‘Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (Sparus aurata L.) fed dietary oxidised oil: attenuation by dietary vitamin E’ by G. Mourente, E. Díaz-Salvago, J. G. Bell and D. R. Tocher. Aquaculture, Volume 214, Issues 1-4, November 2002, pp. 343 – 361.

Published in Aquaculture by Elsevier. Aquaculture, Volume 214, Issues 1-4, November 2002, pp. 343 - 361.

This is the peer reviewed version of this article.

Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L., Osteichthyes, Sparidae) fed dietary oxidised oil: Attenuation by dietary vitamin E

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

*[1] Departamento de Biología, 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)*

Key words: (n-3) HUFA, oxidised oil, vitamin E, antioxidant enzymes, gilthead sea bream, *Sparus aurata*.

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 (?= C_{20} and with = 3 double bonds); 8-isoprostane, 8-*iso*-prostaglandin $F_{2?}$; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances.

Abstract

Previously, we had shown that altering the highly unsaturated fatty acid(HUFA)/vitamin E ratios in sea bream (*Sparus aurata*) livers significantly affected their peroxidation status, with fish fed the diet rich in HUFA and low in vitamin E showing significantly higher values of lipid peroxidation products, without, however, significant effects on liver antioxidant defence enzyme activities. The aim of the present trial was to further characterise the biochemical indicators of peroxidative stress in juvenile sea bream. A high pro-oxidative stress was induced by feeding diets containing around 7% of the dry weight as n-3HUFA. The potential peroxidative stress was increased by oxidising the oil, increasing the peroxide value of the oil some 10-fold. These oils were fed without or with supplemental vitamin E (α-tocopherol acetate at 200 $mg.kg^{-1}$ dry diet) giving four diets in total. Fish were sampled after 30 and 60 days of feeding the experimental diets. None of the diets had any serious deleterious effects on growth and mortality of the fish during the trial. Similarly, there were few significant effects due to dietary oxidised oil or supplementary vitamin E on liver lipid and fatty acid profiles of livers and, in particular, the proportions of HUFA were not decreased by dietary oxidised oil. The vitamin E content of the liver reflected the vitamin E contents of the diets but was also affected by dietary oxidised oil being reduced by oxidised oil in fish fed diets without supplemental vitamin E but, unexpectedly, increased by oxidised oil in fish fed diets supplemented with vitamin E. Liver TBARS levels were significantly lower in fish fed diets supplemented with vitamin E whereas dietary oxidised oil had no major effects on lipid peroxidation products. Catalase and superoxide dismutase activities were both increased in fish fed dietary oxidised oil and reduced by supplementary vitamin E after 30 days feeding. In contrast glutathione peroxidase was less affected by the diets, and the activities of glutathione-S-transferase and glutathione reductase were only reduced by dietary vitamin E after 60 days of feeding. However, all the enzyme activities were significantly affected by the duration of feeding, but the number of interactions between the three factors (time, oil and vitamin E) showed that the relationships were complicated. In conclusion, the present study showed that feeding diets containing oxidized oil significantly affected the activities of liver antioxidant defence enzymes and that dietary vitamin E partially abrogated these effects. Growth and survival of the fish were relatively unaffected suggesting that the responses in sea bream offered effective protection. However, the duration of feeding the diets of high pro-oxidative stress was observed to have a hitherto unknown effect, possibly the result of an adaptive process, but which requires further investigation.

Introduction

Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation is highly deleterious, resulting in damage to cellular biomembranes as a consequence of oxidative deterioration of membrane lipids (Kanazawa, 1991;1993). In fish, *in vivo* lipid peroxidation caused by oxygen radicals is a principal cause of several diseases (Kawatsu, 1969; Sakai et al., 1989; 1998; Watanabe et al., 1970; Murai and Andrews, 1974). Tissue lipid PUFA content and unsaturation index are critical factors in lipid peroxidation (Porter et al., 1995), and as marine fish tissues contain large quantities of n-3 highly unsaturated fatty acids (HUFA) (Sargent et al., 1999), there is a high risk of peroxidative attack and damage. Marine fish must obtain preformed HUFA in their diet as they are unable to synthesise HUFA themselves due to deficiencies in the desaturation and elongation pathway necessary for their biosynthesis (Sargent et al., 1999). Therefore, HUFA are, on one hand, essential for optimal growth and development of marine fish, but they also impose a significant peroxidation burden.

All animals have sysyems to combat *in vivo* oxidation and thus maintain health and prevent oxidation-induced lesions (Jacob, 1995). In consequence, the health of aquatic organisms is linked to overproduction of reactive oxygen species and antioxidants protect cell membranes against the production of free radicals. Thus, normal metabolism depends upon the ratio of free radical production and the activity of lipid peroxidation protection factors. These systems include various antioxidant compounds, principal among them being the dietary micronutrient tocopherol (vitamin E) which serves as a radical sink and antioxidant defence enzymes (Burton, 1990; Buettner, 1993). The enzymes include radical scavenging enzymes such as catalase and superoxide dismutase (SOD) acting on hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\cdot}$), respectively, and glutathione peroxidase (GPX) which scavanges H_2O_2 and lipid hydroperoxides (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1996). Associated enzymes include glutathione reductase (GR) which acts to maintain levels of reduced glutathione, and glutathione-S-transferase (GST) some isoenzymes of which may metabolize lipid hydroperoxides (Halliwell and Gutteridge, 1996; Martinez-Lara et al., 1996).

The overall 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. Our previous work (Mourente et al., 2000) indicated that feeding diets with graded PUFA/vitamin E ratios resulted in graded degrees of peroxidative stress as

evidenced by the leveles of lipid peroxidation products measured in juvenile sea bream, but the responses of the liver antioxidant defence enzyme activities were only moderate. The specific aim of the present trial was to induce a more stressful pro-oxidant status to enable further characterisation of the biochemical indicators of peroxidative stress without causing unneccessary suffering or high mortalities during the trials. Thus, the level of HUFA was increased in the present trial by increasing the content of the diet and by using an oil with a much higher n-3HUFA level (a high quality anchovy oil concentrate). To further increase the potential peroxidative stress, oxidised oil, with a 10-fold higher peroxide value was also used. Therefore, the dietary trial principally had a factorial two design (oxidised (X) v. unoxidised oil and ± vitamin E) giving four diets, HO, HE, HXO and HXE. However, fish were sampled after 30 and 60 days of feeding the experimental diets adding a third factor, time. The effects of dietary oxidised oil, with or without a dietary vitamin E, on survival and growth parameters of the sea bream were determined as well as effects on liver vitamin E, lipid and fatty acid contents and compositions. In addition, the effects of dietary oxidised oil and vitamin E on the activities of the hepatic antioxidant defence enzymes, and liver lipid peroxidation products, including malondialdehyde, measured as thiobarbituric acid reactive substances (TBARS), and isoprostanes, were determined.

