

## The Effects of Oxyclozanide on Survival, Development and Total Protein of *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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### ABSTRACT

We investigated that the effects of oxyclozanide on survival rate, development time, adult longevity and the amount of total protein (TP) in different developmental stages (7<sup>th</sup>-instars larvae, pupae and adult) of greater wax moth *Galleria mellonella* L. First-instar larvae of insects were reared on 0.003, 0.03 and 0.3 g oxyclozanide in 100 g artificial diets. When compared to all tested concentrations of oxyclozanide and control diet without oxyclozanide, it was confirmed that this anthelmintic drug significantly reduced survival rate of 7<sup>th</sup>-instar larval stage, pupal and adult stage of *G. mellonella*. While 7<sup>th</sup>-instars larval rate is 91.25 ± 6.21 % in the control diet without oxyclozanide, this rate has been determined 28.75 ± 3.24 % in the 0.3 % concentration of oxyclozanide. TP of the insect increased in response to all concentration of oxyclozanide in comparison to control diet. This considerable increase was expressed almost two-fold especially at the 0.003 and 0.03 % of oxyclozanide concentrations in all developmental stages. The results obtained from this research support that the oxyclozanide has effects on the survival, development, adult longevity and TP of the insect.

**Key words:** *Galleria mellonella*, oxyclozanide, survivorship, development, total protein.

## INTRODUCTION

Lepidoptera species, especially the larval stage of these insects causes significant economic losses by damaging to agricultural and industrial plants. These species include *Achroia grisella* (lesser wax moth) and *Galleria mellonella* (greater wax moth). Since the larvae of these insects feed on products such as honeycomb, honey, beeswax and pollen residues, they cause serious economic losses in apiculture industry (Uygur & Girişgin, 2008; Ellis, Graham, & Mortensen, 2013). Several studies were conducted on the life cycle, biology, behavior, ecology, molecular biology, physiology of the insect to control this pest with economic significance (Akyol, Yeninar, Şahinler, & Ceylan, 2009; Ellis et al, 2013; Erdem, Küçük, Büyükgüzel, & Büyükgüzel, 2016; Büyükgüzel & Büyükgüzel, 2016).

A variety of physical, chemical, biological and certain other methods are used in the control of agricultural pests to reduce their populations to a certain level and not to eliminate their existence (Kılınçer et al, 2010; Sezer & Ozalp, 2011). Various chemical (sulfur, acetic acid, formic acid, aluminum phosphate, methyl bromide, ethylene dibromide, naphthalene, sulfur and carbon dioxide), physical (hot or cold) applications and biological insecticides (*Bacillus thuringiensis*) are used for the protection of raised honeycombs against the damages caused by greater wax moth (Akyol & Korkmaz, 2008; Akyol et al, 2009; Babarinde et al, 2013). In recent years, studies on the insecticide properties of antibacterial, antiprotozoal, antifungal and anthelmintic drugs with different chemical structures and modes of action by culturing insects with artificial diets in laboratory environment have been increased (Büyükgüzel & Kayaoğlu, 2014; Kılıç, Büyükgüzel, & Büyükgüzel, 2015; Çalık, Büyükgüzel, & Büyükgüzel, 2016; Sugeçti, Büyükgüzel, & Büyükgüzel, 2016; Hız, Erdem, Büyükgüzel, & Büyükgüzel, 2016; Sefer & Büyükgüzel, 2018).

Studies on the effects of various factors on the protein, lipid and carbohydrate content in insects were demonstrated in different insect species (Büyükgüzel & Kalender, 2008; Sönmez & Gülel, 2008; Sharma, Mohan, Dua, & Srivastava, 2011; Clark, Zera, & Behmer, 2015). In a study by Boz & Gülel (2012) who examined the effect of post-parasitizing time and temperature on the total protein, lipid and carbohydrate content of mediterranean flour moth *Ephestia kuehniella* larvae, it was reported that the protein concentration in the hemolymph of mediterranean flour moth parasitized by parasitoid *Venturia canescens* increased with time after parasitizing, while total lipid and carbohydrate content decreased. When the effect of temperature was examined, it was determined that protein and carbohydrate concentrations increased, however the lipid content did not change. Aksoy, Bahadıroğlu, & Kayabaşı (2015a) conducted to determine the effects of different doses of X-ray (50-200 Gy) on the protein, carbohydrate and lipid levels in *Sesamia nonagrioides* larvae were investigated and the best results were obtained as 150 Gy between the applied doses. It was reported that increases in the amount of these molecules (protein, carbohydrate and lipid) caused deterioration in the physiological and biochemical activities in *S. nonagrioides*, and interrupted the biologic stages and prevented the female larvae to enter the pupa stage.

