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RESEARCH PAPER

# Antioxidant Enzyme Activities and Some Biochemical Changes in Rainbow Trout *Oncorhynchus mykiss* (Walbaum 1792) Yolk-Sac Larvae

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**Abstract**

In this work we studied the biochemical status of antioxidant defences of yolk-sac larvae from the commercial fish, rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) over a period of 19 days from hatching. The parameters studied were: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST). Oxidative damage was measured by the formation of malondialdehyde (MDA). Our results showed that the presence of catalytic activities was observed from hatching day and significant changes in the enzymes were seen in the 12 and 19 days yolk-sac larvae, when the larvae finished their endogenous feeding. Our study indicates that catalase activity sharply increased in the 12 and 19 days post-hatch but superoxide dismutase activity sharply decreased in the same period. Glutathione peroxidase regularly decreased with age throughout the 19-day study period. Glutathione reductase showed a significant increase in the 12 and 19 days yolk-sac larvae. There were no significant changes in glutathione S-transferase activity throughout the yolk-sac stage. When the egg yolk-sac was reabsorbed, the total protein content sharply decreased at day 19. The PUFA contents in the 12 and 19 days yolk-sac larvae were found to be higher than in the other yolk-sac stage.

*Keywords*: Oncorhynchus mykiss, antioxidant enzymes, yolk-sac larvae, lipid peroxidation, Glutathione (GSH).

**Gökkuşağı Alabalığı Oncorhynchus mykiss (Walbaum 1792)'in Keseli Larvalarında Antioksidan Enzim Aktiviteleri ve Bazı Biyokimyasal Değişimler**

**Özet**

Bu çalışmada ticari olarak yetiştirilen gökkuşağı alabalığı *Oncorhynchus mykiss* (Walbaum 1792)'in keseli yavrularında, yumurtadan çıktıktan sonraki 19 gün boyunca antioksidan savunma ve biyokimyasal durum çalışıldı. Çalışılan parametreler: süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSHpx), glutatyon redüktaz (GR) ve glutatyon-s-transferaz (GST)'dır. Oksidatif hasar malondialdehit (MDA) oluşumu ile ölçüldü. Sonuçlarımız yumurtaların açıldığı günden itibaren katalitik aktivitenin mevcut olduğunu ve larvalar endojen besini kullanırken, 12 ve 19 günlük keseli larvalarda bulunan enzimlerde önemli değişimlerin olduğunu gösterdi. Çalışmamız yumurtaların açılmasından sonraki 12. ve 19. günlerde katalaz aktivitesinde hızlı bir artma, aynı periyotta süperoksit dismutaz aktivitesinde ise hızlı bir azalma gösterdi. Glutatyon peroksidaz 19 günlük çalışma süresi boyunca düzenli bir şekilde azaldı. Glutatyon redüktaz 12 ve 19 günlük keseli larvalarda önemli bir artış gösterdi. Keseli safha boyunca glutatyon-s-transferaz aktivitesinde önemli bir değişim olmadı. 19. günde yumurta kesesinin emilimiyle total protein içeriği hızla düştü. 12 ve 19 günlük keseli larvalardaki PUFA içeriği, diğer keseli safhalardan yüksek bulundu.

*Anahtar Kelimeler*: *Oncorhynchus mykiss*, Antioksidan enzimler, Keseli larvalar, Lipit peroksidasyonu, Glutatyon (GSH).

## Introduction

Oxygen in its molecular state, $O_2$, is essential for many metabolic processes that are vital to aerobic life. This dependence on oxygen forces aerobic life to withstand considerable toxicity. This gives rise to the so-called ''aerobic-life paradox'' or the ''oxygen paradox'', where aerobic organisms, which cannot exist without oxygen, are nevertheless inherently at risk due to oxidative stress (Davies, 2000; Valko *et al*., 2004; Martínez-Álvarez *et al*., 2005; Díaz *et al*., 2010).

As a consequence of normal oxygen utilization by aerobes, various reactive oxygen species (ROS) or free radicals are produced. These radicals can then attack cell membranes or their constituent

lipoproteins, also attack the DNA, the chemical constituent of genes, causing mutations. Its deleterious effects include oxidation of proteins and steroid components, as well as peroxidation of unsaturated lipids in cell membranes. This produces unstable lipid hydroperoxides, the products of which, on decomposing, are highly reactive, threatening the cell integrity (Chaudıere and Ferrari-Ilıou, 1999; Martínez-Álvarez *et al*., 2005; Halliwell and Gutteridge, 2007; Rahman, 2007; Lushchak, 2014).