Materials and methods

Experimental diets

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 anchovy oil concentrate (both oxidised and unoxidised) 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). Vitamin E was removed from the anchovy oil concentrate by charcoal absorption and half of the oil was pre-oxidised by heating at 50 ºC with vigorous aeration for 24 h, with the degree of oxidation monitored by determination of the peroxide value at 8 h intervals. Four diets of two pellet sizes (500 and 1500 μm, respectively) were produced. The lipid contents, PUFA and vitamin E levels, peroxide values and the fatty acid content of the diets as g fatty acid per Kg of dry diet of the diets are shown in Table 2.

Experimental fish and dietary trial conditions

Sea bream from the same batch, completely weaned, with a functional swimbladder, were obtained from CUPIMAR S. A. (Cadiz, Andalucia, Spain). After acclimatisation to the experimental diet and conditions in the aquarium facilities of the University of Cadiz, Cadiz, for two weeks, the 80 days post-hatch fish with a live mass of 1.52 ± 0.21 g were randomly stocked at an initial density of 5 fish/l into rectangular tanks of 100 l each. Each diet was fed to triplicate tanks. 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 daylight hours by hand. The experiment was established at a terminated after 1200 days-degree (60 days) with a final sampling after an intermediate sampling at the mid-point, 600 days-degree (30 days). The tanks were in an open system continuously supplied with running borehole water of 39 ppt salinity at a temperature of 19.4 ± 0.2ºC. Light was natural photoperiod conditions. The water was treated with biological filters to eliminate ammonia by nitrification processes. Water samples were assayed weekly after filtration through a 0.45 μm membrane prior to analysis. Water quality (NH_3/NH_4^+, NO_2^-, NO_3^-) variables in the rearing tanks were determined with a Technicon Traacs 800 Autoanalyser and water maintained 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}).

Sample collection and biometric determinations

There were three sampling points, at the beginning (initial), mid-point (day 30) and final end-point of the experiment (day 60). 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 percentage of fish surviving.

Three samples of liver per treatment were collected for lipid and fatty acid analysis, vitamin E content, lipid peroxidation status (lipid oxidation products, TBARS and

isoprostane) and liver antioxidant defence 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 homogenisation 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-catalysed 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 ionisation 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 homogenised 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 were determined in the same homogenates of liver and whole fish that were prepared for TBARS analyses. Samples were assayed after storage at –80ºC, to avoid artifacts of prolonged storage at temperatures above –80ºC. Most of the 8-isoprostane is esterified in lipids, so an extraction and hydrolysis was 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 4ºC 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 40ºC. 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 homogenised 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 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 ?l 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 basically to a 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. However, a third factor, time (duration of feeding of diets) was introduced due to the two sampling points (intermediate and end of trial). Results are presented as means ± SD (n = 3 or as otherwise stated). 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 analysed by two- and, three-way analysis of variance (ANOVA). Differences were reported as statistically significant when $P < 0.05$ (Zar, 1984).

Results

The diets had a high PUFA content of around 80 g.kg-1 of diet and a consequently

high unsaturation index of around 345. The high PUFA content of the diets was primarily due to very high levels of n-3HUFA (Table 2). The TBARS contents were all similar but the peroxide values were significantly higher (up to 9-fold) in the diets containing the oxidised oil. The vitamin E contents of the unsupplemented diets were around 36 $mg.kg^{-1}$ diet whereas they were significantly greater in the supplemented diets at between 200 and 260 $mg.kg^{-1}$ diet and, as a result, the PUFA/vitamin E molar ratios were 5 to 8-fold higher in the unsupplemented diets (Table 2).

None of the diets had any serious deleterious effects on growth and mortality of the fish during the trial (Table 3). Dietary oxidised oil decreased growth slightly after 60 days of feeding whereas it had a slight stimulatory effect after 30 days. Conversely, dietary vitamin E tended to increase growth at 30 days but depressed growth at the end of the trial. Mortality was very low during the experiment but Vitamin E supplementation slightly decreased mortality at 30 days although the effect was not apparent at 60 days (Table 3). Liver weights and HIS tended to be lower in fish fed the vitamin E supplemented diets (Table 3).

Dietary oxidised oil increased the proportions of triacylglycerol total neutral lipid in sea bream livers after 30 days feeding but not at 60 days (Table 4). There was virtually no significant dietary effects due oxidised oil or supplementary vitamin E on the total lipid fatty acid profiles from livers of fish fed the different dietary treatments (Table 4). In particular, the proportions of PUFA were not decreased by dietary oxidised oil.

The vitamin E content of the liver was significantly affected by the diets and reflected the vitamin E contents of the diets, being significantly increased in the livers of fish fed the diets supplemented with vitamin E (Table 5). Similarly, the liver PUFA/vitamin E molar ratio was significantly reduced by feeding the diets supplemented with vitamin E. Dietary oxidised oil also significantly affected liver vitamin E levels although the precise effects were dependent upon dietary vitamin E as evidenced by the significant interaction. Thus, dietary oxidised oil tended to result in lower liver vitamin E levels in fish fed the diets without supplemental vitamin E whereas the opposite was the case in fish fed diets supplemented with vitamin E (Table 5). All these effects on liver vitamin E levels were observed at both 30 and 60 days.

