

Vegetable lipid sources affect *in vitro* biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*)

Maria José Caballero^{1*}, Germán Gallardo², Lidia Robaina³, Daniel Montero³, Antonio Fernández¹ and Marisol Izquierdo³

¹Department of Comparative Pathology, Trasmontaña, s/n, 35416 Arucas, Las Palmas de Gran Canaria, Canary Islands, Spain

²Department of Biochemical, Cellular Biology and Physiology, Edificio Ciencias de la Salud, Campus Universitario de San Cristóbal, Las Palmas de Gran Canaria, Canary Islands, Spain

³Grupo de Investigación en Acuicultura, ULPGC & ICCM, PO Box 56, 35200, Telde, Las Palmas de Gran Canaria, Canary Islands, Spain

(Received 7 February 2005 – Revised 19 May 2005 – Accepted 23 May 2005)

Despite the good growth performance of several fish species when dietary fish oil is partly replaced by vegetable oils, recent studies have reported several types of intestinal morphological alterations in cultured fish fed high contents of vegetable lipid sources. However, the physiological process implied in these morphological changes have not been clarified yet, since alterations in the physiological mechanisms involved in the different processes of lipid absorption could be responsible for such gut morphological features. The objective of the present study was to investigate the activities of reacylation pathways in fish, the glycerol-3-phosphate and the monoacylglycerol pathways, in order to clarify the intestinal triacylglycerol (TAG) and phospholipid biosynthesis to better understand the morphological alterations observed in the intestine of fish fed vegetable oils. Intestinal microsomes of sea bream fed different lipid sources (fish, soyabean and rapeseed oils) at three different inclusion levels were isolated and incubated with L-[¹⁴C(U)]glycerol-3-phosphate and [¹⁴C]palmitoyl CoA. The results showed that in this fish species the glycerol-3-phosphate pathway is mainly involved in phospholipid synthesis, whereas TAG synthesis is mainly mediated by the monoacylglycerol pathway. Feeding with rapeseed oil reduced the reacylation activity in both pathways, explaining the high accumulation of lipid droplets in the supranuclear portion of the intestinal epithelium, whereas soyabean oil enhanced phosphatidylcholine synthesis, being associated with the increase in VLDL found in previous studies.

Intestinal microsomes: Vegetable oils: Sea bream: Monoacylglycerol pathway: Glycerol-3-phosphate pathway: Cytidine diphosphate nucleotides: Triacylglycerols: Phospholipids

Although traditionally fish oil has been the main lipid source in commercial fish diets, it constitutes a limited natural resource highly appreciated for other uses including terrestrial animal feeds and pharmacology. Besides, the high growth rate of aquaculture to cope with increasing fish consumption further constrains the availability of this oil. Hence the increased interest in the inclusion of vegetable oils in diets for marine fish to partially replace and reduce the dependency on fish oil. However, while several fish species seem to be able to grow well when dietary fish oil is partly replaced by vegetable oils (Bell *et al.* 2003; Regost *et al.* 2003; Izquierdo *et al.* 2003, in press), recent studies have reported several histological variations in different tissues such as liver (Caballero *et al.* 2004) and intestine (Olsen *et al.* 1999, 2000; Caballero *et al.* 2002, 2003).

In sea bream, Caballero *et al.* (2003) reported an enhanced accumulation of lipid droplets in the enterocytes of fish fed rapeseed oil. Furthermore, modifications in the size and type of intestinal lipoproteins were detected when fish were fed with soyabean

oil (Caballero *et al.* 2003). However, the reasons for these tissue abnormalities have not been clarified yet. Alterations in the physiological mechanisms involved in the different processes of lipid digestion and absorption could be responsible for such gut morphological features.

In contrast to mammals, dietary lipids, primarily triacylglycerols (TAG), are mainly hydrolysed in the lumen of the gut through the action of non-specific bile salt-activated lipase, whereas the mammalian pancreatic lipase enzyme does not seem to be the main enzyme responsible for neutral lipid (NL) digestion in marine fish (Iijima *et al.* 1998; Izquierdo & Henderson, 1998; Izquierdo *et al.* 2000). After intraluminal hydrolysis, dietary fat is taken up on the epithelial cells of the intestine by diffusion of a micellar form of monoglycerol and NEFA. Two pathways are involved in the re-esterification process of the lipids in enterocytes. The first one, the monoacylglycerol pathway, utilizes monoacylglycerols for diacylglycerol (DAG) and TAG biosynthesis through the enzymes monoacylglycerol acyltransferase

Abbreviations: BSA, bovine serum albumin; CDP-choline, cytidine diphosphate choline; CDP-ethanolamine, cytidine diphosphate ethanolamine; DAG, diacylglycerol; DGAT, diacylglycerol transferase; DGCP, diacylglycerol choline phosphotransferase; DGEPT, diacylglycerol ethanolamine phosphotransferase; DTT, dithiothreitol; NL, neutral lipids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, polar lipids; TAG, triacylglycerol.

*Corresponding author: Dr Maria José Caballero, fax +34 928451141, email mcaballero@dmor.ulpgc.es

and diacylglycerol acyltransferase (DGAT). In mammals, monoacylglycerol acyltransferase shows marked preference for the acylation of the sn-1-position, resulting in the synthesis of 80–100% of sn-1,2-DAG (Mansbach *et al.* 2001). DGAT acylates DAG at the sn-3 position using either long- or medium-chain acyl-CoA (Coleman *et al.*, 2000). The second one, the glycerol-3-phosphate pathway, utilizes α -glycerophosphate as an acceptor to yield lysophosphatidic acid, phosphatidic acid (PA) and DAG. DAG is the common precursor for the biosynthesis of TAG and for the production of nitrogenous phospholipids, such as phosphatidylcholine (PC; catalysed by diacylglycerol choline phosphotransferase (DGCPT)) or phosphatidylethanolamine (PE; catalysed by diacylglycerol ethanolamine phosphotransferase (DGEPT)). In mammals, it has been reported that DGCPT has preference for the sn-1,2-DAG as substrate for PC.