Fish tissues contain large quantities of polyunsaturated fatty acids (PUFA) essential for membrane function (Zengin and Akpınar, 2006; Kiron *et al*.,2011). Nevertheless, their richness in PUFA makes them highly susceptible to lipid peroxidation produced during normal cellular aerobic functioning (Matés, 2000), resulting in deleterious deterioration of cellular membranes with pathological effects on cells, tissues and morphogenesis (Fontagné *et al*., 2006; 2008).

Important antioxidants in organisms include the glutathione system and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). The tripeptide (γ-glutamatic acid, cysteine and glycine) reduced glutathione (GSH) plays a key role in protection against oxidative stress by directly scavenging ROS (Leggatt and Iwama, 2009**)** and by regenerating the antioxidant vitamins C and E from their oxidized products (Lee and Dabrowski, 2003; Gao *et al*., 2014). GSH is synthesised intracellulary and oxidized to GSSG in a reaction catalysed by selenium-dependent glutathione peroxidase (Se-GSHpx) and the GSHpx activity of certain isoforms of glutathione S-transferase (GSH) (Solé *et al*., 2004; Halliwell and Gutteridge, 2007). Both sources of GSHpx activity are able to detoxify organic peroxides produced by lipid peroxidation. Glutathione reductase (GR) is required to regenerate GSH from GSSG and regarded as essential to maintain intracellular GSH redox status. The antioxidant SOD catalyses dismutation of $O_2^{\cdot-}$ to $H_2O_2$, and the resulting $H_2O_2$ is converted water and oxygen by CAT or detoxified by GSHpx activity (Ritola *et al*., 2002; Kalaimani *et al*., 2008).

The body has evolved antioxidant defences to protect against free radical–induced damage; for example, cell manufacture repair enzymes that can "destroy free-radical-damaged proteins, remove oxidized fatty acids from membranes, and repair free-radical damage to DNA (Rahman, 2007) and additional, extracellular, antioxidant defences also exist. Vitamins E and C, the carotenoids, and coenzyme Q10 are potentially involved in these antioxidant defences. However, these defences may not be totally effective. Thus, the concentration of free radicals in the body may continue to increase, more damage can occur, and, as a result, the body suffers oxidative stress. Severe oxidative stress may result in cell injury and destruction (Neeraj *et al*., 2013 ).

Life starts with the unification of male and female gametes. As soon as the egg is fertilized by a sperm, the zygote is formed and embryonic development starts and ends up at hatching. Embryonic development varies in different species. Stages of embryonic development in eggs can be summarized as follows: fertilized egg, cleavage, morula, blastula, gastrula, embryonic body formation, optic vesicle and auditory vesicle formation, blastopore closing, tail formation and hatching stages. The hatchlings further undergo organogenesis and appear as like as their parents, thus end the larval stages. Prelarval phase is the period which begins with hatching to the end of absorption of the yolk sac. The most important characteristic of prelarval stage is the existence of a yolk sac. This yolk sac is located in the anterior and ventral sections of the body. At the beginning of the prelarval phase, mouth, anus and digestive tube are like a straight pipe. Head is smaller than the body, eyes are big and nonpigmented. The double-walled sacs in the form of external sensory organ of balance otoliths are on both sides of the head. Nostrils are not developed under the eyes. Towards to the end of this phase, the mouth and anus open. Eyes are pigmented and nutrition mouth starts at the outside part. In Salmonidae fishes, after completing the prelarval phase the fish take the characteristic shape of an adult, called alevin. Postlarval stage is the period which starts after absorbtion and finishes at the end of metamorphosis (Şahinöz *et al*., 2007; Rahman *et al*., 2009; Aral *et al*., 2011).

During fish devolopment, early embryonic stages are the most vulnerable stages to environmental stress, such as changes in temperature or oxygen content of the water and also to the additional stress due to increasing pollution (Von Westernhagen, 1988). It is well known that yolk-sac stage represents an important developmental period for all fish larvae. At this stage, significant changes in the larval body take place before exogenous feeding. Also the energy in the yolk is used for growth, development and activity, additionally lipids are major energy fuels during the embryonic and yolk-sac stages of fish larvae (Fyhn, 1993; Polat *et al*., 1995; Sargent, 1995; Verreth *et al*., 1995; Díaz *et al*., 2010). Stene and Lönning, (1984) have shown the yolk-sac stage to be the most sensitive one, followed by the embryonic stage prior to completion of gastrulation. Hatching up to yolk-sac reserves absorption is one of the most important stages of larval development because high survival phase can affect survival and growth in subsequent stages (Gawlicka *et al*., 2000). However, information about lipid peroxidation and antioxidant defences either in wild or cultured fish species is quite limited (Cowey *et al*., 1985; Radi *et al*., 1987; Stéphan *et al*., 1995; Mourente *et al*., 1999).

