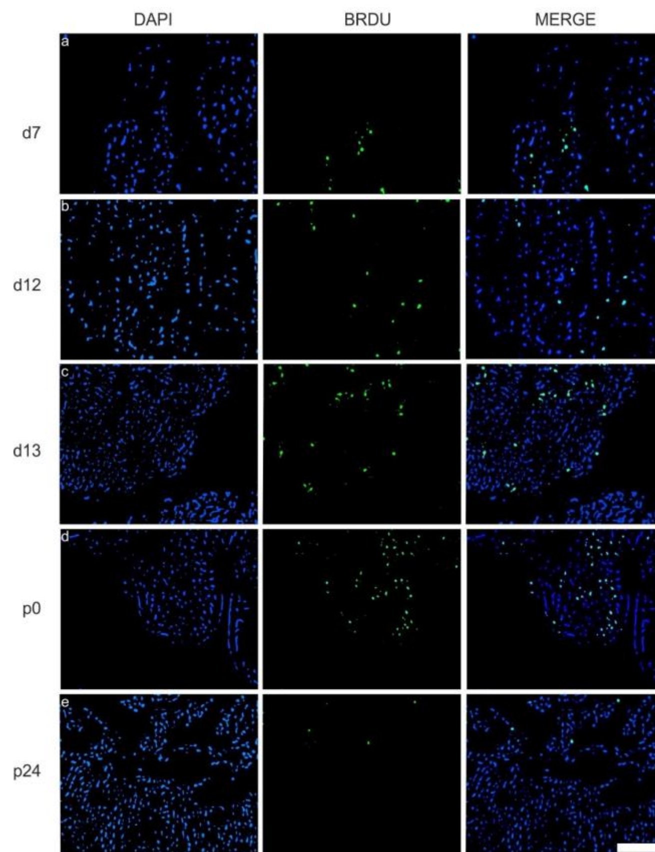

Figure 5. The proliferation rate of stem cells in the Group 1 visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 12 (d12); c) day 13 (d13); d) at pupation (p0); e) pupa 24 h (p24) (scale bar 150 µm).

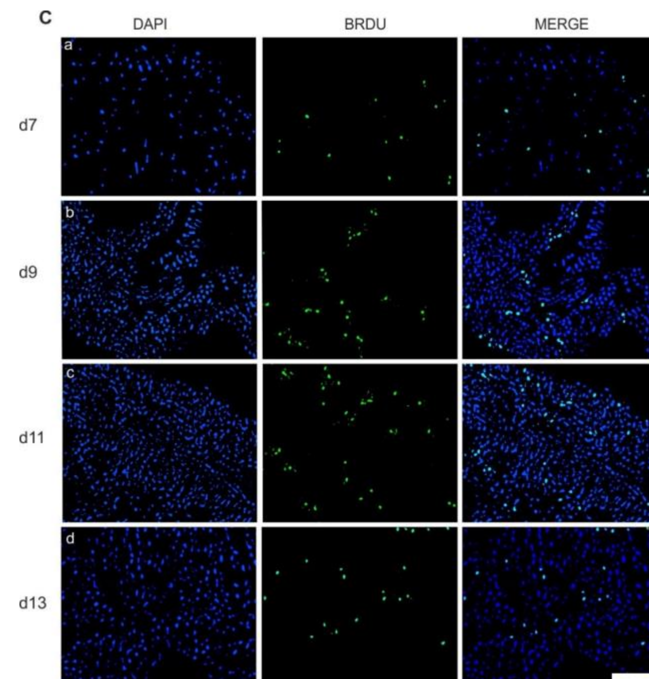

Figure 6. The proliferation rate of stem cells in the Group 2 visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 9 (d9); c) day 11 (d11); d) day 13 (d13) (scale bar 150 µm).

