The Effects of Dietary Vitamin E on the Oxidative Stress and Antioxidant Enzyme Activities in Their Tissues and Ovarian Egg Numbers of Freshwater Crayfish *Astacus leptodactylus* (Eschscholtz, 1823)

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Abstract: The effects of dietary vitamin E on the oxidative stress (lipid peroxidation (as Malondialdehyde (MDA)) and antioxidant enzyme activities (as Glutathione Peroxidase (GSH-Px)) of hepatopancreas, ovarian and muscle tissues and the ovarian egg numbers and size of freshwater crayfish *Astacus leptodactylus* was investigated. Crayfish were fed daily 2% of their total wet weight with vitamin E supplemented diets and a control for 72 days. The vitamin E contents of the control diet, diet 1-3 were 65.83, 100, 150 and 200 mg kg^{-1}, respectively. Results showed that diet 2 containing 150 mg kg^{-1} supplemental vitamin E was associated with a significant increase ($p < 0.001$) in the number of ovarian eggs (48.68%) of crayfish. In addition, the females fed with diet 2 and diet 3 produced significantly bigger ovarian eggs (10.93 and 5.47%) ($p < 0.001$). It was determined that MDA activity decreased significantly in the hepatopancreas, ovarian and muscles of the crayfish fed with vitamin E supplemented diets. While GSH-Px activity was significantly elevated in the hepatopancreas and muscle by vitamin E, its activity decreased in the ovarian.

Key words: *Astacus leptodactylus*, crayfish, vitamin E, ovarian egg, MDA, GSH-Px

INTRODUCTION

Lipid peroxidation is used as an indicator of oxidative stress in cell and tissues. Lipid peroxides derived from polyunsaturated fatty acids are unstable and can decompose to form a complex series of compounds. These include reactive carbonyl compound, which is the most abundant Malondialdehyde (MDA). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation (Winston and Giulio, 1991; Karatepe, 2004; Barim, 2005). Fish and crustacean against this oxidation have built up extensive defence systems, consisting of antioxidant enzymes, endogenous antioxidants, such as Glutathione Peroxidase (GSH-Px), superoxide dismutase and catalase, nutritional antioxidants, such as vitamin E and carotenoids (Winston and Giulio, 1991).

Early development in aquatic organism is dependent on the internal complement of essential nutrients that are present in the egg. These nutrients are, in turn, determined by the maternal diet prior to and during oogenesis. The antioxidant enzyme systems are not synthesized until late in the embryonic development. This makes early antioxidant protection by maternally derived non-enzymatic antioxidants essential (Palace and Werner,

2006). Reduced reproduction, reported in several marina fish species, could be caused either by the influence of a nutrient ambalance or by the restriction in the availability of a biochemical component for egg formation (Izquierdo *et al.*, 2001). Because of these reasons, minimum dietary concentrations of non-enzymatic antioxidants during the reproductive cycle of aquatic organisms are to be regarded as a tenuous guideline.

Vitamin E is a important antioxidant in the lipid phase, of crustacean tissues. This vitamin reacts with the lipid peroxide radical produced by a cycle of auto-oxidation, preventing it to react with a new PUFA and is therefore called a chain breaking antioxidant. However, vitamin E is an indispensable nutrient required to maintain normal health and life functions, such as growth, development, reproduction in fish and crustacea (Cay and King, 1980; Gatlin *et al.*, 1986; He *et al.*, 1992; Fernandez-Palacios *et al.*, 1998; Dandapat *et al.*, 2000; Emata *et al.*, 2000; Tokuda *et al.*, 2000; Izquierdo *et al.*, 2001; Harlioglu *et al.*, 2002; Cavalli *et al.*, 2003; Harlioglu and Barim, 2004; Barim, 2005; Erisit *et al.*, 2006; Palace and Werner, 2006). Vitamin E is usually supplemented in feed as DL-α-tocopheryl acetate, which is stable with respect to oxidation during feed processing and storage (Harlioglu and Barim, 2004; Barim, 2005).

