Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.)

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Abstract

In order to enhance growth, survival and quality during early juvenile stages of marine fish it is important to avoid lipid oxidation problems that are known to cause pathologies and disease. The aim of the present study was to characterize and compare the antioxidant systems in juvenile marine fish of commercial importance in European aquaculture, namely turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and gilthead sea bream (*Sparus aurata*). The experiment investigated the interaction of the dietary antioxidant micronutrient, vitamin E, with antioxidant defence systems. Fish were fed with diets of identical unsaturation index supplemented with graded amounts of vitamin E. The relationships between dietary and subsequent tissue vitamin E levels were determined as well as the effects of vitamin E supplementation on lipid and fatty acid compositions of both liver and whole fish, on the activities of the liver antioxidant defence enzymes, and on the levels of liver and whole body lipid peroxidation products, malondialdehyde (thiobarbituric acid reactive substances, TBARS) and isoprostanes. Growth and survival was only significantly affected in sea bream where feeding the diet with the lowest vitamin E resulted in decreased survival and growth. A gradation was observed in tissue vitamin E and polyunsaturated fatty acid (PUFA)/vitamin E levels in response to dietary vitamin E levels in all species. The activities of the main radical scavenging enzymes in the liver, catalase, superoxide dismutase and glutathione peroxidase generally reflected dietary and tissue vitamin E levels being highest in fish fed with the lowest level of vitamin E. The indicators of lipid peroxidation gave consistent results in all three species, generally being highest in fish fed with the unsupplemented diet and generally lowest in fish fed with the diet with highest vitamin E. In this respect, isoprostane levels generally paralleled TBARS levels supporting their value as indicators of oxidative stress in fish. Overall the relationships observed were logical in that decreased dietary vitamin E led to decreased levels of tissue vitamin E, and generally higher activities of the liver antioxidant enzymes and higher levels of lipid peroxides.

KEY WORDS: antioxidant enzymes, α-tocopherol, *Hippoglossus hippoglossus*, lipid peroxidation, *Scophthalmus maximus*, *Sparus aurata*, vitamin E

Abbreviations AA, all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4n-6); 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; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidized glutathione; GST, glutathione-S-transferase; 8-isoprostane, 8-iso-prostaglandin $F_{2\alpha}$; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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Introduction

Under physiological conditions there is continuous production of reactive oxygen species (ROS), particularly in mitochondria, microsomes, nuclear membranes and phagocytes (Halliwell & Gutteridge 1996). Increased cellular levels of ROS are proposed to occur in marine organisms when the pro-oxidant processes are not adequately balanced by the antioxidant defences and, as a result, ROS are not entirely removed. This can occur when antioxidants are depleted and/or the rate of ROS production surpasses that of enzymatic defences. As a consequence, increased ROS may lead to increased oxidative stress that can result in damage to molecules including DNA in the cell nucleus, inactivation of enzymes and damage to other proteins and lipids within cell membranes and other vital cell components. Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation, is acknowledged as being highly deleterious resulting in damage to cellular biomembranes which contain large amounts of PUFA and is possibly the most extensively studied component of oxidative damage to biological systems. Autoxidation of PUFA produces compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons and is implicated in several pathological conditions in fish (Kawatsu 1969; Watanabe *et al.* 1970; Murai & Andrews 1974; Sakai *et al.* 1989).

Physiological antioxidant protection involves a variety of chemical systems, of endogenous and exogenous origins, both water and fat-soluble. The endogenous components include compounds such as NADH/NADPH, glutathione (GSH), protein sulphydryl (-SH) groups, uric acid and free radical scavenging enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) (Winston & Di Giulio 1991; Halliwell & Gutteridge 1996). Exogenous dietary micronutrients such as vitamins E and C as well as carotenoids also play important roles and recent studies suggest that components can interact to spare or replace each other, particularly when dietary intake of antioxidant vitamins are depleted (Hamre *et al.* 1997; Bell *et al.* 2000). Tissue lipid PUFA content and composition are critical factors in lipid peroxidation. As fish tissues and fish diets typically contain large quantities of highly unsaturated (n-3) series fatty acids they are potentially more at risk from peroxidative attack than those of mammals (Sargent *et al.* 1999). However, information in relation to *in vivo* lipid peroxidation and antioxidant defences either in wild or cultured marine fish species is quite limited (De Silva & Anderson 1995; Stéphan *et al.* 1995; Murata *et al.* 1996; Peters & Livingstone 1996; Mourente *et al.* 1999a, b).

Therefore, in order to enhance growth, survival and quality during the early developmental stages of marine fish it is important to understand, and thus avoid, lipid oxidation problems that can cause pathologies, disease and subsequent mortalities. The aim of the present study was to characterize and compare the antioxidant systems in juvenile marine fish of commercial importance in European aquaculture, namely turbot (*Scophthalmus maximus*), halibut (*H. hippoglossus*) and gilthead sea bream (*S. aurata*). The present experiment investigated the interaction of the dietary antioxidant micronutrient, vitamin E, with the antioxidant defence systems in juvenile fish. Fish were fed with diets of identical unsaturation index and PUFA composition supplemented with various concentrations of vitamin E. The relationships between dietary and subsequent tissue vitamin E levels were determined as well as the effects of vitamin E supplementation on lipid and fatty acid compositions of both liver and whole fish. The effects of dietary vitamin E on the activities of the liver antioxidant defence enzymes were determined. In addition, liver and whole body lipid peroxidation products, including malondialdehyde, measured as thiobarbituric acid reactive substances (TBARS), and isoprostanes, prostaglandin-like compounds that are produced non-enzymatically by free radical catalysed peroxidation of PUFA (Morrow & Roberts 1997; Roberts & Morrow 1997), were determined.

