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Effects of dietary oxidized fish oil and antioxidant deficiency on histopathology, haematology, tissue and plasma biochemistry of sea bass *Dicentrarchus labrax*

Jean-Luc Messager, Germaine Stéphan, Claire Quentel and Félix Baudin Laurencin,

Centre National d'Etudes Vétérinaires et Alimentaires, Laboratoire de Pathologie des Animaux Aquatiques, (CNEVA-LPAA), B.P. 70, 29280 Plouzané, Brest, France.

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Abstract

Two groups of sea bass (mean initial weight 78 and 250 g) were given for 23 weeks a commercial diet, whose composition was modified or not (control). The modified diet was: − supplemented with highly oxidized fish oil, − poorly supplemented with ascorbic acid, − not supplemented with butylated hydroxytoluene, DL-alpha-tocopheryl acetate and choline. At the end of the experiment, fish fed the modified diet displayed skeletal muscle degeneration, decreased red blood cell count, haemoglobin content and haematocrit, and increased erythrocyte fragility and plasma enzyme activity (aspartate aminotransferase and creatine kinase). Simultaneously, low DL-alpha-tocopheryl acetate levels and increased amounts of thiobarbituric acid-reactive substances were observed in muscle and liver. On the other hand, growth disorders, liver lesions, changed plasma enzyme activity (alanine aminotransferase, glutathione peroxydase), or altered conjugated dienes in perivisceral fat were not evidenced. The pathological observations were more pronounced on the smaller fish group, but the disease could already be detected by measuring haematological and biochemical criteria, the interest of which in the diagnosis of such nutritional disease is discussed.

Keywords: Sea bass, nutritional pathology, oxidation stress, diagnosis.

Influence d'un aliment supplémenté en huile de poissons oxydée et appauvri en anti-oxydants, sur l'histopathologie, l'hématologie, la biochimie tissulaire et plasmatique du bar, Dicentrarchus labrax.

Résumé

Deux lots de bars (de 78 et 250 g de poids moyen initial), d'origine différente, reçoivent chacun, pendant 23 semaines, un aliment commercial dont la composition est modifiée ou non (témoin) : − par l'incorporation d'huile de poisson fortement oxydée, − par la diminution de la supplémentation en acide ascorbique, − par la suppression de la supplémentation en butyl-hydroxytoluène, en acétate de tocophérol et en choline. A la fin de l'expérience, les poissons nourris avec l'aliment modifié montrent des lésions de dégénérescence du muscle squelettique, une diminution du nombre de globules rouges, du taux d'hémoglobine et de l'hématocrite, une augmentation de la fragilité érythrocytaire et de fortes activités enzymatiques plasmatiques (aspartate, aminotransferase et créatine kinase). Parallèlement, de faibles valeurs en DL-alpha-tocophérol et une augmentation des teneurs en substances réagissant à l'acide thiobarbiturique sont observées dans le muscle et le foie. Par contre, aucun trouble de croissance, aucune lésion hépatique, aucune modification des activités enzymatiques plasmatiques alanine aminotransférase et glutathione-péroxydase, ni aucune altération des diènes conjugués des lipides de la graisse mésentérique n'est mis en évidence. La pathologie développée est plus accentuée chez le lot de bars de poids moyen inférieur mais reste toutefois modérée; la maladie

peut cependant être déjà détectée par la mesure de divers paramètres hématologiques et biochimiques dont l'intérêt dans le diagnostic de ce type de maladie nutritionnelle est discuté.

Mots-clés : Bar, pathologie nutritionnelle, stress oxydatif, diagnostic.

INTRODUCTION

Most pathological symptoms observed in mammals and birds due to a vitamin E and/or selenium deficiency (Green and Bunyan, 1969; Johnson, 1979) have been reproduced and experimentally studied in several fish species (Woodall *et al.*, 1964; Watanabe *et al.*, 1970; Murai and Andrews, 1974; Poston *et al.*, 1976; Smith, 1979; Hung *et al.*, 1981; Cowey *et al.*, 1984; Lovell *et al.*, 1984; Moccia *et al.*, 1984; Wilson *et al.*, 1984; Miyazaki, 1986). These works show that the effects induced in fish may, as in higher vertebrates, vary according to the species, the experimental conditions and the dietary levels of vitamin C, of polyunsaturated lipids (oxidized or not), of proteins, amino acids containing sulfur, choline and synthetic antioxidants. Despite the difficulties in interpretation related to the complexity and variation of the experimental methods used, it has been clearly established that there is a close interdependence of action between selenium and vitamin E (Poston *et al.*, 1976; Bell *et al.*, 1985) and that the effects of a deficiency in one or the other, or both at the same time, are always exacerbated when the feed contains peroxidized lipids (Murai and Andrews, 1974; Watanabe *et al.*, 1977, 1981; Hung *et al.*, 1981; Cowey *et al.*, 1984). A high supplementation of antioxidants is, however, capable of preventing or alleviating the appearance of this pathology in fish, even if the feed does contain notable quantities of peroxidized lipids (Smith, 1979; Moccia *et al.*, 1984).

