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Antioxidant Enzymatic Defences during Embryogenesis of Rainbow Trout *Oncorhynchus mykiss* (Walbaum 1792)

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Abstract

In this study antioxidant enzymes activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST) were investigated in unfertilized and fertilized eggs, 12, 22 and 32 day embryos of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792). Vitamin E, lipid peroxidation products and fatty acids levels were also determined. A significant decrease in vitamin E level was found in fertilized eggs of *O. mykiss*. In contrast, the level of malondialdehyde (MDA) was very high in fertilised eggs and 12 day embryo. The PUFA contents in fertilized eggs and 12 day embryo were found to be lower than in the other embryonic stages. Measurable amounts of all the SOD, CAT, GSHpx, GR and GST enzymes activities were present in *O. mykiss* eggs and embryos. The most intense decrease was detected in the activities of SOD, GR and GST in fertilized eggs, whereas the most intense increase in the CAT activity was detected in the same stage of *O. mykiss*. GSHpx activity showed a significant increase in fertilized eggs and in 32 day embryos before hatching. GST activity showed the most significant increase in 22 day embryos of eyed eggs stage.

Keywords: Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, vitamin E, embryo, lipid peroxidation.

Gökkuşağı Alabalığı *Oncorhynchus mykiss* (Walbaum 1792)'in Embriyonik Gelişimi Esnasında Enzimatik Antioksidan Savunma

Özet

Bu çalışmada süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSHpx), glutatyon redüktaz (GR) ve Glutatyon S-transferaz (GST) gibi antioksidan enzimlerin aktiviteleri gökkuşağı alabalığı *Oncorhynchus mykiss* (Walbaum 1792)'in döllenmemiş ve döllenmiş yumurtaları, 12, 22 ve 32 günlük embriyolarında araştırılmıştır. E vitamini, lipid peroksidasyon ürünleri ve yağ asidi düzeyleri de belirlenmiştir. *O. mykiss*'in döllenmiş yumurtalarında E vitamini düzeyinde önemli bir azalma bulunmuştur. Aksine malondialdehid (MDA) düzeyi döllenmiş yumurtalar ve 12 günlük embriyolarda çok yüksek bulunmuştur. Döllenmiş yumurtalar ve 12 günlük embriyoların PUFA içerikleri diğer embriyonik safhalardan düşük bulunmuştur. SOD, CAT, GSHpx, GR ve GST enzim aktivitelerinin *O. mykiss*'in yumurta ve embriyolarında ölçülebilir miktarda olduğu belirlenmiştir. *O. mykiss*'in döllenmiş yumurtalarında SOD, GR and GST aktivitelerinde çok önemli bir azalma belirlenirken, aynı safhada CAT aktivitesinde oldukça önemli bir artış tespit edilmiştir. GSHpx aktivitesi döllenmiş yumurtalarda ve yumurtaların açılmasından önce 32 günlük embriyolarda önemli bir artış göstermiştir. GST aktivitesi gözlenme safhasında bulunan 22 günlük embriyolarda önemli bir artış göstermiştir.

Anahtar Kelimeler: Süperoksit dismutaz, katalaz, glutatyon peroksidaz, glutatyon redüktaz, glutatyon S-transferaz, E vitamini, embriyo, lipit peroksidasyonu.

Introduction

Early development in larval fish is dependent on the internal complement of essential nutrients that are present in the egg (Laevens *et al*., 1999). For teleosts, much of the energy required for oocyte development is derived from lipid reserves accumulated by the female prior to or during the spawning season (Wiegand, 1996). Hence nutrition is vital for the production of high quality eggs and larvae, with optimal fatty acid compositions that will provide the best chance of success for developing embryos and larvae (Tocher, 2003).

Fish eggs are known to contain relatively high

concentrations of a particular class of lipids, polyunsaturated fatty acid (PUFA). PUFA are essential to normal development of fish and are incorporated into cellular and subcellular membranes, helping to maintain the fluidity of those membranes. While imparting fluidity is essential, the structure of PUFA makes them particularly susceptible to attack from reactive oxygen species (ROS) (Palace and Werner, 2006). Tissue lipid PUFA content and composition are critical factors in lipid peroxidation, and as fish tissues contain large quantities of highly unsaturated n-3 series fatty acids they may be more at risk from peroxidative attack than are mammals (Bell and Cowey, 1985).

ROS are highly reactive oxidants and can initiate lipid peroxidation, a chain reaction process that can damage PUFA lipids. Lipid peroxidation produces damaging lipid by-products including fatty acid hydroperoxides, aldehydes and hydrocarbons. These by-products can damage cell membranes, inactivate enzymes and damage macromolecules including DNA and RNA within the developing embryo (Mourente *et al*., 1999). They are free radicals and/or oxygen derivatives produced from the normal activity of living cell and also during lipid metabolism. When present at abnormal levels they are harmful to the cells and may alter the physiological mechanisms and in turn adversely affect the health of an animal. The ROS production in animals is regulated through their antioxidant defences. Under homeostasis, equilibrium exists between ROS production and antioxidant mechanisms (Bello *et al*., 2000; Kiron *et al*., 2011). It is also proposed that animals have evolved mechanisms that utilize the toxic properties of ROS to resist pathogens (Rodriguez and Redman, 2005).

