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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

The Effect of Different Cold Storage Period on Total Lipid Amount of *Tenebrio molitor* (Coleoptera: Tenebrionidae) Larvae

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Abstract: The ever-increasing world population indicates that it is inevitable to consider insects such as *Tenebrio molitor*, which are used as live feed and even human food in some countries, as an alternative food source. Especially *T. molitor* larvae are a source of food with high nutritive value for including high lipid and protein. This study seeks answer to the question "will the insect continue to keep its lipid sources during the periods in which it is kept in the cold, or will it continue to use its energy sources since the physiological adaptations it develops are not enough?" The main material of this study was *T. molitor* cultures. Flour: wheat flour (250 g: 250 g) in a ratio of 1:1 was used as food. 25 g wheat germ and 5 g dry yeast was put in it. Larvae at stages 13-15th were grouped as control and trial groups and kept for 5, 10, 15 and 20 days at specified temperatures. The weights, total lipid content and percentages of the larvae whose storage period was over were determined. This study evaluates the total lipid amount and percentages of *T. molitor* larvae stored in refrigerator for different periods. Total lipid amount and percentages of the larvae stored in the cold for 5, 10 and 15 days were found to be higher when compared with the control group. A tendency to decrease was observed in larvae kept for 20 days. As a result, it is recommended for producers not to keep in the refrigerator for more than 15 days. Otherwise, it should be considered that there may be a decrease in important energy and food sources.

Keywords: Fat, low temperature, mealworm, rearing insects, temperature physiology.

Farklı Sürelerde Soğukta Depolamanın *Tenebrio molitor* (Coleoptera: Tenebrionidae) Larvalarının Toplam Lipid Miktarına Etkisi

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Öz: Gittikçe artan dünya nüfusu *Tenebrio molitor* gibi canlı yem hatta bazı ülkelerde insan yiyeceği olarak kullanılan böcekleri alternatif besin kaynağı olarak değerlendirmemizin kaçınılmaz olduğunun habercisidir. Özellikle *T. molitor* larvaları fazlaca yağ ve protein içermesinden dolayı besleyici değeri yüksek bir besin kaynağıdır. Bu çalışma 'soğukta bekletildiği sürelerde böcek lipit kaynaklarını korumaya devam mı edecektir ya da geliştirdiği fizyolojik adaptasyonlar yeterli kalmayıp enerji kaynaklarını kullanmaya devam mı edecektir' sorularına cevap aramaktadır. Bu çalışmanın ana malzemesini *T. molitor* kültürleri oluşturdu. Besin olarak 1:1 oranında un:buğday unu (250 g:250 g) kullanıldı. İçerisine 25 gr rüşeym, 5 gr kuru maya konuldu. 13-15. larval aşamadaki larvalar kontrol ve deneme grupları oluşturularak belirtilen sıcaklıklarda 5, 10, 15 ve 20 gün süre ile bekletildi. Depolama süreleri biten larvaların ağırlıkları, toplam lipid miktarı ve yüzdeleri tespit edildi. Bu çalışmada farklı sürelerde buzdolabında depolanan *T. molitor* larvalarının toplam lipid miktarları ve yüzdeleri değerlendirildi. Soğukta 5, 10 ve 15 gün depolanan larvaların toplam lipid miktarları ve yüzdelerinin kontrol grubuna göre daha fazla olduğu belirlendi. 20 gün bekletilen larvalarda ise azalma eğilimi gözlendi. Sonuç olarak yetiştiricilere 15 günden fazla buzdolabında bekletmemeleri önerilmektedir. Aksi takdirde lipid gibi önemli enerji ve besin kaynaklarının azalabileceği göz önüne alınmalıdır.

Anahtar kelimeler: Yağ, düşük sıcaklık, un kurdu, böcek yetiştiriciliği, sıcaklık fizyolojisi

INTRODUCTION

It is estimated that the world population will exceed 9 billion by 2050 (Mirzaeva et al., 2020). This situation increases the demand for alternative food sources (Sogari et al., 2019). *Tenebrio molitor* (Coleoptera: Tenebrionidae) is used as both live feed for pets and also as human food for humans in some countries due to its high nutritional value and easy farming (Adamkova et al., 2017; 2020). It is the most farmed insect in Europe among edible insects (Costa et al., 2020; Kelemu et al., 2015; Ravzanaadii et al., 2012). Considering the rapidly increasing world population, it is no longer just a dream that in the future our food sources will be obtained mostly from insects rather than traditional meat sources (Belluco et al., 2013; Halloran et al., 2014).