The activity of these enzymes is regulated by several nutritional and physiological factors (Mansbach *et al.* 2001). For instance, ATP and cytidine triphosphate concentrations seem to maintain the proportional cellular synthesis of phospholipids and TAG. Thus, increased concentrations of cellular ATP in yeast favour phospholipid synthesis at the expense of TAG synthesis (Carman, 1997). When cytidine triphosphate decreases in H9C2 cells, phospholipid biosynthesis is attenuated and concentrations of DAG and TAG increase (Hatch & McClarty, 1996). In fish, the activity and regulation of these pathways as well as the physiological implications of possible alterations by nutritional factors are poorly understood. Only Iijima *et al.* (1983) have reported alterations on the conversion of glycerol-3-phosphate into PA in intestinal extracts of carp fed with oxidized lipids.

A series of experiments were conducted in the present study to examine the activity of the monoacylglycerol and the glycerol-3-phosphate pathways in intestinal microsomes of sea bream, determining their regulation by different cytidine nucleotides, as well as the influence of dietary vegetable oil inclusion on both pathways to understand better the morphological changes observed in the intestine of fish fed different lipid sources.

Material and methods

Feeding experiments

The experiments were carried out at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain) and all fish (79 ± 8.0 g mean initial body weight) were obtained from a local fish farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain). The first trial was carried out with sixty gilthead sea bream (*Sparus aurata*) juveniles, which were distributed in three fibreglass tanks of 500 litres in groups of twenty fish. Fish were fed with a commercial diet (Proaqua S.A., Dueñas, Spain). After this experiment, a trial was carried out with 1560 sea bream, which were distributed in twenty-four fibreglass tanks of 500 litres in groups of sixty-five fish. Fish were fed with four different diets. These were combinations of one type of fish oil (anchovy) with two plant oils (rapeseed and soyabean). The fat sources were combined in order to have a wide variety of fatty acid profiles. The 100% anchovy oil diet (100FO) was used as the control diet. In diets 60SO and 60RO, 60% of the anchovy oil was replaced by soyabean and rapeseed oils, respectively, whereas in diet 80SO 80% of the anchovy oil was replaced by soyabean oil. Each diet was assayed in triplicate. Ingredients and fatty

acid composition of the diets are shown in Tables 1 and 2, respectively.

In both experiments fish were fed with the respective diets until apparent satiation three times a day, at 9.00, 12.00 and 15.00 hours, for 3 months, until fish reached a weight of about 200 g. Each tank was provided with continuous seawater and air flows. Water temperature ranged between 21 and 24°C and dissolved oxygen ranged between 6.0 and 8.0 mg/l. Fish were subjected to a natural photoperiod of approximately 12 h light/12 h darkness.

Preparation of microsomal fraction

After the feeding experimental periods, fish were anaesthetized with chlorobutanol, whole intestine was extracted, mesenteric fat was removed and immediately frozen and kept at -80°C . For microsomal preparation, the whole intestine was rinsed with ice-cold 50 mM-Tris-HCl, 0.225 M-sucrose, 1 mM-EDTA, 2 mM-dithiothreitol (DTT) (pH 7.4). The anterior gut portion was immediately cut into small pieces and homogenized (2000 rpm) in four volumes of ice-cold 50 mM-Tris-HCl, 0.225 M-sucrose, 1 mM-EDTA, 2 mM-DTT (pH 7.4) for 2 min. Homogenate was centrifuged at 1200 g for 10 min. Supernatant was centrifuged at 5900 g for 10 min, and then 2 ml postmitochondrial supernatant was further centrifuged at 106 000 g for 60 min to pellet microsomal membranes. Microsomes were washed with 50 mM-Tris-HCl, 0.225 M-sucrose, 1 mM-EDTA, 2 mM-DTT (pH 7.4) and sedimented by recentrifugation at 106 000 g for 60 min. All steps were carried out at 4°C. Washed microsomes were finally suspended in 50 mM-Tris-HCl, 0.225 M-sucrose, 1 mM-EDTA, 2 mM-DTT (pH 7.4) and aliquots were either used the same day or quickly frozen at -80°C . Frozen microsomes were used within 1 week with no significant change in enzymatic activity. Protein content was determined by Lowry assay (Lowry *et al.* 1951).

Reagents

[1- ^{14}C]Palmitoyl CoA (specific activity 55 mCi/mmol) and L-[^{14}C (U)]glycerol-3-phosphate (specific activity 100 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA).

Table 1. Ingredients (g/kg) of the experimental diets containing different lipid sources*

Ingredient	Diet			
	100FO	60SO	60RO	80SO
Fish meal	381	381	381	381
Corn gluten	260	260	260	260
Wheat	150.6	150.6	150.6	150.6
Lysine (99%)	7.23	7.23	7.23	7.23
Premix†	25	25	25	25
Anchovy oil	176	70.4	70.4	35.2
Soyabean oil‡		105.6		140.8
Rapeseed oil‡			105.6	

* The 100% anchovy oil diet (100FO) was used as the control diet. In diets 60SO and 60RO, 60% of the anchovy oil was replaced by soyabean and rapeseed oils, respectively, whereas in diet 80SO 80% of the anchovy oil was replaced by soyabean oil.

