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Effects of alpha-tocopherol and dietary oxidized fish oil on the immune response of sea bass *Dicentrarchus labrax*

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ABSTRACT: Sea bass weighing about 35 g were reared on 6 experimental diets differing in the level of alpha-tocopherol (vitamin E) supplementation (0, 40 or 300 mg kg^{-1}) and fish oil quality (fresh or oxidized). After 35 wk, group comparisons of haematological parameters were made and alpha-tocopherol levels in anterior kidney, spleen, and thymus were assessed in pooled samples. Non-specific immune factors assayed were: (1) plasma lysozyme and complement activities, (2) natural haemolysis of sheep red blood cells (SRBC), and (3) chemiluminescence (CL) response of head kidney phagocytes. Humoral antibody production was also assessed for each group after immunisation with *Vibrio anguillarum* 408. Resistance to bacterial infection was assessed comparatively according to diet. The levels of alphatocopherol in the different tissues were a function of the dietary vitamin E concentrations and were lower when oxidized fish oil was added to the diet. Erythrocyte fragility was raised in vitamin E depleted groups as well as in fish fed diets containing oxidized oil. Disease resistance, antibody response to *V. anguillarum* antigen, and haemolytic activity of sera were not affected by dietary treatment. Plasma lysozyme activity was lower in groups fed diets containing oxidized oil or not supplemented with vitamin E, and complement activity was higher in the fish that were fed the diet containing fresh oil and 300 mg vitamin E kg^{-1}. One month after vaccination, the CL peak response of head kidney phagocytes, stimulated by opsonized zymosan, was significantly lower in the fish that were fed the diet containing oxidized oil and not supplemented with vitamin E.

INTRODUCTION

Diets for cultured marine fish contain high levels of polyunsaturated fatty acids (PUFA) derived from marine fish oils. These fatty acids are especially prone to oxidative damage during diet preparation and storage. Products derived from lipid peroxidation may be absorbed after decomposition in the intestinal tract and transported to tissues, where they may induce adverse effects (Hata et al. 1986). In homeotherms, these effects are characterized by deterioration of tissue lipids, inactivation of enzymes, and destruction of biological membranes (Kaneda & Miyazawa 1987). Because fish contain high concentrations of highly unsaturated fatty acids, they are vulnerable to lipid peroxidation and to tissue damage resulting from lipid peroxidation (Lall 1988).

Alpha-tocopherol (vitamin E) is best known for its antioxidant properties and has been demonstrated to be an essential dietary component for several fish species (Woodall et al. 1964, Watanabe et al. 1970, Murai & Andrews 1974, Poston et al. 1976). Requirements for alpha-tocopherol are affected by other dietary factors such as the presence of oxidized oil in the diet and the dietary lipid level (Hung et al. 1981, Cowey et al. 1984).

In recent years, vitamin E has been demonstrated to be important for the immune response in various species of animals in which it appears to influence both humoral and cellular factors. Supplemental vitamin E enhanced antibody production against a variety of particulate or soluble antigens by promoting increased proliferation of antibody-producing cells (Tengerdy et al. 1973) and by stimulating the response to T-cell mitogens as well as the mixed lymphocyte response to murine spleen cells (Corwin & Gordon 1982).

Supplementation with alpha-tocopherol also increased the phagocytic activity of peritoneal macrophages (Heinzerling et al. 1974) and resistance to bacterial infections (Nockels 1979, Likoff et al. 1981). In fish, the impact of vitamin E on immune functions has also been studied. Blazer & Wolke (1984) showed that both T-cell (migration inhibition factors) and B-cell (plaque-forming cell)-mediated responses were compromised in rainbow trout *Oncorhynchus mykiss* W. fed low vitamin E diets. Humoral immunity and phagocytic ability of the peritoneal macrophages were also impaired in this species. Complement function was only affected in vitamin E depleted Atlantic salmon *Salmo salar* L. (Hardie et al. 1990). The impairment of this function was related to increased mortality rate after challenge with *Aeromonas salmonicida*.

The present study was designed to evaluate the effect of diets containing oxidized or fresh oils and various levels of vitamin E on the immune response and disease resistance of sea bass *Dicentrarchus labrax* L. Complementary studies were conducted in our laboratory (Stéphan et al. 1991) to investigate the effect of such diets on histopathology as well as on plasma and tissue biochemistry.

