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Effects on oxidative stress and some non-enzymatic antioxidants of male crayfish (*Pontastacus leptodactylus*) in moulting period of trout diet.

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The aim of this study was to investigate the effects of trout and control diet on malondialdehyde (MDA), vitamin E (VE), vitamin C (C), vitamin A (VA) and beta carotene (βC) levels in tissues (hepatopancreas, muscle and gills) of *Pontastacus leptodactylus* during moulting period. The control diet was formulated to contain approximately 37.0% crude protein, 7.6% crude fat. The trout diet was obtained from the feed factory as ready-made. The crude protein content of trout feed was 53.0% and crude fat content was recorded as 15.0%. The experiment was carried out with 3 replicates for each dietary treatment. 15 males were used for each replicate (90 females in total). Statistically significant were detected that the levels of MDA increased in the hepatopancreas, muscle and gills tissues of crayfish fed with trout diet according to control diet. In this study, VE, VA, VC and βC levels were found to be lower in trout diet group compared to control diet group. In addition, toward the end of the study, although the crayfish during molting died in trout diet group, no deaths were recorded in control diet group.

Key words: *Pontastacus leptodactylus, MDA, Vitamin E, A, C, β-caroten*

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1. Introduction

Lipid peroxidation (LPO) is naturally produced by the reactive oxygen species (ROS) during the survival of organism. This peroxidation, biggest indicator of OS, is a non-enzymatic chain reaction based on oxidation of mainly unsaturated fatty acids and is associated with the presence of ROS. It leads to the creation of lipid peroxides and other intermediates. These intermediates may influence the properties of cell membranes and their physiological functions. The most common of these intermediates are malondialdehyde (MDA) and 4-hydroxynonenal. During the biochemical events in organism, to cope with the continuous generation of ROS, there are antioxidants that consist of enzymatic antioxidants and non-enzymatic antioxidants. Especially, the key antioxidant players in this antioxidant defense system include superoxide dismutase, catalase, glutathione peroxidase, vitamin E, β-caroten and so on. In a healthy body, ROS and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs [1-4]. During normal life, reactive oxygens are always produced during metabolic activities. Depending on various factors, some organism is less affected by these events. But, aquatic organism are more susceptible to the attack of ROS according to other aerobic organisms, because they have rich source of polyunsaturated fatty acid lipids [5]. Studies have shown that the antioxidant defences and oxidative stress in these organism can be affected by several stressors, including intrinsic (age and phylogenetic position, reproduction, feeding habits, etc) or extrinsic (salinity and temperature changes, pathogens, starvation, etc.) factors [6-8]. The induction of antioxidant defence enzymes may provide sensitive early-warning signals of incipient oxidative stress conditions. These enzymes can be induced by various environmental pro-oxidant conditions (such as pollution), endogenous/exogenous factors (such as age, reproductive, moult, diet and temperature variations) [9,10]. In crayfish, the exoskeleton (shell) is a support organ and acts as a protective armor against external factors. This hard

layer, consisting of a mixture of chitin and lime, completely covers the body. It is seen that the armor is thin in the joint area and thickens in the other parts of the body. The crust consists of four layers. Chemical analysis of the shell revealed chitin, calcium carbonate and calcium phosphate. When this shell, which forms the skeleton of the crayfish, hardens, growth stops. Growth is made possible by the falling of the bark. After the old shell completely falls off, a soft new shell that has previously developed emerges under this shell. This event is called "shell switching" [11-13].

