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Fatty Acid Pattern, Oxidation Product Development, and Antioxidant Loss in Muscle Tissue of Rainbow Trout and *Dicentrarchus labrax* during Growth

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The levels of hydrophilic, lipophilic, and enzymatic antioxidants, the oxidative damage to lipids and proteins, and the fatty acid patterns of triglyceride and phospholipid fractions were assayed in fresh muscle tissue of rainbow trouts (*Oncorhynchus mykiss*) and sea basses (*Dicentrarchus labrax*) during aging, to investigate the correlation between oxidative stress and aging processes in fish. The present studies suggests that lipid peroxidation and accumulation of oxidized proteins during in vivo aging are most likely to be linked with an age-dependent decline of lipophilic antioxidants ($CoQH_2$, CoQ, and vitamin E) and vitamin C contents in muscle tissue, whereas fish aging is not linked to a decline in antioxidant enzymes and reduced glutathione levels. Lipophilic antioxidant and vitamin C levels represent a reliable marker of oxidative stress during aging, and their determination might be useful for the assessment of fish age.

KEYWORDS: Rainbow trout; sea bass; oxidative stress; aging; lipophilic, hydrophilic, and enzymatic antioxidants; fatty acids; lipid, protein; HPLC-DAD; GC-MS

INTRODUCTION

Aging is the progressive deterioration in physiological functions and metabolic process. In recent years, the reactive oxygen species (ROS) have become an active field in aging research because of their potential involvement in many degenerative processes (*1*). According to the "free radical theory", aging results from random oxidative damage to tissues by ubiquitous and deleterious reactive oxygen and nitrogen species and other radicals (R•), formed by both normal metabolic processes and environmental attacks such as radiation, ozone, and other atmospheric pollutants (*2*). It is well-known that utilization of oxygen represents an efficient mechanism for aerobic organisms to generate energy, but ROS, as the byproducts during this process and the other unfavorable events, are also produced within the biological system. These ROS are highly reactive and capable of damaging many biological macromolecules such as DNA, RNA, protein, and lipids (*3*).

In living organisms, the oxidative damage to macromolecules is controlled by two types of antioxidant systems. One is represented by enzymes that remove ROS such as superoxide, hydrogen peroxide, and lipid hydroperoxides and include superoxide dismutase (SOD), catalase (CAT), and the peroxidases. The other group of antioxidative compounds scavenges free radicals; these compounds are generally of low molecular weight and may be water or lipid soluble. Examples of water soluble free radical scavengers are ascorbate and glutathione, while tocopherol and ubiquinol (reduced coenzyme Q) represent lipid soluble low molecular weight free radical scavengers.

A more recent version of the free radical theory is the "oxidative stress hypothesis" of aging, which states that the level of oxidative stress (unbalance between pro-oxidants and antioxidants) increases during cell differentiation and aging (*4*). This was further confirmed by recent data indicating that also in marine invertebrates the aging process is accompanied by increased oxidative stress conditions, which are characterized by a reduction of some antioxidant levels, such as vit E, carotenoids, and reduced glutathione (GSH), and by a loss in the activity of SOD, CAT, and glutathione peroxidase (GPx) (*5*). Similar studies in fish are relatively few, and it is worthy mentioning that fish contains many oxidation sensitive substances, such as polyunsaturated fatty acids (PUFA), that easily undergo oxidative processes, resulting in alterations in smell, taste, texture, color, and nutritional value. In this context, different works compared postmortem quality losses with the rate of change of the antioxidants components (*6*, *7*).

To investigate the link between oxidative stress and aging processes in fish, the levels of hydrophilic, lipophilic, and

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Table 1. Biometric Data of Differently Aged Rainbow Trouts and Sea Basses under Study[a]

Rainbow Trouts

	age groups			
	3 months (I)	1 year (II)	2 years (III)	3 years (IV)
length (cm)	5.4 ± 0.3	23.6 ± 0.9[b]	34.2 ± 2.9[b,c]	39.1 ± 3.5[b,c,e]
weight (g)	1.3 ± 0.2	141.8 ± 22.6[b]	422.8 ± 35.7[b,c]	662.7 ± 219.2[b,c,d]

Sea Basses

	age groups		
	≈1 year (I)	≈3 years (II)	≈5 years (III)
length (cm)	15.9 ± 0.8	28.4 ± 1.5[f]	44.2 ± 4.7[f,g]
weight (g)	64.5 ± 10.2	388.3 ± 44.9[f]	1217.0 ± 260.4[f,g]

[a] Each result represents the mean ± SEM of 15 samples for each group. [b] $p < 0.001$ vs 3 month old rainbow trouts. [c,d] $p < 0.001$ vs 1 and 2 year old rainbow trouts, respectively. [e] $p < 0.01$ vs 2 year old rainbow trouts. [f] $p < 0.001$ vs ≈1 year old sea basses. [g] $p < 0.001$ vs ≈3 year old sea basses.

enzymatic antioxidants, the oxidative damage to lipids and proteins, and the fatty acid patterns of triglyceride (TG) and phospholipid (PL) fractions have been assayed in the fresh muscle tissue of differently aged rainbow trouts (*Oncorhynchus mykiss*) and sea basses (*Dicentrarchus labrax*).

