Effects of dietary polyunsaturated fatty acid/vitamin E (PUFA/tocopherol) ratio on antioxidant defence mechanisms of juvenile gilthead sea bream (*Sparus aurata* L., Osteichthyes, Sparidae)

G. Mourente[1]*, E. Díaz-Salvago[1], D. R. Tocher[2] and J. G. Bell[2]

*[1] Departamento de Biología Animal, Biología Vegetal y Ecología, Facultad de Ciencias del Mar, Universidad de Cádiz, Polígono Rio San Pedro, Apartado 40, ES-11510 Puerto Real (Cádiz), Andalucía, España; [2] Nutrition Group, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK; *Author for correspondence (Tel: +34956016013 Fax: +34956016019; E-mail:Gabriel.Mourente@uca.es)*

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Abbreviations: AA, all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4n-6); CAT, catalase; CDNB, chlorodinitrobenzene; DHA, all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (22:6n-3); EPA, all-*cis*-5,8,11,14,17-eicosapentaenoic acid (20:5n-3); GPX, glutathione peroxidase Se dependent; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidized glutathione; GST, glutatione-S-transferase; HUFA, highly unsaturated fatty acids ($\geq C_{20}$ and with ≥ 3 double bonds); 8-isoprostane, 8-*iso*-prostaglandin $F_{2\alpha}$; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances.

Abstract

Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation is highly deleterious, resulting in damage to cellular biomembranes, and may be a principal cause of several diseases in fish including jaundice and nutritional muscular dystrophy. Tissue lipid PUFA content and composition are critical factors in lipid peroxidation, as is the level of endogenous antioxidant molecules such as vitamin E. The primary objective of the present study was the characterization of antioxidant systems in a cultured juvenile marine fish, gilthead sea bream (*Sparus aurata*) with the underlying aim to understand how to avoid oxidation problems that may cause pathologies and disease and so to enhance growth and quality of early ongrowing stages. Juvenile sea bream were fed diets having either high or low levels of fish oil and supplemented or basal levels of vitamin E with PUFA/vitamin E ratios ranging from 117 ± 12 in the diet with low PUFA supplemented with vitamin E to 745 ± 48 in the diet with high PUFA with no additional vitamin E. None of the diets had serious deliterious effects on growth or survival of the fish, but the different dietary regimes were effective in significantly altering the PUFA/vitamin E ratios in the fish livers with values ranging from 5.7 ± 0.4 in fish fed the diet with low PUFA supplemented with vitamin E to 91.1 ± 13.2 in fish fed the diet with high PUFA with no additional vitamin E. This had effects on the peroxidation status of the fish as indicated by the significantly altered levels of in vivo lipid peroxidation products measured in liver, with fish fed the diet rich in PUFA and low in vitamin E showing significantly higher values of thiobarbituric acid reactive substances (TBARS) and isoprostanes. The isoprostane levels generally followed the same pattern as the TBARS levels supporting its value as an indicator of in vivo oxidative stress in fish, as it is in mammals. However, few significant effects on antioxidant enzyme activities were observed suggesting that more severe conditions may be required to affect these activities such as increasing the PUFA/vitamin E ratio or by increasing peroxidative stress through the feeding of oxidized oils.

Introduction

Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation is acknowledged as being highly deleterious, resulting in damage to cellular biomembranes, particularly to those of subcellular organelles, which contain relatively large amounts of PUFA (Halliwell and Gutteridge, 1996). Tissue lipid PUFA content and unsaturation index are critical factors in lipid peroxidation, and as fish, particularly marine fish, tissues contain large quantities of n-3 highly unsaturated fatty acids (HUFA) (Sargent et al. 1999), they may be more at risk from peroxidative attack than are mammals. However, marine fish are unable to synthesise HUFA due to a relative deficiency in the $\Delta 5$ fatty acyl desaturase and/or the C_{18-20} elongase enzyme activities necessary for the desaturation and elongation of dietary PUFA and so marine fish must obtain preformed HUFA in their diet (Sargent et al. 1999). Therefore, although HUFA are essential for optimal growth and development of marine fish, they also impose a significant peroxidation burden. In fish, *in vivo* lipid peroxidation caused by oxygen radicals is a principal cause of several diseases such as jaundice (Sakai et al. 1989), nutritional muscular dystrophy (Watanabe et al. 1970; Murai and Andrews, 1974) and haemolysis (Kawatsu 1969).

To maintain health and prevent oxidation-induced lesions and mortalities, there must be effective antioxidant systems operating in fish. The components of these systems involve antioxidant compounds such as NADH/NADPH, glutathione (GSH), protein sulphydryl (-SH) groups and uric acid, and dietary micronutrients such as vitamins E and C, and carotenoids. Other components of the antioxidant defence system include enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) and associated enzymes such as glutathione-S-transferase (GST) and glutathione reductase (GR) (Winston and Di Giulio 1991; Halliwell and Gutteridge 1996). The basic biochemistry of these enzyme systems is well documented. CAT and SOD are scavengers of active oxygen species, acting on hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\cdot}$), respectively (Miller et al. 1993). GPX also scavanges H_2O_2 as well as lipid hydroperoxides, which leads to the production of oxidised glutathione (GSSG). GR acts to maintain levels of reduced glutathione (GSH) via the reduction of GSSG at the expense of NADH (Winston and Di Giulio 1991; Halliwell and Gutteridge 1996). Some isoenzymes of GST can also metabolize lipid hydroperoxides although this activity has not been confirmed in fish (Halliwell and Gutteridge 1996). Research into oxidative stress and antioxidant systems in marine fish is increasing (Stéphan et al. 1995; Merchie et al. 1996; Nunes et al. 1996; Bai and Lee 1998; Henrique et al. 1998). However, despite its obvious importance, characterization of the activities of the antioxidant defence enzymes are only rarely included in these studies (Murata et al. 1996; Peters and Livingstone 1996; Mourente et al. 1999a,b). Even the requirements for dietary antioxidants such as

vitamin E, vitamin C and selenium are poorly studied in marine fish (De Silva and Anderson 1995).

The primary objective of the present study was the characterization of antioxidant systems in a cultured juvenile marine fish, gilthead sea bream (*Sparus aurata*), of commercial importance in Europe. The underlying aim is to understand how to avoid oxidation problems that may cause pathologies and disease and so to enhance growth and quality of life during early ongrowing stages in marine fish. In consequence, an experiment was designed to test the capacity of preventing oxidative stress in this species. Juvenile sea bream were fed diets having a high unsaturation index due to n-3HUFA combined with the presence and absence of the principle lipid-soluble antioxidant in vivo, vitamin E, in comparison with diets having a low unsaturation index achieved by substituting n-3HUFA with oleic acid, 18:1n-9, and supplemented or unsupplemented with vitamin E. This design resulted in four diets displaying graded PUFA/vitamin E ratios ranging from 117 ± 12 in the diet with low PUFA supplemented with vitamin E to 745 ± 48 in the diet with high PUFA with no additional vitamin E. The pattern of activities for the antioxidant defence enzymes under these experimental conditions could be postulated. Thus, the activities of SOD, CAT and GPX could be expected to increase with inreasing PUFA/vitamin E ratio in the liver as a result of increased fatty acid peroxidation and increased production of radicals. Similarly, GR activity may be expected to increase with increasing PUFA/vitamin E ratio as a result of increased production of GSSG from the increased activity of GPX. GST may also be increased, especially in diets with low vitamin E, as it is thought to form GSH conjugates with peroxy radicals (Miller et al. 1993).

This study also presented a novel approach in the study of lipid peroxidation products in marine fish. In addition to the measurement of liver thiobarbituric acid reactive substances (TBARS), we determined the levels of isoprostanes, prostaglandin-like compounds that are produced non-enzymatically by free radical catalyzed peroxidation of PUFA (Roberts and Morrow 1997; Morrow and Roberts 1997).

Materials and methods

Experimental design: fish and diets

The experiment was performed during early spring 1999. Fish from the same batch, 70 days post-hatch, completely weaned, with a functional swimbladder, and a live mass of 300-500 mg/fish were purchased from CUPIMAR S. A. (Cadiz, Andalucia, Spain). Fish were allocated randomly in rectangular tanks of 100 l each provided with central drainage, in an open system continuously supplied with running borehole water of 39 ppt salinity at a temperature of 19.4 ± 0.2ºC. The water

had been previously treated with biological filters to eliminate ammonia, by nitrification processes, to sea water quality criteria (1 μg/l NH_3-N maximum). Oxygen was supplied by aeration with the minimum level observed during trials being 5.6 mg/l or 77.8% saturation. Water renewal was set at 10 times total volume per day (0.7 $l.min^{-1}$). Light was natural photoperiod conditions. Water quality (NH_3/NH_4^+, NO_2^-, NO_3^-) variables in the rearing tanks were determined with a Technicon Traacs 800 Autoanalyser. Water samples were filtered through a 0.45 μm membrane prior to analysis and these variables were measured weekly.

After acclimatization of fish to the experimental diet and conditions for two weeks, the fish were stocked at an initial density of 5 fish/l. This represented an initial stocking density of 2.5 kg · m^3 with the final stocking densities varying between 7.8 and 9.6 kg · m^3 depending on the survival of the experimental treatment. The ration varied from 4% to 3% of the biomass/day between the beginning and end of the experiment and was offered to fish 6 times during the light hours by hand. The length of the experiments was established at 550 degree.days or around 30 days for gilthead sea bream at a rearing temperature of approximately 19ºC.

The experimental diets were based on a modified commercial extruded formulation utilizing fishmeal as protein source (Table 1) and having a proximate composition as shown in Table 2. Mineral and vitamin premixes, vitamin E-stripped oils and vitamin E (tocopheryl acetate) were prepared and supplied by the Lipid Nutrition Group, Institute of Aquaculture, University of Stirling and the diets were manufactured by a commercial feed producer (Ewos Ltd., Livingston, Scotland) (Table 1). Four diets of two pellet sizes (500 and 1500 μm, respectively) were produced with either high fish oil or low fish oil (oil level maintained with oleic acid, Fisher Scientific, Loughborough, England) each containing either no added vitamin E or vitamin E added to 200 mg/Kg. A further diet, a commercially-produced starter for marine fish used previously with gilthead sea bream, was also included as a control (Trouw Aquaculture, Spain).

