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Blood antioxidant defenses and hematological adjustments in crowded/uncrowded rainbow trout (*Oncorhynchus mykiss*) fed on diets with different levels of antioxidant vitamins and HUFA

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ABSTRACT

Rainbow trout maintained at crowding or noncrowding conditions were fed on five experimental diets that were formulated considering two levels of vitamin E (25.6 and 275.6 mg/kg diet), vitamin C (0 and 1000 mg/kg diet) and HUFA (12.5 and 30.5 g/kg diet): −E−HUFA, −E+HUFA, +E−HUFA, +E+HUFA, −C+E+HUFA. Hematological parameters, the activity of some antioxidant enzymes and lipid peroxidation from RBC were evaluated. The SOD isoenzyme pattern was analyzed by nondenaturing PAGE. Hematological response to crowding stress was manifested by increased hemoglobin and RBC count in most of the crowded groups. Antioxidant enzyme activity was clearly affected by dietary HUFA levels, with uncrowded fish fed on +HUFA diets showing a higher SOD activity compared to those fed on −HUFA diets. In uncrowded groups, only one CuZn-SOD isozyme was detected, whereas in the crowded fish a great variability was revealed with up to five isozymes. G6PDH activity was increased in uncrowded −E+HUFA fish compared to the remaining groups. Lipid peroxidation was significantly increased in −E+HUFA fish regardless of fish density. Data supported the negative correlation of lipid peroxidation and hematocrit or hemoglobin explained by decreased erythrocyte stability. Dietary imbalances in vitamin E and HUFA supplementation may promote oxidative stress which triggers hematological deterioration, which in turn would affect the whole hematological status and ultimately fish welfare.

1. Introduction

The aerobic condition of fish makes them prone to the generation of reactive oxygen species (ROS) as a result of an exacerbated oxidative metabolism. Under normal circumstances, the antioxidant defenses of fish prevent the uncrontrolled generation of ROS through enzymes such as: superoxide dismutase (SOD), which catalyzes the O_2^- dismutation to H_2O_2; catalase, involved in H_2O_2 reduction to H_2O and O_2; and glutathione peroxidase (GPX), which detoxifies H_2O_2 and organic peroxides by converting reduced glutathione (GSH) to its oxidized form (GSSG). Glucose-6-phospate dehydrogenase (G6PDH) is the first enzyme of the pentose phosphate pathway involved in the NADPH regeneration, a molecule that plays a crucial role in the regeneration of reduced glutathione. Also, there are non-enzymatic molecules such as vitamins E and C involved in the antioxidant defense system thus preventing the reaction of reactive oxygen species with large biomolecules and cell structures. When the ROS-generation rate exceeds that of their removal, oxidative stress occurs, leading to the oxidation of proteins and DNA, as well as the peroxidation of unsaturated lipids from cell membranes (Chaudière and Ferrari-Iliou, 1999; Davies, 2000; Morales et al., 2004; Martínez-Álvarez et al., 2005; Halliwell and Gutteridge, 2007).

Erythrocytes are constantly exposed to ROS because of their role transporting oxygen in blood via its reversible union to hemoglobin (Saltman, 1989). In general, approximately 3% of oxyhemoglobin undergoes autooxidation, thus generating the superoxide radical (O_2^-; Nagababu and Rifkin, 2000). Highly unsaturated fatty acids (HUFA), greatly prone to oxidation, are essential components of fish tissues playing a central role in cell-membrane function. It has been reported that erythrocyte stability is affected by lipid peroxidation under circumstances where ROS generation led to oxidative damages (Ito et al., 1999; Nagasaka et al., 2004).