Pathological characteristics reported in fish are mainly: mortality, growth disorders, nervous symptoms (Poston *et al.*, 1976; Lovell, 1984), skeletal and cardiac muscular dystrophy, liver, pancreatic and kidney degeneration, inflammation (ascites, oedema, pericarditis). Haematological and biochemical changes are also observed: erythrocyte fragility, anaemia, increase in the amount of thiobarbituric acid-reactive substances in tissues, increased plasma enzyme activity (lactate dehydrogenase, aldolase).

Over that last few years, some of these symptoms and lesions have been observed in marine fish, notably in sea bass *Dicentrarchus labrax* farmed in tropical environments (Gallet de Saint-Aurin, 1987; Raymond, 1988): mortality, drop in growth, anorexia, loss of equilibrium, skeletal myopathy, liver, pancreatic and kidney degeneration, deposits of ceroid and hemosiderin found in the liver and spleen. The hypothesis of a disease of nutritional aetiology was therefore put forward, but the incorporation of oxidized lipids in the experimental feeds was not alone able to give clinical signs of the disease (Stéphan, 1988; Baudin Laurencin *et al.*, 1990).

Taking into account these results, the present experiment has twin objectives: on the one hand, to attempt to reproduce in a marine fish, the sea bass, notable pathological effects characteristic of a nutritional disease induced by oxidative phenomena; on the other hand, to evaluate the interest of various haematological and biochemical indices for the detection and diagnosis of this type of disease. As suggested by Moccia *et al.* (1984) fish status is able to modify fish response when they are fed oxidized or vitamin E deficient diets. Therefore two groups of sea bass, differing in origin, size, genetic make up and dietary history are compared in the experiment.

MATERIAL AND METHODS

Experimental design

Two feeds (a control feed and a modified feed) were given for 23 weeks to 2 groups of sea bass (P and G) of different weight and origin, distributed each in 4 tanks located at random in a factorial experimental design (2×2) with two replicates per treatment. The "modified" feed was poor in antioxidants and supplemented with highly oxidized oil. Blood and organ samples were analysed at the start (all fish had been fed the control feed for 1 month) and at the end of the experiment.

Fish

Two groups of sea bass P and G of 78 and 250 g mean respective weight, from two different local farms were randomly distributed (20 fish per tank) in 8 experimental tanks (EWOS, 1×1 m) containing about 300 l of seawater. The same temperature conditions (20 ± 1°C), salinity ($35\,^{0}/_{00}$), aeration (4 compressed air diffusors per tank), and water renewal (150 l/hr.) were maintained throughout the experiment.

Feeds-feeding regime

The control feed was prepared by adding to a IFREMER rehydratable expanded pellet (Person-Le Ruyet *et al.*, 1990) an oily suspension representing

9.55% of the dry weight of the complete feed (composition in *table* 1 *a*).

The modified feed was prepared in the same way, but the phase of oil addition was modified (composition in *table* 1 *b*):

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

a. Control feed		b. Modified feed
6.800	Fresh cod liver oil	0
0	Oxidized cod liver oil	6.800
1.000	Soya lecithin	1.000
1.000	Vitamin Premix 1 (*)	0
0	Vitamin Premix 2 (**)	1.000
0.600	Choline Chloride 50%	0
0.010	B.H.T. (Butyl-Hydroxytoluene)	0
0.140	Ascorbic acid	0.005

(*) Composition of vitamin Premix 1: (per kg Premix).
- Vit. A acetate: 1 000 000 IU
- DL-alpha-tocopheryl acetate: 4 000 mg
- Thiamin: 1 000 mg
- D calcium pantothenate: 5 000 mg
- Vit. B12: 6 mg
- Folic acid: 500 mg
- Meso-inositol: 100 000 mg
- Vit. D3: 100 00 IU
- Vit. K3: 100 mg
- Riboflavin: 2 500 mg
- Pyridoxine: 1 000 mg
- Niacin: 10 000 mg
- Biotin: 100 mg

(**) Composition of vitamin Premix 2
Identical to that of vitamin Premix 1 but does not contain DL-alpha-tocopheryl acetate

– through previous oxidation of the oil with compressed air bubbles at ambient temperature until approximately 30% of the polyunsaturated fatty acids have disappeared.

– through removal of the supplementation of synthetic antioxidants (butyl-hydroxytoluene), tocopherol acetate and choline.

– through reducing the supplementation of ascorbic acid.

The main features of lipids contained in the oils and feeds are presented in *table* 2. The feeds, once prepared, were kept frozen until use (maximum of 3 months). The daily ration, periodically adjusted throughout the experiment, corresponded to approximately 1% of the body weight of the fish.

Table 2. – Main features of the lipids contained in oils and feeds: analytic results.