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A. leptodactylus is a native freshwater crayfish species in Turkey. 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 *A. leptodactylus* after 1985 decreased dramatically (from 5000 tons annually to 200 tons) in most Turkish lakes as a result of the crayfish plague, over-fishing, water pollution and water withdraws for agricultural irrigation (Koksal, 1988; Harlioglu and Barim, 2004; Barim, 2005). 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 *A. leptodactylus* populations are require for this good quality diet.

Therefore, the present study was designed to examine the effect of vitamin E on the oxidative stress (MDA) and antioxidant defence (Glutathione Peroxidase (GSH-Px)) in the hepatopancreas, ovarian and muscle tissues and the ovarian eggs number and size of the *A. leptodactylus.*

MATERIALS AND METHODS

This study was conducted between August 18th and November 05th (79 days) at the crayfish reproduction unit of Aquaculture Faculty of Firat University, Elazig, Turkey. The crayfish used in the present study was provided from Keban Dam Lake population of *A. leptodactylus.*

Practical control diet used in this study (Table 1) was modified after Harlioglu and Barim (2004) and Barim (2005). The control diet was formulated to contain approximately 37% crude protein on a dry-weight basis and 3.25 kcal g^{-1} gross energy. Levels of dietary vitamin E (supplied as DL-α-tocopherol acetate) were 100, 150 and 200 mg kg^{-1} diet for diets 1, 2 and 3, respectively. No vitamin E was added to the control diet, except that supplied by the vitamin premix and feed ingredients. DL-α-tocopherol acetate was donated by Roch Ltd. The ingredients for each 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°C for up to 24 h and then stored in a refrigerator at 4°C until further use.

The vitamin E contents of the control diet, diet 1-3 were 65.83, 100, 150 and 200 mg kg^{-1}, respectively on a dry weight basis. Vitamin E level of the control diet was analysed by High Performance Liquid Chromatography (Miller *et al.*, 1994). The crude protein content was

Table 1: Composition and proximate analysis of the control diet

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
Proximate composition	
Crude protein	37.40
Crude fat	7.60
Crude fibre	4.00
Crude ash	15.00
Nitrogen free extract	29.60
Moisture	6.40
Gross energy (kcal g^{-1})	3.25

[1]Kavilamycine, [2]Antioxidant (mg kg^{-1} dry diet): butylated hydroxytoluene 12.5, [3]Vitamin premix (IU or mg kg^{-1}): 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^{-1} dry diet): Mn 80, Fe 35, Zn 50, Cu 5, I 2, Co 0,4, Se 0,15

analysed by Kjeldahl's method; 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; dry matter after the sample was dried at 105°C for 6 h, ash content after 24 h at 550°C in the furnace and lipid analysed by an ether extraction method (AOAC, 1990).

The experiment was carried out with 3 replicates for each dietary treatment. The 8 males and 15 females were used for each replicate (96 males and 180 females in total). Crayfish were housed in 12 concrete tanks (2×2×0.5 m). Plastic pipes (20 cm in length and 7 cm in diameter) were provided as shelters for the crayfish. *A. 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 3 separate feedings (Harlioglu and Barim, 2004; Barim, 2005). Supplemental water flow was 0.5 L sec^{-1} for each tank. During the trial, mean dissolved oxygen was 7.16±0.52 mg L^{-1}; mean pH was 7.68±0.12; mean water temperature was 14.00±2.59°C.

Crayfish were exposed to natural photoperiod: The range in photoperiod (through outside tanks) during the study was approximately, 10 D-14 L in August, 12 D-12 L in September, 12 D-12 L in October, 13 D-11 L in November.

The crayfish selected from each replicate on November 05 were placed on ice in plastic bags and transported to the laboratory. Sample of 27 crayfish from each of the 4 dietary treatments was randomly selected for analysis. The carapace length (mm) and weight (g) were recorded. The ovarian eggs of each female were counted. The size of ovarian eggs were measured by use of a light microscope and graticule. The tissues of the crayfish for biochemical assays were surgically removed and stored at -80°C.

Assay of lipid peroxidation: An aliquot portion of (1,0 g) crayfish tissue samples were homogenized in a glass-glass homogenizer in mixture of 0.5 mL of $HClO_4$ (0.5 M), 4.5 mL distilled water and 100 μL-500 ppm Butylated Hydroxytoluene (BHT) (Cerhata *et al.*, 1994). 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.