Materials and methods

Experimental diets

The experimental diets contained 56% protein and 14% lipid. They consisted of a common basal extruded nucleus (92.45% of the diet), based on a standard reference diet (Coutteau *et al.* 1996) but slightly modified for this experiment (Table 1), on which the different lipid fractions (7.55% of the diet) were coated (Table 2). The diet ingredients and premixes were blended over 12 h in an industrial pilot-scale vertical single screw blender (INVE Aquaculture N.V., Dendermonde, Belgium). While blending, the fixed oil fraction (4%) the basal pellet was sprayed into the mixer by a nozzle connected to a compressor. The mixed ingredients were stored in bags, transported, extruded and dried (fluidized bed dryer) at the Department of Quality of Animal Products and Transformation Technology (Center of Agricultural Research, Melle, Belgium). Diets were crumbled and sieved (200–300, 300–500, 500–800, 800–1200, 1200–2000 μm) at INVE Technologies N.V. (Baasrode, Belgium). The E0 diet contained no supplemental vitamin E whereas

Table 1 Composition of the experimental basal diet

Ingredient	Complete coated diet (g kg^{-1})
Codfish powder[1]	25
Egg albumin[2]	12
Whey protein concentrate[3]	12
Isolated soy protein[4]	12
Wheat gluten[5]	3
Alpha-cellulose[6]	2.955
Native corn starch[7]	14.8
Oleic acid[8]	2
Hydrogenated coconut oil[9]	2
Choline chloride 50% silica[10]	0.4
Vitamin premix 3%[11]	3
Attractant premix[12]	3
Calcium propionate[13]	0.3
Butylated hydroxytoluene	0.005
Butylated hydroxyanisole	0.005
Total	92.465

[1] Code 0271, Rieber & Son A/S, Norway.
[2] Type HG/LW, Orffa Belgium N.V., Belgium.
[3] LACPRODAN-80, Orffa Belgium N.V., Belgium.
[4] SUPRO 500E, Protein Technologies International, Belgium.
[5] BIOGLUTEN, Amylum N.V., Belgium.
[6] Sigma C8002.
[7] SNOWFLAKE 03401, Orffa Belgium N.V., Belgium.
[8] Code O/0200/17, S.L.R.grade, Fisher Scientific, UK.
[9] Cocos 32/34, Vandemoortele N.V., Belgium.
[10] 50% purity, INVE Aquaculture N.V., Belgium.
[11] Vitamin E- and C-free, INVE Technologies N.V., Belgium.
[12] According to Kanazawa *et al.* (1989).
[13] Orffa Belgium N.V., Belgium.

the E100 and E1000 diets had additional supplemental vitamin E added, as DL-α-tocopheryl acetate, at 100 and 1000 mg kg^{-1}, respectively.

Experimental fish and dietary trial conditions

The turbot experiment was performed in the facilities of the Laboratory of Aquaculture and Artemia Reference Center (Ghent University, Belgium). Juvenile turbot were obtained from a commercial hatchery (France Turbot, Noirmoutier, France) and were maintained in a 1000-L tank for acclimation to laboratory conditions during which time they were fed with the uncoated basal diet. The dietary trial was conducted in three separate recirculating water units with each unit consisting of three 30 L rectangular tanks with separate biofilter. The fish were stocked in the experimental system at an initial weight of 1.80 ± 0.28 g, at a density of 120 individuals per tank. Each diet was fed to triplicate tanks (one tank in each system) with the feed supplied by automatic feeders for 750 degree.days at a water temperature of 19 ± 1 °C. Water quality parameters in each block were monitored daily (temperature, NH_3, NO_2^-, salinity) and excess feed siphoned off, and any dead fish removed.

Table 2 Composition of the coatings (g kg^{-1} of complete coated diet)

Ingredient	E0	E100	E1000
Vitamin E[1]	0.00	0.01	0.11
Oleic acid[2]	0.25	0.24	0.14
DHA65E[3]	5.80	5.80	5.80
Ascorbyl palmitate[4]	0.0025	0.0025	0.0025
Phospholipids[5]	1	1	1
Emulsifier blend[6]	0.5	0.5	0.5

[1] dl-α-tocopherol acetate, Roche, Belgium.
[2] Code O/0200/17, S.L.R. grade, Fisher Scientific, UK.
[3] Ethyl ester concentrate containing 65% n-3 HUFA DHA (ITOCHU, Japan).
[4] 40% active INVE Technologies N.V., Belgium.
[5] Emulpur N, Lucas Meyer GmbH & Co, Germany.
[6] Glycerolmono-oleate/sorbitan monostearate (1:1).

The halibut experiment was performed in the facilities of the Norwegian University of Science and Technology (NTNU) in Trondheim. Normally pigmented halibut fry were obtained from a commercial fish farm (Norwegian Halibut AS, Rørvik, Norway). At NTNU, halibut fry of initial weight 0.64 g were randomly divided into six groups (each diet in duplicate) (100–120 individuals in each unit) in polyethylene tanks (1 × 1 m, 40 cm depth) with a water flow of 6.3–7.5 L min^{-1}. The dietary trial was performed at a temperature of 14 ± 1 °C for a period of 750 degree.days. The fish were fed 6–8 g day^{-1} per tank for approximately 2 weeks after which the ration was altered to between 8 and 11.5 g day^{-1} depending upon the growth rate of the different groups. The tanks were rinsed every day and there were always food particles in excess at the bottom of each unit.

The sea bream experiment was performed in the facilities of the University of Cadiz, Cadiz, Spain. Sea bream from the same batch, 70 days posthatch, completely weaned, with a functional swimbladder, and a live mass of 1.1 ± 0.4 g each were obtained from CUPIMAR S. A. (Cadiz, Andalucia, Spain). After acclimatization to the experimental diet and conditions for 2 weeks, the fish were randomly stocked at an initial density of 5 fish L^{-1} 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^{-1} between the beginning and end of the experiment and was offered to fish six times during the daylight hours (natural photoperiod) by hand. The length of the experiment was established at 750 degree.-days. The tanks were in an open system continuously supplied with running borehole water of 39 ppt salinity at a temperature of 19.4 ± 0.2 °C. The water was treated with biological filters to eliminate ammonia, by nitrification processes, to sea water quality criteria (1 μg L^{-1} NH_3-N maximum). Oxygen was supplied by aeration with the

minimum level observed during trials being 5.6 mg L^{-1} or 77.8% saturation. Water renewal was set at 10 times total volume per day (0.7 L min^{-1}).

Sampling

In all trials, survival was determined by counting the number of fish remaining at the end of the experiment and was expressed as a percentage of the initial number. Fish were sampled and dissected on ice after a 24-h starvation period to avoid interference of gut contents in the analysis. For morphometric measurements, a minimum of 25 specimens were used from each treatment. The head-tail length and live mass were determined for whole fish while dry mass was determined for both whole fish and liver. Live masses were determined by blotting fish and liver on filter paper before weighing, and dry masses were determined after heating in an oven at 60 °C for 24-h. After removal, the liver was carefully cleaned of adhering tissue before weighing and the hepatosomatic index (HSI) calculated [$HSI = 100 \times (\text{liver weight}) \times (\text{body weight})^{-1}$]. Growth was assessed by determining the specific growth rate (SGR) as percentage weight gain per day ($100 \times [(\ln \text{final weight}) - (\ln \text{initial weight})] \times \text{days}^{-1}$) (Wootten 1990). Samples of diets, whole fish and dissected livers were immediately frozen in liquid nitrogen and stored under nitrogen at –80 °C prior to analysis for lipid and fatty acids, vitamin E content, lipid peroxidation products (TBARS and isoprostane) and hepatic antioxidant defence enzyme activities. All liver samples were triplicates of pooled livers, the number of livers dependent upon the size of the fish and weight of individual livers varying between three and six livers per sample.