Free radicals and reactive species are continuously generated in all aerobic biological systems, including fish. Consequently, adequate protection systems are necessary in order to avoid and/or repair the damage these compounds could cause. These systems establish a fragile balance between the physiological properties of free radicals and their capacity to cause damage. To maintain health and prevent oxidation-induced lesions and mortalities, there must be effective antioxidation systems operating in fish. A wide range of antioxidant mechanisms are present in fish, including enzymatic and non-enzymatic defenses (Martínez-Álvarez *et al*., 2005). The main enzymes with antioxidant activity are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST). Together with glutathione, these provide a primary defence as endogenous physiological antioxidants. A second line of defence is established by antioxidants, which can be provided only by nutritional supplements (Sen, 1995), such as vitamin C, vitamin E, ubiquinones, and β-carotenes (Ahmad, 1995; Halliwell and Gutteridge, 2000).

The antioxidant defence system is widely studied in mammals, especially in humans, but hardly investigated in fish. In addition, most of the studies have been done in marine fish (Stéphan *et al*., 1995; Murata *et al*., 1996; Mourente *et al*., 1999) with few studies in freshwater fish (Radi *et al*., 1987; Roy *et al*., 1995). The study of antioxidant enzymes during embryogenesis in fish can be vital to understand both the mechanisms against ROS during the lives of organisms and the changes associated to environmental stress such as temperature, oxygen and pollution (Von Westernhagen, 1988).

The aim of the present study was to determine the antioxidant enzymes activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST) and their relationships with each other as well as other components of the antioxidant system such as vitamin E and lipid peroxidation products and fatty acids levels in unfertilized and fertilized eggs, 12, 22 and 32 day embryos within the egg of rainbow trout *Oncorhynchus mykiss*. Such investigation may be useful to understand how to avoid oxidation problems that may cause pathologies and disease and to enhance growth and quality during embryogenesis.

Materials and Methods

Experimental Fish and Sample Collection

Oncorhynchus mykiss eggs and sperm samples used in the present study were obtained from three females and males aged 4 and 3 years, respectively at a local and commercial fish farm in Sivas, Turkey. Mature *O. mykiss* were artificially spawned and unfertilized eggs samples were taken from each of the three female *O. mykiss* during spawning on the farm and then the eggs were fertilized by conventional procedures and immediately transported to a hatchery. The temperature, pH and oxygen level of the water were 9.7°C, 7.5 and 8.6 mg L^{-1} respectively. The water flow rate was 26 $L.min^{-1}$. The samples of fertilized eggs were taken on days 1, 12, 22 and 32 post fertilization until hatching and stored at -80°C until analysis. Eyed eggs were determined on 22^{nd} day of embryonic development. Eggs were hatched after 35^{th} day of embryonic development.

Analytical Methods

Samples of eggs and embryos (1g x 3 replicates) were homogenised in ice-cold buffer (20 mM phosphate buffer pH 7.4, 1 mM EDTA and 0.1% Triton X-100). Homogenates were centrifuged at 30,000×g for 30min. After centrifugation, the debris was removed, and the supernatant was collected and frozen at -80°C for further enzyme assays.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed in terms of its ability to inhibit the oxygen-dependent oxidation of adrenalin

(epinephrine) to adenochrome by xanthine oxidase plus xanthine (Panchenko *et al*., 1975). The reaction was followed at 480 nm and one unit of SOD activity is defined as the amount of the enzyme causing 50% inhibition of the rate of adenochrome production at 26°C. Solutions used in SOD activity measurement was made fresh daily. The assays were run by adding to the cuvette sequentially 0.05 M potassium phosphate buffer pH 7.8/0.1 mM EDTA, 100 µl adrenaline, 100 µl xanthine and 200 µl sample. The reaction was then initiated by adding 20 µl xanthine oxidase.

Catalase (CAT) (EC 1.11.1.6) activity was measured by following the reduction of hydrogen peroxide (H_2O_2) at 30°C and 240 nm using the extinction coefficient 0.04 mM^{-1} cm^{-1} (Beers and Sizer, 1952). Immediately before assay, a stock solution was prepared. The quartz assay cuvette contained 50µl sample solution in a final volume of 250 µl containing 67 mM phosphate buffer pH 7.0 and 20 mM H_2O_2. One unit of CAT represents the amount of enzyme that decomposes 1 µmol of H_2O_2 per minute.

