



## Changes in Lipids, Fatty Acids, Lipid Peroxidation and Antioxidant Defence System During the Early Development of Wild Brown Trout (*Salmo trutta*)

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Received 14 December 2015  
Accepted 2 June 2016

### Abstract

Generation of radical oxygen species (ROS) is a natural consequence of aerobic metabolism and it becomes more critical during the early development of fish due to the rapid tissue growth resulting in high oxygen consumption. The present study was conducted as the first to evaluate the changes in lipids, fatty acids, lipid peroxidation and antioxidant defense system during the early development of wild brown trout (*Salmo trutta*). Total lipids dramatically decreased from 9.3% (egg) to 4.3% (swim-up). Docosahexaenoic acid (DHA; 22:6n-3) was the predominant fatty acid in all cases and was preferentially conserved during the early development. Non-enzymatic antioxidant scavengers (vitamin E, C and B1) were abundant in egg and decreased dramatically after hatching while the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) increased at the same circumstance. The lowest malondialdehyde (MDA), lipid peroxidation product, was in unfertilized eggs and eyed-stage embryo while the highest level was observed in yolk-sac larvae. Our overall results suggest that high level of non-enzymatic free radical scavengers detoxify ROS during the embryonic development and elevated antioxidant enzymes take this duty over after hatch, protecting embryo and fry from oxidative stress.

**Keywords:** Lipids, fatty acids, antioxidant enzymes,  $\alpha$ -tocopherol, ascorbic acid, thiamine.

### Yabani Kahverengi Alabalığın (*Salmo trutta*) Erken Gelişim Döneminde Lipit, Yağ Asidi, Lipit Peroksidasyonu ve Antioksidan Savunma Sistemindeki Değişimler

#### Özet

Reaktif oksijen türlerinin (ROS) üretimi oksijenli solunumun bir sonucu olup, balıkların erken gelişim dönemlerindeki hızlı büyüme ve buna bağlı yüksek düzeyde oksijen tüketiminden dolayı kritik duruma gelmektedir. Mevcut çalışma, ilk kez doğal sulara yaşayan kahverengi alabalığın (*Salmo trutta*) erken gelişim dönemi boyunca lipit, yağ asitleri, lipit peroksidasyonu ve antioksidan savunma sistemindeki değişimleri incelemektedir. Toplam lipitler yumurtadan (%9,3) serbest yüzmeye geçen yavrulara (%4,3) kadarki dönemde önemli derecede düşüş göstermiştir. Dokozahekzaenoik asit (DHA; 22:6n-3) embriyonik gelişim boyunca tercihi olarak vücutta tutulan yağ asidi olmuştur. Enzimatik olmayan antioksidanlar (vitamin E, C ve B1) yumurtada bol miktarda bulunurken embriyonik gelişim boyunca önemli derecede düşüş gösterirken, katalaz (CAT), süperoksit dismutaz (SOD) ve glutatyon S-transferaz (GST) gibi antioksidan enzimlerin aktivitesi artış göstermiştir. Lipit peroksidasyon ürünü olan malondialdehit (MDA) en düşük seviyede yumurta ve gözlenmiş embriyoda bulunurken, en yüksek seviyede keseli yavru safhasında bulunmuştur. Çalışmamızın sonucuna göre enzimatik olmayan antioksidanlar embriyonik gelişim boyunca reaktif oksijen türlerinin zararlı etkilerini giderirken, yumurtadan çıkıştan sonra bu görevi antioksidan enzimler devralmaktadır.

**Anahtar Kelimeler:** Lipitler, yağ asitleri, antioksidan enzimler,  $\alpha$ -tokoferol, askorbik asit, tiyamin.

#### Introduction

Essential nutrients which are required for growth and energy provision in the early development of fish are dependent on endogenous yolk reserves and they are allocated to the eggs by females (Cejas *et al.*,

2004). These nutrients are determined by maternal diet prior to and during oogenesis (Lavens *et al.*, 1999). Among these nutrients, lipids have a high importance in reproductive performance as they were reported to have positive effect in reproductive fitness (Izquierdo *et al.*, 2001). Lipid reserves in fish eggs

secured by maternal allocation are used through embryogenesis and larval development. Lipids and their constituent fatty acids in fish have several physiological functions as they play vital roles in energy production, membrane structure and functions, eicosanoids synthesis, transcriptional control of lipid homeostasis, and providing essential fatty acids through the life span (Tocher, 2003). Fish lipids are characterized with high amount of long chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA; 20:4n-6), eicosapentanoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). PUFAs are essential for normal development of fish and incorporated into cellular and subcellular membranes, securing the maintenance of the fluidity of those membranes. Membrane fluidity is specifically important at low temperatures, including those brown trout *Salmo trutta* eggs typically develop (Cowey et al., 1985). While imparting fluidity is essential, the structure of PUFAs makes them prone to oxidative stress which is a natural consequence of oxidative metabolism. Fish, like other aerobic animals, need O<sub>2</sub> to survive and to develop normal physiological functions; however, reactive oxygen species (ROS) generated by the use of oxygen can damage biological molecules. This situation is known as the "oxygen paradox" due to the fact that aerobic organisms need oxygen but they are under the risk of oxidation at the same time (Davies, 2000). The harmful effects of ROS can be experienced as DNA damage, enzyme inhibition, structural protein degradation and unsaturated lipid peroxidation resulting in pathologies and alteration of normal development (Halliwell and Gutteridge, 2000). Pathological consequences of oxidative stress in fish include depleted growth rate, low survival, muscular dystrophy, anemia and liver degeneration (Betancor et al., 2012). Oxidative stress happens when the ROS generation rate is higher than the rate of their removal. All aerobic organisms have two types of antioxidant defense system for decreasing ROS levels thence their detrimental effects. One is called enzymatic antioxidant defense system including the enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione -S-transferase (GST). The other group of antioxidant defense system is non-enzymatic system which is composed of free radical scavengers. These low molecular weight compounds are basically represented by glutathione, phenolic compounds,  $\beta$ -carotene, vitamin A, E ( $\alpha$ -tocopherol), B1 (thiamine) and C (ascorbic acid) (Halliwell and Gutteridge, 2000; Lukienko et al., 2000; Fontagne et al., 2008).