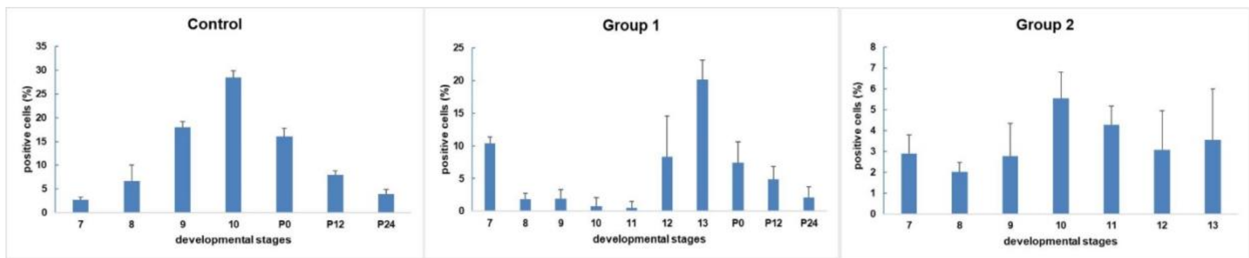

Figure 7. The percentage of BrdU positive nuclei determined using Image J software (http://imagej.nih.gov/ij) for Control, Group 1 (day 0 treated insects), and group 2 (day 3 treated insects) (mean ± SD for the three independent experiments are shown).

High amounts of EcR B1 transcripts were detected in the stem cells of the control insects on days 7 and 8, and the amount of transcripts decreased after day 9 (Figure 8b). EcR B1 transcripts in stem cells of group 1 were mostly at low levels, and a significantly higher level was detected only in the first half of day 12 (Figure 8b). In stem cells of group 2, EcR B1 mRNA levels were almost undetectable until day 11, and only a small increase occurred on this day (Figure 8b). The highest USP 1 mRNA levels in the stem cells of control group insects were detected on days 7 and 8 (Figure 8c). In stem cells of group 1, low USP1 transcripts began to increase from day 9 and peaked on day 11. Following the peak, mRNA levels decreased (Figure 8c). In the stem cells of group 2, USP1 mRNA levels were low until day 13 and then peaked sharply (Figure 8c). The USP 2 levels in stem cells of the control group were relatively high during feeding and early prepupal stage and peaked in the first half of day 10. Following this, the amount of transcripts decreased (Figure 8d). USP 2 transcripts in the stem cells of group 1 were at moderate levels until day 9 of the fifth instar and then peaked on this day. Following the peak, mRNA levels decreased to moderate levels again (Figure 8d). In the stem cells of group 2, USP2 expression was completely suppressed until the end of the day 13 of the fifth instar (Figure 8d).

Moderate E74A mRNA levels in the stem cells of the control group peaked just before pupation (Figure 8e). In stem cells of group 1, moderate levels of E74A decreased until day 10 and remained low until day 13 of the fifth instar. The most striking E74A expression were detected on day 13 just before pupation, followed by a gradual decrease in the early pupal period (Figure 8e). In the stem cells of group 2, relatively high E74A mRNA levels peaked on day 11 and maintained high levels until day 13 (Figure 8e). In the stem cells from the control group, relatively high E74B levels detected on day 8 of the fifth instar gradually increased after pupation and peaked 24 h after pupation (Figure 8f). In the stem cells of group 1, mostly suppressed E74B expression until day 11 of the fifth instar increased at the beginning of day 11 and moderate transcript levels were maintained until pupation. The highest E74B mRNA levels were detected at pupation, followed by a decrease during the early pupal period (Figure 8f). In stem cells of group 2, E74B transcripts were at almost undetectable levels until day 13 of the fifth instar (Figure 8f). In the stem cells of the control group, moderate E75A mRNA levels peaked in the second half of day 9. Although a small decrease occurred on day 10, relatively high transcript levels remained until 24 h after pupation (Figure 9a). In the stem cells of group 1, quite low E75A mRNA levels until day 13 peaked sharply on this day, and then decreasing transcripts were maintained at moderate levels throughout the early pupal stage (Figure 9a). In the stem cells of group 2, E75A levels were almost undetectable until day 13 when a small increase occurred (Figure 9a). E75B transcripts in the stem cells of the control group began to rise after cessation of feeding and the highest mRNA levels were detected in the second half of day 9 and then maintained at moderate levels till 24 h after pupation (Figure 9b). In the stem cells of group 1, low E75B mRNA levels began to increase and peaked on day 13 of the fifth instar. Relatively moderate transcript levels were maintained until 24 h after pupation (Figure 9b). Day 3 fenoxycarb treatment resulted in very low E75B mRNA levels until day 12; however, a small increase occurred in the second half of day 12 and day 13 of the fifth instar (Figure 9b).

