

**Original article (Orijinal araştırma)**

**Effects of pyriproxyfen and *Bacillus thuringiensis* Berliner, 1915 on enzymatic antioxidant defense system and hemocytes of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)<sup>1</sup>**

Pyriproxyfen ve *Bacillus thuringiensis*'in *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)'nın enzimatik antioksidan savunma sistemi ve hemosit sayılarına etkileri

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**Abstract**

With the increasing uses of biological control methods, knowing the physiological and immunological effects of these insecticides on insects is essential for them to be used safely in agricultural areas. The aim of the study is to determine the effects of pyriproxyfen and *Bacillus thuringiensis* subsp. *kurstaki* individually and as a mixture on malondialdehyde levels (MDA), glutathione-s-transferase, acetylcholinesterase, cytochrome P450 enzyme activities in hemolymph, midgut, and fat body and total (THC) and differential hemocyte counts (DHC) of fifth instar larvae of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) 24, 48 and 72 h after exposure under laboratory conditions (30 ± 1°C, 65 ± 5% RH). The study was conducted in the Animal Physiology Research Laboratory of the Department of Biology, Faculty of Science and Letter, University of Çukurova between 2016-2018. Effects of these insecticides on antioxidant, detoxification enzyme activities and MDA levels were changed depends on exposure time and the differences of tissues. THC decreased after 24 h, whereas it had increased after 48 and 72 h. DHC induced depends on exposure time and applied insecticide. This study revealed that pyriproxyfen and *B. thuringiensis* applications caused biochemical, physiological reactions and effected the immune defense system of larvae by the alterations in hemocyte counts.

**Keywords:** Antioxidant defense, detoxification, *Galleria mellonella*, immune defense, pyriproxyfen

**Öz**

Biyolojik mücadele yöntemlerinin artan kullanımları nedeniyle, bu insektisitlerin böcekler üzerindeki fizyolojik ve immünolojik etkilerinin bilinmesi, tarımsal alanlarda güvenle kullanılabilmesi için büyük önem taşımaktadır. Çalışmanın amacı, pyriproxyfen ve *Bacillus thuringiensis* subsp. *kurstaki* nin tek başına ve karışım halinde 24,48 ve 72 saatlik etkileri sonucunda, *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)'nın 5. dönem larvalarının hemolenf, orta barsak ve yağ dokusunda malondialdehid (MDA) miktarı, glutatyon-s-transferaz, asetilkolinesteraz, sitokrom P450 aktiviteleri ile total ve diferansiyel hemosit sayıları üzerine etkilerini laboratuvar koşulları altında (30 ± 1°C, %65 ± 5 RH) belirlemektir. Çalışma, Çukurova Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü Hayvan Fizyolojisi araştırma laboratuvarında 2016-2018 yılları arasında gerçekleştirilmiştir. Pyriproxyfen ve *B. thuringiensis*'in etkisinde, larvaların hemolenf, orta barsak ve yağ dokusunda antioksidan ve detoksifikasyon enzim aktiviteleri ile MDA seviyesinde uygulama süresine ve doku farklılıklarına bağlı olarak değişimler belirlenmiştir. Total hemosit sayısında uygulamadan 24 saat sonra azalma, 48 ve 72 saat sonra ise artış meydana gelmiştir. Diferansiyel hemosit sayısı üzerine etkilerinde ise, pyriproxyfen ve *B. thuringiensis* uygulamasına ve uygulama zamanına bağlı olarak değişiklikler meydana geldiği belirlenmiştir. Bu çalışma ile pyriproxyfen ve *B. thuringiensis* uygulamalarının biyokimyasal ve fizyolojik reaksiyonlara neden olduğu ve hemosit sayılarında değişikliğe yol açarak bağışıklık savunma sistemini etkilediği ortaya konmuştur.

**Anahtar sözcükler:** Antioksidan savunma, detoksifikasyon, *Galleria mellonella*, immun savunma, pyriproxyfen

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## Introduction

*Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) larvae, which adversely affects beehives, are widely used as model organisms for studies in insect physiology and immunology, due to the similarities with the mammalian organisms in the immune response and gastrointestinal tracts. Moreover, *G. mellonella* larvae, in contrast to many other alternative models, can be reared easily in a wide range of temperature and in a short time. Due to the adverse effects of chemical insecticides used in management with *G. mellonella* on human health, problems such as leaving residues in honey and products grown in the surrounding lands, beekeepers are adopting integrated management. A key purpose of integrated management is to use methods that are not harmful to the environment and have minimal non-target effects. In particular, insecticides can be used in combinations due to the restriction of the use of new products in order to prevent the development of resistance of pests against some insecticides. Insecticides with different effect mechanisms used in the mixture increase the effect of each other and consequently this leads to a decrease in cost (Martinez et al., 2004; Attique et al., 2006). The use of combinations of these insecticides at low doses provides different ecological, biological and economic benefits.