Catalase and SOD activities were both increased by dietary oxidised oil and reduced by dietary vitamin E after 30 days feeding (Table 5). At 60 days, oxidised oil had no significant effect on these enzyme activities but vitamin E still reduced catalase activity whereas it now increased SOD activity. Catalase activity increased significantly from 30 to 60 days feeding in fish fed fresh oil and decreased significantly in fish fed oxidised oil, independent of the level of dietary vitamin E. In contrast, glutathione peroxidase was less

affected by the diets and glutathione-S-transferase was only reduced by dietary vitamin E after 60 days of feeding. Liver TBARS levels were significantly lower in fish fed the diets supplemented with vitamin E whereas dietary oxidised oil had no major effects (Table 5). In contrast, the levels of liver isoprostanes showed no consistent patterns.

Table 6 shows the results of three-way ANOVA of selected data from Tables 4 and 5. Combining the data for both time points (as occurs with ANOVA) showed that the significant effect on growth parameters and lipid peroxidation were primarily due to dietary vitamin E whereas the overall effects on enzyme activities were primarily due to dietary oxidised oil. The three-way ANOVA was also able to quantify the effects of time with the biometry highly dependent on time, obviously due to fish growth but also that the fatty acid composition was affected significantly by time with liver PUFA tending to increase and monoenes decrease from 30 to 60 days. All the enzyme activities were significantly affected by time but the large number of interaction between the three factors shows that the relationships were complicated and as aresult difficult to interpret. Liver TBARS were lower after 60 days compared to 30 days when measured relative to mass but were higher per liver due to the increased size of the liver.

Discussion

Our previous study investigated the antioxidant systems in cultured juvenile gilthead sea bream (*S. aurata*) by feeding diets varying greatly in PUFA/vitamin E ratio through having either high or low levels of fish oil (n-3HUFA) combined with the presence or absence of vitamin E (Mourente et al., 2000). None of the diets in that study had serious deleterious 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. However, no significant effects on antioxidant enzyme activities were observed suggesting that more severe conditions of oxidative stress were required to affect the enzyme activities. In the present study, feeding diets containing oxidized oil, has had significant effects on the antioxidant enzyme activities. The present study also showed that time or duration of feeding was an important additional factor to consider in relation to determiniong the biochemical responses to oxidative stress.

As in the previous study, none of the diets in the present study had any grossly

deleterious effects on the overall health and well-being of the fish, which showed excellent growth with very low levels of mortality. The different diets had only relatively minor affects on lipid and fatty acid compositions. In an earlier study in juvenile African catfish (*Clarius gariepinus*), dietary oxidised oil (cod liver oil/corn oil, 1:1) in the presence of low vitamin E reduced growth and supplementation with vitamin E partially abrogated this effect (Baker and Davies, 1997). The growth of fish fed fresh oil was not increased by elevated dietary vitamin E. The level of vitamin E in the livers of catfish fed the oxidised oil was greatly reduced (Baker and Davies, 1997). This effect had been observed previously in sea bass (*Dicentrarchus labrax*) where tissue α-tocopherol levels were reduced in fish fed diets containing oxidised fish oil (Obach et al., 1993). In the present study, the level of hepatic α-tocopherol was reduced in sea bream fed the diets without supplemental vitamin E, consistent with the previous data. Baker and Davies (1996) also observed apparently increased production of docosahexaenoic acid (22:6n-3; DHA) in response to feeding oxidised oils in catfish. However, in the present study, there was no evidence of increased production of DHA, or PUFA in general, in response to dietary oxidised oil which could be due to environmental and dietary differences and/or differences in fatty acid metabolism among fish species (Sargent et al., 1995; 1999).

Overall, many of the effects on the enzyme activities observed in the present study were generally consistent with the commonly perceived biochemical mechanisms of these enzyme systems (Winston and Di Giulio, 1991; Miller et al., 1993; Halliwell and Gutteridge, 1996). Previous studies in which these enzyme activities have been measured in fish have often focussed on their role in pollutant detoxification (Livingstone et al., 1992, 1993; Peters et al., 1994; Martinez-Lara et al., 1996) or developmental aspects (Aceto et al., 1994; Otto and Moon, 1996; Peters and Livingstone, 1996). No interactions were observed between dietary vitamin E and antioxidant defence enzyme activities in Atlantic salmon (Lygren et al., 2000) and no clear relationship between dietary or tissue PUFA/vitamin E ratios and liver antioxidant enzyme activities were observed in gilthead sea bream (Mourente et al., 2000). The present study has indicated that a more direct peroxidative stress, such as that induced by feeding oxidised oil is required before clear effects on the liver antioxidant enzyme activities are observed.

However, both dietary oxidised oil and supplemental dietary vitamin E had significant effects on liver antioxidant defence enzyme activities and lipid peroxidation products. The activities of the liver enzymes were significantly affected by dietary oxidised oil and vitamin E supplementation. As a result of enzymic activity, feeding oxidised oil did not generally increase lipid peroxidation products in liver of sea bream but they were generally reduced by

dietary vitamin E. Interestingly, the activities of the liver enzymes were also significantly affected by the time of exposure to the dietary treatments. Thus, the activities of the two primary radical scavenging enzymes, catalase and SOD, were increased by dietary oxidised oil and reduced by supplementary vitamin E after 30 days of feeding. The activities of these two enzymes would be expected to parallel each other based on the known mechanisms of the enzymes and the fact that superoxide anions are known to be efficiently scavenged by vitamin E in biological systems (Cay and King, 1980). However, after 60 days, the effects on catalase and SOD activities were less apparent, due to a diminished effect of oxidised oil in the case of catalase, and vitamin E no longer reducing the activity of SOD. It was also interesting that the effects of dietary oxidised oil on the activities of GPX and GST, which, based on their probable roles in the antioxidant system may be expected to be more influenced by lipid peroxides, were inconsistent, but also significantly affected by time of exposure. In particular, GST activity was not significantly different between treatments at 30 days, whereas both dietary vitamin E and oxidised oil decreased it's activity at 60 days. Effects on GR activity were also highly dependent on the time of exposure and, as with GST activity, both dietary vitamin E and oxidised oil decreased the activity of GR after feeding the experimental diets for 60 days.