Glutathioneperoxidase (GSHpx) (EC 1.11.1.9) was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Bell *et al*., 1985). The GSSG generated by GSHpx was reduced by GR and NADPH oxidation was monitored at 340 nm. The quartz assay cuvette contained the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.6 mM reduced glutathione (GSH), 3.6 mM sodium azide, 1 IU ml^{-1} glutathione reductase, 0.2 mM NADPH and 0.05 mM H_2O_2. Moreover, 0.05 mM cumene hydroperoxide was used as substrate instead of hydrogen peroxide. Sample was added and specific activities were determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm using the extinction coefficient 6.22 mM^{-1} cm^{-1}. Reaction mixture in quartz assay cuvette consisted of 0.1 M potassium phosphate buffer (pH 7.2), 2 mM EDTA, 0.63 mM NADPH and 0.15 mM oxidised glutathione (GSSG). The reaction initiated by the addition of the sample.

Glutathione S-transferase (GST) (EC 2,5,1,18) activity was measured at 340 nm with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM glutathione (GSH) in 100 mM potassium phosphate buffer, pH 6.5. The quartz assay cuvette containing 100 mM potassium phosphate buffer pH 6.5. 100 ml GSH and 100 ml CDNB were prepared and the reaction initiated by the addition of 50 ml sample. Specific activities were determined using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Fatty Acid Analyses

Total lipid contents of the eggs and embryos were extracted after homogenization in 3:2 (v/v) hexane isopropanol mixtures according to procedures described by Hara and Radin (1978). All solvents contained 0.01% butylated hydroxytoluene as antioxidant. Fatty acid methyl esters were prepared from total lipid by acid-catalyzed transmethylation at 55°C for 15 h according to method of Christie, 1990. They were analyzed in a GC-17A Shimadzu gas chromatography equipped with SPTM-2380 fused silica capillary column 30 m x 0.25 mm x 0,2 µm film thicknes.

The level of Vitamin A, D, E, K and Cholesterol, Stigmasterol and β-sitosterol were analysed by Shimadzu full VP series HPLC according to the method of Katsanidis and Addis, 1999. Total protein levels were spectrophotometrically measured at 750 nm according to the method of Lowry *et al*., 1951 with bovine serum albumin as a standard. Glutathione (GSH) levels were spectrophotometrically measured according to the method of Teare *et al*. 1993. This method is based on the reduction of GSSG to GSH in the presence of glutathione reductase and NADPH, and formation of the colored product which is formed by the reaction of GSH with DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), followed by its absorbance at 412 nm. Malondialdehyde (MDA) levels were spectrophotometrically measured. Thiobarbituric acid-reactive substances (TBARs) were assayed at 532 nm according to the method of Salih *et al*. (1987).

Statistical Analysis

The statistical analyses were performed using a commercial statistical program (SPSS 15.0) for Windows. All analytical determinations were performed in triplicate and the mean values were reported. All data are statistically compared by one way variance analysis (ANOVA) and comparisons between means were performed with Tukey's test. Differences between means were reported as significant if $P<0.05$.

Results

Fatty acid compositions from unfertilized *O. mykiss* eggs to 32 day embryo are shown in Table 1. Throughout the embryogenesis, total polyunsaturated fatty acid (ΣPUFA) were dominated by C22:6n-3, C22:5n-3, C20:5n-3, C20:4n-6, C18:2n-6, and C18:3n-3. C16:0, C18:0 and C14:0 were the most common saturated fatty acids. The major constituents of monounsaturated fatty acids (MUFA) were C18:1n-9 and C16:1n-7.

The most significant ($P<0.05$) depletion was observed in total monounsaturated fatty acid (ΣMUFA), which fell from 26.19% in 12 day embryo to 21.78% in 22 day embryo found in eyed eggs stage. The percentages of ΣMUFA decreased significantly ($P<0.05$) due to the decrease in the content of the

most abundant unsaturated fatty acid, C18:1n9 while a significant increase was observed in the total saturated fatty acids (ΣSFA), due to the increase in the content of the most abundant saturated fatty acid, C16:0 which rose from 13.82% in unfertilized eggs to 15.05% in 22 day embryo.

Throughout the period of embryogenesis, total polyunsaturated fatty acid (ΣPUFA) formed 54.21% of the total fatty acid in 22 day embryo and consisted mainly of total n-3 fatty acid, followed by total n-6 fatty acid. The individual fatty acids contribution to the total polyunsaturated fatty acid was represented by C20:5n-3, C22:5n-3 and C22:6n-3 as total n-3 and C20:4n-6 as total n-6 fatty acids. The PUFA content in 12 day embryo was found to be lower than in the other embryonic stages ($P<0.05$).