Sample collection and biometric determinations

There were two sampling points, at the beginning (day 0) and at the end of the experiment (day 30). The fish were sampled after 24 h starvation to avoid interference of gut contents in the analysis. All measurements and determinations were performed in triplicate. Length was measured, and live mass/dry mass ratios determined for both whole fish and liver. Live masses were determined by blotting fish and liver on filter paper before weighing, and dry mass was determined after heating in an oven at 60ºC for 24 h and cooling in vacuo before weighing. Hepatosomatic index (HSI) was calculated and growth assessed by measuring the specific growth rate (SGR) as %weight gain·day^{-1} (Wootten, 1990). Mortality was measured at the end of the experiment and

expressed as % values.

Three samples of liver per treatment were collected for lipid and fatty acid analysis, vitamin E content, peroxidation (lipid oxidation products, TBARS and isoprostane) status and antioxidant enzyme activities. Each sample consisted of the pooled livers from ten fish to provide enough material for all the required analyses. The diets and dissected livers were immediately frozen in liquid nitrogen and stored at –70ºC before analysis.

Gross composition, total lipid extraction, lipid class separation and quantification

Protein content was determined by the Folin-phenol reagent method, according to Lowry et al. (1951). Total lipid contents were determined gravimetrically after extraction as described below. Carbohydrate contents were determined by a colorimetric method using the phenol-sulphuric acid reagent (Dubois et al., 1956). Ash contents were measured gravimetrically after total combustion in a furnace at 550ºC.

Total lipid was extracted after homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) on silica gel 60 plates, using the single-dimension double-development method described previously (Tocher and Harvie, 1988; Olsen and Henderson, 1989). The classes were quantified by charring (Fewster et al., 1969) followed by calibrated densitometry using a Shimadzu CS-9001PC dual-wavelength flying spot scanner (Olsen and Henderson, 1989).

Total lipid fatty acid analyses

Fatty acid methyl esters (FAME) from total lipids were prepared by acid-catalyzed transmethylation for 16 h at 50ºC, using tricosanoic acid (23:0) as internal standard (Christie, 1989). FAME were extracted and purified as described previously (Tocher and Harvie, 1988) and were separated in a Hewlett-Packard 5890A Series II gas chromatograph equipped with a chemically bonded (PEG) Supelcowax-10 fused silica wall coated capillary column (30 m x 0.32 mm i. d., Supelco Inc., Bellefonte, USA), "on column" injection system and flame ionization detection. Hydrogen was used as the carrier gas with an oven thermal gradient from an initial 50ºC to 180ºC at 25ºC/min and then to a final temperature of 235ºC at 3ºC/min with the final temperature maintained for 10 min. Individual FAME were identified by comparison with known standards and quantified by means of a direct-linked PC and Hewlett-Packard ChemStation software.

Measurements of thiobarbituric acid reactive substances (TBARS)

The measurement of TBARS was carried out using a method adapted from that used by Burk et al. (1980). Up to 20-30 mg of tissue per sample was homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this was added 2.95 ml of freshly prepared 50 mM thiobarbituric acid solution. The reagents were mixed in a stoppered test tube and heated at 100º C for 10 min. After cooling the tubes and removing protein precipitates by centrifugation at 2000 x g, the supernatant was read in an spectrophotometer at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBARS expressed as nmol TBARS/g of tissue, was calculated using the extinction coefficient 0.156 $\mu M^{-1}cm^{-1}$.

Determination of 8-isoprostane levels

The levels of 8-isoprostane, a novel lipid peroxidation product formed non-enzymatically, and thus a potentially good indicator of lipid peroxidation in tissue, were determined by enzyme immunoassay (EIA). Isoprostanes are determined in the same homogenates of liver and whole fish that are prepared for TBARS analyses. Samples should be assayed immediately after collection or, as in this case, stored at –80ºC, as they can also appear in samples as an artifact of prolonged storage at temperatures above –80ºC. Most of the 8-isoprostane will be esterified in lipids, so an extraction and hydrolysis must be performed in order to determine total amounts of 8-isoprostane. Briefly, 2ml ethanol was added to 1.5 ml of sample, mixed, and allowed to stand for 5 minutes at 4^{o}C before precipitated protein was removed by centrifugation. The supernatant was decanted into a clean test tube and 3.5ml 15% KOH added and incubated for 60 minutes at 40^{o}C. The solution was diluted to 10ml with ultrapure water and the pH lowered to below 4.0 with 2 ml concentrated formic acid. Isoprostanes were purified by applying the solution to a C_{18} reverse-phase mini-column ("Sep-Pak", Millipore UK, Watford, UK) after rinsing with 5ml methanol followed by 5ml ultrapure water and 5 ml HPLC grade hexane. Isoprostanes were eluted with 5ml ethyl acetate containing 1% methanol, solvent evaporated under a stream of nitrogen and 1ml EIA kit buffer added. Recovery of prostaglandins and prostaglandin-like compounds after extraction by this method was reported as being 95-100% (Powell, 1982). Total isoprostane is quantified using an EIA kit and 8-isoprostane standard as per manufacturers instructions (Cayman Chemical Co., Ann Arbor, USA).

Determination of vitamin E content

Vitamin E concentrations (as tocopherol plus α-tocopheryl esters) were measured in tissue samples using high-performance liquid chromatography (HPLC). Samples were weighed, homogenized and saponified as described by Bieri (1969), but using a single-step hexane extraction (Bell et al., 1987). Recovery of α-tocopherol using this method was reported as 100.5% ± 1.3. (Huo et al., 1996). HPLC analysis was performed using a 250 x 2 mm reverse phase Spherisorb ODS2 column (Sigma Chemical Co, St. Louis MO, USA) essentially as described by Carpenter (1979). The isocratic mobile phase was 98% methanol pumped at 0.2 ml/min, the effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (±)-α-tocopherol (Sigma Chemical CO, St. Louis, MO, USA) as external standard (10 μg/ml).

Determination of enzyme activities in liver homogenates.

Samples of liver were homogenized in 9 volumes of 20 mM phosphate buffer pH 7.4, 1 mM EDTA and 0.1% Triton X-100, the homogenates were centrifuged at 600 x g, to remove debris, and the resultant supernatants used directly for enzyme assays. Catalase (CAT) activity was measured by following the reduction of hydrogen peroxide at 240 nm using the extinction coefficient 0.04 mM^{-1} cm^{-1} (Beers and Sizer, 1952). Immediately before assay, 50 ml of 67 mM potassium phosphate buffer pH 7.0 was mixed with 80 μl of 30% (v/v) hydrogen peroxide. The assay cuvette (quartz) contained 3.0 ml of above buffered hydrogen peroxide solution plus 25 μl of sample.

Total superoxide dismutase (SOD) activity was assayed by measuring the inhibition of the oxygen-dependent oxidation of adrenalin (epinephrine) to adenochrome by xanthine oxidase plus xanthine (Panchenko et al., 1975). Plastic mini-cuvettes containing 0.5 ml of 100 mM potassium phosphate buffer pH 7.8 / 0.1 mM EDTA, 200 μl adrenaline, 200 μl xanthine and 50 μl distilled water (uninhibited control) or 50 μl sample were prepared and the reaction initiated by the addition of 10 μl xanthine oxidase. The reaction was followed at 480 nm and 1 unit of superoxide dismutase activity is described as the amount of the enzyme which inhibits the rate of adenochrome production by 50%.

Glutathione peroxidase (GPX) was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Bell et al., 1985). Plastic mini-cuvettes containing 0.75 ml of 60 mM potassium phosphate buffer pH 7.4/1 mM EDTA/2 mM sodium azide, 50 μl reduced glutathione, 100 μl NADPH and 5 μl glutathione reductase were prepared. The basal reaction was initiated by the addition of 50 μl hydrogen peroxide solution and the non-enzymic rate without sample added was measured for later subtraction. Sample (50 μl) was then added and the assay continued by measuring absorbance at 340 nm with specific activity

determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione-S transferase (GST) activity was determined by following the formation of glutathione-chlorodinitrobenzene (CDNB) adduct at 340 nm. Standard plastic cuvettes containing 2.5 ml of 120 mM potassium phosphate buffer pH 6.5, 100 µl GSH and 100 µl CDNB were prepared and the reaction initiated by the addition of 50 µl sample. Specific activities were determined using an extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ (Habig et al., 1974).

Glutathione reductase (GR) activity was assayed as described by Racker (1955) by measuring the oxidation of NADPH at 340 nm using the extinction coefficient 6.22 $mM^{-1}.cm^{-1}$. Plastic mini-cuvettes containing 0.6 ml of 0.2 M potassium phosphate buffer pH 7.0 /2 mM EDTA, 200 µl oxidised glutathione and 100 µl NADPH were prepared and the reaction initiated by the addition of 100 µl of sample.

Protein content in the homogenate supernatants was determined by the Folin-phenol reagent method, according to Lowry et al. (1951) following digestion in NaOH/SDS.

Experimental design and statistical analysis

The experiment was performed to a simple two factorial design with the factors being dietary HUFA and vitamin E resulting in four experimental diets containing high HUFA +/- vitamin E and low HUFA +/- vitamin E. Results are presented as means ± SD (n = 3 or 4). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were arc-sin transformed before further statistical analysis. Differences between mean values were analyzed by two-way analysis of variance (ANOVA). In addition, differences between mean values, including the control diet, were analyzed by one-way analysis of variance (ANOVA), followed, when pertinent, by a multiple comparison test (Tukey). Differences were reported as statistically significant when $P < 0.05$ (Zar, 1984).

Results

Vitamin E levels were 2-3 times higher in the diets supplemented with vitamin E, and the control diet, compared to the unsupplemented diets (Table 2). Most importantly, the molar ratio of PUFA/vitamin E was significantly different between the four experimental diets, as planned, with the rank order being H0 > L0 >H200 > L200 (Table 2). The high fish oil diets (H0 and H200) contained about 3.2 % n-3HUFA per dry mass unit, whereas the low fish oil diets (L0 and L200) contained about half this level, between 1.4% to 1.7% n-3HUFA per dry mass unit (Table 3). The high fish oil diets also contained more saturated fatty acids, specifically 16:0. The higher n-3HUFA

and 16:0 was compensated in the low fish oil diets by between 3 and 4 times more 18:1n-9 and total monousaturated fatty acids than the high fish oil diets. The control diet had an intermediate level of n-3HUFA (2.4% per dry mass unit) and much higher 18:2n-6 and total lower monousaturated fatty acids compared to the experimental diets (Table 3). The unsaturation index was significantly higher in the high fish oil diets (187.6 and 184.6) than in the low fish oil diets (140.9 and 143.5) (Table 3).