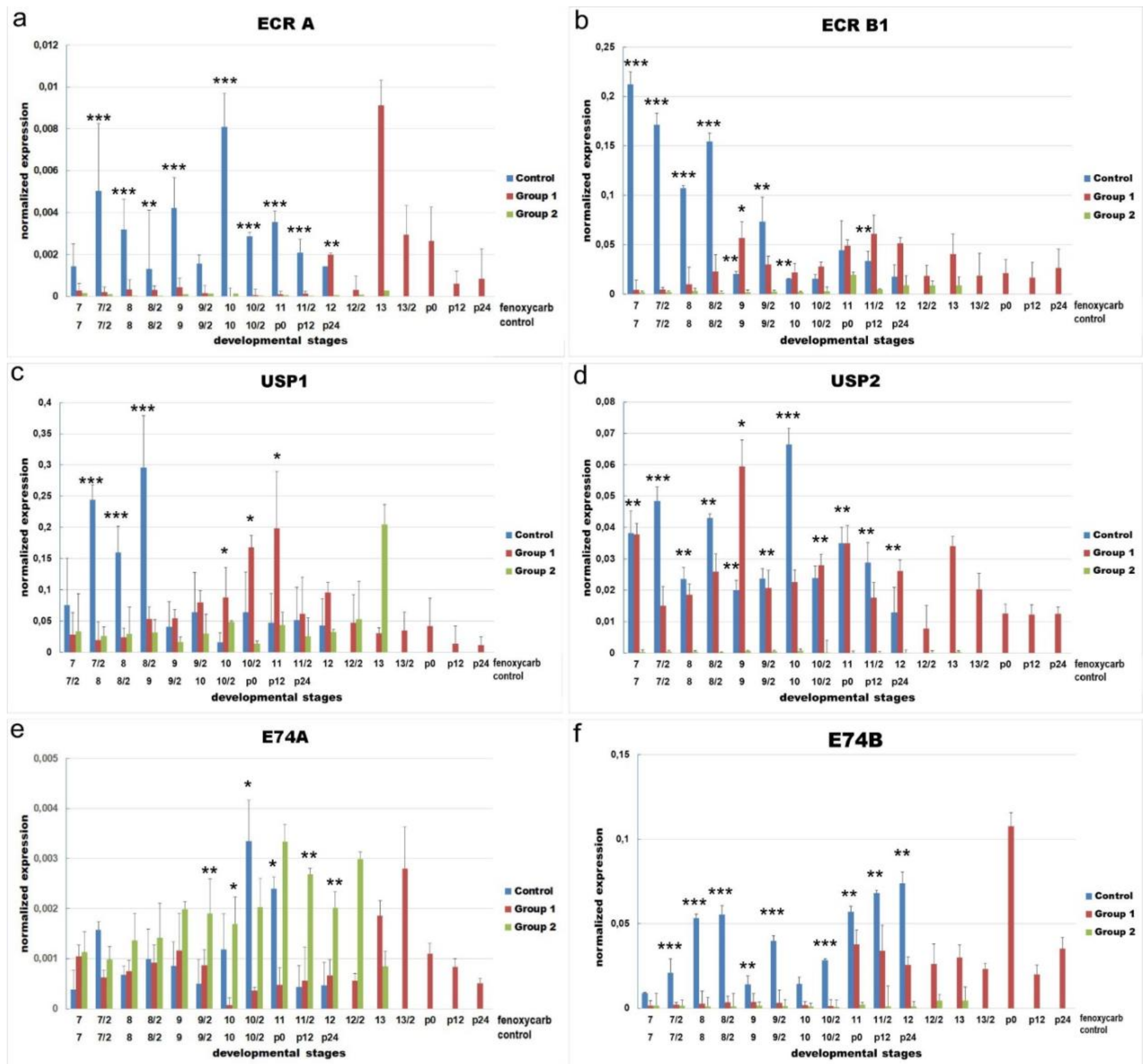

Figure 8. Expression profiles of ecdysone receptors and ecdysone-related transcription factors in the stem cell fractions of untreated and fenoxycarb treated insects (Group 1, day 0 treated insects, and group 2, day 3 treated insects): a) EcR A; b) EcR B1; c) USP1; d) USP2; e) E74A; f) E74B. Expression levels of the genes were normalized using *Bombyx mori* Actin 3 RNA as a housekeeping gene. Asterisks show significantly different expression ($P \leq 0.05$) by one-way ANOVA. Stars above bars into graphs indicate a significant difference between control and treatment groups ($P \leq 0.05$) by ANOVA. Significant differences between * control and day 0 treatment group; ** control and day 3 treatment group; *** control and all treatment groups.

In the stem cells of the control group, BRC Z1 levels showed a fluctuating expression pattern, and the highest level was detected just before pupation (Figure 9c). Relatively high BRC-Z1 levels were detected in the stem cells of group 1 until day 11. After a sudden drop that occurred in the second half of day 11, transcript levels rose sharply on day 13 and then dropped to quite low levels after pupation (Figure 9c). In the stem cells of group 2, the fluctuating expression pattern of BRC Z1 was detected until day 11 but its amount was considerably reduced on days 12 and 13 (Figure 9c).