MATERIAL AND METHODS

Animals and feeding. Sea bass, 35 g mean weight, were distributed randomly by groups of 32 into 12 experimental tanks (EWOS 1 × 1). These tanks were supplied with filtered and well-oxygenated seawater (salinity: 35 ‰; temperature: 20 ± 1 °C). Water renewal (150 l h^{-1}) was maintained throughout the experiment. The diets were prepared just before the start of the experiment by coating IFREMER rehydratable expanded pellets with an oily mixture (Table 1) representing 9.43 % of the dry weight of the complete feed (Person-Le Ruyet et al. 1990). The composition of the basal diet is given in Table 2. Six experimental diets (F-0, F-40, F-300, Ox-0, Ox-40, Ox-300) were prepared, the compositions of which differed in the quality of added fish oil [fresh (F) or oxidized (Ox)] and the level of tocopherol acetate: 0, 40 or 300 mg kg^{-1} of feed. Oxidized oil was obtained by heating at 40 °C with constant aeration (compressed air) for 30 d, and this led to a marked increase in peroxide value (280 mEq kg^{-1} total dietary lipids) and a 16 % decrease in n-3 fatty acids in the feed. The main features of the lipids contained in the experimental diets are shown in Table 3. Diets were considered as isoenergetic and the calculated energy content was 15.86 MJ of metabolizable energy per kg diet. Diets were sealed in plastic bags and stored at −20 °C until use. The daily ration, periodically adjusted throughout the experiment, corresponded to approximately 1 % of the body weight. Each diet was fed to duplicate tanks of fish during a 35 wk period.

Table 1. Composition of the oil suspension (% of dry matter contained in the complete feed)

Cod liver oil[a]	6.800
Soya lecithin	1.000
Vitamin premix[b]	1.000
Choline (50 %)	0.600
Ascorbic acid	0.030
Tocopherol acetate[c]	-

[a] Fresh or oxidized

[b] Composition of vitamin premix (per kg premix)

– Vit. A (Acetate): 1000000 IU	– Vit. D_3: 100000 IU
– Vit. B_1 (thiamin): 1000 mg	– Vit. K_3: 100 mg
– D Calcium pantothenate: 5000 mg	– Vit. B_2 (riboflavin): 2500 mg
– Vit. B_{12}: 6 mg	– Vit. B_6 (pyridoxine): 1000 mg
– Folic acid: 500 mg	– Vit. PP (niacin): 10000 mg
– Meso-inositol: 100 000 mg	– Biotin: 100 mg

[c] dl-alpha-tocopherol acetate: 0, 40 or 300 mg kg^{-1} of feed

General protocol. After 35 wk of feeding, blood samples were taken from 14 fish per dietary treatment (7 fish per tank) for determination of haematological and plasma parameters. Ten other fish from each diet

Table 2. Composition of the IFREMER rehydratable expanded pellet (% of dry matter contained in the complete feed)

Norwegian capelin meal	42.0
Fish protein concentrate	8.0
Blood meal	4.0
Hydrolysed feather meal	3.0
Brewers' yeast	3.0
Corn gluten	7.0
Wheat meal	4.0
Wheat germ meal	3.0
Wheat middlings	4.0
DL-Methionine	0.45
Potato alpha starch liagel®	11.0
Mineral premix[a]	1.0

[a] Composition of the mineral premix (%)

Potassium chloride	9.000
Potassium iodide	0.004
Dicalcium phosphate	50.000
Sodium chloride	4.000
Copper sulphate (25 % Cu)	0.300
Zinc sulphate (34 % Zn)	0.400
Cobalt sulphate (21 % Co)	0.002
Iron sulphate (20 % Fe)	2.000
Manganese sulphate (30 % Mn)	0.300
Calcium carbonate	21.500
Magnesium carbonate	12.400
Sodium fluoride	0.100

Table 3. Main features of the lipids contained in the 6 experimental diets: analytic results

	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300
Total lipids (% of wet feed)	10.7	10.8	11.0	10.8	10.8	10.5
Vitamin E (mg kg^{-1} wet feed)	2.0	49.8	321.4	2.2	41.9	283.9
Peroxide value (POV) (mEq kg^{-1} of lipids)	14.7	15.4	14.9	274.0	286.0	281.0
Main fatty acids (% of total fatty acids)						
C14:0	5.16	5.01	5.06	5.43	5.48	5.49
C15:0	0.51	0.52	0.52	0.55	0.58	0.56
C16:0	16.16	16.16	16.25	17.43	17.59	17.56
C18:0	3.57	3.62	3.63	3.91	3.94	3.96
Total saturates	25.92	25.85	26.00	27.92	28.18	28.14
C16:1	5.96	5.82	5.90	6.30	6.34	6.35
C18:1	2.60	2.56	2.61	2.78	2.86	2.75
C20:1	0.24	0.24	0.24	0.26	0.27	0.28
Total n-7	8.80	8.62	8.75	9.34	9.47	9.38
C18:1	13.53	13.47	13.47	14.46	14.46	14.54
C20:1	4.07	3.94	3.87	4.19	4.20	4.12
C22:1	0.46	0.46	0.36	0.51	0.48	0.44
C24:1	0.76	0.73	0.92	0.90	0.85	0.83
Total n-9	18.82	18.60	18.62	20.06	19.99	19.93
C18:2	8.18	8.25	8.28	8.78	8.80	8.83
C20:2	0.17	0.19	0.18	0.16	0.16	0.18
C20:4	0.78	0.81	0.82	0.73	0.73	0.76
Total n-6	9.13	9.25	9.28	9.67	9.69	9.77
C18:3	1.33	1.36	1.35	1.40	1.39	1.38
C18:4	1.56	1.54	1.55	1.39	1.40	1.42
C20:4	0.70	0.72	0.68	0.63	0.64	0.64
C20:5	9.32	9.35	9.39	7.73	7.66	7.82
C22:5	1.86	1.89	1.85	1.53	1.64	1.56
C22:6	13.13	13.18	13.21	10.78	10.80	10.73
Total n-3	27.90	28.04	28.03	23.46	23.53	23.55
C22:1n11	4.50	4.57	4.45	4.79	4.75	4.60

