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# Effects of dietary oxidized fish oil supplementation on oxidative stress and antioxidant defense system in juvenile rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The objective of the study was to characterize the response of the antioxidant defense system against dietary prooxidant conditions in rainbow trout juveniles. Fish (initial mean weight: 62 ± 1 g) were fed three fishmeal and plant-derived protein-based diets supplemented with 15% fresh fish oil (CTL diet), 15% fresh fish oil from tuna by-products (BYP diet) or 15% autooxidized fish oil (OX diet) over a 12-week growth trial at 17.5 ± 0.5 °C. No significant differences in growth performance were recorded between dietary groups. Muscle lipid content was reduced and n-6 PUFA levels were increased in rainbow trout fed diets BYP and OX compared to CTL. After 12 weeks of feeding, the level of lipid peroxidation products in muscle was not affected whereas the 8-iso-prostane content in liver was increased in fish fed diet OX as well as plasma total and oxidized glutathione contents. The hepatic and muscle contents for α-tocopherol were decreased in fish fed BYP and OX. Hepatic antioxidant enzyme activities and mRNA levels were not affected after 12 weeks of feeding, except for catalase and glutathione peroxidase 1b2 mRNA levels that were decreased in trout fed diet OX. Fish fed diet OX and BYP displayed also reduced cytosolic Nrf2 and both cytosolic and nuclear NF-κB protein levels in liver. The present work indicates that feeding rainbow trout juveniles with fresh fish oil from by-products or moderately oxidized lipid appears not to be detrimental to the growth performance of fish. The mechanisms beyond the control of the antioxidant defense system by moderately oxidized lipid require further investigations in rainbow trout juveniles.

## 1. Introduction

High concentrations of n-3 polyunsaturated fatty acids (PUFA), in particular docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), that have potential benefits for human health [1] are found in the tissues of fish [2]. In the absence of suitable antioxidant protection, due to the high degree of unsaturation, the long-chain n-3 PUFA are prone to in vivo lipid peroxidation within the fish tissues leading to pathologies, including muscular dystrophy [3]. In the feeds also, these fatty acids are readily susceptible to autoxidation when exposed to atmospheric oxygen and if adequate care is not taken in the preparation and storage of feeds, the positive nutritional value of long-chain n-3 PUFA derived from fish oils and meals can become a negative factor for fish [4].

To limit lipid peroxidation, all aerobic organisms possess two types of antioxidant defense system: free radical scavengers, generally of low molecular weight, and antioxidant enzymes [5]. In rainbow trout, antioxidant enzymes have been shown to be modulated by feeding moderate or high levels of oxidized lipid [6,7]. At a molecular level, some of the genes coding for antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST), contain binding sites for redox-sensitive transcription factors, such as the nuclear factor erythroid-2 p45-related factor-2 (Nrf2) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), in their regulatory region [8,9]. Under conditions of oxidative stress, Nrf2 and NF-κB dissociate from their cytosolic inhibitors, Kelch-like ECH-associated protein 1 (Keap1) and NF-κB inhibitor α (IκBα), and translocate to the nucleus, where they modulate the expression of genes involved in antioxidant defense and inflammatory processes [10–12].

The objective of the study was hence to characterize the response of the antioxidant defense system against dietary prooxidant conditions in rainbow trout juveniles.