The rainbow trout is the cold freshwater species mostly reared in European countries, and its age can be easily monitored. In Italian freshwater, the maximum size of this species was reported to be approximately 70 cm in total length and 7 kg in body weight, while the maximum life span is around 4−5 years. Rainbow trouts of four age groups were utilized as follows: I = 3 months old, II = 1 year old, III = 3 years old, and IV = 5 years old. Sea bass has white flesh, a mild taste, and low fat content. These attributes have made several bass species popular around the world, and it actually is the most common fish caught and consumed in the Mediterranean area. The average life span of a sea bass is 6 years; for this species, three age groups were utilized as follows: I = ≈1 year old, II = ≈3 years old, and III = ≈5 years old.

MATERIAL AND METHODS

Standards and Solvents. GPx, CAT, SOD, GSH, oxidized glutathione (GSSG), vitamin C, d-RRR-α-tocopherol, ubiquinones (CoQ_n, $n = 9$ or 10), vitamin A (vit A), retinyl acetate, β-carotene, fatty acids, and other chemicals were purchased from Sigma-Aldrich S.r.l. (Milan, Italy). Organic solvents were products of Carlo Erba, Milano (Italy). All reagents were of analytical grade or of the highest grade available. Ubiquinols (CoQ_nH_2, $n = 9$ or 10) were prepared by reduction of ubiquinones with sodium borohydride. Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Materials. Hatchery rainbow trout were obtained from an aquaculture farm (Stabilimento Ittiogenico of Rome). Sixty fish of four different ages (I = 3 months, II = 1 year old, III = 3 years old, and IV = 5 years old), with the same genetic origin, were maintained in the same rearing and feeding conditions. Live trout were transferred at rearing facilities of University "Tor Vergata" of Rome. Then, fish, 15 for each group, were sacrificed and dissected. Sea basses were caught by trawling in Central Tyrrhenian Sea (Anzio, Rome, Italy), sampled directly on board, and stored in dry ice while transferred to the laboratory within 8 h. Three age groups were selected according fishers' experience: I = ≈1 year old, II = ≈3 years old, and III = ≈5 years old.

Edible tissue from white muscle tissue portions of trouts and sea basses, excised by a scalpel from the central part of the dorsal musculature, was divided into approximately 1 g aliquots and deep frozen at −80 °C until analyzed. Size and weight are shown in **Table 1**.

Evaluation of Lipid Oxidation. Thiobarbituric acid reactive substances (TBARS) were performed on fish muscle according to the procedure of Buege (*8*). BHT was added to reduce lipid oxidation during the assay procedure. Measurement was at 532 nm using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The TBARS data were expressed as μmol of malonaldehyde per g of tissue.

Assay for Protein Carbonyls. Muscle tissues were homogenized in 10 mL of phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 12 000*g* for 10 min. The supernatant was recovered, and the protein concentration was determined using Pierce BCA protein assay kit. The protein carbonyl contents were analyzed by the 2,4-dinitrophenylhydrazine (DNPH) method as described by Levine et al. (*9*). Briefly, an aliquot of tissue supernatant containing 0.5 mg of protein was pipetted into a tube, to which 4.0 mL of 5 mM DNPH in 2.5 M HCl was added. The blank was made by adding 4.0 mL of 2.5 M HCl without DNPH. Samples were incubated at room temperature for 1 h. Then, proteins were precipitated by adding 5 mL of 20% trichloroacetic acid and the precipitate was washed three times with 4 mL of ethanol:ethyl acetate (1:1). Precipitated proteins were redissolved in 2.0 mL of 6 M guanidine HCl in 20 mM potassium phosphate, pH 6.5, and insoluble substances were removed by centrifugation. The carbonyl content was calculated from the maximum absorbance (360−370 nm), using a molar absorption coefficient of 22.000 $M^{-1}\ cm^{-1}$. The results were expressed as nmol carbonyls per mg protein.

Enzyme Assays. Each sample of muscle (≈1 g) was homogenized under a flow of nitrogen for 3 min with a Teflon homogenizer at the maximum speed in the presence of 6.0 mL of 0.01 M PBS, containing 1 mM EDTA, pH 7.0. Homogenates were centrifuged at 12 000*g* for 30 min at 4 °C. The supernatant fraction (assay solution) was collected for enzyme assays, GSH and GSSG, and vitamin C.

GPx was determined on 500 μL of the assay solution according to the method of Paglia and Valentine (*10*), which couples hydrogen peroxide reduction to oxidation of NADPH by glutathione reductase. An enzyme unit was defined as that activity, which oxidized 1 μmol of NADPH per minute.

