Lipid peroxidative stress and antioxidant defence status during ontogeny of rainbow trout (Oncorhynchus mykiss)

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The objective of the present study was to characterise some important antioxidant enzymes and their relationships with retinoids and lipid peroxidation during rainbow trout (Oncorhynchus mykiss) early development. Eggs were incubated at 7°C until the swim-up stage whereupon fry were fed two semi-purified diets with 0% (CO) and 8% (OX) oxidised lipid respectively for 2 months at 17°C. The activities and gene expression of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were determined as well as the levels of retinoids, F2-isoprostanes and lipid-soluble fluorescent products (LSFP) at various developmental stages. Only SOD had a detectable activity in embryos which increased during development and was linked with an increase of mitochondrial (SOD2) and cytosolic (SOD1) gene expression. SOD1 and SOD2 mRNA were more abundant in fry fed OX than in fry fed CO. CAT activity and gene expression also increased during development and were higher in fry fed OX compared with fry fed CO. Activity of Se-dependent GPX (Se-GPX) increased during development. The gene expression of cytosolic Se-GPX (GPX1) increased from hatching to 2-month-fed fry. Both phospholipid-hydroperoxide GPX and GPX1 genes were more expressed in fry fed OX than in fry fed CO. Retinoids decreased during development and, by 2 months, were lowered in fry fed OX compared with those fed CO. The levels of LSFP were higher in fry fed OX compared with fry fed CO. The present study demonstrates that antioxidant defence systems are active all through the development of rainbow trout and modulated by feeding oxidised lipid.

Rainbow trout: Larval development: Lipid peroxidation: Antioxidant enzymes

Under physiological conditions, aerobic tissues continuously generate reactive oxygen species as a byproduct of oxidative metabolism(1). In some specific cell types, reactive oxygen species generation may be beneficial against a pathogen. However, in most cases, high amounts of reactive oxygen species are harmful to cells and cause DNA damage, enzyme inactivation and structural protein degradation as well as peroxidation of PUFA leading to pathologies and alteration of development(2). As fish contain high concentrations of highly unsaturated fatty acids, they are vulnerable to lipid peroxidation and to tissue damage resulting from lipid peroxidation(3). Pathology related to oxidative stress in fish includes reduced growth, poor survival, liver degeneration, anaemia and muscular dystrophy(4–6). Oxidative stress is due to the overwhelming of antioxidant defences of cells by pro-oxidants. This imbalance can be aggravated by diet composition. Feeds for fish and especially feeds for fish larvae contain high levels of PUFA derived from marine fish oils and meals. These fatty acids are particularly prone to oxidative damage during diet preparation and storage. Products derived from lipid peroxidation may be absorbed and transported to tissues, where they may induce oxidative stress(7).

To decrease reactive oxygen species levels and avoid lipid peroxidation, all aerobic organisms possess two types of antioxidant defence systems. One is represented by enzymes and includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). SOD is a metalloenzyme that catalyses the dismutation of the superoxide anion (O2•−) into oxygen and H2O2(8). The Cu/Zn-SOD is located in the cytosol and nucleus, while Mn-SOD is localised within the mitochondrial matrix. Subsequently, H2O2 is reduced to water by CAT in the peroxisomes. GPX catalyses the same reaction as CAT in the cytosol and also converts lipid hydroperoxides into lipid hydroxides, which are more stable products. The other group of antioxidant defence systems is composed of free radical scavengers. These compounds, generally of low molecular weight, lipid or water soluble, are mainly represented by vitamins E and C. However, vitamin A has also been shown to reduce lipid peroxidation(9). If the antioxidant actions of vitamins E and C are well known, the antioxidant action of vitamin A remains still unclear. It could be due to the ability of the free alcohol form of vitamin A to quench singlet oxygen(10) or to the ability of retinoic acid to increase expression and activity of antioxidant enzymes(11). Moreover,

Abbreviations: CAT, catalase; CO, semi-purified diet with 0% oxidised lipid; CT, cycle threshold; dpf, days post-fertilisation; EF1α, elongation factor 1α; GPX, glutathione peroxidase; HPGPX, phospholipid-hydroperoxide glutathione peroxidase; LSFP, lipid-soluble fluorescent products; OX, semi-purified diet with 8% oxidised lipid; Se-GPX, Se-dependent glutathione peroxidase; SOD, superoxide dismutase.