group (5 fish per tank) were vaccinated with *Vibrio anguillarum* bacterin and placed in 6 new separate tanks. From these fish, blood samples for antibody titration were taken 4 wk after vaccination. Head kidney, spleen, and thymus were also removed to determine vitamin E levels and to perform chemiluminescence assays. The 20 fish remaining in each of the original tanks were experimentally infected with *Vibrio anguillarum* serotype 1 (strain 408, Baudin Laurencin 1981) to compare their resistance to the pathogen.

Blood sampling: Blood samples were collected in heparinized vacuum tubes (vacutainer® Becton Dickinson Vacutainer System) from the caudal vessel. Approximately 2 ml of whole blood was taken from each fish. The blood was centrifuged (3000 × *g*, 10 min) and the resulting plasma samples were collected and stored at −70 °C until analysis.

Experimental infection: *Vibrio anguillarum* broth culture was prepared as previously described by Obach et al. (1990). Fish were first anaesthetized with ethylene glycol monophenyl ether and then intraperitoneally injected with 0.2 ml of a dilution of the virulent suspension (5.5×10^6 cells per fish). Deaths were recorded over an 8 d period. Dead fish were collected daily and necropsied. Kidney samples were cultured on tryptic soy agar (TSA), supplemented with 1.5 % NaCl, to verify the presence of *V. anguillarum*.

Vaccination: Ten fish from each dietary treatment

were immunised intraperitoneally with 0.2 ml of the vaccine 'Vibriffa injectable' (Mérieux). The vaccine is a formalized inactivated suspension of *Vibrio anguillarum* containing about 2×10^{10} bacterial cells ml^{-1}. Vaccinated fish were placed in 6 separate tanks and fed as before with the appropriate test diets for 4 wk. Water temperature at vaccination was 20 °C. A few days before sampling, all 10 fish in one tank fed the diet containing fresh oil and 300 mg vitamin E kg^{-1} diet died after a brief accidental increase in water temperature (45 °C for 10 to 15 h). Temperature remained constant in the other tanks.

Test methods. ***Haematological determinations:*** Haematocrit values were determined using the microhaematocrit technique and by centrifugation at 12 000 × *g* for 5 min. Haemoglobin concentration (Hb) was measured spectrophotometrically with a Compur M 1000 D_1 using the cyanmethemoglobin method. The red blood cell count (RBCC) was made by adding blood to Gowas' solution and then analysed photometrically with a Compur M 1000 D_1 by measuring the turbidity. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated.

The erythrocyte fragility test was carried out as described by Drapers & Csallany (1969). Fifty µl of whole blood was added to test tubes containing pH 7.4 phosphate buffered saline (PBS), the tubes were incubated at 20 °C for 24 h, and the resulting haemolysis was measured photometrically.

Antibody production: Agglutination antibody titers were determined according to the microtitration technique described by Maisse & Dorson (1976) for anti-*Aeromonas salmonicida* antibodies, but with slight modifications: the antigen, *Vibrio anguillarum*, was inactivated by heating (100 °C, 60 min) and resuspended in sterile physiological saline to an optical density of 1 at 625 nm.

Lysozyme assay: The turbidimetric assay for lysozyme was carried out according to Parry et al. (1965) as modified by Grinde et al. (1988). Briefly, test plasma (100 µl) was added to 2 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg per ml) in a 0.05 M sodium phosphate buffer, pH 6.2. The reactions were carried out at 20 °C and absorbance at 520 nm was measured between 1 and 4 min. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} at 20 °C.