Lipid extraction and lipid class composition

Total lipid was determined gravimetrically after extraction by homogenization in chloroform:methanol (2:1v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch *et al*. (1957). Separation of lipid classes was performed by high-performance thin-layer chromatography (HPTLC). Approximately 10 μg of lipid extract was loaded as a 2-mm streak and the plate developed to two-thirds distance with methyl acetate:isopropanol:chloroform: methanol:0.25% aqueous KCl (25:25:25:10:9, by vol.). After desiccation, the plate was fully developed with hexane:diethyl ether:acetic acid (85:15:1, by vol.). The classes were quantified by charring at 160 °C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, followed by calibrated densitometry using a Shimadzu CS-9000 dual-wavelength flying spot scanner and DR-13 recorder (Henderson & Tocher 1992).

Total lipid fatty acid analyses

Fatty acid methyl esters (FAME) from total lipids were prepared by acid-catalysed transmethylation for 16 h at 50 °C, using heptadecanoic acid (17:0) as internal standard (Christie 1989). Fatty acid methyl esters were extracted and purified as described previously (Tocher & Harvie 1988) and were separated in a Fisons GC8000 gas chromatograph equipped with a chemically bonded CP Wax 52CB fused silica wall coated capillary column (30 m × 0.32 mm i.d., Chrompack UK Ltd, London), on-column injection system and flame ionization detection. Hydrogen was used as the carrier gas with an oven thermal gradient from an initial 50 °C to 150 °C at 40 °C min^{-1} and then to a final temperature of 230 °C at 2 °C min^{-1}. Individual FAME were identified by comparison with known standards, a well-characterized fish oil and by reference to published data as described previously (Tocher & Harvie 1988) and quantified using a PC with chromcard software (Thermoquest Italia SPA, Milan, Italy).

Measurements of thiobarbituric acid reactive substances (TBARS)

The measurement of TBARS was carried out using a method adapted from that of Burk *et al*. (1980). Up to 20–30 mg of tissue per sample was homogenized in 1.5 mL of 20% (w/v) trichloroacetic acid (TCA) containing 0.05 mL of 1% BHT in ethanol. 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 100 °C for 10 min. After cooling the tubes and removing protein precipitates by centrifugation at 12 000 × ***g***, the supernatant was read in a spectrophotometer at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBARS, expressed as nmol TBARS g^{-1} of tissue, was calculated using the extinction coefficient 0.156 μM^{-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 tissues, 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 should be

assayed immediately after collection or, as in this case, stored at –80 °C, as they can also appear in samples as an artefact of prolonged storage at temperatures above –80 °C (Morrow & Roberts 1997; Roberts & Morrow 1997). Most 8-isoprostanes are found esterified in lipids, and so an extraction and hydrolysis was performed in order to determine total amounts of 8-isoprostane. Briefly, 2 mL ethanol was added to 1.5 mL of homogenate, mixed, and allowed to stand for 5 min at 4 °C before precipitated protein was removed by centrifugation. The supernatant was decanted into a clean test tube and 3.5 mL of 15% KOH added and incubated for 60 min at 40 °C. The solution was diluted to 10 mL 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 C18 reverse-phase mini-column ('Sep-Pak', Millipore UK, Watford, UK) after activating the column with 5 mL methanol followed by 5 mL ultrapure water. The columns were then washed with 5 mL ultrapure water and 5 mL HPLC grade isohexane before eluting isoprostanes with 5 mL ethyl acetate containing 1% methanol. The solvent was evaporated under a stream of nitrogen and 1 mL EIA kit buffer added. Total isoprostane was quantified using an EIA kit and 8-isoprostane standard as per manufacturer's instructions (Cayman Chemical Co., Ann Arbor, USA).

Determination of vitamin E contents

Vitamin E (α-tocopherol) was determined by HPLC with fluorescence detection, as described in Huo *et al.* (1996). Samples were homogenized in 2 mL of methanol containing 1 mg mL^{-1} BHT, and tocol added as an internal standard, using a Potter Elvehjem tube. The samples were then centrifuged for 2 min at $1500 \times g$ and the supernatant transferred to a polypropylene tube. The solid residue was homogenized in 2 mL methanol/BHT and the extract combined with the first one, and with 1 mL methanol/BHT used to rinse the Potter tube. The combined extracts were centrifuged for 10 min at $12\,000 \times g$ and an aliquot of 100 μL was injected. Column and elution details were as in Huo *et al.* (1996). Quantitation was based on peak height ratios (analyte vs. the internal standard tocol).

Determination of enzyme activities in liver homogenates

Samples of liver were homogenized in 9 volumes of 20 mM phosphate buffer pH 7.41 mM ethylene diamine tetraacetic acid (EDTA) and 0.1% Triton X-100, the homogenates were centrifuged at $600 \times g$ 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 & 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 the above buffered hydrogen peroxide solution plus 25 μL of sample.

Total superoxide dismutase activity was assayed by measuring the inhibition of the oxygen-dependent oxidation of adrenaline (epinephrine) to adenochrome by xanthine oxidase plus xanthine (Panchenko *et al.* 1975). Plastic semimicrocuvettes 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 was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Bell *et al.* 1985). Plastic semimicrocuvettes 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 nonenzymic 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 semimicrocuvettes containing 0.6 mL of 0.2 M potassium phosphate buffer pH 7.0/2 mM EDTA, 200 μL oxidized 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 for 1 h at 60 °C in 1 M NaOH/0.25% SDS.

Statistical analysis

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 analysed 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

All the diets all had an unsaturation index of approximately 290 and contained between 85 and 107 mg fatty acids per g of dry diet with 56% being PUFA, predominantly docosahexaenoic acid (DHA, 22:6n-3) (Table 3). There was a graded level of vitamin E ranging from 43 μg g^{-1} dry weight in the unsupplemented diets to 169 and 1247 μg g^{-1} in the E100 and E1000 diets, respectively. Importantly, the molar ratio of PUFA/vitamin E varied from >900 in the unsupplemented diet to 240 in diet E100 and only 28 in diet E1000 (Table 3).