In addition to the use of plant-based insecticides, hormones and analogs, predators and parasitic insects, entomopathogens and some microorganisms are also used in pest management. The most important of these methods of pest management is the use of insect growth regulators and microbial insecticides. Insect growth regulators are insecticides that mimic hormones in insects. Pyriproxyfen, belonging to the group of juvenile hormone analogs that is excreted by the corpus allatum in insects, is an organic and heterocyclic compound. Zhao et al. (2020) demonstrated that growth and development of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) were significantly affected by pyriproxyfen and the fat body tissues were damaged after treatment. The detoxification enzyme activities were also increased by pyriproxyfen and affect the immune signaling pathway. *Bacillus thuringiensis* Berliner, 1915, it is a gram-positive entomopathogenic bacteria that has been used successfully against agricultural pests. It is known that in some cases, the exposure to insecticides results in the increased production of reactive oxygen species and cause oxidative stress in insects (Livingstone, 2001). To understand the detoxification metabolism of organisms against insecticides, some biomarkers are usually used to evaluate the physiological effects of environmental stressors (Boily et al., 2013; Carvalho et al., 2013; Badawy et al., 2015). Lipid peroxidation can be determined by measuring malondialdehyde (MDA) concentration which has been used as a biomarker for oxidative stress in living organisms (Meng et al., 2009). Glutathione-S-transferase (GST), one of the potential biomarkers, is also produced to assess the environmental impact of insecticides. Acetylcholinesterase (AChE) is an important enzyme in the nervous system that is inhibited by many pesticides. The Cytochrome P450 (Cyt P450) superfamily consists of important monooxygenase enzymes that are among the most useful biomarkers of exposure due to its role in biotransformation of xenobiotic (Goksoyr & Farlin, 1992).

The hematological studies are important in the field of insect physiology due to certain vital activities which are performed by hemocytes. Coagulation, phagocytosis, encapsulation, detoxification, storage and distribution of nutritive materials are the primary functions of hemocytes. There is an inherent variability of hemocytes within a species depending on the developmental and physiological stages (Sanjayan et al., 1996; Beetz et al., 2008; Abd el-Aziz & Awad, 2010). Although many studies conducted on the effects of pyriproxyfen and *B. thuringiensis* in insects, there is little information concerning the mixture effects of these insecticides. Consequently, the aim of this study was to evaluate whether sublethal concentrations of pyriproxyfen and *B. thuringiensis* alter cellular and biochemical parameters in insects. To test this hypothesis, detoxification enzymes (AChE, GST and Cyt P450), the amount of the lipid peroxidation products in different tissues of *G. mellonella* and hemocyte counts (total and differential; THC and DHC) in hemolymph were determined.

## Materials and Methods

### Experimental designs

The larvae used in the experiment were obtained from *G. mellonella* stock cultures reared in the Animal Physiology Research Laboratory of Çukurova University, Faculty of Science and Letters. *G. mellonella* larvae were reared at  $30 \pm 1^\circ\text{C}$ ,  $65 \pm 5\%$  RH on a diet composed of bran, honey, glycerol, honeycomb and distilled water (Bronksill, 1961). The study was conducted in the Animal Physiology Research Laboratory of the Department of Biology between 2016-2018. Before bran was added to the food, it was sterilized at  $150^\circ\text{C}$  to prevent contamination. Juvenile hormone analog (10% EC), Pyriproxyfen (Admiral; Sumitomo Chemical, Kwinana, Australia) and larvicide based on *Bacillus thuringiensis* subsp. *kurstaki* (Delfin WG, consisting of 32,000 IU mg<sup>-1</sup> spores; Agrikem, Antalya, Turkey consisting of 32,000 IU mg<sup>-1</sup> spores) were used for determining the activity of antioxidant enzymes and hemocyte counts. LD<sub>50</sub> concentrations of *B. thuringiensis* and pyriproxyfen were defined in our previous study (Tunçsoy Sezer & Ozalp, 2016). Fifth instar larvae were reared on honeycomb with LD<sub>50</sub> concentrations of *B. thuringiensis* and pyriproxyfen (*B. thuringiensis* LD<sub>50</sub> 359 µg mL<sup>-1</sup> and pyriproxyfen LD<sub>50</sub> 2.39 µg mL<sup>-1</sup>) individually and a 1:1 mixture at these concentrations. For enzymatic analysis, hemolymph, midgut, and fat body tissues were dissected after 24, 48 and 72 h from larvae into a chilled eppendorf tubes with cold homogenization buffer (20 mM Tris buffer, pH 7.6). A few crystals of phenylthiourea were added to each sample to prevent melanization (Li et al., 2012). Resulting homogenates were centrifuged at 500 g for 15 min (4°C) and supernatants recentrifuged at 12,000 g for 45 min (4°C). Fat body tissues were homogenized at 50 W, 40-50 s. with ultrasonic homogenizer (Bandelin Sonoplus HD 2070, Berlin, Germany). Experiments were run in triplicate being 20 fifth instar larvae in each replicate. The samples for biochemical assays were frozen at  $-80^\circ\text{C}$  until use.

### Enzyme activities

The TBA assay was used to assess the MDA concentration and the absorbance of the chromophore was measured at 535 nm. The MDA concentration is presented as nmoles of MDA produced per mg protein using a molar extinction coefficient of  $1.56 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (Dubovskiy et al., 2008). For AChE activity, the midgut and fat body tissues were homogenized on ice in five volumes of a Tris-HCl buffer (100 mM, pH 8.0) containing 10% Triton and centrifuged at 12,000 g for 30 min (4°C). This calorimetric method is based on the coupled enzyme reaction of acetylthiocholine as the specific substrate for AChE and 5,5' dithiobis-2-nitrobenzoate as an indicator for the enzyme reaction at 450 nm (Ellman et al. 1961). GST activity was measured spectrophotometrically using 1-chloro-2,4 dinitrobenzene and reduced glutathione as co-substrate, according to the method of Habig et al. (1974) by using an extinction coefficient of  $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ . For the determination of Cyt P450 enzyme activity, p-nitroanisole was used as the substrate (Rose et al., 1995). The tests were conducted with three replicates. Protein amount was measured according to the Bradford (1976) method using bovine serum albumin as a substrate.