The levels of total protein, glutathione (GSH) and MDA in fish *O. mykiss* unfertilized and fertilized eggs, 12, 22 and 32 day embryos are reported in Table 2. The amount of proteins decreased significantly in fertilized eggs and 12 day embryo ($P<0.01$), the amount of protein in 32 day embryo was detected to increase significantly ($P<0.001$), to reach a maximum value (47.64±1.66). The difference in the protein content between unfertilized eggs and 22 day embryo was found statistically insignificant ($P>0.05$). The

Table 1. Changes in fatty acid composition in unfertilized and fertilized eggs, 12, 22 and 32 day embryos of *Oncorhynchus mykiss*[*]

Fatty acids	Unfertilized eggs	Fertilized eggs	12 day embryo	22 day embryo	32 day embryo
C14:0	1.70±0.01 [a]	1.69±0.04[a]	1.76±0.04[a]	1.57±0.03[b]	1.70±0.04[a]
C15:0	0.32±0.00[a]	0.34±0.01[ab]	0.36±0.00[b]	0.36±0.00[b]	0.32±0.02[a]
C16:0	13.82±0.02[a]	14.92±0.01[b]	14.54±0.03[c]	15.05±0.05[b]	14.49±0.07[c]
C17:0	0.39±0.01[a]	0.40±0.01[ab]	0.39±0.00[a]	0.43±0.01[b]	0.40±0.01[a]
C18:0	5.60±0.01[a]	5.42±0.01[b]	5.09±0.05[c]	5.81±0.07[d]	5.60±0.06[a]
C22:0	0.67±0.00[a]	0.81±0.03[b]	0.95±0.02[c]	0.56±0.04[d]	0.71±0.05[a]
C24:0	0.22±0.02[a]	0.19±0.00[ab]	0.16±0.01[b]	0.22±0.00[a]	0.21±0.03[ab]
Σ SFA	22.71±0.01[a]	23.78±0.06[b]	23.25±0.05[c]	24.01±0.06[b]	23.43±0.16[c]
C16:1n-7	3.48±0.01[a]	3.61±0.02[b]	3.79±0.04[c]	3.18±0.02[d]	3.45±0.05[a]
C17:1	0.15±0.00[a]	0.14±0.01[a]	0.18±0.02[b]	0.12±0.00[a]	0.13±0.01[a]
C18:1n-9	19.85±0.06[a]	19.20±0.05[b]	20.31±0.02[c]	16.50±0.03[d]	18.84±0.05[e]
C20:1n-9	1.52±0.01[a]	1.43±0.01[b]	1.44±0.02[b]	1.46±0.01[b]	1.47±0.02[b]
C22:1	0.43±0.00[a]	0.47±0.03[ab]	0.48±0.02[ab]	0.52±0.01[b]	0.42±0.03[a]
Σ MUFA	25.43±0.05[a]	24.85±0.09[b]	26.19±0.07[c]	21.78±0.03[d]	24.31±0.01[e]
C18:3n-3	2.45±0.02[a]	2.29±0.02[b]	2.43±0.03[a]	2.09±0.02[c]	2.58±0.06[d]
C20:5n-3	8.28±0.07[a]	8.52±0.04[b]	8.33±0.04[a]	9.40±0.05[c]	8.08±0.07[d]
C22:5n-3	3.52±0.02[ab]	3.59±0.06[a]	3.43±0.03[b]	3.87±0.04[c]	3.60±0.02[a]
C22:6n-3	24.81±0.02[a]	23.88±0.05[b]	22.77±0.03[c]	27.34±0.02[d]	24.64±0.04[e]
Σ n-3	39.06±0.08[a]	38.28±0.13[b]	36.96±0.03[c]	42.70±0.06[d]	38.90±0.09[a]
C18:2n-6	7.81±0.04[a]	8.17±0.03[b]	8.75±0.07[c]	6.46±0.07[d]	8.34±0.05[e]
C18:3n-6	0.26±0.00[a]	0.28±0.01[b]	0.29±0.00[bc]	0.30±0.01[c]	0.20±0.00[d]
C20:2n-6	1.36±0.01[a]	1.29±0.01[a]	1.35±0.03[a]	1.10±0.01[b]	1.51±0.04[c]
C20:3n-6	0.71±0.01[a]	0.80±0.02[b]	0.90±0.04[c]	0.63±0.01[d]	0.70±0.03[ad]
C20:4n-6	2.66±0.03[a]	2.55±0.02[a]	2.32±0.03[b]	3.02±0.06[c]	2.61±0.07[a]
Σ n-6	12.80±0.02[a]	13.09±0.05[b]	13.60±0.03[c]	11.51±0.05[d]	13.36±0.09[e]
Σ PUFA	51.86±0.06[a]	51.38±0.14[b]	50.56±0.05[c]	54.21±0.03[d]	52.26±0.17[e]
Σ n-3/ Σ n-6	3.05±0.01[a]	2.92±0.01[b]	2.72±0.01[c]	3.71±0.02[d]	2.91±0.02[b]

[*]The data are expressed as percentages of total fatty acids. Each value is the mean±S.E. (standard error) of 3 repetitions. Superscripts after values in a same line with different letters represent significant difference ($P<0.05$). Σ: Total. ΣSFA: Total Saturated Fatty Acid. ΣMUFA: Total Monounsaturated Fatty Acid. Σn-3: Total n-3 Fatty Acid. Σn-6: Total n-6 Fatty Acid. ΣPUFA: Total Polyunsaturated Fatty Acid.

