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Dietary lipid level affects fatty acid composition and hydrolase activities of intestinal brush border membrane in seabass

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Abstract: Triplicate groups of juvenile seabass (initial weight of 241 g) were fed during 13 weeks three isonitrogenous experimental diets containing different lipid levels, 12% (LL group), 21% (ML group) and 30% (HL group). At the end of the experiment, fish weight gain was similar among the 3 dietary groups. Intestinal brush border membranes were purified for each dietary group; one part of the brush border fraction was dedicated to enzyme assays, the remaining fraction being used for lipid extraction followed by fatty acid analysis. The fatty acid composition of the brush border membrane differed among the 3 groups, although the 3 experimental diets had the same fatty acid composition. The increase in dietary lipid level resulted in a lowering in (n-3) polyunsaturated fatty acid (PUFA) paralleled with an increase in monounsaturated fatty acid. A significant reduction in the brush border enzyme activities, namely alkaline phosphatase, aminopeptidase N, gamma-glutamyl transpeptidase and maltase, was also observed with the elevation of the dietary lipid level. The change in activity of intestinal digestive enzymes, which are membrane-bound proteins, could be attributed to the modification of fatty acid composition and fluidity of the brush border membranes (BBM). Such lowering in PUFA and increase in monounsaturated fatty acid in BBM, concomitant with a decline in membrane enzymatic activity, has been described as a malnutrition indicator in mammals. It raises the question of possible disorders of gut functions in fish fed increasing lipid levels.

Keywords: brush border hydrolases - dietary lipids - fatty acids - fish - intestine - membrane lipid

Introduction

During the last decade, commercial feeds for fish farming have incorporated increasing lipid levels. The high energy diets have been considered beneficial for growth, feed efficiency, protein sparing and for reducing phosphorus and nitrogen losses (Kaushik and Oliva-Teles 1985). Recently, some studies focused on the effect of dietary fat content on fat deposition and sensory characteristics of fish flesh (Bjerkeng et al. 1997; Nortvedt and Tuene 1998). Most of the physiological studies dealt with lipogenesis regulation in liver and adipose tissues (Likimani and Wilson 1982; Dias et al. 1998), but little is known about physiological effects in other tissues in fish.

Studies conducted on mammals have shown that the lipid composition of cell membranes in other tissues (such as intestinal cells) are highly affected by the dietary lipid source and content. The change of some fatty acid incorporated into the membrane phospholipid can alter the physicochemical characteristics of the membrane, which in turn can influence conformation, mobility and function of a wide variety of membrane-bound proteins (Ruiz-Gutierrez and Vasquez 1997; Lopez-Pedrosa et al. 1998). In particular, the lipid composition of enterocyte membranes influences the digestive functions of the intestine (Goda and Takase 1994a,b, Duddley et al. 1994; Wang et al. 1996). Raul et al. (1982) assumed that the activity of membranous digestive enzymes are a precocious indicator of the nutritional status of the animal

The aim of this study was to determine to what extent the increase in dietary lipid level affects the lipid composition of intestinal brush border membranes and the functioning of the membrane-bound enzymes in seabass (*Dicentrarchus labrax*).

Materials and methods

Diets and animals

Three isonitrogenous experimental diets were formulated to contain different lipid levels: low (12% of dry matter), medium (21%) or high (30%) lipid level, designated as LL, ML and HL respectively. Whole wheat was varied in inverse relation to fish oil. Diet composition is described in table 1 and fatty acid profiles in table 2. Extruded diets of size 4 mm were manufactured by F.K.H. (Felleskjøpet Havbruk AS, Norway).

