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Effect of dietary vegetable lipid sources in gilthead seabream (*Sparus aurata*) immune status and stress resistance

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SUMMARY – In order to evaluate the effect of vegetable oils on gilthead seabream health, fish were fed different diets that were formulated substituting 60% of fish oil by soybean oil (60SO), rapeseed oil (60RO) or linseed oil (60LO). 80% of fish oil substitution by either soybean oil (80SO) or linseed oil (80LO) plus a mix of vegetable oils at 60% substitution were also assayed. A 100% fish oil diet (FO) was used as a control diet. After 7 months of feeding, basal levels of different immunological parameters were determined. Besides, response to a confinement stress and fatty acid composition of liver and head kidney macrophages was also studied. The inclusion of soybean oil decreased both ACH50 and head kidney phagocytic activity. Rapeseed oil decreased also phagocytic activity. Linseed altered stress response. Soybean oil produced steatosis in liver hepatocytes. A selective incorporation of some EFAs such as DHA and ARA was observed.

Keywords: Seabream, fishoil, vegetable oils, stress resistance.

RESUME – "Effet des sources alimentaires de lipides végétaux sur l'état immunitaire et la résistance au stress de la daurade royale (*Sparus aurata*)". Afin d'évaluer l'effet des huiles végétales sur la santé de la daurade royale, les poissons recevaient différents régimes qui étaient formulés en remplaçant 60% de l'huile de poisson par de l'huile de soja (60SO), de l'huile de colza (60RO) ou de l'huile de lin (60LO). On a testé également un taux de substitution de 80% de l'huile de poisson par de l'huile de soja (80SO) ou de l'huile de lin (80LO) en plus d'un mélange d'huiles végétales à un taux de substitution de 60%. Le régime témoin était à 100% huile de poisson (FO). Après 7 mois d'alimentation, les niveaux de base des différents paramètres immunologiques ont été déterminés. En outre, on a également étudié la réponse au stress de confinement et la composition en acides gras des macrophages du foie et du rein antérieur. L'inclusion d'huile de soja a réduit l'ACH50 et l'activité de phagocytose du rein antérieur. L'huile de colza a également réduit l'activité de phagocytose. Le lin a altéré la réponse au stress. L'huile de soja a produit une stéatose des hépatocytes du foie. On a observé une incorporation sélective de certains acides gras essentiels tels que les DHA et les ARA.

Mots-clés : Daurade, huile de poisson, huiles végétales, résistance au stress.

Introduction

Traditionally, fish oil has constituted the main lipid source in marine fish diets, on the one hand due to its high content in n-3 HUFA (highly unsaturated fatty acids), essential fatty acids for marine fish species, and on the other hand, to the acceptable market price of fish oil. The relative lipid content in marine fish diets has increased during the past years due to a general trend to produce more energetic diets. But the increase in the global demand for fish oil for human and animal consumption, together with the rather stable production of fish oil have produced a steady increase in the market price for this ingredient. As a consequence, there is an increased interest in the inclusion of vegetable oils in marine fish compound diets to partially substitute and reduce reliance on a sole lipid source, such as fish oil.

Variations in the dietary fatty acid profiles caused by the inclusion of vegetable oil sources may considerably alter fish metabolism, which in critical cases may affect health and stress resistance of fish.

The inclusion of vegetable oils causes an inadequate balance of n-3 and n-6 fatty acids, which has

been shown to alter the synthesis of eicosanoids (Bell *et al.*, 1993), compounds directly involved in the immune response.

However, the role of n-3 and n-6 fatty acids in fish immunity is unclear and reports are not conclusive and, very often, contradictory. Some authors have reported negative effects of high dietary levels of n-3 PUFAs (polyunsaturated fatty acids) in channel catfish (*Ictalurus punctatus*) (Erdal *et al.*, 1991, Fracalossi and Lovell, 1994; Li *et al.*, 1994). However, other reports show positive effects of n-3 fatty acids on the immune response of fish. High levels of dietary n-3 fatty acids in the diet increase the activity of head kidney macrophages of channel catfish (Sheldon and Blazer, 1991) or rainbow trout (Ashton *et al.*, 1994). Inadequate levels of n-3 PUFAs in the diet reduce antibody production and *in vitro* killing of bacteria by macrophages in rainbow trout (Kiron *et al.*, 1995) and deplete alternative complement pathway activity in gilthead seabream (*Sparus aurata*) (Montero *et al.*, 1998). Replacement of marine fish oils with alternative lipids of vegetable origin in feed for farmed fish must be studied not only to supply the correct amount of dietary lipids with the adequate essential fatty acid balance for optimum growth, but also to maintain the optimum immune function. Thus, the objective of the present study was to examine the effect of replacing dietary fish oil with vegetable oils on gilthead seabream health.