The mobile phase was 30 mM KH_2PO_4 -Methanol (82.5 + 17.5, v v^{-1} %, pH 3.6) and the flow rate was 1.2 mL min^{-1}. Chromatograms were monitored at 250 nm and injection volume was 20 μL. A Wakosil II 5C18 RS 5 μm (150×4.6 mm SS, SGE, AUS) column was used at room temperature (Karatepe, 2004).

Assay of glutathione peroxidase: The homogenization of tissues was carried out in Glass-glass homogenizer with a buffer containing 1.15% KCl to obtain 1/10 (w v^{-1}) whole homogenate. Homogenates were centrifuged 15 min at 105.000 g at +4°C for GSH-Px activities. The supernatant was used for the assay.

GSH-Px activity in the tissues was measured by the method of Beutler (1975) in which cumene hydroperoxide was used as substrate. Oxide Glutathione (GSSG) produced by the action of GSH-Px in the tissues and cumene hydroperoxide, was reduced by glutathione reductase and NADPH. The decrease of the NADPH concentration was measured at 340 nm. The enzyme activity in the tissues was expressed as units per gram of protein (U g^{-1} protein). The protein content of the samples was measured according to the method of Lowry *et al.* (1951) using BSA as standard.

The results are statistically analyzed by calculating the Standard Error of the mean (mean±SE). Analysis of Variance and Duncan's new multiple range test were employed for the statistical analysis of data.

Differences were considered statistically significant when p<0.05. Tests for data normality and analyses of variance were carried out in duplicate.

RESULTS

The carapace length of males and females among the experimental groups (control, diet 1-3) 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 carapace length of females was 46.19±0.24 mm for control, 46.07±0.19 mm for diet 1, 46.40±0.22 mm for diet 2, 46.47±0.23 mm for diet 3. The mean carapace length of males was 46.67±0.25 mm for control, 46.42±0.29 mm for diet 1, 46.29±0.31 mm for diet 2, 46.60±0.31 mm for diet 3, respectively. There were no significant differences between the experimental groups (44.48±0.34 mm for control, 47.22±0.35 mm for diet 1, 47.33±0.34 mm for diet 2 and 47.26±0.40 mm for diet 3) in the mean carapace length of females that biochemical analysis, the mean ovarian egg numbers and the mean ovarian egg size were determined.

The present study showed that the use of vitamin E (150 mg kg^{-1}) in supplemental feeding gave rise to a significant increase in the number of the mean ovarian eggs (p<0.001). The diet 1-3 in comparison to the control diet increased number of the mean ovarian egg (4.52, 48.68 and 25.45%, respectively). The mean ovarian egg number from crayfish fed diet 2 (169.44±3.24) was significantly higher than those for the crayfish fed with the control diet (113.96±3.74), diet 1 (119.11±3.47) and diet 3 (142.96±3.68) (Fig. 1a).

Number of the mean ovarian egg of 1 mm female carapace length (eggs no mm^{-1} of females) was 1.56±0.01 for control, 2.02±0.09 for diet 1, 2.92±0.06 for diet 2, 2.16±0.05 for diet 3 (Fig. 1b).

The present study showed that the use of 150 mg kg^{-1} vitamin E resulted in a significant increase in the egg size of crayfish.

The diet 1-3 in comparison to the control diet increased size of the mean ovarian egg (4.69, 10.93 and 5.47%). The mean ovarian egg size of crayfish fed with 150 mg kg^{-1} (1.42±0.02) and 200 mg kg^{-1} (1.35±0.01) vitamin E was significantly (p<0.001) bigger than those of the crayfish fed with the control diet (1.28±0.02), diet 1 (1.34±0.03) (Fig. 1c).