Table 1. Composition of the experimental diets (g per 100g dry matter)

	LL	ML	HL
Ingredients			
Fish meal	57.5	57.5	57.5
Whole wheat	27.4	16.5	11.0
Wheat gluten	7.7	9.3	5.6
Fish oil	3.8	13.1	22.3
Potato starch	1	1	1
Mineral premix	0.3	0.3	0.3
Vitamin premix	0.15	0.15	0.15
Stay C 25%	0.06	0.06	0.06
Betaine 97%	0.1	0.1	0.1
Binder (suprex corn)	2	2	2
Proximate composition			
Dry matter	90.4	90.5	95.2
Protein	53.8	53.6	53.8
Lipid	11.3	21.3	30.0
Starch	17.1	11.7	5.4
Energy (kJ)	2160	2390	2580

Table 2. Common fatty acid composition of the three experimental diets (% of total fatty acids).

C14:0	13.0
C16:0	19.2
C18:0	1.9
∑ saturated	35.6
C16:1(n-9)	8.9
C18:1(n-9)	14.7
C20:1(n-9)	8.6
C22:1(n-9)	7.9
∑ monoinsaturated	41.1
C18:2(n-6)	3.3
C20:4(n-6)	0.3
∑ (n-6)	8.3
C18:3(n-3)	0.4
C18:4(n-3)	1.9
C20:5(n-3)	5.7
C22:5(n-3)	0.4
C22:6(n-3)	4.4
∑ (n-3)	13.3

Each diet was randomly assigned to triplicate groups of sea bass having a mean initial weight of 241 ± 9.6 g, stocked in 1 m^3 tanks (50 fish per tank) supplied with continuous flow of sea water. The experiment lasted 13 weeks, temperature was maintained at 22 ± 0.7°C, salinity at 39 ± 1.3 ppm, and dissolved oxygen above 6.2 mg/l. Feed was distributed on demand by means of electronic self-feeders connected to a microcomputer (Husky Hunter, Husky Computers limited) as described in Boujard et al. (1999).

At the end of the experiment, fish unfed since two days were sampled in triplicate from each tank and their intestinal tract was collected.

Analytical methods

After flushing with 5 ml of ice cold NaCl 0.9%, the mucosa of each intestine was scraped, pooled for fish sampled from the same tank, and stored at -80°C until assayed. The

purification of brush border membranes (BBM) was performed on 300 to 900 mg of wet weight mucosa according to Crane et al. (1979). Intestinal scrapings were homogenized in 30 volumes of Tris 2mM-Mannitol 50mM buffer, pH=7; 1ml of this homogenate (H) was taken. The remaining volume was added $CaCl_2$ to a final concentration of 10mM, then was centrifuged at 9000g during 10 minutes. The supernatant was centrifuged at 34000g during 20 minutes in order to obtain a pellet (BBM).
BBM fraction was homogenized in about 100µl of [KCl 0.1M, dithiothreitol 1mM, tris-HEPES 5mM, pH=7.5] buffer. The state of purity of the BB fraction was appreciated by the assay of alkaline phosphatase (Bessey et al. 1946) and Na^+-K^+-ATPase, measured according Trigari et al. (1985) with the following modifications: the incubation buffer contained Tris-ATP 5mM, MgCl2 5mM, NaCl 100mM, KCl 20mM, EDTA 5mM and Tris-HCl 75mM pH=6.5. For the measurement of ouabain insensitive ATPase, 1mM ouabain was added to this buffer. The enzyme reaction was terminated by the addition of perchloric acid 2.5M. The Na^+-K^+-ATPase was calculated by subtracting the ouabain-insensitive ATPase from the total ATPase. 20% of each total brush border preparation was dedicated to enzymatic assays. Aminopeptidase N, γ-glutamyl transpeptidase and maltase were assayed according to Maroux et al. (1973), Meister et al. (1981) and Dahlqvist (1970) respectively. Enzyme activities were expressed at specific activities, i.e., mU/mg protein. Protein was determinated by the Bradford procedure (Bradford 1976).