Fish antioxidant defenses depend, in part, on dietary supply of essential antioxidants, such as vitamins E and C. Vitamin E (α-tocopherol) is the main soluble lipid antioxidant that is crucial in protecting cell membranes from oxidation. This vitamin can scavenge free radicals such as lipid peroxyl radicals and acts as a chain breaker (Hamre et al., 1994; Chaudière and Ferrari-Iliou, 1999). This explains the direct relationship between the dietary levels of HUFA and vitamin E requirements (Sargent et al., 1989; Udilova et al., 2003; Martínez-Álvarez et al., 2005). In this sense, in rainbow trout it has been

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reported that vitamin E deficiencies associated to increased HUFA levels in the diet may affect red-blood-cell stability (Puangkaew et al., 2005). Vitamin E requirements depend not only on dietary lipids but also on its interaction with vitamin C (Olsen et al., 1999). Vitamin C (ascorbic acid) is able to regenerate vitamin E from its oxidized form what renders a synergistic effect of both vitamins in the protection of water and lipid phases respectively against oxidation (Hamre et al., 1997; Chaudière and Ferrari-Iliou, 1999). Recent studies have reported a beneficial role of dietary vitamin C supplementation improving blood oxidative status although no synergistic effect of vitamin E supplementation was manifested (Andrade et al., 2007; Menezes et al., 2006).

ROS generation in fish may be influenced by variations in the concentration of dissolved oxygen of the aquatic environment (Ross et al., 2001), water salinity (Kolayli and Keha, 1999; Martínez-Álvarez et al., 2002), developmental stage (Rudneva, 1999), nutritional challenges (Kiron et al., 2004; Morales et al., 2004; Puangkaew et al., 2005), and the presence of physical stress or xenobiotics (Lindström-Seppä et al., 1996; Tort et al., 1996), as deduced from the changes observed in the activity of antioxidant enzymes. Also, changes in the isoenzymatic pattern of antioxidant enzymes such as SOD have been reported (Pedrajas et al., 1995, 1998). A rise in the energy demands, imposed by crowding, is offset by metabolic adjustments (van der Boon et al., 1991; Wedemeyer, 1996; Vijayan et al., 1990), and this may enhance the generation of ROS. On the other hand, it has been reported that crowding induces a hematological response to compensate for such increased oxygen demands (Barcellos et al., 2004, Trenzado et al., 2006). This response might be compromised if HUFA present in the erythrocyte membrane are not protected from oxidation.

Furthermore, vitamins E and C and HUFA, besides their antioxidant role, are credited with modulating the stress response in fish (Merchie et al., 1997; Montero et al., 2001, 2003; Kolkovski et al., 2000; Ortuño et al., 2003). It has been reported that fish fed vitamin E-deficient diets manifested lower stress resistance, a fast cortisol elevation, hematological and immunological alterations and decreased growth and survival (Montero et al., 2001; Chen et al., 2004; Belo et al., 2005). A possible relation between dietary ascorbate and the physiological response to hypoxia would have been established, since fish fed on a non-supplemented vitamin C diet showed higher plasma glucose levels and a tendency to have wider plasma cortisol variations than fish fed on the supplemented diets (Henrique et al., 1998). Chen et al. (2004) reported the modulatory effect of vitamins E and C preventing a stress-related hematological response and immunosuppression of fish which suggests that both vitamins would be involved in the hypothalamic-sympathetic-chromaffin cell axis and therefore interfering in the stress response (Ortuño et al., 2003).

In the present study, blood parameters were evaluated in fish under crowding stress, considering the possible influence of HUFA and antioxidant vitamin levels present in diet. The purpose of this investigation was to evaluate whether changes in antioxidant enzymes and tissue lipid peroxidation may be indicative of the blood oxidative status of fish and therefore if fish welfare should be improved by diet composition.

2. Materials and methods

2.1. Experimental diets

Five experimental diets (−E−HUFA, −E+HUFA, +E−HUFA, +E+HUFA, −C+E+HUFA) were prepared in the laboratory according to the nutritional requirements of rainbow trout. Diets were formulated considering two levels of vitamin E (25.6 and 275.6 mg/kg diet), vitamin C (0 and 1000 mg/kg diet), and HUFA (12.5 and 30.5 g/kg diet). Fish oil or soybean oil were used as lipid sources for +HUFA and −HUFA diets respectively. Since the vitamin E content from soybean oil was higher than that from fish oil, the vitamin E level present in the −E −HUFA diet (25.6 mg/kg diet) was used as the reference to adjust all the other diets which were supplied with a fixed amount of vitamin E. Additionally, 250 mg vitamin E/kg diet was added to +E diets (see Table 1). The fatty-acid profile was analyzed in both fish and soybean oil and in the lipid fraction of diets after transesterification with METH-PREP-II (Alltech, USA). Fatty-acid methyl esters were identified by gas chromatography (CARLO ERBA 8000, mod. 8060, Thermo Quest Instruments) equipped with a HP (Hewlett Packard)-5MS capillary column (25 m length×0.25 mm internal diameter×0.25 μm film thickness) with split 1:75 and coupled to a Platform II mass spectrometer detector (Micromass Instruments, Manchester, UK). Individual fatty acids were identified by search in the Wiley (6th edition) and NIST/NBS mass spectra library and represented as a percentage area of the total lipids. Vitamins E and C were analyzed in fish meal, lipid sources and diets by reverse-phase high-performance liquid chromatography (HPLC, Waters 600) according to Trenzado et al. (2007).