SOD activity was measured on 500 μL of the assay solution by using a RANSOD kit (RANDOX, Grumlin, U.K.), which was performed according to the supplier's directions. One SOD unit inhibits the rate of 2-(4-iodophenyl)-3-(nitrophenol-5-phenyl tetrazolium chloride) reduction by 50%, at pH 7.0 for 1 min.

CAT activity was assayed on 500 μL of the assay solution according to Aebi (*11*). One CAT unit is defined as the enzyme activity necessary to convert 1 μmol of H_2O_2 to $H_2O + O_2$ at 25 °C and pH 7 in 1 min.

Glutathione Analysis. GSH and GSSG in muscle tissue were assayed by high-performance liquid chromatography (HPLC) according to Reed (*12*). The procedure is based on the initial formation of S-carboxymethyl derivative of GSH with iodoacetic acid, followed by conversion of free amino group to 2,4-dinitrophenyl derivative by reaction with 1-fluoro-2,4-dinitro benzene. A 500 μL amount of assay solution and 10 μg of γ-L-glutamyl glutamic acid (reference standard) were used. A 500 μL amount of assay solution was added with 10 μg of γ-L-glutamyl glutamic acid (reference standard) and 400 μL of 50% metaphosphoric acid. The dinitrophenyl derivatives were separated and measured using a gradient HPLC system (10 A VP Shimadzu liquid chromatograph) equipped with an analytical Supelcosil NH_2 column (25 cm × 4.6 mm, 5 μm, Supelco) and a photodiode array detector set at 350 nm (SPD-M Shimadzu). Mobil phase A: 65% methanol; mobile phase B: 2 volumes of sodium acetate stock solution with 8 volumes of 80% methanol. Gradient: 90% A−10% B for 15 min followed by 45 min linear gradient to 90% B at a flow rate of 1 mL/min. GSH and GSSG were quantified by comparison of areas to those of authentic standards, including the reference standard.

Ascorbic and Dehydroascorbic Acid Analysis. A 500 μL amount of assay solution was treated with 2 μg of hypoxanthine (reference standard) and 2 volumes of 2% metaphosphoric acid for vit C analysis (a) and with 2 volumes of 2% metaphosphoric acid supplemented with 6 mg/mL dithiothreitol for total vit C (ascorbic + dehydroascorbic acids) analysis (b). Both samples were stored at −80 °C under argon and centrifuged before HPLC analysis. The supernatant was collected, and the volume was adjusted to 1 mL with water. To determine total

vit C content, the supernatant containing dithiothreitol was incubated at 45 °C for 2 h prior to HPLC analysis. Samples of 50 μL were injected into a Shimadzu liquid chromatograph on an analytical Supelcosil LC-18-DB column (24 cm × 4.6 mm, 5 μm, Supelco) plus its guard column, by using in line both a photodiode array detector set at 265 nm and an ESA CoulArray (oxidation potential: +400 mV). The mobile phase consisted of 0.02 M NaH_2PO_4/CH_3CN, 99.5/0.5, v/v, containing 0.6 g/L metaphosphoric acid; flow, 0.6 mL/min. Ascorbic acid was quantified by comparison of areas to those of authentic standards, including the reference standard.

Extraction Procedure for α,δ,γ-Tocopherol, Ubiquinols, and Ubiquinones. Lipid soluble antioxidants were extracted using the procedure of Burton et al. (*13*).

Determination of CoQ_{10}-H_2/CoQ_{10}, and CoQ_9-H_2/CoQ_9. Muscle ubiquinols (CoQ_{10}-H_2 or CoQ_9-H_2) and ubiquinones (CoQ_{10} or CoQ_9) were quantified simultaneously by a 10 A VP Shimadzu liquid chromatograph on an analytical Supelcosil LP-18 column (24 cm × 4.6 mm, 5 μm, Supelco) plus its guard column, by using in line both photodiode array (SPD-M Shimadzu) and electrochemical detectors, as previously reported (*14*). The photodiode array detector was set at 275 nm. The electrochemical detection was accomplished by using an ESA CoulArray (Bedford, MA), which allows the postcolumn electrochemical reduction of ubiquinone to ubiquinol (reduction potential, −600 mV) and the quantitation of ubiquinol with high sensitivity and selectivity (oxidation potential, +600 mV). The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol, 55/45, v/v, at flow rates of 0.7 mL/min. The injection volume of the samples was 10 μL.