Figure 2 show the effects of dietary supplementation with vitamin E on MDA of the hepatopancreas, ovarian and muscle of *A. leptodactylus*. The diet 1-3 in
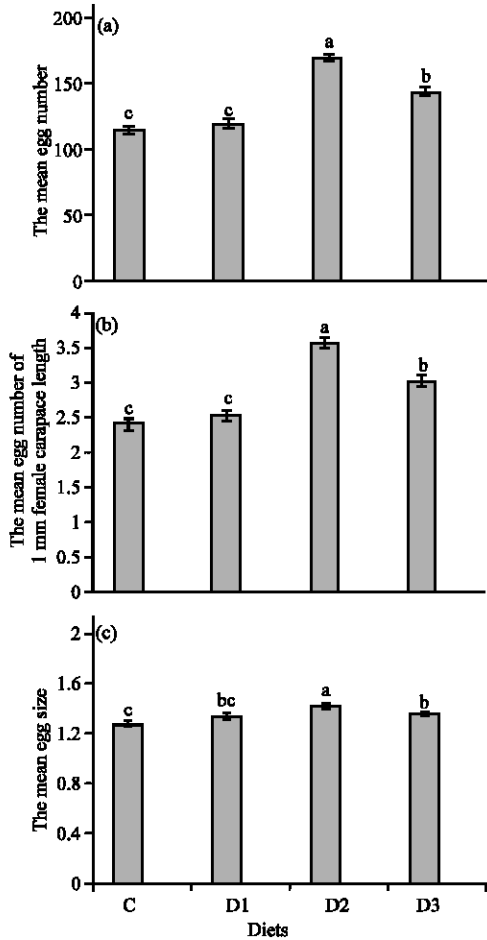
Fig. 1: Effect of different levels of dietary DL-α-tocopherol acetate on the mean ovarian egg number (a), number of the mean ovarian egg of 1 mm female carapace length (eggs no/mm of females) (b) and mean ovarian egg size (c) of *A. leptodactylus*. Values followed by the same letters are not significantly different (p>0.05) and values followed by the different letters are significantly different (p<0.001)

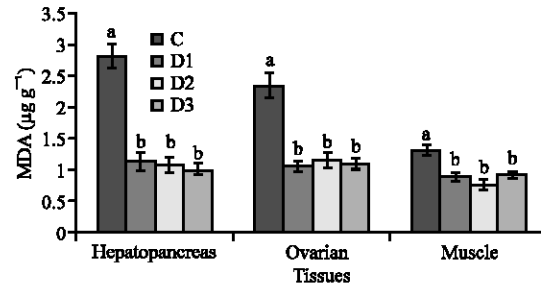
Fig. 2: Effect of different levels of dietary DL-α-tocopherol acetate on MDA level of hepatopancreas, ovaian and muscle tissues of *A. leptodactylus*. Values followed by the same letters are not significantly different (p>0.05) and values followed by the different letters are significantly different (p<0.001)
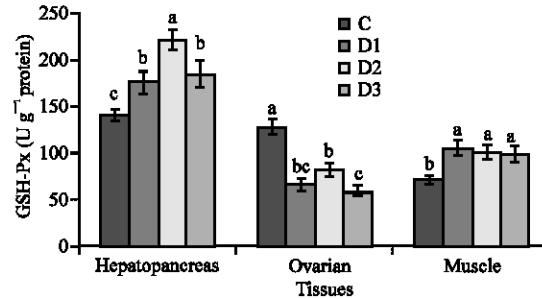

Fig. 3: Effect of different levels of dietary DL-α-tocopherol acetate on GSH-Px activity of hepatopancreas (p<0.001), ovaian (p<0.001) and muscle (p<0.05) tissues of *A. leptodactylus*. Values followed by the same letters are not significantly different (p>0.05) and values followed by the different letters are significantly different

comparison to the control diet decreased the MDA level of hepatopancreas (59.28, 61.78 and 65.00%, respectively) (p<0.001), ovarian (57.40, 50.43 and 53.85%, respectively) (p<0.001) and muscle tissue (35.61, 42.42 and 31.06%, respectively) (p<0.001).

Supplementary vitamin E in the diet resulted in an increase in the activity of GSH-Px in the hepatopancreas and muscle of the crayfish. The percentage increase in GSH-Px activity was 24.70, 56.37 and 30.53%, respectively for hepatopancreas (p<0.001) and 46.78, 40.20 and 37.25%, respectively for muscle (p<0.05) at the diet 1-3 compared to the control diet. But, this activity decreased

in the ovarian tissues (48.24, 36.07 and 53.94%, respectively) (p<0.001) at the diet 1-3 compared to the control diet (Fig. 3).