The precise reasons for the effects of length of exposure to the experimental diets on the liver enzymes was unclear. However, the data perhaps suggest that the mid-point sampling (30 days) occurred while the enzymes were in a supra-induced state and that the end-point (60 days) represents an adapted state. Additionally, the data suggest that the level of hydrogen peroxide may play a critical role in the regulation and expression of antioxidant defence enzymes, at least in sea bream. Thus removal of hydrogen peroxide by catalase and GPX (primarily catalase due to it's much higher affinity and specificity and, thus, overall activity) may be rapid in sea bream, due to the fact that both these enzymes activities were high irrespective of the dietary treatment. However, the different responses of catalase and GPX may also indicate a different mechanism for regulation of gene expression for GPX, perhaps related to the fact that GPX is more involved in the removal of organic peroxides and, only to a much lesser extent, hydrogen peroxide.

Furthermore, the regulation through gene expression of antioxidant defence enzymes, particularly catalase, differs from one cell system to another (Rohrdanz and Kahl, 1998). It has been established that the activities of antioxidant defence enzyme activities in fish vary in different organs of fresh water and marine fish (Wdzieczak et al., 1982; Lemaire et al., 1993), depending upon feeding behaviour (Radi and Markovics, 1988) and other ecological conditions (Winston and Di Giulio, 1991). Furthermore, it can be assumed that low

molecular weight antioxidants (urea, ascorbic acid and α-tocopherol) appeared earlier in the process of evolution than antioxidant defence enzymes, and antioxidant systems in fish depend not only on taxonomic position of the fish species but also on their ecological peculiarities, particularly natural mobility (Rudneva, 1997). These are perhaps other reasons why studies with fish have generally failed to reveal consistent responses in antioxidant defence enzyme activities (Di Giulio et al., 1995).

In conclusion, the present study showed that feeding diets containing oxidised oil significantly affected the activities of liver antioxidant defence enzymes, in particular increasing the activities of two of the primary radical scavenging enzymes, catalase and superoxide dismutase, and that dietary vitamin E partially abrogated these effects. Growth and survival of the fish were relatively unaffected indicating that the responses gave effective protection against this pro-oxidative dietary stress. This suggested that gilthead sea bream has a well-developed and efficient liver antioxidant defence enzyme system and mechanisms for a marine teleost. However, the duration of feeding the diets of high pro-oxidative stress was observed to have a hitherto unknown effect, possibly the result of a further adaptive process, but which warrants further investigation.

Acknowledgements

This work was funded by a European Community Research Programme in the Fisheries Sector (FAIR), Contract nº FAIR CT97-3382.

References

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

Baker, R.T.M., Davies, S.J., 1996. Changes in tissue α-tocopherol status and degree of lipid peroxidation with varying α-tocopheryl-acetate inclusion in diets for African catfish. Aquaculture Nutr. 2, 71-79.

Baker, R.T.M., Davies, S.J., 1997. Modulation of tissue α-tocopherol in African catfish, *Clarias gariepinus* (Burchell), fed oxidized oils, and the compensatory effect of supplemental dietary vitamin E. Aquacult. Nutr. 3, 91-97.

Beers, R.F., 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., 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., 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.

Buettner, G.R., 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, α- tocophenol, and ascorbate. Arch. Biochem. Biophys. 300, 535-543.

Burk, R.F., Trumble, M.J., 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.

Burton, G.W., 1990. Vitamin E: Antioxidant activity, biokinetics, and bioavailability. Annu. Rev. Nutr. 10, 357-82.

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

Cay, P. B., King, M. M., 1980. Vitamin E: It's role as a biological free radical scavenger and it's relationship to the microsomal mixed function oxidase system. In: Machlin, L.J. (Ed.), Vitamin E, A Comprehensive Treatise: Basic and Clinical Nutrition 1. Marcel Dekker Inc., New York, pp. 289-317.

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

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical Mechanisms: Metabolism, Adaptation and Toxicity. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environment Fate, and Risk Assessment. Taylor and Francis, London, pp. 523-562.

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

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

Folch, J., Lees, M., 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., Jacoby, W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercaturic acid formation. J. Biol. Chem. 249, 7130-7139.

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

Huo, J-Z., Nelis, H.J., Lavens, P., Sorgeloos, P., 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

Jacob, R.A. 1995. The integrated antioxidant system. Nutr. Res. 15: 755-766.

Kanazawa, K. 1991. Hepatotoxicity caused by dietary secondary products originating from lipid peroxidation. In: Friedman, M. (Ed.), Nutritional and Toxicology Consecuences of Food Procesing. Plenium Press, New York, pp. 237-253.

Kanazawa, K. 1993. Tissue injury induced by dietary products of lipid peroxidation. In: Corongiu, F. (Ed.), Free Radicals and Antioxidants in Nutrition. Richelieu Press, pp 383-399.

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.

Lemaire, P., Viarengo, A., Canesi, L., Livingstone D.R. 1993. Pro-oxidant and antioxidant processes in gas gland and other tissues of cod (*Gadus morhua*). J. Comp. Physiol. 163, 477-486.

Livingstone D.R, Archibald S., Chipman J.K., Marsh J.W. 1992. Antioxidant enzymes in liver of dab *Limanda limanda* from the North Sea. Mar. Ecol. Prog. Ser. 91, 97-104.

Livingstone D.R, Lemaire, P., Matthews, A., Peteres, L., Bucke, D., Law, R.J. 1993. Pro-oxidant and antioxidant responses in liver of dab (*Limanda limanda*) exposed to sediment contaminated with hydrocarbons and other chemicals. Mar. Pollut. Bull. 26, 602-606.

Lowry, O.H., Roseborough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lygren, B., Hamre, K., Waagbo, R. 2000. Effect of induced hyperoxia on the antioxidant status of Atlantic salmon *Salmo salar* L. fed three different levels of dietary vitamin E. Aquaculture Res. 31, 401-407.

Martinez-Lara, E.; Toribio, F.; López-Barea, J., Barcena, J.A. 1996. Glutathione-S-transferase isoenzyme patterns in the gilthead seabream (*Sparus aurata*) exposed to enviromental contaminants. Comp. Biochem. Physiol. 113C, 215-220.

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

Mourente, G., Díaz-Salvago, E., Tocher, D.R., J.G. Bell. 2000. 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). Fish Physiol. Biochem. 23, 337-351.