† Premix of vitamins and minerals according to the National Research Council (1993) recommendations for fish.

‡ Crude vegetable oils.

Table 2. Fatty acid composition of the experimental diets (g/100 g fatty acids)*

	Diet			
	100FO	60SO	60RO	80SO
14:0	6.21	3.18	3.11	1.10
16:0	18.51	15.45	12.43	14.24
16:1 <i>n</i> -7	6.46	3.41	3.39	2.20
16:2 <i>n</i> -6	1.15	0.60	0.57	0.37
17:1	1.28	0.63	0.63	0.37
16:4 <i>n</i> -3	1.85	0.88	0.85	0.44
18:0	3.18	3.21	2.35	3.30
18:1 <i>n</i> -9	16.26	18.48	36.84	21.07
18:1 <i>n</i> -7	2.47	1.84	ND	ND
18:2 <i>n</i> -6	13.07	31.28	17.04	38.54
18:3 <i>n</i> -6	0.21	0.12	0.10	0.07
18:3 <i>n</i> -3	1.41	3.24	4.64	4.03
18:4 <i>n</i> -3	1.43	0.79	0.76	0.48
20:1 <i>n</i> -9	1.89	1.63	2.08	1.49
20:4 <i>n</i> -6	0.54	0.34	0.33	0.25
20:4 <i>n</i> -3	0.43	0.25	0.26	0.22
20:5 <i>n</i> -3	11.19	5.88	5.84	3.73
22:1 <i>n</i> -11	1.95	1.49	2.13	2.01
22:1 <i>n</i> -9	0.14	0.22	0.03	0.03
22:3 <i>n</i> -6	0.53	0.33	0.29	0.22
22:4 <i>n</i> -6	0.25	0.21	0.17	0.16
22:4 <i>n</i> -3	0.19	0.15	0.12	0.10
22:5 <i>n</i> -3	1.16	0.75	0.63	0.50
22:6 <i>n</i> -3	4.99	3.20	3.26	2.36
Σ Saturated	27.9	21.84	17.89	18.64
Σ Monoenes	30.45	27.70	45.10	27.17
Σ <i>n</i> -3	22.65	15.14	16.36	11.86
Σ <i>n</i> -6	15.75	32.88	18.50	39.61
Σ <i>n</i> -9	18.29	20.33	38.95	22.59
Σ <i>n</i> -3 HUFA	17.96	10.23	10.11	6.91
Σ <i>n</i> -6 HUFA	1.32	0.88	0.79	0.63
<i>n</i> -3- <i>n</i> -6	1.44	0.46	0.88	0.30

HUFA, Highly unsaturated fatty acids; ND, not detected.

*The 100% anchovy oil diet (100FO) was used as the control diet. In diets 60SO and 60RO, 60% of the anchovy oil was replaced by soyabean and rapeseed oils, respectively, whereas in diet 80SO 80% of the anchovy oil was replaced by soyabean oil.

Palmitoyl CoA, glycerol-3-phosphate, 1-monoacylglycerol, Trisma Base, bovine serum albumin (BSA; fatty acid-free), MgCl₂, DTT, EDTA, cytidine diphosphate choline (CDP-choline), cytidine diphosphate ethanolamine (CDP-ethanolamine), cytidine triphosphate, and standards of TAG, DAG, PC, PE and PA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Silica gel H and G were obtained from Fluka (Buchs, Switzerland). All other chemicals and solvents were of reagent high quality and were obtained from local suppliers.

Glycerol-3-phosphate pathway assay

Microsomes (0.8–1 mg protein) from sea bream fed 100FO, 60RO, 60SO and 80SO were used to investigate the incorporation of L-[¹⁴C(U)]glycerol-3-phosphate into the different lipid classes. These were incubated at 37°C for 10 min (temperature and time was previously determined according to the highest activity found but always beneath radiolabelling saturation) with the Basal Solution 1 containing: 0.002 μmol L-[¹⁴C(U)]glycerol-3-phosphate (specific activity 100 mCi/mmol), 0.200 μmol glycerol-3-phosphate, 0.020 μmol palmitoyl CoA, 70 mM-Tris-HCl

(pH 7.4), 1 mM-DTT, 3.0 mM-MgCl₂, 2 mg/ml BSA in a final volume of 0.5 ml. The reaction was terminated by addition of 4 ml chloroform-methanol (2:1) containing 0.01% butylated hydroxytoluene. Lipids were extracted by the method of Folch *et al.* (1957). The extracted lipids were dissolved in 3 ml chloroform and developed on TLC plates. The TLC plates were activated at 100°C for 2 h before use.

NL (TAG and DAG) were developed with hexane-diethyl ether-acetic acid (80:20:2, by vol). Polar lipids (PL; PC and PE) were developed with chloroform/methanol/ammonium hydroxide/water (65:35:1:3, by vol). PA was separated on silica gel G plate which was prepared by using 0.25 M-oxalic acid. In this case, the solvent was petroleum ether-acetone-formic acid (74:26:0.25, by vol). After developing, the plates were neutralized with iodine vapour and then viewed under UV light. The separated bands were scraped and transferred directly into scintillation vials and suspended in 3 ml Optiphase '2' (Wallac, Turku, Finland) and the amount of radioactivity was determined by scintillation counting (1211 Rackbeta, Wallac).

