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Incorporation and metabolism of ^{14}C-labelled polyunsaturated fatty acids in juvenile gilthead sea bream *Sparus aurata* L. *in vivo*

Gabriel Mourente[1] and Douglas R. Tocher[2]
[1]*Departmento de Biología, Facultad de Ciencias del Mar, Universidad de Cádiz, Polígono Río San Pedro, Apartado 40, 11510-Puerto Real, Cádiz, Spain;* [2]*NERC Unit of Aquatic Biochemistry, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, U.K.*

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Abbreviations: AA, 5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4n-6); CPL, diradyl (diacyl + alkenylacyl + alkylacyl) glycerophosphocholine; DHA, 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3); EPA, 5,8,11,14,17-eicosapentaenoic acid (20:5n-3); EPL, diradyl (diacyl, alkenylacyl + alkylacyl) glycerophosphoethanolamine; HUFA, highly unsaturated fatty acids ($\geq C_{20}$ and with $\geq$ 3 double bonds); LA, 9,12-octadecadienoic acid (linoleic acid, 18:2n-6); LNA, 9,12,15-octadecatrienoic acid (α-linolenic acid, 18:3n-3) PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid(s).

Abstract

The incorporation, and the capacity for desaturation and elongation *in vivo*, of intraperitoneally-injected, ^{14}C-labelled n-3 and n-6 C_{18} and C_{20} PUFAs were investigated in juvenile gilthead sea bream, *Sparus aurata*. The results indicate that juvenile gilthead sea bream have only limited ability to convert C_{18} PUFAs to C_{20} and C_{22} HUFAs *in vivo*. The data are consistent with the results from nutritional studies on larvae, post-larvae and fingerlings that have shown that gilthead sea bream require the provision of preformed eicosapentaenoic and docosahexaenoic acids in the diet. The impairment in the desaturase/elongase pathway was quantitatively and qualitatively similar to that found in turbot, *Scophthalmus maximus*, being at the level of the Δ5-desaturase. The low activity of Δ5-desaturase combined with the consistent finding that arachidonic acid is selectively retained in membrane phosphatidylinositol suggests that, in addition to eicosapentaenoic and docosahexaenoic acids, gilthead sea bream may also have a requirement for preformed arachidonic acid in the diet.

Introduction

Animals, including fish, do no possess the Δ-12 and Δ-15 fatty acid desaturase enzymes necessary for the biosynthesis of linoleic (LA,18:2n-6) and linolenic (LNA, 18:3n-3) acids, respectively, from oleic acid, 18:1n-9 (Henderson and Tocher 1987; Sargent *et al.* 1989). The requirement for dietary C_{18} PUFA is related to the ability of the fish to convert the C_{18} PUFA, *via* the desaturation/elongation pathways, to the longer-chain more unsaturated C_{20} and C_{22} PUFA. Freshwater fish, or at least salmonids, have the ability to bioconvert C_{18} PUFA to a great extent (Henderson and Tocher 1987), whereas marine fish, such as the turbot, *Scophthalmus maximus*, appear to lack one or more of the desaturase enzymes required to synthesize highly unsaturated fatty acids (HUFA,

$\geq C_{20}$ and with ≥ 3 double bonds) (Sargent *et al.* 1989). Rainbow trout, *Oncorhynchus mykiss*, have a substantial capability to produce C_{20} and C_{22} PUFA from C_{18} PUFA (Owen *et al.* 1975), whereas other freshwater species may not be so active in this conversion process, although the rates were still greater than those observed with marine fish species (Owen *et al.* 1975; Kanazawa *et al.* 1979). In consequence, the general inability of marine fish to desaturate and elongate C_{18} PUFA dictates the essential requirement of these fish for preformed C_{20} and C_{22} PUFA in their diet (Sargent *et al.* 1989).