Total haemolytic complement assay: Complement activity was assayed using the procedure of Mayer (1971), with some modifications: total reaction volume and SRBC count were reduced 50-fold (from 7.5 to 0.15 ml and from 5×10^8 to 1×10^7 SRBC), to permit using microplates. SRBC were washed 3 times with phosphate buffered saline and resuspended to yield a 2 % concentration (2×10^8 cells ml^{-1}) in Hank's balanced salt solution (HBSS). SRBC were sensitised with heat-inactivated sea bass anti-SRBC serum, diluted to 1:70, a dilution permitting complete haemolysis (pers. obs.). SRBC were incubated with occasional shaking at 30 °C for 30 min. Test plasma samples were first diluted to 1/200, then volumes of the diluted plasma (0.01, 0.02, ... 0.09 ml) were combined with HBSS (0.09, 0.08, ... 0.01 ml) in the microplate and 0.05 ml of sensitised SRBC were added per well. Complete haemolysis was obtained by mixing 0.05 ml of sensitised SRBC with 0.1 ml distilled water (positive control). After incubation at 20 °C for 60 min, microplates were centrifuged at 600 × *g* for 5 min. Optical density of supernatants was measured in a microplate reader (Argus 300 – Packard) at 490 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of plasma added (ml). The volume which yielded 50 % haemolysis was determined and used for calculating the complement activity of the sample (CH50 units ml^{-1}) as follows (Sakai 1981):

$$\text{CH-50 unit ml}^{-1} = \frac{\text{Dilution coefficient of plasma}}{\text{ml of 50\% haemolysis}} \times \frac{0.15 \text{ ml}}{7.5 \text{ ml}}$$

Natural haemolytic activity: The technique used was based on that of Chiller et al. (1969). Serial 2-fold dilutions of each sample (50 µl) from sea bass were made on microtitration plates (Plaque microwell 96 U Nunclon Delta) with HBSS. Fifty µl of a 2 % uncoated SRBC suspension was added to each well. The plates were incubated for 1 h at 20 °C and then overnight at 4 °C. Natural haemolytic activity was expressed as the reciprocal of the highest dilution yielding complete haemolysis.

Chemiluminescence assay: Head kidney cell suspensions were prepared as previously described by Angelidis et al. (1988). Head kidney was removed and pounded on a metallic filter (filtres Cellector Belco – 100 µm). Cells were harvested, suspended in HBSS without phenol red, and kept on crushed ice. Leukocyte concentration was adjusted to 5×10^6 viable cells ml^{-1}. Cell viability was evaluated by the trypan blue exclusion method. Luminol was prepared according to the method described by Scott & Klesius (1981) to give a stock solution of 8 mM, and stored for a week at 4 °C in tubes wrapped in aluminium foil. Stock solutions were diluted 4 to 6 h before use in HBSS to obtain working solutions of 0.05 mM. The procedure for opsonizing zymosan was a modification of that used by Scott & Klesius (1981). Zymosan and pooled serum from normal (non-experimental) fish were incubated together at 30 °C for 45 min. Zymosan

Table 4. *Dicentrarchus labrax*. Vitamine E concentration (µg g^{-1} of fresh tissue) in head kidney, spleen, and thymus of sea bass fed 6 experimental diets for 39 wk. Data are expressed as the vitamin E concentration for pooled organs from 5 fish per diet (2 replicates)

Organ	Replicate	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300
Head kidney	1	6.39	11.95	ND	1.10	4.73	33.75
	2	4.27	14.92		0.40	4.06	33.42
Spleen	1	6.90	11.60	ND	0.96	7.80	29.05
	2	6.45	13.49		1.18	5.90	30.23
Thymus	1	5.31	11.00	ND	0.65	3.32	37.23
	2	4.55	11.54		0.12	3.34	34.88
		c	b		d	c	a

Columns not sharing a common letter are significantly different at $p \leq 0.01$
ND: not determined

final concentration was 1 mg ml^{-1}. Duplicate assay vials received 0.1 ml cell suspension, 0.1 ml opsonized zymosan, and 0.05 ml luminol. Control vials were prepared by replacing zymosan with HBSS. Chemiluminescence (CL) values were recorded 6 times at 10 min intervals on a 1250 LKB luminometer, to determine CL peak intensities.

Vitamin E determination: Endogenous dl-alpha-tocopherol levels were determined by the Buttriss & Diplock (1984) method, with some modifications, previously described by Messager et al. (1992).

Statistical methods. For most of the parameters studied, the effect of each of the 2 factors quality of oil (F-1: fresh or oxidized) and level of vitamin E supplementation (F-2: 0, 40 or 300 mg kg^{-1} diet), as well as their interaction, were tested by a 2-way analysis of variance (ANOVA) based on the average of 7 fish per tank and 2 tanks per treatment (n = 2) using a computerized STATITCF statistical system (ITCF, Boigneville, France). For endogenous vitamin E levels and antibody titers, due to the accidental loss of one entire experimental group (F-300), the effect of only 1 factor (diet) was tested by a 1-way ANOVA. When $p \leq 0.05$ a Newman-Keuls test was applied. CL peak responses were compared using a Kruskall-Wallis non-parametric test, after rank transformation. When $p \leq 0.05$, a Dunn test (Dunn 1964) was applied.