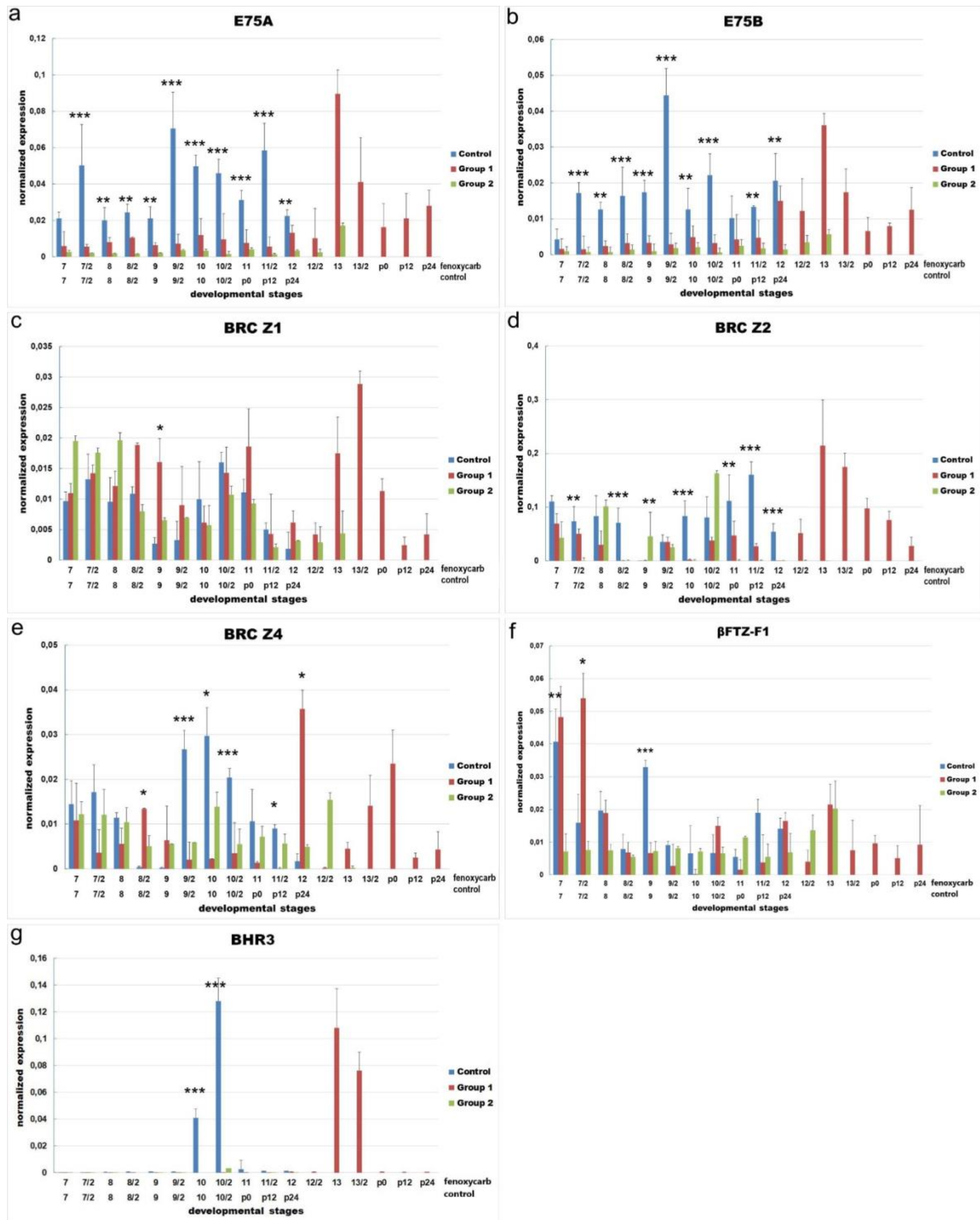

Figure 9. Expression profiles of ecdysone receptors and related transcription factors in the stem cell fractions of untreated and fenoxycarb treated insects (Group 1, day 0 treated insects, and group 2, day 3 treated insects): a) E75A; b) E75B; c) BR-C Z1; d) BR-C Z2; e) BR-C Z4; f) βFTZ-F1; g) BHR3. Expression levels of the genes were normalized using *Bombyx mori* Actin 3 RNA as a standard. Asterisks show significantly different expression ($P \leq 0.05$) by one-way ANOVA. Stars above bars into graphs indicate a significant difference between control and treatment groups ($P \leq 0.05$) by ANOVA. Significant differences between * control and day 0 treatment group; ** control and day 3 treatment group; *** control and all treatment groups.

In the stem cells of the control group, moderate BRC-Z2 mRNA levels decreased suddenly to an undetectable level on day 9. Following this decline, it started to rise again and peaked 12 h after pupation (Figure 9d). In stem cells of group 1, the fluctuating expression pattern of BRC-Z2 was detected until day 13. mRNA levels dropped to an undetectable level on some days. The highest transcript level was detected on day 13, followed by a gradual decline until 24 h after pupation (Figure 9d). In the stem cells of group 2, BRC Z2 expression was detected in only 5 of 13 developmental days, and the highest mRNA level was determined in the second half of day 10 (Figure 9d). In the stem cells of the control group, moderate BRC-Z4 levels on days 