In crayfish, the body is covered by the cuticle and growth is possible only by molting. Moulting period is a process involving the formation of gastrolith and growth of internal organs and tissues, change of the animal's outer shell. The crayfish change their shell 8 times in the first year and reach a length of 5 cm, length of 8 cm in the second year changing the shell 5 times, they reach length 10-12 cm in the third year. Females molt once and males twice a year when they reach sexual maturity. Moulting success in crayfish is vital for sustaining an aquaculture system. One main factor affecting the moulting in crayfish in aquaculture organism is the condition. Crayfish condition is largely affected by their nutrition and environmental conditions. It is very important to investigate the biochemical changes in this period in order to create the conditions suitable for the natural environment of the crayfish during the moulting period. Nutrition is one of the biggest and inevitable metabolic activities of organisms. The diet content during feeding causes the change of oxidative stress depending on the interaction of many biochemical parameters of the organism [11,13-15]. For this reason, it is of great importance to feed the organism with feeds that will meet the vital period and needs of the organism.

Crayfish are collected in the Decapoda order of the Crustacea (crustaceans) class of the Arthropoda phylum [12,16]. As a result of the genetic research, it was reported that this species in Turkey is *Pontastacus leptodactylus* [17]. Crayfish can be obtained by catching and aquaculture in the world. According to the aquaculture statistics data, the amount of crayfish, which cannot be farmed in Turkey, obtained by catching was determined to be 1233 tons in 2020 and 1011 tons in 2021 [18]. It is widely distributed in lakes, pounds and rivers in many parts of Turkey. This species has commercial importance in Turkey and were exported to a number of European countries until 1986. The production of *P. leptodactylus* after 1985 decreased dramatically (from 5000 tonnes annually to 200 tonnes) in most Turkish lakes as a result of the crayfish plague, over-fishing, water pollution, and water withdraws for agricultural irrigation [19]. For these reasons, crayfish especially, broodstocks have to be fed with good quality diet for growth, production and reproduction. So the knowledge of how the biochemical and physiological systems changes during the mating period of *P. leptodactylus* populations are require for this good quality diet.

The current study aimed to determine the resistance to the trout diet of male *P. leptodactylus*. In the meantime, it was explored the effect of the trout diet on oxidative stress (malondialdehyde (MDA)), the vitamin A, E, C and β-carotene levels in the tissues (hepatopancreas, gills, muscle) of male *P. leptodactylus* during moulting period.

2. Experimental

This investigation was carried out in the crayfish reproduction unit of Aquaculture Faculty of Fırat University, Elazığ, Turkey. It was conducted between April 15 and May 20. The crayfish used in the present study was provided from Keban Dam Lake population of *P. leptodactylus*.

Control diet used in this study (Table 1) was modified after Barim [19]. This diet was formulated to contain approximately 37.0% crude protein, 7.6% crude fat, The ingredients for diet were thoroughly mixed, before adding water, in a commercial food mixer, cold-pelleted by forcing through 3-mm holes using a laboratory pellet mill, air-dried at 55 ^{o}C for up to 24 h, and then stored in a refrigerator at 4 ^{o}C until further use.

The trout diet used in the study was obtained from the feed factory as ready-made. This diet contain fish meal, soybean meal, fish oil, corn gluten, wheat flour, vitamin and mineral premix. Crude protein content of trout feed was 53.0% and crude fat content was recorded as 15.0%. This diet included Ca (2.5%), P(1.7%), Na (0,5%), vitamin A (24.000 IU kg^{-1}), vitamin D3 (5000 IU kg^{-1}), vitamin E (500 mg kg^{-1}), vitamin C(400 mg kg^{-1}).

Table 1. Composition and proximate analysis of the control diet [19].

Ingredient	Percent of dry weight
Fish (anchovy) meal	35.78
Soybean meal	38.64
Wheat flour	19.30
Sunflower oil	4.00
Dicalcium phosphate	1.00
Sodium phosphate	0.40
Avilamycine[1]	0.10
Antioxidant [2]	0.10
Vitamin premix [3]	0.50
Mineral premix [4]	0.18

(1) Kavilamycine
(2) Antioxidant (mg/kg dry diet): butylated hydroxytoluene 12.5.
(3) Vitamin premix (IU or mg/kg): vitamin A 2,000,000 IU, vitamin D_3 200,000 IU, vitamin E 20,000 IU, vitamin K 3,000 mg, vitamin B_1 1,000 mg, vitamin B_2 3,000 mg, Niacin 30,000 mg, Calcium D-Pantothenate 10,000 mg, vitamin B_6 2,000 mg, vitamin B_{12} 4 mg, Folic Acid 600 mg, D-Biotin 200 mg, Choline Chloride 100,000 mg and vitamin C 60,000 mg.
(4) Mineral premix (mg/kg dry diet): Mn 80, Fe 35, Zn 50, Cu 5, I 2, Co 0,4, Se 0,15.