Tenebrio molitor is actually an insect that damages stored products. It may take up to 18 months for larvae, which emerge 10-12 days after hatching, to mature depending on temperature and humidity. Adults may reach a weight of 300 mg and they live up to 2-3 months. Temperature and humidity are very important environmental factors especially in their life cycles (Finkel, 1948; Liu et al., 2009; 2020; Mirzaeva et al., 2020 Siemianowska et al., 2013). The insect needs 28 °C for its optimum growth, it can live only 48 hours in under 10°C (Mirzaeva et al., 2020). Low or high temperatures cause important physiological reactions in insects, as in other living beings (Dooremalen & Ellers, 2010; Helgadóttir et al., 2017). They can change the protein, carbohydrate or lipid amounts or compounds they contain (Rathee & Ram, 2018; Zou et al., 2010). This situation is one of the most important adaptations they gain to survive in changing climatic conditions (Duman et al., 1991; Lee et al., 1996). During the mass farming of this insect, producers keep larvae and their pupae with cold storage method when there is no demand from consumers during the periods when population is high (Levie et al., 2015; Tiencheu et al., 2013). Cold storage method extends the shelf life of insects. However, prolongation of cold storage period has negative effects on insects' life cycles (Errico et al., 2021). In addition to its negative effects such as larval and pupal development time, adult weight, deformation rate, it also affects important energy sources such as carbohydrate, protein and lipid. Cold storage can prolong the development time and adult weight of insects. It also can increase the rate of deformation. Substances such as carbohydrates, fats and proteins may also decrease or increase depending on the low temperatures and developmental stages to which they are exposed (Melis et al., 2018; Mirzaeva et al., 2020; Rathee & Ram, 2018).

Adipose tissue plays a very important physiological role in the life of insects. It regulates a large number of physiological and metabolic activities (Arrese & Soulages, 2010). Lipid metabolism is essential for growth and reproduction and it provides the energy needed during periods when feeding is stopped (Lee et al., 1996). Insects store their energy reserves in the form of glycogen and triglyceride in the main fat cells adipocytes (Arrese & Soulages, 2010). In addition, they synthesize most of the metabolites in the circulation with adipose tissue hemolymph proteins (Graham et al., 2000). The lipid content of insects can vary according to rearing conditions, temperature, feed content and developmental stages. Especially temperature is a factor that has a direct effect on insects' developmental stages and the energy sources they include (Aman et al., 2017; Azeez et al., 2014; Pant & Gupta, 1979; Patterson & Duman, 1978). They react to changes in temperature by providing different physiological adaptations specific to species. In low temperatures, they slow their metabolism and generally increase their energy stores such as lipid and protein (Sinclair & Marshall, 2018).

Tenebrio molitor larvae have been found to include more fat than pupae and adults (Jajić et al. 2019; 2020). Morales-Ramos et al., (2015) found 32.1% lipid in pupae and 35.9% lipid in larvae. The amount of lipid they contain may differ according to developmental stages, food, humidity and temperature (Stanley-Samuelson et al., 1988; Van Broekhoven et al., 2015). Van Broekhoven et al., (2015) found that *T. molitor* larvae fed with low quality food in terms of protein and starch content also reduced the lipid reserves required for energy. Jones et al., (1972) found total lipid percentage as 14.96% in larvae. Kröncke et al., (2019) found lipid amount as 27.13 g in 100 g when fresh, as 27.27 g when rock oven dried, as 29.57 g when vacuum dried and as 26.80 g when freeze dried. Mlček et al., (2019) found lipid rate by dry weight as 16.7 g, Siemianowska et al., (2013) found as 22%, Jajić et al., (2019) found as 25.19%, Danthine et al., (2013) found as 30% in those sold in the market and as 36% when bred in laboratory, Ravzanaadii et al., (2012) found as 32.7%, Finkel, (1948) found as 0.24 g, Adamkova et al., (2017) found as 24.56%. Selaledi & Mabelebele, (2021) found the content of sun-dried larvae higher than those oven-dried or freeze-dried. There are a large number of studies on the total lipid amount and the fatty acids contained in *T. molitor* in literature. However, the number of studies on how the cold storage period affects the total lipid content of this species during mass production is limited. The hypothesis in this study is that the weights, total lipid amount and percentages of larvae will not change during the period they are kept in the refrigerator. This is because temperature is one of the factors that affect insect