## Oxyclozanide and *G. mellonella*

*Galleria mellonella* is commonly used as a model organism in several fields such as clinical drug trials and investigation of the activity of disease factor microorganisms due to its high ecological adaptation, short life cycle, high egg-laying capacity and it is a species that could breed high number of healthy individuals (Junqueira, 2012; Harding, Schroeder, Collins, & Frankel, 2013; Jacobsen, 2014; Champion, Wagley, & Titball, 2016). Furthermore, similar to certain other species, the larvae and pupae of these insects are also used as pseudo-hosts in propagation of parasitoid insects under laboratory conditions (Bernardi, Haddad, & Parra, 2000; Büyükgüzel, 2006).

The oxyclozanide that was used in the study is a salicylanilide derivative anthelmintic drug (Kang, Wakabayashi, & Kim, 2016). Oxyclozanide is effective against adults of *Fasciola hepatica* and *Fasciola gigantica* (liver flukes), which belong to trematodes, an important group of parasites (Coles & Stafford, 2001; Power et al, 2013). Its effect on parasites is demonstrated by breaking down the oxidative phosphorylation chain and degrading the energy metabolism (Hrckova & Velebny, 2013). There is no information on the use of oxyclozanide, a salicylanilide derivative anthelmintic drug, in the control of agricultural pest species. Thus, the greater wax moth *G. mellonella*, a Lepidoptera species, was used as a model organism in the present study to investigate the effect of oxyclozanide on insects, which constitute a significant group of invertebrates. The final aim of the present study was to contribute to the control methods against this species and to investigate the availability of anthelmintic drugs for chemical control of the species in the Lepidoptera order. Furthermore, information on the availability of oxyclozanide as an insecticide in pest control is significant since it would contribute to the preservation and the yield of agricultural crops, improvement their quality and development of new methods. In the present study, the effects of oxyclozanide on the survival rate, growth period, adult longevity and total protein content of the larvae were investigated by supplementing the diets that were provided for *G. mellonella* with oxyclozanide in the laboratory environment.

## MATERIAL AND METHODS

### Culturing *G. mellonella* in laboratory environment

*G. mellonella* (Lepidoptera: Pyralidae), the greater wax moth, was cultured in the university insect culture laboratory to maintain the stock insect culture. For the propagation of the insect culture, newly hatched larvae were grown in artificial medium (Bronskill, 1961). The culture was conducted in an incubator (Nüve, FN 400) that was set to  $28 \pm 2^\circ\text{C}$  temperature and  $65 \pm 5\%$  relative humidity and continuously in darkness.

### Obtaining the *G. mellonella* larvae

The *G. mellonella* larvae to be used in diet experiments were obtained by the hatching of the eggs laid by female individuals grown in the stock culture. The first stage larvae that were freed with the hatching of the eggs were transferred to medium size wire-mesh glass jars with a metal lid (60x120 mm) using a soft-tip moistened brush (No: 0, Goya Toray). Thus, the effects of different concentrations of oxyclozanide,

ingested directly by the larvae via diets, on the survival rate until adult stage, growth period, adult longevity and total protein (TP) content of the larvae were investigated.

### **Determination of oxyclozanide concentrations**

Concentration of the quantities tested in diet tests conducted by adding oxyclozanide [2,3,5-trichloro-N-(3,5-dichloro-2-hydroxyphenyl)-6-hydroxybenzamide] (Jo et al, 2011), a salicylanilide derivative, was determined as gram quantity (%) supplemented to 100 grams of diet. Three different concentrations, namely 0.003, 0.03 and 0.3 g were used to investigate the effect of oxyclozanide on the insect except for the control diet (without oxyclozanide). The concentrations were determined based on the preliminary experiments that were conducted based on the concentrations utilized in previous studies that investigated the effect of certain antibiotics on *G. mellonella* (Büyükgüzel & Kalender, 2007, 2008, 2009). Twenty 1<sup>st</sup> stage larvae were transferred into each jar prepared for the experiment using a fine-tipped brush. The jars were covered with lids that were cut out and replaced with thin metal mesh and placed in incubators where insects were cultured. Experiments were repeated four times.