With sea bream, the survival rate and growth as determined by body weights and SGR were lower in fish fed with diet E0 compared with diet E1000 (Table 4). In contrast, there were no significant differences in survival rates or growth parameters between the dietary groups with turbot or halibut. The slightly lower survival rates with halibut were

Table 3 Fatty acid (mg g^{-1} dry weight) and vitamin E (mg kg^{-1} dry weight) contents, unsaturation index and molar ratio of PUFA/vitamin E of experimental diets

	E0	E100	E1000
14:0	5.0	4.4	3.9
16:0	10.4	10.2	8.3
18:0	4.9	4.5	3.9
Total saturates	21.0	19.7	17.1
16:1(n-7)	1.6	1.8	1.3
18:1(n-9)	21.0	19.1	16.3
18:1(n-7)	1.6	1.6	1.4
20:1(n-9)	0.5	0.2	0.4
Total monoenes	25.1	23.1	19.8
18:2(n-6)	9.7	8.8	7.7
20:4(n-6)	2.3	2.0	1.8
22:4(n-6)	0.3	0.2	0.2
22:5(n-6)	2.0	1.7	1.5
Total (n-6)PUFA	14.7	13.0	11.5
18:3(n-3)	1.4	1.7	1.4
18:4(n-3)	0.8	0.6	0.5
20:4(n-3)	0.3	0.3	0.3
20:5(n-3)	8.4	7.5	6.8
22:5(n-3)	1.6	1.4	1.3
22:6(n-3)	32.2	28.0	25.0
Total (n-3) PUFA	45.0	39.7	35.5
Total PUFA	59.7	52.7	47.0
Total fatty acids	106.9	97.0	84.9
Unsaturation index	296	288	292
Total vitamin E*	43 ± 2^{c}	169 ± 1^{b}	1247 ± 17^{a}
PUFA/vitamin E	904 ± 20^{a}	240 ± 2^{b}	28 ± 1^{c}

*Predominantly α-tocopheryl acetate; PUFA, polyunsaturated fatty acids. Unsaturation index, no. of double bonds x content (percentage). The significance of differences between dietary vitamin E levels and PUFA/vitamin E ratios were analysed by one-way analysis of variance. Mean values with different superscript letters are significantly different ($P < 0.05$) as determined by Tukey's multiple range test.

Table 4 Effect of dietary vitamin E on survival (%) and growth of turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and sea bream (*Sparus aurata*)

	Turbot			Halibut			Sea bream		
	E0	E100	E1000	E0	E100	E1000	E0	E100	E1000
Survival (%)	93 ± 6	93 ± 3	88 ± 16	78	85	77	93.9 ± 1.1^{b}	95.1 ± 1.2^{a,b}	97.0 ± 0.6^{a}
Body WW (g)	3.9 ± 0.9	3.9 ± 0.7	3.9 ± 0.7	3.4 ± 1.5	3.5 ± 1.2	3.1 ± 1.0	3.4 ± 0.3^{b}	3.7 ± 0.5^{a}	3.7 ± 0.6^{a}
Body DW (mg)	648.9	665.6	761.1	830 ± 120	810 ± 140	760 ± 90	615 ± 53^{b}	670 ± 150^{a,b}	807 ± 219^{a}
Liver WW (mg)	29.3 ± 5.4	32.1 ± 3.3	31.5 ± 4.0	92.3 ± 25.6	77.2 ± 20.4	71.4 ± 21.8	72.8 ± 5.5	82.4 ± 15.2	72.0 ± 15.8
Liver DW (mg)	5.0 ± 1.1	7.0 ± 4.5	7.2 ± 0.1	26.1 ± 5.3	21.3 ± 5.2	19.5 ± 4.7	22.9 ± 2.1	27.3 ± 5.8	23.0 ± 4.9
SGR (%)	1.8 ± 0.6	1.8 ± 0.5	1.8 ± 0.5	2.0 ± 0.4	2.0 ± 0.4	1.8 ± 0.4	3.8 ± 0.3^{b}	4.1 ± 0.5^{a}	4.0 ± 0.6^{a}
HSI (%)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	2.7 ± 0.3^{a}	2.2 ± 0.3^{b}	2.3 ± 0.3^{b}	1.9 ± 0.2^{a,b}	2.0 ± 0.1^{a}	1.7 ± 0.2^{b}

Results are mean ± SD (survival, $n = 3$ except for halibut where the data are the means of two; body WW and SGR, $n = 75$, 25 and 90 for turbot, halibut and sea bream; Liver WW and HSI, $n = 12$, 25 and 9 for turbot, halibut and sea bream; DWs, $n = 3$, 3 and 9 for turbot, halibut and sea bream). The significance of differences between dietary treatments for each species were analysed by one-way analysis of variance. Mean values with different superscript letters within a given row for each individual species are significantly different ($P < 0.05$) as determined by Tukey's multiple range test. DW, dry weight; HSI, hepatosomatic index; SGR, specific growth rate; WW, wet weight.

well within the expected range for this species. Liver masses were not significantly affected by dietary vitamin E level in any species although HSI was significantly lower in halibut fed with the diets supplemented with vitamin E compared with the unsupplemented diet (Table 4). Similarly, the HSI was lower in sea bream fed with the E1000 diet compared with fish fed with diet E100.

The dietary vitamin E level had no significant effect on the lipid contents of the fish or of their livers in turbot and halibut but liver lipid content increased with increasing dietary vitamin E in sea bream (Table 5). This effect of dietary vitamin E on liver lipid content in sea bream was reflected in the lipid class composition, with the proportion of TAG and total neutral lipids increasing with increasing dietary vitamin E, but dietary vitamin E had no effect on the lipid class composition of liver lipids in halibut and turbot (data not shown). There were few significant differences in the fatty acid compositions of liver total lipids between fish fed with the different diets irrespective of species (Table 6). The percentages of EPA, 22:5n-3, DHA, total n-3PUFA and total PUFA were lower in halibut fed the diet without supplemental vitamin E compared with fish fed with the diets supplemented with vitamin E although only the values for EPA and 22:6n-3 were significant because of the intradiet variation in DHA levels (Table 6). The proportions of AA, EPA, DHA, total n-3PUFA and total PUFA were lower in sea bream fed with diet E100 compared with fish fed with the other diets E0 and E1000 although the reason for this was unclear.