Lipids were extracted from the remaining fraction of each BBM sample, according to a micro-method described in Jones et al. (1992). BBM were homogenized in 1 ml buffer (0.1 M phosphate, pH 7.2) and lipids were extracted by adding 1 ml acetic acid 0.15 M and 7.5 ml chloroform/methanol (1:2 vol/vol) followed by a gentle mixing. After 2 mn, 2.25 ml chloroform and 2.25 distilled water were added. After 5 mn low speed centrifugation (4000 g), the lower chloroform layer containing neutral and polar lipids was collected. The chloroform was reduced to dryness under nitrogen and the residue dissolved in 1 ml chloroform and stored at -80°C under nitrogen.

Fatty acid esters were prepared in dry methanol containing 2.5% sulfuric acid and were quantified by gas-liquid chromatography in a Varian.3400 gas chromatograph equipped with a DB Wax fused silica capillary column (30m x 0.25mm i.d., film thickness: 0.25µm, J & W Scientific) using helium as carrier gas (1.4 ml/mn). The thermal gradient was 100 to 180 °C at 8°C/mn, 180 to 220°C at 4°C/mn and a constant temperature of 220°C during 20 min. Injector and flame ionization detector temperatures were 260 and 250°C, respectively. Fatty acid methyl esters were identified by comparison with known standard mixtures (Sigma 189-19) and quantified using a Spectra.Physics 4270 integrator.

Statistical analysis

Results are given as mean ± SD (n=3). Fatty acid values, expressed in percent of total fatty acids, were arcsin ($x^{1/2}$) transformed before analysis of variance. The variance homogeneity of the data were checked using Bartlett's test (Dagnelie, 1975). Data were compared by one-way ANOVA followed by Neuman-Keuls multiple range test when significant differences were found at the 0.05 level.

Results

Juveniles sea bass have doubled their weight during the 13 weeks of experiment. Weight gain was similar among the three experimental groups. Moreover, no difference have been evidenced in feed intake data per fish for the total duration of the experiment. Considering the last 4 weeks of the experiment, weight gain remained similar for the 3 groups, but feed intake was lower as the dietary lipid level was elevated (Table 3). The groups fed the highest lipid level ingested 30% less food than the group fed the lowest lipid level.

Table 3. Growth and feed intake* in sea bass fed the three lipid levels

	LL	ML	HL	
Total period				
Weight gain (g)	209 ± 15.3	213 ± 23.4	231 ± 13.8	ns
Feed intake (g/fish)	291 ± 24.7	266 ± 37.7	253 ± 14.7	ns
4 last weeks				
Weight gain (g)	66 ± 2.8	71 ±6.6	69 ± 12.4	ns
Feed intake (g/fish)	102 ± 8.1[a]	93 ± 8.6[ab]	77 ± 9.4[b]	

Means ± S.D. (n=3) with different superscript letter in a same row are significantly different ($P<0.05$).
* calculated on 3 tanks per diet, each tank containing 50 fish.

Values obtained during the different steps of purification of brush border membrane from sea bass intestinal scraping are given in Table 4 and show the effectiveness of the method. BBM fraction was very poor in protein (less than 2% of the recovered proteins), nevertheless it contained around 25% of the recovered alkaline phosphatase; this represented a 14.5 fold increase in the specific activity over the starting activity in the homogenate. The contamination level was low considering that only 2% of the recovered Na^+-K^+-ATPase was contained in this fraction.

Table 4. Purification of brush border membranes from a scraping of sea bass intestine.

Fraction	Protein	Alkaline phosphatase		(Na^+-K^+)-ATPase	
	Recovery %	specific activity	Recovery %	specific activity	Recovery %
LL					
H	100	623 ± 89.9	100	7.9 ± 1.63	100
BBM	1.8 ± 0.28	9348 ± 2779	31.5 ± 4.02	13.2 ± 3.99	1.8 ± 0.65
		15.0		*1.7*	
ML					
H	100	445 ± 71.7	100	8.3 ± 2.61	100
BBM	1.8 ± 0.37	6357 ± 1595	23.2 ± 3.52	12.2 ± 2.84	1.3 ± 0.36
		14.3		*1.5*	
HL					
H	100	362 ± 53.2	100	8.9 ± 3.25	100
BBM	1.5 ± 0.34	5303 ± 1273	26.5 ± 2.79	14.2 ± 3.13	1.6 ± 0.45
		14.6		*1.6*	

Data are given as means ± S.D. (n=3); Values in italics represent the enrichment factor.
H: homogenate, BBM: brush border membranes.