Monoacylglycerol pathway assay

Microsomes (0.8–1 mg protein) from sea bream fed 100FO, 60RO, 60SO and 80SO were used to investigate the incorporation of [1-¹⁴C]palmitoyl CoA. These were incubated at 37°C for 10 min with Basal Solution 2 containing: 0.001 μmol [1-¹⁴C]palmitoyl CoA (specific activity 55 mCi/mmol), 0.040 μmol 1-monopalmitoyl-rac-glycerol, 0.020 μmol palmitoyl CoA, 70 mM-Tris-HCl (pH 7.4), 1 mM-DTT, 3.0 mM-MgCl₂, 2 mg/ml BSA in a final volume of 0.5 ml. The reaction was terminated by addition of 4 ml chloroform-methanol (2:1) containing 0.01% butylated hydroxytoluene. Lipids were extracted by the method of Folch *et al.* (1957). The extracted lipids were dissolved in 3 ml chloroform and developed on TLC plates for NL and PL as described earlier for the glycerol-3-phosphate pathway assay. The respective lipid areas were scraped into vials, and radioactivity was determined by scintillation counting.

Incubation of cytidine nucleotides in the glycerol-3-phosphate and monoacylglycerol pathway assays

Microsomes (0.8–1 mg protein) from sea bream fed commercial diet were used to investigate the incorporation of L-[¹⁴C(U)]glycerol-3-phosphate and [1-¹⁴C]palmitoyl CoA into the different lipid classes in the presence or absence of cytidine nucleotides. Both Basal Solution 1 and Basal Solution 2 were assayed with CDP-choline and CDP-ethanolamine using the method described earlier for the glycerol-3-phosphate and monoacylglycerol pathway assays, respectively. The incubation mixture is listed in Table 3.

Statistics

The results are expressed as means and standard deviations. Data were compared by one-way ANOVA. Tukey's test for comparison of means was applied ($P \leq 0.05$). When the variances were not normally distributed, the Kruskal-Wallis non-parametric test was applied to the data (Sokal & Rolf, 1995).

Table 3. Cytidine nucleotide incubation mixture for triacylglycerol and phospholipid biosynthesis from L-[¹⁴C(U)]glycerol-3-phosphate (glycerol-3-phosphate pathway assay) and [¹⁻¹⁴C]palmitoyl CoA (monoacylglycerol pathway assay)

Material	Incubation mixture		
	Control	CDP-choline (0-600 μmol)	CDP-ethanolamine (0-600 μmol)
L-[¹⁴ C(U)]Glycerol-3-phosphate			
Basal Solution 1*	+	+	+
CDP-choline	-	+	-
CDP-ethanolamine	-	-	+
[¹⁻¹⁴ C]Palmitoyl CoA			
Basal Solution 2*	+	+	+
CDP-choline	-	+	-
CDP-ethanolamine	-	-	+

CDP-choline, cytidine diphosphate choline; CDP-ethanolamine, cytidine diphosphate ethanolamine.

* For details of Basal Solutions 1 and 2, see p. 449.

Results

Effect of dietary lipids on the glycerol-3-phosphate pathway

The incorporation of L-[¹⁴C(U)]glycerol-3-phosphate into the NL and PL in the intestinal microsomes of sea bream fed different experimental diets (100FO, 60SO, 60RO, 80SO) is shown in Fig. 1. In general, the order of incorporation of L-[¹⁴C(U)]glycerol-3-phosphate into the different lipid classes was: PC >

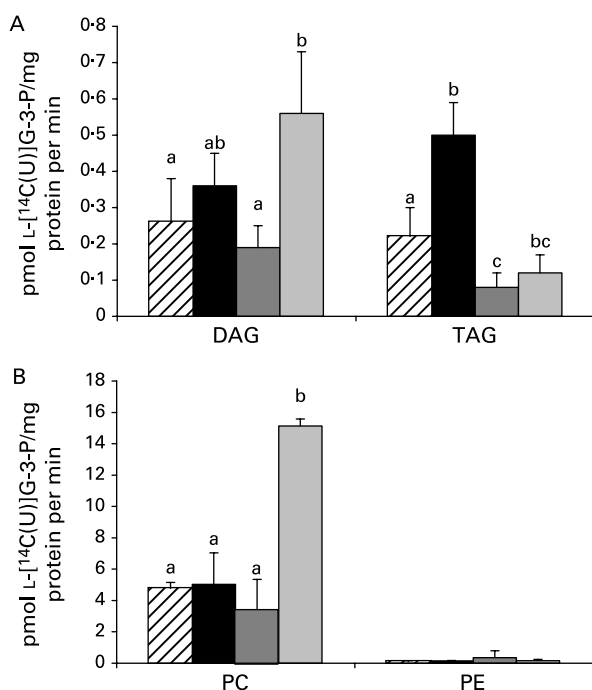


Fig. 1. Effect of the dietary lipids (▨, 100FO; ■, 60SO; ■, 60RO; □, 80SO diets) in the incorporation of L-[¹⁴C(U)]glycerol-3-phosphate (L-[¹⁴C(U)]G-3-P) into neutral lipids (A, diacylglycerol (DAG); triacylglycerol (TAG)) and polar lipids (B, phosphatidylcholine (PC); phosphatidylethanolamine (PE)). For details of diets and Basal Solution 1, see Table 1 and p. 449. Cytidine nucleotides were not added. Values are means with standard deviations depicted by vertical bars ($n=4$). ^{a,b,c} Mean values among diets for each lipid class with unlike superscript letters were significantly different ($P \leq 0.05$).