Table 5 Effect of dietary vitamin E on lipid content (mg mg^{-1} wet weight) of whole fish and livers of turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and sea bream (*Sparus aurata*)

	Whole fish	Liver
Turbot		
E0	18.1 ± 4.8	66.5 ± 9.0
E100	22.7 ± 2.6	67.4 ± 16.4
E1000	20.0 ± 1.2	53.0 ± 4.1
Halibut		
E0	21.7 ± 6.6	41.1 ± 8.5
E100	25.3 ± 4.8	41.7 ± 4.9
E1000	29.2 ± 2.3	48.4 ± 10.2
Sea bream		
E0	n.d.	34.6 ± 4.5^{b}
E100	n.d.	46.0 ± 3.8^{a,b}
E1000	n.d.	63.0 ± 11.3^{a}

Results are mean ± SD (n = 3). The significance of differences between dietary treatments for each species were analysed by one-way analysis of variance. Mean values with different superscript letters within a given column for each individual species are significantly different ($P < 0.05$) as determined by Tukey's multiple range test.
n.d., not determined.

In all three species, liver and whole body vitamin E levels generally reflected the dietary level of vitamin E (Table 7). There were graded increases in body and liver vitamin E levels between the fish fed with the E0, E100 and E1000 diets with fish fed with the highest level of vitamin E (E1000) showing significantly higher levels of vitamin E. The molar ratio of PUFA/vitamin E in liver and whole fish also showed significant downward gradations between the fish fed with the E0, E100 and E1000 diets clearly reflecting the dietary vitamin E input (Table 7). The only exception being the low level of vitamin E in the liver of halibut fed with diet E100, although halibut tended to show lower levels of vitamin E compared with turbot and sea bream.

The liver antioxidant enzyme activities showed some notable differences between the dietary treatments. Catalase and SOD activities were significantly lower, but GST and GR activities significantly higher, in sea bream fed with diet E1000 compared with E0 (Table 7). Similarly the activities of catalase and GPX were lowest in halibut fed with the E1000 diet. The activity of GPX was not affected by dietary vitamin E in sea bream whereas its activity was significantly higher in turbot fed with diet E0 compared with turbot fed with the vitamin E supplemented diets. Interestingly, in halibut, the activities of GPX, GST and catalase were highest in the fish fed with diet E100 which showed surprisingly low liver vitamin E levels (Table 7). In all species, the levels of liver TBARS were significantly higher in fish fed with the E0 diet compared with fish fed with the vitamin E supplemented diets (Table 7). Similarly, in whole turbot and halibut, the levels of TBARS were significantly lower in fish fed with the diet supplemented with the highest level of vitamin E (E1000). In all species, liver isoprostane levels were generally significantly lower in fish fed with the E1000 diet, and similarly in halibut, isoprostane levels in whole fish were significantly lower in fish fed with the diet with the highest level of vitamin E (Table 7).

Discussion

Significant effects of dietary vitamin E levels on mortality or growth of the fish were observed in the present trial although there were differences among the species. Sea bream survival and growth was increased by supplementing vitamin E to the diet, whereas, these effects were not observed with turbot or halibut. In a previous dietary trial with turbot varying dietary PUFA and vitamin E levels, there were no significant effects of either parameter on growth (Stéphan *et al*. 1995). Growth was not affected by dietary α-tocopheryl acetate inclusion in diets for African (*Clarius gariepinus*) (Baker & Davies 1996a)

Table 6 Effect of vitamin E on the fatty acid composition (percentage of total fatty acids by weight) of total lipid of livers from turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*) and sea bream (*Sparus aurata*)

	Turbot			Halibut			Sea bream		
	E0	E100	E1000	E10	E100	E1000	E10	E100	E1000
Total saturated	21.3 ± 1.2	19.8 ± 0.8	20.4 ± 1.3	25.8 ± 2.6	22.9 ± 1.8	21.3 ± 1.9	22.8 ± 1.1	22.9 ± 0.9	22.9 ± 0.5
Total monoenes	22.5 ± 0.4^{b}	26.0 ± 1.7^{a}	23.4 ± 1.1^{a,b}	50.8 ± 14.1	37.0 ± 4.6	34.7 ± 4.9	31.2 ± 1.6^{b}	40.8 ± 1.4^{a}	32.3 ± 1.3^{b}
18:2n-6	7.8 ± 0.7	8.2 ± 0.3	8.3 ± 0.4	3.3 ± 2.1	4.9 ± 1.0	6.9 ± 0.8	5.9 ± 0.3	5.4 ± 0.5	5.3 ± 0.4
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
20:2n-6	1.1 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	0.7 ± 0.6	1.0 ± 0.3	1.1 ± 0.0	0.3 ± 0.3	0.7 ± 0.1	0.6 ± 0.0
20:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	3.0 ± 0.3	2.5 ± 0.5	2.9 ± 0.2	0.4 ± 0.4	0.6 ± 0.1	0.6 ± 0.1	1.3 ± 0.1^{a}	0.7 ± 0.0^{b}	1.1 ± 0.1^{a}
22:4n-6	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
22:5n-6	1.8 ± 0.1	2.1 ± 0.4	1.9 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.0^{a,b}	0.3 ± 0.1^{b}	0.5 ± 0.0^{a}
Total n-6PUFA	14.3 ± 0.3	14.8 ± 0.5	15.0 ± 0.5	4.7 ± 3.4	6.9 ± 1.6	9.1 ± 0.8	8.9 ± 0.3	8.4 ± 0.5	9.0 ± 0.4
18:3n-3	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
18:4n-3	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.0
20:3n-3	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	tr	tr	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
20:5n-3	4.8 ± 0.5	4.1 ± 0.1	4.5 ± 0.4	4.9 ± 2.8^{b}	9.0 ± 0.4^{a}	8.6 ± 0.5^{a}	5.4 ± 0.2^{a}	3.2 ± 0.2^{b}	5.0 ± 0.1^{a}
22:5n-3	2.5 ± 0.1	3.2 ± 0.5	2.9 ± 0.1	1.5 ± 1.0^{b}	2.8 ± 0.8^{a,b}	3.7 ± 0.3^{a}	2.0 ± 0.2^{a}	1.2 ± 0.1^{b}	2.3 ± 0.2^{a}
22:6n-3	32.8 ± 1.4	30.1 ± 1.2	32.0 ± 0.5	11.5 ± 8.8	20.3 ± 3.7	21.4 ± 4.9	16.1 ± 0.6^{a}	8.5 ± 0.7^{b}	14.8 ± 0.7^{a}
Total n-3PUFA	41.9 ± 1.8	39.4 ± 0.6	41.2 ± 0.5	18.7 ± 12.9	33.1 ± 4.2	35.0 ± 5.2	25.5 ± 0.6^{a}	14.8 ± 0.7^{b}	23.9 ± 0.7^{a}
Total PUFA	56.2 ± 1.6	54.2 ± 1.0	56.2 ± 0.2	23.4 ± 16.3	40.0 ± 5.8	44.1 ± 5.8	37.5 ± 1.3^{a}	27.7 ± 0.0^{b}	36.7 ± 0.8^{a}