### Total and differential hemocyte counts

For determining the THC and DHC, hemolymph was obtained by piercing the cuticle for blood smear slide preparation. Air dried smear was then fixed in neat alcohol for 10 min, stained with Giemsa stain and mounted in Entellan (Sigma Aldrich, Darmstadt, Germany). Phase contrast microscope (Nikon Eclipse E200; Tokyo, Japan) was used to determine the hemocyte types and morphology (Sezer & Ozalp, 2015). Four µL of hemolymph was obtained from larvae by using microcapillary tube (Sigma Aldrich, Darmstadt, Germany) for total hemocyte count. Hemolymph was then transferred to eppendorf tubes that contained 36 µL of anticoagulant buffer (9.8 mM NaOH, 186 mM NaCl, 17 mM Na<sub>2</sub>EDTA, and 41 mM citric acid, pH 4.5). The number of circulating hemocytes per mm<sup>3</sup> was calculated using the formula of Jones (1962).

### Data analysis

The statistical analyses of the data were performed at  $P < 0.05$  by a series of analysis of variance and Student-Newman Keul's test using SPSS 21.00.

## Results

MDA level in hemolymph had significantly decreased 24 and 48 h after application of all treatments, however it increased after 72 h when the larvae exposed to pyriproxyfen, *B. thuringiensis* and mixed relative to the control (Figure 1a). In midgut, MDA level had significantly increased after 24 h in all treatments and 72 h after exposure to *B. thuringiensis* and mixed treatments relative to the control (Figure 1b). MDA level in fat body had significantly increased after 24 h in all treatments, nevertheless it significantly decreased after 48 and 72 h relative to the control (Figure 1c).

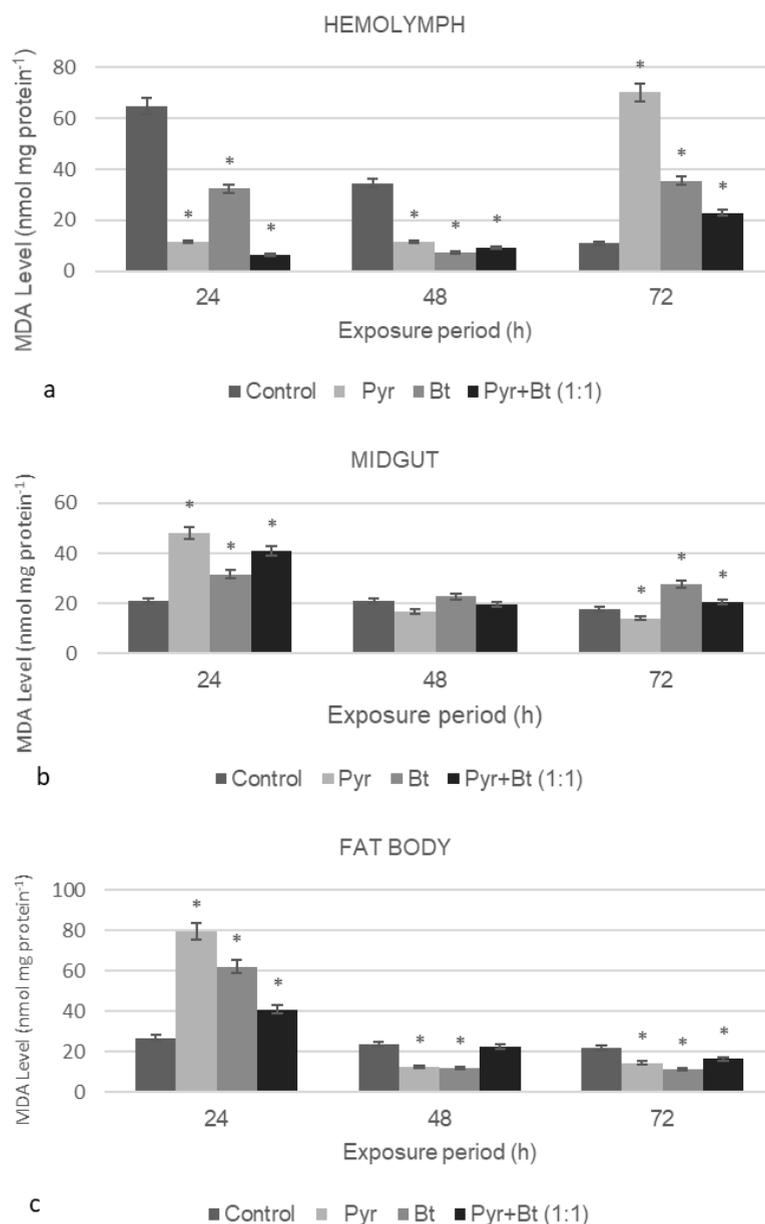


Figure 1. MDA levels of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) MDA level in hemolymph of *Galleria mellonella*, b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

AChE activity in hemolymph had significantly increased after 24 and 72 h after exposure to pyriproxyfen, however at the same time point it had significantly decreased in *B. thuringiensis* treatment relative to the control. In the mixed treatment, the enzyme activity had significantly decreased after 24 h, nevertheless it had increased after 72 h relative to the control (Figure 2a). AChE activity in midgut had significantly 24 h after exposure to pyriproxyfen, however it had decreased after 48 h relative to the control. In *B. thuringiensis* treatments, AChE activity had significantly increased after 48 and 72 h relative to the control (Figure 2b). In fat body, AChE activity had significantly increased 24 h after exposure to pyriproxyfen, however it decreased after 48 h relative to the control (Figure 2c).