The three experimental diets exhibited the same fatty acid composition, but the fatty acid composition of the intestinal brush border membranes of the three groups exhibited large differences, as a result of the difference in dietary lipid level (Table 5). The most notable differences were observed in monounsaturated fatty acids (MUFA) in which a 100% increase was observed between group LL and HL, when (n-3) and (n-6) polyunsaturated fatty acids (PUFA) showed a 30% decrease. The proportion of saturated acid slightly decreased (14%) with the elevation of the dietary lipid level, but this trend was not followed by each saturated fatty acid. Among the most represented MUFA, oleic acid reached 15.3% of total fatty acids in group fed HL when it was only 11.5% in group fed LL. Concerning the (n-6) PUFA, the proportion of linoleic acid and arachidonic acid drew near to the diet fatty acid composition when dietary lipid level increased. Specially, it can be pointed out that arachidonic acid in HL group fell down a very low value, 0.9% of the total fatty acids. Moreover, the ratio linoleic acid/arachidonic acid was 38% higher in group HL (4.0±0.50), compared to groups LL (2.9±0.32) and ML (2.8±0.31). In the (n-3) PUFA family, eicosapentaenoic acid remained at a constant level in the three groups when docosahexaenoic acid showed a 50% decrease between LL and HL groups. Docosahexaenoic acid value in HL group was close to that of the diet.

Table 5. Fatty acid composition (% of total fatty acids) of the brush border membranes of enterocytes in sea bass fed the three lipid levels.

	LL	ML	HL
C14:0	2.9 ± 0.58^{c}	4.9 ± 0.65^{b}	8.5 ± 1.68a
C16:0	25.2 ± 0.65^{a}	23.8 ± 2.24^{a}	20.6 ± 1.43^{b}
C18:0	12.5 ± 0.795^{a}	9.7 ± 1.81^{b}	5.5 ± 0.92^{c}
∑ saturated	42.8 ± 0.37^{a}	40.7 ± 3.15^{ab}	36.6 ± 1.92^{b}
C16:1(n-9)	2.9 ± 0.20^{c}	4.9 ± 0.71^{b}	7.0 ± 0.80^{a}
C18:1(n-9)	11.8 ± 0.99^{b}	13.9 ± 1.62^{ab}	15.3 ± 0.36^{a}
C20:1(n-9)	3.0 ± 0.31^{c}	5.2 ± 1.15^{b}	8.3 ± 0.28^{a}
C22:1(n-9)	1.3 ± 0.37^{c}	2.7 ± 0.89^{b}	6.1 ± 0.69^{a}
∑ monoinsaturated	19.2 ± 0.23^{c}	27.0 ± 4.08^{b}	37.2 ± 1.90^{a}
C18:2(n-6)	5.3 ± 0.76^{a}	3.9 ± 0.14^{b}	3.2 ± 0.18^{c}
C20:4(n-6)	1.8 ± 0.11^{a}	1.4 ± 0.32^{a}	0.9 ± 0.25^{b}
∑ (n-6)	9.9 ± 1.06^{a}	8.1 ± 0.41^{b}	7.0 ± 0.76^{b}
C18:3(n-3)	5.2 ± 1.62^{a}	5.5 ± 2.70^{a}	1.4 ± 0.48^{b}
C18:4(n-3)	0.4 ± 0.02^{c}	0.6 ± 0.08^{b}	1.1 ± 0.19^{a}
C20:5(n-3)	6.9 ± 0.58^{a}	7.0 ± 0.77^{a}	6.2 ± 0.48^{a}
C22:5(n-3)	0.4 ± 0.09	0.4 ± 0.02	0.4 ± 0.06
C22:6(n-3)	13.8 ± 1.97^{a}	9.8 ± 1.94^{b}	7.1 ± 1.19^{c}
∑ (n-3)	26.7 ± 1.70^{a}	23.4 ± 1.09^{a}	18.2 ± 3.41^{b}

Means ± S.D. (n=3) with different superscript letter in a row are significantly different (P<0.05).