Results are mean ± SD (n = 3). The significance of differences between dietary treatments for each species were analysed by one-way analysis of variance. Mean values with different superscript letters within a given row for each individual species are significantly different ($P < 0.05$) as determined byTukey's multiple range test. PUFA, polyunsaturated fatty acids; tr, trace value (<0.05%).

and channel catfish (*Ictalurus punctatus*) (Bai & Gatlin 1993). Lygren *et al*. (2000) found no differences in SGR of Atlantic salmon (*Salmo salar*) fed with three different levels of dietary vitamin E. On the other hand, amago salmon (*Oncorhynchus rhodurus*) fed with a diet without vitamin E supplementation showed lower appetite with a significant decrease in body weight although survival rate was not significantly different from that of control fish fed with diets supplemented with 400 mg α-tocopherol kg^{-1} (Taveekijakarn *et al*. 1996). Juvenile Korean rockfish (*Sebastes schlegeli*) fed with a diet without vitamin E showed a significantly lower weight gain and feed conversion ratio than those fed with diets containing 20–120 mg vitamin E kg^{-1} but fish fed with a diet containing high (500 mg kg^{-1}) vitamin E also showed adverse responses in terms of growth (Bai & Lee 1998).

Livers from fish fed with the requirement, or higher doses, of tocopherol, possibly may be expected to be larger than livers of fish fed with diets low in vitamin E, because some degeneration of liver tissues through lipid peroxidation would have been possible. This degeneration has been observed in salmonids through histological examination (Smith 1979). Hower, often the opposite is true with lipoid liver degeneration as a result of lipid accumulation (Tacon 1996). Different dietary levels of vitamin E did not affect the HSI in Atlantic salmon (Lygren *et al*. 2000). In the present study, the HSI was not affected by dietary vitamin E in turbot but it was increased in halibut fed with the diet with the lowest vitamin E content and similarly it was lowest in sea bream fed with the diet containing the highest vitamin E level. The HSI was not influenced by the dietary α-tocopheryl acetate inclusion level in African catfish (Baker & Davies 1996a) but a correlation was found between HSI and dietary vitamin E dose in juvenile catfish fed with diets containing fresh or rancid oil and a supplementation of 20 or 100 mg α-tocopherol kg^{-1} dry diet (Baker & Davies 1996b).

In the present study, a gradation was observed in liver vitamin E levels in response to dietary vitamin E levels in all species, although generally only the level of vitamin E in the liver of fish fed with the E1000 diet was significantly different from those of fish fed with the other diets. The same effect was observed when examining the levels of vitamin E in whole turbot and halibut. This is consistent with several previous studies showing an increase in the α-tocopherol content in plasma or in body tissues as dietary vitamin E increased. The α-tocopherol content in turbot liver and muscle tissue was significantly influenced by dietary intake of α-tocopherol and to a lesser extent by dietary lipid as the α-tocopherol content was lower in the muscle tissue of fish

Table 7 Effect of dietary vitamin E on the activities of antioxidant enzymes in liver and lipid peroxidation products in liver and whole fish of turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and sea bream (*Sparus aurata*)

	Turbot			Halibut			Sea bream		
	E0	E100	E1000	E10	E100	E1000	E10	E100	E1000
Vitamin E*									
Liver	160 ± 15^{b}	706 ± 766^{b}	6205 ± 3423^{a}	71 ± 45^{b}	25 ± 22^{b}	1484 ± 794^{a}	54 ± 4^{c}	495 ± 20^{b}	6661 ± 631^{a}
Fish	62 ± 6^{b}	135 ± 69^{b}	256 ± 98^{a}	2 ± 1	7 ± 6	56 ± 97	n.a.	n.a.	n.a.
PUFA/vit E									
Liver	145 ± 20^{a}	54 ± 16^{b}	5 ± 0^{c}	103 ± 40^{b}	637 ± 155^{a}	14 ± 5^{b}	583 ± 70^{a}	75 ± 4^{b}	7 ± 1^{c}
Fish	292 ± 46^{a}	117 ± 18^{b}	44 ± 2^{c}	4400 ± 1773^{a}	1500 ± 333^{b}	235 ± 23^{b}	n.a.	n.a.	n.a.
Catalase	157 ± 9	163 ± 13	153 ± 17	101 ± 46^{a,b}	165 ± 17^{a}	96 ± 36^{b}	466 ± 73^{a}	456 ± 7^{a}	331 ± 13^{b}
SOD	4.7 ± 0.7	4.3 ± 1.7	4.9 ± 0.6	5.4 ± 0.4	5.8 ± 0.2	5.8 ± 0.6	5.4 ± 0.1^{a}	4.9 ± 0^{b}	5.0 ± 0.1^{b}
GPX	615 ± 41^{a}	170 ± 18^{b}	244 ± 120^{b}	51 ± 14^{b}	95 ± 9^{a}	25 ± 5^{c}	162 ± 16	176 ± 7	165 ± 4
GST	158 ± 12	162 ± 15	137 ± 12	178 ± 9^{b}	240 ± 23^{a}	206 ± 27^{a,b}	86 ± 3^{b}	85 ± 1^{b}	99 ± 2^{a}
GR	7.8 ± 0.5	8.6 ± 0.3	8.5 ± 0.5	19.7 ± 3.6	15.5 ± 0.3	16.6 ± 1.1	21.4 ± 0.8^{b}	23 ± 0.0^{b}	29.8 ± 1.1^{a}
TBARS									
liver	0.35 ± 0.02^{a}	0.27 ± 0.05^{b}	0.21 ± 0.01^{b}	6.47 ± 1.01^{a}	0.78 ± 0.17^{b}	0.49 ± 0.12^{b}	2.06 ± 0.20^{a}	1.01 ± 0.20^{b}	1.56 ± 0.22^{a}
fish	24.1 ± 0.7^{a}	23.2 ± 1.9^{a}	17.5 ± 2.1^{b}	4.45 ± 0.77^{a}	2.73 ± 0.60^{b}	2.29 ± 0.42^{b}	n.a.	n.a.	n.a.
Isoprostane									
Liver	17.2 ± 2.5^{a}	20.0 ± 6.5^{a}	13.5 ± 0.4^{b}	16.0 ± 6.4^{a}	11.4 ± 4.2^{a,b}	7.3 ± 1.7^{b}	48.1 ± 6.5^{a,b}	56.9 ± 8.1^{a}	33.6 ± 4.2^{b}
Fish	36.9 ± 15.1	33.4 ± 13.6	38.3 ± 14.6	19.1 ± 1.7^{a}	18.6 ± 4.2^{a}	9.8 ± 1.1^{b}	n.a.	n.a.	n.a.