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

Obach, A., Quentel, C., Laurencin, F.B. 1993. Effects of alpha-tocopherol and dietary oxidized fish oil on the inmune response of sea bass *Dicentrarchus labrax*. Dis.aquat.org. 15, 175-185.

Olsen, R.E., 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., 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., Loktaeva, T.D. 1975. Intramitochondrial localization and release of rat liver superoxide dismutase. FEBS Lett. 55, 84-87.

Peters, L.D., 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., 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.

Porter, N.A., Caldwell, S.E., Mills, K.A. 1995. Mechanisms of free radical oxidation of unsaturated lipids. Lipids 30, 277-290.

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

Radi, A.A.R., Markovics, B. 1988. Effects of metal ions on the antioxidant enzyme activities, proteins contents and lipid peroxidation of carp tissues. Comp. Biochem. Physiol. 90C, 69-72.

Rohrdanz, E., Kahl, R. 1998. Alteration of antioxidant enzyme expression in response to hydrogen peroxide. Free. Rad. Biol. Med. 24, 27-38.

Rudneva, I.I. 1997. Blood antioxidant system of Black Sea elasmobranch and teleosts. Comp. Biochem. Physiol. 118C, 255-260.

Sakai, T., Murata, H., Endo, M., Yamauchi, K., Tabata, N., 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.

Sakai, T., Murata, H., Endo, M., Shimomura, T., Yamauchi, K., Ito, T., Yamaguchi, T., Nakajima, H., Fukudome, M. 1998. Severe oxidative stress is thought to be a principal cause of jaundice of yellowtail *Seriola quinqueradiata*. Aquaculture 160, 205-214.

Sargent, J. R., Bell, J. G., Bell, M. V., Henderson, R. J., D. R. Tocher. 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11, 183-198.

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

Tocher, D.R., 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., Hibiya, T. 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi 36, 623-630.

Wdzieczak, J., Zalesna, G., Wujec, E., Peres, G. 1982. Comparative studies on superoxide dismutase, catalase and peroxidase levels in erythrocytes and livers of different freshwater and marine fish species. Comp. Biochem. Physiol. 73B, 361-365.

Winston, G.W., 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 basal diet and oil coatings

Component	HO	HXO	HXE	HE
Pellet				
Fishmeal[1]	72	72	72	72
Starch[2]	10	10	10	10
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	2	2	2
Coating				
Vitamin E-stripped fish oil[5]	12.6	-		-
Oxidised oil[6]	-	12.6	-	-
Oxidised oil + Vitamin E[7]	-	-	12.6	-
Stripped fish oil[5] + 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; $FeSO_4.7H_2O$, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg; 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] Incromega TG3525 (Croda Universal Ltd., Hull, U.K.), stripped by activated charcoal (Sigma Chemical Co. Ltd.) adsorption in hexane.

[6] Vitamin E-stripped tuna orbital oil oxidised by heating at 50 °C in an oxygen-rich atmosphere for 24 h.

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

Table 2. Gross composition (percent dry mass), unsaturation index, peroxide value, TBARS content (μmol MDA.mg^{-1} dry mass), vitamin E content (mg.kg-1 diet), ratio of PUFA/Vitamin E (mol/mol) and total lipid fatty acid content (μg fatty acid · mg-1 dry mass) of experimental diets.

	HO		HXO		HXE		HE	
Gross composition								
Protein	65.7 ± 0.7		67.5 ± 2.7		67.6 ± 2.1		65.9 ± 2.2	
Carbohydrates	12.7 ± 0.3		11.1 ± 0.2		10.7 ± 0.3		13.3 ± 0.1	
Ash	4.7 ± 5.3		5.3 ± 0.5		4.5 ± 0.4		4.1 ± 0.4	
Total lipid	16.9 ± 0.2		16.1 ± 1.0		17.2 ± 0.5		16.7 ± 0.4	
Unsaturation index	343.1 ± 0.1		346.1 ± 1.7		342.5 ± 2.3		346.3 ± 3.0	
Peroxide value	9.2 ± 0.5	b	42.0 ± 1.6	a	42.0 ± 1.6	a	4.5 ± 0.4	b
TBARS content	6.2 ± 0.1		6.2 ± 0.4		6.0 ± 0.2		6.2 ± 0.1	
Tocopherol (vitamin E) content	37.9 ± 3.0	b	35.3 ± 7.3	b	260 ± 38	a	197 ± 5	a
PUFA content	86.2 ± 3.8		82.9 ± 2.5		76.5 ± 0.3		78.3 ± 2.7	
Molar ratio PUFA/Vitamin E	3063 ± 609	a	3795 ± 926	a	467 ± 64	b	628 ± 11	b
Fatty acid composition								
16:0	10.0 ± 0.5		8.8 ± 0.1		9.4 ± 0.2		9.5 ± 0.1	
18:0	1.8 ± 0.2		1.6 ± 0.1		1.7 ± 0.0		1.7 ± 0.0	
Total saturated	15.8 ± 0.4		14.2 ± 0.2		15.5 ± 0.7		15.6 ± 0.2	
16:1(n-7)	3.5 ± 0.2		3.1 ± 0.0		3.4 ± 0.1		3.4 ± 0.1	
18:1	16.3 ± 1.1		14.2 ± 0.2		15.3 ± 0.2		15.4 ± 0.3	
20:1(n-9)	6.3 ± 0.5		5.5 ± 0.1		6.0 ± 0.1		6.2 ± 0.2	
22:1	9.1 ± 0.8		8.0 ± 0.1		8.5 ± 0.4		8.9 ± 0.1	
24:1(n-9)	0.6 ± 0.2		0.6 ± 0.0		0.7 ± 0.1		0.8 ± 0.1	
Total monoenes[1]	32.3 ± 2.5		28.3 ± 0.4		30.9 ± 1.0		30.8 ± 0.3	
18:2(n-6)	2.6 ± 0.2		2.3 ± 0.0		2.4 ± 0.0		2.5 ± 0.0	
20:4(n-6)	2.3 ± 0.1		2.0 ± 0.0		2.3 ± 0.1		2.1 ± 0.0	
Total (n-6)	7.1 ± 0.5		6.6 ± 0.1		6.8 ± 0.2		6.7 ± 0.1	
18:3(n-3)	1.3 ± 0.1		1.3 ± 0.1		1.3 ± 0.0		1.3 ± 0.0	
18:4(n-3)	4.1 ± 0.3		3.7 ± 0.1		4.0 ± 0.1		3.9 ± 0.1	
20:4(n-3)	2.5 ± 0.2		2.2 ± 0.0		2.5 ± 0.1		2.3 ± 0.0	
20:5(n-3)	38.0 ± 2.8		33.5 ± 0.5		36.5 ± 0.3		35.5 ± 0.6	
22:5(n-3)	5.0 ± 0.7		3.9 ± 0.1		4.3 ± 0.1		4.2 ± 0.1	
22:6(n-3)	29.1 ± 1.9		25.3 ± 0.3		27.5 ± 0.4		27.2 ± 0.4	
Total (n-3)	83.7 ± 6.1		72.9 ± 0.8		79.4 ± 0.5		77.7 ± 1.2	
Total polyenes	91.2 ± 6.7		79.7 ± 0.9		86.6 ± 0.4		84.9 ± 1.3	