metabolism most. Therefore, it is expected that the metabolism of insect will slow and lipid amount will not change in low temperature. However, it is not known exactly what will become of these lipid sources with the prolongation of storage time. Will the insect continue to keep its lipid sources or will it continue to use its energy sources since the physiological adaptations it develops will not be enough? For this reason, the present study evaluated the effects of storage time on the weight, total lipid amount and percentages of *T. molitor* larvae and the points that should be considered by mass producers.

MATERIALS AND METHODS

This study was carried out in the Biology Laboratory of Sinop University, Faculty of Education, Sinop-Turkey in 2021. The main material of the trials consisted of *T. molitor* cultures which were available in the laboratory since 2017. Flour : wheat flour (250 g:250 g) in a ratio of 1:1 was given to the larvae and adults as food. 25 g wheat germ and 5 g dry yeast was put in it.

Trials were performed at 28 ± 2 and + 4 ºC, 60 ± 5% relative humidity and continues darkness. The insects were reared in plastic containers (size 30 × 20 × 5 cm). Wooddust was added in the containers to ease movement on the foodstuff. Small pieces (2 for each container, 4 × 4 × 6 cm) were cut from egg boxes for providing convenience for adults to mate and lay eggs. While the plastic containers were covered to prevent entrance of other living beings, small holes were opened on the top side to enable gas exchange. Potato was used for humidity (3 × 3 × 3 cm). They were wrapped into aluminium foil in order to prevent their contact and moisturing and decaying the food. The potatoes were changed every 3 d for the food layer not to get mouldy. The food layer was adjusted 4 - 5 cm thick. The food was renewed with intervals of 10 d. The larvae in the old food were separated by using a sifter and they were transferred on the new food. The containers were checked every day.

After 130-160 mg (in larval stage of 13-15th) larvae were obtained, they were weighed one by one. Control groups were also weighed. This was called the 'first weighing'. They were put in separate petri dishes one by one and given food. Control groups (0, 5, 10, 15 and 20 days) and trial groups (5, 10, 15 and 20 days) were formed. They were stored under specified laboratory conditions and in the refrigerator. The larvae, storage period of which was over, were taken out of the refrigerator and weighed. Control groups were also weighed. This value was called 'second weighing' and they were put in the freezer (Profilo 6600) until they were analyzed. The method applied by Folch et al., (1957) was used to find out the total lipid amount larvae included. Each larva was homogenized (Pro 2000) in 1:2 chloroform-methanol solution. The solution obtained was filtered with Whatman 41 paper. The volatile solution was evaporated under nitrogen gas. The sample obtained was weighed ('initial value') and placed in a desiccator containing silica gel. It was weighed until constant weigh ('final value'). At constant weigh, the total lipid amount was calculated in mg from the difference between the 'initial value' and the 'final value'. Total lipid percentage was found by dividing the mg value of the total lipid calculated to the 'first' and 'second' weight values of the larvae. Each trial group was replicated three times. 10 larvae were used for each trial. A total of 270 larvae were used for the whole study.

Statistical Analysis: SPSS 21.0 was used to evaluate the data. Data were tested for normality. They were found to be normally distributed according to Shapiro-Wilk test. It was found with One-way Anova whether there were differences between groups and with Tukey HSD test between which groups (control, cold storage for 5, 10, 15, 20 days). Paired comparisons between the 'first' and 'second' weighing of the control group and cold storage groups were determined with Independent samples t test ($P < 0.05$ was considered as significant).