Being a natural consequence of aerobic metabolism, ROS generation is further enhanced in several conditions including exercise, exposure to UV light, cold temperatures, ischemia reperfusion, exposure to environmental contaminants and bacterial and fungal infections (Aruoma et al., 1991). Rapid tissue growth also induces higher aerobic metabolism

and the production of ROS. Thus, early development of fish, the period of the highest growth rate through life cycle, could be expected to increase the generation of ROS. As the antioxidant enzymes which are in function in the liver and other tissues of adult fish are not efficiently synthesized until the late embryonic development of larval fish, maternally allocated non-enzymatic antioxidants such as vitamin E and C become essential (Cowey et al., 1985; Ciarcia et al., 2000). Vitamin E is the major membrane-bound lipid soluble antioxidant while vitamin C is an important water soluble antioxidant which protects low density lipoproteins from oxidation and is required for the normal formation of cartilage (Rudneva, 1999; Fontagne et al., 2008). Thiamine is a water soluble vitamin which is important for energy metabolism and proper functioning of several enzymes in mitochondria (Desjardins and Butterworth, 2005). This vitamin serves as a coenzyme and is essential for cellular metabolic processes, metabolism of fatty acids and carbohydrates (Brown et al. 1998), and neural activities (Amcoff et al., 2000). Previous studies showed that thiamine is an important nutrient in early life stages of fish (Fitzsimons et al., 2007; Lee et al., 2009). Regarding this aspect, some diseases including early mortality syndrome were identified as a result of thiamine deficiency, leading to major mortality in early life stages (Honeyfield et al., 2005). Induced thiamine deficiency also disorders oxidative metabolism causing oxidative stress and leads to neurodegenerative alters (Sharma et al., 2013).

Brown trout naturally distributes in Turkish fresh waters and it has been exploited heavily wherever it lives for the last 3-4 decades (Arslan and Aras, 2007; Arslan et al., 2007). This heavy exploitation as well as the ecological damages such as impaired water quality and disruption of the nursery areas has put the species in danger (Alp et al., 2003; Arslan and Aras, 2007; Arslan et al., 2007). Several studies were conducted in order to better understand the biology and structure of wild brown trout populations for proper management of the natural stocks (Alp et al., 2003; Arslan and Aras, 2007; Arslan et al., 2007) but works focused on the early life stages of this species are very limited (Alp et al., 2010). The embryonic and larval development of brown trout, which naturally occurs in the gravel of redds in winter, is one of the most critical periods of its life span in terms of survival. During this period high mortalities are caused by several factors including reduced efficiency of the dispersal of toxic metabolic wastes (Dumas et al., 2007). During embryogenesis, oxygen consumption of developing eggs is accelerated and thence, the possible risk of oxidative damage is increased. It could be proposed that antioxidant enzyme system is induced as a response to these unfavorable conditions (Rudneva, 1999).

As reviewed by Martinez-Alvarez et al. (2005),

a number of studies were conducted to evaluate antioxidant defense system affected by biotic and abiotic factors in fish. However, only a few studies focused on early development of fish in this sense (Aceto *et al.*, 1994; Peters and Livingstone, 1996; Rudneva, 1999; Mourente *et al.*, 1999; Fontagne *et al.*, 2008; Kalaimani *et al.*, 2008; Diaz *et al.*, 2010). To the best of our knowledge, specifically, no studies were performed to evaluate the antioxidant system during the early development of wild brown trout living in Turkish freshwaters or anywhere else. Therefore, we aimed to disclose how the antioxidant defense system works during the early development of wild brown trout from the Stream Yagli (Coruh Basin, Turkey).

## Materials and Methods

### Broodstocks Management, Hatchery Conditions and Sampling

A total of 40 female brown trout were caught by electrofishing from the Stream Yagli (Coruh River Basin, Turkey, 40°22'18.3"N 41°06'54.1"E) during the 2 consecutive reproductive seasons (October-November of 2011 and 2012). Fish were ranged between 16.0-30.0 cm and 44.7-244.0 g in total length and weight, respectively. Fish were spawned on the site and eggs were transferred to the hatchery of the Faculty of Fisheries at Ataturk University, Erzurum, Turkey, where eggs from each female were separately fertilized in 3 replicates with freshly obtained sperm of male brown trout from the same stream. After fertilization, eggs were placed in small baskets with a nylon screen bottom (3 baskets per female) and placed in a vertical incubator with flow-through water system (FET, Kayseri, Turkey) fed by well water with constant temperature (9.5°C). Dead embryos were removed at eyed-stage and hatching. Fish were kept in the incubation baskets through the embryonic development till swim-up stage following the completion of yolk-sac absorption. All experimental conditions were same for the 2 consecutive years. In order to observe the changes in lipids, fatty acids, lipid peroxidation and antioxidant defense system during the early development of brown trout, sampling was done at 4 different stages: egg before fertilization, eyed-stage embryo (30 days post fertilization, dpf), yolk-sac larvae (newly hatched; 46 dpf) and swim-up fry (75 dpf). Eggs, embryos, larvae and fries were used as a whole for all analyses. Data from 2 consecutive years were combined as there were no statistical differences between 2 years in terms of any of the parameters determined.

### Lipid and Fatty acid Analysis

Lipids were extracted according to Folch *et al.* (1957) procedure using approximately 1 g of sample.

Separation of total lipids into polar (phospholipids) and neutral (mostly triglycerides) lipids were done using Sep-Pak silica cartridges (Waters, Milford, MA, USA). The mobile phases were chloroform and methanol for neutral and phospholipids, respectively (Juaneda and Rocquelin, 1985). Fatty acid methyl esters (FAME) were prepared in total lipids according to protocols established by Metcalfe *et al.* (1966). The FAMES obtained were determined by gas chromatography (Agilent 6890 N), equipped with a flame ionization oven and fitted with a DB 23 capillary column (60 m, 0.25 mm i.d. and 0.25 µm) ejector. The detector temperature program was set at 190°C for 35 minutes, then increased at a rate of 30°C minute<sup>-1</sup> up to 220°C, where the temperature was maintained for five minutes. Carrier gas was hydrogen (2ml min<sup>-1</sup> and split ratio was 30:1). The individual fatty acids were identified by comparing their retention times to that of a standard mix of fatty acids (Supelco 37 component FAME mix), and quantification of the individual fatty acids (% of total detected), were made against a C19:0 internal standard from Sigma (USA).