DAG > TAG for all the experimental groups. Fish fed 100FO and 60SO diets had similar TAG–DAG (0.6:1) and PC–DAG ratios (12.7:1 and 13.8:1, respectively) (Table 4), whereas a decrease in the synthesis of TAG was observed in the fish fed 60RO diet (Fig. 1(A)), resulting in a reduced TAG–DAG ratio (0.3:1), not affecting the PC–DAG ratio (13:1) (Table 4). A substantial increased synthesis of PC was observed in fish fed 80SO diet (Fig. 1(B)), showing a PC–DAG ratio of 18.7:1 (Table 4), and a decrease in the incorporation of TAG. The incorporation of L-[¹⁴C(U)]glycerol-3-phosphate in other phospholipids, such as PE, was not significant.

Effect of the dietary lipids on the monoacylglycerol pathway

The incorporation of [¹⁻¹⁴C]palmitoyl CoA into the NL and PL in the intestinal microsomes of sea bream fed the different experimental diets (100FO, 60SO, 60RO, 80SO) is shown in Fig. 2.

In general, the order of incorporation of [¹⁻¹⁴C]palmitoyl CoA was: DAG > TAG > PC > PE. Fish fed 100FO diet showed a high incorporation of [¹⁻¹⁴C]palmitoyl CoA into DAG, with significant differences when compared to the 60SO and 80SO diets. A similar pattern was followed in the synthesis of TAG (Fig. 2(A)). However, fish fed 60RO diet showed the lowest incorporation of [¹⁻¹⁴C]palmitoyl CoA into DAG and TAG (Fig. 2(A)). Fish fed 80SO diet showed a high synthesis of PC (Fig. 2(B)), having a PC–DAG ratio of 0.3:1 (Table 5). Incorporation of [¹⁻¹⁴C]palmitoyl CoA was observed into PE although it was lower than into PC for all experimental groups.

Effect of different cytidine nucleotides on glycerol-3-phosphate pathway

Figure 3 shows the incorporation of L-[¹⁴C(U)]glycerol-3-phosphate into the lipid classes by the intestinal microsomes of sea bream fed commercial diet in the presence or absence of CDP-choline and CDP-ethanolamine. In the absence of cytidine diphosphate nucleotides (Control incubation mixture), 15.6 pmol of L-[¹⁴C(U)]glycerol-3-phosphate was incorporated into PC, and approximately 1.5 pmol was found in PE and PA. The incorporation of L-[¹⁴C(U)]glycerol-3-phosphate into DAG was 8.1 pmol and into TAG was 3.9 pmol (TAG–DAG ratio was 0.5:1).

In presence of CDP-choline, substantial increase in PC synthesis was observed (48.3 pmol), being approximately three times that found in the Control. The incorporation of radioactivity into TAG was significantly lower than in the Control incubation mixture (1.4 pmol in CDP-choline v. 3.9 pmol in the Control). In this case, the TAG–DAG ratio was 0.13:1.

Table 4. Ratios of incorporation of L-(¹⁴C(U))glycerol-3-phosphate in intestinal microsomes of sea bream fed the experimental diets*

Diets	TAG–DAG	PC–DAG	TAG–PC
100FO	0.6	12.7	0.05
60SO	0.6	13.8	0.04
60RO	0.3	13.0	0.02
80SO	0.2	18.7	0.01

DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerol.

* For details of diets, see Table 1.

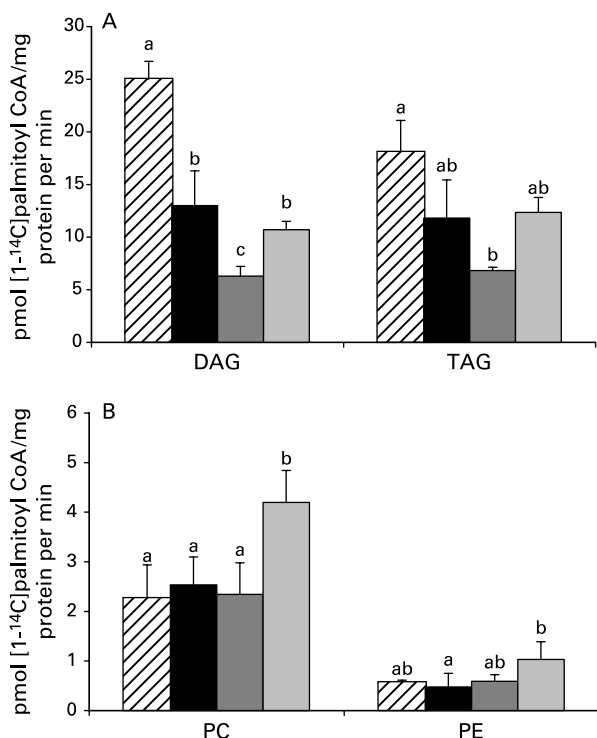


Fig. 2. Effect of the dietary lipids (▨, 100FO; ■, 60SO; ▒, 60RO; □, 80SO diets) in the incorporation of [1-¹⁴C]palmitoyl CoA into neutral lipids (A, diacylglycerol (DAG); triacylglycerol (TAG)) and polar lipids (B, phosphatidylcholine (PC); phosphatidylethanolamine (PE)). For details of diets and Basal Solution 2, see Table 1 and p. 449. Cytidine nucleotides were not added. Values are means with standard deviations depicted by vertical bars (*n*=4). ^{a,b,c} Mean values among diets for each lipid class with unlike superscript letters were significantly different (*P*≤0.05).

In the presence of CDP-ethanolamine, incorporation into PE and PA was not affected, whereas the incorporation into PC increased up to 21.2 pmol in comparison with the Control, but was lower than the one obtained with CDP-choline. The TAG–DAG ratio was similar to that found in the presence of CDP-choline (0.13 : 1).