*Oncorhynchus mykiss* has been recognized as a main aquaculture species in the world with high economic value. As the main aquaculture species,

knowledge of the antioxidant enzyme activities, lipid peroxidation and the biochemical compositions in early embryonic and yolk-sac stages are extremely important in understanding the formation of protective mechanisms during early stages of larval development at the start of exogenous feeding. In fish, lipid peroxidation caused by oxygen radicals is a principal cause of several diseases such as jaundice, nutritional muscular dystrophy and hemolysis (Mourente *et al*., 1999). Consequently, the understanding of antioxidant enzyme dynamics could provide an important contribution for the enhancement of larval and juvenile quality. Previously we showed the correlation between antioxidant enzyme activities and lipid peroxidation during embryogenesis of rainbow trout *Oncorhynchus mykiss* (Zengin *et al*., 2015). The overall aim is the characterization of the antioxidant systems in cultured yolk-sac larvae of *Oncorhynchus mykiss* in order to enhance the growth and quality of early life stages by avoiding oxidation problems that may cause pathologies and diseases. This is within the line of research that seeks to ascertain the concrete aspects of the physiology and nutrition of this salmonid species, which is of great scientific as well as commercial interest.

## Materials and Methods

Experimental fish and sample collection was described in our previous study (Zengin *et al*., 2015). The *O. mykiss* used were obtained from the local and commercial fish farm Yeşilova in Zara (Sivas-Turkey). Fish were kept in the pool under natural environmental conditions. Eggs were fertilized in February. The water temperature was 9.7°C during embryogenesis and was 10.9°C during yolk-sac larvae in March. pH and oxygen level of the water were 7.6 and 8.5 mg $L^{-1}$ respectively. Hatching occurred after 35 days. The yolk-sacs were exhausted 19 days post-hatching. Samples of yolk-sac *O. mykiss* larvae (3x1g) were taken on days 0 (when the eggs hatched), 5, 12 and 19 until yolk absorption and stored at -80°C until analysis. The pool in which the fish were reared had a flow-through water supply originating from an underground natural spring. The water flow rate was 26 $L.min^{-1}$.

### *Analytical methods*

Whole yolk-sac larvae (1g x 3 replicates) were homogenized 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. The supernatant was collected and frozen at -80 °C until analysed. The resultant supernatants were used directly for 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 were 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 250µl containing 67mM phosphate buffer pH 7.0 and 20 mM $H_2O_2$. One unit of CAT represents the amount of enzyme that decomposes 1µmol of $H_2O_2$ per minute.

Glutathione peroxidase (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 containing 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 oxidized glutathione (GSSG). The reaction was 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 cuvettes 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 yolk-sac larvae 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 analysed 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 thickness.

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, glutathione (GSH) and malondialdehyde (MDA) levels were spectrophotometrically measured. They were assayed at 750 nm according to the method of Lowry *et al*. (1951) with bovine serum albumin as a standard, 412 nm according to the method of Teare *et al*. (1993) and 532 nm according to the method of Salih *et al*. (1987) respectively.

***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 were statistically compared by one way variance analysis (ANOVA) and comparisons between means were performed with Tukey's test. Differences between means were reported as insignificant if P>0.05, significant if P<0.05, more significant if P<0.01 and most significant if p P<0.001.

## Results

Fatty acid compositions from 0 day *O. mykiss* yolk-sac larvae to 19 day yolk-sac larvae are shown in Table 1. Throughout the yolk-sac stage, the PUFA were dominated by C22:6n-3, C22:5n-3, C20:5n-3, C20:4n-6, C18:2n-6, and C18:3n-3. Total polyunsaturated fatty acids (ΣPUFA) were at a minimum (51.91%) at 5 days yolk-sac larvae and reached a maximum (54.08%) at 12 days yolk-sac larvae of *O. mykiss*, mainly due to the increased percentage of C22:6n-3. C14:0, C16:0 and C18:0 were the most common saturated fatty acids. Total saturated fatty acid (ΣSFA) values increased during the yolk-sac stage and changed from 23.13% in 5 days yolk-sac larvae to 26.84% in 12 days yolk-sac larvae of *O. mykiss*, mainly due to the increased percentage of C16:0 and C18:0. 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 the ΣMUFA, which fell from 24.96% in 5 days yolk-sac larvae to 19.08% in 12 days yolk-sac larvae. The percentages of the ΣMUFA decreased significantly (P<0.05) due to decrease in the content of the most abundant unsaturated fatty acid, C18:1n-9. According to C18:1n-9, a small but statistically significant (P<0.05) decrease in the percentages of C16:1n-7 was also observed.