	Oil		Feed	
	Fresh	Modified	Control	Modified
Total lipids (% of the wet feed)	-	-	11.55	11.05
Fatty acids/total lipids	-	-	88.60	81.00
Vitamin E (mg/kg wet feed)	-	-	38.00	5.00
Peroxide value (POV) (mEq/kg of lipids)	8.2	780.0	11.80	348.00
Main fatty acids (% of total fatty acids)				
C14:0	6.8	7.7	5.5	5.9
C15:0	0.4	0.5	0.4	0.4
C16:0	13.7	15.6	13.9	15.1
C17:0	0.3	0.4	0.3	0.3
C18:0	2.3	2.6	2.5	2.7
TOTAL	23.5	26.8	22.6	24.4
C16:1	8.1	9.1	6.3	6.6
C18.1	3.2	3.6	2.8	2.9
C20:1	0.6	0.6	0.4	0.5
TOTAL n – 7	11.9	13.3	9.5	10.0
C18:1	9.7	10.9	10.5	11.4
C20:1	8.0	9.1	7.4	7.9
C22:1	1.1	1.3	0.9	1.0
C24:1	0.7	0.7	0.6	0.8
TOTAL n – 9	19.5	22.0	19.4	21.0
C18:2	1.2	1.2	8.0	8.7
C20:2	0.2	0.2	0.2	0.2
C20:2	0.6	0.5	0.5	0.4
TOTAL n – 6	2.0	1.9	8.7	9.3
C18:3	0.5	0.5	1.3	1.3
C18:4	2.1	1.7	2.1	1.9
C20:4	0.6	0.4	0.6	0.4
C20:5	11.1	7.6	9.4	7.2
C22:5	1.4	1.0	1.1	0.9
C22:6	10.8	6.7	10.1	7.8
TOTAL n – 3	26.5	17.9	24.6	19.5
C22:1 n – 11	8.0	9.1	7.9	8.5

Samples

Samples were taken from 9 fish per tank.

1 ml of blood was sampled, with a vacutainer(r) system (lithium heparinate) from the *ductus Cuvieri* of every fish, previously fasted for 48 hours. Blood was immediately refrigerated in crushed ice and samples were taken to measure the haematocrit, the haemoglobin content, the red blood cells count (RBC) and erythrocyte fragility. The remaining blood was centrifuged (3 000 g, 7 min). Part of the plasma was kept at 4°C to determine in the following hours the enzyme activities of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and creatine kinase (CK). Another part of the plasma was frozen at –20°C for ulterior determination of glucose and protein concentrations and was then frozen at –80°C (for 24 hours) the measure the glutathione peroxidase activity (GPX).

As soon as the blood sample had been obtained, the fish were killed, measured and dissected. For each

fish, the liver and spleen were weighed to determine the hepato-somatic index (HSI) and the splenosomatic index (SSI). Then, samples of liver, muscle and adipose tissue were taken. The liver and muscle samples were immediately placed in a Bouin's fluid for ulterior histological examination. Other sections were conserved for immediate determination of the liver thiobarbituric acid-reactive substances (TBA) or were frozen at −80°C to determine the liver and muscle vitamin E content and the TBA reactive substances content in muscle.

Finally, the mesenteric fat was removed, its lipids extracted and immediately analysed by spectrophotometry to measure the conjugated dienes.

Methods of measurement and analysis

Growth

Growth was expressed by the specific growth rate G $(\%.d^{-1}) = (\ln P_f - \ln P_i)/(f-i) \times 100$. P_f and P_i were the mean weights of the fish at days f (final sample) and i (initial sample).

Haematology

The following parameters were studied:

– Haematocrit through centrifugation of capillary tubes for 5 min at 12 000 g.

– The haemoglobin content by the cyanmethemoglobin method and the red blood cells count (RBC) by turbidimetry were determined with a photometer Compur(r) M 1 000 D (Quentel and Aldrin, 1986).

From these parameters were calculated: the mean corpuscular volume (MCV), the mean corpuscular haemoglobin (MCH), the mean corpuscular haemoglobin concentration (MCHC).

– Erythrocyte fragility, through the micromethod according to a concentration of NaCl: 10 µl of heparinized blood were placed successively into 12 cupules in a microtitration plate Nunclon(r) with a round base, each containing a saline solution of NaCl. The NaCl concentration varied, from one cupule to another, from 1 to $7^0/_{00}$ and by steps of $0.5^0/_{00}$. After shaking, the plate was placed in an oven at 20°C for 4 hours. The reading was then made: two concentrations of the saline were recorded. The first corresponded to when hemolysis began, *i.e.* the degree of concentration which produced hemolysis in a few erythrocytes, partial hemolysis. The second, total hemolysis, which provoked hemolysis of all cells.

Plasma biochemistry

Except for the determination of GPX activity, all analyses (at 30°C) were carried out with the help of an automaton ISAMA(r) (Isabiologie, France). Plasma glucose was measured colorimetrically with the Biomerieux(r) (kit N° 61273) method (glucose oxidase) and reagents. Plasma protein was determined through colorimetry (540 nm) by the biuret method.

The ALAT, ASAT and CK activity were determined through the kinetic UV method using reagents marketed by Biomerieux(r) (respectively: kits N° 63301; 63201; 63151).