All data are presented as mean ± SD (n = 3). The significance of differences between dietary treatments for each species were analysed by one-way analysis of variance. Mean values with different superscript letters within a row for each species are significantly different ($P < 0.05$) as determined by Tukey's multiple range test. Catalase (mmol min^{-1} mg protein^{-1}); GPX, total glutathione peroxidase (nmol min^{-1} mg protein^{-1}); GR, glutathione reductase (nmol min^{-1} mg protein^{-1}); GST, glutathione-S-transferase (nmol min^{-1} mg protein^{-1}); SOD, total superoxide dismutase (SOD Units min^{-1} mg protein^{-1}); Vitamin E (μg g tissue^{-1}). TBARS (thiobarbituric acid-reactive substances; mg mg protein^{-1}); Isoprostane (pg mg protein^{-1}). *α-Tocopherol (small amounts of γ and δ were also detected); n.a., not analysed.

fed with cod liver oil, compared with those fed with peanut oil which will have a higher vitamin E content than cod liver oil (Stéphan *et al*. 1995). Also in Atlantic salmon, the hepatic α-tocopherol level significantly reflected the dietary vitamin E input (Lygren *et al*. 2000). Plasma α-tocopherol levels increased as dietary vitamin E increased in juvenile African catfish (Baker & Davies 1996b), and Gatta *et al*. (2000) found a correlation between dietary vitamin E level and flesh deposition in sea bass (*Dicentrarchus labrax*). Bai & Lee (1998) found a linear relation between dietary levels of α-tocopheryl acetate and liver α-tocopherol concentration in Korean rockfish, but the highest level of α-tocopherol supplementation (500 mg kg^{-1}) did not result in an elevated level of liver α-tocopherol compared with other groups, but did result in higher muscle and plasma tocopherol concentrations than those of fish fed with the other diets. This finding may suggest that the high level of α-tocopheryl acetate could have a negative effect on liver α-tocopherol concentration in this fish species. This is contrary to most fish species where the liver is the main site of deposition with increasing dietary supplement.

A useful way of expressing vitamin E levels is by relating them to the level of the PUFA molecules which protect from autoxidative damage. The PUFA/α-tocopherol ratio was found to be important in determining tissue susceptibility to fatty acid peroxidation in an earlier study in which vitamin E supplementation limited the effect of increased dietary fish oil on increased susceptibilty of tissues to peroxidation (Stephan *et al*. 1995). In the present study, the molar ratio of PUFA/vitamin E in the unsupplemented diet was around 9- and 32-fold higher than in the diets supplemented with 100 and 1000 mg vitamin E kg^{-1}, respectively. This was closely reflected in the molar ratios of PUFA/vitamin E in the turbot and sea bream livers, which were around 11-fold lower in fish fed with the E100 diet and 29- (turbot) to 83- (sea bream) fold lower in fish fed with the E1000 diet compared with the unsupplemented diet. The molar ratio of PUFA/vitamin E in the whole fish showed a similar trend in turbot and halibut but it was noteworthy that vitamin E levels in halibut were generally lower resulting in higher PUFA/vitamin E ratios.

The lipid contents and lipid class compositions of liver and whole fish were unaffected by dietary vitamin E levels in turbot and halibut but in sea bream the liver total lipid and triacylglycerol contents showed significant positive correlations with dietary vitamin E. Clearly the sea bream livers

were storing more lipid when fed with diets with higher vitamin E levels although the precise mechanism for this or its physiological significance is unclear. However, these fish grew better and so possibly ate more resulting in increased lipid deposition. The PUFA status may be expected to be more influenced by different levels of tissue vitamin E. Consistent with halibut showing the lowest levels of liver vitamin E and the highest PUFA/vitamin E ratios, there were significantly lower percentages of EPA, 22:5n-3, DHA, total n-3PUFA and total PUFA in fish fed with the diet with the lowest vitamin E level. Similarly, in the liver of juvenile Korean rockfish fed with different levels of α-tocopheryl acetate (20–500 mg vitamin E kg^{-1}), lower levels of PUFA and a lower PUFA/saturated fatty acid ratio were found when the fish were fed with the diet with low vitamin E (Bai & Lee 1998). By contrast, in the present study, there was no effect of dietary vitamin E on liver PUFA compositions in turbot, and the lower proportions of PUFA in sea bream fed with diet E100 compared with fish fed with the other diets was a curious result that was not obviously related to tissue vitamin E levels. The fatty acid composition of Atlantic salmon liver and fillet were not influenced by dietary α-tocopheryl acetate levels and length of feeding (Scaife *et al.* 2000). Watanabe *et al.* (1977) suggested that dietary deficiency of tocopherol exerts some effects on fatty acid composition, but α-tocopherol supplementation exceeding the requirement level had little effect on fatty acid composition of fish. Dietary vitamin E deficiency, coupled with selenium deficiency or feeding oxidized oil has been shown to increase liver PUFA levels in rats and African catfish (Buttriss & Diplock 1988; Baker & Davies 1996b). The mechanism proposed was stimulation of hepatic fatty acid desaturation and elongation activities as confirmed by Bell *et al.* (2000) in Atlantic salmon depleted in vitamin E and astaxanthin.