The dietary treatments had relatively few effects on the growth and survival of the fish although fish fed diet L0 were significantly smaller and had smaller livers than fish from the other treatments but mortality rates and SGR were not significantly different (Table 4). The proportions of total lipid and polar lipid in the livers of the fish were significantly lower in fish at the end of the dietary trial compared to initial fish whereas the proportions of triacylglycerol (TAG) and total neutral lipids were higher, especially in the experimental diets (Table 5). The ratio of TAG to cholesterol was highest in fish from treatment H200 (high fish oil and high vitamin E) indicating the greatest accumulation of TAG in liver from fish fed this diet. The total lipid of the fish livers reflected the fatty acid composition of the diets. Thus, total lipid of livers from fish fed the high fish oil diets (H0 and H200) showed two-fold more n-3HUFA than livers from fish fed the low fish oil diets and 1.3-fold more n-3HUFA more than livers from fish fed the control diet (Table 6). In contrast, liver from fish fed low fish oil diets (L0 and L200) had higher levels of 18:1(n-9) than liver from fish fed high fish oil (H0 and H200) diets and control diet. Livers from fish fed the control diet showed high levels of 18:2n-6 and lower levels of monounsaturated fatty acids than any of the experimental diets (Table 6).

The livers from the fish at the beginning of the trial showed quite high levels of vitamin E and this level was significantly reduced in fish fed diets that were not supplemented with vitamin E and the diet supplemented with vitamin E but containing high HUFA (Table 7). Livers from fish fed the diets supplemented with vitamin E diet showed higher contents of vitamin E compared to fish fed the experimental diets unsupplemented with vitamin E, significantly so with the H0 diet although the level of vitamin E in the livers of fish fed the L0 and H200 diets were not significantly different (Table 7). However, liver from fish fed low fish oil with added vitamin E (L200) showed the highest vitamin E content. The molar ratio of PUFA/vitamin E in liver was significantly highest in fish fed diet H0 and significantly the lowest in fish fed diet L200 (Table 7). The TBARS content ($\mu mol \cdot mg^{-1}$ liver mass) was higher in liver from fish fed high fish oil diets and control diet compared to fish fed the low fish oil diets (Table 7). The highest value for liver TBARS being found in fish fed the H0 diet and lowest in fish fed the L0 and L200 diets, with fish fed the H200 and control diets giving intermediate values. The level of 8-isoprostane was also significantly higher in liver from fish fed the H0 diet compared to all the other diets (Table 7).

The activities of CAT and SOD in liver were higher in fish at the end of the dietary trial in comparison with the initial fish but there were no significant differences between the experimental diets although the activities were generally lower than in fish fed the control diet (Table 7). The activities of GPX and GST were not different between any of the treatments whereas GR activity was highly variable between tratments and tended to be higher in fish fed the low fish oil diets (Table 7).

Table 8 shows the results of two-way ANOVA of the data for diets H0, L0, H200 and L200. These data clearly show that liver vitamin E levels were highly dependent upon both dietary vitamin E levels and dietary HUFA levels. However, the lipid peroxidation products in liver were influenced differently by the diets. TBARS levels were only affected by the level of dietary HUFA and not dietary vitamin E, whereas isoprostane levels were significantly affected by dietary vitamin E and not by dietary HUFA levels (Table 8). Two-way ANOVA did not discern any effect of diet on the activities of the enzymes other than GR being significantly affected by dietary HUFA level.

Discussion

In a similar dietary trial in turbot (*Scophthalmus maximus*) varying dietary PUFA and vitamin E levels, there were no significant effects of either variable on growth (Stephan et al. 1995). In the present trial there were also few significant effects of the diets on growth parameters other than an effect of both fish oil and vitamin E on the dry mass of the fish with the fish fed the high fish oil having higher dry masses, especially in combination with vitamin E. The liver wet and dry masses in absolute terms were also significantly higher in fish fed the high fish oil diets. However, the livers from fish fed all the experimental diets showed increased proportions of TAG and increased TAG/cholesterol ratios with no significant differences between the diets. Therefore, none of the experimental diets used in the present study had any gross deliterious effect on the fish in terms of growth performance and survival.

As expected, the different dietary fatty acid compositions were reflected in the fatty acid compositions of the livers from the fish. Thus, the high fish oil diets resulted in increased levels of total PUFA and n-3HUFA, particularly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) whereas the low fish oil diets resulted in lower levels of PUFA and HUFA, and high levels of 18:1n-9 in the liver. Therefore, the potential for lipid peroxidation was theoretically higher in the fish fed the H0 and H200 diets. Similarly, the dietary vitamin E levels were, at least partly, reflected in the liver vitamin E levels despite the level of vitamin E in livers from fish fed diet L0 being higher than expected. However, the diets were formulated to significantly vary the molar ratio of PUFA/vitamin E and this was achieved with the rank order of

the ratio in the diets (H0 > L0 > H200 > L200) being highly significant. This was largely reflected in the PUFA/vitamin E ratios in the fish livers at the end of the trial where the rank order for the ratio was H0 > L0 = H200 > L200. Therefore, the data show that the dietary trial was largely successful in altering the prooxidant/antioxidant (PUFA/vitamin E or α-tocopherol) ratio, and hence the potential susceptibility of the tissue to lipid peroxidation, in a graded manner as planned. The importance of the α-tocopherol/PUFA ratio in determining tissue susceptibility to fatty acid peroxidation had been stressed in an earlier study in which increased dietary fish oil increased the susceptibilty of turbot tissues to peroxidation, with supplementation of vitamin E limiting that effect (Stephan et al. 1995). A recent study has also shown that muscle homogenates from sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) fed high fish oil diets were significantly more susceptible to oxidation in vitro than homogenates from fish fed low oil diets (Alvarez et al. 1998).

Despite the above, there was no obvious or logical pattern discernable in the data obtained for the activities of the liver antioxidant enzyme systems. The pattern of activities for the enzymes under the experimental conditions in the present study was not as predicted and a clear relationship between liver PUFA/vitamin E ratio and the activities of the liver antioxidant enzymes was not observed. Previously, we had shown that SOD and GST activities decreased as the PUFA/vitamin E ratio decreased during early development in unfed common dentex (*Dentex dentex*) larvae (Mourente et al. 1999a). Conversely, the activities of CAT and, to a lesser degree, GPX actually increased with decreasing PUFA/vitamin E ratio in that study. In a subsequent study on larval dentex, there were no significant effects on the antioxidant enzyme activities of increasing dietary HUFA, but in this study the level of dietary vitamin E increased in parallel with dietary HUFA (Mourente et al. 1999b).

There are few data in the literature on fish with which to compare the results of the present study. The activities of the antioxidant enzymes have been measured in marine fish including sardine (*Sardina pilchardus*) (Peters et al. 1994) and turbot larvae (Peters et al. 1996) and freshwater fish such as rainbow trout and black bullhead (*Ameiurus melas*) (Aceto et al. 1994; Otto and Moon 1996). However these studies focussed on the role of the enzymes in pollutant detoxification (Peters et al. 1994) or developmental aspects (Aceto et al. 1994; Otto and Moon 1996; Peters and Livingstone 1996) rather than the effects of dietary nutrients. In mammals, the effects of dietary PUFA, including fish oil, on the activity of the antioxidant enzymes in liver are contradictory, but in a recent study, supplementation of PUFA, including EPA, to Swiss 3T3 cells resulted in increased levels of PUFA in phospholipids and the activities of SOD, GPX and GST increased with degree of unsaturation of the phospholipids (Benito et al. 1997).

There are perhaps two main reasons for lack of significant effects on the liver antioxidant

enzymes in the present study. Firstly, the level of HUFA fed in the high fish oil diets may not be sufficiently high and the resultant peroxidative load not sufficiently different to that in the low fish oil diets or, alternatively, the difference in HUFA levels between the diets used in the present study could also be described as reduced HUFA versus normal HUFA. However, one of the main differences between the fatty acid compositions of the diets was the DHA content and there are data to suggest that DHA does not exert as high a peroxidative burden as expected based on peroxidizability index (Kubo et al. 1997, 1998). Secondly, and alternatively, the levels of dietary vitamin E were not low enough to deplete tissue vitamin E to a sufficiently low level to significantly increase peroxidative stress. However, as described above, the dietary treatments significantly altered the PUFA/vitamin E ratios in livers and there were significant differences between the diets in the levels of in vivo peroxidation peroducts. Both TBARS and isoprostane levels were highest in fish fed the H0 diet which had the highest PUFA/vitamin E ratio in the liver. These data support the conclusion that, despite the lack of effect on antioxidant enzyme activities, the different dietary treatments did have effects on the peroxidation status in the liver which resulted in differential production of lipid peroxidation products. It is possible that the antioxidant enzyme systems only get switched on in more severe conditions of peroxidative stress such as occurs when oxidized oils are fed but this is not known. As the antioxidant enzyme systems are also involved in detoxification of xenobiotics, and are often used to assess environmental contamination by certain organic pollutants, these alternative functions may additionally complicate the interpretation of the data.

As described above, the dietary treatments resulted in significant differences in the levels of in vivo peroxidation products measured in the liver. Two-way ANOVA showed that the levels of the lipid peroxidation products, TBARS, were significantly influenced by dietary HUFA levels, being significantly increased in fish fed the high fish oil diets with vitamin E having no significant effect. It was clear that there was no effect of vitamin E in fish fed the low fish oil diets but the data suggested that TBARS were reduced by vitamin E in fish fed the high fish oil diets, but the high standard deviation for the data for H200 diet made this non-significant. The TBARS assay measures aldehydes that are secondary (and end-) products of the lipid peroxidation chain reactions and so could be expected to be influenced by the major chain-breaking antioxidant, vitamin E, although there are several other antioxidants that act similarly including ubiquinone, carotenoids, urate and glutathione. In addition, vitamin C (ascorbate) functions to regenerate vitamin E from the tocopheryl radical and so , as above there are other factors that could affect the results and complicate the interpretation of the data.