### α-Tocopherol Analysis

The α-tocopherol concentrations of samples were determined by an HPLC method (Cort *et al.*, 1983; Zaspel and Csallany, 1983). The mobile phase (consisting of 93% methanol, 6.5% HPLC distilled water and 0.5% phosphoric acid) was delivered at a flow rate of 1.2 mL min<sup>-1</sup>. Each sample was combined with 4.5 ml methanol containing 1% phosphoric acid, and 0.45 ml methanol with 5% of 1,2,3-trihydroxybenzene (pyrogallol), and homogenized twice for 45 s on ice. The homogenate was centrifuged at 2800 g for 5 min at 4 °C. Supernatants were transferred to glass tubes and methanol was added to each tube to a volume of 5 ml. After vortexing, a 1-mL aliquot of each sample was stored at -20°C and assayed within 10 days.

### Ascorbic Acid Analysis

Samples (approximately 200 mg) were homogenized in 50 g L<sup>-1</sup> trichloroacetic acid (TCA) in 250 mmol L<sup>-1</sup> HClO<sub>4</sub> containing 0.8 g L<sup>-1</sup> ethylenediaminetetraacetic acid (EDTA). Homogenized samples were centrifuged at 29,000 g for 30 min at 4°C. Supernatants were tested for ascorbic acid level using the dinitrophenylhydrazine (DNPH) method modified by Dabrowski and Hinterleitner (1989).

### Thiamine Analysis

Free thiamine (THCl), thiamine pyrophosphate (TPP) and thiamine monophosphate (TMP) were extracted from the samples according to Brown *et al.*

(1998). Vitamins were then quantified using a high-performance liquid chromatograph (HPLC) system as described by Brown *et al.* (1998) and Mancinelli *et al.* (2003) with slight modifications. The HPLC system consisted of a delivery system pump (1200 Series, Agilent Technologies, CA, USA) equipped with a 100- $\mu$ l automatic injection unit connected to a 4.6 mm x 150 mm (Zorbax, Agilent Technologies, CA, USA) column coupled with NH<sub>2</sub> packed guard column. Fluorescent detector (G1321A FLD) was set at 375 nm for excitation and at 430 nm for emission. Mobile phase was composed of potassium phosphate buffer (pH 7.5, 85 mM) + acetonitrile (65:35, v:v). Flow rate was 0.5 ml per min. The column thermostat was set at 30°C. Each external standard curve for THCl, TMP and TPP was prepared using 1 mM of each standard stock solution in 0.01 M HCl. Each standard concentration ranged from 1.0 to 100 nmol L<sup>-1</sup> for linearity. Extraction recovery rates were  $94.7 \pm 3.0$  % (n = 4) for THCl and 100 % for both TMP and TPP. For the recovery, known amounts of each thiamine hydrochloride, TMP and TPP standards were added into running samples at the beginning of the extraction and followed by extraction procedure as described above. Thiamine level was expressed as total thiamine.

#### Antioxidant Enzymes Activities and Lipid Peroxidation Level

Samples were ground with liquid nitrogen in a mortar to prepare the tissue homogenates. The ground tissues (0.5 g each) were then treated with 4.5 mL of appropriate buffer. The mixtures were homogenized on ice using an ultra-turrax homogenizer for 15 min. Homogenates were filtered and centrifuged by using a refrigerated centrifuge at 4°C. Then the supernatants were used to determine the enzymatic activities. All assays were carried out at room temperature in triplicate.

CAT activity was determined according to Aebi (1984). Decomposition of H<sub>2</sub>O<sub>2</sub> in the presence of CAT was followed at 240 nm. CAT activity was defined as the amount of enzyme required to decompose 1 nanomole of H<sub>2</sub>O<sub>2</sub> per minute, at 25°C and pH 7.8.

SOD activity was measured according to Sun *et al.* (1988). The estimation was based on the generation of O<sub>2</sub><sup>-</sup> produced by xanthine and xanthine oxidase, which react with nitro blue tetrazolium (NTB) to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction.

Total GST activity was determined according to Habig and Jakoby (1981). The activity of enzyme was assayed spectrophotometrically at 340 nm in a 4 mL cuvette containing 0.1M PBS (pH 6.5), 30 mM GSH, 30 mM 1-chloro-2,6-dinitrobenzene and tissue homogenate.

Lipid peroxidation level was determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test (Ohkawa *et al.*, 1979). Samples were weighed and homogenized in 10 mL of 100 g L<sup>-1</sup> KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g L<sup>-1</sup> sodium laurylsulfate, 1.5 mL of 200 g L<sup>-1</sup> acetic acid, 1.5 mL of 8 g L<sup>-1</sup> 2-thiobarbiturate and 0.3 mL distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 mL of n-butanol: pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 1875 g. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The recovery was over 90%.

Protein concentration was determined according to Bradford (1976) method using bovine serum albumin as a standard.

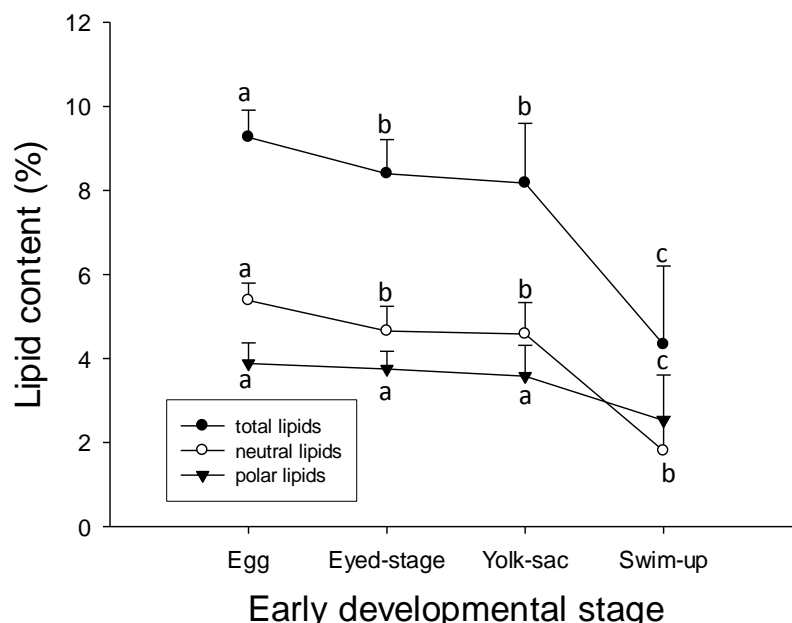
#### Statistics

Results were expressed as mean $\pm$ SD. For each developmental stage (egg, eyed-stage embryo, yolk-sac larvae, swim-up fry) average values of 3 incubation baskets for 40 different females were used (n=40). Data tested for normality and homogeneity of variances with Levene's were further subjected to analysis of variance (ANOVA) and subsequent comparison of means by Duncan's multiple range test was applied when early developmental stages had significant effect on a target parameter. Percentage data were arcsin transformed prior to statistical analysis. Correlation coefficients were calculated to evaluate correlations between estimated parameters and early developmental stages. Differences were considered statistically significant at P<0.05.