The plasma glutathione peroxydase activity was determined through the method described by Paglia and Valentine (1967) employing 2 successive enzyme substrates: cumene hydroperoxide and hydrogen peroxide (0.2 mmol/l H_2O_2 in the presence of 20 mmol/l of sodium azide).

Histology

After inclusion in paraffin, sections of 7 µm were made and coloured by hematoxylin-eosin, the reaction to the periodic acid Schiff (PAS) and by Sudan black.

Tissue biochemistry

Tissue moisture content (%): determined through a gravimetric method, with desiccation of tissues for 24 hours in an oven at 100°C.

Thiobarbituric acid-reactive substances (TBA): The liver content was determined with the (slightly modified) Ohkawa *et al.* (1979) method: with the aim of protecting the lipids from possible oxidation, whilst heating, 100 µg of butylhydroxytoluene were added to each test portion of 200 µl of a tissue homogenate (10% W/V in distilled water). The muscular content was determined by the Witte *et al.* (1970) method.

Spectrophotometric analysis of the lipids of the mesenteric fat

• The lipids were extracted according to the Folch *et al.* (1957) method. They were placed in cyclohexane (chromasol quality, SDS France) and the coefficient of absorption of a solution at 1% (W/V) (Crastes de Paulet, 1988) was determined at 232 nm (1 cm wide cell) with a BECKMAN-DU 40 spectrophotometer.

• *Liver and muscle vitamin E content.* The DL-alpha-tocopherol contents were determined by the

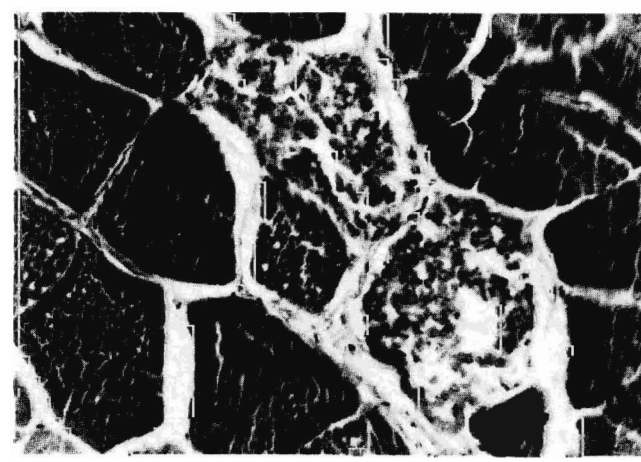

Figure 1. – Typical granular degeneration of the muscular fibres of sea bass fed the modified feed.

(slightly modified) Buttriss and Diplock (1984) method: to approximately 100 mg of liver of 1 g of muscle were added 1 ml of a methanolic solution of ascorbic acid (20 mg/ml) the 5-7 dimethyltocol at the rate of 1 to 10 µg according to the presumed alpha-tocopherol content of the sample. Saponification is effected with 1 ml of 2 mol/l solution of KOH in methanol for 30 min in a 90°C water bath. After cooling and the addition of 1 ml of a mixture of ethanol-water (4/5, V/V), the unsaponifiable part was extracted twice with 1.5 ml hexane; it was then dry evaporated and silylated with 100 µl of the silyl mixture (ref. 3.3038 - SUPELCO 17, rue Saint-Gilles, 75003 Paris) for 15 min at 50°C. After evaporation under nitrogen, the silyl derivatives were dissolved in hexane and were quantified by using gas-chromatography with a GIRDEL (551, rue de Verdun, 92150 Suresnes) apparatus coupled to an integrator. A capillary column OV1701 grafted onto silica ($L = 32$ m; $\varphi = 0.32$ mm) was used. The carrier gas was helium ($Pe = 0.6$ bars). The temperature of the oven was 280°C and of the detector and injector 300°C.

Statistical methods

The effect of each of the two factors: the dietary factor (F1: modified versus control feed), the group-origin factor (F2: P versus G group fish) as well as their interaction were first studied by a two-way analysis of variance (ANOVA), with the help of the software "STATITCIF" (ITCF, Boigneville, France). When a significant interaction ($p \leqslant 0.05$) of the two factors was noted, a Newman-Keuls test was applied to compare the means of the 4 treatments. In some instances (body weight, RSS, RBC, MCHC, plasma glucose and ALAT activity) data required a logarithmic transformation before ANOVA.

Non-homogeneous data (ASAT, CK and GPX activity, partial and total hemolysis, muscular amount of vitamin E, content of TBA reactive substances in muscle and liver) where compared using a Kruskall-Wallis non-parametric test, after rank transformation. When $p \leqslant 0.05$, a Dunn test (Dunn, 1964) was applied to compare rank sums of the different treatments.

RESULTS

Mortality-gross pathology-histopathology

No mortality was recorded throughout the experiment. On dissection, no fish presented notable external or internal macroscopical lesions.