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vitamin A has many other functions that are more prominent than its activity as an antioxidant especially during early development\(^{12}\).

The objective of the present study was hence to characterise some important antioxidant enzymes and their relationships with retinoids and lipid peroxidation during rainbow trout (Oncorhynchus mykiss) early development. The response of antioxidant defence systems to dietary pro-oxidant conditions was also evaluated by feeding fry with oxidised lipid.

Materials and methods

Experimental fish and diets

Rainbow trout (O. mykiss) eggs were obtained from fertilisation of ova collected from eight females by a common pool of sperm from eight males in the INRA experimental fish farm in Léès-Athas (Pyrénées-Atlantiques, France). Eggs were incubated in a large tray supplied by spring water at 7 ± 1°C. At the eyed stage (32 d post-fertilisation (dpf)) and hatching (44 dpf), dead embryos were removed. At the swim-up stage (70 dpf), fry were transferred to the INRA experimental fish farm in Donzacq (Landes, France) and randomly distributed into six tanks (600 larvae per 50 litre fibreglass tank) supplied by spring water at 17 ± 1°C. From the swim-up stage, which corresponds to the beginning of exogenous feeding, fish were hand-fed four or six times per d to apparent satiation. Two semi-purified diets with 0 % (CO) and 8 % (OX) oxidised lipid respectively were tested in triplicate for 2 months (Table 1). Diets were isoproteic (56 %) and isolipidic (16 %) with 8 % soyabean lecithin and 8 % fresh or oxidised salmon oil. Oxidised lipid was obtained by bubbling air through fish oil (La Lorientaise, France; specially prepared from crude salmon oil, additive free) for 90 h at 50°C. Fresh and oxidised oils were stabilised immediately after reception or oxidation with 300 parts per million ethoxyquin as in common usage for fish oil for animal nutrition, resulting in a final concentration of 24 parts per million ethoxyquin in diets, in order to spare the other nutrients such as vitamins and test only the effect of oxidised lipid and not of oxidised diet. Oils were both assayed for oxidative state before incorporation into the diets (Table 2).

**Table 1.** Formulation and composition of experimental diets (g/100 g dry weight)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CO</th>
<th>OX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Casein–dextrin basis</strong></td>
<td>75·0</td>
<td>75·0</td>
</tr>
<tr>
<td>Soyabean lecithin</td>
<td>8·0</td>
<td>8·0</td>
</tr>
<tr>
<td>Fresh salmon oil</td>
<td>8·0</td>
<td>–</td>
</tr>
<tr>
<td>Oxidised salmon oil</td>
<td>–</td>
<td>8·0</td>
</tr>
<tr>
<td>Mineral mixture‡</td>
<td>4·5</td>
<td>4·5</td>
</tr>
<tr>
<td>Vitamin mixture§</td>
<td>4·5</td>
<td>4·5</td>
</tr>
<tr>
<td><strong>Proximate composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (%)</td>
<td>93·6</td>
<td>92·7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>49·3</td>
<td>49·5</td>
</tr>
<tr>
<td>Total lipids</td>
<td>16·0</td>
<td>15·8</td>
</tr>
<tr>
<td>Ash</td>
<td>6·1</td>
<td>6·5</td>
</tr>
<tr>
<td>Gross energy (kJ/g DM)</td>
<td>22·7</td>
<td>22·6</td>
</tr>
</tbody>
</table>

CO, semi-purified diet with 0 % oxidised lipid; OX, semi-purified diet with 8 % oxidised lipid.

Chemical analyses of diets and oils

Proximate composition of diets was determined according to the following procedures: DM after drying at 105°C for 24 h, protein (N × 6·25) by the Kjeldahl method after acid digestion, ash by incineration at 550°C for 16 h and gross energy in an adiabatic bomb calorimeter. Total lipid was extracted and measured gravimetrically according to Folch et al.\(^{13}\) using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids according to Shantha & Decker\(^{15}\) and analysed in a Varian Chrompack CP-3800 gas chromatograph equipped with a DB Wax fused silica capillary column (30 m × 0·25 mm internal diameter, film thickness 0·25 mm; Varian, Les Ulis, France) using He as the carrier gas (1·4 ml/min). The thermal gradient was 100 to 180°C at 8°C/min, 180 to 220°C at 4°C/min and a constant temperature of 220°C during 20 min. Injector and flame ionisation detector temperatures were 260 and 250°C, respectively. The unsaturation index (UI) was calculated according to the formula: UI = \(\sum f \times 100\) (number of double bonds). Peroxide value of fish oil was determined by colorimetric determination of Fe-thiocyanate according to Shantha & Decker\(^{15}\). Conjugated dienes and trienes were measured as specific extinctions at the wavelengths of 232 and 268 nm, respectively\(^{16}\). Anisidine value was determined according to the European Committee for Standardization\(^{17}\).