#### Results

##### Lipids and Fatty Acids

Total lipids decreased dramatically from 9.3% (egg) to 4.3% (swim-up) during the early development where main decrease was in neutral lipids part of the total lipids (P<0.05) (Figure 1). The twenty one fatty acids (FA) detected across all sampling intervals are presented in Table 1. The proportion of saturated fatty acids (SFA) was significantly influenced by the early developmental stages. The highest value was observed in swim-up stage ( $25.95 \pm 0.87\%$ ) while it did not differ significantly during the period from egg ( $25.14 \pm 0.86\%$ ) to yolk-sac stage ( $25.20 \pm 0.82\%$ ). The most abundant fatty acid in SFAs was 16:0 in all cases. This fatty acid was significantly influenced by the early developmental stages having the highest value at swim-up stage ( $15.75 \pm 0.90\%$ ). The other SFA influenced was 14:0 which had the lowest value at swim-up stage ( $2.36 \pm 0.43\%$ ), showing opposite



**Figure 1.** Changes in total lipids and lipid classes (neutral and polar lipids) during the early development of wild brown trout. Data are presented as mean $\pm$ SD ( $n=40$ ). ANOVA was applied to test the effect of different developmental stages on lipids. Duncan's multiple range test was applied when early developmental stages had significant effect on lipids. Means with different superscript letters in a line are significantly different ( $P<0.05$ ).

**Table 1.** Fatty acid composition (% of total fatty acids) of wild brown trout through the early development

Fatty acids	Early developmental stages			
	Egg	Eyed-stage	Yolk-sac	Swim-up
14:0	2.55 $\pm$ 0.37 <sup>a</sup>	2.76 $\pm$ 0.49 <sup>a</sup>	2.65 $\pm$ 0.38 <sup>a</sup>	2.36 $\pm$ 0.43 <sup>b</sup>
14:1	0.24 $\pm$ 0.11	0.22 $\pm$ 0.10	0.24 $\pm$ 0.10	0.19 $\pm$ 0.08
15:0	0.13 $\pm$ 0.03	0.14 $\pm$ 0.03	0.14 $\pm$ 0.05	0.12 $\pm$ 0.02
15:1	0.20 $\pm$ 0.05	0.19 $\pm$ 0.04	0.18 $\pm$ 0.03	0.19 $\pm$ 0.03
16:0	14.71 $\pm$ 0.80 <sup>b</sup>	14.49 $\pm$ 0.80 <sup>b</sup>	14.52 $\pm$ 0.79 <sup>b</sup>	15.75 $\pm$ 0.90 <sup>a</sup>
16:1n-7	7.06 $\pm$ 0.92 <sup>a</sup>	6.66 $\pm$ 1.45 <sup>b</sup>	6.79 $\pm$ 0.57 <sup>b</sup>	6.14 $\pm$ 0.46 <sup>b</sup>
17:0	0.75 $\pm$ 0.18	0.83 $\pm$ 0.14	0.79 $\pm$ 0.13	0.78 $\pm$ 0.14
17:1	0.44 $\pm$ 0.13	0.49 $\pm$ 0.14	0.50 $\pm$ 0.11	0.51 $\pm$ 0.16
18:0	5.66 $\pm$ 0.62	5.66 $\pm$ 0.47	5.63 $\pm$ 0.38	5.61 $\pm$ 0.31
18:1n-9	15.98 $\pm$ 2.28	15.89 $\pm$ 1.97	15.82 $\pm$ 1.65	15.29 $\pm$ 1.61
18:2n-6	6.86 $\pm$ 0.92 <sup>a</sup>	6.40 $\pm$ 0.57 <sup>b</sup>	6.31 $\pm$ 0.55 <sup>b</sup>	5.38 $\pm$ 0.37 <sup>b</sup>
18:3n-3	7.08 $\pm$ 1.64	6.74 $\pm$ 1.51	6.84 $\pm$ 1.34	6.22 $\pm$ 1.44
20:0	1.35 $\pm$ 0.41	1.47 $\pm$ 0.40	1.48 $\pm$ 0.35	1.38 $\pm$ 0.36
20:1n-9	0.99 $\pm$ 0.14 <sup>a</sup>	1.01 $\pm$ 0.23 <sup>a</sup>	0.98 $\pm$ 0.22 <sup>a</sup>	0.79 $\pm$ 0.26 <sup>b</sup>
20:2n-6	0.70 $\pm$ 0.16	0.69 $\pm$ 0.15	0.67 $\pm$ 0.13	0.65 $\pm$ 0.19
20:3n-6	0.64 $\pm$ 0.12	0.65 $\pm$ 0.12	0.64 $\pm$ 0.10	0.66 $\pm$ 0.16
20:3n-3	1.55 $\pm$ 0.21 <sup>b</sup>	1.61 $\pm$ 0.19 <sup>b</sup>	1.63 $\pm$ 0.16 <sup>b</sup>	2.04 $\pm$ 0.26 <sup>a</sup>
20:4n-6	3.56 $\pm$ 0.98	3.79 $\pm$ 0.94	3.81 $\pm$ 0.80	3.18 $\pm$ 0.66
20:5n-3	8.89 $\pm$ 1.55	9.33 $\pm$ 1.01	9.42 $\pm$ 1.13	9.48 $\pm$ 1.01
22:5n-3	4.08 $\pm$ 0.56	4.10 $\pm$ 0.62	4.07 $\pm$ 0.54	4.06 $\pm$ 0.42
22:6n-3	16.58 $\pm$ 1.66 <sup>b</sup>	16.78 $\pm$ 1.43 <sup>b</sup>	16.98 $\pm$ 1.42 <sup>b</sup>	20.01 $\pm$ 1.99 <sup>a</sup>
$\Sigma$ SFA <sup>¶</sup>	25.14 $\pm$ 0.86 <sup>b</sup>	25.35 $\pm$ 1.06 <sup>b</sup>	25.20 $\pm$ 0.82 <sup>b</sup>	25.95 $\pm$ 0.87 <sup>a</sup>
$\Sigma$ MUFA <sup>§</sup>	24.92 $\pm$ 2.38	24.46 $\pm$ 2.22	24.52 $\pm$ 1.72	23.12 $\pm$ 1.78
$\Sigma$ PUFA <sup>¶</sup>	49.94 $\pm$ 2.03	50.19 $\pm$ 1.76	50.28 $\pm$ 1.38	50.93 $\pm$ 1.74
$\Sigma$ LC-PUFA <sup>‡</sup>	36.00 $\pm$ 3.41 <sup>b</sup>	36.95 $\pm$ 2.28 <sup>b</sup>	37.22 $\pm$ 2.08 <sup>b</sup>	40.08 $\pm$ 2.47 <sup>a</sup>
n3	38.19 $\pm$ 2.02 <sup>b</sup>	38.65 $\pm$ 1.51 <sup>b</sup>	38.85 $\pm$ 1.38 <sup>b</sup>	41.01 $\pm$ 1.92 <sup>a</sup>
n6	11.76 $\pm$ 0.77 <sup>a</sup>	11.54 $\pm$ 0.53 <sup>a</sup>	11.43 $\pm$ 0.49 <sup>a</sup>	9.88 $\pm$ 0.44 <sup>b</sup>
n3/n6	3.26 $\pm$ 0.28 <sup>b</sup>	3.36 $\pm$ 0.17 <sup>b</sup>	3.41 $\pm$ 0.20 <sup>b</sup>	4.16 $\pm$ 0.33 <sup>a</sup>