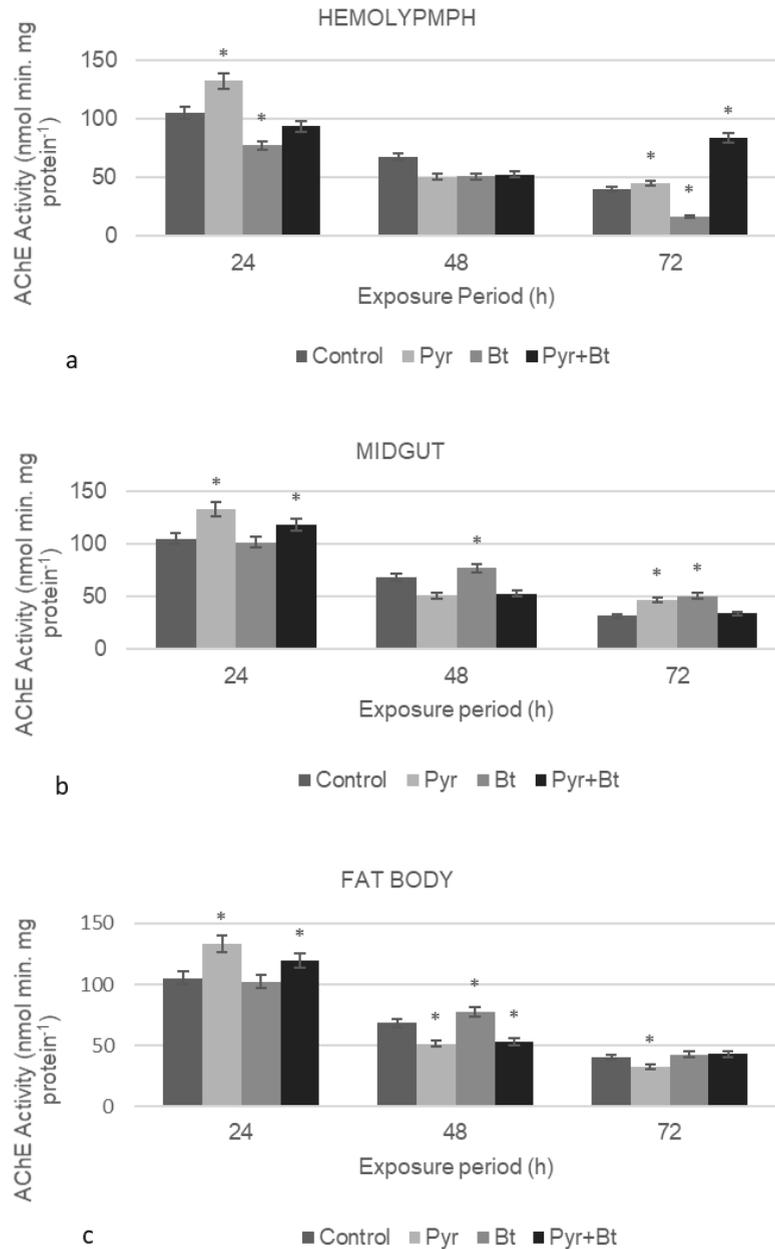


Figure 2. AChE activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) AChE activity in hemolymph of *Galleria mellonella*, b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

GST activity in hemolymph and midgut had significantly decreased 24 h after application of all treatments. In fat body, GST activity had increased after 24 h in *B. thuringiensis* treatment, however it had decreased in mixed treatment relative to the control. Forty-eight h after exposure to pyriproxyfen and mixture the GST activity had significantly decreased in hemolymph, nevertheless it had increased in *B. thuringiensis* group relative to the control. In midgut, GST had significantly increased in all treatments. After 72 h, the enzyme activity had also significantly increased after exposure to pyriproxyfen and *B. thuringiensis*, however it had decreased in mixed treatment relative to the control. GST activity in fat body had significantly decreased after 72 h after application of all treatments (Figure 3).