Histological examinations (livers and muscles of 72 fish, 18 from each group, removed at the end of the experiment) show the absence of any significant liver or pancreatic lesions (inter-hepatocytary pancreas). Muscular lesions were on the contrary observed. They only appeared in fish fed the modified feed. Granular degeneration of the muscular fibres (*fig.* 1) was noted in 12 of the 18 fish of group P and 7 of 18 of group G. Up to 5% of superficial muscle fibres were injured. In group P only, the white muscle was also affected, but to a lesser extent.

Feeding behaviour-growth

The daily ration (1% of the body weight) was very quickly ingested (<2 min) by the fish of group P. Independent of the quality of the feed (control and

Table 3. – Biometric parameters (mean ± standard deviation) at days 0 ($n = 23$) and 162 ($n = 18$) of sea bass fed two different diets. Statistical significance: (1) two-way ANOVA and Newman-Keuls test results ($p \leqslant 0.05$). F1: dietary factor; F2: group origin factor; I: interaction F1*F2. (2) Kruskall-Wallis test and Dunn test results ($p \leqslant 0.05$). NS: not significant; S: significant ($p \leqslant 0.05$). Values with common letters between parentheses are not significantly different.

	Day	Feed					Statistical significance ($p \leqslant 0.05$) at day 162	
		Modified		Control				
Group origin		P	G	P	G			
Body weight	0			86 ± 15	266 ± 66		F1	NS
(g)	162	204 ± 35	419 ± 86	225 ± 48	421 ± 76	(1)	F2	S
							I	NS
Condition index	0			1.29 ± 0.12	1.41 ± 0.11		F1	NS
(W/L^3)	162	1.50 ± 0.15	1.40 ± 0.12	1.49 ± 0.12	1.40 ± 0.09	(1)	F2	S
							I	NS
Hepato-somatic	0			2.49 ± 0.63	2.27 ± 0.40		F1	NS
index	162	3.53 ± 0.66	2.40 ± 0.46	3.11 ± 0.47	2.36 ± 0.54	(1)	F2	S
							I	NS
Spleno-somatic	0			0.17 ± 0.06	0.11 ± 0.04		F1	S
index	162	0.13 ± 0.03	0.16 ± 0.10	0.11 ± 0.04	0.12 ± 0.05	(1)	F2	NS
							I	NS

modified feed), the appetite of the fish of group G was slightly lower: the feed sometimes remained in the water for up to 10 min and was occasionally not all ingested (up to 10%). The specific growth rate was markedly superior in the fish of group P (0.56%.d^{-1} instead of 0.28%.d^{-1} in G-group fish) but did not appear to ge greatly influenced by the quality of the feed. P group fish fed the control feed, did, however, show an apparently slightly higher growth rate (0.59%.d^{-1}) than those fed the modified feed (0.53%.d^{-1}).

Biometry (*table* 3)

The dietary factor only influenced the splenosomatic index, higher in fish fed the modified diet.

The group origin factor explained the other differences: a higher body weight in G-group but higher condition factor (W/L^3) and HSI in P-group fish.

Haematology (*table* 4)

Haematocrit, haemoglobin content, RBC, lower in fish fed the modified diet, were affected by the dietary factor.

The group origin factor also had significant effects on RBC, the haemoglobin content and MCHC, higher in P-group and on MCV, higher in G-group fish; but no interaction between the two factors was noted.

The partial hemolysis index was highly increased in fish fed the modified feed, but only significantly in P-group fish and MCH was found slightly higher in G-group fish fed the modified feed.

Plasma biochemistry (*table* 5)

A significant dietary effect on plasma ASAT and CK activies, increased in fish fed the modified diet, was revealed by the Kruskall-Wallis test. The protein level was higher in P-group fish, but was not affected by the dietary factor. Glyceamia seemed to be affected by both factors, without significant interaction but was higher mainly in P-group fish fed the control diet. GPX activity, whatever the substrate used, and ALAT activity were not significantly influenced by either factor.

Table 4. – Haematological parameters (mean ± standard deviation) at days 0 (n=23) and 162 (n=18) of sea bass fed two different diets. Statistical significance: (1) two-way ANOVA and Newman-Keuls test results ($p \leq 0.05$). F1: dietary factor; F2: group origin factor; I: interaction F1*F2. (2) Kruskall-Wallis test and Dunn test results ($p \leq 0.05$). NS: not significant; S: significant ($p \leq 0.05$). Values with common letters between parentheses are not significantly different.