Thiobarbituric acid-reactive substances were measured according to Salih et al.\(^{18}\) with modifications. Briefly, samples were homogenised in 20 % TCA (w/v) containing 0·02 % of butylated hydroxytoluene as antioxidant using ultra-turrax and centrifuged at 5000 g for 20 min at 4°C. The resulting supernatant fractions were incubated with 0·8 % thiobarbituric acid solution (w/v) at 100°C for 30 min, cooled in ice and centrifuged at 800 g for 10 min. Absorbance was measured at 532 nm and the quantification was achieved by comparison with a standard curve of malondialdehyde equivalents generated by acid-catalysed hydrolysis of 1,1,3,3-tetraethoxypropane.

Sample collection

Samples were taken on 0, 21, 44 and 70 dpf stages and from each tank on 81, 98 and 133 dpf stages after starvation for 24 h. These stages correspond respectively to oocytes, embryos, hatchedy fry, swim-up fry, complete yolk-sac resorbed fry, 1-month-fed fry and 2-month-fed fry. Fish were anaesthetised.
in diluted 2-phenoxethanol for wet weight determination, frozen in liquid N₂ and stored at −80°C until analysis.

**Determination of total 8-isoprostane levels and lipid-soluble fluorescent products**

The level of oxidative stress was assessed by measuring larval concentration of 8-isoprostanes, the products of non-enzymic peroxidation of arachidonic acid by reactive oxygen species. Isoprostanes were assayed in all withdrawn samples except for measurement of lipid-soluble fluorescent products (LSFP), total lipid of oocytes and fry from the swim-up stage onwards was extracted according to Folch et al. (1957). For measurement of lipid-soluble fluorescent products (LSFP), total lipid of oocytes and fry from the swim-up stage onwards was extracted according to Folch et al. (1957) and diluted in chloroform–methanol (7:3, v/v). Fluorescence intensity was determined in a spectrophotometer Triad Dynex (Sercalab Technologies, Bonneuil sur Marne, France) using excitation/emission wavelengths of 360/465 nm and quinine sulfate was used as a standard at a concentration of 1 μg/ml in 0.05 M-sulfuric acid.

**Determination of retinoid levels**

Retinoid extraction was conducted by homogenising 2.5 g of oocytes, swim-up fry, 1-month fry or 2-month fry in 10 volumes of 10 mM-PBS. The homogenate was divided into two equivalent volumes (mixtures A and B). The pH of mixture B was adjusted to 4.2 with acetic acid before sonication for 3 min. Proteins were precipitated by adding 4.5 ml ethanol containing 2% pyrogallol (w/v) as antioxidant. After addition of 10 ml hexane (mixture A) or ethyl acetate–methyl acetate (8:1, v/v) (mixture B), mixtures were centrifuged (10 min; 10000 g; 4°C). Samples were extracted two other times with 4 ml hexane or ethyl acetate–methyl acetate (8:1, v/v) and the upper organic phases were pooled for each mixture and evaporated to dryness under N₂. The residues were then dissolved in 200 μl isopropanol–methanol–acetonitrile–tetrahydrofuran (40:30:15:15, by vol.). After centrifugation at 10000g for 3 min, a 100 μl sample was subjected to HPLC on a 2695 Alliance Separation Module equipped with a Waters 2487 dual λ absorbance detector and a Waters 2475 multi-wavelength fluorescence detector (Waters, Saint-Quentin-en-Yvelines, France). Instrument control, and data acquisition and processing were achieved by the use of Waters Empower software.