### **Experiments on *G. mellonella* survival rate, developmental time and adult longevity**

All experiments (jars media where the first-stage larvae were kept and containers prepared for the 7<sup>th</sup> stage larvae to become pupae) were kept in the dark at all times except for a brief daily inspection period. The number of mature (7<sup>th</sup> stage) *G. mellonella* larvae that completed the development was counted and noted and they were placed in 30 ml plastic sample containers (Orlab, L190030, 35x55 mm) that were layered with tissue paper to provide a dry environment for pupae. After the number of individuals that reached the pupal stage and the number of individuals that matured from these pupae were noted and the survival rates of the larvae, pupae and adults were calculated. The longevity of individuals who reached the maturity stage was also calculated.

### **Determination of TP in *G. mellonella* 7<sup>th</sup> stage larvae, pupae and adults**

*G. mellonella* 7<sup>th</sup> stage larvae, pupae and adult individuals cultured with diets that contained different concentrations of oxyclozanide were taken to 1.5 ml Eppendorf tubes and stored in a deep freeze (-80°C) until the analyses were conducted. Experiments were repeated four times using five 7<sup>th</sup> larvae, pupae and adults for each trial. All development stage of *G. mellonella* collected for each repeat were placed in 1.5 ml phosphate buffer ( $K_2HPO_4$ ) (pH: 8) and initially grinded in 24000 rpm tissue grinder homogenizer (Ultra Turrax, Ika T18 basic NC, USA), and then in an ultrasonic homogenizer (10 sec, 30 w) (Bandelin Sonoplus HD2070, Berlin, Germany). Homogenized 7<sup>th</sup> stage larvae and others were transferred to 2 ml Eppendorf tubes and centrifuged at +4°C for 10 minutes at 1000x g. The supernatant was used to determine the TP after the sediment was discarded. Bovine serum albumin solutions were prepared for use as standard protein solution and a standard graph was plotted. TP content was calculated based on this standard graph. The absorbances of the

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samples were measured with the Folin-Lowry (Lowry, Rosebroug, Farr, & Randall, 1951) method at 600 nm with a spectrophotometer (Shimadzu 1700 UV / Vis, Kyoto, Japan).

### Statistical analyses

Percentages of *G. mellonella* individuals that reached different stages (7<sup>th</sup> stage, pupa and adult) were defined as the survival rate, development as the time required to reach these stages in days, the time they survived as adults after they reached the adult stage in days and TP content in mg in tissue extract.

One-way analysis of variance (ANOVA) (SPSS 1997) was used to evaluate the data on the duration of development, adult longevity and TP content of the insect, and the "LSD Test" was used to determine the significance of the difference between the mean values. In the evaluation of the survival rate data, "χ<sup>2</sup> (Chi square) test" (Snedecor and Cochran, 1989) was used. Correlation analysis was also performed to test the correlation between tested concentration and survival rate of insect (SPSS 1997). The significance of the means was assessed at the 0.05 level of significance.

## RESULTS

### The effect of oxyclozanide on the survival rate and developmental time of *G. mellonella* larvae, pupae and adults

Comparison of the concentrations of oxyclozanide, a anthelmintic substance, used in the study group with the control group demonstrated that the increase in oxyclozanide concentration decreased the larval survival rate (Figs. 1-3). While 91.25 ± 6.21 % of the larvae in the control diet that did not contain oxyclozanide reached 7<sup>th</sup> stage, in 0.003, 0.03 and 0.3 g oxyclozanide concentrations, only 77.50 ± 8.19 %, 61.25 ± 7.97 % and 28.75 ± 3.24 % of the larvae reached the 7<sup>th</sup> stage, respectively (Fig. 1). We obtained significantly relationship between the tested concentration of oxyclozanide and survival rate. Tested concentrations were negatively correlated with survival rate of 7<sup>th</sup>-instar larva ( $R^2 = 0.97$ ,  $P > 0.05$ ), pupa ( $R^2 = 0.95$ ,  $P > 0.05$ ) and adult ( $R^2 = 0.96$ ,  $P > 0.05$ ) stages of *G. mellonella*.