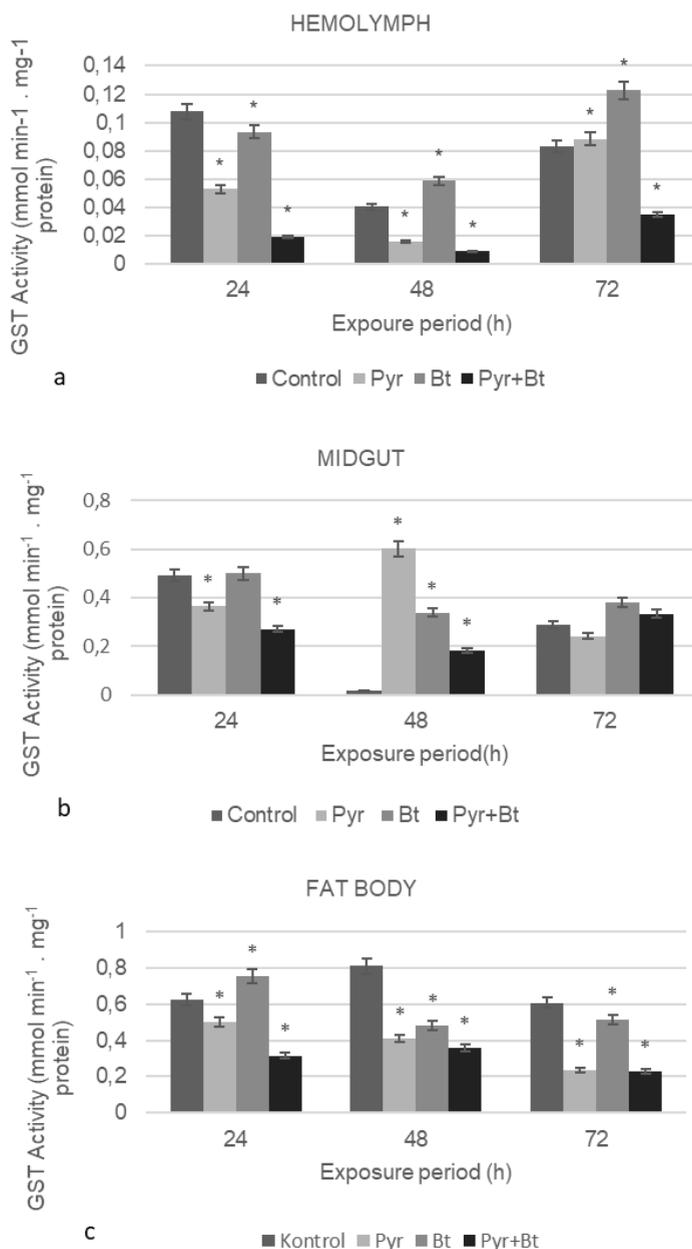


Figure 3. GST activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) GST activity in hemolymph of *Galleria mellonella*, b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

The Cyt P450 activity in hemolymph had significantly decreased after 24, 48 and 72 h in all treatments, except pyriproxyfen. After 72, Cyt P450 activity in midgut had significantly increased after exposure to *B. thuringiensis* relative to the control (Figure 4).

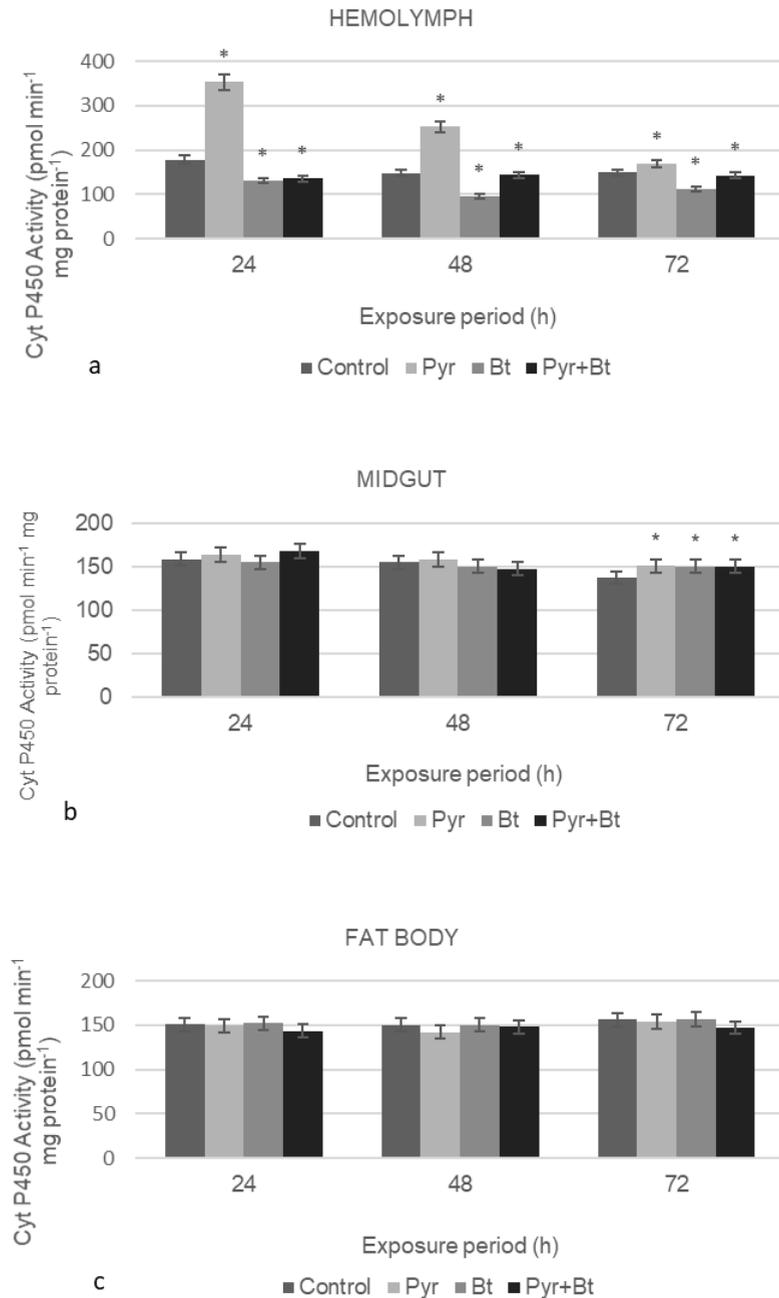


Figure 4. Cyt P450 activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) Cyt P450 activity in hemolymph of *Galleria mellonella*, b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

The THC in pyriproxyfen, *B. thuringiensis* and mixed treatments had decreased significantly after 24 h, however it increased after 48 and 72 h in all treatments. After 72 h, THC decreased in mixed treatment relative to the pyriproxyfen and *B. thuringiensis* treatments (Figure 5). The highest increase after 48 h was observed with the mixture of pyriproxyfen and *B. thuringiensis*.