RESULTS

Endogenous vitamin E levels. Head kidney, spleen, and thymus vitamin E levels reflected dietary input and were significantly decreased by the addition of oxidized oil to the diet (Table 4).

Haematology. The haematological measurements for fish fed the 6 experimental diets are shown in Table 5. Although fish fed the diet containing oxidized oil and no supplemental vitamin E had lower haematocrit, haemoglobin, and RBCC values, no statistical differences were noted between the 6 experimental groups. Erythrocyte fragility (EF) was significantly affected by both factors: vitamin E depleted groups showed an increased EF when compared to those fed diets supplemented with 40 or 300 mg vitamin E kg^{-1} diet. EF was also increased in fish fed diets containing oxidized oil.

Plasma parameters (Table 6). Plasma lysozyme and complement activities were affected by both factors: fish oil quality and level of vitamin E supplementation. In fact, lysozyme activity was markedly higher in groups fed diets containing fresh oil and supplemented with 40 or 300 mg vitamin E kg^{-1} (significant interaction: F-300 > F-40 > F-0 = Ox-0 = Ox-40 = Ox-300) and complement activity was higher in fish fed the diet containing fresh oil and 300 mg vitamin E kg^{-1} (significant interaction: F-300 > F-40 = F-0 = Ox-0 = Ox-40 = Ox-300). Natural haemolytic activity was not influenced by either factor.

Experimental infection. Following challenge, deaths occurred from the 2nd to the 9th day. All dead fish were positive for *Vibrio anguillarum* and classic signs of haemorrhagic septicaemia were observed. The post-challenge mortality rates ranged from 78 to 90 % and no statistically significant differences between the various diet groups were detected (Table 7).

Serum antibody. Before vaccination none of the fish sampled had detectable serum antibody (log_2 titer ≥ 2) specific for *Vibrio anguillarum*. Vaccination resulted in substantial antibody production in all diet groups (mean log_2 titer = 8.3) but there were no significant differences in antibody responses among fish given the dietary treatments (Table 8).

Chemiluminescence. The CL response of head kidney phagocytes stimulated by opsonised zymosan increased progressively, reached a peak between 30 and 40 min, and then decreased slightly. Zymosan-stimulated CL responses were widely variable in all the experimental dietary groups. Basal activities for non-

Table 5. *Dicentrarchus labrax*. Haematological parameters (mean + standard deviation) of sea bass after 35 wk on 6 experimental diets (2 replicates, n = 7). Statistical significance : F-1 = supplemented vitamin E level factor; F-2 = lipid quality factor; I = interaction F-1 × F-2

Parameter	Replicate	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300	Statistical significance	
Haematocrit	1	26.0 ± 3.4	23.7 ± 3.1	24.1 ± 3.3	21.1 ± 2.9	31.8 ± 3.9	24.9 ± 2.4	F-1	NS
(%)	2	27.4 ± 6.5	29.1 ± 4.1	33.7 ± 2.4	19.1 ± 3.2	25.7 ± 3.4	32.7 ± 4.5	F-2	NS
								I	NS
Haemoglobin	1	6.64 ± 0.77	6.27 ± 0.91	6.59 ± 0.76	5.81 ± 1.01	8.38 ± 0.57	6.81 ± 0.78	F-1	NS
concentration	2	6.93 ± 1.63	7.74 ± 1.13	8.86 ± 0.61	4.99 ± 0.86	6.95 ± 0.80	8.01 ± 0.71	F-2	NS
(g 100 ml^{-1})								I	NS
Red blood cell	1	2533 ± 337	2409 ± 418	2388 ± 390	2098 ± 392	3261 ± 357	2544 ± 337	F-1	NS
counts (10^3 mm^{-3})	2	2600 ± 743	2979 ± 399	3493 ± 200	1886 ± 353	2567 ± 354	3080 ± 295	F-2	NS
								I	NS
MCV (μm^3)[a]	1	102.7 ± 1.0	99.1 ± 7.3	101.6 ± 5.7	101.8 ± 6.6	97.3 ± 3.4	98.1 ± 4.1	F-1	NS
	2	107.5 ± 18.2	97.8 ± 4.5	96.5 ± 4.2	101.1 ± 4.2	100.4 ± 5.4	106.0 ± 6.8	F-2	NS
								I	NS
MCH (pg)[b]	1	26.3 ± 1.1	26.2 ± 1.9	27.8 ± 1.5	27.7 ± 0.9	25.8 ± 1.1	26.9 ± 0.8	F-1	NS
	2	27.0 ± 1.9	26.0 ± 1.1	25.4 ± 1.1	26.5 ± 1.0	27.0 ± 1.5	26.1 ± 1.3	F-2	NS
								I	NS
MCHC (%)[c]	1	25.6 ± 0.9	26.5 ± 1.2	27.4 ± 1.2	27.3 ± 1.2	26.5 ± 1.7	27.4 ± 0.6	F-1	NS
	2	25.6 ± 3.9	26.6 ± 0.7	26.3 ± 0.6	26.3 ± 1.2	26.9 ± 1.1	24.5 ± 1.7	F-2	NS
								I	NS
Haemolysis (24 h)	1	33.3 ± 6.7	26.4 ± 5.1	29.7 ± 7.5	58.3 ± 28.94	31.75 ± 2.6	29.0 ± 5.2	F-1	••
(%)	2	30.1 ± 18.0	18.2 ± 6.2	23.3 ± 12.1	94.3 ± 10.4	25.03 ± 4.1	35.5 ± 13.3	F-2	•
								I	NS