In apparent contrast to TBARS levels, two-way ANOVA indicated that concentrations of liver isoprostanes were significantly affected by the level of dietary vitamin E but not HUFA. However,

in this case interaction between the two parameters was indicated by ANOVA and it was clear that supplementary vitamin E significantly lowered the concentration of isoprostanes measured in fish fed the high fish oil diets, but not in fish fed the low fish oil diets. This was similar to the non-significant trend observed with TBARS levels. In addition, both TBARS and isoprostane levels were significantly highest in livers of fish fed the H0 diet (high HUFA and low vitamin E) as would be expected. Therefore, in vivo production of isoprostanes and TBARS followed a broadly similar pattern in the present study.

The data in the present study support the potential of isoprostane measurements as indicators of in vivo lipid peroxidation in fish. This is an important finding as the use of isoprostane as measures of in vivo oxidative stress has only been reported for mammalian species rich in arachidonic acid (AA; 20:4n-6) (Roberts and Morrow 1997). Therefore, studies to date have concentrated on the production of isoprostanes from AA, the major products being F_2-isoprostanes (i.e. containing the F-type prostane ring) with the most important regioisomer being 8-isoprostane (8-*iso*-prostaglandin $F_{2\alpha}$). However, other HUFA will also be peroxidized by non-enzymatic free radical mechanisms to produce isoprostanes, and F_3- and F_4-isoprostanes from EPA and DHA, respectively, have been identified, characterized and reported to correlate with other markers of lipid peroxidation (Nourooz-Zadeh et al. 1997, 1998). The EIA kit used in the present study uses antibodies directed specifically against 8-*iso*-prostaglandin $F_{2\alpha}$ but 8-*iso*-prostaglandin $F_{3\alpha}$, the equivalent isoprostane from EPA, has 21% reactivity with this antibody whereas all enzymatically produced prostaglandins have a reactivity of at least 20-fold lower or, for most prostaglandins, considerably less. Enzymatically produced prostaglandins are dominated by those derived from AA, even in fish tissues where EPA is normally present in far greater amounts than AA (Tocher 1995). However, there is no reason to believe this would also be the case for non-enzymatically produced isoprostanes. Indeed, it is more likely that isoprostanes are produced in quantities reflecting the concentrations of their HUFA precursors. Irrespective of what the isoprostane profile was in sea bream liver, the present study has shown that isoprostane analyses will be useful in assessing oxidative stress in fish.

The present study investigated the antioxidant systems in a cultured juvenile marine fish, gilthead sea bream (*S. aurata*) by feeding diets varying greatly in vitamin E/PUFA ratio through having either high or low levels of fish oil (n-3HUFA) combined with the presence or absence of vitamin E. None of the diets had serious deliterious effects on growth or survival of the fish, but the different dietary regimes were successful in significantly altering the PUFA/vitamin E ratios in the fish livers. This had effects on the peroxidation status of the fish as evidenced by the significantly altered levels of in vivo lipid peroxidation products measured in liver, with fish fed the diet rich in HUFA and low in vitamin E showing significantly higher values of TBARS and isoprostane. The

isoprostane levels generally followed the same pattern as TBARS levels supporting its value as an indicator of in vivo oxidative stress in fish as well as mammals. However, no significant effects on antioxidant enzyme activities were observed suggesting that more severe conditions may be required to affect these activities such as further lowering the PUFA/vitamin E ratio or by increasing peroxidative stress through the feeding of oxidized oils.

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References

Aceto, A., Amicarelli, F., Sacchetta, P., Dragani, B., Bucciarelli, T., Masciocco, L., Miranda, M. and Di Ilio, C. 1994. Developmental aspects of detoxifying enzymes in fish (*Salmo iridaeus*). Free Rad. Res. 21: 285-294.

Alvarez, M.J., Lopez-Bote, C.J., Diez, A., Corraze, G., Arzel, J., Dias, J., Kaushik, S.J. and Bautista, J.M. 1998. Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (*Onchorynchus mykiss*) and sea bass (*Dicentrarchus labrax*). B. J. Nutr. 80: 281-289.

Bai, S.C. and Lee, K.J. 1998. Different levels of dietary DL-α-tocopheryl acetate affect the vitamin E status of juvenile Korean rockfish, *Sebastes schlegeli*. Aquaculture 161: 405-414.

Beers, R.F. and Sizer, I.W. 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140.

Bell, J.G., Cowey, C.B., Adron, J.W. and Shanks, A.M. 1985. Some effects of vitamin E and selenium deprivation on tissue enzyme levels and indices of tissue peroxidation in rainbow trout (*Salmo gairdneri*). Br. J. Nutr. 53: 149-157.

Bell, J.G., Cowey, C.B., Adron, J.W. and Pirie, B.J.S. 1987. Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in Atlantic salmon parr (*Salmo salar*). Aquaculture 65: 43-54.

Benito, S., Fernandez, Y., Mitjavila, S., Moussa, M., Anglade, F. and Periquet, A. 1997. Phospholipid fatty acid composition affects enzymatic antioxidant defenses in cultured Swiss 3T3 fibroblasts. Redox Rep. 3: 281-286.

Bieri, J.G. 1969. Chromatography of tocopherols. *In*: Lipid Chromatographic Analysis, Vol. 2.

p.459. Edited by G.V. Marinetti. Marcel Dekker Inc., New York.

Burk, R.F., Trumble, M.J. and Lawrence, R.A. 1980. Rat hepatic cytosolic GSH-dependent enzyme protection against lipid peroxidation in the NADPH microsomal lipid peroxidation system. Biochim. Biophys. Acta 618: 35-41.

Carpenter, A.P. 1979. Determination of tocopherols in vegetable oils. J. Am. Oil Chem. Soc.56: 668-671.

Christie, W.W. 1989. Gas Chromatography and Lipids: A Practical Guide, 1st edn. The Oily Press, Ayr, Scotland.

De Silva, S.S. and Anderson, T.A. 1995. Fish Nutrition in Aquaculture. Chapman & Hall, London.

Dubois, M., Gilles, G.A., Hamilton, J.K., Rebels, P.A. and Smith, F. 1956. Colorimetric methods for determination of sugars and related substances. Anal. Chem. 3: 350-356.

Fewster, M.E., Burns, B.J. and Mead, J.F. 1969. Quantitative densitometric thin layer chromatography of lipids using cupric acetate reagent. J. Chromatogr. 43: 120-126.

Folch, J., Lees, M. and Sloane-Stanley, G.H.S. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Habig, W.H., Pabst, M.J. and Jacoby, W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercaturic acid formation. J. Biol. Chem. 249: 7130-7139.

Halliwell, B. and Gutteridge, J.M.C. 1996. Lipid peroxidation: a radical chain reaction. *In*: Free Radicals in Biology and Medicine. pp. 188-266. Edited by B. Halliwell and J.M.C. Gutteridge. Clarendon Press, Oxford.

Henrique, M.M.F., Gomes, E.F., Gouillou-Coustans, M.F., Oliva-Teles, A. and Davies, S.J. 1998. Influence of supplementation of practical diets with vitamin C on growth and response to hypoxic stress of seabream, *Sparus aurata*. Aquaculture 161: 415-426.

Huo, J-Z., Nelis, H.J., Lavens, P., Sorgeloos, P. and De Leenheer, A.P. 1996. Determination of vitamin E in aquatic organisms by High-Performance liquid chromatography with fluorescence detection. Anal. Biochem. 242: 123-128

Kawatsu, H. 1969. Studies on the anemia of fish-III. An example of macrcytic anemia found in brook trout, *Salvelinus fontinalis*. Bull. Freshwater Res. Lab. 19: 161-167.

Kubo, K., Saito, M., Tadokoro, T. and Maekawa, A. 1997. Changes in susceptibility of tissues to lipid peroxidation after ingestion of various levels of docosahexaenoic acid and vitamin E. B. J. Nutr. 78: 655-669.

Kubo, K., Saito, M., Tadokoro, T. and Maekawa, A. 1998. Dietary docosahexaenoic acid does not promote lipid peroxidation in rat tissue to the extent expected from peroxidizability index of the lipids. Biosci. Biotechnol. Biochem. 62: 1698-1706.

Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the

Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Merchie, G., Lavens, P., Storch, V., Übel, U., Nelis, H., De Leenheer, A. and Sorgeloos, P. 1996. Influence of dietary vitamin C dosage on turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*) nursery stages. Comp. Biochem. Physiol., 114A: 123-133.

Miller, J.K., Brzezinska-Slebodzinska, E. and Madsen, F.C. 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76: 2812-2823.

Mourente, G., Tocher, D.R., Díaz, E., Grau, A. and Pastor, E. 1999a. Relationships between antioxidant enzyme activities and lipid peroxidation products during early development in *Dentex dentex* eggs and larvae. Aquaculture 179: 309-324.

Mourente, G., Tocher, D.R., Díaz-Salvago, E., Grau, A. and Pastor, E. 1999b. Study of the n-3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex* at *Artemia* feeding stage. Aquaculture 179: 291-307.

Morrow, J.D. and Roberts, L.J. 1997. The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. 36: 1-21.

Murata, H., Sakai, T., Yamauchi, K., Ito, T., Tsuda, T., Yoshida, T. and Fukudome, M. 1996. *In vivo* lipid peroxidation levels and antioxidant activities of cultured and wild yellowtail. Fisherie Science 62: 64-68.

Murai, T. and Andrews, J.W. 1974. Interaction of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). J. Nutr. 104: 1416-1431.

Nourooz-Zadeh, J., Halliwell, B. and Anggard, E.E. 1997. Evidence for the formation of F_3-isoprostanes during peroxidation of eicosapentaenoic acid. Biochem. Biophys. Res. Commun. 236: 467-472.

Nourooz-Zadeh, J., Liu, E.H.C., Anggard, E.E. and Halliwell, B. 1998. F_4-Isoprostanes: A novel class of prostanoids formed during peroxidation of docosahexaenoic acid (DHA). Biochem. Biophys. Res. Commun. 242: 338-344.