Determination of α-, δ-, and γ-Tocopherols. Analyses were performed by gas chromatography−mass spectrometry (GC-MS) (Shimadzu MS-QP5050) in selected ion monitoring mode using methoxy-δ-tocopherol as the reference standard. Conditions: column DB1 J&W (25 m × 0.2 mm × 0.33 μm); injection, 1 μL; split ratio, 2; oven temperature, 100 °C for 1 min to 280 °C at 30 °C/min and hold for 25 min; injector temperature, 250 °C; carrier gas, helium; and flow, 1 mL/min. The electron impact at 70 eV was used for ionization of the compounds; the following ions were monitored: metoxy-δ-tocopherol: *m*/*z* 151, 191, 417; δ-tocopherol-TMS: *m*/*z* 209, 249, 475; γ-tocopherol-TMS: *m*/*z* 223, 489; α-tocopherol-TMS: *m*/*z* 237, 503. Tocopherols were quantitated by comparison of areas to those of authentic standards, including the reference standard.

Determination of Vit A and β-Carotene. Vit A and β-carotene were extracted as previously reported (*15*) and assayed by a Shimadzu liquid chromatograph on an analytical Restek LC-18 ODS amine column (24 cm × 4.6 mm, 5 μm) plus its guard column, by using a photodiode array detector set at 325 (vit A) and 453 nm (β-carotene). Mobile phase: A = 20 μm $NaClO_4$ in MeOH/H_2O (96/4, v/v), B = MeOH/2-propanol (55/45, v/v); gradient program: 5% B for 5 min, 20% B in 15 min, and then 90% B in 25 min; flow, 1 mL/min. Retinyl acetate (20 μg) was used as the standard.

Fatty Acid Patterns of TG and PL Fractions. Extraction of lipids from approximately 1 g of muscle sample was performed according to the procedure of Bligh and Dyer (*16*). Fatty acid patterns of TG and PL fractions were analyzed by GC-MS (Shimadzu MS-QP5050) following purification of lipid fractions by thin-layer chromatography according to Passi et al. (*17, 18*).

Statistical Analysis. Three independent analyses were carried out on 15 samples for each age group for antioxidant concentrations, fatty acids quantification, and TBARS and protein carbonyls determinations. Statistical analyses were performed by Mann−Whitney U Test. The $p < 0,05$ was selected as the point of minimal acceptance statistical significance. All data in the tables are expressed as the mean ± standard error (SEM).

RESULTS

Biometric data of differently aged rainbow trouts and sea basses under study are reported in **Table 1**. As expected, their weight and length were strictly age-dependent.

Protein and Lipid Oxidation. Carbonyl content was measured as a marker of protein oxidation. As shown in **Table 2**, protein carbonyl content began to increase in the muscle tissue of the trouts at age group III with a 26.6% increase when compared with the I age group; however, only age group IV showed a statistically significant elevation of the protein carbonyl content with a 73.3, 63.5, and 36.8% increase when compared to groups I, II, and III, respectively. Also in the muscle tissue of sea bass, protein carbonyl content showed an age-dependent behavior with a 41.2% enhancement of age group III when compared with group II.

Table 2. Total Lipids, TG, and PL Fractions (% of Total Lipids), TBARS, and Protein Carbonyl Values in the Muscle Tissue of Differently Aged Rainbow Trouts and Sea Basses[a]

Rainbow Trouts

	age groups			
	3 months (I)	1 year (II)	2 years (III)	3 years (IV)
total lipids (%)	1.71 ± 0.18	1.84 ± 0.23	1.79 ± 0.16	1.85 ± 0.24
TG (%)	59.3 ± 5.5	62.4 ± 4.8	60.8 ± 6.2	61.4 ± 7.4
PL (%)	11.1 ± 2.5	12.2 ± 2.8	10.9 ± 2.2	11.5 ± 1.4
TBARS (μmol/g)	3.26 ± 0.62	3.51 ± 0.83	4.78 ± 1.12[c]	6.65 ± 1.44[b,d,e]
protein carbonyls (nmol/mg)	0.15 ± 0.06	0.16 ± 0.03	0.19 ± 0.07	0.26 ± 0.05[c,f]

Sea Basses

	age groups		
	≈1 year (I)	≈3 years (II)	≈5 years (III)
total lipids (%)	1.68 ± 0.21	1.77 ± 0.19	1.71 ± 0.22
TG (%)	44.6 ± 4.5	45.4 ± 4.1	45.8 ± 5.2
PL (%)	16.3 ± 2.5	17.2 ± 2.8	16.6.8 ± 2.5
TBARS (μmol/g)	2.30 ± 0.16	2.47 ± 0.28	3.15 ± 0.51[g,h]
protein carbonyls (nmol/mg)	0.17 ± 0.06	0.18 ± 0.05	0.24 ± 0.08

[a] Each result represents the mean ± SEM of 15 samples for each group. [b,c] $p < 0.001$ and 0.01, respectively, vs 3 month old rainbow trouts. [d,e] $p < 0.001$ vs 1 and 2 year old rainbow trouts, respectively. [f] $p < 0.01$ vs 2 year old rainbow trouts. [g] $p < 0.001$ vs ≈1 year old sea basses. [h] $p < 0.001$ vs ≈2 year old sea basses.