Material and methods

Experimental diets

Seven isoenergetic and isoproteic experimental diets with a lipid content of about 22% were formulated. Peruvian anchovy oil was the only added lipid source in the control diet (Diet FO). All the other diets contained vegetable oils to substitute either 60 or 80% of the anchovy oil used in diet FO by soybean oil in diets 60SO and 80SO, linseed oil in diets 60LO and 80LO, rapeseed oil in diet 60RO and a mixture of the three vegetable oils in diet Mix (Table 1). Fish oil was included in diets 60SO, 60RO, 60LO and Mix at a sufficiently high level to as to cover the EFA requirements for this species (Montero *et al.*, 1996) (Table 2). Diets with two different sizes (3 and 5 mm) were prepared and extruded by Nutreco ARC (Stavanger, Norway).

Table 1. Lipid sources of experimental diets (% of oil inclusion)

	Diet						
	FO	60SO	60RO	60LO	Mix	80SO	80LO
Fish oil	100	40	40	40	40	20	20
Soybean oil	-	60	-	-	6	80	-
Rapeseed oil	-	-	60	-	18	-	-
Linseed oil	-	-	-	60	36	-	80

Feeding trials

One thousand five hundred and sixty gilthead seabream juveniles (85 g initial body weight) were distributed in 24 tanks of 500 l (65 fish/tank, each diet assayed by triplicates) supplied continuously with seawater with temperature ranging from 19.5 to 23.8°C, continuous aeration (oxygen ranging from 6.9 to 8.3 mg/l during the experimental period) and natural light cycle (around 12 h light/12 h dark). Fish were fed the experimental diets until apparent satiation (3 times per day, six days per week) for 204 days, when they reached the commercial size.

Biochemical analysis

At the end of the experimental period, samples of liver and head kidney macrophages were obtained from 6 fish per tank for biochemical analysis.

Lipids from the experimental diets, liver and head kidney macrophages were extracted with a chloroform:methanol (2:1 v:v) mixture as described by Folch *et al.*, (1957). The fatty acid methyl esters were obtained by transesterification with 1% sulphuric acid in methanol and purified by absorption chromatography on NH₂ Sep-pack cartridges (Waters, S.A., Milford, Massachusetts) and separated and quantified by gas-liquid chromatography (Christie, 1982).

Table 2. Main fatty acid (FA) composition (g FA/100 g Total FA) of the experimental diets

	Diet						
	FO	60SO	60RO	60LO	Mix	80SO	80LO
16:0	17.51	14.26	11.24	11.48	11.86	13.45	9.58
18:1n-9	13.64	17.07	37.10	16.32	22.29	18.24	15.99
18:2n-6	4.70	30.42	14.69	11.48	14.53	38.52	13.24
18:3n-3	0.64	3.42	5.03	28.09	19.97	4.39	36.69
20:4n-6	0.74	0.42	0.36	0.32	0.30	0.30	0.25
20:5n-3	16.16	7.85	7.10	6.94	6.92	5.02	4.30
22:6n-3	7.09	4.24	4.31	3.98	3.84	2.43	3.31
Saturated	28.31	21.14	18.99	18.46	18.51	19.13	15.26
Monounsaturated	29.47	26.46	43.66	25.05	31.25	26.42	23.54
Total n-3	31.56	18.54	19.89	42.23	33.59	13.83	46.07
Total n-6	7.98	32.65	16.34	13.15	15.55	39.79	14.71
Total n-9	16.48	19.05	39.66	18.32	24.44	19.87	17.84
n-3 HUFA	26.17	13.09	12.98	12.24	11.72	8.16	8.23

Histological studies

Livers from 6 fish from each tank were fixed in 10% buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin. Sections series of 4 µm were stained with hematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1979).