Table 2. Changes in Total Protein, GSH and MDA in unfertilized and fertilized eggs, 12, 22 and 32 day embryos of *Oncorhynchus mykiss*[*]

	Unfertilized eggs	Fertilized eggs	12 day embryo	22 day embryo	32 day embryo
Total Protein mg/g	37.22±1.49 [a]	28.67±2.92 [c]	28.54±0.91 [c]	34.55±1.53 [a]	47.64±1.66 [d]
GSH µg/g	28.07±1.45 [a]	35.55±1.47 [c]	26.50±0.75 [a]	41.56±2.02 [d]	54.72±1.28 [d]
MDA nmol/g	49.30±1.44 [a]	57.44±1.70 [d]	57.44±1.76 [d]	48.23±1.34 [a]	44.51±1.42 [b]

*Each value is the mean±S.E. (standard error) of 3 repetitions. Superscripts after values in a same line with different letters represent significant difference. a: $P>0.05$, b: $P<0.05$, c: $P<0.01$, d: $P<0.001$.

a: Values of $P>0.05$ is not statistically significant.

b: Values of $P<0.05$ is statistically significant.

c: Values of $P<0.01$ is statistically more significant

d: Values of $P<0.001$ is statistically most significant

difference in the glutathione concentration between unfertilized eggs and 12 day embryo was found statistically insignificant (P>0.05). While the glutathione concentration in the fertilized eggs exhibited significantly (P<0.01) high values, the most significant increase was detected in 22 day embryo and 32 day embryo of both groups (P<0.001)

The level of lipid peroxides, as indicated by assayable MDA, was significantly (P<0.001) higher in fertilised eggs and 12 day embryo (57.44±1.70 and 57.44±1.76, respectively) than in unfertilized eggs and 22 and 32 day embryos (Table 2). The level of MDA showed an important decrease in 32 day embryo (P<0.05). Statistical difference between unfertilized eggs and 22 day embryo was not observed (P>0.05).

The specific activity of the antioxidant enzymes is shown in Table 3. Considering the change in the superoxide dismutase (SOD) activity, an important decrease was detected in fertilized eggs, 12 day embryo and 22 day embryo compared to unfertilized eggs (P<0.001). There was no significant SOD activity difference in 32 day embryo (P>0.05). The activity of catalase (CAT) was found insignificant between unfertilized eggs and 32 day embryo (P>0.05). However, it was found significantly higher in fertilized eggs, 12 day embryo and 22 day embryo (P<0.001, P<0.01). Compared to unfertilized eggs, GSHpx activity was found to increase slightly in fertilized eggs, but decrease in 12 day embryo (P<0.05). While there was no difference between unfertilized eggs and 22 day embryo (P>0.05), GSHpx activity was found to increase at a significant amount in 32 day embryo (P<0.001).

The highest GR activity (P<0.01) was observed in 22 day embryo, the lowest in fertilized eggs (P<0.001). Similar GR activity values were examined between unfertilized eggs and 12 day embryo (P>0.05). Compared to unfertilized eggs, GR activity (P<0.05) was found to decrease slightly in 32 day embryo (P<0.01). The highest GST activity was found in 22 day embryo, the lowest in fertlized eggs (P<0.001). Compared to unfertilized eggs, it decreased in 12 day embryo and 32 day embryo (P<0.05, P<0.01).

Table 4 shows the leves of Retinol (µg/g), Vitamin D_3 (µg/g), δ-Tocopherol (µg/g), α-Tocopherol (µg/g), Vitamin K_2 (µg/g), Cholesterol (mg/g), Stigmasterol (µg/g) and β-sitosterol (µg/g).

When retinol levels were examined throughout the embryogenesis, there were no significant differences from unfertilized eggs to 12 day embryo. Similarly, there were no differences in the level of retinol between 22 and 32 day embryos of *O. mykiss*, but an increase was observed in 22 day embryo. A steady increase in vitamin D_3 level was noted throughout the development (P<0.05, P<0.01 P<0.001). δ-Tocopherol level remained relatively constant throughout the experimental period before increasing sharply in 32 day embryo (P<0.001). α-Tocopherol did not show any marked change during the embryonic development except for fertilized eggs where a significant (P<0.01) decrease was noted (Table 4). In the development stage of *O. mykiss*'s

Table 3. Changes in antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST) activities in unfertilized and fertilized eggs, 12, 22 and 32 day embryos of *Oncorhynchus mykiss*[*]