The experiment was carried out with 3 replicates for each dietary treatment. 15 males were used for each replicate (90 males in total). Crayfish were housed in tanks (2 x 2 x 0.5 m). Plastic pipes (20 cm in length and 7 cm in diameter) were provided as shelters for the crayfish. *P. leptodactylus* were acclimatised to

temperature and flow conditions and starved for one week to standardize their nutritional conditions and to ensure that they were in good health prior to the start of the experiment. After one week, crayfish were weighed and were fed 2 % of their total wet weight daily, divided into three separate feedings [19]. Supplemental water flow was 0,5 l/sec for each tank. During the trial, mean dissolved oxygen was 7,34±0,25 mg/L; mean pH was 7,18±0,06; mean water temperature was 13,76±1,12 ºC.

The crayfish selected from each replicate were placed on ice in plastic bags and transported to the laboratory. Sample of crayfish taken from each of the two dietary treatments was randomly selected for analysis. The weight (g) were recorded. The tissues of the crayfish for biochemical assays were surgically removed and stored at -80^0C.

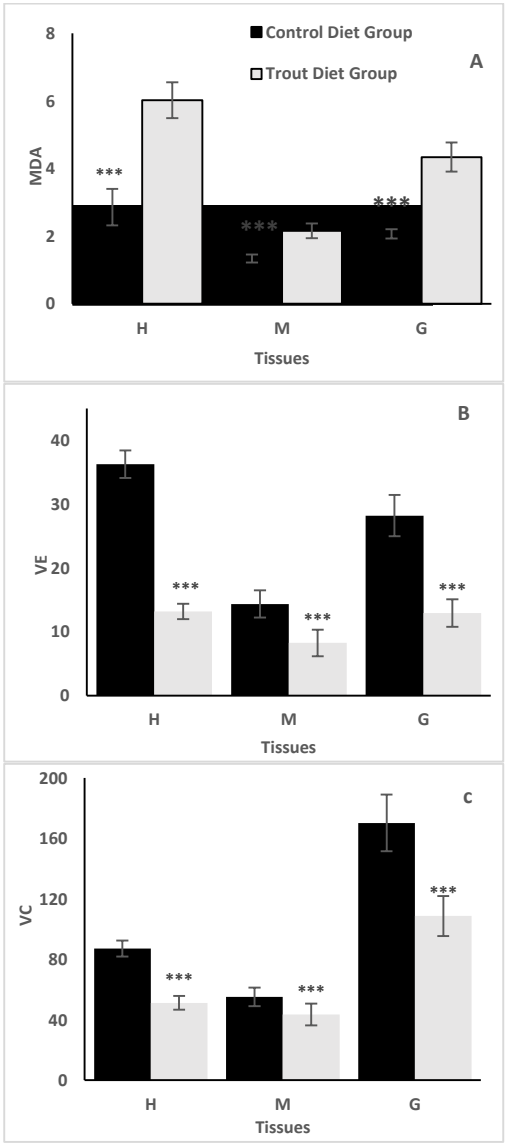
All analyses for determination of tissue βC, VA and VE levels were carried out on three aliquots, ranging from 200 to 1000 mg weight. Samples were homogenized in a glassglass homogenizer in 1 ml of cold acetone. Homogenized samples were transferred into polyethylene tubes and 2 ml ethanol was added to the tubes. After 0.3 ml n-hexane was filled into tubes for vitamins extractions, they were centrifuged. This step was repeated two times. N-hexane in tubes was evaporated using the nitrogen. Then the residues were solved in mobile phase (methanol: acetonitrile: chloroform; 47: 42: 11, v/v) [20]. The crayfish tissue samples (1,0 g) were homogenized in a glass-glass homogenizer in mixture of 0.5 ml of HClO4 (0.5 M), 4.5 ml distilled water and 100µl-500 ppm butylated hydroxytoluene (BHT) for determination of tissues VC and MDA. Then, the samples were centrifuged at 4500 rpm for 5 min and supernatants were injected into HPLC system. Addition of acid was necessary to precipitate proteins and release the MDA bound to the amino groups of proteins and other amino compounds. Acid addition was also needed to maintain the stability of vitamin C [21,22].