Data are mean ± SD (n=3). Values within a row with different superscript letters are significantly different ($p < 0.05$). PUFA, polyunsaturated fatty acid.

[1] Includes some minor fatty acids not shown.

Table 3. Growth-related performance and survival rates of gilthead sea bream (*Sparus aurata*) after being fed the experimental diets for 30 and 60 days.

	Initial	HO	HXO	HXE	HE	Significance		
						ox. oil	Vit. E	inter.
30 days								
Fish length (mm) [1]	4.8 ± 0.2	6.5 ± 0.3	6.6 ± 0.3	6.8 ± 0.1	6.6 ± 0.3	yes	yes	no
Fish live mass (g) [1]	1.5 ± 0.2	4.1 ± 0.5	4.2 ± 0.3	4.6 ± 0.0	4.0 ± 0.6	yes	yes	yes
Fish dry mass (mg) [2]	0.4 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.2	no	no	yes
Fish dry mass % [2]	23.9 ± 0.5	26.8 ± 0.7	26.5 ± 0.5	27.2 ± 0.7	29.1 ± 10.1	no	no	no
Liver live mass (mg) [1]	29.5 ± 5.0	90.2 ± 23.7	88.6 ± 6.5	88.8 ± 7.0	73.4 ± 14.6	yes	yes	yes
Liver dry mass (mg) [2]	17.2 ± 1.8	32.3 ± 9.3	32.9 ± 3.5	32.1 ± 2.4	27.1 ± 3.8	no	no	no
Liver dry mass % [2]	33.4 ± 0.6	35.7 ± 0.9	37.0 ± 1.5	37.5 ± 2.8	37.0 ± 2.1	no	no	no
HSI [1]	2.0 ± 0.3	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	1.8 ± 0.1	yes	yes	yes
Mortality (nº of fish) [2]		2.0 ± 1.7	1.7 ± 2.1	1.0 ± 1.0	1.0 ± 1.7	no	no	no
Mortality (%) [2]		1.0 ± 0.9	0.8 ± 1.0	0.5 ± 0.5	0.5 ± 0.9	no	yes	no
SGR (%day^{-1}) [2]		3.3 ± 0.4	3.4 ± 0.3	3.7 ± 0.0	3.2 ± 0.5	yes	yes	yes
60 days								
Fish length (mm) [1]	4.8 ± 0.2	8.3 ± 0.0	8.3 ± 0.2	8.0 ± 0.1	8.1 ± 0.2	yes	yes	yes
Fish live mass (g) [1]	1.5 ± 0.2	8.1 ± 0.1	8.2 ± 0.6	7.2 ± 0.3	7.5 ± 0.5	yes	yes	yes
Fish dry mass (mg) [2]	0.4 ± 0.0	2.1 ± 0.1	2.3 ± 0.4	1.9 ± 0.1	1.9 ± 0.2	no	no	no
Fish dry mass % [2]	23.9 ± 0.5	28.0 ± 0.5	28.0 ± 0.3	28.0 ± 0.4	28.4 ± 0.3	no	no	no
Liver live mass (mg) [1]	29.5 ± 5.0	129.0 ± 19.2	121.9 ± 17.5	114.0 ± 15.3	107.7 ± 5.1	no	yes	yes
Liver dry mass (mg) [2]	17.2 ± 1.8	42.4 ± 5.9	40.8 ± 6.7	38.8 ± 4.1	37.1 ± 2.3	no	no	no
Liver dry mass % [2]	33.4 ± 0.6	32.9 ± 0.6	33.3 ± 1.2	34.2 ± 1.0	34.4 ± 1.9	no	no	no
HSI [1]	2.0 ± 0.3	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	no	yes	no
Mortality (nº of fish) [2]		2.0 ± 2.0	3.3 ± 4.0	2.3 ± 2.5	1.3 ± 0.6	no	no	no
Mortality (%) [2]		4.0 ± 3.6	5.0 ± 4.0	3.3 ± 3.2	2.3 ± 1.5	no	no	no
SGR (%day^{-1}) [2]		2.8 ± 0.0	2.8 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	no	yes	no

Data are means ± SD ([1]n=30 and 90 for initial point and experimental diet, respectively; [2]n=3). Significant effects due to dietary oil or vitamin E supplementation as determined by two-way analysis of variance are indicated (yes). no, not significant; HSI, Hepato-somatic index; SGR, specific growth rate.