Serum and plasma studies

Blood, extracted from 10 fish per tank, was obtained by puncture of the caudal sinus. Serum was obtained by blood coagulation at environmental temperature during 1 h, followed by 4°C during the night, and subsequent centrifugation at 3000 rpm. Sera were kept at -80°C until determination of the alternative complement pathway activity (ACH50) as described by Sunyer and Tort, (1995) and lysozyme serum activity as described by Anderson and Siwicki (1994).

After the experimental period, 10 fish per tank were subjected to an acute stress consisting in overcrowding during 2 hours. 2.5 hours after the stress induction, blood was obtained as described above and plasma was obtained after centrifugation at 3000 rpm for 10 min. Plasma cortisol concentration was obtained as described by Molinero and González (1995) for this species, using the trypsin-antitrypsin method.

Macrophage phagocytic activity

Head kidneys from 6 fish per diet were extracted by dissection and macrophages were isolated using a Percoll gradient. Macrophage solution was incubated with *Vibrio anguillarum* as described by Esteban and Mesenger (1997). Phagocytic activity was measured as described by Blazer (1991).

Statistical analysis

All the data were statistically treated using ANOVA and Tukey's multiple range test (Sokal and Rohlf, 1995).

Results and discussion

Analysis of the fatty acid composition of the diets showed the highest saturated fatty acid and n-3 HUFA contents in diet FO, the highest linoleic acid (18:2n-6) in diets 60SO and 80SO, the highest linolenic acid (18:3n-3) in diets 60LO and 80LO, and the highest oleic acid (18:1n-9) in diet 60RO (Table 2).

Fish fed 80S diet showed significantly higher lipid content when compared with fish fed FO diet (Fig. 1). Liver fatty acid composition clearly reflected that of the dietary lipids (Fig. 2), livers of fish fed FO diet being higher in saturated fatty acids and n-3 HUFA, and those of fish fed diets with soybean oil (60SO and 80SO), rapeseed oil (60RO) and linseed oil (60LO and 80LO) higher in linoleic acid (18:2n-6), oleic acid (18:1n-9) and linolenic acid (18:3n-3), respectively, whereas the fatty acid profile of those fish fed Mix diet resembled more the profile of fish fed the FO diet. Linoleic and linolenic acids were accumulated in the liver proportionally to their levels in the diet, suggesting the lower utilization of these fatty acids in comparison to other 18C fatty acids. This agrees with the findings of Argyropoulou *et al.* (1992) when they fed grey mullet on vegetable oils.