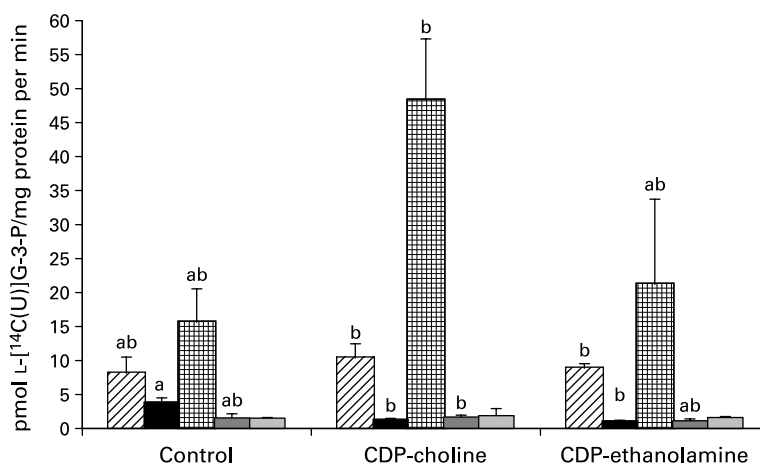


Fig. 3. Effect of the cytidine nucleotides in the incorporation of L-[¹⁴C(U)] (glycerol-3-phosphate (L-[¹⁴C(U)]G-3-P) into diacylglycerol (DAG, ▨), triacylglycerol (TAG, ■), phosphatidylcholine (PC, ▤), phosphatidylethanolamine (PE, ▒) and phosphatidic acid (PA, □) in intestinal microsomes of sea bream fed commercial diet. For details of diets and Basal Solution 1, see Table 1 and p. 449. The addition of 0.600 μmol cytidine diphosphate choline (CDP-choline) and 0.600 μmol cytidine diphosphate ethanolamine (CDP-ethanolamine) was according to the incubation mixture (for details, see Table 3). Values are means with standard deviations depicted by vertical bars (*n*=3). ^{a,b} Mean values in the same series with unlike superscript letters were significantly different (*P*≤0.05). Where no letters are indicated, no significant differences were observed.

Table 5. Ratios of incorporation of [1-¹⁴C]palmitoyl CoA in intestinal microsomes of sea bream fed the experimental diets*

Diets	TAG–DAG	PC–DAG	TAG–PC
100FO	0.7	0.1	5.8
60SO	0.8	0.2	4.1
60RO	0.8	0.4	2.2
80SO	1.2	0.3	2.9

DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerol.
* For details of diets, see Table 1.

Effect of different cytidine nucleotides on the monoacylglycerol pathway

Figure 4 shows the incorporation of [1-¹⁴C]palmitoyl CoA into the lipid classes by the intestinal microsomes of sea bream fed commercial diet in the presence or absence of CDP-choline and CDP-ethanolamine. The highest incorporation of [1-¹⁴C]palmitoyl CoA was quantified in the NL while low radioactivity was found in the polar fraction for any of the three incubation mixtures: Control incubation (89.9 pmol NL *v.* 9.7 pmol PL), CDP-choline incubation (85.7 pmol NL *v.* 6.7 pmol PL) and CDP-ethanolamine incubation (122.3 pmol NL *v.* 14.6 pmol PL). Incorporation of radioactivity into DAG was significantly higher than into TAG, the TAG–DAG ratio being 0.6 : 1 for all the incubation mixtures.

Discussion

The present study has adapted for the first time the method originally described in rats by Lehner & Kuksis (1995) to study enzymatic reacylation activity in fish gut using isolated fish intestinal microsomes. At the same time, it also opens the possibility of investigating TAG and phospholipid biosynthesis in the enterocytes of juvenile cultured fish and allows us to understand better dietary lipid utilization.

On the one hand, the presence of CDP-choline markedly stimulated PC synthesis by the glycerol-3-phosphate pathway, whereas TAG synthesis was suppressed due to a diversion of a significant

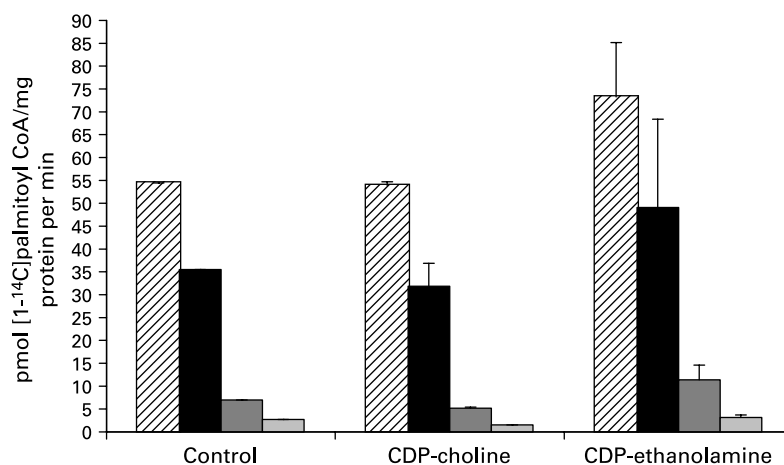


Fig. 4. Effect of cytidine nucleotides in the incorporation of [1-¹⁴C]palmitoyl CoA in diacylglycerol (DAG, ▨), triacylglycerol (TAG, ■), phosphatidylcholine (PC, ■) and phosphatidylethanolamine (PE, □) in intestinal microsomes of sea bream fed commercial diet. For details of diets and Basal Solution 2, see Table 1 and p. 449. The addition of 0.600 μmol cytidine diphosphate choline (CDP-choline) and 0.600 μmol cytidine diphosphate ethanolamine (CDP-ethanolamine) was according to the incubation mixture (for details, see Table 3). Values are means with standard deviations depicted by vertical bars ($n=3$).

fraction of the DAG pool to PC synthesis. This fact suggested that the enzyme DGAT, which catalyses DAG in TAG, and the enzyme DGCPT, which catalyses DAG in PC, share a common pool of DAG, and consequently the availability of this intermediate seems to limit TAG synthesis. Both enzymes also seem to share the DAG pool in rat hepatocytes where, in the presence of sufficient CDP-choline, the affinity of the DGCPT for the DAGs is higher than that of DGAT, favouring PC synthesis (Stals *et al.* 1992).