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## 2. Materials and methods

### 2.1. Experimental fish and dietary trial conditions

The feeding trial was carried out in the INRA experimental fish farm at Donzacq (Landes, France) supplied with flow through spring water at 17.5 ± 0.5 °C. Rainbow trout (*Oncorhynchus mykiss*) juveniles from the same parental stock with a mean body weight of 62 ± 0.6 g were randomly allocated to 150-L fiberglass tanks with 40 fish/tank and acclimatized to the rearing conditions two weeks prior to the start of the experiment. Fish were hand fed twice a day to visual satiation. Each diet was distributed to three replicate groups of fish over a 12-week growth trial. Fish from each tank were bulk weighed every three weeks. Feed was withheld for 16 h before every weighing and sampling. At the start, after 3 weeks and at the end of the 12-week growth trial, 3 fish from each replicate were withdrawn, anaesthetized (benzocaine, 30 mg/L) and blood samples were collected from the caudal vein into heparinized syringes. Fish were euthanized subsequently by a sharp blow to the head and liver and fillet were dissected and weighed for calculating hepato-somatic indice (HSI). Tissues were immediately frozen in liquid nitrogen and stored at −80 °C before analysis. Plasma was recovered from centrifuged (3000 × g for 5 min) blood samples, immediately frozen and stored at −80 °C before analysis. All experimental procedures complied with the European Directive 010/63/EU for the protection of animals used for scientific purposes, and the French Decree no. 2013–118 for animal experimentation.

### 2.2. Experimental diets

Three practical diets based on fishmeal and plant-derived proteins were formulated to contain 49% crude protein and 23% crude lipid with different levels of lipid peroxidation (Table 1). Diet CTL used as control diet contained fresh capelin oil stabilized with 300 mg ethoxyquin/kg. Diet BYP was supplemented with crude tuna oil manufactured exclusively from cooked and raw tuna by-products and stabilized with Barox™ liquid containing butylated hydroxytoluene, butylated hydroxyanisole and ethoxyquin. Diet OX was supplemented with oxidized lipid obtained by bubbling air through crude tuna oil for 7 days at 50 °C with two air stone diffusers. The oxidative state of fish oils was monitored before incorporation into the diets (Table 2). The levels of vitamin E ranged from 161 in diets CTL and BYP to 352 mg D-α-tocopherol equivalents per kg in diet OX (Table 2) or 240 and 524 IU/kg, respectively, thus meeting the vitamin E requirements of rainbow trout as reported by the NRC [14]. Experimental diets were manufactured using a twin-screw extruder (Clextral BC45, Firminy, France) at the INRA experimental facilities in Donzacq just before the feeding trial and stored at 0 °C until each 3-week feeding period.

### 2.3. Determination of proximate and fatty acid composition

Proximate composition of diets was determined according to following procedures: dry matter 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 10 h and gross energy in an adiabatic bomb calorimeter. Total lipid was extracted and measured gravimetrically according to Folch et al. [15] using dichloromethane instead of chloroform. Total lipid was separated into neutral and polar fractions using silica cartridges (Waters, Saint-Quentin-en-Yvelines, France) according to Juaneda and Rocquelin [16]. Fatty acid methyl esters were prepared and analyzed as previously described [6].

### 2.4. Determination of lipid peroxidation, glutathione and vitamin E levels

Peroxide value (PV) was assessed by colorimetric determination of iron-thiocyanate according to Shantha and Decker [17]. Conjugated dienes (E232) and trienes (E268) were measured as specific extinctions at the wavelengths of 232 and 268 nm, respectively [18]. Anisidine value (AV) was determined according to standard procedures [19]. Total carbonyl compounds were determined according to Endo et al. [20] at 420 nm using 2,4-decadienal as standard. Lipid-soluble fluorescent products (LSFP), thiobarbituric acid-reactive substances (TBARs) and 8-isoprostanes (8-isoPT) produced from non-enzymatic peroxidation of arachidonic acid by reactive oxygen species were analyzed as previously described [7]. Oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured in plasma and liver using Cayman glutathione assay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturer's instructions with protein concentration assessed by the method of Lowry et al. [21] using bovine serum albumin as a standard. Tocopherols were extracted according to Folch et al. [15] using dichloromethane instead of chloroform and analyzed by HPLC on a Nova-Pak Silica column (60 Å, 4 µm, 3.9 × 150 mm) with an isocratic mobile phase of hexane-ethyl acetate (98:2 v/v) at a flow rate of 1 mL/min using a Waters Alliance 2695 separation module with a Waters 2487 dual λ absorbance detector set at 292 nm and a Waters 2475 multi wavelength fluorescence detector set at 295/330 nm (excitation/emission).