Retinol and retinyl palmitate were determined by injection of mixture A into a Spherisorb ODS2 C₁₈ reversed-phase column (250 × 4.6 mm, with a particle size of 5 μm; Waters) fitted with a security guard cartridge system. Retinoic acid and retinal were determined by injection of mixture B into the same column. The solvent system consisted of water–acetonitrile–methanol (57:37:6, by vol.) containing 100 mM-ammonium acetate (pH 4.2) (solvent A), methanol (100%) (solvent B) and acetonitrile–methanol–isopropanol (60:35:5, by vol.) (solvent C). A linear gradient from solvent A (100%) to solvent B (100%) was applied over a period of 20 min, followed by isocratic elution with solvent B (100%) for 5 min and isocratic elution with solvent C (100%) for 45 min. Then a linear gradient from solvent C (100%) to solvent A (100%) was applied for 5 min. The flow rate was set at 1 ml/min and the detection wavelength at 350 nm for the detection of retinoic acid and retinal and 325 nm for the detection of retinol and retinyl palmitate. Retinoids were identified by retention times of pure standards (Sigma, Saint-Quentin-Fallavier, France).

**Determination of antioxidant enzyme activities**

The whole embryos or larvae samples were homogenised in 7 volumes of ice-cold 20 mM-phosphate buffer (pH 7.4) containing 1 mM-EDTA using ultra-turrax. The homogenates were centrifuged at 10000g for 10 min at 2°C to remove debris. The resultant supernatant fractions were incubated for 1 h with 0.2 volumes of 20 mM-phosphate buffer (pH 7.4) containing 100 μM-SOD, 0.4% horse liver catalase, 100 μM-hexobarbitone and 100 μM-leupeptin. The level of oxidative stress was assessed by measuring larval concentration of 8-isoprostanes, the products of non-enzymic peroxidation of arachidonic acid by reactive oxygen species.
Antioxidant functions in rainbow trout fry

1 mM-EDTA and 0.5 % Triton X-100 (v/v) before use for antioxidant enzyme assays.

The activity of total SOD (EC 1.15.1.1) was assayed using a reagent kit and SOD from bovine liver as a standard (Sigma). The reaction was based on its inhibitory effect on the rate of superoxide-dependent reduction of a water-soluble tetrazolium salt (WST-1) by xanthine–xanthine oxidase (20). The reaction was monitored at 450 nm and 37°C. One SOD unit was defined as the amount of enzyme required to inhibit the reduction of WST-1 to WST-1 formazan in the presence of superoxide by 50 %.

CAT activity (EC 1.11.1.6) was assayed in a quartz microplate with 5 μl sample solution in a final volume of 250 μl containing 67 mM-phosphate buffer (pH 7), 1 mM-EDTA and 20 mM-H2O2. Activity was determined by following the reduction of H2O2 at 30°C and 240 nm using the extinction coefficient 40 μM⁻¹cm⁻¹(21). One unit of CAT represents the amount of enzyme that decomposes 1 μmol H2O2 per min.

GPX activity (EC 1.11.1.9) was assayed by following the rate of NADPH oxidation at 340 nm and 30°C by the coupled reaction with glutathione reductase using the extinction coefficient 6.22 μM⁻¹cm⁻¹(22). Cumene hydroperoxide and H2O2 were the substrates for measuring total GPX and Se-dependent GPX (Se-GPX), respectively. The assay was realised in a microplate with 20 μl sample solution in a final volume of 240 μl containing 50 mM-phosphate buffer (pH 7.4), 1 mM-EDTA, 2 mM-sodium azide, 2 mM-reduced glutathione, 0.1 mM-NADPH, 0.2 units glutathione reductase and 0.2 mM-cumene hydroperoxide or 50 μM-H2O2. One unit of GPX is defined as the amount of enzyme that catalyses the oxidation of 1 μmol NADPH per min.

Protein concentration was determined according to Lowry et al.(23), using bovine serum albumin as a standard.

Design of polymerase chain reaction primers and probes

PCR primers used for the quantification of the different genes were designed using Primer 3 software (Table 3). The cDNA sequences of the elongation factor 1α (EF1α) and the cytosolic Cu/Zn superoxide dismutase (SOD1) were obtained from GenBank sequences AF498320 and AF469663, respectively. The cDNA sequences of CAT, mitochondrial Mn superoxide dismutase (SOD2), phospholipid-hydroperoxide glutathione peroxidase (HPGPX) and cytosolic glutathione peroxidase (GPX1) were obtained from The Institute for Genomic Research (TIGR) sequences TC99600, TC104653, TC95828 and TC126469, respectively. The PCR products were run on a 2 % agarose gel to check that only one fragment was amplified (i.e. absence of genomic DNA amplification) and sequenced to ensure that the correct mRNA sequences were quantified.