A decrease was observed in all oxyclozanide concentrations when the effect of oxyclozanide on the rate of transformation of greater wax moth *G. mellonella* into pupa was compared between the study and control groups. The highest decrease was observed with 0.3 g oxyclozanide concentration. While 83.75 ± 8.54 % of the larvae grown with the control diet reached the pupal stage, only 77.50 ± 8.19 %, 58.75 ± 7.15 % and 26.25 ± 5.11 % of the larvae cultured with 0.003 %, 0.03 % and 0.3 % oxyclozanide concentrations added to the artificial diet reached the pupal stage, respectively, and it was found that the decrease was statistically significant when compared to the control (Fig. 2).

Oxyclozanide, the anticholinergic substance added to the artificial diets, significantly affected the percentage of pupae that reached adulthood, similar to the rate of larvae that reached the 7<sup>th</sup> stage and pupa stage (Fig. 3). In the control group, 80.00 ± 8.66 %

of the first stage larvae reached adulthood, while in 0.3 g oxytalanide concentration, only  $25.00 \pm 4.67$  % of the larvae reached the adulthood.

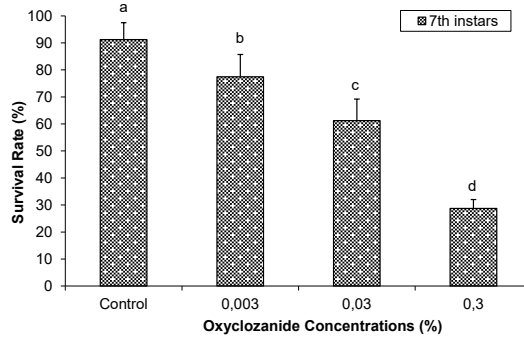


Fig. 1. Effects of oxytalanide on *G. mellonella* 7th stage survival rate. Four replicates with 20 larvae per replicate were used. Values labelled with the same letter are not significantly different from each other within each survival rate,  $P > 0.05$  ( $\chi^2$  test). Control diet (without oxytalanide).

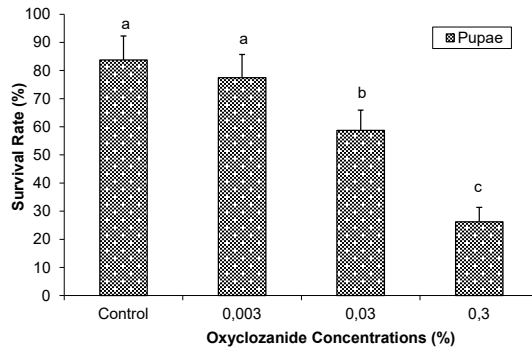


Fig. 2. Effects of oxytalanide on *G. mellonella* pupal survival rate. Four replicates with 20 larvae per replicate were used. Values labelled with the same letter are not significantly different from each other within each survival rate,  $P > 0.05$  ( $\chi^2$  test). Control diet (without oxytalanide).

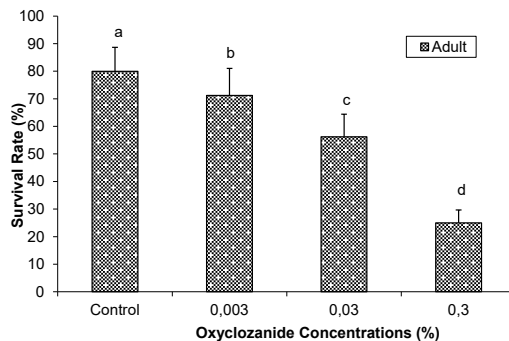


Fig. 3. Effects of oxytalanide on *G. mellonella* adult survival rate. Four replicates with 20 larvae per replicate were used. Values labelled with the same letter are not significantly different from each other within each survival rate,  $P > 0.05$  ( $\chi^2$  test). Control diet (without oxytalanide).