7 and 8 decreased to undetectable levels in the second half of day 8 and the first half of day 9. However, transcripts sharply rose in the second half of day 9 and peaked on day 10. Following the peak, its levels decreased gradually until 24 h after pupation (Figure 9e). In the stem cells of group 1, low levels of BRC- Z4 until day 12 peaked suddenly on this day but followed by a sharp decrease. mRNA levels started to rise again from day 13 and produced a second peak at pupation (Figure 9e). In the stem cells of group 2, a fluctuating expression pattern of BRC-Z4 was detected during the experiment (Figure 9e).

Two βFTZ-F1 peaks were detected in the stem cells of the control group on days 7 and 9, respectively. Transcript levels were low on other days during the experiment (Figure 9f). In the stem cells of group 1, the highest βFTZ-F1 levels were detected on day 7 of the fifth instar. After this day, low βFTZ-F1 mRNA levels were maintained during the experiment (Figure 9f). Low βFTZ-F1 levels in the stem cells of group 2 were maintained during the experiment and the highest level was determined on day 13 of the fifth instar (Figure 9f). In the untreated insects, BHR3 transcripts were only detected on day 10 of the fifth instar and peaked in the second half of the day (Figure 9g). A similar expression pattern was detected in the stem cells of group 1. BHR3 transcripts were detected only on day 13 (Figure 9g). Expression of BHR3 was completely suppressed in the stem cells of group 2 during the experiment (Figure 9g).