The level of total protein, GSH and MDA in mg per gram tissue, µmol per gram tissue and nmol per gram tissue respectively were examined in fish *O. mykiss* 0 day and 5, 12 and 19 days yolk-sac larvae (Table 2). The amount of total protein showed a constant trend in the 0 day and 5 days yolk-sac larvae without a significant difference (P > 0.05) but decreased significantly from 5 days to 19 days yolk-sac larvae (P<0.01, P<0.001), and reached a minimum value (47.55±2.35) in 19 days yolk-sac larvae. The GSH concentration increased linearly over time from 0 day to 12 days yolk-sac larvae before declining sharply in the last yolk-sac stage (Table 2). The level of lipid peroxides, as indicated by assayable MDA, was most significantly (P<0.001) higher in 12 days yolk-sac larvae (30.41±1.75) and in the all other stages yolk-sac larvae did not show any statistical differences.

The specific activity of the antioxidant enzymes is shown in Table 3. The activity of SOD, CAT, GSHpx, GR and GST enzymes (U/g, µg/g/1min, U/g/1min, U/g/1min and µg/g/1min respectively) were examined in 0 day and 5, 12 and 19 days yolk-sac larvae of *O. mykiss*. Considering the changes in the SOD and GSHpx activities, more consistent changes are shown throughout the yolk-sac stage. Both enzymes were considerably decreased in 12 days (P<0.01) and 19 days (P<0.001) yolk-sac larvae, but SOD activity showed a significant decrease in 19 days (P<0.001) yolk-sac larvae, and reached a minimum value (1.51±0.08). Statistically insignificant (P>0.05) decreases in SOD and GSHpx activities were observed in 0 day and 5 days yolk-sac larvae.

Unlike the SOD and GSHpx activities, CAT activity was detected to increase significantly (P<0.001), and reached a maximum value (75.36±5.71) in 19 days yolk-sac larvae. The activity of GR was found insignificant between 0 day and 5 days yolk-sac larvae (P>0.05). However, it was found significantly lower in 12 days and 19 days yolk-sac larvae P<0.01). While the highest GST activity in all stages were found in 19 days yolk-sac larvae, there were no significant differences throughout the yolk-sac stage.

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) in 0 day and 5, 12 and 19 days yolk-sac larvae of *O. mykiss*.

When retinol levels were examined throughout the yolk-sac larvae, there were no significant differences in the level of retinol in between 0 day, 5 days and 19 days yolk-sac larvae of *O. mykiss*, but in