Table 4. Effects of dietary oxidised oil and vitamin E on lipid content (percentage of dry weight), proportions of polar lipid, neutral lipid and triacylglycerol (percentages of total lipid), and fatty acid composition (percentage of total fatty acids by weight) of total lipid of liver from sea bream (*Sparus aurata*) after being fed the diets for 30 days.

	t = 30 days				*t* = 60 days			
	HO	HXO	HXE	HE	HO	HXO	HXE	HE
Total lipid	30.6 ± 5.3	43.3 ± 8.2	37.5 ± 10.5	44.0 ± 3.7	29.2 ± 4.9	21.9 ± 3.6	36.8 ± 7.6	31.1 ± 9.1
Total polar lipid	27.0 ± 2.9	19.5 ± 3.6	18.7 ± 3.0	20.6 ± 4.4	26.0 ± 4.2	31.9 ± 3.7	22.8 ± 3.5	27.7 ± 6.4
Total neutral lipid	73.0 ± 2.9	80.5 ± 3.6	81.1 ± 3.0	79.4 ± 4.4	74.0 ± 4.2	68.1 ± 3.7	77.2 ± 3.5	72.3 ± 6.4
Triacylglycerol	64.2 ± 3.2	72.5 ± 4.0	72.5 ± 3.0	69.7 ± 4.4	63.6 ± 5.8	55.4 ± 3.1	66.3 ± 5.3	60.9 ± 8.6
Fatty acid composition								
16:0	8.5 ± 0.8	7.4 ± 0.4	8.3 ± 0.8	8.0 ± 0.4	8.5 ± 0.7	8.6 ± 0.5	8.2 ± 0.6	8.6 ± 1.4
18:0	2.7 ± 0.5	2.2 ± 0.3	2.5 ± 0.3	2.0 ± 0.2	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.5
Total saturated[1]	13.6 ± 1.2	12.2 ± 1.1	13.4 ± 1.1	12.7 ± 0.6	13.8 ± 0.9	13.4 ± 0.7	13.4 ± 0.9	13.8 ± 2.3
16:1n-7	2.8 ± 0.0	2.6 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.7 ± 0.1	2.4 ± 0.2
18:1	13.0 ± 0.9	12.5 ± 1.1	13.4 ± 0.6	11.6 ± 0.1	10.6 ± 0.6	9.8 ± 0.2	10.7 ± 0.6	9.8 ± 0.2
20:1[2]	3.0 ± 0.2	2.8 ± 0.0	2.8 ± 0.0	2.7 ± 0.0	2.6 ± 0.1	2.6 ± 0.0	2.7 ± 0.1	2.6 ± 0.2
22:1[3]	4.2 ± 0.3	4.0 ± 0.1	4.1 ± 0.4	3.8 ± 0.1	3.5 ± 0.0	3.4 ± 0.1	3.9 ± 0.2	3.4 ± 0.3
Total monoenes	23.8 ± 0.8	22.6 ± 1.4	24.0 ± 0.7	21.4 ± 0.2	19.8 ± 0.7	19.7 ± 0.6	20.8 ± 0.7	19.1 ± 0.6
18:2n-6	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.1	1.9 ± 0.0	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.0	1.6 ± 0.2
20:4n-6	1.9 ± 0.0	1.9 ± 0.2	1.8 ± 0.1	1.8 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.1 ± 0.2
Total n-6PUFA[4]	5.7 ± 0.1	5.6 ± 0.2	5.5 ± 0.0	5.5 ± 0.1	5.9 ± 0.2	6.0 ± 0.7	5.7 ± 0.2	5.8 ± 0.1
20:4n-3	2.1 ± 0.3	2.4 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	2.0 ± 0.3	1.7 ± 0.2
20:5n-3	20.3 ± 1.2	19.3 ± 2.0	19.5 ± 1.6	21.2 ± 0.9	19.8 ± 1.2	19.5 ± 0.4	20.6 ± 1.7	21.7 ± 1.7
22:5n-3	5.6 ± 0.9	8.3 ± 2.2	6.7 ± 0.6	6.3 ± 0.6	4.9 ± 0.9	5.2 ± 1.0	7.2 ± 2.5	3.9 ± 0.2
22:6n-3	21.6 ± 0.9	22.6 ± 1.8	20.7 ± 1.0	23.4 ± 0.8	25.9 ± 1.9	27.6 ± 0.9	22.3 ± 1.2	26.5 ± 1.6
Total n-3PUFA[5]	54.2 ± 1.7	57.1 ± 1.9	53.4 ± 1.6	57.5 ± 0.8	57.2 ± 0.7	57.9 ± 1.0	56.7 ± 0.7	58.2 ± 1.5
Total PUFA[6]	61.1 ± 1.8	63.9 ± 1.6	60.2 ± 1.6	64.2 ± 0.8	64.3 ± 0.6	64.8 ± 0.3	63.3 ± 0.7	65.1 ± 1.6

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05%.
Significant effects due to dietary oil or vitamin E supplementation were determined by two-way analysis of variance. The only significant differences were that dietary oxidised oil increased the proportions of total neutral lipid and triacylglycerol and decreased the percentages of 14:0 and 18:3n-3.
[1]Includes 15:0, 17:0, 20:0, 22:0 and 24:0 present at < 0.05%; [2]predominantly n-9 isomer; [3]predominantly n-11 isomer; [4]includes18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6, present at < 0.5%; [5]includes 20:3n-3, present at < 0.5%; [6]includes C_{16} PUFA; PUFA, polyunsaturated fatty acids.

Table 5. Vitamin E contents, activities of antioxidant enzymes and levels of lipid peroxidation products (TBARS and 8-Isoprostane) in liver from gilthead sea bream (*Sparus aurata*) after being fed the experimental diets for 30 and 60 days.