Discussion

The formation of the pupal midgut is strictly controlled by 20-hydroxyecdysone (20E), juvenile hormone (JH), paracrine factors (Smagghe et al., 2005) and various components from hemolymph (Hakim et al., 2007) and fat body (Smagghe et al., 2003; Hakim et al., 2010). The molecular mechanism of ecdysone during the formation of pupal midgut from stem cells is still unclear. Therefore, this study investigated the critical expression timing of ecdysone-related genes involved in the developmental processes of midgut stem cells by manipulating developmental events. Kamimura & Kiuchi (1998) reported that 1 ng of fenoxycarb administration on day 0 of the fifth instar temporarily suppressed 20E release from the prothoracic gland resulting in a prolonged feeding phase. However, the midgut purge and wandering behavior that emerged in group 1 at the beginning of day 11 were signs of ecdysone release (Sakurai et al., 1998). Due to the prolonged feeding stage, the development of pupal midgut in group 1 was delayed but stem cell proliferation and differentiation occurred almost in the same physiological timing as in the control when considered the spinning activity and gut purge. Based on the morphologic observations and BrdU assay results, proliferation of stem cells occurred in both the control group and group 1 during the late prepupal stage, and following a decrease in cell proliferation these cells began to differentiate into columnar pupal midgut epithelial cells during the early pupal stage. In contrast, fenoxycarb treatment on day 3 of the fifth instar completely inhibited ecdysone release (Kamimura & Kiuchi, 1998). Therefore, pupal midgut formation did not occur in group 2 but proliferated stem cells produced new mature larval epithelial cells.

The type of ecdysone receptors and the expression patterns of ecdysone-related genes are key elements in determining cell fate in insects (Talbot et al., 1993). Our previous study showed the co-expression patterns of EcR A and USP-2 in the midgut stem cells and their high mRNA levels especially during the late prepupal stage (Goncu et al., 2016). The delayed ecdysone secretion due to fenoxycarb treatment on day 0 completely suppressed the first increase of the EcR A; however, achieving critical hemolymph ecdysone levels allowed the EcR A expression to be similar to that of the control group.

Parthasarathy & Palli (2007) reported that EcR A and USP B regulate the differentiation of imaginal diploid cells in *Aedes aegypti* midgut. In *Manduca sexta* L., 1763 (Lepidoptera: Sphingidae) epidermis, EcR A and EcR B1 have been found during the cell divisions but only EcR A isoform has been detected during wing cuticle deposition in wing cells (Jindra et al., 1996). The similarity in the EcR A/USP2 expression patterns in the stem cells of group 1 with the control group and their complete inhibition in group 2 supported the involvement of this heterodimer in the late metamorphic process of stem cells.

Similar expression profiles of EcR B1 and USP 1 showed their involvement as a heterodimeric receptor complex in stem cells. According to the expression timing in the control group and group 1, EcR B1 and USP1 may be responsible for cellular processes such as the reprogramming of stem cells for the formation of the pupal midgut epithelium that occurs just before the initiation of metamorphic events in stem cells. The inhibition of EcR B1 and USP1 in stem cells of group 2 also supports this hypothesis. Riddiford et al. (2000) proposed that the loss of EcR B1 in the general epidermis of *M. sexta* might be critical to switch from the predifferentiation to the differentiation phase. When we evaluate our results and these previous reports, the marked decrease in EcR B1 expression and increase in EcR A expression in the control group and group 1 in the late prepupal stage may be a signal for the onset of differentiation events in midgut stem cells.

Stilwell et al. (2003) found that MsE74A mRNA expression occurs when ecdysteroid titers decrease. In this study, the presence of E74A in the long-term feeding phase of both treated groups both supported this previous report and pointed to a possible relationship between JH and transcriptional regulation of E74A. However, Fletcher et al. (1995) reported that mutated E74A and E74B cause death during the prepupal stage of *D. melanogaster,* and this information indicated their critical role in larval-pupal metamorphosis. In the stem cells of group 1, complete inhibition of E74A just before the termination of feeding suggested that its downregulation might important for the onset of metamorphic events. Maintaining the high expression rate of E74A in stem cells of group 2 supported this possibility. A previous study reported that E74A is produced in most larval and imaginal tissues of *Drosophila* (Boyd et al., 1991) but it can also bind late puffs and activate late genes. Ali et al. (2013) demonstrated that the highest E74A expression in the wing disc of *B. mori* is detected during the late prepupal stage and regulates transcription of late genes like the cuticular protein gene. Therefore, when expression profiles of E74A in stem cells of the control and group 1 insect are considered, E74A may mediate ecdysone for the activation of late genes allowing the stem cell differentiation. In contrast to E74A, almost complete inhibition of E74B expression in the extended feeding periods of treated insects indicated that the expression of this isoform is inhibited by juvenile hormone. Similarly, Stilwell et al. (2003) suggested that MsE74B expression occurs in direct response to ecdysone in the absence of JH in the epidermis of *M. sexta* associating with pupal commitment. In addition to this, its critical expression timing in the control group and group 1 after cessation of feeding and its complete inhibition in group 2 suggested its pivotal role in the preparation of stem cells for remodeling events.