	Unfertilized eggs	Fertilized eggs	12 day embryo	22 day embryo	32 day embryo
SOD (U/g)	8.33±0.37[a]	4.25±0.17[d]	5.05 ±0.12[d]	4.75 ±0.16[d]	7.61 ±0.11[a]
CAT (µg/g/1min)	25.67±2.40[a]	57.01±2.88[d]	34.01±1.11[c]	36.33±1.33[c]	24.01±2.88[a]
GSHpx (U/g/1min)	14.07±0.52[a]	17.01±1.11[b]	11.01±0.58[b]	15.33±1.21[a]	26.67±0.88[d]
GR (U/g/1min)	3.51±0.51[a]	1.57±0.23[d]	3.51±0.51[a]	4.33±0.16[c]	3.17±0.17[b]
GST (µg/g/1min)	475.01±33.29	275.01±34.03[d]	416.67±16.17[b]	510.01±17.28[b]	376.67±13.21[c]

* The meaning of the symbols is given under Table 2.

Table 4. Changes in Vitamin A, D, E, K and Cholesterol, stigmasterol and β-sitosterol contents in unfertilized and fertilized eggs, 12, 22 and 32 day embryos of *Oncorhynchus mykiss*[*]

	Unfertilized eggs	Fertilized eggs	12 day embryo	22 day embryo	32 day embryo
Retinol (vit. A) µg/g tissue	1.24±0.02[a]	1.26±0.04[a]	1.19±0.02[a]	1.43±0.06[b]	1.11±0.01[b]
Vitamin D_3 µg/g tissue	1.31±0.05	1.49±0.11[b]	1.37±0.07[b]	1.69±0.02[c]	1.73±0.08[d]
δ-Tocopherol µg/g tissue	1.48±0.12	1.13±0.07[c]	1.04±0.01[c]	0.96±0.05[c]	5.22±0.07[d]
α-Tocopherol µg/g tissue	28.12±1.85[a]	25.21±0.93[c]	28.98±0.54[a]	30.01±1.13[a]	29.73±1.19[a]
Vitamin K_2 µg/g tissue	11.71±0.32	14.85±0.33[c]	13.78±0.41[b]	15.08±1.11[c]	14.01±0.58[b]
Cholesterol mg/g tissue	1.65±0.02[a]	1.81±0.01[b]	1.71±0.03[a]	1.98±0.09[c]	1.79±0.01[b]
Stigmasterol µg/g tissue	132.69±1.64	98.33±1.23[c]	94.99±1.21[c]	92.67±1.33[c]	97.55±1.18[c]
β-sitosterol µg/g tissue	0.19±0.06	0.26±0.04[b]	0.47±0.01[c]	0.12±0.01[b]	0.11±0.01[b]

* The meaning of the symbols is given under Table 2.

eggs and embryos, an important ($P<0.05$, $P<0.01$) fluctuation with a decreasing and increasing trend in vitamin K_2 levels was observed. This fluctuation seen in the *O. mykiss*'s eggs and embryos, showed a significant ($P<0.01$) increase in 22 day embryo. Similar to vitamin K_2 level, in the development stage of the *O. mykiss*'s eggs and embryos, an important ($P<0.05$, $P<0.01$) fluctuation with a decreasing and increasing trend in cholesterol levels was observed. A significant ($P<0.01$) increase was shown in 22 day embryo.

The highest stigmasterol level was observed in unfertilized eggs. Although there were no significant differences in stigmasterol levels from fertilized eggs to 32 day embryo a slight decrease was observed during embryonic development. The highest β-sitosterol level was observed in 12 day embryo. It was observed that β-sitosterol levels did not show any significant changes in the other embryonic stages.

Discussion

Lipid peroxidation is considered as the main molecular mechanisms involved in the oxidative damage to cell structures and in the toxicity process that lead to cell death. It is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers. Enzymes such as catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase, and non-enzymatic factors such as vitamins A, C and E, glutathione, ubiquinone, iron chelators and thiol-containing proteins all function to neutralise ROS or byproducts within the cell. As long as the cell, tissue, and whole organism can maintain antioxidant concentrations above the level of ROS generation, cellular components are protected. It is expected that supplementation with adequate antioxidants, as for instance, α- tocopherol, will keep sensitive cells and organs in healthy conditions and increase lifespan (Palace and Werner, 2006; Dianzani and Barrera, 2008; Repetto *et al*., 2012).

In this study the *O. mykiss* eggs and embryos contained substantial SOD, CAT, GSHpx, GR and GST activities. All these enzyme activities were also detected in unfertilized eggs. This indicates the presences of compounds that can cause oxidative stress and also suggests that the eggs were well protected against peroxidation despite the very high level of PUFA. The results of Mourente *et al*. (1999) in *Dentex dentex* and Díaz *et al*. (2010) in *Acipenser naccarii* in terms of notable antioxidant-enzyme activity in the embryo within the egg support the findings of the present study.