		Feed					
		Modified		Control			Statistical significance ($p \leq 0.05$) at day 162
Group origin	Day	P	G	P	G		
Haematocrit (%)	0			23.2 ± 2.1	22.9 ± 1.5		F1 S
	162	23.0 ± 3.0	21.3 ± 2.6	27.6 ± 3.7	27.3 ± 2.7	(1)	F2 NS
							I NS
Haemoglobin	0			6.51 ± 0.60	6.67 ± 0.50		F1 S
content (g/100ml)	162	6.75 ± 0.79	6.07 ± 0.64	8.08 ± 1.08	7.60 ± 0.80	(1)	F2 S
							I NS
Red blood cells	0			2431 ± 296	2550 ± 275		F1 S
RBC (in thousand/mm^3	162	2676 ± 390	2261 ± 304	3155 ± 491	2946 ± 304	(1)	F2 S
							I NS
Mean corpuscular volume	0			95.6 ± 5.7	90.3 ± 5.9		F1 NS
MCV (μm^3)	162	86.5 ± 5.3	94.4 ± 2.8	87.7 ± 4.8	92.8 ± 5.4	(1)	F2 S
							I NS
Mean corpuscular haemoglobin	0			26.9 ± 1.6	26.3 ± 1.7		F1 NS
MCH (pg)	162	25.4 ± 1.5	27.0 ± 1.1	25.7 ± 1.6	25.7 ± 1.6	(1)	F2 S
		(*a*)	(*b*)	(*a*)	(*b*)		I S
Mean corpuscular haemoglobin	0			28.2 ± 0.9	29.1 ± 0.8		F1 NS
MCHC (%)	162	29.4 ± 2.1	28.6 ± 0.9	29.4 ± 1.5	27.8 ± 0.7	(1)	F2 S
							I NS
Partial hemolysis	0			4.26 ± 0.27	4.08 ± 0.18		
(NaCl ‰)	162	4.89 ± 0.39	4.64 ± 0.23	4.50 ± 0.17	4.56 ± 0.24	(2)	S
		(*b*)	(*a*)	(*a*)	(*a*)		
Total hemolysis	0			2.67 ± 0.37	2.59 ± 0.40		
(NaCl ‰)	162	2.59 ± 0.39	2.67 ± 0.24	2.61 ± 0.37	2.64 ± 0.33	(2)	NS

Table 5. – Plasma parameters (mean ± standard deviation) at days 0 ($n = 23$) and 162 ($n = 18$) of sea bass fed two different diets. Statistical significance: (1) two-way ANOVA and Newman-Keuls test results ($p \leq 0.05$). F1: dietary factor; F2: group origin factor; I: interaction F1*F2. (2) Kruskall-Wallis test and Dunn test results ($p \leq 0.05$). NS: not significant; S: significant ($p \leq 0.05$). Values with common letters between parentheses are not significantly different.

Group origin	Day	Feed: Modified P	Feed: Modified G	Feed: Control P	Feed: Control G		Statistical significance ($p \leq 0.05$) at day 162	
Alanine aminotransferase	0						F1	NS
ALAT (U/l)	162	5.4 ± 7.9	2.5 ± 1.4	3.3 ± 2.9	2.4 ± 2.3	(1)	F2	NS
							I	NS
Aspartate aminotransferase	0			16.4 ± 12.1	10.0 ± 5.5			
ASAT (U/l)	162	229 ± 210 (*b*)	107 ± 129 (*b*)	13.5 ± 4.3 (*a*)	14.9 ± 9.1 (*a*)	(2)	S	
Creatine kinase	0			88 ± 67	42 ± 22			
CK (U/l)	162	1 202 ± 1 205 (*b*)	125 ± 102 (*b*)	110 ± 68 (*a*)	63 ± 66 (*a*)	(2)	S	
	0			0.82 ± 0.08	0.75 ± 0.10		F1	S
Glucose (g/l)	162	0.73 ± 0.09	0.70 ± 0.06	0.85 ± 0.15	0.75 ± 0.06	(1)	F2	S
							I	NS
	0			42.6 ± 4.0	49.4 ± 3.6		F1	NS
Proteins (g/l)	162	49.0 ± 5.0	45.5 ± 3.0	50.0 ± 7.0	44.0 ± 3.0	(1)	F2	S
							I	NS
GPX (ROOH)								
nmol NADPH oxidized/min/mg	0							
of proteins	162	1.72 ± 054	1.53 ± 0.38	1.82 ± 031	1.64 ± 0.45	(2)	NS	
GPX (H_2O_2)								
nmol NADPH oxidized/min/mg	0							
of proteins	162	1.46 ± 0.40	1.23 ± 0.32	1.54 ± 0.29	1.27 ± 0.41	(2)	NS	

Tissue biochemistry (*table* 6)

No difference was found in the moisture content of muscle. Levels of vitamin E in tissues of fish fed the modified diet were decreased in liver and in muscle. Furthermore, vitamin E levels were higher in the G-group fish, significantly in liver.

The amount of TBA reactive substances was higher in muscle and, at a minor degree, in liver of fish fed the modified feed, while the coefficient of absorption (at 232 nm) of the lipids of the mesenteric fat was only influenced by the group origin factor; it was higher in the P-group fish.