Previous studies have suggested that the E75A gene can be induced directly or indirectly by the juvenile hormone in different insect tissues such as mature midgut cell (Goncu et al., 2016), *Drosophila* S2 cells (Dubrovsky et al., 2004), *Manduca* CH1 cultured cells (Dubrovskaya et al., 2004), but in this study, fenoxycarb treatment inhibited both E75A and E75B expression in the midgut stem cells during prolonged feeding phase of both treated groups. Similar to our results, different publications reported that E75A expression levels decreased in the midgut of *Tribolium castaneum*, Herbst, 1797 (Coleoptera: Tenebrionidae) after methoprene treatment, (Parthasarathy & Palli, 2008), and JH did not increase E75A levels in cultured *M. sexta* wing discs (Keshan et al., 2006). These results suggest that the interaction between JH and E75A is cell-specific. The upregulation of isoforms in control and group 1 insect stem cells during the formation of pupal midgut and their inhibition in stem cells of group 2 strongly suggested the role of E75 isoforms in this process.

Early BR-C gene is known to have an important role in *D. melanogaster* during metamorphosis because a mutation in this gene prevents larva-pupal transformation (Restifo & White, 1992; Fletcher & Thummel, 1995; Kiss et. al., 1998). The presence of all BRC transcripts during the extended feeding period of treated insects showed that JH did not inhibit BR-C gene expression. Similarly, it has been reported that JH does not inhibit the expression of BRC transcripts in *M. sexta* wing discs, moreover, metamorphic competence and production of BRC transcripts occur in the late larval stage when JH is still present in hemolymph (Zhou et al., 1998). Zhou & Riddiford (2002) reported that BR-C Z1 inhibits the activation of pupal cuticle genes in *Drosophila*. In this study, the presence of BR-C mRNAs in midgut stem cells of all groups during the feeding phase suggests that it may have a role in the inhibition of pupa-specific genes. Thus, downregulation of their expression in the middle prepupal stage may be a signal for the activation of genes responsible for pupal midgut formation. However, previous reports along with upregulation in BRC mRNA levels occurring in the stem cells of the control and group 1 and the absence of similar increases in group 2 indicate that they may have a role in the formation of the pupal midgut. BRC isoforms have been reported to be involved in the growth of wing buds by increasing cell division in *Blatta germanica* L., 1767 (Blattodea: Ectobiidae) (Huang et al., 2013) and also imaginal disc differentiation and histoblast proliferation in *Drosophila* (Zhou & Riddiford, 2002).

A previous study on BFTZ-F1 have suggested its role as a cellular competence factor associated with both 20E and JH in many tissues (Bernardo & Dubrovsky, 2012). Bernardo & Dubrovsky (2012) reported that the FTZ-F1 nuclear receptor is involved in JH signaling via JH receptor candidates methoprene-tolerant and germ cell-expressed. In this study, fenoxycarb treatment did not inhibit FTZ-F1 expression, suggesting that βFTZ-F1 is likely associated with JH signaling in stem cells. Our previous study demonstrated that BHR3 is a late prepupal stage-specific gene in the midgut stem cells of *B. mori* (Goncu et al., 2016). Timing of BHR3 expression in the stem cells of control and group 1 indicated that hormonal conditions suitable for BHR3 expression are provided only during the late prepupal stage under high 20E levels (Kamimura & Kiuchi, 1998; Sakurai et al., 1998). The absence of BHR3 transcripts in the stem cells of group 2 also supported this finding. The critical expression timing of BHR3 and its complete inhibition in stem cells of group 2 strongly indicated its possible role (s) for midgut stem cell differentiation. Siaussat et al. (2005) reported that PHR3 and PIE75 are detected as components of a 20E-induced genetic cascade associated with proliferative arrest and chitin precursor synthesis of IAL-PID2 cells.