Real-time quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from embryos and larvae using Trizol reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s instructions, and stored in nuclease-free water (Promega, Charbonnières, France). Genomic DNA was eliminated from the samples using RQ1 Rnase-Free DNase (Promega) and purified RNA was then stored at −20°C. Samples were subjected to electrophoresis on 1 % agarose gels to confirm the integrity of the 28S and 18S rRNA bands and RNA quality was assessed as the 260:280 nm absorbance ratio.

cDNA was generated from 1 μg DNase-treated total RNA using 200 U SuperScript™ III RT (Invitrogen) and 500 ng oligo(dT)15 primers (Promega) in a total volume of 20 μl. The thermal cycle used for RNA samples was: 65°C for 3 min, 25°C for 10 min, 42°C for 1 h and then 99°C for 5 min. For each sample, reverse transcription was performed in duplicate.

Real-time PCR was performed using the iCycler iQ™ (Bio-Rad, Marne-la-Coquette, France) with iQ™ SYBR® Green Supermix (Bio-Rad). All PCR reactions were set up in ninety-six-well plates using 200 nmol/l of each primer and 10 μl cDNA (dilution factor = 32) in a reaction volume of 25 μl. Thermal cycling was initiated with incubation at 95°C for 3 min for hot-start iTaq™ DNA polymerase activation. Thirty-five steps of PCR were performed, each one consisting of heating at 95°C for 20 s and at the annealing temperature for 30 s (Table 3). Following the final cycle of the PCR, melting curves were systematically monitored (increase set point temperature from 55 to 94°C by 0.5°C/10 s) to confirm production of a single product. Each cDNA sample was assayed in duplicate. Controls were included in each plate to test the absence of contamination by genomic DNA or assay reagents. Standard curves were obtained for each cDNA template by plotting the cycle threshold (CT) values against the log10 of five different dilutions (in triplicate) of cDNA sample solutions. CT values corresponded to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit.

Real-time amplification PCR efficiency was determined from standard curves according to E = 10^(−1/slope). The relative expression ratio (R) of each target gene was calculated based on PCR efficiency and CT deviation between sample

Table 3. Sequences of the polymerase chain reaction primers used to assay gene expression by real-time quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1α</td>
<td>TCCCTCTGTGCTGTTCGCTG</td>
<td>ACCCCAGGAGCACATCTGTG</td>
<td>159</td>
<td>59</td>
</tr>
<tr>
<td>SOD1</td>
<td>TGTGCTCTGTGAAAGCTGTATT</td>
<td>TTTGCACTCTGCTGACGTCAC</td>
<td>201</td>
<td>56</td>
</tr>
<tr>
<td>SOD2</td>
<td>TCCTCGACCTGACCTACGAC</td>
<td>GCCTCTTCCATTAAACCTC</td>
<td>201</td>
<td>56-5</td>
</tr>
<tr>
<td>CAT</td>
<td>TGATGTCACACAGAGGTCAGGTA</td>
<td>GTGGGCTAGTGGTGGTAG</td>
<td>195</td>
<td>55</td>
</tr>
<tr>
<td>GPX1</td>
<td>CGAGCCTCGATGACGCTAGGTT</td>
<td>TGCTTCCGCTGACGTCAC</td>
<td>183</td>
<td>59</td>
</tr>
<tr>
<td>HPGPX</td>
<td>TGGAGGCTGAGGAGGAGCAGGGTT</td>
<td>ACCCTTTCCCTTGGGCTGTT</td>
<td>152</td>
<td>59</td>
</tr>
</tbody>
</table>

EF1α, elongation factor 1α; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; HPGPX, phospholipid-hydroperoxide glutathione peroxidase.
and control using the Relative Expression Software Tool (REST)(24). R was expressed in comparison with normalised EF1a as the reference gene according to Essex-Fraser et al. (25) as no reference gene (EF1a, β-actin and glyceraldehyde-3-phosphate dehydrogenase) could be used directly. The level expression of EF1a within each group was normalised to a selected control group (70 dpf) as follows: individual value within a group/(mean value within a group/mean value of control group). The swim-up fry stage was chosen as the control as the mean CT value of this group was close to the mean CT value of all samples for EF1a.