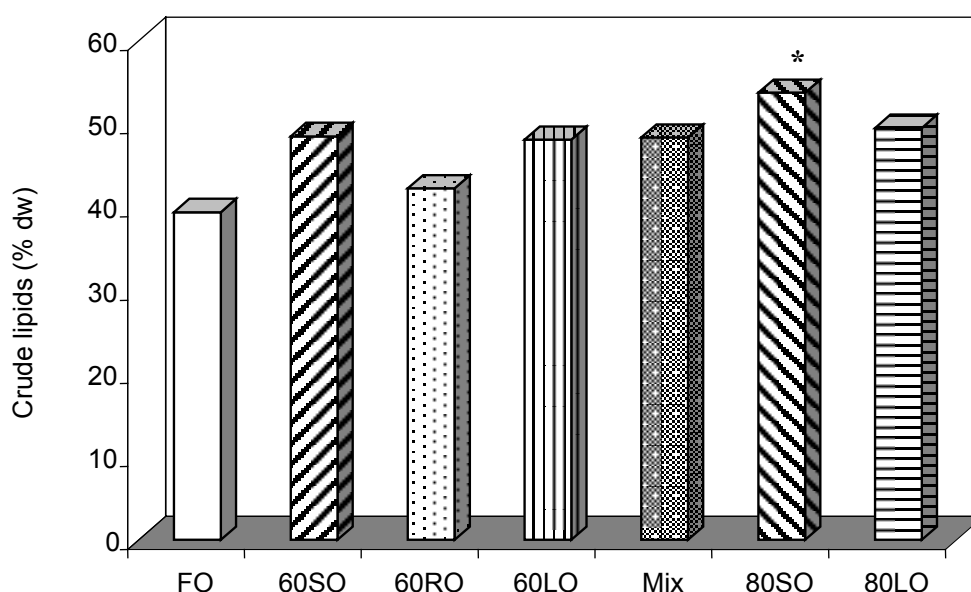


Fig. 1. Liver lipid content of fish fed the experimental diets (% dry weight).

*denotes significant differences ($P < 0.05$).

The increase of dietary fatty acids of the n-6 series (mainly linoleic acid) produced histological alterations in the liver, such as cellular hypertrophy and irregular or displaced cellular nuclei (Fig. 3), as described for other species, such as *Sciaenus ocellatus* (Tucker *et al.*, 1997) as well as gilthead seabream (Alexis, 1997). Lipidosis or abnormal fat droplets deposition is considered a physiological response to a nutritional imbalance, which in some cases may lead to progressive degeneration processes (Caballero *et al.*, 2002).

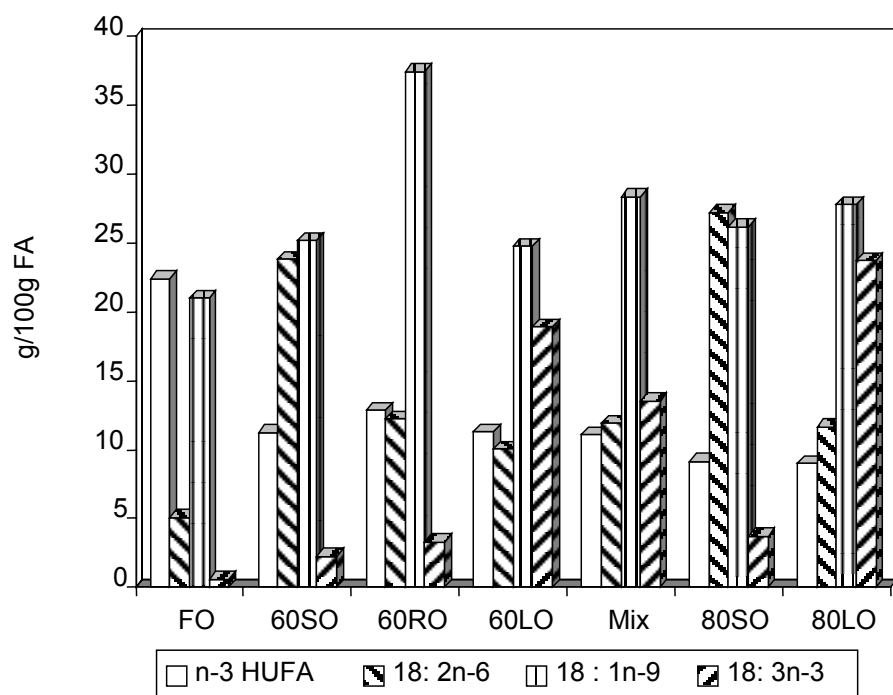


Fig. 2. Main fatty acids (characteristic from each experimental oils) in liver of fish fed the experimental diets (g FA/ 100g Total FA).