In conclusion, we classified the genes firstly according to their susceptibility to fenoxycarb and secondly to their specific expression timings at each developmental stage (Figure 5a, b). The obvious inhibitor effect of fenoxycarb was detected in the expression of EcR A, EcR B1, USP 2, E74B, E75A, E75B and BHR3. Expression of USP1, BRC Z2 and BRC Z4 was not completely inhibited but their mRNA levels are reduced after fenoxycarb treatment. BRC Z1 and βFTZ F1 expression were almost not affected by fenoxycarb treatment and E74A was the only gene whose expression was induced by fenoxycarb (Figure 10a).

The expression of E74A and BRC isoforms in the feeding stage of all groups indicated their roles in the maintaining of larval midgut structure. The high expression status of the EcR B1, USP1, and E75 isoforms during the early prepupal stage suggest that these genes could be important for the involvement of stem cells in metamorphic changes and readiness for proliferation. Expression of EcR A, USP2, BRC Z1 and BHR3 may be important during the late prepupal stage when stem cells highly proliferate and prepare for differentiation. The presence of abundant E74B and BR-C Z2 mRNAs during the early pupal stage revealed their possible role in the pupal midgut. When considering all groups, the results obtained were not sufficient to make any inferences regarding the possible function of BFTZ-F1 in midgut stem cells (Figure 10b).

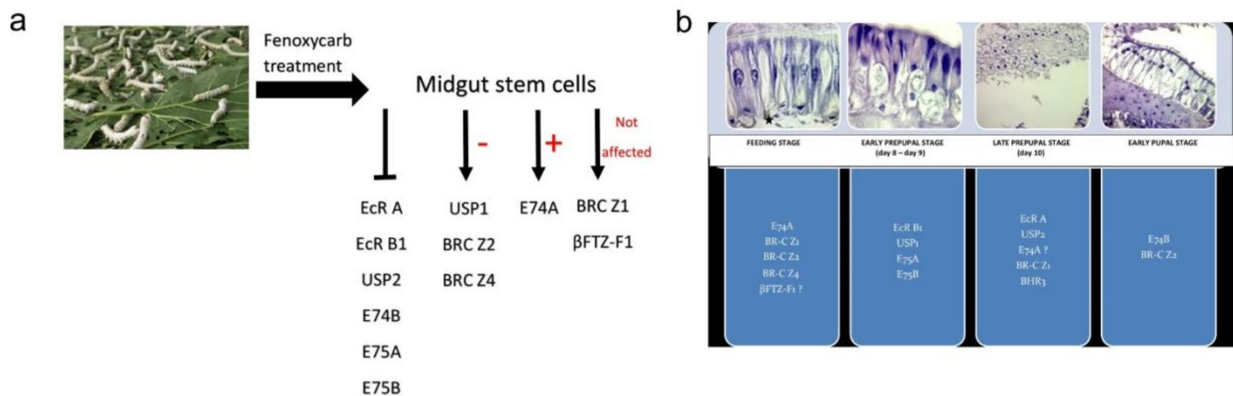

Figure 10. a) Comparison of expression profiles of ecdysone-related genes in the midgut stem cells of untreated and fenoxycarb treated insects showed their sensitivity of fenoxycarb treatment**.** Ecdysone receptors, USP2, E74B, and E75 isoforms were inhibited; however, USP1, BRC Z2 and Z4 were reduced by fenoxycarb. E74A was the only induced gene after fenoxycarb treatment. BRC Z1 and βFTZ-F1 genes seemed non-responsive to the fenoxycarb treatment. b) We grouped ecdysone-related genes in connection with their timing of expression and morphological evaluations of stem cells**.** More evidence is needed for genes with question marks.

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