The enzyme activities investigated in this study showed that the SOD activity in the embryonic development was lower than CAT, GSHpx and GST activities during embryogenesis of *O. mykiss*. The results of this study also showed that the activity of SOD was lowest in fertilized eggs and highest in unfertilized eggs of *O. mykiss* indicating the presence of increased superoxide anion ($O_2^{\cdot-}$) production in unfertilized eggs. Catalase has one of the highest turnover rates for all enzymes. The enzyme very efficiently promotes the conversion of H_2O_2 to water and molecular oxygen. In this study CAT activity was higher than the SOD activity during embryogenesis of *O. mykiss* and both enzymes act in chain to eliminate the superoxide anions formed. Unlike the SOD activity, CAT activity reached the maximum level in fertilized eggs and decreased gradually until the end of embryogenesis. While CAT activity decreased from fertilized eggs to 32 day embryo, GSHpx activity showed an important increase at the same period. GST activities showed the most important increase in 22 day embryo of eyed eggs stage.

Unlike the results of Díaz *et al*. (2010) in *Acipenser naccarii* showing that the activity corresponding to the GR was detected only after hatching, our results showed GR activity in eggs and embryos of *O.mykiss.* The activity of GR in our study, which had the lowest value of all the enzymes assayed is nonetheless essential for the regeneration of reduced GSH (Miller *et al*., 1993). GSHpx activity should be accompanied by activity of GR. The differences in GSHpx and GR activities may indicate an important role for the glutathione system in embryogenesis as a front line of antioxidant defence. All living organisms have evolved protective systems to minimize injurious events that result from bioactivation of chemicals including xenobiotics and oxidative products of cellular metabolism of molecular oxygen. The major protective system is dependent upon GSH. GSH acts both as a nucleophilic " scavenger " of numerous compounds and their metabolites, via enzymatic and chemical mechanisms, converting electrophilic centers to thioether bonds, and as a cofactor in the GSH peroxidase - mediated destruction of hydroperoxides. GSH depletion to about 15 – 20% of total glutathione levels can impair the cell's defense against the toxic actions of such compounds and may lead to cell injury and death. Glutathione peroxidase and glutathione reductase are primary responsible for maintenance of the intracellular concentration of GSH (Tian et al., 1999; Leopold and Loscalzo, 2000; Smart and Hodgson, 2008).

Our results on GR activity agree with those of Mourente *et al*. (1999) in *Dentex dentex.* Related to GSHpx activity our results agree with Wiegand *et al*.'s (2000) studies in zebrafish that showed that from early beginning of ontogenesis (2–4 cells) embryos possess detoxication enzymes like GST and GSHpx. These enzymes seem to be constitutive in the embryos. According there is major activity in

fertilized eggs and 22 day embryo showing the need to face free radicals production.

GSHpx acts in conjunction with the tripeptide glutathione (GSH), which is present in cells in high (2-5 mM) concentrations in almost all eukaryotic cells and many procaryotic organisms. It is, besides conjugation, also used to reduce reactive oxygen species. This reaction is catalysed by the GSHpx and the peroxidase function of the GST. The substrate for the catalytic reaction of GSHpx is H_2O_2, or an organic peroxide ROOH. The GSHpx has a protector role against lipid peroxides and decomposes peroxides to water (or alcohol) while simultaneously oxidising GSH. Significantly, GSHpx competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress (Fahey and Sunquist, 1991; Nakano *et al*., 1999; Valko *et al*., 2006).

It is difficult to interpret changes in the activity of antioxidant enzymes occurring in embryonic development of *O. mykiss*, because the activity of these enzymes is also strongly related to the level of oxidative metabolism and depends on many factors such as conformational changes in enzymes caused by oxidants, change in the ratio between SH- and SS-groups in enzymes, nitrate amino acid residues, and reversibly influence the active sites of enzymes (Sunde and Hoekstra, 1980).

It should be noted that the activity of SOD, which catalyzes dismutation of superoxide radicals to O_2 and to the less reactive species H_2O_2, strongly correlates with the level of redox processes: the more intensive oxygen consumption, the higher the activity of SOD. Changes in the embryonic development occurring under a moderate excess of oxygen in medium indicate that metabolic processes under these conditions are not disturbed. Energy metabolism in embryos is disturbed only under a large excess of oxygen (Bean, 1963; Mc Cord and Fridovich, 1969; Lynch and Fridovich, 1978; Isuev *et al*., 2008). Differential sensitivity of embryos of different animal species to oxygen excess or deficiency is based on different degree of reliability of their antioxidant defense systems (Isuev *et al*., 2008). Janssens *et al*. (2000) reported a correlation between the decline in SOD activity and oxidative metabolism indicating that the enzyme levels of SOD were adjusted to the endogenous source of reactive oxygen species. Decreased production and non-availability of the substrate ($O_2^{\cdot-}$) in response to vitamin E supplementation may be a reason for decreased SOD activities. Palace *et al*. (1993) demonstrated an increase in SOD activity in the tissues of *O. mykiss* fed with vitamin E deficient diets.