The gilthead sea bream, *Sparus aurata*, is an halotolerant species that may thrive in either hypohaline or hyperhaline environments (Audouin 1962; Arias *et al.* 1984; Chervinski 1984). It is also a highly prized food fish in the Mediterranean and eastern Atlantic, and is successfully cultured in a number of countries including Spain, France, Italy, and Israel. The essentially and minimum dietary levels of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids have been investigated in larval and fingerling gilthead sea bream (Koven *et al.* 1989, 1990, 1992; Kalogeropoulos *et al.* 1992; Mourente *et al.* 1992). Dietary n-3 HUFA showed a high correlation with larval growth rate (Koven *et al.* 1992; Mourente *et al.* 1992), although no correlation was found between n-3 HUFA and survival rates (Koven *et al.* 1992). The quantitative requirement for C_{20} and C_{22} n-3 HUFA (mainly EPA and DHA) was estimated to be approximately 0.9% of the diet for gilthead sea bream fingerlings (Kalogeropoulos *et al.* 1992). The requirement for n-3 HUFA of juvenile or adult gilthead sea bream is unknown (Kissil 1991). The conversion of LNA to EPA and DHA *via* desaturase and elongase activities was presumed to be low in most of the previous studies (Mourente and Odriozola 1990; Kissil 1991; Mourente *et al.* 1992). Relatively large proportions of n-6 PUFA, particularly arachidonic acid (AA, 20:4n-6), have been found in phosphatidylinositol (PI) from gilthead sea bream larvae and postlarvae brain lipids (Mourente *et al.* 1992; Mourente and Tocher 1993), suggesting a specific requirement for n-6 PUFA as well as n-3 PUFA.

In the present study, the incorporation, and the capacity for desaturation and elongation, of C_{18} and C_{20} PUFA were investigated in juvenile gilthead sea bream *in vivo*. Fish were sacrificed 2 days after intraperitoneal injections of ^{14}C-labelled n-3 and n-6 PUFA, lipids were extracted from the whole fish, resolved into specific lipid classes and fatty acid fractions, and analyzed for radioactive content.

Materials and methods

Fish

Juvenile gilthead sea bream, *Sparus aurata* L., of 2–5 g, reared on a commercial diet, were obtained from a commercial marine fish farm (Cultivos Piscícolas Marinos S.A., San Fernando, Cádiz, Spain). The fish were maintained in 50 l rectangular fibre-glass tanks, supplied with underground sea water (salinity 32 g/l and temperature 20 ± 1°C) in an open circuit system with aeration. Prior to experimentation, the fish were fed with a commercial dry pelleted diet in a daily ration of 5% of total body weight.

Injection studies and experimental conditions

It was previously established that radioactive fatty acids (3–5 μCi), dissolved in a small volume of ethanol (5–20 μl) can be injected into the intraperitoneal cavity of anaesthetized fish without apparent detrimental effects, yielding sufficient levels of radioactivity in the body lipids to permit subsequent analyses (Linares and Henderson 1991; Olsen and Ringo 1992). In the present study, six groups of three fish were starved for one week prior to injection to ensure no dietary input of C_{20} and C_{22} PUFA and to maximize the rate of bioconversion of the injected ^{14}C-labelled PUFA. The fish in four of the groups were injected with 3 μCi of either [1-^{14}C]LA,[1-^{14}C]LNA,[1-^{14}C]AA or [1-^{14}C]EPA suspended in 5 μl of ethanol. The fish in a fifth group were injected with ethanol alone as an injection control. Fish were injected after anaesthetization by hypothermic shock (Summerfelt and Smith

1990), whereby the fish were placed in a beaker containing a 1:1 mixture of sea water and crushed ice for 1–2 min until immobilized. No mortalities occurred during starvation or during the incubation period after the fish were injected with radioactive PUFA. After injection, the groups of fish were maintained in cylindrical glass aquaria containing 2 l of sea water with aeration. Fish recovered from anaesthesia and injection within 5 min after injection with no apparent ill effects. After 48 hours, fish were removed from the aquaria, carefully blotted with filter paper, killed by immersion in liquid nitrogen and wet weight determined. The sixth group of fish was used for dry weight determination: wet weight was determined as described above, then fish were finely chopped, desiccated at 110°C for 24 hours and then cooled *in vacuo* before dry weight was determined.

