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Original Research

Oxidative stress and some biochemical parameters during starvation and refeeding in *Astacus leptodactylus* (Esch., 1823)

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Abstract: This study was conducted to determine the effect of starvation (78 days) and refeeding (33 days) on oxidative stress (malondialdehyde (MDA)), antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GSH-Px)), arginase (AR) and nitric oxide (NO) in the hepatopancreas, muscle and gills tissues of freshwater crayfish (*Astacus leptodactylus*). Crayfish were maintained at three experimental groups: control (fed), starved (not fed) crayfish for 78 days and refeeding crayfish for 33 days after 78 days starvation. The biochemical analysis in the tissues were measured at 3, 18, 33, 48, 63 and 78 days of starvation and feeding and at 3, 18 and 33 days of refeeding. In the all periods, the MDA levels were significantly higher in tissues of starved crayfish than the control ones. Significant changes in the MDA levels were also observed amongst starvation times. In the tissues of starved crayfish was significantly higher SOD, CAT, GR and AR activities when compared to control crayfish. The GSH-Px and NO activity showed increases and decreases in different starvation periods according to control group. The parameters measured returned to control values after 33 days of the refeeding. Collectively, the present study indicated that starvation induces MDA, SOD, CAT, GSH-Px, GR, AR and NO activity in the tissues, and *A. leptodactylus* exposed to starvation can be withstand 78 days.

Key words: Crayfish, Starvation, Enzymatic Antioxidants, Oxidative stress.

Introduction

The reactive oxygen species (ROS) are naturally produced during the survival of organism. But, ROS is highly deleterious because of cytotoxic oxidants at pathological levels. 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 (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR). 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, 2). The biggest indicator of OS is lipid peroxidation (LPO). 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 (2, 3).

Arginase (AR) and nitric oxide (NO) is one of the changing parameters when the OS in the organism increase. Arginase (L-arginine amidinohydrolase, EC) catalyses the hydrolysis of L-arginine to form urea and L-ornithine in the final reaction of the urea cycle (4). Nitric oxide is produced from arginine by nitric oxide synthase (NOS). Arginine is the sole substrate of NOS (4,5).

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, feeding habits, etc) or extrinsic (salinity and temperature changes, pathogens, starvation, etc.) factors (2, 6, 7). Starvation or undernutrition during life cycle of aquatic organisms can occur either in the natural environment and under culture conditions due to spatial and temporal patchiness of food resources. Because food is one of the most important factors influencing life cycle of organisms, starvation studies may be useful to understanding of the nutritional physiology, metabolic requirements and biochemistry analysis (8, 9, 10). Furthermore, the capacity to withstandand recover from nutritional stress or starvation is an important for maintaining the population (9).

Studies on the effects of starvation has been documented in fish (7, 10, 11) crustaceans (6, 9, 12-16), cephalopod (17, 18) and gastropoda (8). In these study was generally determined that starvation effects growth (11, 14), oxidative stress parameters (7), some antioxidant indexes (14), oxygen consumption and metabolic enzymes (15), blood biochemical parameters (10), immune system (13), protein (6, 17), lipid and fatty acids (8, 12, 16, 18) and reproduction (19) of aquatic organisms. At present, however, the effects of starvation in *Astacus leptodactylus* have yet to be investigated.

The narrow-clawed crayfish (*Astacus leptodactylus*) is among the most important crustacean species in the Turkey. This species is seen in 27 countries and it is also a cultured species. Because of crayfish plague (*Aphamomyces astaci*), overfishing and pollution in the Turkey, the total harvest of the crayfish reduced dramatically (from 5000 tons annually to 200 tons) after 1985 (20,

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21).

The current study aimed to determine the starvation resistance in *A. leptodactylus*. In the meantime, it was explored the effect of starvation on oxidative stress (MDA), the antioxidants (SOD, CAT, GR and GSH-Px), AR and NO in hepatopancreas, gills and muscle of *A. leptodactylus* during starvation and refeeding.

Materials and Methods

This study was conducted between July 25, 2015 and Nowember 22, 2015 (111 days) at the crayfish reproduction unit of the Fisheries Faculty of Fırat University, Elazığ, Turkey. The adult female crayfish (*A. leptodactylus*) were collected from Aydıncık in the Keban Dam Lake. The study was conducted in accordance with European Union Council Directives (2006/88/EC). This crayfish transported to the laboratory and acclimatized in the fiberglass tanks for 10 days ((July 25-August 03) at 20.06±1.39°C with pH of 7.56±0.14, a dissolved oxygen content of 7.64±0.21 mg L^{-1}. During this period, the crayfish were fed with control diet.

Chemicals, feed materials and crayfish

The chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Fish (anchovy) meal, soybean meal**,** sunflower oil, wheat flour, antioxidant, dicalcium phosphate and sodium phosphate was donated by a food producer firm (DSM Nutritional Products).

Feed preparation and experimental design

The control diet (Table 1) was formulated to contain approximately 39% crude protein, 8% lipid on a dry-weight basis and 3.30 kcal g^{-1} gross energy. Gross energy was calculated based on physiological fuel values of 9 kcal g^{-1} for lipid and 4 kcal g^{-1} for protein and carbohydrate; the crude protein content was analysed by Kjeldahl's method; dry matter after the sample was dried at 105 ^{o}C for 6 hours. Lipid analysed by an ether extraction method and ash content was calculated after 24 h at 550 ^{o}C in the furnace (22, 23).