Our data showed that there is an age-related increase of oxidative damage to lipids; in trouts (**Table 2**), TBARS content was significantly different between age group IV and the others, increasing by 103.9, 89.4, and 39.1% when compared with groups I, II, and III, respectively. Also in the muscle tissue of sea bass, the TBARS content showed an age-dependent behavior with an increase of 7.4% from class I to class II and 27.5% from class II to class III.

Lipid Content and Fatty Acids Composition. The total muscle tissue lipids and the percentages of TG and PL fractions of rainbow trout and sea bass species of different age groups are given in **Table 2**. All percentages were similar enough in the muscle tissue of differently aged rainbow trouts and sea basses.

The fatty acid in the PL and TG fractions as well as the proportion of the saturated, monounsaturated, and polyunsaturated acids are given in **Tables 3** and **4**.

TG and PL fractions of the muscle tissue of the differently aged rainbow trouts and sea basses studied were characterized by high levels of n-3 PUFA, considerable amounts of saturated and monounsaturated fatty acids, and minimum levels of n-6 fatty acid, with n-3/n-6 ratios always higher than 5 (**Tables 3** and **4**).

In both lipid fractions of trouts, the percentage of saturated fatty acids was comparable; monounsaturared fatty acids were much more abundant in the TG fraction (39.8−42.6% in TG and 14.5−15.3% in PL) while n-3 PUFA (mainly C20:5 n-3 and C22:6 n-3) were more abundant in the PL fraction (59.3−65.1 vs 38.3−39.2%). C20:4 n-6 prevailed in the PL fraction

Table 3. Fatty Acid Levels (%) of TG and PL Fractions in the Muscle Tissue of Differently Aged Rainbow Trouts[a]

	I 3 months old		II 1 year old		III 2 years old		IV 3 years old	
fatty acid	TG	PL	TG	PL	TG	PL	TG	PL
C14:0	0.1	0.1	0.2	0.2	0.1	0.2	0.1	0.2
C15:0	0.2	0.4	0.3	0.3	0.3	0.2	0.2	0.5
C16:0	13.3	12.5	14.1	11.8	13.4	13.1	13.1	16.2
C17:0	0.1	0.3	0.2	0.1	0.1	0.2	0.1	0.4
C18:0	5.2	6.1	6.2	7.6	6.3	7.0	6.8	8.1
C20:0	0.1		0.1		0.1		0.1	
C24:0	0.1	0.2	0.1	0.2		0.1		0.3
Σ saturated	19.1	19.6	21.2	20.2	20.3	20.8	20.4	25.7[b]
C16:1 n-7	4.1	1.3	3.7	1.5	3.8	1.7	4.0	1.4
C17:1 n-7	0.3	0.3	0.2	0.2	0.2	0.4	0.4	0.2
C18:1 n-9 > n-7	30.4	11.1	29.8	10.7	31.6	10.3	32.8	11.3
C20:1 n-9 > n-7	4.5	1.5	3.5	1.3	3.1	1.1	3.2	1.3
C22:1 n-11 > n-9	3.3	1.1	2.6	1.2	2.8	1.0	1.9	0.8
Σ monoenoic	42.6	15.3	39.8	14.9	41.5	14.5	42.3	15.0
C18:2 n-6	3.9	3.6	3.5	3.8	3.8	4.7	3.3	3.8
C20:2 n-6	0.2	0.1	0.1	0.2	0.1	0.3	0.1	0.2
C20:3 n-6	0.1	0.4	0.2	0.3	0.1	0.2	0.1	0.4
C20:4 n-6	0.9	1.8	1.1	2.0	0.5	1.7	0.8	1.6
C22:4 n-6		0.8		0.7		0.9		0.8
C22:5 n-6		0.3		0.2		0.3		0.2
Σ PUFA n-6	5.1	7.6	4.9	7.3	4.5	8.1	4.3	7.0
C18:3 n-3	0.3	0.8	0.2	0.9	0.4	1.1	0.3	0.6
C18:4 n-3		0.4		0.3		0.2		0.2
C20:3 n-3	0.1	0.3	0.1	0.1	0.2	0.2	0.1	0.1
C20:4 n-3	0.1	0.3	0.2	0.2	0.1	0.1		0.1
C20:5 n-3	8.4	19.6	8.7	20.1	8.1	18.7	7.7	17.3
C22:3 n-3		0.2		0.3		0.1		0.2
C22:5 n-3	1.7	2.7	1.6	2.8	1.5	2.5	1.3	2.3
C22:6 n-3	22.6	33.2	23.3	32.9	25.8	33.7	25.1	31.5
Σ PUFA n-3	33.2	57.5	34.1	57.6	34.7	56.6	34.5	52.3[b]
Σ PUFA	38.3	65.1	39.0	64.9	39.2	64.7	38.8	59.3
n-3/n-6	6.5	8.5	6.9	7.9	7.7	7.0	8.0	7.5

[a] Data are the means of five experiments performed in triplicate. SEMs were below 10%. [b] $p < 0.01$ vs age groups I, II, and III.