RESULTS

In the control group, there was a statistical difference in the 'first weighing' (Control 1) from day 5 ($F_{8,260}$= 5.188, P<0.001, Tukey HSD), in the 'second weighing' (Control 2) from day 15 ($F_{8,260}$= 14.373, P<0.001, Tukey HSD) (Figure 1). Although there was no difference in the cold storage group (CS1 and CS2, Tukey HSD), a difference was found between 'first weighing' and 'second weighing' (F= 0.290, df=58, P= 0.009, Independent t test) (Figure 1).

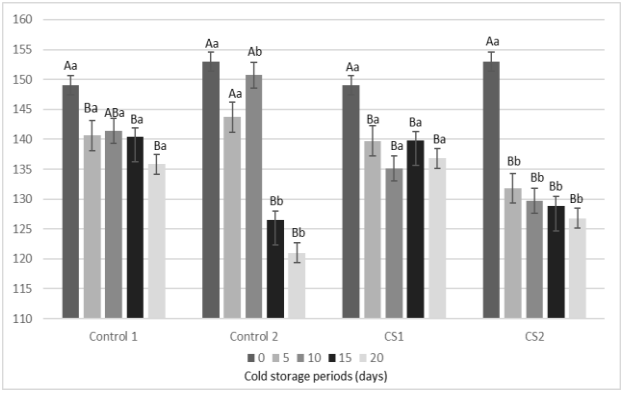

Figure 1. The effects of different periods of cold storage on larval weight before trial and after cold storage (mg). There is no statistical difference between the same uppercase and lowercase letters. Uppercase letters were used for comparison of days 0, 5, 10, 15 and 20 (Tukey HSD). Lowercase letters were used for 'first' and 'second' weighing groups (for exp. comparison of day 15 control group and day 15 cold storage, Independent t test). Control 1, Control 2, CS1: Before cold storage, CS2: After cold storage.

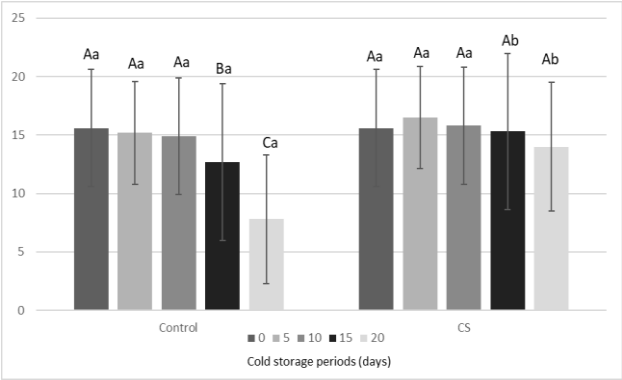

Figure 2. The effects of different periods of cold storage on total lipid amount of *Tenebrio molitor* larvae (mg). There is no statistical difference between the same uppercase and lowercase letters. Uppercase letters were used for comparison of days 0, 5, 10, 15 and 20 (Control and Cold Storage group, Tukey HSD). Lowercase letters were used for the comparison of control group day 15 and cold storage day 15 (Control/Cold Storage, Independent t test). C: Control, CS: Cold Storage.

Total lipid amounts of the control group were found to decrease depending on time (Figure 2). This shows that the control group uses lipids for their metabolic needs, perhaps making the required preparation for pupation. While total lipid amount was 15.6 ± 5.0 mg on day 0, it was found to be 7.8 ± 5.5 mg on day 20 ($F_{4,145}$= 35.688, P<0.001, Tukey HSD). Although there was no statistical difference in the cold storage group, there was a slight tendency to decrease on day 20 in total lipid amount ($F_{4,145}$=, P= 0.248, Tukey HSD). These values were found as 15.6 ± 5.0, 16.5 ± 4.4, 15.8 ± 6.4, 15.3 ± 4.8, 14.0 ± 4.1 on days 0, 5, 10 and 20, respectively. When the control group and the cold storage group were compared, a statistical difference was observed starting from day 15 (Figure 2) (F= 1.173, df=58, P<0.001, Independent t test).