Proximate composition of diets was assayed according to AOAC methods (2000) for dry matter (105 °C until achieving constant weight), ash by combustion in a muffle furnace (500 °C for 16 h), crude protein (N×6.25) after acid digestion by the Kjeldahl method, and lipids through constant diethyl ether extraction by the Soxhlet method. The diet composition and proximate analyses and HUFA content in experimental diets are reported in Tables 1 and 2, respectively.

Fish meal was obtained from ALIMAR S.A. (Chile). The remaining dietary components were obtained from MUSAL & CHEMICAL S.A (Spain), Panreac Química S.A (Spain), Roche Diagnostics S.A. Biochemicals (Mannheim, Germany).

2.2. Experimental design

The fish (rainbow trout, *Oncorhynchus mykiss*, initial mean mass± SEM: 53.14±5.20 g) from a local farm (Loja, Granada), were

Table 1
Composition and proximate values of the experimental diets

	Experimental diets				
	−E−HUFA	−E+HUFA	+E−HUFA	+E+HUFA	−C+E+HUFA
Ingredients (g/100 g diet)					
Fish meal	60	60	60	60	60
Fish oil	–	10	–	10	10
Soybean oil	10	–	10	–	–
Pregelatinized starch	15	15	15	15	15
Dextrin	5	5	5	5	5
Mineral mixture[a]	5	5	5	5	5
Vitamin mixture[b]	2	2	2	2	2
Carboxymethylcellulose	1	1	1	1	1
Betaine	1	1	1	1	1
Cellulose	1	1	1	1	1
Antioxidant vitamins content (mg/kg diet)					
Vitamin E[c]	24.5	23.3	270.8	264.1	269.5
Vitamin C[d]	996.2	975.4	978.2	959.1	0.0
Proximate composition					
Moisture (%)	10.48	10.93	8.29	11.98	11.37
Protein (% dm)	50.18	50.10	49.41	49.81	49.18
Lipid (% dm)	18.39	17.02	16.79	17.57	18.56
Ash (% dm)	12.25	12.64	11.92	11.65	12.69
NFE[e] (% dm)	19.18	20.24	21.88	20.97	19.57

[a] Mineral mixture (g/kg diet) according to De la Higuera et al. (1988): Ca $(PO_4H_2)_2{\cdot}H_2O$ (30), $CaCO_3$ (6.5), KCl (2.5), NaCl (4), $MnSO_4{\cdot}H_2O$ (0.2), $FeSO_4{\cdot}7H_2O$ (1.5), $MgSO_4$ (4.6), KI (0.02), $CuSO_4{\cdot}5H_2O$ (0.05), $ZnSO_4{\cdot}7H_2O$ (0.2), $CoSO_4{\cdot}7H_2O$ (0.05), Na_2SeO_3 ($0.218.10^{-2}$), $Al_2(SO_4)_3{\cdot}18H_2O$ (1.10^{-2}).

[b] Vitamin mixture (mg/kg diet): thiamine (40), riboflavin (60), pyridoxine (30), pantothenic acid (150), niacin (250), folic acid (15), inositol (1000), choline (5000), biotin (3), cyanocobalamin (0.05), vitamin A (1), vitamin D (0.6), menadione (25).

[c] Vitamin E: 250 mg/kg diet was added to +E diets. Since vitamin E content of soybean oil was higher than in fish oil, 8.6 mg/kg diet of this vitamin was added to +HUFA diets.

[d] Vitamin C: except in −C diet, 1000 mg/kg diet was added.

[e] NFE: Nitrogen-free extract, calculated by difference. dm: dry matter.