**Table 1.** Fatty acid composition of 0 day, 5 days, 12 days and 19 days yolk-sac larvae of *Oncorhynchus mykiss*

| Fatty acids | 0 day yolk-sac larvae | 5 days yolk-sac larvae | 12 days yolk-sac larvae | 19 days yolk-sac larvae |
|---|---|---|---|---|
| C14:0 | 1.70±0.05$^{a}$ | 1.69±0.04$^{a}$ | 1.29±0.05$^{b}$ | 1.26±0.05$^{b}$ |
| C15:0 | 0.34±0.01$^{a}$ | 0.34±0.01$^{a}$ | 0.33±0.01$^{a}$ | 0.29±0.00$^{b}$ |
| C16:0 | 14.37±0.10$^{a}$ | 14.29±0.06$^{a}$ | 16,42±0.02$^{b}$ | 16.54±0.09$^{b}$ |
| C17:0 | 0.39±0.01$^{ab}$ | 0.38±0.01$^{ac}$ | 0.41±0.01$^{b}$ | 0.37±0.00$^{c}$ |
| C18:0 | 5.53±0.02$^{a}$ | 5.45±0.09$^{a}$ | 7.54±0.03$^{b}$ | 6.79±0.01$^{c}$ |
| C22:0 | 0.75±0.05$^{ac}$ | 0.80±0.04$^{a}$ | 0.58±0.04$^{b}$ | 0.64±0.02$^{bc}$ |
| C24:0 | 0.22±0.01$^{ab}$ | 0.18±0.00$^{b}$ | 0.28±0.02$^{a}$ | 0.79±0.03$^{c}$ |
| Σ SFA | 23.29±0.03$^{a}$ | 23.13±0.09$^{a}$ | 26.84±0.03$^{b}$ | 26.68±0.14$^{b}$ |
| C16:1n-7 | 3.57±0.06$^{a}$ | 3.65±0.08$^{a}$ | 2.65±0.14$^{b}$ | 2.91±0.05$^{b}$ |
| C17:1 | 0.19±0.01$^{ab}$ | 0.24±0.03$^{a}$ | 0.17±0.00$^{b}$ | 0.20±0.00$^{ab}$ |
| C18:1n-9 | 19.10±0.03$^{a}$ | 19.24±0.12$^{a}$ | 14.54±0.09$^{b}$ | 16.43±0.06$^{c}$ |
| C20:1n-9 | 1.39±0.01$^{a}$ | 1.34±0.02$^{a}$ | 1.24±0.03$^{b}$ | 0.90±0.02$^{c}$ |
| C22:1 | 0.47±0.01$^{a}$ | 0.50±0.02$^{a}$ | 0.49±0.03$^{a}$ | 0.45±0.03$^{a}$ |
| Σ MUFA | 24.71±0.07$^{a}$ | 24.96±0.17$^{a}$ | 19.08±0.19$^{b}$ | 20.90±0.07$^{c}$ |
| C18:3n-3 | 2.65±0.07$^{a}$ | 2.72±0.06$^{a}$ | 1.62±0.05$^{b}$ | 1.92±0.02$^{c}$ |
| C20:5n-3 | 8.00±0.09$^{a}$ | 7.92±0.08$^{a}$ | 9.07±0.06$^{b}$ | 8.02±0.08$^{a}$ |
| C22:5n-3 | 3.77±0.03$^{a}$ | 3.89±0.02$^{a}$ | 3.06±0.07$^{b}$ | 2.20±0.05$^{c}$ |
| C22:6n-3 | 24.17±0.04$^{a}$ | 23.86±0.11$^{b}$ | 30.05±0.08$^{c}$ | 29.16±0.12$^{d}$ |
| Σ n-3 | 38.59±0.11$^{a}$ | 38.39±0.12$^{a}$ | 43.80±0.16$^{b}$ | 41.30±0.18$^{c}$ |
| C18:2n-6 | 8.43±0.06$^{a}$ | 8.61±0.13$^{a}$ | 5.21±0.05$^{b}$ | 6.28±0.06$^{c}$ |
| C18:3n-6 | 0.20±0.00$^{a}$ | 0.21±0.01$^{a}$ | 0.16±0.01$^{b}$ | 0.31±0.00$^{c}$ |
| C20:2n-6 | 1.46±0.03$^{a}$ | 1.42±0.04$^{a}$ | 1.02±0.01$^{b}$ | 0.82±0.02$^{c}$ |
| C20:3n-6 | 0.76±0.01$^{a}$ | 0.79±0.02$^{a}$ | 0.65±0.04$^{b}$ | 0.64±0.02$^{b}$ |
| C20:4n-6 | 2.56±0.07$^{a}$ | 2.48±0.05$^{a}$ | 3.25±0.07$^{b}$ | 3.06±0.08$^{b}$ |
| Σ n-6 | 13.41±0.04$^{a}$ | 13.52±0.06$^{a}$ | 10.28±0.06$^{b}$ | 11.12±0.04$^{c}$ |
| Σ PUFA | 51.99±0.08$^{ab}$ | 51.91±0.16$^{a}$ | 54.08±0.21$^{c}$ | 52.42±0.14$^{b}$ |
| Σ n-3/ Σ n-6 | 2.88±0.01$^{a}$ | 2.84±0.01$^{a}$ | 4.26±0.02$^{b}$ | 3.72±0.03$^{c}$ |

*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 0 day, 5 days, 12 days and 19 days yolk-sac larvae of *Oncorhynchus mykiss*

| | 0 day yolk-sac larvae | 5 days yolk-sac larvae | 12 days yolk-sac larvae | 19 days yolk-sac larvae |
|---|---|---|---|---|
| Total Protein mg/g | 132.34±5.67$^{a}$ | 129.16±7.85$^{a}$ | 93.23±3.77$^{c}$ | 47.55±2.35$^{d}$ |
| GSH µmol/g | 1.76±0.30$^{a}$ | 1.88±0.25$^{b}$ | 1.98±0.18$^{b}$ | 1.01±0.09$^{d}$ |
| MDA nmol/g | 17.23±0.87$^{a}$ | 16.29±0.58$^{a}$ | 30.41±1.75$^{d}$ | 16.15±0.80$^{a}$ |