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There were no significant differences between the oxyclozanide concentration applications based on the rate of *G. mellonella* larvae that reached to 7<sup>th</sup>, pupae and adult stages. Although this ratio of oxyclozanide in the diet delayed the time that was required for the 1<sup>st</sup> stage larvae to reach the 7<sup>th</sup> stage for 4 days, the difference was not statistically significant (Fig. 4).

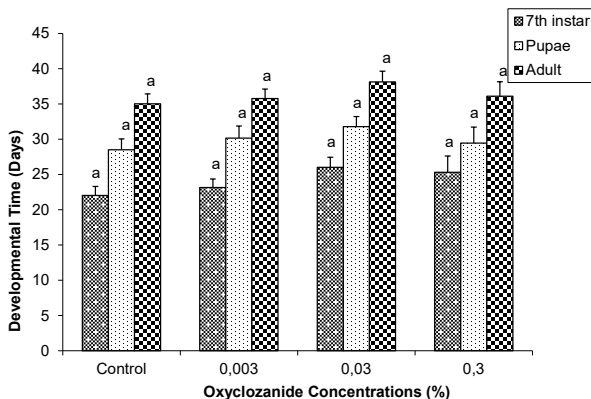


Fig. 4. Effects of oxyclozanide on *G. mellonella* larval, pupal and adult developmental time. Four replicates with 20 larvae per replicate were used. Values labelled with the same letter are not significantly different from each other within each developmental time,  $P > 0.05$  (LSD test). Control diet (without oxyclozanide).

### The effect of oxyclozanide on adult longevity of *G. mellonella*

When the effect of oxyclozanide on adult longevity of the insect was examined, it was determined that there was no statistically significant difference based on the results (Table 1).

Table 1. Mean ( $\pm$ SE) adult longevity of *G. mellonella* treated with Oxyclozanide

Oxyclozanide(%)	Adult Longevity (Day)
	(Mean $\pm$ SE)*
0.000 <sup>§</sup>	10.03 $\pm$ 0.92a
0.003	10.55 $\pm$ 0.50a
0.03	10.56 $\pm$ 0.29a
0.3	9.38 $\pm$ 0.43a

Four replicates with 20 larvae per replicate. \*Means within a column followed by the same lowercase letter are not significantly different,  $P > 0,05$  (LSD Test).<sup>§</sup> Control diet (without Oxyclozanide)

### The effect of oxyclozanide on TP content in *G. mellonella* 7<sup>th</sup> stage larvae, pupae and adults

All oxyclozanide concentrations in the diet increased the TP content in larval, pupal and adult stages of the insects, and the differences were statistically significant. In

the control group, the 7<sup>th</sup> stage larva TP content was recorded as  $39.00 \pm 1.87$  mg / ml, the pupal TP content was  $20.75 \pm 1.08$  mg / ml and the adult TP content was  $12.25 \pm 1.86$  mg / ml.

Comparison of the results obtained with 0.03 % oxyclozanide group with that of the control group demonstrated that the TP content in the larvae was almost double in the oxyclozanide group. It was determined that TP content was  $84.00 \pm 2.57$  mg / ml in the larvae cultured with the diet that contained 0.03 g oxyclozanide (Fig. 5). Similar to the larval TP content, the TP content increased to  $57.00 \pm 1.54$  mg / ml in the pupae cultured with the diet that contained 0.03 % oxyclozanide concentration (Fig. 6). A similar finding was also observed in the adult stage. It was determined that the TP content was  $12.25 \pm 1.86$  mg / ml in adult phase in the control group and this value increased to  $41.25 \pm 1.43$  mg / ml with the diet that contained 0.03 % oxyclozanide (Fig. 7).