The control diet was stored in a deep freeze at -4ºC until further use. Amount of food fed was determined according to the following equation (22) and divided into three separate feedings:

Daily food amount to feed (g food day^{-1}) = (water temperature °C/10) x (crayfish weight/100).

The experiment was carried out with 3 replicates for each treatment. After 10 days of acclimation, 30 active crayfish were used for each replicate (360 crayfish in total). In the tanks of control, starvation and refeeding group were placed 180 (90 for starvation control and 90 for refeeding control), 90 and 90 crayfish, respectively. Crayfish were housed in 12 fiberglass tanks (100 L x 95 W x 60 H cm) and a 12-h light/ 12-h dark photoperiod. Plastic pipes (25 cm in length and 7 cm in diameter) were provided as shelters for the crayfish. Adequate aeration was provided for each aquarium by a air pump.

The crayfish were divided into three groups as follow:

- Group 1, the crayfish were fed with control diet between August 04, 2015 and November 22, 2015 (111 days).
- Group 2, the crayfish were starved between August 04, 2015 and October 20, 2015 (78 days).
- Group 3, the crayfish were refeeding with control diet between October 21, 2015 and Nowember 22, 2015 after starved for 33 days.

Table 1. Composition of the control diet.

Ingredients	Percent of dry weight
Fish (anchovy) meal	35.78
Soybean meal	37.72
Wheat flour	19.40
Sunflower oil	4.00
Dicalcium phosphate	2.00
Sodium phosphate	0.40
Astaxanthin	0.02
Vitamin premix [1]	0.50
Mineral premix [2]	0.18

(1) 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.

(2) Mineral premix (mg kg^{-1} dry diet): Mn 80, Fe 35, Zn 50, Cu 5, I 2, Co 0,4, Se 0,15.

The carapace length and weight of crayfish among the experimental groups and within the replicates of each treatments were not significantly different ($p>0.05$ for each cases) at the beginning of the experiment. The carapace length and weight of crayfish was 17.61±1.76 g wet body, 40.58±0.65 mm carapace length for control group, 18.27±1.94 g wet body, 40.92±0.84 mm carapace length for starved group and 18.01±1.89 g wet body, 40.72±0.91 mm carapace length for refeeding group, respectively.

Starvation time was chosen based on the recorded starvation time of previous studys (1, 7, 8, 9, 14, 24, 25, 26). Starvation periods tested were 3, 18, 33, 48, 63 and 78 days. Refeeding periods tested were 3, 18 and 33 days.

Dissolved oxygen, pH and water temperature were measured daily in the tanks of control (7.49±0.84 mg L^{-1}, 7.59±0.17 and 20.12±1.99° C, respectivelly), starved (7.98±0.58 mg L^{-1}, 7.45±0.17 and 19.69±1.29° C, respectivelly) and refeeding (7.65±0.31 mg L^{-1}, 7.52±0.11 and 20.40±1.17° C, respectivelly) groups.

The crayfish died during the study were saved and removed from tanks.

Sample collection and preparation

The hepatopancreas, muscle (in abdomen) and gills tissues for analysis were washed in ice-cold physiological saline, and stored at -80 °C until the biochemical assays, which were performed within 15 day after extraction. These tissue were homogenised in a Teflon-glass homogenizer in buffer containing 1.15 per cent KCl at a 1:10 (w/v) ratio to the whole homogenate. The homogenate was centrifuged at 18.000g and 4 °C for 30 min before analysis.

Biochemical analyses

The levels of MDA, as indices of the LPO in all tissues, were measured with the thiobarbituric-acid reac-

tion using methods described in Placer et al. (27). The quantification of thiobarbituric acid reactive substances was determined through a comparison with the standard curve of MDA equivalents generated by the acid catalysed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of MDA were expressed as nmol g^{-1} tissue.

The SOD activity was determined using the method described by Sun et al. (28), which is based on the principle that xanthine reacts with xanthine oxidase to generate superoxide radicals that react with nitroblue tetrazolium to form a coloured formazan dye. To analysethe SOD activity, 600 µL of the SOD reaction mixture containing 0.1mM EDTA, 0.1mM xanthine, 25 µmol L^{-1} of nitrobluetetrazolium, and 50mg of bovine serum albumin was added to125 µL of the supernatant or 125 µL of an SOD standard solution. Then, 25 µL of 9.9 nM xanthine oxidase solution was added to each tube at 30-s intervals. The tube was incubated for 20 min at 25 °C. Then, the reaction was terminated by the addition of 0.5 mL of 0.8 mM CuCl2 solution every 30 s. The amount of formazan was determined by measuring the absorbance at 560 nm with a spectrophotometer. The results of this enzymatic assay are provided as U ml^{-1}, where one unit of SOD is defined as the amount of sample causing 50 per cent inhibition of NBT reduction.