DISCUSSION

After 5 months of feeding, the modified feed employed in this experiment led to the production in sea bass of certain characteristic signs of the pathology developed by numerous animal species, including fish, when they are fed diets favouring an *in vivo* lipid peroxidation process. Degeneration of the muscular fibres was observed, as well as elevated ASAT and CK plasma activities due to the release into the blood flow of these enzymes which are particularly abundant in muscle (Gaudet, 1975; Krasnovic-Ozretic and Ozretic 1987). At the same time, the amount of TBA reactive substances, the most commonly used parameter to measure the level of lipid peroxidation *in vivo* (Crastes de Paulet, 1988), was practically doubled in muscle (on average: +120% for P-group, +94% for G-group). An increased erythrocyte fragility, associated with a marked decrease in RBC and haemoglobin content were also observed. In vitamin E deficient fish, haematological changes may indeed originate from a decrease in the protective power that this vitamin has against the peroxidation of the phospholipids of the cell membranes induced by the presence of oxidized oil in the feed (Smith, 1979; Hung *et al.*, 1981; Cowey *et al.*, 1984; Moccia *et al.*, 1984) or by a selenium deficient diet (Bell *et al.*, 1985, 1987).

However, the presence of these characteristic pathological symptoms was very limited: the lesions affected only 1 to 5% of the muscle fibres and were only observed in a fraction of the population. The degree of erythrocyte fragility was also relatively low, the partial hemolysis index being higher by only 8.7 and 1.8% respectively for groups P and G compared to the controls. In the same manner, the RBC and haemoglobin content still remained elevated and were not characteristic of actual anaemia. Furthermore,

Table 6. – Tissue parameters (mean ± standard deviation) at days 0 ($n=23$) and 162 ($n=18$) of sea bass fed two different diets. Statistical significance: (1) two-way ANOVA and Newman-Keuls test results ($p \leqslant 0.05$). F1: dietary factor; F2: group origin factor; I: interaction F1*F2. (2) Kruskall-Wallis test and Dunn test results ($p \leqslant 0.05$). NS: not significant; S: significant ($p \leqslant 0.05$). Values with common letters between parentheses are not significantly different.

	Day	Feed				Statistical significance ($p \leqslant 0.05$) at day 162	
		Modified		Control			
Group origin		P	G	P	G		
Moisture content of muscular tissue (%)	0			74.5 ± 1.0	72.1 ± 1.2		F1 NS
	162	72.0 ± 1.5	71.6 ± 0.8	72.3 ± 1.9	71.8 ± 0.8	(1)	F2 NS
							I NS
Muscular amount of vitamin E (µg/g fresh tissue)	0			3.95 ± 0.78	4.16 ± 0.67		
	162	1.20 ± 0.53 (*a*)	1.74 ± 0.43 (*a*)	3.32 ± 0.68 (*b*)	4.00 ± 0.87 (*b*)	(2)	S
Muscular TBA reactive substances (nmol tetramethoxy-propane/g fresh tissue)	0			2.02 ± 0.78	1.76 ± 0.70		
	162	4.38 ± 1.05 (*c*)	3.12 ± 1.35 (*bc*)	1.98 ± 0.54 (*ab*)	1.60 ± 1.79 (*a*)	(2)	S
Amount of Vitamin E in the liver (µg/g fresh tissue)	0			24.5 ± 4.3	28.6 ± 3.6		F1 S
	162	6.63 ± 1.10 (*a*)	9.38 ± 1.05 (*b*)	28.9 ± 2.3 (*c*)	40.7 ± 2.7 (*d*)	(1)	F2 S
							I S
TBA reactive substance in the liver (nmol tetramethoxy-propane/g fresh tissue)	0			79.8 ± 19.3	67.2 ± 16.1		
	162	113 ± 38 (*b*)	89 ± 22 (*b*)	80 ± 32 (*ab*)	71 ± 18 (*a*)	(2)	S
						(2)	S
Coefficient of absorption (232 nm) of the mesenteric fat lipids	0			7.50 ± 0.46	6.39 ± 0.24		F1 NS
	162	6.59 ± 0.40	5.99 ± 0.24	6.73 ± 0.32	6.27 ± 0.32	(1)	F2 S
							I NS

other symptoms and lesions considered as characteristic of this type of pathology did not appear: appetite, behaviour or growth disorders, signs of exudative diathesis, pancreatic lesions. Finally, neither liver symptoms such as the presence of lipoid degeneration and ceroid deposit described by Corbari *et al.* (1984) in sea bass fed a feed supplemented with oxidized oil, nor a consecutive increase in the plasma ALAT activity were noted.

All these observations correspond to a pathology either moderate or in its first stages. In this case, of the tissue examined, the muscle was where the first signs of lesions appear. This is important and interesting from a diagnostic point of view. It was also in the skeletal muscle that Cowey *et al.* (1984) observed the most significant tissue lesions in rainbow trout and muscular dystrophy is also the most characteristic pathological sign reported in carp (Watanabe *et al.*, 1970; Watanabe *et al.*, 1977).