<sup>¶</sup>SFA: saturated fatty acid; <sup>§</sup>MUFA: monounsaturated fatty acid; <sup>¶</sup>PUFA: polyunsaturated fatty acid; <sup>‡</sup>LC-PUFA: long chain polyunsaturated fatty acid. Data are presented as mean $\pm$ SD ( $n=40$ ). Means with different superscript letters in a line are significantly different ( $p < 0.05$ ). ANOVA was applied to test the effect of different developmental stages on each individual fatty acid. Duncan's multiple range test was applied when early developmental stages had significant effect on each fatty acid. Means with different superscript letters in a line are significantly different ( $P<0.05$ ).

trend with 16:0. Monounsaturated fatty acids (MUFA) dominated by oleic acid (OA; 18:1n-9) were numerically low in swim up stage ( $15.98 \pm 2.28\%$ ) but no significant differences were observed between varying sampling intervals. The second and third dominant MUFAs were 16:1n-7 and 20:1n-9, respectively, both of which were significantly affected by early developmental stages, having the lowest value at swim-up stage ( $6.14 \pm 0.46\%$  and  $0.79 \pm 0.26\%$ , respectively). Polyunsaturated fatty acids (PUFA) showed significant positive correlation with progressing early developmental stages ( $P < 0.05$ ;  $R^2 = 0.88$ ) while the amounts at different sampling intervals were not significantly different. However, among PUFAs, the amount of LC-PUFAs (PUFAs with at least 20C) was significantly higher in swim-up fries ( $39.63 \pm 2.54\%$ ) than those at all other sampling intervals. DHA was the predominant fatty acid of all detected 21 fatty acids, thence PUFAs and LC-PUFAs at all stages. The amount of 22:6n-3 was significantly affected by the sampling intervals with the highest value in swim-up stage ( $20.01 \pm 1.99\%$ ). The other PUFAs significantly affected were linoleic acid (LA; 18:2n-6) and 20:3n-3. While LA was significantly lower at swim-up stage ( $5.38 \pm 0.37\%$ ) than all other stages, 20:3n-3 was significantly higher at the same circumstance ( $2.04 \pm 0.26\%$ ). The amount of total n-3 fatty acids was significantly higher ( $41.01 \pm 1.92\%$ ) and total n-6 fatty acids were significantly lower ( $9.88 \pm 0.44\%$ ) at swim-up stage than the other developmental stages.

#### **$\alpha$ - Tocopherol, Ascorbic Acid, Thiamine Contents, Antioxidant Enzymes Activities and Lipid Peroxidation Values**

Early developmental stages had a significant effect on the levels of  $\alpha$ -tocopherol, ascorbic acid and thiamine ( $P < 0.05$ ). Activities of antioxidant enzymes such as SOD, CAT and GST as well as lipid peroxidation level were also significantly influenced by early developmental stages ( $P < 0.05$ ). The level of  $\alpha$ -tocopherol did not change significantly between egg and eyed-stage embryo, however it showed a dramatic decrease from eyed-stage embryo to swim-up fry (Figure 2A). Ascorbic acid showed a progressive decrease through the developmental stages from  $244.6 \pm 28.8 \mu\text{g}^{-1}$  (egg) to  $129.6 \pm 20.5 \mu\text{g}^{-1}$  (swim-up fry). Statistical differences were significant between all sampling stages (Figure 2B). Thiamine showed the same pattern with  $\alpha$ -tocopherol that constantly decreased through early development except for the interval between egg and eyed-stage embryo (Figure 2C). Thiamine content was around 6 fold lower in swim-up stage than in unfertilized egg ( $8.9$  vs.  $1.5 \text{ nmol g tissue}^{-1}$ ). Lipid peroxidation assessed by the level of MDA showed opposite trend to  $\alpha$ -tocopherol, ascorbic acid and thiamine till yolk-sac stage. It was stable in eggs and eyed-stage, and then increased between eyed-stage embryo and yolk-sac larvae