[a]MCV = mean corpuscular volume
[b]MCH = mean corpuscular haemoglobin
[c]MCHC = mean corpuscular haemoglobin concentration
NS: not significant; • $p \leq 0.05$; •• $p \leq 0.01$; •• $p \leq 0.001$

Table 6. *Dicentrarchus labrax*. Plasma parameters (mean ± standard deviation) of sea bass fed 6 experimental diets for 35 wk (2 replicates, n = 5). Statistical significance (2 way ANOVA) : F-1 = supplemented vitamin E level factor; F-2 = lipid quality factor; I = interaction F-1 × F-2

Parameter	Replicate	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300	Statistical significance
Plasma lysozyme ($U\ ml^{-1}$)	1	320 ± 85	358 ± 63	410 ± 97	298 ± 60	304 ± 68	276 ± 40	F-1 ••
	2	300 ± 45	364 ± 52	416 ± 87	324 ± 95	300 ± 81	288 ± 64	F-2 •••
		c	b	a	c	c	c	I •••
Complement activity ($CH\text{-}50\ units\ ml^{-1}$)	1	143.0 ± 56.3	172.1 ± 59.4	186.2 ± 57.9	148.8 ± 64.1	163.2 ± 52.9	154.5 ± 44.6	F-2 ••
	2	153.4 ± 52.9	160.3 ± 61.4	197.1 ± 53.0	132.5 ± 55.6	169.0 ± 46.8	161.0 ± 54.1	F-2 •
	c	c	a	c	c	c	I	•
Natural haemolytic activity (log_2)	1	3.4 ± 0.9	3.6 ± 0.5	3.8 ± 0.8	2.8 ± 0.4	3.2 ± 1.3	3.4 ± 1.5	F-1 NS
	2	3.8 ± 1.5	4.2 ± 0.4	3.6 ± 0.9	3.6 ± 0.5	3.6 ± 1.1	3.4 ± 0.9	F-2 NS
								I NS

NS: not significant ; • $p \leq 0.05$; •• $p \leq 0.01$; ••• $p \leq 0.001$ Treatments not sharing a common letter are significantly different

stimulated phagocytes were very low (always < 1 mV). CL peak responses were significantly lower in fish fed the diet containing oxidized oil and not supplemented with vitamin E, when compared with fish fed the diets with oxidized oil and 300 mg vitamin E kg^{-1} or fresh oil and 40 mg vitamin E kg^{-1} (Fig. 1).

DISCUSSION

Stéphan et al. (1991) showed that the fish in this experiment exhibited no differences in growth rate and other biometric parameters after being fed the experimental feed for 35 wk. Likewise, no obvious changes in health or behaviour were noted, and necropsy performed upon sampling did not yield any evidence of macroscopic lesions. However, these authors observed a degeneration of the muscle fibers, together with high aspartate amino transferase (ASAT) and creatine kinase (CK) activities, which they related to the level of thiobarbituric acid-reactive substances, a parameter frequently used to assess the oxidation level of lipids *in vivo* (Crastes de Paulet 1988) and to the tissue content of alpha-tocopherol. The muscle levels of alpha-tocopherol reflected dietary input and were clearly decreased by the addition of oxidized oil to the diet. The vitamin E concentrations in the haematopoietic organs were similarly affected.