*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

the level of retinol a partially increase was observed in 12 days yolk-sac larvae. A statistically significant decrease in vitamin $D_3$ level was noted in 5 days, 12 days and 19 days yolk-sac larvae (P<0,001, P<0,01, P<0,001). δ-Tocopherol level showed a significant (P<0.05) decrease in 5 days yolk-sac larvae before increasing sharply in 12 days and 19 days yolk-sac larvae (P<0,001). α-Tocopherol did not show any marked change in between 0 day and 5 days yolk-sac larvae, but showed a significant increase in 12 days (P<0.001) and 19 days (P<0.05) yolk-sac larvae, and reached a maximum value (51.72±1.66) in 12 days yolk-sac larvae. *O. mykiss*'s sac larvae in the yolk-sac stage did not show any remarkable change in vitamin $K_2$ levels from 0 day to 12 days yolk-sac larvae, but a statistically significant decrease in vitamin $K_2$ level was noted in 19 days yolk-sac larvae (P<0.001). Cholesterol showed the most significant (P<0.001) increase in all yolk-sac larvae, except for only 0 day yolk-sac larvae. The highest stigmasterol levels were observed in 12 days and 19 days (P<0.001) yolk-sac larvae. Although there were no significant differences in stigmasterol levels from 0 day to 5 days yolk-sac larvae, a slight decrease was observed during yolk-sac stage. β-sitosterol levels did not show any significant changes in yolk-sac stage.

## Discussion

The primary defences consist of two groups, the antioxidant compounds and the antioxidant scavenging enzymes. Antioxidant compounds include vitamins C and E, as well as glutathione, β-carotene and uric acid. Vitamin E is an important intracellular

**Table 3.** Changes in antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx), glutathione reductase (GR) and glutathione S-transferase (GST) activities in 0 day, 5 days, 12 days and 19 days yolk-sac larvae of *Oncorhynchus mykiss*

| | 0 day yolk-sac larvae | 5 days yolk-sac larvae | 12 days yolk-sac larvae | 19 days yolk-sac larvae |
|---|---|---|---|---|
| SOD (U/g) | 6.21±0.32 | 5.99±0.27 | 5.16±0.49$^{c}$ | 1.51±0.08$^{d}$ |
| CAT (µg/g/1min) | 20.41±1.22 | 18.64±1.51 | 49.99±3.44$^{d}$ | 75.36±5.71$^{d}$ |
| GSHpx (U/g/1min) | 7.56±0.44 | 7.41±0.18 | 6.52±0.11$^{c}$ | 5.37±0.15$^{d}$ |
| GR (U/g/1min) | 2.55±0.06 | 2.63±0.05 | 2.02±0.01$^{b}$ | 2.00±0.04$^{b}$ |
| GST (µg/g/1min) | 218.32±11.23 | 224.22±13.82 $^{a}$ | 196.00±9.45 $^{a}$ | 235.00±2.52 $^{a}$ |

* 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 0 day, 5 days, 12 days and 19 days yolk-sac larvae of *Oncorhynchus mykiss*

| | | 0 day yolk-sac larvae | 5 days yolk-sac larvae | 12 days yolk-sac larvae | 19 days yolk-sac larvae |
|---|---|---|---|---|---|
| Retinol (vit. A) | µg/g | 0.88±0.08$^{a}$ | 0.77±0.03$^{a}$ | 1.33±0.09$^{c}$ | 0.93±0.06$^{a}$ |
| Vitamin $D_3$ | µg/g | 0.88±0.06 | 0.25±0.02$^{d}$ | 0.65±0.04$^{c}$ | 0.16±0.01$^{d}$ |
| δ-Tocopherol | µg/g | 1.23±0.22 | 0.81±0.14$^{b}$ | 2.63±0.74$^{d}$ | 2.34±0.41$^{d}$ |
| α-Tocopherol | µg/g | 30.52±1.12$^{a}$ | 29.31±0.56$^{a}$ | 51.72±1.66$^{d}$ | 32.03±1.38$^{b}$ |
| Vitamin $K_2$ | µg/g | 10.15±0.97$^{a}$ | 9.70±0.61$^{a}$ | 10.82±0.42$^{a}$ | 6.83±0.48$^{d}$ |
| Cholesterol | µg/g | 2.15±0.11 | 4.46±0.02$^{d}$ | 5.95±0.13$^{d}$ | 4.42±0.12$^{d}$ |
| Stigmasterol | mg/g | 95.67±2.23$^{a}$ | 92.96±3.38$^{a}$ | 145.90±5.31$^{d}$ | 141.17±4.34$^{d}$ |
| β-sitosterol | µg/g | 0.09±0.02$^{a}$ | 0.07±0.01$^{a}$ | 0.08±0.02$^{a}$ | 0.07±0.01$^{a}$ |