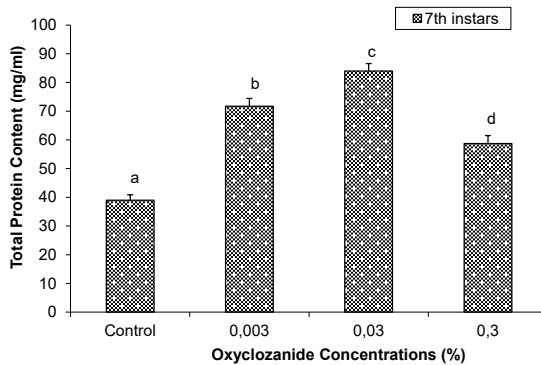


Fig. 5. Effects of oxyclozanide on *G. mellonella* larval total protein content. The data are given as the mean of four replicates. Five larvae were used for each replicate test. There were no differences between the values indicated with the same letter,  $P > 0.05$  (LSD Test). Control diet (without oxyclozanide).

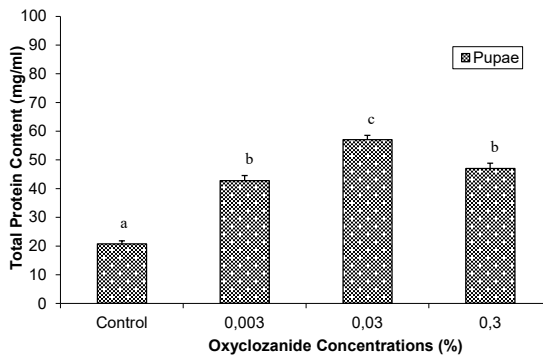


Fig. 6. Effects of oxyclozanide on *G. mellonella* pupal total protein content. The data are given as the mean of four replicates. Five pupae were used for each replicate test. There were no differences between the values indicated with the same letter,  $P > 0.05$  (LSD Test). Control diet (without oxyclozanide).



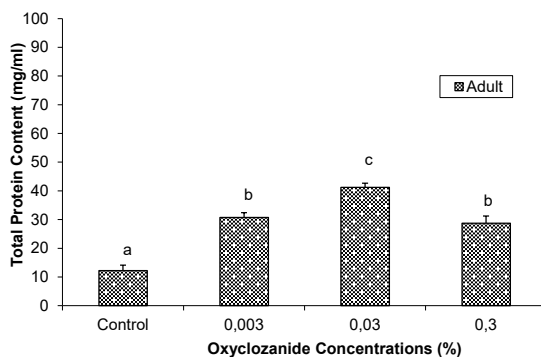
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Fig. 7. Effects of oxyclozanide on *G. mellonella* adult total protein content. The data are given as the mean of four replicates. Five adult were used for each replicate test. There were no differences between the values indicated with the same letter,  $P > 0.05$  (LSD Test). Control diet (without oxyclozanide).

## DISCUSSION

The insects (Insecta), located in the Hexapoda subphylum of the Arthropoda phylum, include approximately 70 % of all species living in the world. Furthermore, new species that are scientifically identified every passing year are added to this count. Several insect species harm one third of the global crops, thus they have both ecological and economical significance (Aydın, 2011).

In the present study, the effects of oxyclozanide that was added to an artificial diet on survival, growth and TP content of *G. mellonella* were investigated. It was determined that all oxyclozanide concentrations demonstrated significant effects on all parameters investigated for the insect. Similar results were obtained with previously studied anthelmintic substances (niclosamide from the salicylanilide group, triclabendazole, mebendazole and oxfendazole from the benzimidazole group) (Büyükgüzel & Kayaoğlu, 2014; Kılıç et al, 2015; Çalık et al, 2016; Sugeçti et al, 2016).

In a study conducted with niclosamide, an anthelmintic substance from the salicylanilide group, it was determined that niclosamide reduced the survival rate of the insect in 7<sup>th</sup> stage larva, pupa and adult stages, 1.0 g concentration increased the adult growth period and increased the longevity of male individuals (Büyükgüzel & Kayaoğlu, 2014). In the present study, it was also determined that the survival rate decreased in the 7<sup>th</sup> stage larvae, pupae and adult insects, however the differences in the growth period and adult longevity of the insect were statistically insignificant. In previous studies that supported the results of the present study, benzimidazole group anthelmintic drugs, namely triclabendazole, mebendazole and oxfendazole, were used and it was concluded that administration of anthelmintic drugs decreased the percentage of the insect larvae that reached the 7<sup>th</sup>, pupal and adult stages (Çalık et al, 2016; Sugeçti et al, 2016). Comparison of the results obtained in 0.3 g oxyclozanide administered and control groups demonstrated that oxyclozanide significantly reduced the rate of the insects that reached larval, pupal and adult stages. Similarly, it was determined that 0.1 % g triclabendazole

concentration significantly decreased the rate of the insects that reached pupal and adult stages (Kılıç et al, 2015).