All results are expressed as mean ± S.E. The data were analyzed with an Independent-Sample T Test SPSS 12.0 for Windows was utilized for statistical analysis. The level of significance was set at $p < 0.05$.

3. Results and Discussion

The mean weight of males crayfish among the experimental groups (control, trout diet) and within the replicates of each dietary treatments were not significantly different ($p>0.05$ for each cases) at the beginning of the experiment. The mean weight of male crayfish was 26.42±2.28 g for control diet group, 25.91±2.42 g for trout diet group.

In this study, the data obtained as a result of the analyzes were statistically evaluated and and given in graphic form (Figure 1 (A,B,C,D)). The results obtained in our study reveal that the VE, VC, VA and βC and MDA levels in hepatopancreas of crayfish in the trout and control diet groups was the most changed (Figure 1).



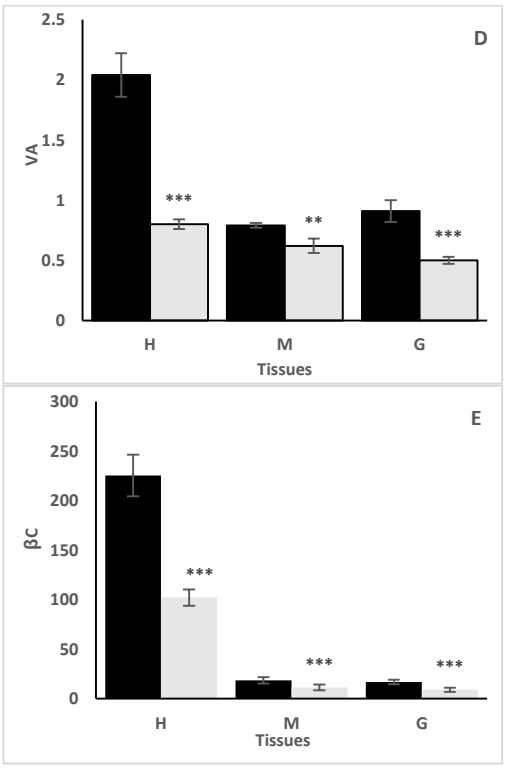


Figure 1. A, B, C, D, E: Comparison of the mean concentration of the tested MDA (µg/g) (A), vitamin E (µg/g) (B), vitamin C (µg/g) (C), vitamin A (µg/g) (D), beta carotene (µg/g) (E) in hepatopancreas (H), muscle (M) and gills (G) tissues of crayfish in the trout diet group and the control diet group. Significance between groups was shown as asterisk (*p < .05, **p < .01, ***p < .001).