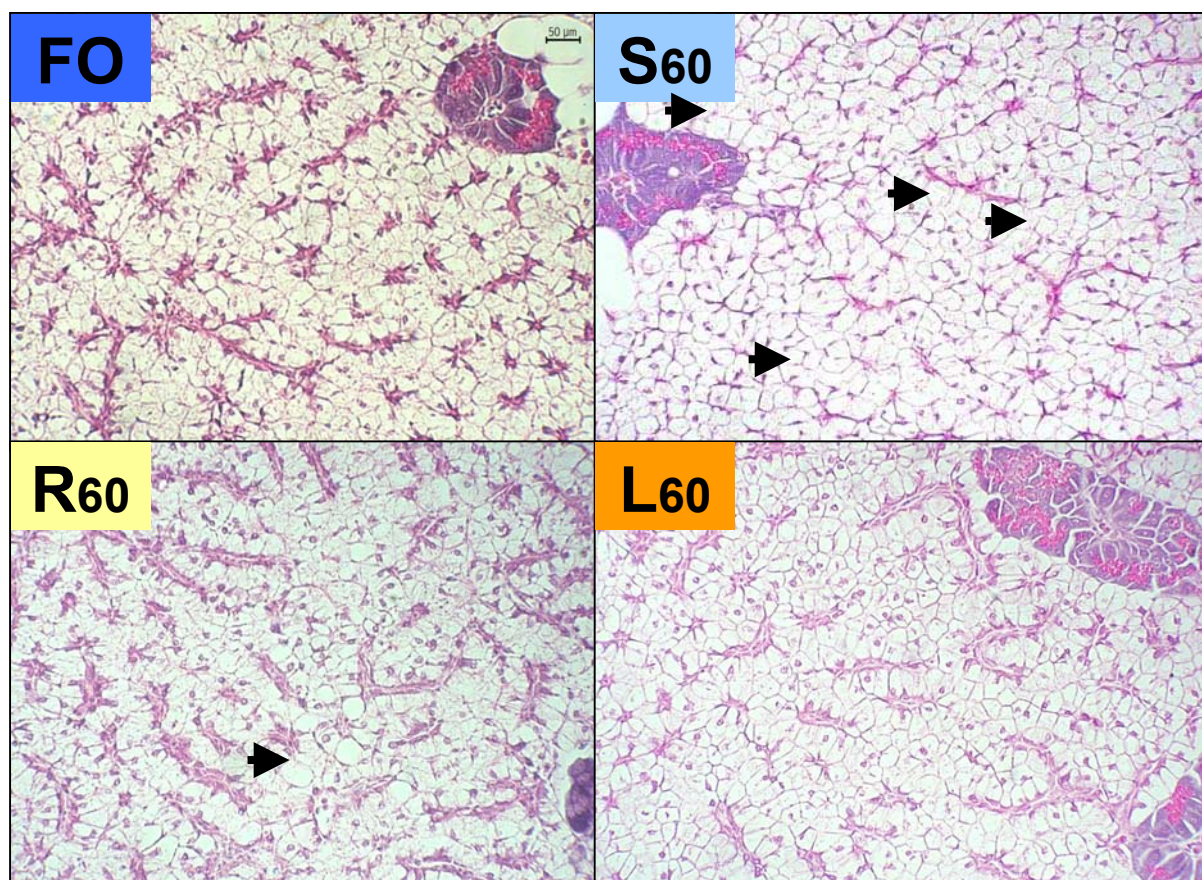


Fig. 3. Hepatocytes from fish fed experimental diets. ► Example of swollen hepatocytes with migrated nuclei.

Regarding humoral immunity, the inclusion of linseed or rapeseed oils in seabream diets did not produce effects on the different immune parameters measured. However, fish fed soybean oil-containing diets (60SO and 80SO) showed lower values of ACH50, this value being significantly lower ($P<0.05$) than in fish fed FO (Fig. 4). Similar effects have been described for cod (*Gadus morhua*), where the complement activity of this species showed a positive correlation with the polyenes present in serum (Waagbø *et al.*, 1995). However, no effect of vegetable oils inclusion was found on seabream serum lysozyme activity, fish fed FO diet showing the highest (but not significant) values of lysozyme activity in serum (Fig. 5). Cellular immunity, in terms of phagocytic activity of head kidney macrophages, was affected in fish fed either rapeseed or soybean oil-containing diets (diets 60RO and 60SO and 80SO, respectively), fish fed these diets showing significantly ($P<0.05$) lower values of phagocytic activity against *Vibrio anguillarum* than those showed by fish fed FO diet (Fig. 6).