The specific activities of some enzymes of intestinal brush border membranes, aminopeptidase N, alkaline phosphatase, γ-glutamyl transpeptidase and maltase are reported in Table 6. A lowering in specific activity for each enzyme was observed, consecutively to an increase in dietary lipid level. In particular, γ-glutamyl transpeptidase exhibited a 56% decrease and phosphatase alkaline a 43% decrease between LL and HL groups.

Table 6. Specific activity of some enzymes of intestinal brush border membranes in sea bass fed the three lipid levels.

	LL	ML	HL
		mU·mg protein^{-1}	
γ-glutamyl transpeptidase	119 ± 36.2^{a}	87 ± 22.7^{b}	53 ± 13.5^{c}
Aminopeptidase	2646 ± 672.8^{a}	2093 ± 515.7^{ab}	1837 ± 697.1^{b}
Alkaline phosphatase	9348 ± 2778.7^{a}	6357 ± 1594.6^{b}	5303 ± 1273.2^{b}
Maltase	301 ± 78.3^{a}	269 ± 48.3^{ab}	224 ± 56.3^{b}

Means ± S.D. (n=3) with different superscript letter in a same row are significantly different ($P<0.05$).

Discussion

Valid physiological data can be drawn from this study since fish doubled their weight during the course of the experiment . The dietary lipid level induced a modulation in feed intake after an adaptation period: during the 4 last weeks, reduced feed intakes were associated with increased dietary lipid concentrations, as described by Boujard et al. (1999). The physiological mechanisms by which fat produce satiety are not well known but an action of endogenous cholecystokinin has been hypothesized in mammals (Guimbaud et al. 1997).

The purification of intestinal brush border membranes could be achieved by this method with satisfactory results. Indeed, taking alkaline phosphatase specific activity as a measure of the extent of BBM purification, we obtained at least a 14-fold purification. Moreover, the contamination of this BBM fraction by basolateral membranes was reduced to traces, based on assays of Na^{+}-K^{+}-ATPase which could be considered as a marker of the basolateral membranes (Mircheff et al., 1985). The degree of purification of the BBM fraction extracted from the intestinal scraping corresponded to that obtained by intestinal scraping in mammals (Malathi et al., 1979) and adult fish (Crane et al., 1979; Bogé et al., 1982). The recovery of brush border membranes, appraised by the relative activity of alkaline phosphatase - brush border membrane/homogenate - was similar in all dietary groups.

Fatty acid composition of trout intestinal brush border membrane has been described in literature (Pelletier et al. 1986; Pelletier et al. 1987), but few studies related the effect of dietary lipid on this composition (Behar et al. 1989). At the opposite, it has been reported in mammals that the fatty acid composition of the brush border membranes reflects that of the diet (Wang et al. 1996; Takase and Goda 1990). In our study, the fatty acid compositions of the 3 diets were similar; surprisingly, the fatty acid composition of the brush border membranes of intestine was affected by the dietary lipid level. Literature reported three examples of modification of BBM fatty acid composition when the fatty acid composition of the diet was similar.

First, lipid composition of small intestine mucosa changes during mammal postnatal development. PUFA concentration increases during the maturation of enterocyte BBM (Furlan and Catala 1996). In particular, Alessandri et al. (1991) reported an increase of arachidonic concentration during enterocyte differentiation. This case cannot be considered in our study since the seabass used were adults.