As in all crutaceans, many changes occur in metabolic functioning during the moulting period in crayfish. Oxidative stress, especially expressed as lipid peroxidation, is one of the prominent parameters in this period. The cell and tissues injury caused by oxidative damage that can occur with high levels of free radicals or ROS is one of the consequences of uncontrolled oxidative stres. It is well established that the main ROS generated by cellular metabolism are the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2), siglet oxygen ($^1O_2$), and hydroxyl radical (HO^*), and these compounds can rapidly react to form other molecules like peroxyl radicals (ROO^*) and alkoxyl radicals (RO^*). Under basal conditions, the advarse effects of oxyradicals in living aerobic organisms are prevented by the antioxidant system. But, the antioxidant and detoxifying systems during high levels of radicals are deficient and not able to neutralise the active intermediates produced by xenobiotics and their metabolites that potential to cause toxin insults to cellular components such as lipids of biological membranes and proteins of enzymes. Lipid peroxidation which is the result of interactions of lipid radicals and/or formation of nonradicals species by ROO^* is used to be a valuable indicator of the oxidative damage of cellular companents [1,3,23,24]. Because of this reason, in the present study was investigated levels of MDA, as a secondary lipid peroxidation product. As a result of the analysis, that the MDA level in tissues increased due to moulting period.

Moulting influences all aspects of crustacean biology (cellular metabolism, physiology and behaviour). Metabolism is elevated because organic reserves such as mineral deposits, glucose, α-chitin-protein, gastrolith matrix protein, glycoprotein and ecdysteroids in tissues (especially hepatopancreas) and hemolymph are conversion and release [11,25]. Aiken and Waddy [11]. reported that tissue metabolism can elevate oxygen consumption by as much as 1900% during premoult. We found that the level of MDA was statistically higher in hepatopancreas according to the muscle and gills. Additionally, the MDA levels were higher in gills according to the muscle. These findings are in agreement with a previous observation that was made by Paital and Chainy [26] who found that the oxidative stress physiology markers were higher in hepatopancreas in comparison to gills and abdominal muscle of *S. serrata* in all seasons. Similarly, Verlecar et al. [27] determined that digestive gland is specific tissues in seasonal variation of ROS level such as H_2O_2 and lipid proxidation of *P. viridis*. It is known that crustacea hepatopancreas, the main digestive gland, contains fat-soluble vitamins, regulates the metabolism of the body and exhibits high oxygen consumption [26,27]. Thus, the generation of O_2^{-*} and H_2O_2 can be comparatively more in this organ than other organs. Yudkovski et al. [28] determined that during late premoult stage occured up-regulation of genes and three additional gene changes effecting oxidative stress in gastrolith disc. This increase in MDA levels could be related to direct damage to biological molecules and tissues of excessive free radical generation due to an increase of the physiological activity caused primarily by the varying metabolic activity during moulting because those described above.

The balance between the production and accumulation of ROS during moulting of decapod crustaceans is affected by ecdysteroids in the endocrine control. Ghanawi and Saoud . [25] reported that ecdysteroids synthesized by the Y-organ and under negative regulatory control of moult-inhibiting hormone (MIH) are primarily involved in regulating the moulting process (ecdysis) of crustaceans. It has been emphasized that in level of ecdysteroid occurs also the increases or decreases in periods of moulting. Ecdysteroid responsive moult-related genes. Some of these ecdysteroid-responsive genes, particularly in the hepatopancreas, revealed differentially expressed genes encoding metabolic and transport enzymes. Epidemiological studies proved genetic variation in the endogenous and exogenous antioxidant defense systems may affect enzymatic antioxidant activity. It was determined that the damages of the mitochondrial membranes and protein structure occurred with effect of variation in the genes coding antioxidant enzymes formed by changes in level of ecdysteroid can, at its turn, enhance ROS [29,30]. In our study, MDA level changed according to tissue characteristic. These changes in levels of analysed parameters can most likely be ascribed to fail in manage and prevent of ROS species in the mitochondria with to be broken of exposure level of enzymes to target cells because of changes in level of ecdysteroid [25,29-31].