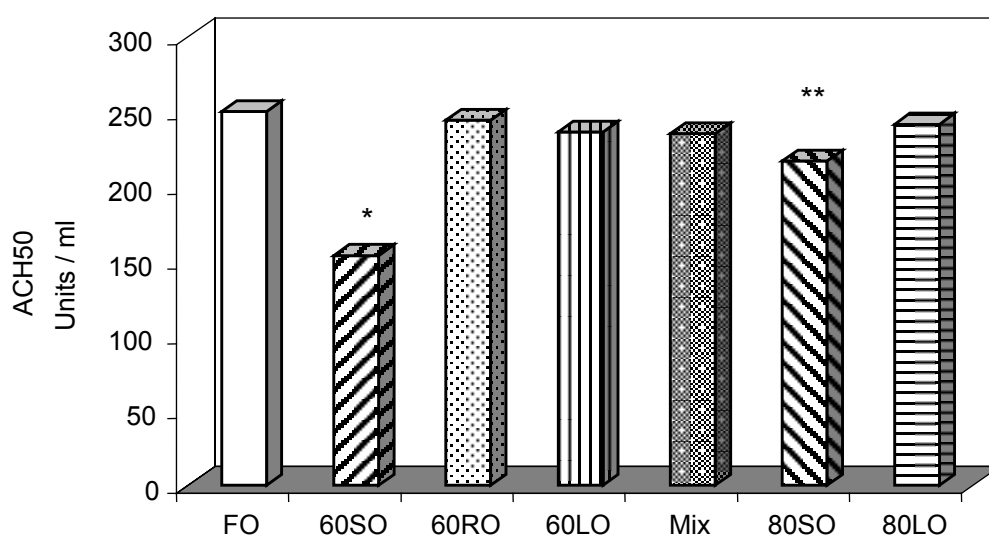


Fig. 4. Serum alternative complement pathway activity of fish fed the different experimental diets (units/ml). *denotes significant differences ($P<0.05$); **denotes significant differences ($P<0.1$).

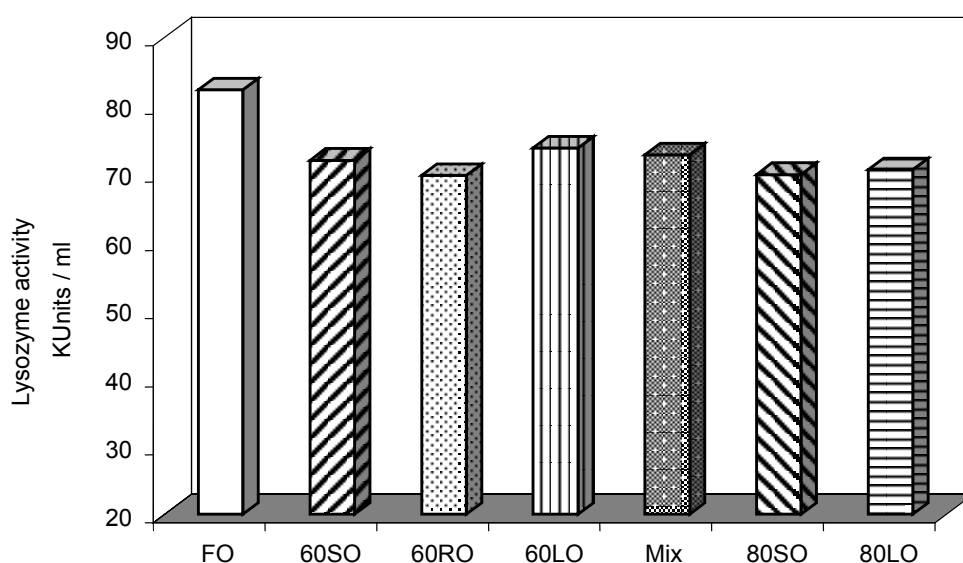


Fig. 5. Serum lysozyme activity of fish fed the experimental diets (K units/ml).