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

oxidant measure of its highly lipophilic properties. It provides antioxidant action in and near lipid membranes by converting $O_2$-·, ·OH, and LOO·- to less reactive forms. Vitamin A and b-carotene are lipophilic and scavenge $O_2$-· or react with peroxyl radicals. Glutathione (GSH) is a hydrophilic tripeptide and is critical to glutathione redox cycling and enzyme regulation. GSH reacts directly with $O_2$-·, ·OH, or organic free radicals (R·, RCOO·) (Yu, 1994; Marks *et al*., 1996; Rabideau, 2001). SOD, CAT, GSHpx, GR, GST and GSH are important components of the antioxidant defence system (Martinez-Alvarez *et al*. 2005). Several studies have indicated that the integrity and viability of cells are often accompanied by an increment in antioxidative capacity (Chen *et al*., 2009; Jiang *et al*., 2009; 2013).

During early development, when the embryo is small and total metabolic activity is low, little if any protein is utilized for energy production; bulk protein quantities remain relatively constant. As growth proceeds and the metabolic rate increase, a large portion of yolk protein is shunted into energy production and bulk protein quantities decline. This is especially evident after hatch, in accordance with the higher levels of activity and energy demand (Hoar and Randall, 1988; Fyhn, 1993; Ronnestad and Fyhn, 1993). In the present study, the amount of total protein decreased significantly throughout the yolk-sac stage. Related to protein quantities, the results found in *O. mykiss* yolk-sac larvae agree with studies given before but did not agree with studies Mourente and Vázquez, (1996), Parra *et al*., (1999) and Mourente *et al*., (1999), Solé *et al*., (2004).

Our study also showed that the catabolism of *O. mykiss*'s larvae increased with body growth during yolk-sac development. The increased catabolism is much higher during yolk-sac development than embryogenesis, probably due to the increased activity and the resultant higher energy requirements following hatch (Gunasekera *et al*., 2001; Johnston *et al*., 2007; Zengin *et al*., 2013; 2015). Tocher (2010) reported that in general, lipid utilization occurs to a greater extent after hatching, possibly reflecting the greater energy demands of the mobile, freeswimming yolk-sac larvae when compared with the embryonic egg stages.

During the development of the *O. mykiss*'s sac larvae, a significant depletion was observed in the ΣMUFA, mainly due to the consumption of the C16:1n-7 and C18:1n-9 in 12 days and 19 days yolk-sac larvae. Similarly *O. mykiss*'s sac larvae showed an important decrease in the Σn-6 fatty acids owing to the consumption of the C18:2n-6. But, the ΣSFA values increased significantly in the same stages of *O. mykiss*. The depletion in the ΣMUFA and Σn-6 contents and the increase in the amount of the ΣSFA investigated in this study showed that the oxidation of fatty acids occurs at different rates in the yolk-sac stage. Studies have indicated that the SFA and MUFA are oxidized at a much faster rate than PUFA (C20:4n-6 and C22:6n-3) (Wiegand *et al*., 1991; Wiegand, 1996). In the present study, ΣPUFA values, especially Σn-3, increased during the yolk-sac stage. Our study showed that PUFA is transferred to swim up fry.

MDA is a key to metabolite production and a useful marker of lipid peroxidation (Mourente *et al*. 2007). Enhanced lipid peroxidation in our study only occurred in the 12 days yolk-sac larvae. This could be due to auto-oxidation of the PUFA as a consequence of increased oxygen presence due to higher metabolism during this stage.

A major role of lipids in fish is for the storage and provision of metabolic energy in the form of ATP provided through the beta-oxidation of fatty acids (Sargent *et al*., 1989; Froyland *et al*., 2000). Electron transport chains in mitochondria and microsomes are the central source of ROS in the cell (Pesonen *et al*., 1999). Antioxidant enzymes were suggested to be more likely activated by an acute oxidative stress (Farooqui and Farooqui, 2012). CAT is a scavenger of hydrogen peroxide ($H_2O_2$), therefore its high activity should indicate the presence of a large amount of $H_2O_2$ in the system (Puangkaew *et al*., 2005). In the present study, a significant increase in the CAT activity observed in 12 and 19 days yolk-sac larvae possibly indicate that these larvae suffered from oxidative stress. A similar phenomenon was seen in CAT activities in *Dentex dentex* larvae (Mourente *et al*. 1999).