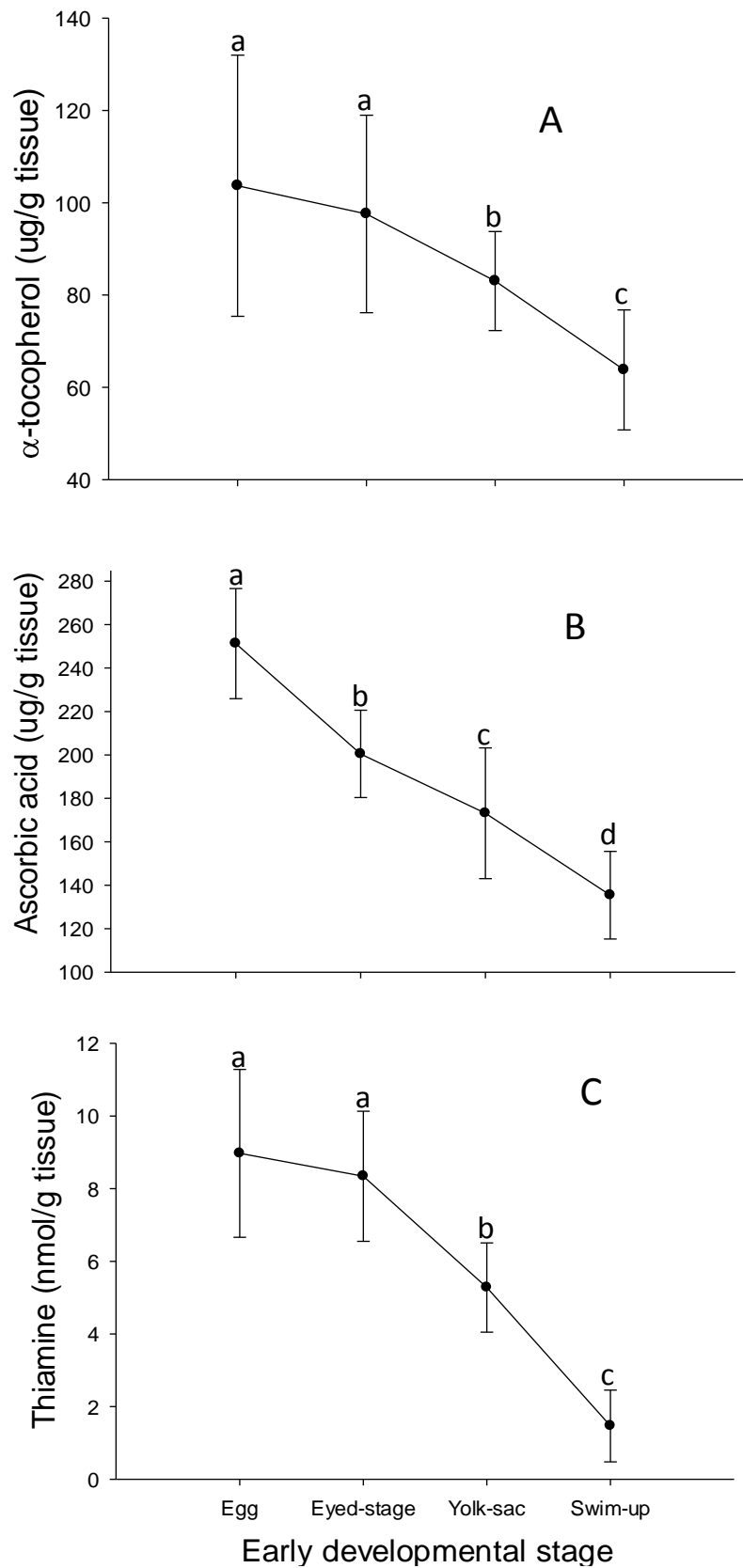
where it started to decrease again (Figure 3). Activities of all three enzymes SOD, CAT and GST were significantly lower in eggs and eyed-stage embryos, and then they increased dramatically in yolk-sac swim-up samples (Figure 4).

#### **Discussion**

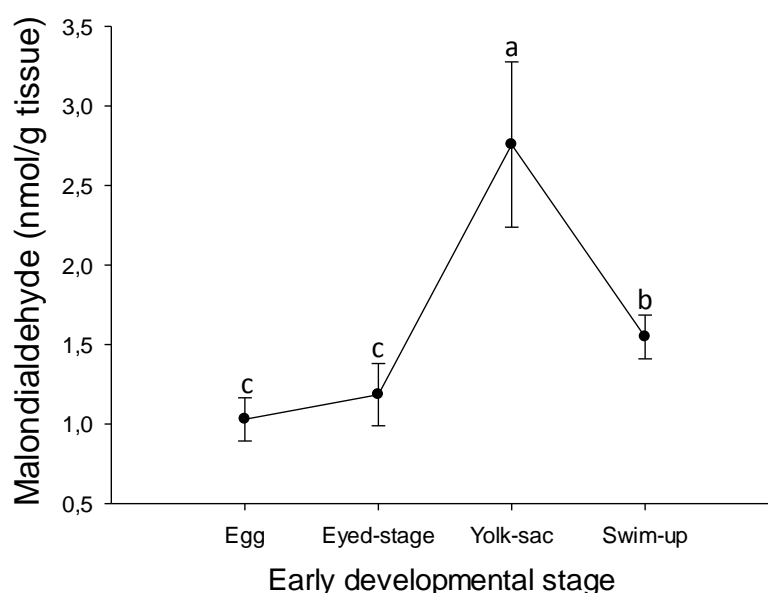
This study is the first to report lipids, fatty acid profile and antioxidant system through early development in wild brown trout. In wild fish, maternal allocation of nutrients under parent-egg-progeny relationships is considered the key aspect in embryonic development, larval viability and recruitment variability (Kamler, 2005). Lipid reserves, the main energy source in fish eggs provided by maternal allocation are used by developing embryo and larvae as not only substrate for energy metabolism but also structural component in membrane formation (Sargent, 1995).