Vitamin E is actually a collective term referring to two structural types of molecule: tocopherols and tocotrienols. Both molecule types consist of a phytyl tail, which is embedded in the lipid membrane of cellular and subcellular membranes, and a chroman head, which protrudes to the inner or outer surfaces of the membrane. The hydroxyl group(s) situated on the chroman head of either tocopherols or tocotrienols are the centre of the vitamin's antioxidant activity. This moiety is typically sacrificed to reduce ROS or lipid peroxyl radicals before they can damage other cellular components (Dimascio *et al*., 1991; Palace and Werner, 2006). By virtue of this ability, one tocopherol molecule can protect up to 1000 lipid molecules in the membrane (Liebler, 1993). The biopotencies of tocopherols, defined as the absorption in the gut, are in the order α-tocopherol >β-tocopherol > γ-tocopherol > δ-tocopherol (Deshpande *et al*., 1996).

The transfer of vitamin E from the yolk to the developing embryo is a gradual process that is probably associated with the assimilation of other lipids from the yolk (Palace and Werner, 2006). King (1985) reported that two weeks after hatching 50-66% of the tocopherol was associated with the yolk in *O. mykiss*. Takeuchi *et al*. (1981) showed that while concentrations of tocopherol changed in the yolk during development from fertilised eggs through embryonic development of ayu (*Plecoglossus altivelis*), the actual total amount of tocopherol in the embryo or larvae stayed the same. However, Cowey *et al*. (1985) found that vitamin E concentrations declined significantly in Atlantic salmon (*Salmo salar*) from the fertilised egg and eye stage to the yolk sac and swim up stages. We have examined an important decrease in vitamin E (α-tocopherol and δ-tocopherol) level in fertilized eggs of *O. mykiss*, but the level of α-tocopherol in unfertilised eggs and 12 day, 22 day and 32 day embryos except that fertilized eggs remained relatively same. Our α-tocopherol results were found to agree with Takeuchi *et al*.'s (1981) studies in *Plecoglossus altivelis* but in disagreement with Cowey *et al*. (1985)'s studies in *Salmo salar*. Cowey *et al*. (1985) found that vitamin E concentrations declined significantly in Atlantic salmon (*Salmo salar*) from the fertilised egg and eye stage to the yolk sac and swim up stages. In our study, δ-tocopherol levels from fertilised eggs to 22 day embryo remained relatively constant, but it increased sharply in 32 day embryo ($P<0.001$).

Lipid peroxidation occurs in presence of α-tocopherol deficiency. In addition to containing high concentrations of polyunsaturated fatty acids and transition metals, biological membranes of cells and organelles are constantly being subjected to various types of damage (Chance *et al*., 1979; Halliwell and Gutteridge, 1984). Autoxidation of PUFA produces compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes such as malondialdehyde (MDA) and hydrocarbons. Malondialdehyde is formed as an end product of lipid peroxidation and therefore, it is accepted as an indicator of lipid damage by ROS (Sumida *et al*., 1989).

The result of the present study illustrate that the MDA level in fertilised eggs and 12 day embryo was highest which is in agreement with studies by

Mourente *et al*. (1999) in *Dentex dentex*. They showed that the level of malondialdehyde (MDA) was very high in eggs but it was 3-fold lower in newly hatched larvae. Our study also showed that there is a significant decrease in vitamin E (α-tocopherol and δ-tocopherol) level in fertilized eggs of *O. mykiss*. When we look at the PUFA content, consisting mainly of total n-3 fatty acid in fertilised eggs and 12 day embryo, it was found to be lower than in the other embryonic stages. A decrease in the percentage of C22:6n-3 in fertilised eggs and 12 day embryo suggests that the egg lipid PUFA content and composition are critical factors in lipid peroxidation and since fish tissues contain large quantities of highly unsaturated n-3 series fatty acids they may be more at risk from peroxidative attack than mammals (Bell and Cowey, 1985).

These results show the levels of lipid peroxidation products, MDA levels, vitamin E levels and the activities of the embryonic antioxidant enzymes during embryogenesis of *O. mykiss*. The nutritive substances nourishing the embryo during embryogenesis are mainly lipids with a high level of unsaturated fatty acids tending towards oxidation. Protection against oxidation is offered by the antioxidant enzymes in this life phase. Besides, during fish development, early embryonic stages are the most vulnerable stages to environmental stress, like changes in temperature or oxygen content of the water let alone the additional stress due to the increasing pollution (Von Westernhagen, 1988).

In conclusion, this study revealed that measurable amounts of all the antioxidant enzymes were present in *O. mykiss* eggs and embryos prior to hatching and the antioxidant enzymatic defence mechanisms in the freshwater fish *O. mykiss* are operating adequately and support the embryonic development. Further studies will aid to explain the antioxidant enzymatic defences during the yolk sac larvae development of *O. mykiss*.

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