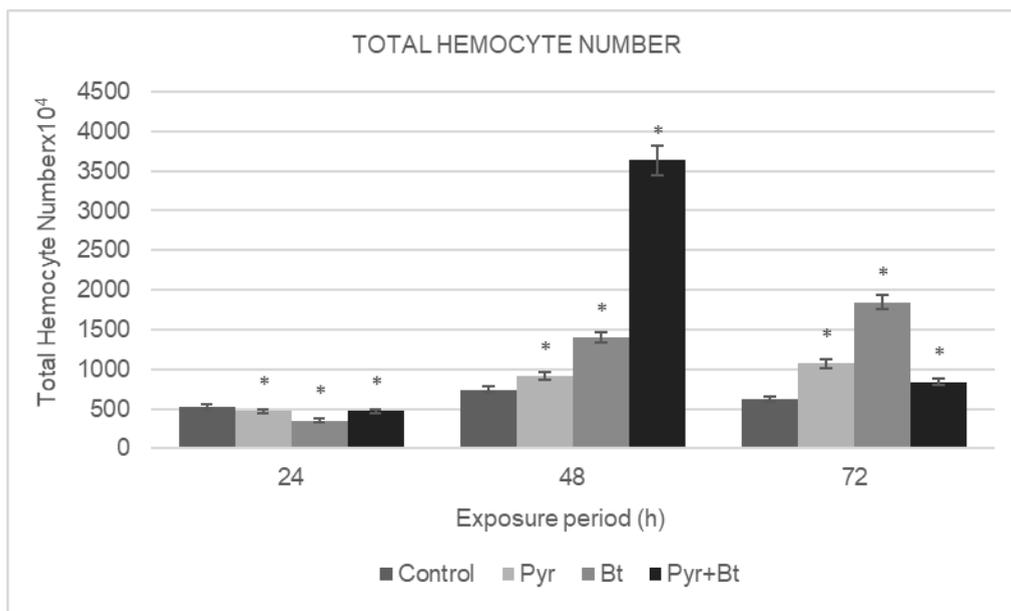


Figure 5. Total hemocyte count of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

DHC in larvae revealed the highest number of plasmatocyte and granulocyte in all treatments. The plasmatocyte count in pyriproxyfen and *B. thuringiensis* treatments decreased significantly after 24 h, however it increased in mixed treatment at the same time interval relative to the control. After 48 h, it also increased significantly relative to the control. The highest increase after 72 h was observed with *B. thuringiensis* ( $p < 0.05$ ; Figure 6a). The highest granulocyte count was observed in pyriproxyfen treatment after 24 and 48 h. After 72 h, it decreased in pyriproxyfen and *B. thuringiensis* treatments relative to the control ( $p < 0.05$ ; Figure 6b). The prohemocyte count decreased in all treatments after 24 and 48 h. After 72 h, an increase was observed in only mixed treatment relative to the control ( $p < 0.05$ ; Figure 6c).

The spherulocyte count increased in *B. thuringiensis* and mixed treatments after 24 h, however it decreased after 72 h ( $p < 0.05$ ; Figure 7a). The oenocytoid count decreased in *B. thuringiensis* treatment after 48 h, however it increased in mixed treatment relative to the control. After 72 h, it increased in all treatments relative to the control ( $p < 0.05$ ; Figure 7b).

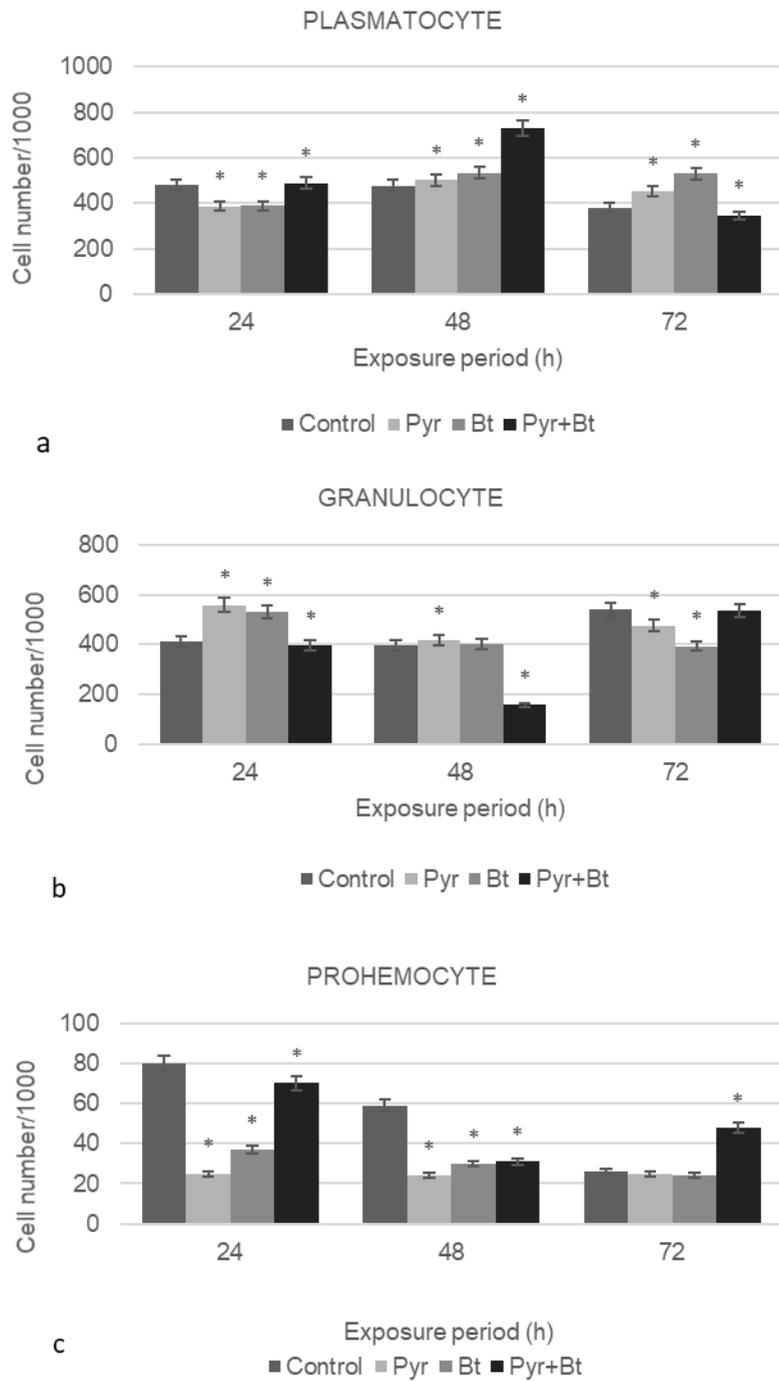


Figure 6. Differential hemocyte counts of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) plasmatocyte, b) granulocyte, c) prohemocyte counts were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