Table 4. Fatty Acids Levels (%) of TG and PL Fractions in the Muscle Tissue of Differently Aged Sea Basses[a]

	I ≈1 year old		II ≈3 years old		III ≈5 years old	
fatty acid	TG	PL	TG	PL	TG	PL
C14:0	0.5	0.4	0.7	0.5	0.6	0.5
C15:0	0.4	0.7	0.6	0.6	0.5	0.6
C16:0	17.0	15.8	18.1	14.0	16.9	16.0
C17:0	0.4	0.6	0.5	0.6	0.6	0.7
C18:0	8.7	6.8	8.4	6.1	8.2	7.3
C24:0	1.8	1.5	1.9	1.6	1.5	1.7
Σ saturated fatty acids	29.8	25.8	30.2	23.4	28.3	26.8
C16:1 n-7	3.5	2.0	3.2	1.9	3.7	2.1
C17:1 n-7	1.4	1.5	1.3	1.7	1.8	1.4
C18:1 n-9 > n-7	18.8	14.3	18.7	15.6	19.2	14.8
C20:1 n-9 > n-7	2.4	0.7	2.1	0.8	2.2	0.6
C22:1 n-11 > n-9	1.6	0.2	1.4	0.3	1.5	0.2
Σ monoenoic fatty acids	28.1	18.7	26.7	20.3	28.4	19.1
C18:2 n-6	1.9	2.2	2.1	2.5	2.3	2.2
C20:2 n-6	0.8	0.4	0.7	0.5	0.8	0.4
C20:3 n-6	0.1	-	0.1	-	0.1	-
C20:4 n-6	1.4	1.7	1.3	1.6	1.5	1.6
C22:4 n-6	2.5	1.9	2.4	2.2	2.4	2.0
C22:5 n-6	0.3	0.4	0.2	0.3	0.2	0.2
Σ PUFA n-6	7.0	6.6	6.8	7.1	7.3	6.4
C18:3 n-3	0.7	0.4	0.8	0.3	0.7	0.3
C20:3 n-3	0.3	0.1	0.2	0.1	0.2	0.1
C20:4 n-3	0.1	0.1	0.1	0.1	0.1	0.1
C20:5 n-3	10.9	12.6	11.0	13.1	11.8	10.5
C22:5 n-3	1.5	2.5	1.2	2.2	1.4	2.1
C22:6 n-3	22.3	33.2	23.0	33.4	21.8	34.6
Σ PUFA n-3	35.1	48.9	36.3	49.2	36.0	47.7
Σ PUFA	42.1	55.5	43.1	56.3	44.3	54.1
n-3/n-6	5.0	7.4	5.3	6.9	5.1	7.5

[a] Data are the means of five experiments performed in triplicate. SEMs were below 10%.

(1.6−2.0 vs 0.5−1.1%). For what concerns n-3 PUFA of the PL fraction, their amount was significantly lower ($p < 0.01$) in the group IV as compared to the other age groups (**Table 3**). Fatty acid patterns of TG and PL fractions of sea basses showed similar behavior as trouts (**Table 4**): the percentage of saturated fatty acids in both TG and PL fractions was comparable enough (28.3−30.2% in TG and 23.4−26.8% in PL); the level of monoenoic fatty acids was much higher in TG than in PL (26.7−28.4 vs 18.7−20.3%); n-3 PUFA were more abundant in PL than in TG (47.7−49.2 vs 35.1−36.3%); conversely, the percentage of n-3 PUFA did not vary significantly with advancing age (**Table 4**). It is noteworthy that the proportion of C22:6 n-3 remained more constant before decreasing in the highest age group, whereas the proportion of C20:5 n-3 decreased in the PL fractions by 6.7% from group II to group III and by 13.9% from group II to group IV in trout and by 19.8% from group II to group III in sea bass. Arachidonic acid (C20:4 n-6) remained constant in PL and TG fractions throughout all age groups in both trouts and sea basses.

Lipophilic antioxidants. The species under study are characterized by two different ubiquinol/ubiquinone redox couples: $CoQ_{10}H_2-CoQ_{10}$ in trouts and $CoQ_9H_2-CoQ_9$ in sea bass. The results indicated a strong and progressive decrease of CoQ_nH_2 and CoQ_n ($n = 9$ or 10) levels with advancing age. In trouts, both $CoQ_{10}H_2$ and CoQ_{10} levels decreased significantly by −45.1 and −37.0%, respectively, in the group II; −50.4 and −39.9% in the group III; and −53.7 and −52.8% in the group IV when compared to group I (**Table 5**). In sea basses, the negative trend was less marked in comparison with trouts: the significant reduction of CoQ_9H_2 and CoQ_9 levels occurred only in group III as compared to groups I and II (−35.7 and −20.0%, respectively).