DISCUSSION

Although, the negative effects of vitamin E deficiency on the reproductive performance of higher vertebrates have been demonstrated since the early 1920s, dietary vitamin E was only conclusively shown to be an important nutrient for fish reproduction in 1990, with its deficiency resulting in immature gonads in carp and ayu and reducing hatching rates and fry survival in ayu. Vitamin E supplementation (up to 2000 mg kg^{-1}) in red seabream diets improved percentages of buoyant eggs, hatching rates and percentage of normal larvae (Izquierdo *et al.*,

2001). Fernandez-Palacious *et al.* (1997) found that increased levels of dietary vitamin E (from 22-125 mg kg^{-1}) reduced the percentage of abnormal gilthead seabream eggs. However, Fernandez-Palacious *et al.* (1998) found that egg viability and percentage of abnormal eggs improved with increasing dietary α-tocopherol levels. In a different study, Emata *et al.* (2000) determined that the total egg production, mean egg number per spawning, number of spawns and mean egg diameter were not affected by the dietary supplementation of vitamin C and E, but broodstock given dietary supplementation of vitamin C alone or in combination with vitamin E caused a higher percentage of spawns with higher percentage (>90%) egg viability, hatching and cumulative survival rate than these of the control in a research on the effect of dietary vitamin C and E in the reproduction of milkfish (*Chanes chanos*). In addition to this study, it was found that *Paralichthys olivaceus* spawned 1.5 times more eggs than the α-tocopherol unsupplemented group (Tokuda *et al.*, 2000). Nevertheless, the beneficial effect of dietary vitamin E supplementation on fish reproduction was not found in some studies (King *et al.*, 1985; Eskelinen, 1989).

There have been not many studies with the effect of vitamin E on reproduction of crayfish and other crustaceans. The effect of dietary vitamin E in *A. leptodactylus* reproduction has been stated in prior studies. It was reported that the bigger and more pleopodal eggs and stage-1 juveniles in freshwater crayfish were improved when fed with diets supplemented vitamin E before spawning (Harlioglu *et al.*, 2002; Harlioglu and Barim, 2004; Barim, 2005). However, Erisir *et al.* (2006) established that the presence of vitamin E higher than 150 mg kg^{-1} in the ovigerous crayfish and 100 mg kg^{-1} in the females with stage-1 juveniles in diets may negatively affect the connective tissue formation by decreasing the muscle arginase activity. In this study, was determined that the use of 150 mg kg^{-1} vitamin E 72 days before breeding period resulted in a significant increase in the ovarian eggs number of *A. leptodactylus*.

The one of primary non-enzymatic antioxidants in fish eggs are vitamins E. Content of this lipid-soluble vitamin in fish eggs has been suggested to permit larger initial egg size, which in turn has been correlated with larger larval size and better early survival (Lavens *et al.*, 1999; Palace and Werner, 2006). The primary function of vitamin E is to serve as an antioxidant and it is by far the most physiologically important one in most vertebrates. As such, vitamin E plays an important role in protecting eggs during early development. Harlioglu and Barim (2004) determined that use of vitamin E (100 mg kg^{-1}) caused a

significant increase in the mean pleopodal egg size of crayfish. In this study, diet containing 150 mg kg^{-1} supplemental vitamin E was associated with a significant increase in the mean ovarian egg size (p<0.001). The differents of active dose of dietary vitamin E on ovarian and pleopodal eggs of *A. leptodactylus* may derive from different of carapace length of crayfish and fertilized by the sperm of male crayfish of pleopodal eggs.