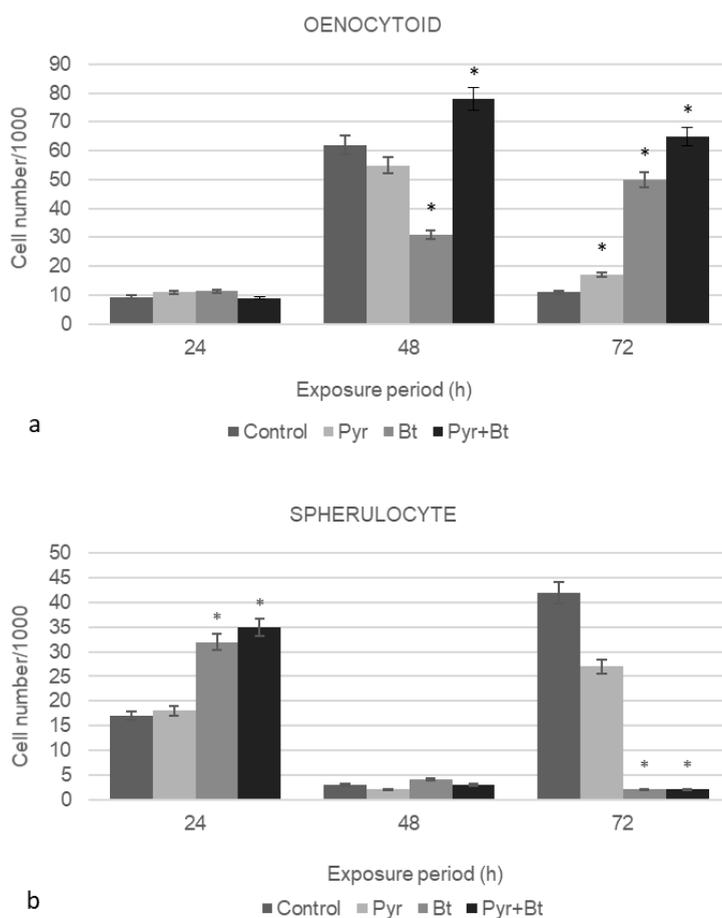


Figure 7. Differential hemocyte counts of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) spherulocyte, b) oenocytoid counts were determined. Data shown are means  $\pm$  SE (Student-Newman Keul's test; asterisks indicate significant differences at  $P < 0.05$ ).

## Discussion

SOD and CAT are major enzymes in the first line of defense against oxidative damage converting the superoxide anion radical to hydrogen and the hydrogen peroxide to water and molecular oxygen, respectively (Hong et al., 2018). In our previous study, the activity of SOD, CAT and GPx in hemolymph and fat body had significantly increased after 72 h for larvae exposed to pyriproxyfen, *B. thuringiensis* and mixture, however in midgut the enzyme activities had significantly decreased relative to the control (Tuncsoy & Ozalp, 2016). Increased activities of antioxidant enzymes in the fat body and hemolymph are consistent with increased rates of adaptive metabolic responses to elevated lipid peroxidation (Hyrsl et al., 2007). In this study, we found a significant increase after 24 h in all treatments in the MDA level of midgut and fat body relative to the control. Similarly, Boctor & Salama (1983) found that *B. thuringiensis* bacteriosis in *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) increased lipid peroxidation. The increase of MDA level in midgut 24 h after exposure of *B. thuringiensis* might be also due to the destruction via endotoxins. Also, an increase after 72 h was observed in hemolymph of *G. mellonella* larvae when exposed to all treatments. It is considered that hemolymph has a vital role in destruction of free radicals in comparison with midgut and fat body. Fahmy (2012) also found that pyriproxyfen increased the CAT activity and MDA level in larvae of *S. littoralis*. Similarly, Dubovskiy et al. (2008) determined that *B. thuringiensis* infection resulted in increased SOD and MDA activity in the *G. mellonella* midgut, however CAT activity decreased on the first and following days after infection with *B. thuringiensis*. The increased MDA level 24

h after application of all treatments in the midgut and fat body of larvae is evidence of oxidative stress. However, the MDA level significantly decreased after 48 and 72 h in midgut and fat body after exposure to pyriproxyfen, *B. thuringiensis* and mixture. These contradictions may be due to the tissue specific differences in antioxidant enzyme activity (Hong et al., 2018).