Erythrocyte fragility was significantly increased by feeding a diet that contained a low level of alpha-tocopherol and/or oxidized lipids. This phenomenon has already been observed (Hung et al. 1981, Cowey et al. 1984) and may be the result of the structural role played by this vitamin in the cell membranes and/or of membrane phospholipid peroxidation. Indeed, beside its anti-oxidant function, alpha-tocopherol, through its structure which features a hydrophilic end and a long hydrophobic chain, is easily integrated within the cell membranes (Jore & Ferradini 1988), where it can establish structural links with PUFA (Lucy 1974). Vitamin E deficiency could therefore be reflected by a change in membrane structure and increased fragility. In addition, as it has been shown in higher vertebrates, peroxidation of dietary PUFA results in the formation of hydroperoxides, followed by secondary auto-oxidation products (Kanazawa et al. 1985, Oarada et al. 1986) which can themselves induce endogenous lipid peroxidation (Minamoto et al. 1985, Kaneda & Minazawa 1987). Lipid peroxides can cause alteration of the cell membranes, as reflected by reduced fluidity, modification of permeability, and decompartmentalization (Jore & Ferradini 1988). The levels of thiobarbituric acid-reactive substances noted in muscle indicated high lipid peroxidation *in vivo*, favourable to erythrocyte membrane degeneration. Such a fragility of eryth-

Table 7. *Dicentrarchus labrax*. Mortality rate (%) after experimental injection with *Vibrio anguillarum* 408 of sea bass fed 6 experimental diets for 35 wk (2 replicates, n = 20). Statistical significance (2-way ANOVA) . F-1 = supplemented vitamin E level factor; F-2 = lipid quality factor; I = interaction F-1 × F-2

	Replicate	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300	Statistical significance	
Mortality rate (%)	1	85.7	78.9	84.2	78.9	85.0	84.2	F-1	NS
	2	77.8	90.5	78.6	89.5	83.3	89.5	F-2	NS
								I	NS

Table 8. Antibody titres (mean + standard deviation) of sea bass fed 6 experimental diets for 39 wk, 4 wk after vaccination with *Vibrio anguillarum* bacterin (2 replicates, n = 5)

	Replicate	F-0	F-40	F-300	Ox-0	Ox-40	Ox-300
Antibody titres (log_2)	1	8.4 ± 0.8	8.8 ± 0.7	ND	8.6 ± 1.4	8.0 ± 0.9	7.8 ± 1.5
	2	9.0 ± 1.1	8.6 ± 0.7	ND	7.0 ± 1.4	8.8 ± 0.7	8.4 ± 0.8
		a	a		a	a	a

ND: not determined

rocytes could facilitate the development of anaemia. While the statistical tests used did not reveal any significant differences, the fact remains that RBCC, haematocrit, and haemoglobin were clearly lower in the fish fed the Ox-0 feed, indicating an anaemic tendency. This phenomenon is in agreement with the results obtained from similar experiments on other species such as rainbow trout (Smith 1979, Moccia et al. 1984) and catfish (Murai & Andrews 1974).

Phagocyte activity of the head kidney leukocytes of fish fed the Ox-0 feed was significantly lower than noted in groups fed the T-40 and Ox-300 diets. A relationship appeared to exist between the chemiluminescence response and the tissue levels of alpha-tocopherol in the groups fed the diets with oxidized lipids, even if this relationship was not always significant. Blazer & Wolke (1984) showed that rainbow trout fed alpha-tocopherol-deficient diets had lower phagocyte activities than those fed a diet containing 250 mg vitamin E kg^{-1}. By contrast, in an experiment conducted by Hardie et al. (1990), the macrophage activity of Atlantic salmon was not altered by feeding a low alpha-tocopherol diet, and these authors suggested that the tissue concentration of vitamin E, although low, may have been sufficient to maintain leukocyte membrane integrity. Indeed, the same oxidative processes as those described for erythrocytes may exist for macrophages, and this may induce cell membrane

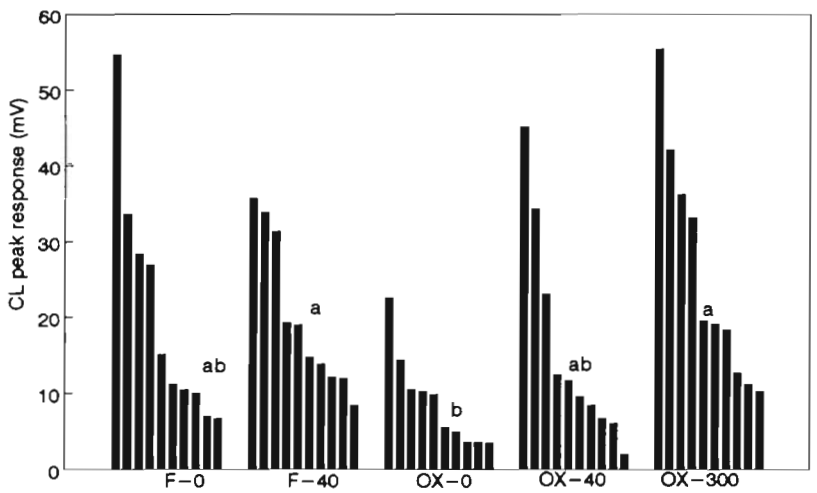

Fig. 1: *Dicentrarchus labrax*. Luminol-enhanced chemiluminescence (CL) peak response of zymosan-stimulated head kidney phagocytes from sea bass fed various experimental diets for 39 wk (10 fish per diet). CL assay was performed 4 wk after vaccination with *Vibrio anguillarum* vaccine. Groups sharing a common letter are not statistically different ($p < 0.05$)