Nunes, G.D., Dinis, M.T. and Bucke, D. 1996. The response to environmental stress in juvenile *Sparus aurata* L. fed diets supplemented with high doses of vitamin C. Bull. Eur. Assoc. Fish Pathol. 16: 208-210.

Olsen, R.E. and Henderson, R.J. 1989. The rapid analysis of neutral and polar lipids using double-development HPTLC and scanning densitometry. J. Exp. Mar. Biol. Ecol. 129: 189-197.

Otto, D.M.E. and Moon, T.W. 1996. Endogenous antioxidant systems of two teleost fish, the rainbow trout and the black bullhead, and the effect of age. Fish Physiol. Biochem. 15: 349-358.

Panchenko, L.F., Brusov, O.S., Gerasimov, A.M. and Loktaeva, T.D. 1975. Intramitochondrial localization and release of rat liver superoxide dismutase. FEBS Lett. 55: 84-87.

Peters, L.D. and Livingstone, D.R. 1996. Antioxidant enzyme activities in embryologic and early

larval stages of turbot. J. Fish Biol. 49: 986-997.

Peters, L.D., Porte, C., Albaiges, J. and Livingstone, D.R. 1994. 7-Ethoxyrosorufin *O*-deethylase (EROD) and antioxidant enzyme activities in larvae of sardine *(Sardina pilchardus*) from the North coast of Spain. Mar. Pollut. Bull. 28: 299-304.

Powell, W.S. 1982. Rapid extraction of arachidonic acid metabolites from biological samples using octadecylsilyl silica. Methods Enzymol. 86: 467-517.

Racker, E., 1955. Glutathione reductase (liver and yeast). *In*: Methods in Enzymology, vol. 2. pp. 722-725. Edited by S.P. Colowick and N.O. Kaplan. Academic Press, New York,

Roberts, L.J. and Morrow, J.D. 1997. The generation and actions of isoprostanes. Biochim. Biophys. Acta 1345: 121-135.

Sakai, T., Murata, H., Endo, M., Yamauchi, K., Tabata, N. and Fukudome, M. 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agric. Biol. Chem. 53: 1739-1740.

Sargent, J.R., Bell, J.G., McEvoy, L., Tocher, D.R. and Estévez, A. 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177: 191-199.

Stéphan, G., Guillaume, J. and Lamour, F. 1995. Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture 130: 251-268.

Tocher, D.R. 1995. Glycerophospholipid metabolism. *In*: Biochemistry and Molecular Biology of Fishes. Vol. 4. Metabolic and Adaptational Biochemistry. pp. 119-157. Edited by P.W. Hochachka and T.P. Mommsen. Elsevier Press, Amsterdam.

Tocher, D.R. and Harvie, D.G. 1988. Fatty acid composition of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. Fish Physiol. Biochem. 5: 229-239.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya, T. 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi 36: 623-630.

Winston, G.W. and Di Giulio, R.T. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquatic Toxicology 19: 137-161.

Wootten, R.J. 1990. Ecology of Teleost Fishes. Chapman and Hall Fish and Fisheries Series 1, 404 pp.

Zar, J. H., 1984. Biostatistical Analysis, 2nd Edn. Prentice-Hall, Englewood Cliffs, NJ.

Table 1. Formulation of experimental diets showing composition of the base pellet and oil coatings.

Component	L0	L200	H0	H200
Pellet				
Fishmeal[1]	72.0	72.0	72.0	72.0
Starch[2]	10.0	10.0	10.0	10.0
Finnstim/Betafin	0.5	0.5	0.5	0.5
Mineral premix M2[3]	2.4	2.4	2.4	2.4
Vitamin E-free premix[4]	0.5	0.5	0.5	0.5
Vitamin E-stripped fish oil[5]	2.0	2.0	2.0	2.0
Coating				
Vitamin E-stripped fish oil[5]	-	-	12.6	-
Oleic acid[6]	12.6	-	-	-
Fish oil + Vitamin E[7]	-	-	-	12.6
Oleic acid + Vitamin E[7]	-	12.6	-	-

[1] LT94, Low temperature fish meal (Ewos Ltd., Livingston, U.K.).

[2] Paselli WA4 (Avebe Ltd., Ulceby, U.K).

[3] Supplied (per kg diet): KH_2PO_4, 22g; $FeSO_4$.7H2O, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4$.7H2O, 2 mg; and KI, 2 mg.

[4] Supplied (mg/kg diet): ascorbic acid, 1000; myo-inositol, 400; nicotinic acid, 150; calcium pantothenate, 44; riboflavin, 20; pyridoxine hydrochloride, 12; menadione, 10; thiamine hydrochloride, 10; retinyl acetate, 7.3; folic acid, 5; biotin, 1; cholecalciferol, 0.06; cyanocobalamin, 0.02.

[5] Stripped by activated charcoal (Sigma Chemical Co. Ltd.) adsorption in hexane.

[6] Fisher Scientific, Loughborough, England.

[7] Tocopherol acetate added to oils at 2000 mg/Kg.

Table 2. Gross composition (% dry mass), tocopherol content (ng · mg^{-1} dry mass), TBARS content (µmol · mg^{-1} dry mass) and molar ratio PUFA/vitamin E in control and experimental diets fed to gilthead sea bream (*Sparus aurata*).

	Control diet	Experimental diet			
		H0	L0	H200	L200
Protein (% dry mass)	49.1 ± 1.5 ^{a}	43.9 ± 0.4 ^{b}	41.9 ± 1.5 ^{b}	42.2 ± 0.2 ^{b}	44.6 ± 0.6 ^{b}
Carbohydrates (% dry mass)	7.1 ± 0.9 ^{c}	10.8 ± 0.4 ^{a}	10.4 ± 0.4 ^{ab}	9.2 ± 0.1 ^{b}	9.9 ± 1.0 ^{ab}
Ash (% dry mass)	12.7 ± 1.2	11.9 ± 0.5	11.2 ± 0.2	12.4 ± 0.7	12.8 ± 1.1
Total lipid (% dry mass)	22.3 ± 0.2 ^{b}	24.3 ± 0.2 ^{a}	24.1 ± 0.6 ^{a}	24.0 ± 0.8 ^{a}	23.8 ± 0.7 ^{a}
TBARS(µmol·mg^{-1} dry mass)	2.9 ± 0.2 ^{b}	2.3 ± 0.0 ^{b}	2.5 ± 0.3 ^{b}	4.9 ± 0.2 ^{a}	2.2 ± 0.1 ^{b}
ng vitamin E mg^{-1} dry mass					
Total tocopherol	323.3 ± 6.3 ^{a}	77.0 ± 4.1 ^{c}	132.6 ± 8.1 ^{b}	340.3 ± 15.9 ^{a}	318.0 ± 3.7 ^{a}
Molar ratio PUFA/vitamin E	183 ± 7.0 ^{bc}	744.9 ± 48.0 ^{a}	239.9 ± 19.8 ^{b}	160.1 ± 15.8 ^{c}	117.4 ± 11.6 ^{d}

Data are means ± SD (n=3). SD=0 implies SD<005. Values within the same row bearing different superscript letter are significantly different ($p<0.05$); PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances.

Table 3. Total lipid fatty acid content (µg fatty acid · mg dry mass) in control and experimental diets fed to gilthead sea bream (*Sparus aurata*).