The CAT activity was measured by measuring the decrease in the hydrogen peroxide concentration according to Aebi (29). The reaction contained 50 mM potassium phosphate buffer (50 mM, pH 7.0; prepared by mixing 1.335 g of Na_2HPO_4-$2H_2O$ in 150 mL and 0.681g of KH_2PO_4 in 100 mL) and 10 mM H_2O_2 (as substrate) and was started by the addition of the sample. This activity was assessed by measuring the absorbance at 240 nm over a period of 3 min with a spectrophotometer. The results of this enzymatic assay are provided as k mg^{-1} of protein.

The GSH-Px activity was determined according to the method described by Matkovics et al., (30), which the oxidation of GSH by H_2O_2 was coupled to NADPH oxidation in the presence of exogenous glutathione reductase to maintain substrate concentration. The reaction contained 50 mM HCl buffer (pH 7.0; prepared by mixing 6.057 g tris-hydroxymethil-aminomethane, 0.372 g EDTA-Na_2 and 3.90 mL HCl in distilled water), reduced glutathione (6 mg in HCl buffer) and cumene-hydroperoxide (5 µL in HCl buffer) was started by the addition of the sample. The tube was incubated for 10 min at 37 °C. The sample was centrifuged at 2500g for 5 min. Then, the tube was added trichoroacetic acid (1 mL), 0.4 M tris buffer (2 mL) and 5,5-dithiobis-2 nitrobenzoic acid (1 mL). The absorbance at 412 nm was recorded over a period of 5 min. The results of this enzymatic assay are provided as U g^{-1} protein.

The GR activity was measured using the method described by Smith et al., (31), which proportional to the amount of the 2-nitrobenzoic acid (TNB) formed by the reaction of a mole GSH with DTNB (5,5'-dithiobis 2-nitrobenzoic acid) within 2 minutes. The reaction contained 0.2 M fosfat tamponu (pH 7.5; prepared by 1 mM EDTA), 3mM DTNB (pH 7.5; prepared by 100 ml 0.01 M fosfat tamponu), 2mM NADPH and 20 mM GSSG. The absorbance was determined at 412 nm with a spectrophotometer. The results of this enzymatic assay are provided as U g^{-1} protein.

The AR level was determined spectrophotometrically in the optimized conditions for crayfish Hartenstein (32) by the thiosemicarbazide diacetylmonoxime urea (TDMU) method of Geyer and Dabich (33) one unit of arginase activity was expressed as the amount of enzyme catalyzing the formation of one mmole of urea h^{-1} at 37ºC. The results are provided as units mg^{-1} of protein.

The NO activity was measured using the method described by Lyall et al. (34) method. NO measurement is very difficult in biological specimens, because it is easily oxidized to nitrite (NO_2) and subsequently to nitrate (NO_3) which serve as index parameters of NO production. Samples were initially deproteinized with NaOH and ZnSO4. Total nitrite (NO_2+NO_3) was determined by spectrophotometer at 545 nm after conversion of NO_2 to NO_3 by assay reactive. A standard curve was established by a set of serial dilutions of sodium nitrite. The results are provided as µmol per gram tissue.

The protein levels in the tissues were found by the method described by Lowry et al. (35).

Statistical analyses

All results were expressed as the means±standard deviations. The data were analyzed with an One-Way Anova (Duncan) and Independent-Sample T Test using the SPSS 21 computer programme (SPSS). P-values<0.05 were considered to be statistically significant.

Results

No mortality was recorded in the control group. But, in the starved groups died 10 crayfish in total on 53, 66, 70, 72, 75, 76 and 78 th days. For this reason, it was started feeding in the crayfish of refeeding groups after 78 th day. In the refeeding group died 7 crayfish in total in the first week (1, 3 and 4 th days), then death did not occur.

The data obtained from the study results was shown in Figure 1-2. Different feeding time in the control group, there were no significant differences in the specific activities for any of the oxidative stress and nonenzymatic antioxidants measured in the hepatopancreas, muscle and gills of *A. leptodactylus*. But, the levels of MDA, SOD, CAT, GSH-Px, GR, arginase and NO in the tissues were significantly different amongst starvation times. The measured parameters in the tissues significantly changed also amongst refeeding times. But, at the end of the refeeding period, these parameters in tissues had recovered to the control levels.

The concentration of MDA in hepatopancreas for 3 (52.22% ($p<0.01$)), 18 (202.29% ($p<0.001$)), 33 (224.44% ($p<0.001$)), 48 (285.89% ($p<0.001$)), 63 (360.47% ($p<0.001$)) and 78 (460.49% ($p<0.001$)) days was determined higher in those crayfish exposed to starvation according to control. The percentage of MDA in muscle and gills were found higher in those crayfish exposed to 18 (32.26% ($p<0.01$), 28.57% ($p<0.01$)), 33 (61.29% ($p<0.001$), 33.33% ($p<0.01$)), 48 (93.33% ($p<0.001$), 88.46% ($p<0.001$)), 63 (183.33% ($p<0.001$), 128.57% ($p<0.001$)) and 78 (169.70% ($p<0.001$), 244.44% ($p<0.001$)) days starvation compared with control, respectively (Figure 1A). After refeeding, the MDA levels in hepatopancreas, muscle and gills were