degradation. The macrophage membrane plays a key role in phagocytosis, and the changes in viscosity and permeability which may occur can alter cellular functions. Moreover, the macrophage membrane contains several receptors for the Fc fragment of immunoglobulins (Griffin 1983, Sakai 1984, Honda et al. 1985) and for the complement factor C3 (Johnson & Smith 1984, Nonaka et al. 1984, Matsuyama et al. 1992). These factors favour antigen adhesion and engulfment and result in phagosome formation. Lastly, the membrane contains enzymes that are involved in the production of active forms of O_2 (Chung & Secombes 1988) and that are the source of chemiluminescence during the respiratory burst.

Plasma lysozyme activity also appears to be dependent on the 2 dietary factors. Vitamin E deficiency on the one hand, and supplementation of diets with oxidized lipids on the other, induce a decrease in the activity of the enzyme. In higher vertebrates, the biological effects of lipid peroxides may not only influence cell membrane function but also lead to the inhibition of certain enzymes (Kaneda & Miyazawa 1987), probably due to the establishment of bonds between proteins and lipids at the level of sulphur amino-acids (Jore & Ferradini 1988). Indeed, some oxidation products, in particular those with low molecular weight, may react with the thiol groups of certain enzymes and inhibit them (Schauenstein et al. 1977). Thus, Kanazawa et al. (1975) showed that linoleic acid oxidation products may inhibit lysozyme, *in vitro*, through selective destruction of certain amino-acids, including methionine.

The complement specific haemolytic activity also varied according to dietary treatment. The sea bass which received a diet containing non-oxidized lipids and supplemented with 300 mg alpha-tocopherol per kg of feed had higher complement activity than found in the other experimental groups. Hardie et al. (1990) observed a similar phenomenon in Atlantic salmon. In this regard, the plasma from fish fed an alpha-tocopherol-deprived diet had lower total specific haemolytic activity and bacterial opsonizing capacity than the fish fed a diet supplemented with 326 mg kg^{-1} vitamin E. The mechanism of action of vitamin E and oxidized lipids on the complement activity is not known. However, the fact that macrophages are the site of production of some of the complement proteins suggests that an alteration of macrophage function caused by peroxidation of the membrane phospholipid fatty acids may alter the synthesis of some of these proteins.

Several studies have suggested that lysozyme (Fänge et al. 1976, Murray & Fletcher 1976, Lundblad et al. 1979, Lindsay 1986, Grinde 1989), complement (Li & Lovell 1985, Hardie et al. 1990, 1991), and phagocyte activity (Ellis 1981, MacArthur & Fletcher 1985) play a major defensive role against infectious diseases and may constitute the first, and sometimes the most important, response of fish against bioaggressors (Blazer 1991). In this study, however, the variations of these 3 parameters due to different dietary treatments did not yield differences in survival of *Vibrio anguillarum* infection. This result can possibly be explained by the high challenge dose used (it killed 78 to 90 % of the fish). Likely, this high dose overwhelmed the protective effect of the foregoing parameters. It is also possible that depression of some aspects of immunocompetence, although significant, may not have been marked enough to induce a measurable decrease in immune protection against the trial bacterium. Indeed, during a similar study on turbot (Obach & Baudin Laurencin 1992), an increase in susceptibility to *V. anguillarum* was observed in fish fed a diet containing oxidized oil and not supplemented with vitamin E. In this case, however, the decrease in the immune parameters measured was much greater. In the present experiment, the influence of the various dietary treatments was not reflected by any variation in specific humoral response. Thus, macrophage and possible lymphocyte alterations were not sufficiently pronounced to prevent these cells from producing antibody. Forster et al. (1988) investigated the influence of dietary lipid oxidation and vitamin E levels on performance and disease resistance in coho salmon *Oncorhynchus kisutch*. Immunocompetence, judged by antibody production in response to *Vibrio* vaccination, and mortality after experimental infection with *V. anguillarum* or *V. ordalii* were unaffected by the dietary treatments. Nevertheless, the fish did not suffer a vitamin E deficiency on any of the diets and the data did not preclude the possibility that significant dietary effects might have been identified if more fish had been employed or if fish had been reared at a higher water temperature.

After 35 wk on the test diets, certain haematological and immunological parameters were modified, but only slightly. Similarly, the histopathological lesions and enzymatic variations observed by Stéphan et al. (1991) in relation to the dietary treatment of this study were moderate. Compared to salmonids (Hung et al. 1981, Cowey et al. 1984), sea bass may have lower needs for vitamin E, and they may be more resistant to dietary lipid oxidation (Baudin Laurencin et al. 1989).

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