AChE is an enzyme that found in many central and peripheral tissues, particularly in the nervous and muscular tissues (Massoulié et al., 1993). AChE inactivates the neurotransmitter acetylcholine in the synapses of the insect's central nervous (Casida & Durkin, 2013; Johnson, 2015; Zhu et al., 2017). In this study, it was determined that pyriproxyfen, *B. thuringiensis*, and mixed treatments caused significant changes in AChE activity in hemolymph, midgut, and fat body of the larvae after 24, 48, 72 h. In another study with *G. mellonella*, it was found that AChE activity decreased in hemolymph after exposure to sodium tetraborate, although an increase was observed in fat body (Durmus, 2007). Similarly, Nathan et al. (2008) determined that lethal concentration of azadirachtin, which is juvenile hormone analog, significantly inhibited the activity of AChE only at the high dose in *Nilaparvata lugens* (Stål, 1854) (Hemiptera: Delphacidae) compared with control. It was concluded that the reason of the reduction of AChE activity owing to increased free radical formation when pyriproxyfen and *B. thuringiensis* was applied may be due to the inactivation of this enzyme by free radicals.

Chemical insecticides, which are frequently utilized in fields against pests, enable to destroying of pest in a short time period, although overuse of these insecticides leads to progressing of resistance. Detoxification enzymes, including esterases, GST, and Cyt P450 has a vital role in resistance development. GST catalyze the secondary metabolism of a vast array of compounds which is oxidized by the Cyt P450 family (Wilce & Parker, 1994). The catalytic reactions transform a wide range of endogenous and xenobiotic compounds, such as insecticides (Armstrong, 1997; Zhu et al., 2017). Weirich et al. (2002) reported that the GST activity in the ventricles of honey bees was five times higher than the hemolymph. It was also determined that GST activity in midgut of *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae increased when nickel and diazinon applied individually and in combination; however, when diazinon was applied individually, a decrease was observed in body wall and when nickel was applied individually. GST activity also decreased in fat body (Zawisza-Raszka & Dolezych, 2008). We found similar results with other studies and it was thought that various results in GST activities in the tissues may be due to the differences between tissue metabolism and defenses against reactive oxygen species. The Cyt P450 is also related to the synthesis of insect hormones, such as 20-hydroxyecdysone and juvenile hormone (Scott, 1999; Warren et al., 2002; Pondeville et al., 2013). In this study, P450 activity increased in hemolymph after 24, 48 and 72 h and in midgut 72 h after exposure to pyriproxyfen, *B. thuringiensis* and mixture. The increase in Cyt P450 activity when exposed to pyriproxyfen may be due to involving of monooxygenases in the synthesis of insect hormones. We also determined that Cyt P450 activity increased in midgut 72 h after application of all treatments, and significant increases in hemolymph of pyriproxyfen treatments after 24, 48 and 72 h. It is considered that this effect in hemolymph might be due to the accumulation of enzymes or proteins in non-target regions such as hemolymph, where these molecules bind to the toxin substances entering the body of the insect. Similar results to our findings have been found in insects responding to other chemicals, for example P450 monooxygenase increased in Malpighian tubules and in midgut of lepidopteran insect during the defense response to allelochemical toxicity (Yorulmaz & Ay, 2010). Qiu et al. (2003) also determined that Cyt P450 enzyme increased in midgut of *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae in proportion to fat body when exposed to pentamethyl benzene and naphthalene.

Hemocytes, which are important in the immune system of insects, decline with the starvation, parasitism, diseases and insecticides as well as changes in the growth of the insects. Thus, it is known that THC may change in stress factors. In this study, we determined that THC had decreased 24 h after exposure to pyriproxyfen, *B. thuringiensis*, and mixture, whereas it increased after 48 and 72 h. Significant

changes in the DHC of five hemocyte types were observed after application of all treatments. Degradation of cellular immune response may be due to the decreased hemocyte count in response to bacterial intoxication. Furthermore, a decrease in the THC may have occurred in starvation, which is an effect of bacteriosis of *B. thuringiensis* (De Block & Stoks, 2008; Lee et al., 2006). Broderick et al. (2010) showed that THC decreased in *Lymantria dispar* (L., 1758) (Lepidoptera: Erebididae) larvae exposed to *B. thuringiensis*. Manachini et al. (2011) also determined that THC in *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Dryophthoridae) hemolymph decreased after exposure to a sublethal concentration of *B. thuringiensis*. Insect hormones, such as 20-hydroxyecdysone and juvenile hormone, act in two ways in the immune system. 20-Hydroxyecdysone causes proliferation of hemocytes, while juvenile hormone and analogs have an adverse effect (James & Xu, 2012). Juvenile hormone analogs such as pyriproxyfen have an inhibitory effect on the ecdysone biosynthesis and suppress the release of hemocytes from hematopoietic organs. Kim et al. (2008) determined that juvenile hormones have an antagonistic effect on hemocyte counts and spreading of hemocytes. Zibae et al. (2012) also showed that THC, plasmatocyte and granulocyte counts decreased in *Eurygaster integriceps* Puton, 1881 (Hemiptera: Scutelleridae) adults after exposure to pyriproxyfen. Ghasemi et al. (2014) also reported that THC, plasmatocyte, prohemocyte and spherulocyte counts decreased in *Ephestia kuehniella* Froggat, 1912 (Lepidoptera: Pyralidae) larvae, while granulocyte and oenocytoid counts increased when exposed to pyriproxyfen.

In conclusion, taking into consideration studies with different insect species, it is thought that juvenile hormone analogs such as pyriproxyfen and entomopathogen *B. thuringiensis* may have an adverse effect on hemocyte counts, inhibit larval hematopoietic functions or cytotoxic effects such as cell proliferation. Moreover, pyriproxyfen and *B. thuringiensis* also negative effects on oxidative stress and detoxification mechanism of *G. mellonella*. Investigation of the toxic effects of *B. thuringiensis* and pyriproxyfen on insects individually and in combination are of useful effects on pest management and improving of new methods which have less adverse effects on environment could overcome potential toxicological effects on non-target organisms.

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