Statistical analyses
Results are given as mean values and standard deviations. Statistical analyses were performed with the computing program Statbox (Grimmer Logiciels, Paris, France) and differences were considered significant when P values were <0.05. Statistical differences in gene expression between the control group and samples were evaluated in group means by 5000 randomisation tests using REST software (24) and differences were considered significant at P<0.05.

Results
Growth performance
Fertilisation rate was very good (96.6 %) and survival remained high from the eyed stage (32 dpf) onwards (Table 4). Mean wet weight increased about 20-fold from the oocyte stage to the 2-month-fed fry stage. No significant differences of survival or growth were observed between fry fed CO and fry fed OX.

Levels of lipid peroxidation products
The levels of 8-isoprostane were the highest in oocytes but no significant effect of dietary oxidised lipid was recorded (Table 5). On the other hand, the levels of LSFP were consistently higher in fry fed OX than in fry fed CO for a given stage. No clear effect of fry development on the evolution of these products of lipid peroxidation was noted.

Retinoid contents in oocytes and fry
Retinoic acid was found to be higher in oocytes than in fry; it decreased during development and was significantly lower in 2-month fry fed OX compared with fry fed CO. Retinal contents in oocytes and fry were the main storage form of vitamin A. A decrease in retinol, peak during development and was significantly lower in 2-month fry fed OX compared with fry fed CO. No change effect of diet on the evolution of these products of lipid peroxidation was noted.

Retinoid contents in oocytes and fry
Retinoic acid was found to be higher in oocytes than in fry; it decreased during development and was significantly lower in 2-month fry fed OX compared with fry fed CO. Retinal contents were less clear. Retinol, peak during development and was significantly lower in 2-month fry fed OX compared with fry fed CO. No change effect of diet on the evolution of these products of lipid peroxidation was noted.

Table 4. Survival (%) and mean wet weight (mg) of oocytes, embryos and fry of rainbow trout (Oncorhynchus mykiss) during the experiment (Mean values and standard deviations of three replicates)

<table>
<thead>
<tr>
<th>Stage (dpf)</th>
<th>0 or 1*</th>
<th>32</th>
<th>44</th>
<th>70</th>
<th>81 CO</th>
<th>81 OX</th>
<th>98 CO</th>
<th>98 OX</th>
<th>133 CO</th>
<th>133 OX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (%)</td>
<td>97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19</td>
<td>--</td>
<td>--</td>
<td>77&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3</td>
<td>111&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
<td>183&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18</td>
</tr>
</tbody>
</table>

* The stage indicated is 0 dpf for survival and 1 dpf for mean wet weight.

<sup>a–e</sup> Mean values within a row with unlike superscript letters are significantly different (P<0.05).
difference was observed according to fry diet. SOD1 and SOD2 genes were expressed from 21 dpf embryos to 2-months-fed fry (Figs. 2(A) and (B)). SOD1 gene expression was not significantly different during development except for 1-month and 2-month fry fed OX where an increase was noted. SOD1 gene expression was significantly higher in fry fed OX than in fry fed CO (1.29-, 1.31- and 1.35-fold for 81, 98 and 133 dpf fry). SOD2 gene expression increased during development particularly during the early stages of development (21 and 44 dpf) and this expression was significantly higher (1.33-fold) in 2-month fry fed OX than in 2-month fry fed CO.

CAT activity, which was not detectable in 21 dpf embryos, greatly increased during development particularly after 2-month feeding (Fig. 1(B)). This activity was affected by dietary levels of oxidised lipid: 2-month fry fed OX showed a higher activity than fry fed CO. CAT gene expression was detectable in 21 dpf embryos with an expression significantly higher than in newly hatched fry. CAT gene expression increased during development from hatching to 2-month-fed fry (Fig. 2(C)). This expression was significantly higher (1.33-fold) in 1-month fry fed OX than in 1-month fry fed CO.