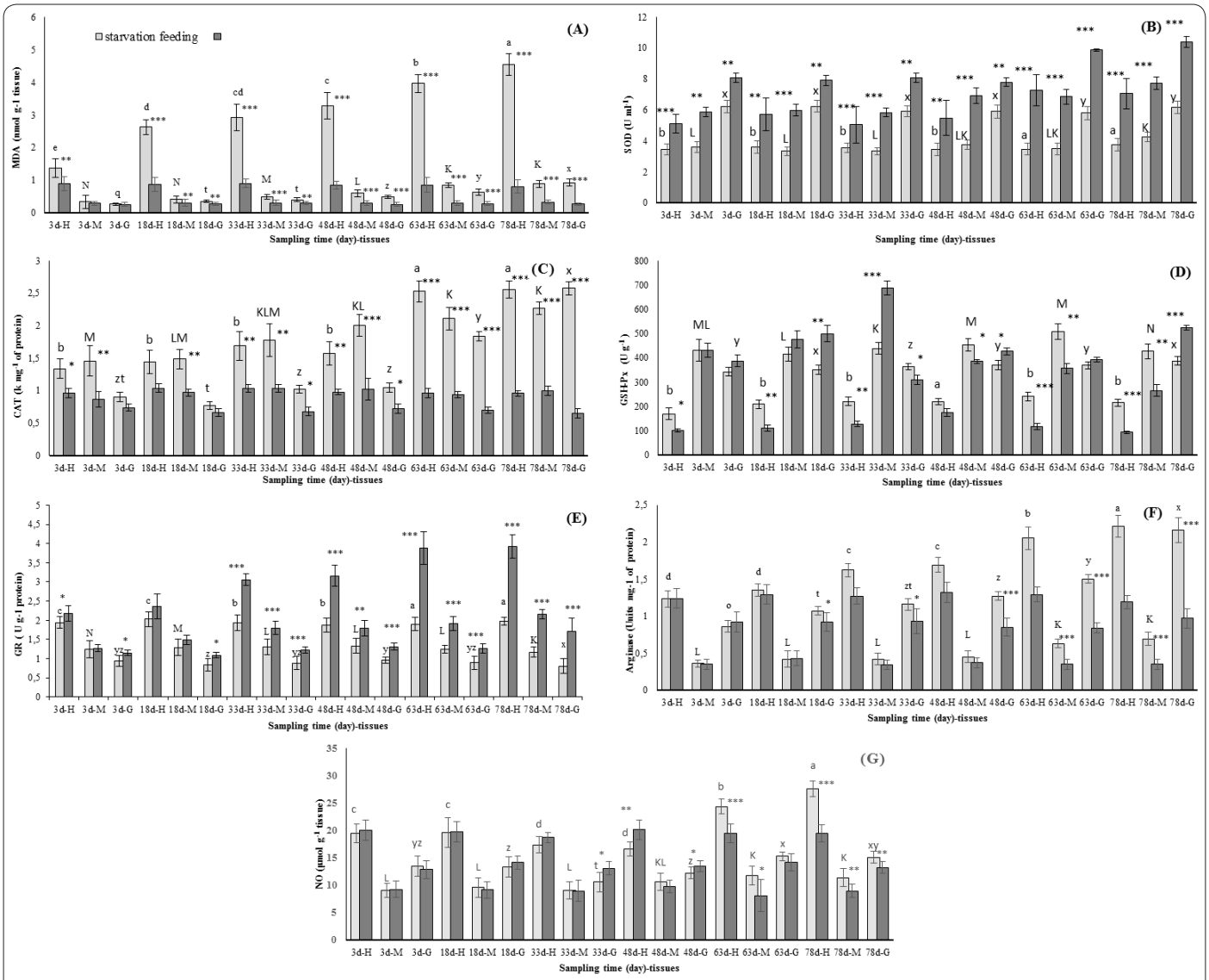

Figure 1. Comparison of the mean concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) according to tissues of the starvation and control group (H: hepatopancreas, M: muscle, G: gills)
Note: Asterisc on the bar shown statistical significance between stavation and control group of the same tissue of the same day (*p<0.05, **p<0.01, ***p<0.001, Independent-Sample T Test). Different letters on the bar show statistical significance amongst different starvation times according to tissues (a, b, c, d, e for hepatopancreas (MDA; p<0.001, SOD; p<0.01, CAT; p<0.001, GSH-Px; p<0.001, GR; p<0.001, AR; p<0.001, NO; p<0.001), K, L, M, N for muscle (p<0.001 for MDA, SOD, CAT, GSH-Px, GR, AR, NO) and x, y, z, t, q for gills (p<0.001 for MDA, SOD, CAT, GSH-Px, GR, AR, NO), One Way Anowa, Duncan Test).

found higher in those crayfish exposed to 3 (278.72% (p<0.001), 126.67% (p<0.001), 125.00% (p<0.001)) and 18 (176.09% (p<0.001), 56.25% (p<0.001), 53.57% (p<0.001)) days refeeding compared with control, respectively (Figure 2A).