It was found in the results of the current study that the levels of MDA increased in the hepatopancreas (110.48%), muscle (61.19%) and gills (109.66%) tissues of trout diet group crayfish according to control diet group crayfish. Many studies have been carried out on the nutritional needs of crayfish. As a result of this study, it was determined that the high fat content in the diets negatively affected the growth of crayfish. For example, Ackefors et al. [32] *Astacus astacus* crayfish in different proportions protein (22,31.40%), carbohydrates (9.2-25.8), fat (5.5-16) and P/E (19-153 mg/day) kcal) have investigated the evaluation of 12 diets. In this study, crayfish with an initial mean body weight of 146±5 mg were fed at a water temperature of 18.3-19.6°C for 394 days. At the end of this period, it was found that crayfish fed with feed containing 40% protein showed approximately 3% live weight gain every day, the optimum P/E ratio was 114-123 mg/kcal, and high fat content (13-16%) caused slower growth and survival rates. Jussila [33] *C. tenuimanus* (11.5±0.3 g) crayfish with diet containing 30% protein, 10% fat, *A. astacus* (21.2±0.5) with food containing 48% protein, 22% fat g) and *Pasifastacus leniusculus* (31.5±0.9 g) crayfish were fed at 22-24 °C water temperature with 6-10% of their body weight weekly for 125-182 days, and their growth performance and HSI were examined. At the end of the research, they determined that high fat content caused slowing in growth. Fotedar et al. [34] crayfish (*C. tenuimanus*, mean starting weight 1.99±0.09 g) with three different feeds containing 25.57% crude protein, 0.28%, 6.28% and 12.28% fat fed for 108 day. At the end of this period, the mean live weight of the crayfish at the end of the trial was 6.34±0.72 g, 5.03±0.55 g, 3.54±5 g for feeds containing 0.28%, 6.28% and 12.28% fat, respectively. They determined SBO as 1.07±0.06, 0.82±0.07, 0.61±0.09 and the high amount of fat added to the crayfish feeds would cause a slowdown in growth. In addition, Hajra et al. [35] fed shrimp (*P. monodon*) with an average weight of 0.510 g with feeds containing different ratios of protein (45.10-46.90%) and fat (25.90-33.0%). As a result of the study, the average weight of the crayfish fed with diet containing 46.30% crude protein and 10.60% fat, which was determined to be the best diet, was determined as 1.3376 g. In the light of these studies, high fat content (%24) in trout feed according to control diet (15.0%) may cause high MDA levels. Furthermore, as a result of feeding crayfish with diets containing high fat content, they cannot utilize this fat with metabolic activities. Thus, the LPO level and oxidative stress may increase with radical as the generation of O_2^{-*}.

In this study, it was observed that the mortality rate (1st repeat group: 46.66%, 2 st repeat group: 53.33%, 3 st repeat group 40.00%) of crayfish in the trout diet group increased. Therefore, the work was stopped. However, there was no death in the crayfish in the control group. It was also observed that the crayfish in the trout diet group died during molting. . It may indicates an the results of excessive production of O2 *-generation in tissues due to increasing metabolic activity and during moulting period [31]. This may be attributed to the increase in oxidative stress as a result of the evaluation of high fat content in the diet in the digestive system.

Vitamin E, VC, VA and βC, has a fundemental role in the normal metabolism of all cells, provides protection against the potentially damaging effects of reactive species of oxygen formed during metabolism or that are encourented in the environment [36]. On the other hand, there have been not many comparative studies on the relationship among oxidative stress, VE, VC, VA and βC levels of aquatic organisms. In a study, the effects of oxidative stress (as pollution) on VE, VC, VA, βC contents and MDA level of *A. leptodactylus* has been documented by Barim and Karatepe [36]. Barim and Karatepe [36] found that the VE, VC and βC levels in the muscle and hepatopancreas decreased with stress of the pollution, and MDA levels increased. The relationship between different storage temperatures, starvation, reproduction with these vitamins has been determined in the studies [36-39]. Barım-Öz et al. [31]. determined that Vitamin E, VC, VA and βC levels in tissues during moulting period decreased. Similarly, in the present study observed that the vitamin E, VC, VA and βC levels in tissues of crayfish in the trout diet group lower than control diet group. The MDA level showed the opposite ratio. These decreases in the tissues VE, VA and βC levels can be attributed to the inhibition of radical formation or the potential free radical scavenging activity of measured parameters.