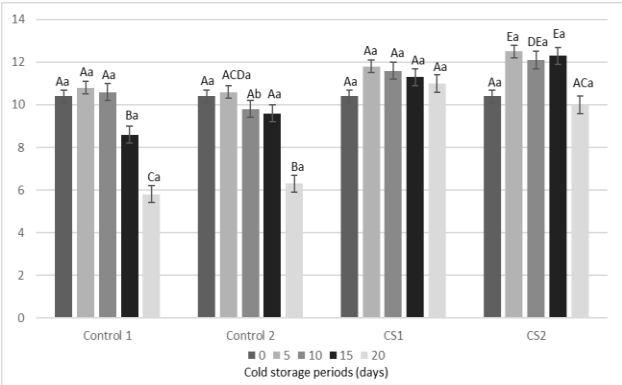

Figure 3. The effects of different periods of cold storage on total lipid percentage of *Tenebrio molitor* larvae (%). There is no statistical difference between the same uppercase and lowercase letters. Uppercase letters were used for comparison of days 0, 5, 10, 15 and 20 (Tukey HSD). Lowercase letters were used for first and second weighing groups (for exp. comparison of day 15 control group and day 15 cold storage, Independent t test).

In the 'first weighing', a statistical difference was found starting from day 15 ($F_{8,261}$= 24.887, P<0.001, Tukey HSD). No difference was found between the cold storage groups (CS1). Statistical difference was found in the 'second weighing' in control group. A decrease was found in total lipid percentage on day 20 in cold storage group ($F_{8,261}$= 31.009, P<0.001, Tukey HSD). No statistical difference was found when the first and second weighing were compared with each other (Figure 3) (F= 0.106, df= 58, P= 0.877, Independent t test). While lipid percentages decreased in parallel with the storage time in control groups (Control 1 and 2), a slight increase was found in the cold storage group on day 5, 10 and 15 (Figure 3).

DISCUSSION

The present study evaluated the effects of different periods of cold storage on the weights, total lipid amounts and percentages of *T. molitor* larvae. It was found that the weights of larvae in the control group decreased until day 20, while no difference was found in the cold storage group (Figure 1). In the control group, lipid use continued for metabolic needs. Resources required for survival and pup formation may have decreased depending on time. The differences between the 'first' and 'second' weighing in the cold storage group suggest that although the larvae slowed their metabolism in the cold, growth and development continued, even though partly. In addition, water loss, which is a method of adaptation to low temperature, might have caused a slight loss of weight. On the contrary, the fact that weights of *T. molitor* larvae stored in the cold for 5, 10, 15 and 20 days suggests that this group did not have enough time to use their reserves. In addition, it is known that insects slow their metabolism in the cold. This decreased metabolism is for protecting energy sources. In the control group, a time dependent loss of weight was seen although they were given food. This result shows that the control group used lipids for its metabolic needs and prepared for pupation, while on the other hand the cold storage group slowed their metabolism and tried to protect their existing sources. In a study conducted on *Cnephasia jactatana* (Lepidoptera: Tortricidae) by using three different temperatures, Ochieng-Odero, (1992) found that the females reared at 15 °C were heavier. The reason for this was explained with insects at 15 °C having a slower growth rate.

Edible insects have received increasing attention as a food product in recent years (Errico et al., 2021). They can be an alternative source of protein and lipid when compared with traditional sources of meat (Costa et al., 2020). The amount of lipid in insects varies depending on orders and species. In terms of dry matter, the highest total lipid amount was found in Lepidoptera and Coleoptera (up to 30%), while the lowest lipid amount was reported in Orthoptera and Odonata (less than 20%) (Dreassi et al., 2017). *T. molitor* larvae are generally rich in fat. Some insects store higher amount of lipid in the early stages of

life (Morales-Ramos et al., 2015; Paul et al., 2017). Jones et al., (1972) found total lipid percentage as 14.96% in their 98.66 mg *T. molitor* larvae. Mlček et al., (2019) found lipid ratio as 16.7% g by dry weight. These results are close to the results of the present study. In the present study, lipid amounts of day 0 control groups was found as 15.6 ± 5.0 mg and 10.4% (Figure 2 and 3). Interestingly, while the highest amount of lipid in the cold storage group was found as 16.5 ± 4.4 mg in day 5 group, the highest lipid percentage was found in the day 5 cold storage group as 12.5% ± 0.2. In the present study, while a decrease was found in the lipid amount of the larvae in the control group depending on time, no changes were found in the cold storage group (Figure 2). When we look at the lipid percentages, there is a slight tendency for increase in the cold storage group (Figure 3). This result shows that the lipids, which will facilitate survival in the cold and work as a source of energy, are protected and even increased (Azeez et al., 2014; Dooremalen & Ellers, 2010).