Among GPX, Se-GPX activity was higher than non-Se-GPX activity (Figs. 1(C) and (D)). Non-Se-GPX activity was detectable from 70 dpf and stayed low, without significant difference among dietary groups. However, Se-GPX activity was detectable from hatching and increased during development. Two Se-GPX were studied by RT-PCR: HPGPX and GPX1 (Figs. 2(D) and (E)). HPGPX mRNA abundance was more or less constant during development. It was significantly higher at the 21 dpf stage and 2-month fry fed OX. The HPGPX gene was 1.31-fold more expressed in fry fed OX than in fry fed CO after 1 month of feeding. GPX1 gene expression decreased from 21 dpf to hatching before increasing significantly from the swim-up stage to the end of the experiment. The GPX1 gene was 1.32-fold more expressed in fry fed OX than in fry fed CO after 2 months of feeding.

**Discussion**

**Evolution of antioxidant defences during larval development**

The present results show that both the activity and expression of genes coding for antioxidant enzymes increased during rainbow trout development. The evolution of the three antioxidant enzymes SOD, CAT and GPX confirm previous results obtained by Aceto et al.\(^\text{(26)}\) in rainbow trout embryos. The same tendencies have also been reported for CAT and GPX in other fish species such as turbot\(^\text{(27)}\) and common dentex\(^\text{(28)}\) whereas the reverse has been observed for SOD. However, the present study has shown that only SOD presented a readily measurable activity in embryos, which seems to confirm that like in turbot and common dentex, SOD is needed in the very early developmental stages to reduce elevated tissue concentration of O\(_2\)(\(^\text{(27)}\)). According to Vernier\(^\text{(29)}\), kidney and liver are present at the rainbow trout embryonic stage analysed in the present study and SOD has been reported to be highly active in these two tissues\(^\text{(26)}\).

The present study is the first one to measure the expression of genes coding for antioxidant enzymes during the early
development of fish. The results of gene expression analyses confirm data of antioxidant enzyme activities. The increase of SOD2 gene expression occurred during the very early stages, between the embryo and swim-up stages, whereas the increases of CAT and GPX1 gene expression were noted later, between hatching and complete yolk-sac resorption. All studied genes coding for antioxidant enzymes have been shown to be expressed from the embryonic stage. The genes coding for HPGPX and GPX1 were even more expressed in rainbow trout embryos compared with swim-up fry.

Contrary to antioxidant enzymes, retinoids declined during rainbow trout early development. A decrease of retinol during embryonic development has also been reported for lake trout (30). This pattern in fish larval stages is similar to what has been described for other antioxidant vitamins such as vitamin E(12,28). These non-enzymic antioxidants in fish eggs are essential to ensure early antioxidant protection. However, retinoids, especially retinoic acids, are also recognized as highly active molecules in developmental processes(31). In higher vertebrate cells, retinoic acids exist in several stereoisomeric forms: predominantly all-trans-retinoic acid and 13-cis-retinoic acid, but also as less-stable isomers such as 9-cis-retinoic acid(32). In the present study, 9-cis-retinoic acid was not detected in any of the developmental stages and 13-cis-retinoic acid was detected only in fry and in very low amounts (data not shown). As in higher vertebrates, all-trans-retinoic acid was the main form of retinoic acid in rainbow trout oocytes and fry. Oocytes displayed significant levels of retinoic acid and these levels declined during embryonic development. Retinyl palmitate was the major retinoid in rainbow trout oocytes and fry with levels ranging from 44 % in fry fed diet CO to 96 % in oocytes. Retinal was reported to be the essential mode of retinoid storage in eggs of teleosts whereas retinyl esters were described as

![Activity of superoxide dismutase (SOD) (A), catalase (B), Se-dependent glutathione peroxidase (Se-GPX) (C) and non-Se-GPX (NS-GPX) (D) in rainbow trout (Oncorhynchus mykiss) embryos and fry fed different diets: \( P \leq 0.05 \).](image_url)

Table 6. Levels of different retinoids in rainbow trout (Oncorhynchus mykiss) oocytes and fry (ng/g)

<table>
<thead>
<tr>
<th>Stage (dpf)</th>
<th>0</th>
<th>70</th>
<th>98 CO</th>
<th>98 OX</th>
<th>133 CO</th>
<th>133 OX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoic acid</td>
<td>213&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21</td>
<td>191&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38</td>
<td>17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>Retinol</td>
<td>1893&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77</td>
<td>502&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84</td>
<td>206&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>48 463&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7866</td>
<td>5785&lt;sup&gt;b&lt;/sup&gt;</td>
<td>458</td>
<td>181&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37</td>
</tr>
</tbody>
</table>

dpf, Days post-fertilisation; CO, semi-purified diet with 0 % oxidised lipid; OX, semi-purified diet with 8 % oxidised lipid.