The results of the present study illustrate that the MDA level in the hepatopancreas and ovarian was higher than muscle. Several studies demonstrated that hepatopancreas is metabolically more active and the oxyradical generating enzymes display comparatively higher activities than other tissues. So, hepatopancreas being lipid rich and for its high metabolic rate, it may undergo spontaneous autooxidation and thus, the generation of $O_2^{- y}$ and H_2O_2 may be comparatively more in this organ than other organs (Arun and Subramanian, 1998; Dandapat *et al.*, 2000, Borkovic *et al.*, 2008). In addition, accumulation of biochemical components, especially lipids, in the maturing ovary has been reported in fish and crustacea (Izquierdo *et al.*, 2001; Palacious *et al.*, 2000; Palace and Werner, 2006). For example, Palacious *et al.* (2000) found that the level of total lipid, acylglycerides, cholesterol and total protein in mature ovaries of *Penaeus vannamei* increased. Bell *et al.* (2000) and Cavalli *et al.* (2003) demonstrated that highly unsaturated fatty acids, which are vital components of cellular membranes, are particularly susceptible to attack by reactive oxygen radicals. Uncontrolled damage to membrane fatty acids and the accumulation of their oxidized breakdown products can have deleterious consequences for cell and organ function and may increase the requirement for antioxidants (Bell *et al.*, 2000; Cavalli *et al.*, 2003). For these reasons, the main cause for these differences could be the different rates of free radical generation and different antioxidant potentials in the tissues.

In the current study was determined that vitamin E supplementation has a profoundly inhibitory effect on MDA in the ovarian, muscles and hepatopancreas of crayfish. Gatlin *et al.* (1986) demonstrated that the hepatic microsomes of fish fed a vitamin E deficient diet were more susceptible to MDA. The results of our study are in accordance with He and Lawrence (1993), who found lowered levels of acid-stimulated mitochondrial and microsomal MDA in the hepatopancreas of shrimps fed diets containing >100 mg of vitamin E kg^{-1} feed. In the present study, it was also, observed that the higher levels of dietary vitamin E (150 and 200 mg kg^{-1}) did not provide additional protection compared to 100 mg kg^{-1} in the tissues.

The level of dietary vitamin E showed significant effects on the activities of the GSH-Px enzymes of the hepatopancreas, ovarian and muscle antioxidant defence system. These effects have to be interpreted within the knowledge of the commonly perceived biochemical mechanism of this enzyme system. For instance, GSH-Px acts in conjunction with other enzymes to $2H_2O_2$ and to terminate lipid peroxidation. Hidrojen peroxid plays a critical role in the regulation and expression of antioxidant enzymes in various cellular systems. Nevertheless, the regulation of the gene expression of antioxidant enzymes differs from one cell system to other (Barman, 1974). Therefore, the observed differential response to vitamin E supplementation of GSH-P$_x$ in the hepatopancreas, ovareian and muscles is not surprising. On the other hand, the amount of H_2O_2 in the tissues may have a positive effect on the regulation of gene expression of GSH-Px. Our results indicate that vitamin E supplementation induces GSH-Px activity in the tissues (Cay and King, 1980). However, GSH-Px plays an important role against the autooxidation of fish lipids in lipid rich organs (Wdzieczak *et al.*, 1982; Jagneshwar *et al.*, 2000).

As the hepatopancreas and ovarian during gonadal maturation is lipid rich and has a high metabolic rate, it may undergo spontaneous autooxidation and thus, the generation of O_2^- and H_2O_2 may be of a comparatively higher level in these tissues than in the muscle. Therefore, the increased GSH-Px activity in the hepatopancreas and muscle protected the organ from the formation of lipid peroxides by reducing H_2O_2 levels, which in turn attenuated OH$^-$ generation. However, low activity of GSH-Px may also, demonstrate the inefficiency of this organ in neutralizing the impact of peroxides during gonadal maturation.

CONCLUSION

The present study showed that 150 mg kg^{-1} supplemental vitamin E resulted in significantly more ovarian eggs and size in freshwater crayfish, *A. leptodactylus*.

However, the results of the present investigation clearly indicated that 100 mg kg^{-1} supplemental vitamin E during gonadal development reduced the degree of tissue MDA and the higher doses of this vitamin (150 and 200 mg kg^{-1}) did not provide additional protection against oxidative stress. The response of antioxidant activity (GSH-Px) in the different levels of dietary vitamin E may be tissue specific.

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