Secondly, it has been shown that an increase in dietary lipid level by triglyceride incorporation resulted in a modification of BBM fatty acid composition. In this case, fatty

acid composition of BBM reflected that of the dietary triglyceride (Dudley et al. 1994). This case coincides with our study since the increase in dietary lipid level was achieved by an elevated incorporation of cod liver oil which is mainly triglycerides. BBM concentration in saturated, monounsaturated and n-6 fatty acids reflected that of the diet in fish fed HL. BBM concentration in n-3 PUFA was maintained at a higher level than in the diet, suggesting that a minimum concentration of linolenic family PUFA was preserved in membrane, probably for sustaining membrane fluidity. This may indicate that dietary n-3 PUFA were preferentially incorporated in BBM membrane. Indeed these n-3 PUFA are probably not the result of some elongase and desaturase activity, since these enzymes are expressed at a very low level in marine fish (Mourente and Tocher 1998). Morever, there are no data in the literature on the effects of dietary factors on the desaturation/elongation pathway in marine fish.

Finally, a lowering in (n-3) and (n-6) PUFA, paralleled with an increase in MUFA (namely oleic acid) in BBM has been reported in piglets suffering protein energy malnutrition. These changes in BBM composition were associated with impaired BBM activities. In our study, the same alterations appeared in BBM fatty acid composition and enzyme activity in fish fed high lipid level. In particular, the linoleic/arachidonic ratio was elevated in fish fed high lipid level, as it was reported by Lopez-Pedrosa et al. (1998) in malnourished piglets. These authors imputed the alteration of the BBM enzymatic activities to the change in lipid composition of membranes in piglets. We can also hypothesized that the decline in enzymatic activity observed in fish fed HL resulted from a modification of fatty acid compstion of BBM. Thus, the hypothesis of a gut perturbation induced by high dietary lipid level must be considered in our study.

Our data, faced to the observations of Lopez-Pedrosa et al. (1998), suggested that the fall in enzymatic specific activities could not be only imputed to the reduction of food intake, as it is generally described in literature (McCarthy et al. 1980; Czernichow et al. 1990). So, the drastic fall in peptidase activities, γ-glutamyl transpeptidase and aminopeptidase N would be in part explained by the reduction in protein ingestion; in the case of maltase, the reduced activity in group fed HL diet can be also attributed to the reduction in starch ingestion. Nevertheless, the obtained data in fatty acid composition of BBM led us to investigate hypothesis not only based on enzyme-substrate interactions. The alteration of BBM hydrolase in fish fed high lipid level could be also due to an alteration of the morphologic structure of intestinal villi. Indeed, Goda and Takase (1994) have shown that rats fed high fat diet exhibited a significantly reduced surface area of microvilli per enterocyte compared to the control animals; this reduction was accompanied by a decrease in the activities of BBM hydrolases. Histological studies would have allow to assess in our experiment the effect of high dietary lipid levels on the villi surface.

The changes in enzymatic activities of membranes can be also attributed to an alteration of membrane fatty acid composition. Duddley et al.(1994) have established a positive correlation between disaccharidase activities and C18:0 concentration in rat BBM. In our study, the decrease in maltase activity could be related to the decrease of C18:0 induced by the high dietary lipid level. In the same way, the increase in alkaline phosphatase activity with (n-3) and (n-6) PUFA level in BBM observed in our study has been previously related in carp by Behar et al.(1989). These changes in alkaline phosphatase activity were mainly attributed to a modification in membrane fluidity which depends mainly on long chain fatty acids. Wahnon et al. (1992) have proposed that (n-3) fatty acids with 20 or more carbon atoms, and particularly docosahexaenoic acid, raise membrane fluidity, affecting the activity of the bound enzymes. In our study, the increase in dietary lipid level led to a 50% decrease in docosahexaenoic acid in BBM. The decrease in activity of the four studied enzymes could be related to changes in membrane fluidity.

Modifications in fatty acid composition and hydrolases in intestinal brush border membrane of fish were induced by increasing dietary lipid level. Similar observations were reported in malnourished mammals. The changes in intestinal functions were evidenced after 3 months of feeding, and it can be wondered whether the use of high dietary lipid level would induce later metabolic perturbations.

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