* Mean values within a row with unlike superscript letters are significantly different \( (P \leq 0.05) \).

![Fig. 1. Activity of superoxide dismutase (SOD) (A), catalase (B), Se-dependent glutathione peroxidase (Se-GPX) (C) and non-Se-GPX (NS-GPX) (D) in rainbow trout (Oncorhynchus mykiss) embryos and fry fed different diets: \( P \leq 0.05 \).](image_url)
additional retinoids that accompany the accumulation of lipid substances. In the present study, total lipid accounted for 12% of oocyte wet weight and 4% of 2-month fry wet weight and could not explain totally the observed difference between the present results with very low levels of retinal in oocytes (about 0.2% of total retinoids) and the results obtained on Chum salmon eggs by Irie & Seki. The high levels of retinol and retinyl palmitate in rainbow trout oocytes could ensure the early antioxidant protection by stabilising peroxyl radicals produced in the developing embryo as suggested by Palace & Werner.

Response of antioxidant defences to dietary oxidised lipid

Feeding rainbow trout fry with oxidised lipid resulted in decreased vitamin A larval contents and increased levels of lipid peroxidation products as well as increased antioxidant enzyme activity and gene expression. However, fry fed oxidised
lipid did not display any significant decrease of growth or survival compared with fry fed the control diet in agreement with what has been reported in juvenile rainbow trout(35,36), European sea bass(37), gilthead sea bream(38) and Atlantic halibut(39). In the last study, some skeletal malformations were noticed. Other malformations such as muscular dystrophy or ‘Sekoke’ disease have been reported in juvenile common carp and Siberean sturgeon larvae fed oxidised lipid(7,19). In the present study, no obvious malformation was evidenced whereas the same level and degree of oxidised lipid as in our previous study(19) with Siberean sturgeon larvae were tested. So, according to growth performance and response of antioxidant defences, rainbow trout fry fed oxidised lipid displayed a moderate oxidative stress. No deleterious effect was noted probably due to the clear response of the enzyme-dependent antioxidant defence system at the molecular level. Also Tocher et al. (40) showed in three marine species that the extent of peroxidative stress and deleterious effects appeared inversely proportional to the responses of hepatic antioxidant defence enzyme activities.

In the present study, among antioxidant enzymes, CAT displayed the most pronounced increase of activity after 2 months of feeding with dietary oxidised lipid. The increase in gene expression occurred earlier, after 1 month of feeding. The response of other antioxidant enzymes to dietary oxidised lipid was noticed at the molecular level with an increase in gene expression as soon as 11 d feeding for cytosolic Cu/Zn SOD and 2 months of feeding for GPX1. The gene expression analysis of the two SOD isoforms revealed that SOD1 was more sensitive to dietary oxidised lipid than SOD2. The mRNA levels of antioxidant enzymes constitute a valid biomarker of oxidative stress as suggested by Olsvik et al. (41).

Levels of lipid peroxidation products such as LSFp and 8-isoprostane positively correlated to antioxidant enzyme activities and gene expression and negatively correlated to retinoid levels. However, a high level of 8-isoprostane was found in oocytes compared with fry whereas no significant difference of LSFp was noted between oocytes and fry. This discrepancy between results could be due to an overestimation of isoprostanes in oocytes, which then masked significant differences between fry groups. Lipid peroxidation is difficult to evaluate and since none of the different analytical methods taken individually is ideal(2), the use of a combination of different measurements is preferable.

In conclusion, the present results suggest that rainbow trout fry antioxidant defence systems are active all through development with a predominance of antioxidant vitamins in the earliest stages and then a predominance of antioxidant enzymes. The antioxidant defence systems can be modulated by feeding oxidised lipid. The activation of antioxidant enzymes and the use of antioxidant vitamins such as vitamin A to eliminate free radicals in rainbow trout fry allow counteraction in part of the oxidative stress which can be then described as moderate. Dietary oxidised lipid resulted in increased levels of larval lipid peroxidation products leading to a decrease a flesh quality, but no depression of growth or survival or high occurrence of deformed fish was recorded.

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References

Antioxidant functions in rainbow trout fry