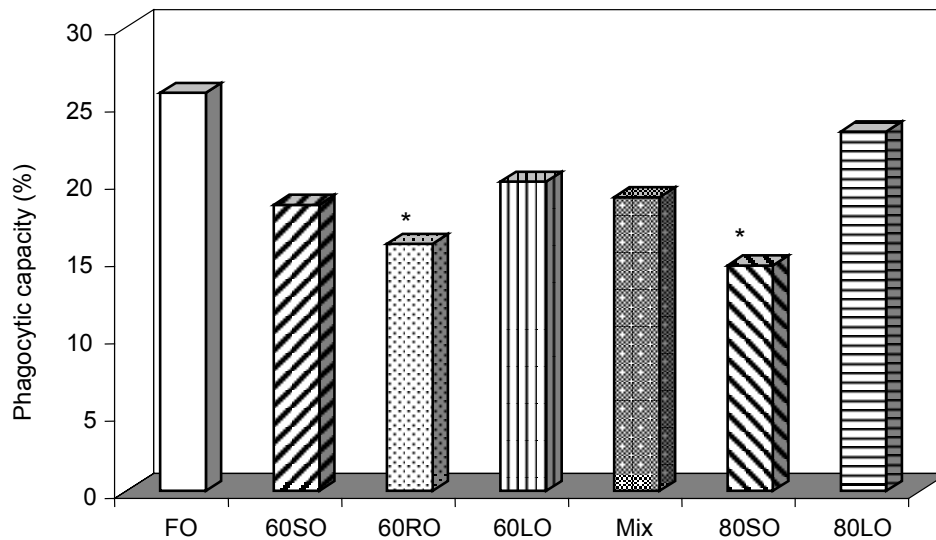


Fig. 6. Head kidney macrophages phagocytic activity against *Vibrio anguillarum* (% of activated cells). *denotes significant differences ($P<0.05$).

These effects of dietary oils on both macrophage-dependent humoral and cellular immunity could be produced by an imbalance in the fatty acid profile of immune cells. The nature of the dietary oils consumed determines the fatty acid profile of macrophages, these immune cells fatty acid profile being characterized by those fatty acid typical of the vegetable oil contained in each diet (Fig. 7). Dietary fatty acid from vegetable oils increased both linoleic (diets 60SO and 80SO) and linolenic (diets 60LO and 80LO) acids in macrophages of fish fed diets with soybean or linseed oil, respectively. Oleic acid increased in macrophages of fish fed rapeseed oil (60RO), whereas fish fed FO diets showed the highest n-3 HUFA proportion in macrophages as described for other species fed with different oils (Waagbø *et al.*, 1995; Farndale *et al.*, 1999).

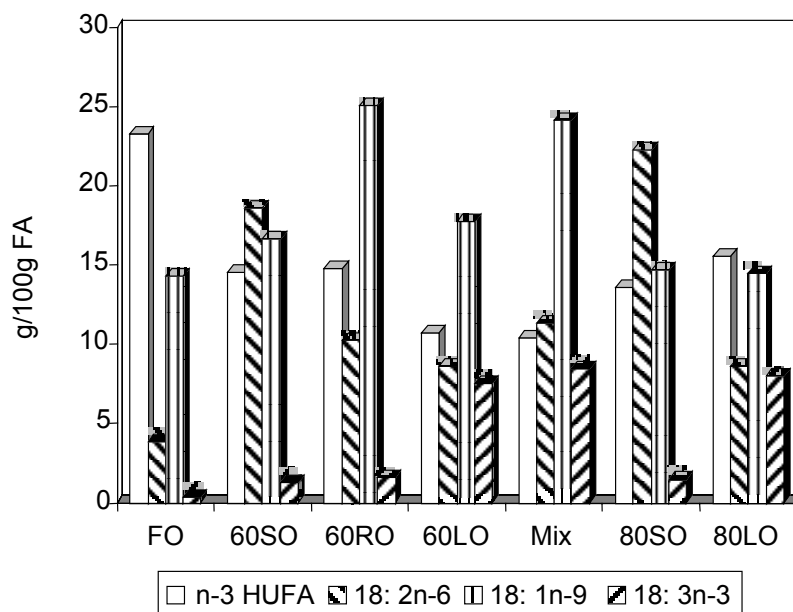


Fig. 7. Main fatty acids (FA) (Characteristic from each experimental oils) in head kidney macrophages of fish fed the experimental diets (g/100 g FA).