The percentage of SOD in hepatopancreas, muscle and gills were found higher in those crayfish exposed to 3 (47.26% (p<0.001), 62.88% (p<0.01) and 30.06% (p<0.01)), 18 (58.17% (p<0.01), 79.34% (p<0.001) and 26.72% (p<0.01)), 33 (41.74% (p<0.001), 74.85% (p<0.001) and 36.89% (p<0.01)), 48 (57.93% (p<0.01), 84.31% (p<0.001) and 32.03% (p<0.01)), 63 (109.20% (p<0.001), 97.42% (p<0.001) and 70.34% (p<0.001)) and 78 (87.27% (p<0.001), 81.07% (p<0.001) and 68.99% (p<0.001)) days starvation compared with control, respectively (Figure 1B). The levels of SOD in hepatopancreas, muscle and gills were found higher in those crayfish exposed to 3 (85.96% (p<0.001), 89.91% (p<0.001) and 47.89% (p<0.001)) and 18 (54.86% (p<0.001), 41.86% (p<0.01) and 25.12% (p<0.01)) days refeeding compared with control, respectively (Figure 2B).

The concentration of CAT in hepatopancreas for 3 (39.58% (p<0.05)), 33 (62.50% (p<0.01)), 48 (62.20% (p<0.01)), 63 (163.54% (p<0.001)) and 78 (166.67% (p<0.001)) days was determined higher in those crayfish exposed to starvation according to control. The activity of CAT in muscle were higher in those crayfish exposed to 3 (67.82% (p<0.05)), 18 (53.61% (p<0.01)), 33 (71.15% (p<0.01)), 48 (97.06% (p<0.001)), 63 (124.46% (p<0.001)) and 78 (127.00% (p<0.001)) days starvation compared with control. The CAT levels in gills tissue of those crayfish exposed to 33 (50.00%) , 48 (45.83%), 63 (162.85%) and 78 (296.92%) day starvation were significantly higher than controls (p<0.05, p<0.05, p<0.001 and p<0.001 respectively) (Figure 1C). After refeeding, the percentage of CAT in hepatopancreas and muscle were found higher in those crayfish exposed to 3 (148.51% (p<0.001), 161.96% (p<0.001)) and 18 (46.94% (p<0.05), 54.64% (p<0.01)) days re-