Fatty acid	Control diet	Experimental diet			
		H0	L0	H200	L200
14:0	5.9 ± 0.2 ^{b}	7.7 ± 0.3 ^{a}	4.1 ± 0.1 ^{c}	8.0 ± 1.2 ^{a}	5.0 ± 0.2 ^{bc}
15:0	4.9 ± 0.8	4.2 ± 0.6	5.0 ± 0.4	5.5 ± 1.1	4.6 ± 1.3
16:0	20.0 ± 0.9 ^{ab}	22.6 ± 0.5 ^{a}	12.5 ± 0.3 ^{c}	22.3 ± 3.0 ^{a}	15.1 ± 1.8 ^{bc}
17:0	0.8 ± 0.0	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	1.3 ± 0.5
18:0	4.7 ± 0.2 ^{a}	4.1 ± 0.1 ^{a}	2.5 ± 0.1 ^{b}	4.1 ± 0.5 ^{a}	3.0 ± 0.4 ^{b}
20:0	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.6 ± 0.2	0.2 ± 0.1
22:0	0.3 ± 0.0	0.1 ± 0.1	nd	0.3 ± 0.2	nd
24:0	0.1 ± 0.1	nd	nd	nd	nd
Total saturated	37.1 ± 1.0 ^{a}	40.2 ± 1.7 ^{a}	25.3 ± 0.3 ^{b}	41.6 ± 5.0 ^{a}	29.3 ± 1.5 ^{b}
16:1(n-9)	0.1 ± 0.1	nd	nd	0.1 ± 0.1	nd
16:1(n-7)	5.9 ± 0.3 ^{b}	8.2 ± 0.2 ^{a}	7.6 ± 0.2 ^{ab}	7.9 ± 1.1 ^{ab}	9.0 ± 0.8 ^{a}
18:1(n-9)	18.9 ± 0.8 ^{b}	20.2 ± 0.4 ^{b}	67.0 ± 2.1 ^{a}	19.8 ± 2.4 ^{b}	78.1 ± 8.4 ^{a}
18:1(n-7)	3.1 ± 0.1 ^{b}	4.1 ± 0.0 ^{b}	7.7 ± 0.2 ^{a}	4.1 ± 0.5 ^{b}	9.1 ± 1.0 ^{a}
20:1(n-9)	3.9 ± 0.2 ^{c}	12.5 ± 0.2 ^{a}	5.3 ± 0.1 ^{bc}	12.4 ± 1.7 ^{a}	6.4 ± 0.6 ^{b}
20:1(n-7)	0.3 ± 0.0 ^{ab}	0.5 ± 0.0 ^{a}	nd	0.6 ± 0.1 ^{a}	0.1 ± 0.1 ^{b}
22:1(n-11)	5.7 ± 0.3 ^{b}	19.3 ± 0.3 ^{a}	6.4 ± 0.2 ^{b}	19.9 ± 2.6 ^{a}	7.9 ± 0.9 ^{b}
22:1(n-9)	nd	1.2 ± 0.1	nd	nd	nd
24:1(n-9)	0.9 ± 0.1 ^{b}	1.5 ± 0.0 ^{a}	0.6 ± 0.0 ^{c}	1.5 ± 0.2 ^{a}	0.8 ± 0.1 ^{bc}
Total monoenes	38.8 ± 1.7 ^{c}	67.5 ± 1.3 ^{b}	94.7 ± 2.5 ^{a}	66.2 ± 8.4 ^{b}	111.3 ± 11.8 ^{a}
16:2(n-6)	1.3 ± 0.3	1.3 ± 0.0	1.6 ± 0.0	1.0 ± 0.2	1.6 ± 0.1
16:3(n-3)	1.0 ± 0.1 ^{abc}	0.5 ± 0.1 ^{c}	1.7 ± 0.8 ^{ab}	0.6 ± 0.1 ^{bc}	2.2 ± 0.2 ^{a}
16:4(n-3)	0.7 ± 0.0	0.9 ± 0.1	0.9 ± 0.3	0.9 ± 0.0	0.9 ± 0.3
18:2(n-6)	18.1 ± 0.8 ^{a}	3.0 ± 0.1 ^{c}	5.8 ± 0.2 ^{b}	2.9 ± 0.3 ^{c}	6.2 ± 0.4 ^{b}
18:3(n-3)	3.8 ± 0.2 ^{a}	1.5 ± 0.0 ^{b}	1.1 ± 0.0 ^{c}	1.5 ± 0.2 ^{bc}	1.1 ± 0.1 ^{c}
18:4(n-3)	2.2 ± 0.0 ^{b}	3.4 ± 0.1 ^{a}	1.8 ± 0.1 ^{b}	3.3 ± 0.4 ^{a}	2.2 ± 0.2 ^{b}
20:2(n-6)	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.5
20:4(n-6)	1.0 ± 0.0 ^{a}	1.0 ± 0.0 ^{a}	0.4 ± 0.0 ^{b}	0.9 ± 0.1 ^{a}	0.5 ± 0.1 ^{b}
20:3(n-3)	nd	0.2 ± 0.0	nd	0.2 ± 0.1	nd
20:4(n-3)	0.6 ± 0.0 ^{b}	1.1 ± 0.0 ^{a}	0.4 ± 0.0 ^{c}	1.1 ± 0.1 ^{a}	0.5 ± 0.0 ^{bc}
20:5(n-3)	9.2 ± 0.4 ^{a}	10.8 ± 0.2 ^{a}	4.6 ± 0.1 ^{b}	10.7 ± 1.5 ^{a}	5.8 ± 0.7 ^{b}
22:5(n-6)	0.4 ± 0.0 ^{ab}	0.5 ± 0.0 ^{a}	nd	0.5 ± 0.1 ^{a}	0.1 ± 0.1 ^{b}
22:5(n-3)	1.5 ± 0.1 ^{a}	1.8 ± 0.0 ^{a}	0.5 ± 0.0 ^{b}	1.7 ± 0.3 ^{a}	0.7 ± 0.1 ^{b}
22:6(n-3)	13.0 ± 0.5 ^{b}	18.6 ± 0.4 ^{a}	8.1 ± 0.2 ^{c}	18.3 ± 2.5 ^{a}	10.1 ± 1.2 ^{bc}
Total polyenes	52.9 ± 2.1 ^{a}	45.0 ± 0.8 ^{a}	27.2 ± 1.2 ^{b}	44.1 ± 5.8 ^{a}	32.6 ± 3.1 ^{b}
Total (n-9)	23.8 ± 1.0 ^{c}	35.4 ± 0.8 ^{b}	72.9 ± 2.2 ^{a}	33.8 ± 4.2 ^{bc}	85.2 ± 9.1 ^{a}
Total (n-7)	9.3 ± 0.4 ^{c}	12.8 ± 0.3 ^{b}	15.3 ± 0.2 ^{ab}	12.5 ± 1.7 ^{b}	18.2 ± 1.7 ^{a}
Total (n-6)	21.0 ± 0.8 ^{a}	6.1 ± 0.2 ^{c}	8.0 ± 0.2 ^{b}	5.8 ± 0.7 ^{c}	9.1 ± 0.8 ^{b}
Total (n-3)	31.9 ± 1.3 ^{a}	38.9 ± 0.6 ^{a}	19.1 ± 0.9 ^{b}	38.3 ± 5.1 ^{a}	23.5 ± 2.4 ^{b}
HUFA(n-6)	1.3 ± 0.1 ^{a}	1.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{c}	1.4 ± 0.2 ^{a}	0.6 ± 0.0 ^{b}
HUFA(n-3)	24.3 ± 1.1 ^{b}	32.5 ± 0.6 ^{a}	13.6 ± 0.4 ^{c}	32.0 ± 4.5 ^{ab}	17.0 ± 2.0 ^{c}
Unsaturation index	188.4 ± 1.4 ^{a}	187.6 ± 0.8 ^{a}	140.9 ± 0.9 ^{b}	184.6 ± 1.9 ^{a}	143.5 ± 1.8 ^{b}

Data are mean ± SD (n=3). SD = 0.0 implies SD < 0.05. Values within the same row bearing different superscript letter are significantly different ($p<0.05$). tr, trace < 0.05; nd, not detected; HUFA, highly unsaturated fatty acids.

Table 4. Growth-related performance and survival rates of gilthead sea bream (*Sparus aurata*) fed control and experimental diets.

	t=0 days	t=30 days				
	Initial	Control	H0	L0	H200	L200
Fish length (mm)	35.5 ± 3.5 ^{c}	52.5 ± 5.1 ^{a}	51.5 ± 1.6 ^{a}	49.0 ± 0.7 ^{b}	51.5 ± 0.6 ^{a}	52.3 ± 0.7 ^{ab}
Fish live mass (g)	0.5 ± 0.2 ^{c}	2.0 ± 0.6 ^{ab}	1.9 ± 0.2 ^{a}	1.6 ± 0.1 ^{b}	2.0 ± 0.1 ^{a}	1.9 ± 0.1 ^{ab}
Fish dry mass (mg)	133.1 ± 22.7 ^{c}	434.1 ± 46.6	323.6 ± 58.6	300.2 ± 86.3	399.5 ± 24.7	362.4 ± 24.0
Fish dry mass %	25.1 ± 0.4 ^{ab}	25.4 ± 0.3 ^{ab}	25.0 ± 0.5 ^{ab}	24.3 ± 1.3 ^{a}	26.5 ± 0.7 ^{b}	25.2 ± 0.5 ^{ab}
Liver live mass (mg)	9.7 ± 3.4 ^{c}	54.0 ± 20.2 ^{ab}	67.5 ± 3.5 ^{a}	55.9 ± 4.2 ^{b}	67.7 ± 6.0 ^{a}	60.5 ± 1.3 ^{b}
Liver dry mass (mg)	3.0 ± 0.4 ^{c}	19.7 ± 4.4 ^{b}	25.6 ± 2.0 ^{a}	21.3 ± 1.6 ^{b}	25.6 ± 2.1 ^{a}	23.1 ± 0.0 ^{b}
Liver dry mass %	30.9 ± 0.8 ^{b}	36.5 ± 0.8 ^{a}	37.8 ± 1.2 ^{a}	37.9 ± 1.0 ^{a}	37.8 ± 0.2 ^{a}	38.2 ± 0.9 ^{a}
HSI	1.9 ± 0.3 ^{c}	2.6 ± 0.2 ^{a}	3.1 ± 0.1 ^{b}	3.1 ± 0.2 ^{b}	3.2 ± 0.0 ^{b}	2.8 ± 0.1 ^{a}
Mortality (nº of fish)		19.5 ± 7.5	15.3 ± 7.7	13.3 ± 4.9	12.0 ± 4.5	19.7 ± 9.7
Mortality (%)		3.9 ± 1.5	3.1 ± 1.5	2.7 ± 1.0	2.4 ± 0.9	3.9 ± 1.9
SGR (%day^{-1})		4.5	4.4± 0.3	3.8± 0.2	4.5± 0.2	4.4± 0.2

Data are mean ± SD (n =30 for initial point and control diet; n=90 for experimental diets). Values within the same row bearing different superscript letter are significantly different ($p<0.05$). HSI, Hepato-somatic Index; SGR, Specific Growth Rate.

Table 5. Gross composition (% dry mass) and lipid class composition (% total lipid) of livers from gilthead sea bream (*Sparus aurata*) fed control and experimental diets.