VE is potent antioxidants with lipoperoxyl radical-scavenging activities. Lipid peroxyl radicals react approximately 10^5 times faster with α-TOH than with PUFA. The one molecule of α-TOH can protect approximately 1000 molecules of PUFA against oxidation. These finding are corroborated by in vivo studies. In many studies have been determined that the requirement of VE increases at high levels of unsaturated lipid [31,40,41]. The data from the present study indicated that the level of VE in the hepatopancreas (63.65%), muscle (42.54%) and gills (54.09%) was statistically lower in those crayfish of trout diet groups compared with control (Figure 1B). These decreases in the tissues can be most likely responses to increased ROS generation because of high of lipid and fatty acid levels during moulting period.

Vitamin C or L-ascorbic acid is considered to be the most powerful water-soluble antioxidant in extracellular fluids. Especially, this vitamin can protect biomembranes against peroxidative damage by trapping peroxylradicals in the aqueous phase before they can initiate [1,30]. In this study was determined that the decreases in the tissues (hepatopancreas; 41.26%, muscle; 21.31%, gills; 36.18%) VC levels occured in the crayfish of the trout diet group (Figure 1C). Vitamin C can also act to protect membranes against peroxidation by enhancing the activity of tocopherol, chain-breaking antioxidant. With in vitro studies it was found that ascorbic acid reduces the tocopheroxyl radical and thereby restores the radical-scavenging activity of tocopherol.

Vitamin A (retinol) is one of the fat soluble vitamins, works as a good antioxidative agent as it neutralizes the free radicals [42,43]. Li et al. [43]. determined that VA status and its metabolism in the fasting and refeeding cycle contribute to the regulation of the expression of genes in hepatic glucose and lipid metabolism in rats. In the current study was found that the levels of VA

in the trout diet group significantly decreased in hepatopancreas (60.78%), muscle (21.51%) and gills (45.05%) according to control group (Figure 1D). The decrease in the VA levels are most likely a response to the increased ROS generation induced by starvation. In many studies was reported that the carotenes are converted to VA as it is essential for the epithelial cells differentiation [42,31]. The antioxidant activity of carotenoids when reacting with the O_2^{-*} anion through oxidation of this radical to oxygen arises in the capacity to prevent the formation of peroxides (H_2O_2/O_2^{2-}) and other reactive oxygen species such as HO^* derived from peroxides through Fenton-type reactions [31]. The data presented here indicate that βC levels in hepatopancreas (54.67%), muscle (38.16%) and gills (46.27%) tissues of crayfish in trout diet group decreased according to control (Figure 1D).

In this study, the decreases in VE, VC, VA and βC levels in tissues of trout diet group according to control diet groups may be a consequence of i) differentiation oxygen concentration in the tissues with the change of oxygen values in the plasma during glycogenolysis or gluconeogenesis under moulting stres that causes an excessive reactive oxygen species generation ii) release of these non-enzymatic antioxidants (VE, VC, VA and βC) from plasma and PUFA in tissues to prevented of LPO that occurs during moulting [31, 44].

4. Conclusions

This investigation was planned to give a concrete answer to the question of whether crayfish (*P. leptodactylus*) can be grown with trout diet. For this, the crayfish were fed with trout diet obtained from the factory as ready-made and a control diet prepared for the needs of the crayfish. The results of the study illustrated that the levels of MDA increased at a high level in the hepatopancreas, muscle and gills tissues of trout diet group crayfish according to control diet group crayfish. But, VE, VC, VA and βC levels decreased. In addition, toward the end of the study, although the crayfish during molting died in trout diet group, no deaths were recorded in control diet group. For these reason, it was concluded that crayfish farming cannot be done with trout diet.

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