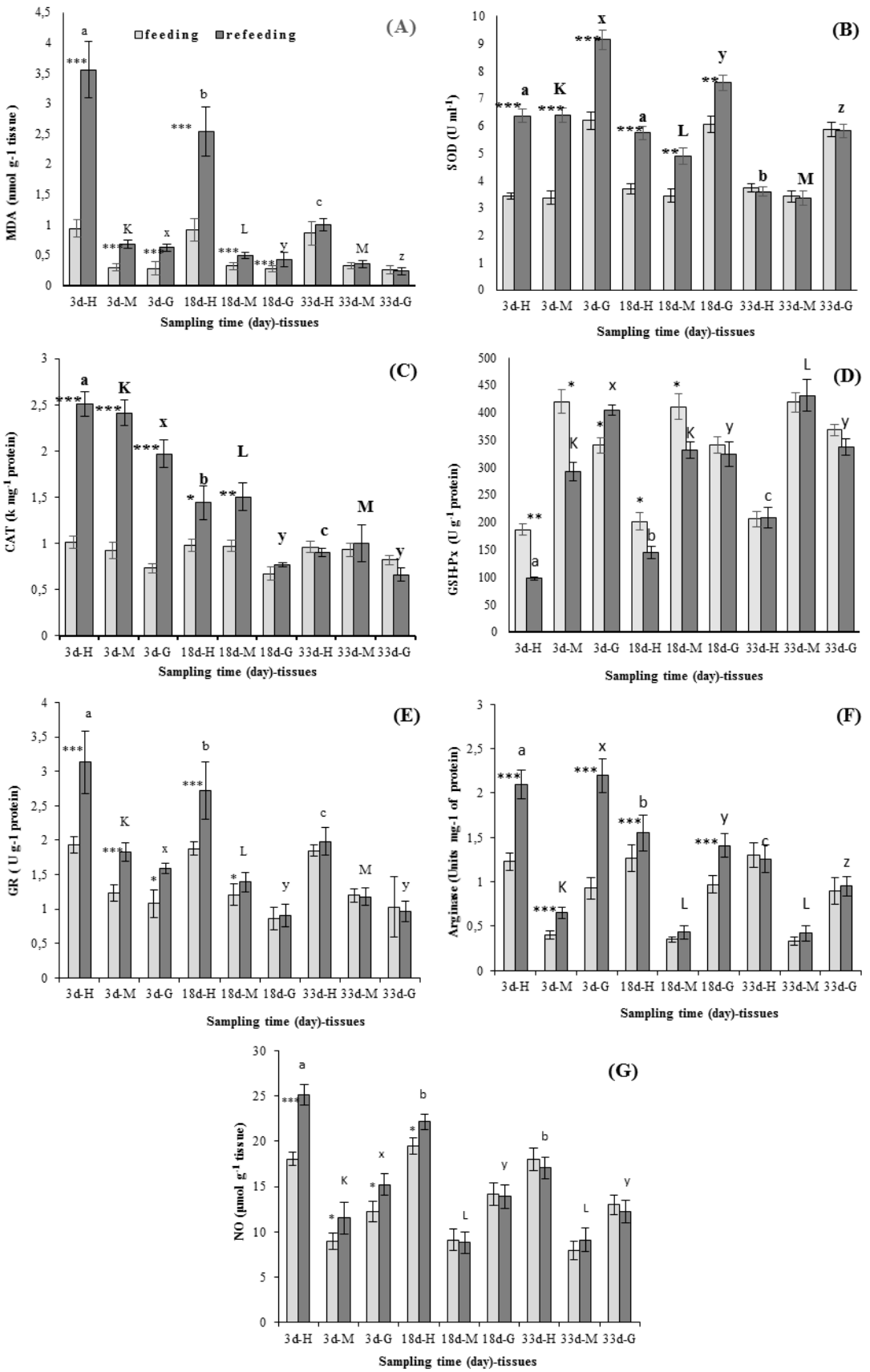

Figure 2. Comparison of the mean concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) according to tissues of the refeeding and control group (H: hepatopancreas, M: muscle, G: gills) **Note:** Asterisc on the bar shown statistical significance between refeeding and control group of the same tissue of the same day (*p<0.05, **p<0.01, ***p<0.001, Independent-Sample T Test). Different letters on the bar show statistical significance amongst different refeeding times according to tissues (a, b, c for hepatopancreas (MDA; p<0.001, SOD; p<0.01, CAT; p<0.001, GSH-Px; p<0.001, GR; p<0.001, AR; p<0.001, NO; p<0.001), K, L, M for muscle (MDA; p<0.001, SOD; p<0.001, CAT; p<0.001, GSH-Px; p<0.01, GR; p<0.001, AR; p<0.001, NO; p<0.05) and x, y, z for gills (MDA; p<0.001, SOD; p<0.001, CAT; p<0.001, GSH-Px; p<0.05, GR; p<0.001, AR; p<0.001, NO; p<0.01), One Way Anowa, Duncan Test).

feeding compared with control, respectively, but this level in the gills was higher only for 3 day (169.84% ($p<0.001$) according to control (Figure 2C).

The level of GSH-Px in the muscle was statistically higher in those crayfish exposed to 33 (56,52%) day starvation compared with control ($p<0.001$), but this level in those crayfish exposed to 48, 63 and 78 day starvation was lower (15.28% ($p<0.05$), 29,91% ($p<0.01$) and 37.35% ($p<0.001$), respectively). The percentage of GSH-Px in gills were determined higher in those crayfish exposed to 18 (42.79%), 48 (14.99%) and 78 (35.14%) days starvation according to control ($p<0.01$, $p<0.05$ and $p<0.001$ respectively), but this level in those crayfish exposed to 33 day starvation was lower (15.12% ($p<0.05$)). The GSH-Px levels in hepatopancreas tissue of those crayfish exposed to 3 (39.94%), 18 (47.26%), 33 (41.98%), 63 (51.66%) and 78 (56.39%) day starvation were significantly lower than controls ($p<0.05$, $p<0.01$, $p<0.01$, $p<0.001$ and $p<0.001$ respectively) (Figure 1D). After refeeding, the percentage of GSH-Px in hepatopancreas and muscle were found lower in those crayfish exposed to 3 (47.54% ($p<0.01$), 30.26% ($p<0.05$)) and 18 (28.13% ($p<0.05$), 19.22% ($p<0.05$)) days refeeding compared with control, respectively. But, this level in the gills was higher only for 3 day (18.94% ($p<0.05$) according to control (Figure 2D)

The concentration of GR in hepatopancreas for 3 (12.37% ($p<0.05$)), 33 (58.55% ($p<0.001$)), 48 (68.98% ($p<0.001$)), 63 (104.74% ($p<0.001$)) and 78 (97.98% ($p<0.001$)) days was determined higher in those crayfish exposed to starvation according to control. The percentage of GR in gills were found higher in those crayfish exposed to 3 (22.34% ($p<0.05$)), 18 (29.76% ($p<0.05$)), 33 (39.77% ($p<0.001$)), 48 (37.89% ($p<0.001$)), 63 (42.70% ($p<0.001$)) and 78 (112.50% ($p<0.001$)) days starvation compared with control, respectively. The concentration of GR in muscle for 33 (38.46% ($p<0.001$)), 48 (34.59% ($p<0.01$)), 63 (52.80% ($p<0.001$)) and 78 (84.62% ($p<0.001$)) days was determined higher in those crayfish exposed to starvation according to control (Figure 1E). After refeeding, the GR levels in hepatopancreas, muscle were found higher in those crayfish exposed to 3 (62.18% ($p<0.001$), 48.78% ($p<0.001$)) and 18 (44.68% ($p<0.001$), 14.88% ($p<0.05$)) days refeeding compared with control, respectively. But, this level in the gills was higher than control for 3 day (47.22% ($p<0.05$) (Figure 2E)

The activity of AR in hepatopancreas for 33 (27.56% ($p<0.001$)), 48 (28.03% ($p<0.001$)), 63 (58.91% ($p<0.001$)) and 78 (85.71% ($p<0.001$)) days was determined higher in those crayfish exposed to starvation according to control. The AR activity in muscle were higher in those crayfish exposed to 63 (80.00% ($p<0.001$)) and 78 (97.14% ($p<0.001$)) days starvation compared with control. The AR levels in gills tissue of those crayfish exposed to 18 (16.30%), 33 (24.73%) , 48 (49.41%), 63 (78.57%) and 78 (122.68%) day starvation were significantly higher than controls ($p<0.05$, $p<0.05$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively) (Figure 1F). After refeeding, the percentage of AR in hepatopancreas and gills were found higher in those crayfish exposed to 3 (70.73% ($p<0.001$), 136.56% ($p<0.001$)) and 18 (22.05% ($p<0.001$), 45.36% ($p<0.001$)) days refeeding compared with control, respectively, but this level in the muscle was higher only for 3 day (62.50% ($p<0.001$) according to control (Figure 2F).