However, the "oxidizing power" of the modified feed appeared strong: the main natural (vitamins E and C) and synthetic (BHT) antioxidants are contained in very small quantities whilst the degree of oxidation of the incorporated oil appeared to be significant. In relation to several aspects (POV of oxidized oil, degradation rate of polyunsaturated fatty acids in oil and feed, vitamin E levels measured in the feed), this oxidizing power resembled that of the highly oxizided feed which enabled Hung *et al.* (1981) and Moccia *et al.* (1984), to induce, in 24 weeks, significant pathological symptoms and notably marked liver lesions in rainbow trout (it has to be said that the fish were small sized). In addition, the modified feed considerably reduced the vitamin E levels in sea bass tissues which, at the end of the experiment, were at levels at least as low as those determined by Hung *et al.* and Cowey *et al.* (1984), in rainbow trout which presented marked pathological symptoms.

With the hypothesis of a moderated or late developed disease, the farmed sea bass in grow-out may appear to be a species particularly resistant to the phenomenon of oxidation or of which the vitamin E requirements would be limited. However, the incidence of the modified feed may have been alleviated due to insufficient food intake or even possible diffusion throughout the medium during feed distribution of part of the altered products of the oxidized oil. Moreover, fish have other means of defence, notably enzyme mechanisms (Cowey, 1986) which are not apprehended in this study. These means of defence could be predominant in certain species like sea bass. Numerous other factors inherent to feed, experimental conditions, the biological stage and to the size of the fish, seem to be able to modify fish response and

may explain the differences in the results obtained for the same species as, for example in trout, according to the works of Cowey *et al.* (1981, 1983, 1984) and Watanabe *et al.* (1984). In our experiment, the impact of the modified feed was effectively stronger on one of the two experimental populations. The frequency of muscular lesions, the partial hemolysis index and the plasma CK activity clearly illustrated this situation which also tended to show through most of the other parameters. Indeed, compared to their respective controls, the P-group fish generally resulted in a stronger increase than G-group fish in the amount of liver (37.1% instead of 25.3%) and muscular (120.4% instead of 94.1%) TBA reactive substances and in the plasma ASAT activity (1 586% instead of 621%) compared to those of group G.

The age, size, origin and the status of body stores naturally differentiated the two populations from the beginning of the experiment. Subsequently, the differences in the quantities of feed ingested and in growth rate (initial weight multiplied by 2.6 for group P and by 1.6 for group G), provide further reasons for a quicker and more marked response for P-group fish).

Although restrained and little externalized, the disease developed by sea bass could, however, be detected by certain biochemical or haematological parameters, relatively easy to use and which may be applied to important numbers of fish. This observation reveals its interest in the detection and diagnosis of this type of nutritional pathology in fish.

The increase in CK and ASAT activity appears to be a sensitive, early and particularly useful criterion in the detection of muscle lesions, at least in sea bass. Associated with an increase in the amount of muscular TBA reactive substances, this increased enzyme activity should incite chemical analyses to be made, notably of vitamin E, on tissue samples from fish and on the feed distributed. Thus, 68% of sea bass fed the modified feed, compared to 0% in the control group, had both a TBA reactive muscular substance level higher than 2.5 nmol of equivalent tetra methoxy propane/g and an ASAT activity higher than 20 U/l. Additionally, the observation in P-group fish, compared to G-group fish, of higher plasma ASAT and CK activities in conjunction with more frequent tissue lesions and at a higher extent leads us to think that these enzyme parameters may help to extimate the degree of severity of the disease of the population concerned. Finally, through the study of the serum isosenzymes, it may be possible to specify the skeletal and/or cardiac origin of the muscular lesions.

The haematological parameters also reveal the influence of the modified feed: tendency towards anaemia and erythrocyte fragility. These typical symptoms (Smith, 1979; Cowey *et al.*, 1984; Moccia *et al.*, 1984; Woodall *et al.*, 1984) are not however, very pronounced in this study on sea bass. The number of erythrocytes and the haemoglobin content remained at a level comparable to that determined at the beginning of the experiment and the degree of erythrocyte fragility is little different to that of the controls, although sufficient enough to be significant in P-group fish. On the other hand, the stability of MCV, MCH, and MCHC values indicated that the tendency towards anaemia indifferently affects the entire erythrocyte population whereas Woodall *et al.* (1964) and Moccia *et al.* (1984) observe in salmonids an increase in the proportion of immature cells which they attribute to a blockage in cellular maturation. The results obtained in the present study seem to minimize the interest of these haematological parameters in the diagnosis of this type of disease in sea bass although they may be only an expression of the native of moderated feature of the pathological phenomenon.

The influence of the modified feed did not provoke the formation of conjugated dienes (as expressed as a variation in the coefficient of absorption at 232 nm) in the lipids of the adipose perivisceral tissue nor a modification in plasma GPX activity, contrary to previously obtained results (Stéphan, 1988). The absence of an increase in the ALAT activity, dominant enzyme in the liver of fish (Gaudet *et al.*, 1975; Krajnovic-Ozretic and Ozretic, 1987) tallies with the absence of lesions, even microscopic, in this organ. On the other hand, the liver level of TBA reactive substances is slightly higher than that of the controls. If an increased value in this parameter is related to liver lipid peroxidation, it will still be kept at a subclinical level. These remarks lead us to believe that lipids may be better protected from peroxidation in liver and mesenteric fat than in the muscle.

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