The physical structure of holometabolous insects have the ability to store diets ingested during the whole larval period. The diets ingested and stored during the larval period are utilized during the metamorphosis and the adult stages (Aksoy, Bahadırođlu & Torođlu, 2015b). Furthermore, previous studies demonstrated that the chemical substances added to insect diets had an adverse effect on the biologic properties of the insect by damaging the quality of the diets (Nielsen & Toft, 2000; Kılıç et al, 2015). The holometabolous *G. mellonella* utilizes the diet intake in the larval stage until the adult stage. Based on the results of the present study, the fact that the chemical substance ingested with the diet had negative effects on the survival and development of the insect could be due to the negative effects of this chemical substance on the consumption of the diets by reducing the quality of the diet.

Lipids, carbohydrates and proteins are the major biomolecules that required for the physiological functions of insects (growth, development and reproduction) (Van der Horst, Vroemen, & Van Marrewijk, 1997). It was demonstrated that oxyclozanide had positive effects on the TP content in 7<sup>th</sup> stage larvae, pupae and adult *G. mellonella*. This increase could be due to the active utilization of the diets ingested with oxyclozanide by the insect. The determination of changes in the TP content in the whole body or a specific tissue of an insect is important in determining whether the substances in the diet were effectively used by the insect and whether it was effective on the growth of the insect (Büyükgüzel, 2002; Büyükgüzel & İçen, 2004; Sak, Uçkan, & Ergin, 2006; Sak, Ergin, Uçkan, Rivers, & Er, 2011). Some studies have shown that synthetic chemicals, herbal pesticides and biopharmaceuticals are effective on the body weight of insect by altering the amount of lipid, carbohydrate and protein (Vijayaraghavan & Chitra, 2002; Wang et al, 2005; Sezer & Ozalp, 2011).

It is known that chemical substances have important effects on proteases that provide free amino acids in insects and also these substances have important effects on the formation of enzymes which help to eliminate the toxic effect (Ramaswamy, 1987). Many studies have shown that there is a relationship between the tolerance of toxic properties of insecticidal chemical substances and total body protein (Nath, Suresh, Varma, & Kumar, 1997; Ahmed, Wilkins, & Mantle, 2002; Guedes, Oliveira, Guedes, Ribeiro, & Serrao, 2006). All tested oxyclozanide concentrations demonstrated a positive effect on the TP levels in larvae, pupae and adult stages of the insect, which could be due to the development of a tolerance for the chemical substance added to the diet.

The total amount of protein is known to be an important biomolecule in all stages of development of the insect. Our total protein content results have clearly showed that the effects of oxyclozanide on insect. It was determined that increasing concentrations of the tested chemical substance had adverse effects on the biological parameters of the insect. Since the tested 0.3 % oxyclozanide concentration had significant negative effects on the survival and developmental stages of the insect, the said substance could be evaluated for use as an alternative insecticide.

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Increasing number of studies are conducted on the effects of anthelmintic substances on insects (Büyükgüzel & Kayaoğlu, 2014; Kılıç et al, 2015; Çalık et al, 2016; Sugeçti et al, 2016). In the present study, where the salicylanilide group anthelmintic oxyclozanide was used, it was determined that increasing oxyclozanide concentrations had negative effects on important parameters of greater wax moth *G. mellonella*, an agricultural pest, such as survival rate and development and also the changes in TP content in all developmental stages were identified in the study. The fact that the results were obtained with very low oxyclozanide concentrations (0.003, 0.03 and 0.3 g / 100 g) could make it possible to control the target pest without harming humans, the environment and non-target organisms. These results are also significant in terms of identifying the capacity oxyclozanide as an insecticide with further studies. However, in order to fully identify and understand the effects of this substance on *G. mellonella* and its uses in the field in practice as an insecticide, further detailed experiments should be conducted.

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