	HO	HXO	HXE	HE	Significance		
					OX	VE	inter.
30 days							
Vitamin E							
ng Vit E.mg^{-1} live mass	73 ± 10	52 ± 2	456 ± 49	263 ± 65	yes	yes	yes
ng Vit E.mg^{-1} dry mass	204 ± 27	141 ± 5	1215 ± 131	709 ± 177	yes	yes	yes
μg Vit E.Liver^{-1}	6.6 ± 0.9	4.6 ± 0.2	40.5 ± 4.4	19.3 ± 4.8	yes	yes	yes
Molar ratio PUFA/Vitamin E	1042 ± 130	2030 ± 62	206 ± 23	448 ± 111	yes	yes	yes
Antioxidant enzyme activities							
Catalase	437 ± 10	667 ± 23	520 ± 25	307 ± 7	yes	yes	no
Superoxide dismutase	5.7 ± 0.0	6.5 ± 0.2	6.0 ± 0.8	2.8 ± 0.1	yes	yes	yes
Glutathione peroxidase	102 ± 6	85 ± 4	90 ± 6	86 ± 1	yes	no	yes
Glutathione S-transferase	895 ± 80	802 ± 100	944 ± 61	833 ± 15	no	no	yes
Glutathione reductase	26.3 ± 1.6	59.2 ± 2.7	71.3 ± 7.2	77.8 ± 28.9	no	yes	no
TBARS							
μmol.mg^{-1} live mass	1.9 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	1.2 ± 0.0	no	yes	yes
μmol.mg^{-1} dry mass	5.4 ± 0.5	5.0 ± 0.5	4.2 ± 0.3	3.3 ± 0.1	no	yes	yes
μmol.liver^{-1}	174 ± 16	162 ± 16	141 ± 10	90 ± 4	yes	yes	yes
8-Isoprostane	110 ± 6	188 ± 3	125 ± 22	177 ± 3	no	no	yes
60 days							
Vitamin E							
ng Vit E.mg^{-1} live mass	72 ± 13	58 ± 11	891 ± 86	486 ± 29	yes	yes	yes
ng Vit E.mg^{-1} dry mass	217 ± 39	173 ± 34	2609 ± 253	1412 ± 83	yes	yes	yes
μg Vit E.Liver^{-1}	8.6 ± 1.6	6.9 ± 1.4	107.5 ± 10.4	58.6 ± 3.4	yes	yes	yes
Molar ratio PUFA/Vitamin E	880 ± 158	825 ± 165	96 ± 9	159 ± 9	no	yes	no
Antioxidant enzyme activities							
Catalase	575 ± 28	490 ± 59	363 ± 8	365 ± 16	no	yes	no
Superoxide dismutase	5.2 ± 0.5	6.3 ± 0.3	6.9 ± 0.0	7.5 ± 0.3	no	yes	yes
Glutathione peroxidase	48.9 ± 8.2	55.2 ± 6.1	45.8 ± 5.8	58.2 ± 8.9	no	no	no
Glutathione S-transferase	979 ± 45	728 ± 49	647 ± 44	835 ± 132	yes	yes	no
Glutathione reductase	49.3 ± 6.3	38.3 ± 7.0	25.6 ± 7.5	35.1 ± 3.0	yes	yes	no
TBARS							
μmol.mg^{-1} live mass	1.5 ± 0.2	1.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	no	yes	yes
μmol.mg^{-1} dry mass	4.6 ± 0.6	5.5 ± 0.3	2.8 ± 0.2	3.4 ± 0.6	no	yes	yes
μmol.liver^{-1}	195 ± 24	224 ± 14	110 ± 9	124 ± 23	no	yes	no
8-Isoprostane	169 ± 2	121 ± 9	168 ± 30	84 ± 4	no	no	yes

Data are presented as means ± SD (n = 3). SD=0.0 implies SD < 0.05. Significant effects due to dietary oil or vitamin E supplementation as determined by two-way analysis of variance are indicated (yes). no, not significant. Catalase, (μmol.min^{-1}.mg protein^{-1}); total glutathione peroxidase, glutathione reductase and glutathione-S-transferase all nmol.min^{-1}.mg protein^{-1}; superoxide dismutase (units.min^{-1}.mg protein^{-1}); 8-isoprostane (pg.mg protein^{-1}). PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid-reactive substances.

ble 6. Results (F-values) of three-way analysis of variance for selected data from Tables 4 and 5.

	F- values						
				Interactions			
	A) Vitamin E	B) Oxidised Oil	C) Time	A x B	A x C	B x C	A x B x C
ometry							
Fish length	ns	ns	1174.2***	ns	19.1***	5.0*	ns
Fish live mass	7**	ns	917.9***	ns	15.7***	ns	3.9*
Fish dry mass	ns	ns	167.9***	ns	9.3**	ns	ns
Fish dry mass %	ns	ns	ns	ns	ns	ns	ns
Liver live mass	11.2***	4.6*	91.4***	ns	ns	ns	ns
Liver dry mass	4.3*	ns	29.4***	ns	ns	ns	ns
Liver dry mass %	ns	ns	33.8***	ns	ns	ns	ns
Hepato-somatic index	13.0***	ns	236.8***	ns	ns	ns	ns
tal lipid							
live mass %	ns	ns	11.8**	ns	ns	ns	5.5*
dry mass %	ns	ns	6.8*	ns	ns	ns	5.4*
tty acid (Mass %)							
Total Monoenes	ns	ns	65.3***	ns	ns	ns	ns
Total PUFA	ns	ns	9.3**	ns	ns	ns	ns
Total n-3PUFA	ns	ns	9.2**	ns	ns	ns	ns
Total n-6HUFA	ns	ns	16.9**	ns	ns	ns	ns
Total n-3HUFA	ns	ns	9.7**	ns	ns	ns	ns
tamin E							
per live mass	3773.9***	251.8***	443.0***	343.1***	442.0***	9.8*	ns
per dry mass	3630.9***	229.1***	587.1***	335.8***	527.5***	ns	ns
per liver	4137.9***	286.3***	1304.0***	418.8***	1036.7***	ns	10.7**
PUFA/vitamin E	148.7***	ns	ns	ns	ns	17.2**	6.3*
ıtioxidant enzyme activitiy							
Catalase	215.9***	72.3***	10.7**	ns	ns	160.2***	5.6*
Superoxide dismutase	ns	27.1***	27.8***	ns	47.5***	15.5**	20.6**
Glutathione peroxidase	ns	8.1**	2087.9***	4.2*	ns	ns	29.9***
Glutathione S-transferase	ns	10.1**	4.7*	ns	5.4*	12.1**	ns
Glutathione reductase	ns	ns	22.6***	4.4*	24.7***	6.6*	5.0*
BARS							
μg.mg^{-1} live mass	38.3***	ns	10.3**	ns	ns	ns	8.0*
μg.mg^{-1} dry mass	45.7***	ns	ns	ns	ns	ns	8.1*
μg.liver^{-1}	64.7***	ns	5.7*	ns	5.0*	ns	8.7*
oprostane							
pg.mg^{-1} protein	ns	7.8*	6.7*	ns	ns	ns	138.1***

gnificance levels are ***p < 0.001, ** p< 0.01, * p < 0.05, ns p > 0.05.

JFA, highly unsaturated fatty acids; PUFA, polyunsaturated fatty acids.