However, a selective incorporation of certain fatty acids can be observed in head kidney macrophages. Docosahexaenoic acid (22: 6n-3, DHA) is preferentially incorporated into cells, since macrophage DHA/dietary DHA ratio values are close to 2, denoting the importance of this fatty acid in this type of cells (Table 3). Similar results have been described for cod (Waagbø *et al.*, 1995).

Table 3. Relation between essential fatty acid (FA) in macrophages and diets (FA macrophage/dietary FA ratios)

	Diet						
	FO	60SO	60RO	60LO	Mix	80SO	80LO
EPA (20:5n-3)	0.66	0.69	0.94	0.81	0.83	1.19	1.73
DHA (22:6n-3)	1.91	1.51	1.94	1.34	1.23	3.36	2.58
ARA (20: 4n-6)	4.38	3.10	5.89	5.19	5.03	6.03	7.67

Arachidonic acid (ARA) is also preferentially incorporated into macrophages. ARA macrophage/dietary ARA ratios ranged from 3 to 7, the highest ratios being from those fish fed the diets with lower ARA content (Table 3). These cells have a higher affinity for this fatty acid than other cells, due to the role of this fatty acid in eicosanoid production (Thompson *et al.*, 1995). ARA is selectively incorporated into phosphatidylinositol of plaice neutrophils (*Pleuronectes platessa*) (Tocher and Sargent, 1986).

Higher contents of linolenic acid (60SO and 80SO) or oleic acid (60RO) in macrophages induced by high contents in the diet could be producing a decrease in macrophage membrane flexibility and hence a decrease in phagocytic ability and other processes associated with macrophage activity such as complement activity. High contents of linolenic acid do not affect macrophage function, but seem to alter the stress response, since fish fed linseed-containing diets (60LO and 80LO) showed significantly higher plasma cortisol basal levels (Fig. 8) and a strong response to overcrowding stress. Further experiments are required to clarify the role of n-3 fatty acid on ACTH receptors and/or cortisol release in interrenal cells of the anterior kidney of seabream.

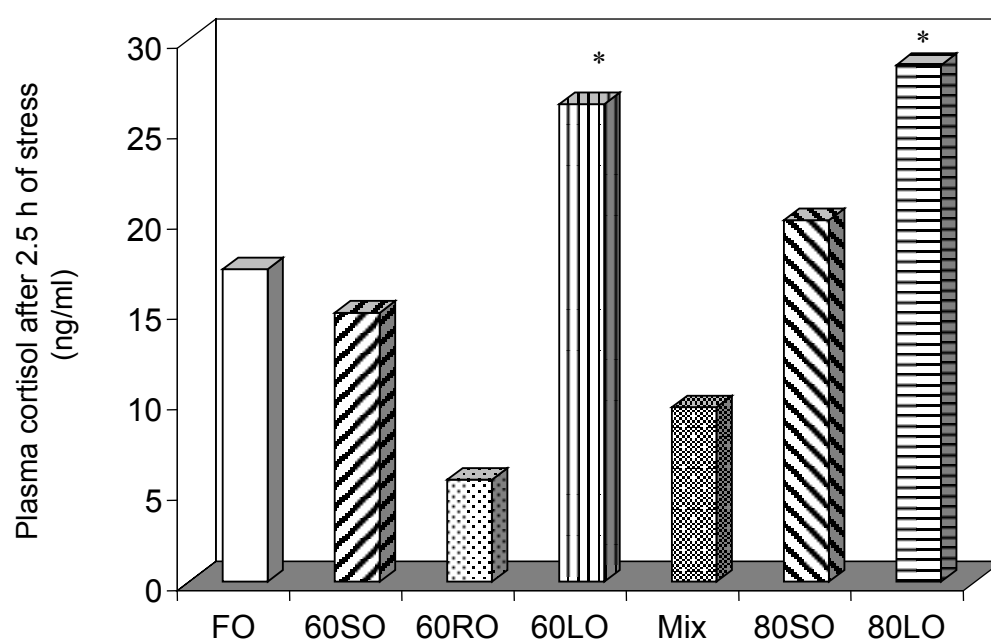


Fig. 8. Plasma cortisol concentration (ng/ml) after 2.5 of stress induction (2h of overcrowding). *denotes significant differences ($P < 0.05$).

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