Lipid extraction

Lipids were extracted from weighted fish by homogenization in chloroform/methanol (2:1, v/v), containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant, according to Folch *et al.* (1957). Solvent was evaporated under a stream of nitrogen and lipids extracts desiccated overnight *in vacuo* before weighing. Lipid extracts were redissolved in chloroform/methanol (2:1, v/v) at a concentration of 50 mg/ml and stored under an atmosphere of nitrogen at -20°C until analysis. An aliquot of the total lipid was taken for determination of radioactivity as described below.

Lipid class quantification

Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using a single-dimension double-development method described previously (Tocher and Harvie 1988). The classes were quantified by charring followed by calibrated densitometry using a Shimadzu CS-9000 dual-wavelength flying spot scanner and DR-13 recorder (Olsen and Henderson 1989).

Fatty acid analysis

Individual glycerophospholipid classes were separated by thin-layer chromatography (TLC) essentialy according to the method of Vitiello and Zanetta (1978), using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) as developing solvent. For the separation of neutral lipid classes hexane/diethyl ether/acetic acid (80:20:2), v/v/v) was used (Christie 1989). Fatty acid methyl esters, from total lipids and individual glycerophospholipid or neutral lipid classes, were prepared by acid-catalyzed transmethylation for 16h at 50°C, using nonadecanoic acid (19:0) as internal standard (Christie 1989). Methyl esters were extracted and purified as described previously (Tocher and Harvie 1988). The fatty acid methyl esters were analyzed in a Hewlett-Packard 5890 A Series II gas chromatograph equipped with a chemically-bonded (PEG) Omegawax 320 fused-silica wall-coated capillary column (30 m × 0.32 mm i.d.) (Supelco Inc., Bellefonte, USA), using hydrogen as carrier gas with a thermal gradient from 185°C to 235°C. Individual fatty acid methyl esters were identified and quantified by reference to authentic standards and to a well characterized fish oil, using a Hewlett-Packard 3394 recording integrator (Tocher and Harvie 1988). All solvents contained 0.01% BHT as an antioxidant.

Incorporation of radioactivity into total lipids and individual lipid classes

Samples of total lipids (3 mg) from ^{14}C-PUFA-injected juvenile gilthead sea bream were applied in 4 cm streaks to TLC plates and polar lipid and neutral lipid classes separated as above. Lipid classes were visualized by brief exposure to iodine vapour, bands marked and the iodine removed under vacuum (Tocher and Harvie 1988). Individual classes were scraped into scintillation mini vials, 4 ml of liquid scintillation cocktail (Ready Safe, Beckman) added and radioactivity determined in a Beckman LS 5000 CE liquid scintillation spec-

trophotometer. Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Incorporation of radioactivity into fatty acid methyl esters from total lipids

Fatty acid methyl esters from total lipids were prepared as described above. Methyl esters were separated by argentation-TLC, using 2% silver nitrate-impregnated TLC plates with toluene/acetonitrile (95:5,v/v) as developing solvent (Wilson and Sargent 1992). This system resolves the methyl esters into discrete bands based on both degree of unsaturation and chain length (Christie 1989; Wilson and Sargent 1992). Developed TLC plates were subjected to autoradiography for 14 days using Kodak X-OMAT AR-5 X-ray film, and the labelled bands scraped into scintillation vials and radioactivity determined as above. Identification of labelled bands was confirmed by using authentic unlabelled standards run on parallel plates, with visualization by charring as above.

Materials

[1-^{14}C]PUFA (all 50–53 mCi. mmol^{-1} and 99% pure) were obtained from NEN Dupont, Investigación Técnica Inductrial S. A. (ITISA), Madrid, Spain. BHT, silver nitrate and potassium chloride were from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). TLC (20 × 20 cm × 0.25 mm) and HPTLC (10 × 10 × 0.15 mm) glass plates, precoated with silica gel 60 (without fluorescent indicator), were purchased from Merck (Darmstadt, Germany). All solvents were from HPLC grade and were obtained from Fluka Chemical Co. (Glossop, Derbyshire, U.K.).