The level of NO in the hepatopancreas was statistically lower in those crayfish exposed to 48 (17.63%) day starvation compared with control ($p<0.01$), but this level in those crayfish exposed to 63 and 78 day starvation was higher (25.40% ($p<0.001$) and 41.94% ($p<0.001$), respectively). The percentage of NO in gills were determined lower in those crayfish exposed to 33 (19.30%) and 48 (9.50%) days starvation according to control ($p<0.05$, and $p<0.05$ respectively), but this level in those crayfish exposed to 78 day starvation was higher (13.92% ($p<0.01$)). The NO levels in muscle tissue of those crayfish exposed to 63 (45.79%) and 78 (26.98%) day starvation were significantly higher than controls ($p<0.05$ and $p<0.01$ respectively) (Figure 1G). After refeeding, the percentage of NO in hepatopancreas was found lower in those crayfish exposed to 3 (39.09% ($p<0.001$)) and 18 (13.73% ($p<0.05$)) days refeeding compared with control, respectively. But, this level in the muscle and gills was higher only for 3 day (24.69% ($p<0.05$) and (23.94% ($p<0.01$)) according to control (Figure 2G).

Discussion

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 compenents (2). The results of the study illustrated that starvation increased the levels of MDA as a secondary lipid peroxidation product in the hepatopancreas, muscle and gills of the crayfish. Similar findings have also been reported in *A. naccarii*, *O. mykiss* (7), *D. dentex* (24), *M. nipponense* (14), *S. trutta* (1), *D. labrax* (25) and *H. littorale* (26) after different starvation periods. These increases in MDA depending on starvation can most likely be associated to that antioxidant defenses were inadequate for effective scavenging of the generated ROS.

In the present study, after 53 days of starvation were observed mortality in crayfish, and the mortality rose steadily. In the mean time, the levels of MDA in the all tissue were higher in those crayfish exposed to 63 and 78 days starvation compared to data of 3, 18, 33 and 48 days starvation. Seifried et al. (36) reported that low doses of ROS can be mitogenic, whereas medium doses lead to temporary or permanent growth arrest, and high doses usually result in cell death either by opoptosis or necrosis. The data presented here show that the MDA levels in the tissues examined recovered to normal values when the crayfish in refeeding group were returned to the control diet for 33 days. Similar findings have also been reported for some aquatic species (14, 37, 38). Nevertheless, oxidative stress indications remained after refeeding period in tissues of aquatic organisms in some studies (1, 7, 25). For example; Furne et al. (7) determined that oxidative stress indications didn't changed after 60 day refeeding period in the hepatic tissues and red blood cell in rainbow trout and sturgeon. Oxidative stress caused by a decline in oxygen consumption, differs among aquatic organisms and may be a consequence of lower activity by these organisms in a attempt to conserve body energy reserves during

periods of food shortage (25).

The findings of present study showed that the hepatopancreas was more sensitive to starvation and refeeding than the muscle and gills. It was found in many studies that hepatopancreas being lipid rich and for its high metabolic rate, it may undergo spontaneous autooxidation and thus the generation of O_2^{-*} and H_2O_2 may be comparatively more in this organ than other organs (39). The highly unsaturated fatty acids, that are vital components of cellular membranes, are particularly susceptible to attack by reactive oxygen radicals. It was reported that aquatic organisms have rich source of polyunsaturated fatty acid lipids (2). A different behaviour during starvation depending on the tissue have been determined in aquatic organisms (3, 7, 14, 24, 25, 26, 37, 38). For example, Furne et al. (7) found that LPO levels in white muscle and heart of *A. naccarii* and *O. mykiss* indicate no oxidative stress during the 72 days of starvation, but this levels in muscle increased in both species during refeeding. In this study, starvation induced a significant increase in the levels of MDA in the muscle tissue, especially from 33, 63 and 78 days. The main cause for increased MDA levels may be related to increase of free radical generation. In this study, the levels of MDA in the gills tissue were found higher in those crayfish exposed to 3, 63 and 78 days starvation compared to data of 18, 33 and 48 days starvation. Contrary to the results of the present study, wehere no significant changes in the levels of MDA were observed in the gills of *H. littorale* during starvation (26). These increases in MDA can most likely be ascribed to excessive ROS production because the gills are the respiratory organs and exposed to ambient oxygen. Addition, the main cause for these differences in the gills could be the different rates of free radical generation due to different antioxidant potentials depending on different period of starvation tested.