Panta & Gupta, (1979) found a significant increase in the protein, total lipid, phospholipid and triacylglycerol amounts of silkworm (*Philosamia ricini*) (Lepidoptera: Saturniidae) larvae at low temperature. In a study they exposed the larvae of Hepialus sp. (Lepidoptera: Hepialidae) insects to low temperature (0-3 °C), Zou et al., (2010) found that as unsaturated fatty acids increased in the total lipid content, saturated fatty acids decreased. They also reported that linoleic acid synthesis increased in larvae. In a study conducted with *Aedes aegypti* (Diptera: Culicidae) larvae, Sasmita et al., (2019) found that while the developmental period of larvae farmed at low temperatures decreased, energy reserves increased. The data of the present study also support the hypothesis that larvae increase their lipid reserves at low temperature, while they decrease energy consumption. However, it was found that with the prolongation of storage time, the larvae could not continue this resistance they developed.

Larval, pupal or adult developmental stages of the insect are also effective on this resistance (Irwin & Lee, 2003). They can store different amounts of lipid, protein or carbohydrates according to different developmental stages, or even if they are in the same developmental stage, large pupae, larvae or adult insects store more lipid (Scaccini et al., 2019). Therefore, the insect has more energy reserve and can easily overcome these extreme conditions (Irwin & Lee, 2003; Sinclair & Marshall, 2018). In a study conducted at very low temperatures in Mid-Western North America, it was found that *Eurosta solidaginis* (Diptera: Tephritidae) spent less energy when compared with those insulated under a layer of snow (Irwin & Lee, 2003). It has been found that Woolly Bear Caterpillar (*Pyrrharctia isabella*) (Lepidoptera: Noctuoidae), which is a freezing-resistant species, suppresses lipid consumption and metabolic speed in extremely low temperatures (Marshall & Sinclair, 2012). The lipids stored in larval period were found to continue their presence until adult (Aguila et al., 2007). These results show that protecting energy and lipids is one of the most important components of the success of surviving in low temperatures. In a study which examined the fatty acid compound of membrane and storage lipids of two different life stages (larvae and adult) of *Orchesella cincta* (Collembola: Entomobryidae) at low temperature, it was found that adult biomembranes had much more fatty acid compound than the larvae (Dooremalen & Ellers, 2010). The ability to adjust lipid compound to changes in ambient temperature is an improved adaptation to literature.

CONCLUSIONS

In this study, of the hypotheses, only the hypothesis that weight of larvae would not change with the prolongation of cold storage period was supported. With the prolongation of storage period, a slight decrease was found especially in the total lipid percentages of insects stored for 20 days. According to these data, the physiological adaptations developed by the insect did not show resistance to prolonged storage period. In the total lipid amount and percentages of cold storage groups, it was found that especially the insects stored on days 5, 10, and 15 slowed their metabolism, increased or kept their existing sources of energy. There was a slight tendency to decrease in total lipid amount and percentages only after day 15. It is an important result that the total lipid amount and percentages tended to decrease as of day 20 for mass producers. Considering that the amount of lipid started to decrease after day 15 and the quality will decrease in terms of the lipid amounts they included, it is recommended not to store these larvae for periods longer than 15 to 20 days. For this reason, future studies should store in longer periods of time and compare the total lipid amount in different developmental stages (for exp. pupa and adult). It will be useful for understanding how they react to low temperature at which stage and to understand the physiology of the insect. However, considering that they keep their lipid content until day 15, it can be thought that *T. molitor* larvae stored up to 15 days can be used as live feed and even become a source of lipid for humans in the future.

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