The level of dietary vitamin E also showed some significant effects on the activities of the enzymes of the liver antioxidant defence system. These effects have to be interpreted within the knowledge of the commonly perceived biochemical mechanisms of these enzyme systems. For instance, catalase and SOD are scavengers of active oxygen species, acting on hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively (Miller *et al.* 1993). Glutathione peroxidase also scavenges H_2O_2 as well as lipid hydroperoxides, which leads to the production of oxidised glutathione (GSSG). Glutathione reductase acts to maintain levels of reduced GSH via the reduction of GSSG at the expense of NADPH (Winston & Di Giulio 1991; Halliwell & Gutteridge 1996). Some isoenzymes of GST may also metabolize lipid hydroperoxides (Halliwell & Gutteridge 1996). Thus, the higher activity of GPX in turbot fed with the E0 diet, the lower levels of catalase and GPX in halibut fed with the E1000 diet and the lower level of catalase in sea bream fed with the E1000 diet are all consistent with the expected pattern. Interestingly, the highest levels of catalase, GPX and GST in halibut were observed in fish fed with diet E100, which of course was not the diet with the lowest vitamin E level. However, the activities were perfectly correlated with tissue (liver) vitamin E levels as these were the halibut with the lowest liver vitamin E levels. In contrast, the activity of GST and GR were not related to dietary or tissue vitamin E levels in turbot or sea bream. Glutathione-S-transferase is thought to form GSH conjugates with peroxy radicals but this activity has not been confirmed in fish (Miller *et al.* 1993). Few studies in fish have focused on the effects of dietary nutrients on the activities of the liver antioxidant defence enzymes with most studies investigating the role of the enzymes in pollutant detoxification (Peters *et al.* 1994) or developmental aspects (Aceto *et al.* 1994; Otto & Moon 1996; Peters & Livingstone 1996). No clear relationship between PUFA/vitamin E ratios and liver antioxidant enzyme activities were observed in gilthead sea bream fed with diets varying in PUFA/vitamin E ratios (Mourente *et al.* 2000) or during early development in unfed common dentex (*Dentex dentex*) larvae (Mourente *et al.* 1999a). Similarily, no interactions were observed between dietary vitamin E and antioxidant enzyme activities in Atlantic salmon but liver SOD and GPX activities were significantly reduced in hyperoxygenated fish although catalase activity was unaffected (Lygren *et al.* 2000). Peters & Livingstone (1996) investigated the antioxidant enzyme activities in embryologic and early larval stages of turbot. They found that SOD activity decreased progressively with age, while the activities of catalase, GPX and GR increased. The decreasing SOD activity may be indicative of a decreasing need to detoxify the superoxide radical. The increasing activities of catalase, GPX and GR may indicate a progressive need to remove hydrogen peroxide and lipid peroxides from the tissues.

The data assessing the level of lipid peroxidation in the present study gave consistent results in all three species with both TBARS and isoprostane levels generally being highest in fish fed with the E0 diet and generally lowest in fish fed with the E1000 diet. Previously, it was shown that dietary vitamin E, as well as the nature of dietary lipid, had a significant influence on the level of TBARS in both fresh and frozen turbot tissue with the content of TBARS higher in fish fed with diets containing cod liver oil than in diets containing peanut oil, and lower with α-tocopherol supple-

mentation (Stéphan *et al.* 1995). It was also found that peroxidation of PUFA is more efficiently inhibited by α-tocopherol supplementation in liver than in muscle, probably simply because of the fact that liver usually attains a much higher level of vitamin E than muscle, and so liver will be better protected from peroxidation than muscle. In juvenile African catfish, elevated dietary vitamin E resulted in decreased levels of TBARS (Baker & Davies 1996a, 1997), although doses of α-tocopherol above the requirement only marginally improved the protection against peroxidation. In contrast, Olsen *et al.* (1999) found that α-tocopherol did not influence the tissue TBARS content in juvenile Arctic char (*Salvelinus alpinus* L.), while high dietary PUFA increased the content of TBARS in liver and in muscle.

That the two separate assays used to estimate lipid peroxidation status in the present study gave consistent results was noteworthy as the TBARS assay is a chemical assay measuring aldehydes that are secondary (and end-) products of the lipid peroxidation chain reactions whereas the 8-isoprostane assay is an immunoassay measuring nonenzymatically produced peroxidized derivatives of specific PUFA (Burk *et al.* 1980; Roberts & Morrow 1997). That the production of isoprostanes and TBARS showed a similar pattern in the present study supports the potential of isoprostane measurements as indicators of *in vivo* lipid peroxidation in fish. This is consistent with previous studies which showed that isoprostane levels broadly paralleled TBARS levels in sea bream fed with diets varying in PUFA/vitamin E ratio (Mourente *et al.* 2000) and that levels of 8-isoprostane were increased in plasma of Atlantic salmon fed with diets deficient in astaxanthin or vitamin E and astaxanthin (Bell *et al.* 2000). Prior to these studies, isoprostanes as measures of *in vivo* oxidative stress had only been reported for mammalian species rich in arachidonic acid (AA; 20:4n-6) and, consequently, 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 regio-isomer being 8-isoprostane (8-iso-prostaglandin $F_{2\alpha}$) (Roberts & Morrow 1997). Additional studies showed that other PUFA were also peroxidized by nonenzymatic free radical mechanisms to produce isoprostanes, and F_3- and F_4-isoprostanes from EPA and DHA, respectively, were identified, characterized and reported to correlate with other markers of lipid peroxidation (Nourooz-Zadeh *et al.* 1997, 1998). Although enzymatically produced prostaglandins are dominated by those derived from AA, even in fish tissues where EPA is normally present in far greater amounts (Tocher 1995), there is no reason to believe this would also be the case for nonenzymatically produced isoprostanes. Indeed, it is more likely that isoprostanes are produced in quantities reflecting the concentrations of their PUFA precursors. The present study on three marine fish species suggests that the commercially available EIA kit, which uses antibodies towards 8-iso-prostaglandin $F_{2\alpha}$, but which also has 21% reactivity towards 8-iso-prostaglandin $F_{3\alpha}$, the equivalent isoprostane from EPA, can be used effectively in fish.

The present study has demonstrated relationships between dietary vitamin E levels, liver vitamin E levels, the activities of the liver antioxidant enzymes and the levels of liver lipid peroxidation products. The overall balance of the data showed that these relationships were logical in that decreased dietary vitamin E and increased dietary PUFA/vitamin E levels led to decreased levels of vitamin E and increased PUFA/vitamin E in liver, and generally higher activities of the liver antioxidant enzymes and higher levels of lipid peroxides. Studies investigating more severe peroxidizing conditions, including the feeding of oxidized oils, are in progress in order to induce a more stressful pro-oxidant status and to further characterize the biochemical indicators of peroxidative stress.

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