On the other hand, in agreement with the results found by Iijima *et al.* (1983) in carp intestinal preparations, in the present study the addition of CDP-ethanolamine also produced a slight increase in PC biosynthesis, whereas PE synthesis was not affected, suggesting that the conversion of PE into PC through the enzyme phosphatidylethanolamine methyltransferase is faster than the particular biosynthesis of PE by the DGEPT enzyme, denoting the importance of PC in this tissue. Phosphatidylethanolamine methyltransferase activity has also a high significance in PC synthesis in rat liver, where it accounts for 20–40% of the total PC synthesized by the hepatocytes (Tijburg *et al.* 1989).

Regarding the monoacylglycerol pathway, the addition of CDP-choline or CDP-ethanolamine to the substrate [1-¹⁴C]palmitoyl CoA did not stimulate PC or PE biosynthesis, whereas synthesis of DAG and TAG through this pathway was significantly higher when compared to the glycerol-3-phosphate pathway. Thus, PC synthesis from the monoacylglycerol pathway was 53% lower than in the glycerol-3-phosphate pathway, and the synthesis of NL was 86% higher than in the glycerol-3-phosphate pathway.

It is important to notice that *in vitro* products of the monoacylglycerol pathway differ from those obtained *in vivo*. Thus, while *in vivo* little DAG is accumulated in the enterocytes of sea bream (Caballero *et al.* 2003), accumulation of DAG was readily observed *in vitro* in the present experiment when microsomal or purified enzyme fractions are employed. The present results suggest the presence of some cellular factors *in vivo* involved in the enzymatic activation. For instance, it has been reported (Lehner & Kuksis, 1995) that the intestinal enzyme which

catalyses DAG into TAG requires a cytoplasmic protein of low molecular mass for its activation. In addition, the lower availability of NEFA *in vitro* could be another cause of the higher DAG–TAG ratio found in *in vitro* assays.

In general, the present results showed that in sea bream the glycerol-3-phosphate pathway is mainly implicated in PL synthesis whereas TAG synthesis is clearly mediated by the monoacylglycerol pathway, the DGCPT and DGAT enzymes being the two main points in the intestinal biosynthesis of PL and NL. The results of the present study agree well with Johnston *et al.* (1970, 1967), showing that PC can be synthesized via monoacylglycerol in microsomes of sea bream, although the amount of PC synthesized is lower than the TAG or DAG biosynthesis, and much less than in the glycerol-3-phosphate pathway. This could be partly due to a different stereospecificity of the DAG formed in each pathway, being mainly sn-1,2-DAG in the glycerol-3-phosphate pathway and a mixture of sn-1,2-DAG and sn-1,3-DAG in the monoacylglycerol pathway. This ratio of enantiomeric DAG might interfere with the production of PC through DGCPT since the possibility of the catalysis of this enzyme with its specific substrate decreases (sn-1,2-DAG).

Histological studies carried out in sea bream fed different vegetable oils have shown several gut morphological alterations depending on the dietary fatty acid composition (Caballero *et al.* 2002, 2003), which could be related to the intestinal mechanisms involved in the reacylation of the absorbed lipids. For instance, 60% substitution of fish oil by rapeseed reduced *in vitro* TAG acylation synthesis in sea bream intestinal microsomes in both the glycerol-3-phosphate and monoacylglycerol pathways, which could be due to decreased expression of the acyltransferases or to changes in membrane fatty acid composition that may exert its effect on the enzymes. This reduction in reacylation activity would explain the high accumulation of lipid droplets in the supranuclear portion of the intestinal epithelium, namely absorbed non-reacylated lipids in sea bream fed this type of diet (Caballero *et al.* 2003), and this agrees with the high percentage of oleic acid found in the intestinal NEFA fraction of this fish.

On the contrary, feeding sea bream with soyabean oil enhanced PC synthesis, particularly when substitution levels increased up to 80%. The present results could be related to the alterations in the enterocyte membrane fatty acid composition shown in previous studies (Caballero *et al.* 2003), since many of the enzymes of these pathways are associated with the cell membranes (Mansbach *et al.* 2001). As a consequence, the TAG–PC ratio was reduced by the addition of soyabean oil in diets 60SO and 80SO and this would be responsible for the high amount of VLDL found in this fish (Caballero *et al.* 2003) in comparison with the fish oil-fed ones where chylomicrons were the main lipoprotein observed. These facts agree with results shown in rainbow trout, where the production of surface materials, such as PC, is superior to the synthesis of TAG, resulting in the production of VLDL that are constituted by a high amount of PL (Sire *et al.* 1981).

In summary, the results of the present study showed that for sea bream the main pathway for PL biosynthesis is the glycerol-3-phosphate pathway, while the biosynthesis of TAG is mainly mediated by the monoacylglycerol pathway. High levels of oleic acid in diets reduced the reacylation activity in both intestinal pathways, explaining the high accumulation of lipid droplets in the supranuclear portion of the intestinal epithelium, whereas high content of 18:2n-6 in the diets enhanced PC synthesis and is responsible for the increase in VLDL found in previous studies.