SOD enzyme catalyses the transformation of O_2^{-*} to H_2O_2 and water. CAT located in the peroxisomes is an enzyme that is facilitates the removal of H_2O_2 that is metabolised to molecular oxygen and water (2). In the present study, the activity of SOD and CAT in hepatopancreas, muscle and gills were higher in crayfish of the starvation group compared with control group. The increases in this enzyme activities may be meant to neutralise the overproduction of O_2^- anions and H_2O_2 due to the oxidative stress induced by starvation. This idea was corroborated by the observations of Bayir et al. (1), who the increased SOD activity would result in a higher generation of H_2O_2, which may be the reason for high hepatic and branchial CAT activities of *S. trutta*. Similar findings have also been determined in the liver of *D. dentex* (24) and in the gills of *H. littorale* (26). But, this activity decreased in the liver and blood of *A. naccarii* and *O. mykiss* (7), in the liver of *Macrobrachium nipponense* (14), in the liver, intestine and gills of *D. labrax* (25). The present data and former information reveal that such discrepancies may be explained by differential species response, culture conditions, age, duration and degree of food availability (25).

GSH-Px is mainly involved in the removal of organic peroxides (2, 40). Studies showed that GSH-Px activity in the liver decreased in the *A. naccarii* and *O. mykiss* (7) during starvation, but it increased in *D. dentex* (24) and *S. trutta* (1). On the other hand, this activity during starvation increased in the intestine of *D. labrax*, but decreased in the white muscle (25). The present study illustrates that hepatopancreas GSH-Px activity in *A. leptodactylus* decreased in all periods of starvation. Reduced GSH-Px activity may be related to the production of O_2^{-*} or to the direct action of starvation on enzyme synthesis (40, 39). In this study was found that this activity showed fluctuations in muscle and gills according to starvation period tested. It was reported that H_2O_2 is neutralized by two different enzymes present in the cellular system, they are GSH-P_x and CAT. Each differs in its affinity for H_2O_2, and intracellular H_2O_2 concentration is one of the factors in deciding which of these enzymes will be functional. Furthermore, It was reported that the regulation of the gene expression of antioxidant enzymes differs from one cell system to other (2, 39). Therefore, the observed differential response to starvation of GSH-P_x in the gills, hepatopancreas and muscles is not surprising**.** Furthermore, the increased GSH-Px activity in the muscle and gills protected the organ from the formation of lipid peroxides by reducing H_2O_2 levels, which in turn attenuated OH^{-*} generation.

Among the enzymatic antioxidants, GR catalyzes the NADPH-dependent regeneration of GSH from the oxidized form (GSSG) generated by GSH-Px (24, 41). The present study showed that hepatopancreas, muscle and gills GR activities increased in parellel with starvation. A similar increase in the hepatic and branchial GR activity was reported in *S. trutta* deprived of food (1). Additionally, an increase in GR activity was also determined in the liver of *S. aurata* (42), in the muscle of the *A. naccarii* and *O. mykiss* (7) and in the kidney, gills and brain of the *H. littorale* (26). On the contrary, prolonged starvation induced a decreased activity of this enzyme in the liver of the *D. dentex* (24). The increases in GR activity might reflect a possible antioxidant defense failure responsible for the reported increase in LPO. In this study determined that the GR activity recovered to the control levels at the end of the refeeding period. Thethis result obtained in *A. leptodactylus* are consistent with those reported for other fish species (7, 24, 42).

The AR enzymes convert L-Arg into L-ornithine and urea, precursors for L-proline and polyamines compounds, that are vital to tissue homeostasis and wound repair. The AR competes with inducible NOS, the high-output, inducible pathway for increased production of ·NO, for L -Arg, their common substrate in multiple cell types including endothelial cells. In cases in which L -Arg is limited, ·NO levels can reduction, and this may be the result of increased AR activity (5, 43). In the some crustaceans has been determined AR activities in the midgut, gill, muscle and hemolymph besides hepatopancreas (44, 45). The effect of stress on the AR and NO in the aquatic organisms was obtained by Barim et al. (40), Barim and Erisir (46) and Barim-Oz et al. (47), who determined that stress occurred with pollution and reproduction changed arginase and NO levels. But, the study to compare the effect on these parameters of starvation was not found. In the present study, the activity of AR in all tissues were higher in crayfish of the starvation group compared with control group. But, in NO activity was determined increases and decreases depending on starvation. The increases in arginase activity, to

detoxify the ammonia and for the successful survival can be a metabolic adaptation of crustaceans. Additionally, Camargo and Alonso (48) determined that the variations in NO activity can be correlated with damage to mitochondria in cells by cause a shortage of tissue O_2 of nitrite produced as an oxidative metabolite of NO on fish and crayfish. The physiological significance and damage of these changes have to be analysed in further experiments.

This is the first comprehensive report of levels of MDA, some enzymatic antioxidants, arginase and NO in the tissues of *A. leptodactylus* during starvation. Especially, the effect on arginase and NO levels of starvation in aquatic organisms has previously been studied. Knowing the physiological strategies activated by *A. leptodactylus* to refeeding and prolonged periods of starvation may have important implications in understanding how crayfish are well adapted to environments with highly variable food availability. Additionally, it is important to highlight that the scenario reported in the study may be worsened by the ongoing climatic changes, such as heat waves and prolonged droughts, therefore, the level of LPO and activity of antioxidants, AR and NO related to starvation can be used as useful tools to monitor future environmental issues. The addressed molecular responses of farmed crayfish under the stressful effect of starvation but also refeeding should be used in the aquaculture section as evaluation tools for the design of feeding schedules to decrease feed costs and the maintenance of crayfish adequate healt and stock (25, 26).

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