Also, vitamin E contents showed a progressive decrease with age: −33.3, −58.3, and −73.1%, respectively, in the age groups II, III, and IV of trouts as compared to the group I and −27.2 and −22.6% in the group III of sea bass as compared to the age groups I and II, respectively. These data showed that aging is associated with a change in the mean values of lipophilic antioxidants (**Table 5**). β-Carotene and retinol have not been detected in the muscle tissue of all groups analyzed by our analytical methods.

Hydrophilic and Enzymatic Antioxidants. Table 5 also showed that the muscle levels of GSH and GSSG remained unchanged in the four different age groups of rainbow trouts and in three age groups of sea basses. On the contrary, vitamin C levels showed a significant decreasing trend in both trouts and sea basses during aging (−47.3 and −73.6%, respectively, in age groups III and IV of trouts as compared to group II and −29.5 and −34.5%, respectively, in the age groups II and III of sea basses as compared to the group I).

The activity of GPX (GPX units/mg protein) in sea bass showed a downward trend (−14.8% on going from groups II to III), whereas a weak increasing trend was observed for the activity of this enzyme in trouts with aging. Cu,Zn−SOD activity (SOD units/mg protein) in trouts showed no significant differences between the different groups, while in sea bass it increased from group I to group III by 38.3% (**Table 5**). CAT activity results were very low in the muscle tissue of all fish groups (<0.0005 units/mg protein) and were not tabulated.

Table 5. Antioxidant Enzyme Activities and Hydrophilic and Lipophilic Antioxidant Levels in Muscle Tissue of Differently Aged Rainbow Trouts and Sea Basses[a]

Rainbow Trouts

	age groups			
	3 months (I)	1 year (II)	2 years (III)	3 years (IV)
GPx (U/mg protein)	0.11 ± 0.04	0.13 ± 0.05	0.12 ± 0.05	0.15 ± 0.06
Cu–Zn SOD (U/mg protein)	7.31 ± 0.5	7.13 ± 0.8	6.63 ± 0.7	7.5 ± 0.71
VIT C (μg/g)	10.2 ± 1.8	12.9 ± 0.4[c]	6.8 ± 0.3[b]	3.4 ± 0.6[b,d,e]
GSH (μg/g)	47.1 ± 6.6	50.3 ± 8.4	46.2 ± 5.3	45.6 ± 5.7
GSSG (μg/g)	1.41 ± 0.4	1.82 ± 0.6	1.31 ± 0.5	1.83 ± 0.8
$coQ_{10}H_2$ (μg/g)	8.09 ± 0.96	4.44 ± 0.69[c]	4.01 ± 0.46[c]	3.74 ± 0.57[c]
coQ_{10} (μg/g)	2.78 ± 0.51	1.75 ± 0.39[b]	1.67 ± 0.48[b]	1.31 ± 0.29[c]
VIT E (μg/g)	10.8 ± 2.8	7.2 ± 1.2[c]	4.5 ± 0.5[c]	2.9 ± 0.6[c,d,e]

Sea Basses

	age groups		
	≈1 year (I)	≈3 years (II)	≈5 years (III)
GPx (U/mg protein)	0.52 ± 0.11	0.54 ± 0.16	0.46 ± 0.17
Cu–Zn SOD (U/mg protein)	4.70 ± 1.8	5.81 ± 1.6	6.52 ± 1.5
VIT C (μg/g)	6.1 ± 0.8	4.3 ± 0.5[c]	4.0 ± 0.6[c]
GSH (μg/g)	29.6 ± 4.6	28.7 ± 5.3	28.1 ± 7.1
GSSG (μg/g)	2.9 ± 0.6	3.1 ± 0.5	3.3 ± 0.6
coQ_9H_2 (μg/g)	5.15 ± 0.64	4.72 ± 0.23	3.31 ± 0.39[f,h]
coQ_9 (μg/g)	2.51 ± 0.33	2.09 ± 0.31	2.01 ± 0.17[g]
VIT E (μg/g)	6.6 ± 0.7	6.2 ± 0.8	4.8 ± 0.9[f,h]

[a] Each value represents the mean ± SD of 15 samples for each group. [b,c] $p <$ 0.001 and 0.01, respectively, vs 3 month old rainbow trout. [d,e] $p <$ 0.001 vs group I and group II, respectively, of trouts. [f,g] $p <$ 0.001 and 0.01, respectively, vs ≈1 year old sea basses. [h] $p <$ 0.01 vs group II of sea basses.

DISCUSSION

Our data on fish support the hypothesis that the level of oxidative stress increases during the aging process. Is it related due to a decline in antioxidant defenses or an increase in the rate of prooxidant generation? Both of these conditions take place during fish aging, apart from the species and the living conditions.

Aerobic cells contain various amounts of the three main antioxidant enzymes: SOD, CAT, and GPx. These three enzymes are necessary for cell survival since inhibition of their activity leads to the arrest of cell mitosis and to cell death.