Lipid content of brown trout egg in the present study was 9.3%, in accordance with wild lake trout egg (9.1-9.9%) from Lake Michigan (Czesny *et al.*, 2012) and it was slightly higher than wild brown trout egg (7.8-8.6%) from some Norwegian streams (Jonsson and Jonsson, 1997). The latter study reported that the energetic investment in gonads to relative soma in female brown trout during the maturation was estimated at 67%. In the present study, total lipid reserves were utilized to a great extent from 9.3% (egg) to 4.3% (swim-up) during the early development with the main decrease during the period between yolk-sac (hatching) and swim-up stages. As we observed in our study, in general, lipid utilization intensively occurs after hatching, especially in species having eggs rich in neutral lipid, possibly reflecting the greater energy requirements of mobile yolk-sac larvae in comparison to the embryonic egg stage (Tocher, 2003). Utilization of lipids during the early development differs among the species. Fish such as Atlantic herring (*Clupea harengus*) and cod (*Gadus morhua*) with eggs rich in phospholipids, having moderate neutral lipid storage, mainly consumed phospholipids in early development (Rainuzzo *et al.*, 1997) whereas neutral lipids were the main substrate for the aerobic energy metabolism in white seabream (*Diplodus sargus*) from hatching to 3 day old larvae (Cejas *et al.*, 2004). Our results were in accordance with Atlantic salmon (*Salmo salar*) in which both neutral and polar lipids were utilized markedly during the early development from fertilized egg to swim-up fry (Cowey *et al.*, 1985), but neutral lipids were spent in a greater amount.

During development, preferential utilization of some fatty acids seemed to be species specific. In gilthead seabream (*Sparus aurata*), the pattern of fatty acid loss was  $n-6 > n-9 > n-3$  (Koven *et al.*, 1989). In the same study, the authors reported that, among n-3 fatty acids, DHA was more strongly conserved than EPA. In turbot (*Scophthalmus maximus*), ARA was also



**Figure 2.** Changes in  $\alpha$ -tocopherol (A), ascorbic acid (B) and total thiamine (C) during the early development of wild brown trout. Data are presented as mean $\pm$ SD ( $n=40$ ). ANOVA was applied to test the effect of different developmental stages on  $\alpha$ -tocopherol, ascorbic acid and total thiamine. Duncan's multiple range test was applied when early developmental stages had significant effect on vitamins. Means with different letters are significantly different at  $P<0.05$ .



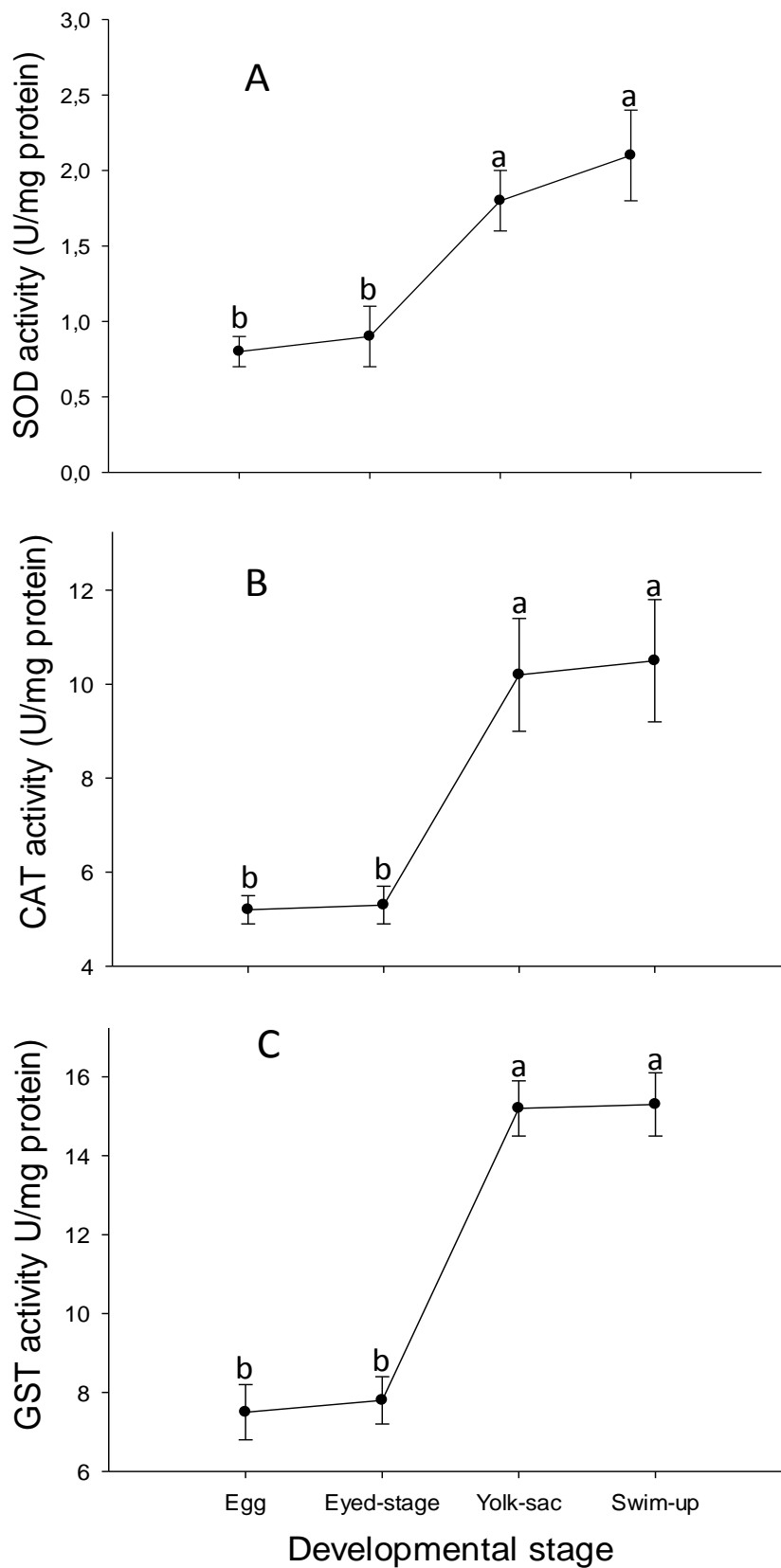
**Figure 3.** Changes in lipid peroxidation assessed by the level of MDA during the early development of wild brown trout. Data are presented as mean $\pm$ SD ( $n=40$ ). ANOVA was applied to test the effect of different developmental stages on MDA. Duncan's multiple range test was applied when early developmental stages had significant effect on MDA. Means with different letters are significantly different at  $P<0.05$ .

tended to be conserved in addition to DHA, (Rainuzzo *et al.*, 1994) while only ARA was conserved in white seabream (Cejas *et al.*, 2004). In our study, DHA was the most abundant fatty acid through all stages. This fatty acid was followed by OA and 16:0. In general, n-3 LC-PUFAs together with saturated fatty acids were preserved while n-6 PUFAs were utilized during the development. Regarding individual fatty acids, our results clearly demonstrated the preferential preservation of the fatty acids such as 14:0, 16:0, 20:3n-3 and DHA, and the preferential utilization of those such as 16:1n-7, 18:2n-6 and 20:1n-9. The ratios of the other individual fatty acids were not significantly affected by the early developmental stages. Among the preserved fatty acids DHA is a very important structural component in cell membranes, particularly in the process of synaptogenesis and retinogenesis during early development of fish (Mourente *et al.*, 1991; Sargent, 1995). 16:0 is also one of the main fatty acids of cell membrane and preferential conservation could be attributed to its structural function (Sargent, 1995).