	t=0 days	t=30 days				
Sample	Initial	Control	H0	L0	H200	L200
Protein	40.9 ± 0.5 ^{cd}	45.3 ± 0.6 ^{a}	42.2 ± 0.7 ^{bc}	41.3 ± 0.3 ^{cd}	39.6 ± 1.8 ^{d}	44.5 ± 0.4 ^{ab}
Lipid	48.7 ± 1.9 ^{a}	32.4 ± 8.3 ^{b}	34.1 ± 1.5 ^{b}	37.8 ± 4.7 ^{b}	35.5 ± 2.1 ^{b}	33.7 ± 2.3 ^{b}
Carbohydrate	6.2 ± 0.2 ^{d}	18.0 ± 0.8 ^{c}	19.6 ± 0.0 ^{ab}	18.0 ± 0.6 ^{c}	20.4 ± 0.6 ^{a}	18.4 ± 0.5 ^{bc}
Ash	4.2 ± 1.1	4.3 ± 0.4	4.1 ± 0.2	2.9 ± 0.3	4.5 ± 1.0	3.4 ± 0.6
Sphingomyelin	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	tr	nd	0.1 ± 0.1
Phosphatidylcholine	12.8 ± 0.6 ^{a}	9.9 ± 1.9 ^{ab}	8.9 ± 0.4 ^{ab}	9.1 ± 0.6 ^{ab}	8.0 ± 0.6 ^{b}	9.8 ± 1.7 ^{ab}
Phosphatidylserine	1.7 ± 0.1	1.6 ± 0.3	1.1 ± 0.4	0.8 ± 0.4	1.0 ± 0.2	1.1 ± 0.2
Phosphatidylinositol	3.3 ± 0.6 ^{a}	2.5 ± 0.5 ^{ab}	2.0 ± 0.2 ^{b}	1.5 ± 0.3 ^{b}	1.6 ± 0.1 ^{b}	1.9 ± 0.2 ^{b}
Phosphatidic acid/cardiolipin	2.7 ± 0.5 ^{a}	1.6 ± 0.4 ^{ab}	1.1 ± 0.3 ^{b}	1.4 ± 0.2 ^{b}	1.2 ± 0.2 ^{b}	1.1 ± 0.4 ^{b}
Phosphatidylethanolamine	7.4 ± 0.5 ^{a}	5.3 ± 0.9 ^{ab}	4.6 ± 0.4 ^{b}	4.4 ± 0.3 ^{b}	4.0 ± 0.3 ^{b}	5.2 ± 1.3 ^{ab}
Lyso-phosphatidylcholine	2.3 ± 0.3 ^{a}	1.5 ± 0.4 ^{ab}	1.6 ± 0.1 ^{ab}	1.3 ± 0.2 ^{b}	1.1 ± 0.2 ^{b}	1.4 ± 0.3 ^{ab}
Lyso-phosphatidylethanolamine	0.9 ± 0.4	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
Sulphatides	0.7 ± 0.1 ^{a}	0.7 ± 0.2 ^{a}	0.3 ± 0.0 ^{b}	0.4 ± 0.2 ^{ab}	0.3 ± 0.0 ^{b}	0.6 ± 0.1 ^{ab}
Cerebrosides	nd	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	nd	nd
Pigments	1.7 ± 0.3 ^{a}	1.0 ± 0.3 ^{ab}	1.2 ± 0.4 ^{ab}	0.8 ± 0.1 ^{b}	0.7 ± 0.1 ^{b}	0.5 ± 0.2 ^{b}
Unknown	0.7 ± 0.3 ^{ab}	0.6 ± 0.2 ^{ab}	nd	0.2 ± 0.2 ^{a}	0.2 ± 0.3 ^{a}	1.4 ± 0.3 ^{b}
Cholesterol	5.4 ± 0.3 ^{a}	5.4 ± 0.9 ^{a}	4.1 ± 0.3 ^{ab}	4.4 ± 0.4 ^{ab}	3.6 ± 0.7 ^{b}	5.0 ± 1.1 ^{ab}
Free fatty acids	0.5 ± 0.1 ^{ab}	0.3 ± 0.1 ^{b}	0.9 ± 0.3 ^{a}	0.5 ± 0.1 ^{b}	0.5 ± 0.2 ^{ab}	0.5 ± 0.1 ^{b}
Triacylglycerol	56.8 ± 2.6 ^{b}	67.3 ± 5.7 ^{ab}	70.9 ± 1.8 ^{a}	71.4 ± 1.3 ^{a}	75.2 ± 2.0 ^{a}	67.9 ± 5.6 ^{ab}
Steryl esters	2.8 ± 0.7	2.4 ± 0.5	2.4 ± 0.7	3.1 ± 0.5	2.5 ± 0.3	3.2 ± 0.6
Total polar lipid	33.8 ± 1.9 ^{a}	25.0 ± 4.3 ^{ab}	21.6 ± 1.2 ^{b}	20.4 ± 1.9 ^{b}	18.0 ± 1.5 ^{b}	22.1 ± 3.6 ^{b}
Total neutral lipid	66.2 ± 1.9 ^{b}	75.0 ± 5.5 ^{ab}	78.4 ± 1.2 ^{a}	79.6 ± 1.9 ^{a}	82.0 ± 1.5 ^{a}	77.9 ± 3.6 ^{a}
Triacylglycerol/Cholesterol	10.5 ± 0.9 ^{a}	12.9 ± 2.2 ^{ab}	17.3 ± 1.4 ^{ab}	16.3 ± 1.4 ^{ab}	21.8 ± 4.7 ^{b}	14.5 ± 3.9 ^{ab}

Results are means ± SD of triplicate samples (n = 3) of pooled livers. SD=0.0 implies SD<0.05. Values within the same row bearing different superscript letters are significantly different ($p<0.05$). tr , trace<0.05; nd, not detected.

Table 6. Total lipid fatty acid composition (mass %) of liver from gilthead sea bream (*Sparus aurata*) fed control and experimental diets.

	t=0 days		t=30 days			
Fatty acid	Initial	Control	EH-0	EL-0	EH-200	EL-200
14:0	2.1 ± 0.1 ^{bc}	2.3 ± 0.1 ^{abc}	3.1 ± 0.2 ^{a}	1.8 ± 0.1 ^{c}	2.9 ± 0.5 ^{ab}	1.9 ± 0.1 ^{c}
15:0	5.5 ± 1.3 ^{a}	1.3 ± 0.6 ^{b}	1.4 ± 0.3 ^{b}	1.2 ± 0.2 ^{b}	1.4 ± 0.4 ^{b}	1.1 ± 0.4 ^{b}
16:0	10.7 ± 0.4 ^{b}	12.7 ± 0.4 ^{a}	13.2 ± 0.5 ^{a}	8.5 ± 0.1 ^{c}	13.0 ± 0.2 ^{a}	8.6 ± 0.2 ^{c}
17:0	0.5 ± 0.0 ^{a}	0.3 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	0.2 ± 0.0 ^{c}	0.2 ± 0.0 ^{c}	0.2 ± 0.0 ^{c}
18:0	4.3 ± 0.1 ^{b}	5.4 ± 0.1 ^{a}	3.8 ± 0.2 ^{bc}	2.6 ± 0.2 ^{d}	3.8 ± 0.1 ^{c}	2.4 ± 0.1 ^{d}
20:0	0.3 ± 0.1 ^{a}	0.2 ± 0.0 ^{ab}	0.1 ± 0.0 ^{ab}	0.2 ± 0.1 ^{ab}	0.1 ± 0.0 ^{b}	0.2 ± 0.1 ^{ab}
22:0	0.3 ± 0.3	0.1 ± 0.0	nd	tr	tr	nd
24:0	0.1 ± 0.1	0.1 ± 0.0	nd	nd	nd	nd
Total saturated	24.0 ± 0.9 ^{a}	22.5 ± 1.1 ^{ab}	22.0 ± 0.4 ^{ab}	14.6 ± 0.2 ^{c}	21.5 ± 0.4 ^{b}	14.4 ± 0.3 ^{c}
16:1(n-9)	nd	nd	0.1 ± 0.0	tr	0.2 ± 0.0	tr
16:1(n-7)	3.3 ± 0.6 ^{b}	5.1 ± 0.3 ^{ab}	6.3 ± 0.2 ^{a}	4.9 ± 1.0 ^{ab}	6.4 ± 0.2 ^{a}	4.4 ± 0.8 ^{ab}
18:1(n-9)	12.4 ± 0.5 ^{c}	20.6 ± 1.2 ^{b}	19.2 ± 0.8 ^{b}	42.8 ± 0.3 ^{a}	19.4 ± 0.8 ^{b}	42.1 ± 0.9 ^{a}
18:1(n-7)	2.6 ± 0.2 ^{c}	3.8 ± 0.2 ^{b}	4.0 ± 0.0 ^{b}	5.0 ± 0.2 ^{a}	4.0 ± 0.0 ^{b}	4.9 ± 0.0 ^{a}
20:1(n-9)	1.0 ± 0.1 ^{d}	1.8 ± 0.3 ^{c}	5.3 ± 0.3 ^{a}	2.3 ± 0.1 ^{b}	5.5 ± 0.1 ^{a}	2.5 ± 0.1 ^{b}
20:1(n-7)	nd	0.2 ± 0.1 ^{ab}	0.3 ± 0.0 ^{a}	0.1 ± 0.0 ^{c}	0.3 ± 0.0 ^{a}	0.2 ± 0.0 ^{bc}
22:1(n-11)	0.4 ± 0.6 ^{c}	1.2 ± 0.1 ^{bc}	5.2 ± 0.2 ^{a}	1.4 ± 0.1 ^{b}	5.3 ± 0.0 ^{a}	1.7 ± 0.1 ^{b}
22:1(n-9)	nd	0.4 ± 0.0 ^{b}	1.1 ± 0.1 ^{a}	0.5 ± 0.0 ^{b}	1.1 ± 0.1 ^{a}	0.5 ± 0.0 ^{b}
24:1(n-9)	0.2 ± 0.3 ^{b}	0.7 ± 0.0 ^{a}	1.2 ± 0.1 ^{a}	0.7 ± 0.0 ^{a}	1.1 ± 0.1 ^{a}	0.6 ± 0.0 ^{a}
Total monoenes	19.9 ± 2.1 ^{d}	33.7 ± 2.1 ^{c}	42.7 ± 0.8 ^{b}	57.8 ± 0.4 ^{a}	43.2 ± 1.0 ^{b}	56.9 ± 0.4 ^{a}
16:2(n-6)	1.8 ± 0.2 ^{a}	0.1 ± 0.0 ^{d}	0.3 ± 0.0 ^{c}	0.5 ± 0.0 ^{b}	0.3 ± 0.0 ^{bc}	0.4 ± 0.0 ^{bc}
16:3(n-3)	1.8 ± 0.3 ^{a}	0.7 ± 0.1 ^{c}	0.6 ± 0.0 ^{c}	1.3 ± 0.1 ^{ab}	0.6 ± 0.0 ^{c}	1.3 ± 0.1 ^{b}
16:4(n-3)	nd	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{a}	0.1 ± 0.0 ^{b}	0.3 ± 0.0 ^{a}	0.2 ± 0.0 ^{b}
18:2(n-6)	9.7 ± 0.5 ^{b}	11.4 ± 0.4 ^{a}	2.1 ± 0.3 ^{d}	3.9 ± 0.1 ^{c}	1.9 ± 0.1 ^{d}	4.1 ± 0.0 ^{c}
18:3(n-6)	2.6 ± 0.3	nd	nd	nd	nd	nd
18:3(n-3)	1.5 ± 0.1 ^{b}	1.8 ± 0.0 ^{a}	0.7 ± 0.1 ^{c}	0.6 ± 0.0 ^{c}	0.7 ± 0.1 ^{c}	0.8 ± 0.0 ^{c}
18:4(n-3)	1.2 ± 0.1 ^{a}	1.4 ± 0.2 ^{a}	1.5 ± 0.1 ^{a}	0.8 ± 0.0 ^{b}	1.6 ± 0.1 ^{a}	0.8 ± 0.0 ^{b}
20:2(n-6)	0.2 ± 0.0	0.4 ± 0.2	0.2 ± 0.0	1.0 ± 0.8	0.2 ± 0.0	0.2 ± 0.0
20:3(n-6)	0.4 ± 0.1 ^{a}	0.6 ± 0.1 ^{a}	0.2 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}	0.2 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}
20:4(n-6)	1.2 ± 0.1 ^{a}	1.0 ± 0.1 ^{ab}	0.8 ± 0.1 ^{b}	0.4 ± 0.0 ^{c}	0.8 ± 0.1 ^{b}	0.5 ± 0.0 ^{c}
20:3(n-3)	nd	0.1 ± 0.0	0.1 ± 0.0	tr	0.1 ± 0.0	0.1 ± 0.0
20:4(n-3)	0.5 ± 0.0 ^{c}	0.7 ± 0.1 ^{b}	1.0 ± 0.0 ^{a}	0.4 ± 0.1 ^{c}	1.0 ± 0.1 ^{a}	0.4 ± 0.0 ^{c}
20:5(n-3)	4.5 ± 0.2 ^{a}	4.5 ± 0.3 ^{a}	5.2 ± 0.3 ^{a}	2.4 ± 0.0 ^{b}	5.3 ± 0.3 ^{a}	2.8 ± 0.1 ^{b}
22:5(n-6)	0.4 ± 0.1 ^{a}	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{a}	0.1 ± 0.0 ^{c}	0.3 ± 0.0 ^{a}	0.1 ± 0.0 ^{b}
22:4(n-3)	nd	nd	nd	nd	0.1 ± 0.0	nd
22:5(n-3)	1.0 ± 0.1 ^{b}	1.6 ± 0.2 ^{a}	1.7 ± 0.1 ^{a}	0.7 ± 0.1 ^{b}	1.8 ± 0.2 ^{a}	0.8 ± 0.1 ^{b}
22:6(n-3)	11.6 ± 0.5 ^{ab}	9.7 ± 1.0 ^{b}	13.1 ± 0.5 ^{a}	6.1 ± 0.2 ^{c}	13.1 ± 1.0 ^{a}	7.5 ± 0.4 ^{c}
Total polyenes	38.4 ± 0.9 ^{a}	34.5 ± 1.4 ^{b}	28.1 ± 0.6 ^{c}	18.6 ± 0.2 ^{d}	28.4 ± 2.0 ^{c}	20.1 ± 0.6 ^{d}
Unknown	9.1 ± 1.3	9.3 ± 0.8	7.2 ± 0.6	9.1 ± 0.4	7.0 ± 0.8	8.7 ± 0.4
Total (n-9)	13.5 ± 0.9 ^{d}	23.5 ± 1.4 ^{c}	26.9 ± 0.9 ^{b}	46.3 ± 0.4 ^{a}	27.2 ± 0.8 ^{b}	45.8 ± 0.9 ^{a}
Total (n-7)	6.0 ± 0.6 ^{b}	9.0 ± 0.5 ^{a}	10.6 ± 0.2 ^{a}	10.1 ± 0.8 ^{a}	10.6 ± 0.3 ^{a}	9.4 ± 0.8 ^{a}
Total (n-6)	16.3 ± 0.9 ^{a}	13.8 ± 0.1 ^{b}	3.9 ± 0.2 ^{d}	6.0 ± 0.7 ^{c}	3.8 ± 0.2 ^{d}	5.4 ± 0.0 ^{c}
Total (n-3)	22.1 ± 0.4 ^{ab}	20.7 ± 1.4 ^{b}	24.2 ± 0.7 ^{ab}	12.6 ± 0.5 ^{c}	24.6 ± 1.8 ^{a}	14.6 ± 0.6 ^{c}
HUFA(n-6)	2.0 ± 0.1 ^{a}	1.8 ± 0.2 ^{a}	1.3 ± 0.1 ^{b}	0.6 ± 0.0 ^{c}	1.3 ± 0.1 ^{b}	0.7 ± 0.0 ^{c}
HUFA(n-3)	17.6 ± 0.5 ^{b}	16.7 ± 1.3 ^{b}	21.1 ± 0.6 ^{a}	9.7 ± 0.4 ^{c}	21.3 ± 1.6 ^{a}	11.6 ± 0.6 ^{c}