In the muscle tissue of all fish age groups, CAT activity results were very low (<0.0005 units/mg protein), and these data were in accordance with our previous work on antioxidant levels in different Mediterranean marine species of fish and shellfish indicating that in fish the CAT activity is largely lower than that reported in mammals and birds (*18*). In our fish species, no significant changes were shown for SOD and GPX. This was in accordance with our finding of similar GSH levels in all age groups. With age, the level of antioxidant enzymes does not change, so that it is not possible to explain the aging process by a lack of protection due to a decrease in the activity of the antioxidant enzymes.

Our data showed that lipid peroxidation increased with aging when expressed as TBARS. The TBARS values were significantly increased in the age groups III and IV of trouts and age group III of sea basses with respect to the respective group I. Certainly the most direct approach for the assessment of lipid peroxidation is the quantification of the primary (hydroperoxides) products, but in practice, it is very difficult because of their labile, fleeting nature. Consequently, detection of lipid peroxidation has relied largely on indirect methods, that is, analyses of secondary or end products derived from hydroperoxides such as malondialdehyde (MDA). TBARS is very sensitive but poor in specificity, and many compounds, other than MDA, could react with TBA. The lack of specificity may account for no perfect correspondence between increased TBARS levels and analogous PUFA decrease.

Fatty acids in the PL and TG fractions of both species did not show significant differences among the selected age groups, except in the PUFA of the PL fraction in trouts, where their amount is significantly lower in age group IV as compared to the other age groups (**Tables 3** and **4**). On the other hand also, feed utilization, hormone production, and general changes in metabolism may modify PUFA levels with growth of the fish.

Protein carbonyl content, an indicator of oxidative protein damage, increased in an age-related pattern, with significantly higher levels in age group IV of trouts and in age group III of sea basses as compared to the other age groups. This parameter could be useful as a potential biomarker of oxidative damage in fish muscle tissue. Our findings are in agreement with the animal and human studies from other investigators (*19*, *20*). After water, which accounts for about 80% (w/w), proteins are the major constituents (15−20%) of fish flesh. It is well-known that proteins are susceptible to damage by ROS in vitro and in vivo, and oxidative modification of proteins may lead to the structural alternation and functional inactivation of many enzyme proteins. Normally, oxidatively modified proteins are degraded more rapidly than native proteins by a proteolitic system, which is proposed as a secondary free radical defense system (*21*). Thus, the age-related accumulation of oxidatively modified proteins is due to either excessive oxidation of proteins or decreased capacity to clear up oxidatively damaged proteins.

Protein oxidation is thought to be one of the major sources of quality deterioration in muscle food products leading to loss of protein functionality, texture, and nutritional quality (*22*). Whereas no significant differences were found in the muscle tissue concentrations of enzymatic antioxidants (Cu,Zn−SOD, GPx, and CAT) and of GSH, the levels of the main lipophilic antioxidants and vitamin C are dramatically depleted, and these results clearly show that oxidative stress occurs in fish during aging. In particular, the marked drop of $CoQH_2$, not counterbalanced by a concomitant increase of its oxidized form CoQ, indicates that the total coenzyme Q ($CoQH_2$ + CoQ) biosynthesis is decreased in aging fishes (**Table 3**).

According to Yamashita and Yamamoto (*23*), the imbalance of the ratio $CoQH_2$/CoQ in biological systems can be considered an early marker of oxidative stress in vivo. Besides its activities in the electron transport chain, coenzyme Q (in reduced form) has also been implicated as the only endogenously synthesized lipid soluble antioxidant protecting cellular membranes and plasma lipoproteins from free radical damage (*24*). Furthermore, it is able to sustain efficiently the chain breaking antioxidant capacity of vit E, by regenerating it from tocopheroxyl radical (*25*), which otherwise would need the cooperation of a hydrophilic antioxidant such as vit C or GSH. It has been suggested that the cooperation between vitamin E and vitamin C is probably the most important mechanism against biomolecules oxidation. In our opinion, also, the protective role played by the couple vitamin E−ubiquinol should be carefully evaluated. A derangement of these reductive mechanisms, due to an overproduction of pro-oxidant reactive species, coupled to a reduced CoQ_{10} biosynthesis, represents an important fingerprint of oxidative stress.

The present studies suggest that in freshwater and marine fishes lipid peroxidation and accumulation of oxidized proteins

during aging are most likely to be linked with an age-dependent decline of vitamin C and lipophilic antioxidants ($CoQH_2$, CoQ, and vitamin E) contents in muscle tissue, whereas fish aging is not linked to a decline in antioxidant enzymes and GSH levels. The significant depletion of the levels of the above-mentioned antioxidants and the concomitant increase of TBARS and protein carbonyls represent a reliable marker of oxidative stress, which supports the "free radical theory of aging" also in fish. In addition, the quantification of lipophilic antioxidants and vitamin C in muscle tissues of fish might be particularly indicated for the assessment of their age.

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