Acknowledgements

This study was partially supported by research grants from the Spanish Government under the FEDER programme, project no. 1FD1997-1774 and from the European Union under 'Quality of Life and Management of Living Resources' through the project Q5RS-2000-30 058 'RAFOA'.

References

- Bell JG, McGhee F, Campbell PJ & Sargent JR (2003) Rapeseed oil as an alternative to marine fish oil in diets of post-smolt Atlantic salmon (*Salmo salar*): changes in flesh fatty acid composition and effectiveness of subsequent fish oil 'wash out'. *Aquaculture* **218**, 515–528.
- Caballero MJ, Izquierdo MS, Kjörsvik E, Fernández AJ & Rosenlund G (2004) Histological alterations in liver of sea bream (*Sparus aurata*) caused by feeding for a short or long-term with vegetable oils. Recovery of normal morphology after feeding fish oil as a solely lipid source. *J Fish Dis* **27**, 531–541.
- Caballero MJ, Izquierdo MS, Kjörsvik E, Montero D, Socorro J, Fernández AJ & Rosenlund G (2003) Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* **225**, 325–340.
- Caballero MJ, Obach A, Rosenlund G, Montero D, Gisvold M & Izquierdo MS (2002) Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **214**, 253–271.
- Carman GM (1997) Phosphatidate phosphatases and diacylglycerol pyrophosphate phosphatases in *Saccharomyces cerevisiae* and *Escherichia coli*. *Biochim Biophys Acta* **1348**, 45–55.
- Coleman RA, Lewin TM & Muoio D (2000) Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. *Annu Rev Nutr* **20**, 77–103.
- Folch J, Lees M & Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.
- Hatch GM & McClarty G (1996) Regulation of cardiolipin biosynthesis in H9C2 cardiac myoblasts by cytidine 5'-triphosphate. *J Biol Chem* **271**, 25810–25816.
- Iijima N, Zama K & Kayama M (1983) Effect of the oxidized lipids on the metabolic pathway of lipid biosynthesis in the intestine of carp (*Cyprinus carpio*). *Nippon Suisan Gakkaishi* **49**, 1465–1470.
- Iijima N, Tanaka S & Ota Y (1998) Purification and characterization of bile-salt activated lipase from the hepatopancreas of red sea bream, *Pargus major*. *Fish Physiol Biochem* **18**, 56–61.
- Izquierdo MS & Henderson RJ (1998) The determination of lipase and phospholipase activities in gut contents of turbot (*Scophthalmus maximus*) by fluorescent-based assays. *Fish Physiol Biochem* **19**, 153–162.
- Izquierdo MS, Montero D, Robaina L, Caballero MJ, Rosenlund G & Ginés R (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead sea bream (*Sparus aurata*) fed vegetable oils for a long-term period. Recovery of fatty acids profile by fish oil. *Aquaculture* **250**, 431–444.
- Izquierdo MS, Obach A, Arantzamendi L, Robaina L, Montero D & Rosenlund G (2003) Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquacult Nutr* **9**, 397–407.
- Izquierdo MS, Socorro J, Arantzamendi L & Hernández-Cruz CM (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol Biochem* **22**, 97–107.
- Johnston JM, Paltauf F, Schiller CM & Schultz LD (1970) The utilization of the α -glycerophosphate and monoglyceride pathways for phosphatidylcholine biosynthesis in the intestine. *Biochim Biophys Acta* **218**, 124–133.
- Johnston JM, Rao GA & Lowe PA (1967) The separation of the α -glycerophosphate and monoglyceride pathways in the intestinal biosynthesis of triglycerides. *Biochim Biophys Acta* **137**, 578–580.
- Lehner R & Kuksis A (1995) Triacylglycerol synthesis by purified triacylglycerol synthetase of rat intestinal mucosa: role of acyl-CoA acyltransferase. *Biol Chem* **270**, 13630–13636.
- Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Mansbach CM, Tso P & Kuksis A (2001) *Intestinal Lipid Metabolism*. New York: Kluwer Academic.
- National Research Council (1993) *Nutrient Requirements of Fish*. Washington, DC: National Academy Press.
- Olsen RE, Myklebust R, Kaino T & Ringø E (1999) Lipid digestibility and ultrastructural changes in the enterocytes of Arctic charr (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin. *Fish Physiol Biochem* **21**, 35–44.
- Olsen RE, Myklebust R, Ringø E & Mayhew TM (2000) The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic charr (*Salvelinus alpinus* L.): a quantitative ultrastructural study. *Fish Physiol Biochem* **22**, 207–216.
- Regost C, Arzel J, Robin J, Rosenlund G & Kaushik SJ (2003) Total replacement of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta maxima*): 1. Growth performance, flesh fatty acid profile, and lipid metabolism. *Aquaculture* **217**, 465–482.
- Sire MF, Lutton C & Vernier JM (1981) New views on intestinal absorption of lipids in teleostean fishes: an ultrastructural and biochemical study in the rainbow trout. *J Lipid Res* **22**, 81–94.
- Sokal RR & Rolf FJ (1995) *Biometry. The Principles and Practice of Statistics in Biological Research*, 3rd ed, New York: WH Freeman & Co.
- Stals HK, Mannaerts GP & Declercq PE (1992) Factors influencing triacylglycerol synthesis in permeabilized rat hepatocytes. *Biochem J* **283**, 719–725.
- Tijburg LBM, Huelen MJH & Van Golde LMG (1989) Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biochim Biophys Acta* **1004**, 1–19.