Superoxide dismutase is one of the antioxidant enzymes found in most biological systems. There is 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 (Palace *et al*., 1993; Janssens *et al*., 2000; Puangkaew *et al*., 2005). In *Oncorhynchus mykiss* yolk-sac larvae, SOD activity showed a significant decrease from 12 days to 19 days, and reached a minimum value at 19 days, but α-Tocopherol level showed a significant increase in 12 and 19 days, and reached a maximum value in 12 days yolk-sac larvae. Decreased production and non-availability of the substrate ($O_2^-$) in response to plenty of α-Tocopherol may be a reason for decreased SOD activities in 19 days yolk-sac larvae. The results of Palace *et al*. (1993) and Puangkaew *et al*. (2005) in *Oncorhynchus mykiss* in terms of notable antioxidant enzyme activity support the findings of the present study. When the vitamin E is present at high levels, the fish is under mild oxidative stress.

The tripeptide reduced glutathione (GSH) plays a key role in protection against oxidative stress by directly scavenging ROS (Leggatt, and Iwama, 2009) and by regenerating the antioxidant vitamin E from their oxidized products (Gao *et al*., 2014). It is one of the most abundant and most important molecular antioxidant in cellular cytoplasm. GSH is also used as a reducing equivalent in the metabolism of reactive intermediates, for example reduction of lipid peroxides by the action of GSHpx. Levels of total glutathione and GR activity have all been proposed as biomarkers of oxidative stress in fish (Dalton *et al*., 1999; Stephensen *et al*., 2002). In *O. mykiss* we observed that from hatching (day 0), all enzymatic activities were already measurable and in the 12 days yolk-sac larvae, the concentration of GSH was found to be about two fold higher than that of the 19 days yolk-sac larvae. The concentration of MDA also showed similar result with the GSH in the same stage. The specific activity of GSHpx and GR were markedly high until the 12 days yolk-sac larvae in the course of yolk-sac stage. Our findings were in agreement with Dalton *et al*., (1999) and Stephensen *et al*., (2002) studies.

In our study, the increases in CAT activity and antioxidant vitamin E content that remove $H_2O_2$ and organic peroxides over time, was enough to counteract ROS formation. As a result, lipid peroxidation in the 19 days yolk-sac larvae was significantly lower. Similarly, in *Dentex dentex* larvae, the prooxidant status and lipid peroxidation declined over the 9-day period study (Mourente *et al*., 1999) but, in *Solea senegalensis*, lipid peroxidation in the 19 and 28 days was significantly higher during early larval stages (Solé *et al*., 2004).

An improvement in broodstock nutrition and feeding should be reflected by high quality eggs, sperms and also the quality and quantity of seed produced. Vitamins have been linked with broodstock fish growth and egg quality. Broodstock fish dietary vitamins and trace elements are transferred to the eggs, where it is stored to support growth and development of the larvae until the first feed intake. Vitamin E (α-Tocopherol) is an important micronutrient that affects the reproduction performance of farmed fishes. Increasing vitamin E in the diet increases spawning and fertilization success, egg diameter, egg survival hatchability and larval survival (Mourente *et al*., 2000; Gammanpila *et al*., 2007).

In conclusion, we determined the prooxidant (PUFA), antioxidant (vitamin E), peroxidation (MDA) levels and the activities of the antioxidant enzyme systems in the early developmental stages of *Oncorhynchus mykiss* yolk-sac larvae without

exogenous feeding. The activities of the antioxidant enzymes were expressed throughout the 19-day study period of the early yolk-sac larvae, the levels of catalase were initially low whereas SOD and GSHpx were high in yolk-sac larvae. Overall, the total activities of catalase increased, whereas the total SOD and GSHpx activities decreased during the early stages of development. These data suggested that CAT was relatively more important in the yolk-sac larvae; CAT activity, which is very high in comparison to the other activities, is important at all stages. The enzyme activities appeared to be directly related to the prooxidant and the levels of lipid peroxidation products as measured by MDA levels. There was a direct relationship between antioxidant (vitamin E) levels and peroxidation products in the present study. The evidence suggested that lipid peroxidation may be a significant additional stress in fresh water fish yolk-sac larvae under conditions with no exogenous dietary input. This study will have further application on biochemical studies of the early developmental stages of *Oncorhynchus mykiss* yolk-sac larvae for optimal rearing in aquaculture. Further studies will aid to explain the antioxidant enzymatic defences during the actively feeding rainbow trout *Oncorhynchus mykiss* larvae after yolk-sac absorption.

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