Results are means ± SD of triplicate samples (n = 3) of pooled livers. SD = 0.0 implies SD < 0.05. Values within the same row bearing different superscript letter are significantly different ($p<0.05$). tr, trace < 0.05; nd, not detected; HUFA, highly unsaturated fatty acids.

Table 7. Levels of lipid peroxidation products (TBARS and 8-Isoprostane), vitamin E content and activities of antioxidant enzymes of liver from gilthead sea bream (*Sparus aurata*) fed control and experimental diets.

	t=0 days	t=30 days				
	Initial	Control	H0	L0	H200	L200
Vitamin E (alpha-tocopherol)						
ng · mg^{-1} live mass	222.2±10.1 ^{b}	237.1±8.1 ^{b}	52.6±5.9 ^{c}	185.3±9.4 ^{b}	193.1±30.0 ^{b}	554.7±24.1 ^{a}
ng · mg^{-1} dry mass	718.3±32.5 ^{b}	649.8±22.1 ^{bc}	139.4±15.5 ^{e}	489.1±24.9 ^{d}	510.9±79.3 ^{cd}	1452.1±63.0 ^{a}
µg · liver^{-1}	12.0±0.5 ^{b}	12.8±0.4 ^{b}	3.6±0.4 ^{c}	10.4±0.5 ^{b}	13.1±2.0 ^{b}	33.6±1.5 ^{a}
Molar ratio PUFA/vitamin E	22.9±2.9 ^{bc}	30.9±1.2 ^{b}	91.1±13.2 ^{a}	17.5±1.2 ^{c}	25.1±6.3 ^{bc}	5.7±0.4 ^{d}
TBARS						
µmol · mg^{-1} live mass	2.3±0.2 ^{ab}	2.4±0.2 ^{ab}	2.7±0.1 ^{a}	1.6±0.1 ^{b}	2.2±0.5 ^{ab}	1.7±0.2 ^{b}
µmol · mg^{-1} dry mass	7.5±0.6 ^{a}	6.6±0.7 ^{ab}	7.1±0.2 ^{a}	4.2±0.4 ^{b}	5.9±1.2 ^{ab}	4.5±0.5 ^{b}
µmol · liver^{-1}	22.6±1.9 ^{a}	129.2±13.1 ^{bcd}	182.0±3.9 ^{b}	89.8±8.0 ^{d}	151.0±30.9 ^{bc}	105.0±11.6 ^{cd}
Isoprostane (pg · mg protein $^{-1}$)	18.3±0.0 ^{b}	16.1±5.4 ^{b}	64.9±17.3 ^{a}	31.9±4.2 ^{b}	19.7±2.2 ^{b}	36.9±3.6 ^{b}
Antioxidant enzyme activities						
CAT [1]	38.6±6.6 ^{c}	81.1±5.9 ^{a}	66.8±9.2 ^{b}	65.8±7.1 ^{b}	67.2±5.7 ^{b}	61.6±4.0 ^{b}
SOD [2]	2.8±0.0 ^{b}	3.7±0.4 ^{a}	3.0±0.1 ^{ab}	3.2±0.3 ^{ab}	3.0±0.3 ^{ab}	3.3±0.2 ^{ab}
GPX [3]	5.8±0.0	3.1±1.2	5.0±1.2	5.2±0.9	4.4±2.1	5.2±0.6
GST [3]	363.6±43.8	323.9±87.5	329.6±32.0	293.1±47.6	320.4±38.6	371.1±38.7
GR [3]	1.9±0.2 ^{bc}	0.3±0.0 ^{c}	3.8±1.3 ^{abc}	6.0±1.0 ^{a}	3.7±1.4 ^{abc}	4.2±1.4 ^{ab}

Results are means ± SD of triplicate samples (n = 3) of pooled livers. Values bearing different superscript letter are significantly different ($p<0.05$). SD = 0.0 implies SD<0.05. nd, not detected; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione transferase; PUFA polyunsaturated fatty acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances. [1] (µmol · min^{-1} · mg protein^{-1}). [2] (SOD units · min^{-1} · mg protein^{-1}) [3](nmol · min^{-1} · mg protein^{-1}).

Table 8. Results (F-values) of two-ways analysis of variance for the data from the four experimental diets (H0, L0, H200, L200)

	F-values		
	A) Vitamin E	B) Fish oil	Interaction AxB
TBARS			
µmol/mg live mass	ns	13.74**	ns
µmol/mg dry mass	ns	15.85**	ns
µmol/Liver	ns	26.79**	ns
Isoprostane (pg mg protein $^{-1}$)	15.5**	ns	22.6**
Vitamin E (alpha-tocopherol)			
ng /mg live mass	324.47***	304.84***	65.40***
ng /mg dry mass	320.05***	299.51***	62.88***
µg /Liver	320.86***	223.33***	56.14***
Liver molar ratio PUFA/vitamin E	55.78***	79.49***	27.13***
Antioxidant enzyme activity			
Catalasc	ns	ns	ns
SOD	ns	ns	ns
GPX-Se	ns	ns	ns
GSH S-transferase	ns	ns	5.37*
GSSG reductase	ns	4.79*	ns
Biometry			
Fish length (mm)	ns	ns	ns
Fish live mass (g)	ns	ns	ns
Fish dry mass (mg)	ns	ns	ns
Fish dry mass %	6.20*	8.04**	ns
Liver live mass (mg)	ns	9.60*	ns
Liver dry mass (mg)	ns	9.82**	ns
Liver dry mass %	ns	ns	ns
HSI	ns	ns	ns
Mortality (nº of fish)	ns	ns	ns
Mortality (%)	ns	ns	ns
Liver gross composition (% dry mass)			
Protein			
Lipid	ns	ns	ns
Carbohydrate			
Ash			

PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances. Significance levels are *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns $p>0.05$.