Being rich in PUFA, fish lipids are prone to oxidative stress during the early development and whole life span. In the present study, PUFAs which are considered pro-oxidants represented half of the fatty acids during the early development. However, lipid peroxidation product MDA was still low till yolk-sac (hatching) stage where it reached the highest level. It is evident that protection of PUFAs from oxidation during the embryonic development was secured by non-enzymatic antioxidant scavengers such as  $\alpha$ -tocopherol, ascorbic acid and thiamine

which were abundantly present in unfertilized egg and eyed-stage embryo. The increase in lipid peroxidation (MDA) at yolk-sac larvae could be also considered that hatching may require an increase in environmental oxygen concentration resulting in an imbalance between ROS production and antioxidant system as it was observed in sturgeon *Acipenser naccarii* (Diaz *et al.*, 2010). In our study, having completed the embryonic development, brown trout fry showed dramatic decrease in  $\alpha$ -tocopherol, ascorbic acid and thiamine and these compounds reached the lowest level at swim-up fry as it was observed in several aquatic organisms from Black Sea (Rudneva, 1999). This dramatic decrease in non-enzymatic free radical scavengers corroborates that these are actively involved in detoxification process of free radicals, protecting embryos against possible damages. Dietary vitamin E significantly decreased lipid peroxidation products in seabass larvae fed high DHA diets and oxidative stress indicators which were further reduced by dietary vitamin C supplementation (Betancor *et al.*, 2012). In developing *Dentex dentex* larvae, dramatic collapse in vitamin E indicated that this vitamin was consumed rapidly at this stage in the process of quenching ROS and chain-breaking. The collapse in vitamin E level only occurred after yolk-sac resorption and disappearance of oil globes. These process were argued by the authors that there might be transient increase in PUFA and that cannot be incorporated into membranes in starving animals and thus must be oxidised (Mourente *et al.*, 1999). In our study, we also observed the decrease in vitamin E content with decreasing lipid content. Regarding





**Figure 4.** Changes in the activity of antioxidant enzymes SOD (A), CAT (B) and GST (C) during the early development of wild brown trout. Data are presented as mean $\pm$ SD ( $n=40$ ). ANOVA was applied to test the effect of different developmental stages on SOD, CAT and GST. Duncan's multiple range test was applied when early developmental stages had significant effect on antioxidant enzymes. Means with different letters are significantly different at  $P<0.05$ .

antioxidant function, thiamine was also reported as a free radical scavenger as the deficiency of thiamine was suggested an inducing factor for oxidative stress in brain mitochondria of house mouse, *Mus musculus* (Sharma et al., 2013). Moreover, thiamine improved antioxidant defense and inhibited lipid peroxidation and protein oxidation of juvenile Jian carp *Cyprinus carpio* var. Jian (Li et al., 2014)

SOD catalysis the dismutation of the superoxide anion ( $O_2^-$ ) into the oxygen and hydrogen peroxide ( $H_2O_2$ ) which is further reduced to water by CAT in the peroxisomes (Fontagne et al., 2008). GST plays an important role in detoxification of lipid peroxides and demonstrates the functions such as glutathione peroxidase activity towards reactive oxygen species in the cell in the case of oxidative stress (Rudneva et al., 2010). In the present study, during the embryonic development from egg through yolk-sac embryo at hatching, antioxidant enzymes such as CAT, SOD and GST were already present at low amount in brown trout and their activity increased with early development in consistent with the results observed in rainbow trout embryos (Aceto et al., 1994; Fontagne et al., 2008). This finding suggests that, after hatching, antioxidant defense was compensated by the elevated activity of antioxidant enzymes instead of non-enzymatic scavengers which showed dramatic decrease during the yolk-sac abortion as it was established by Rudneva (1999). However, some contradictive results for antioxidant enzyme activities during the early development of fish were reported in different studies. The activity of SOD decreased through the early development in turbot (Peters and Livingstone 1996) and *Dentex dentex* (Mourente et al., 1999) while the activity of CAT and GPx increased. In some other species such as Asian seabass (*Lates calcarifer*) SOD showed no difference during early developmental stages (Kalaimani et al., 2008). Early development is the critical stage for the fish as a lot of factors including oxidative stress cause high mortalities (Dumas et al., 2007). During the embryogenesis and early development after hatching, fish have fast growth rate and it stimulates the ROS generation during the rapid tissue growth. Therefore, the antioxidant defense system is of a high importance during early development of fish.

In conclusion, our results showed that high amounts of lipids as an essential nutrient, rich in PUFAs, were allocated to the egg by female brown trout. On the other hand, molecular antioxidant scavengers such as  $\alpha$ -tocopherol, ascorbic acid and thiamine were also allocated to the egg at an abundant level. The antioxidant enzymes such as CAT, SOD and GST were present but at low amount in unfertilized egg, as well. Molecular scavengers dramatically decreased after hatching while activity of antioxidant enzymes increased at the same circumstance. Our overall results suggest that high level of non-enzymatic free radical scavengers detoxify ROS during the embryonic development and

elevated antioxidant enzymes take this duty over after hatching.

## Acknowledgements

Funding for this study was provided by The Scientific and Technological Research Council of Turkey (TUBITAK) under project 110O338. Ali Yurdakul, Ertem Yazici, Tugce Sensurat, Fatih Coteli and Muhammet Coteli provided assistance sampling the broodstocks from the Stream Yagli. Yasar Gunbeyi helped in hatchery activities.

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