**Table 1**
Formulation and composition of the experimental diets.

| Diet | CTL | BYP | OX |
|---|---|---|---|
| *Ingredients (%)* | | | |
| Norwegian herring meal[a] | 32 | 32 | 32 |
| Wheat gluten[b] | 10 | 10 | 10 |
| Corn gluten meal[c] | 9 | 9 | 9 |
| Soybean protein concentrate[a] | 8.5 | 8.5 | 8.5 |
| Soybean meal[d] | 2.5 | 2.5 | 2.5 |
| White lupin meal[e] | 2.5 | 2.5 | 2.5 |
| Rapeseed meal 00[d] | 2 | 2 | 2 |
| Dehulled pea meal[f] | 1.5 | 1.5 | 1.5 |
| Whole wheat[d] | 14 | 14 | 14 |
| Soybean lecithin[g] | 1 | 1 | 1 |
| Vitamin premix[h] | 1 | 1 | 1 |
| Mineral premix[i] | 1 | 1 | 1 |
| Fresh fish oil[a] | 15 | – | – |
| Fresh fish oil from tuna by-products[j] | – | 15 | – |
| Oxidized fish oil[j] | – | – | 15 |
| *Proximate composition* | | | |
| Dry matter (DM, %) | 97 | 97 | 97 |
| Crude protein (% DM) | 48 | 49 | 50 |
| Total lipid (% DM) | 23 | 23 | 22 |
| Gross energy (kJ/g DM) | 24 | 24 | 24 |
| Ash | 7 | 7 | 7 |

[a] Norse LT94, crude fish oil and Estrilvo from Sopropêche (Wimille, France).
[b] Supplied by Roquette (Lestrem, France).
[c] Supplied by Inzo (Argentan, France).
[d] Supplied by Sud-Ouest Aliment (Haut-Mauco, France).
[e] Farilup 500 from Terrena (Martigné-Ferchaud, France).
[f] Primatex from Sotexpro (Berméricourt, France).
[g] Supplied by Louis François (Croissy-Beaubourg, France).
[h] Vitamin premix (IU or g/kg premix): retinyl acetate, 500,000 IU; cholecalciferol, 250,000 IU; DL-α-tocopheryl acetate, 5000 IU; sodium menadione bisulfate, 1 g; thiamin-HCl, 0.1 g; riboflavin, 0.4 g; niacin, 1 g; D-calcium pantothenate, 2 g; pyridoxine-HCl, 0.3 g; D-biotin, 20 mg; folic acid, 0.1 g; cyanocobalamin, 1 mg; L-ascorbyl-2-polyphosphate, 5 g; *myo*-inositol, 30 g; choline, 100 g. All ingredients were diluted with α-cellulose.
[i] Mineral mixture (g/kg premix): $CaHPO_4{\cdot}2H_2O$, 500; $CaCO_3$, 215; $Mg(OH)_2$, 124; KCl, 90; NaCl, 40; $FeSO_4{\cdot}7H_2O$, 20; $ZnSO_4{\cdot}7H_2O$, 4; $MnSO_4{\cdot}H_2O$, 3; $CuSO_4{\cdot}5H_2O$, 3; NaF, 10; KI, 0.04; $Na_2SeO_3$, 0.03; $CoCl_2{\cdot}6H_2O$, 0.02. All ingredients were diluted with α-cellulose.
[j] Supplied by Marine Biotechnology Products (Port Louis, Mauritius).

### 2.5. Determination of antioxidant enzyme activities and gene expressions

SOD (EC1.15.1.1), CAT (EC1.11.1.6), total and selenium-dependent GPX (EC 1.11.1.9), GR (EC1.6.4.2) and GST (EC2.5.1.18) activities were assayed in liver as previously described with a minimum of two