Statistical analysis

Results are presented as means ± SD of triplicate experiments. The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were arc-sin transformed before further statistical analyses. Differences between mean values for incorporation of radioactivity into total lipids and individual lipid classes were analyzed by one-way ANOVA followed (where appropriate) by a multiple comparison test (Tukey) (Zar 1984).

Table 1. Lipid content and lipid class composition of juvenile gilthead sea bream after starvation for 1 week.

Lipid content (percentage of dry weight)	16.1 ± 3.1
Lipid class composition (percentage of total lipid)	
Total polar lipids	23.6 ± 1.4
Diradyl glycerophosphocholines	10.6 ± 0.5
Diradyl glycerophosphoethanolamines	6.8 ± 0.3
Phosphatidylserine	1.8 ± 0.2
Phosphatidylinositol	1.5 ± 0.2
Phosphatidic acid/Cardiolipin	1.2 ± 0.1
Sphingomyelin	1.2 ± 0.1
Lyso-phosphatidylcholine	0.3 ± 0.0
Sulfatide/Cerebroside	0.3 ± 0.0
Total neutral lipids	76.3 ± 1.4
Cholesterol	8.9 ± 0.4
Free fatty acid	0.7 ± 0.1
Triacylglycerol	58.5 ± 1.5
Steryl ester	8.2 ± 0.2

Data are means ± SD (n = 3). SD = 0.0 implies an SD < 0.05.

Results

Total lipid accounted for 16.1% of the dry body weight with total neutral lipids accounting for over 75% of the total lipid. Triacylglycerol was the predominant neutral lipid (58.5% of total lipid), followed by cholesterol (8.9%) and steryl esters (8.2%) (Table 1). The predominant polar lipid classes were total diradyl (diacyl + alkenylacyl + alkylacyl) glycerophosphocholines (CPL) (10.6% of total lipid) and total diradyl glycerophosphoethanolamines (EPL) (6.8%), followed by phosphatidylserine (PS) (1.8%) and PI (1.5%) (Table 1).

The proportions of n-3 PUFA were greater than those of n-6 PUFA and the level of DHA was higher than that of EPA in all lipid classes studied. Total lipids were rich in monounsaturated fatty acids (39.4%), whereas total polar lipids, CPL, PS

Table 2. Fatty acid compositions of total lipid, total polar lipids and individual glycerophospholipid classes in gilthead sea bream after starvation for 1 week.

Fatty acid	TL	TPL	CPL	EPL	PS	PI
14:0	3.3 ± 0.3	0.7 ± 0.0	1.0 ± 0.2	0.5 ± 0.1	1.3 ± 0.3	2.0 ± 0.5
16:0DMA	0.1 ± 0.0	0.5 ± 0.0	nd	1.5 ± 0.1	nd	nd
16:0	14.6 ± 0.4	17.7 ± 0.1	24.5 ± 1.2	6.8 ± 0.2	6.9 ± 0.9	6.2 ± 0.4
16:1(n-7)	6.4 ± 0.1	2.3 ± 0.1	2.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.5	0.6 ± 0.1
18:0DMA	0.6 ± 0.0	1.2 ± 0.1	nd	3.8 ± 0.1	nd	nd
18:1(n-9)DMA	0.3 ± 0.0	0.7 ± 0.0	nd	2.4 ± 0.1	nd	nd
18:1(n-7)DMA	0.2 ± 0.0	0.2 ± 0.0	nd	0.8 ± 0.0	nd	nd
18:0	4.0 ± 0.2	7.8 ± 0.2	4.0 ± 0.0	10.5 ± 0.3	23.5 ± 1.1	24.0 ± 1.2
18:1(n-9)	17.7 ± 0.7	11.8 ± 0.1	14.4 ± 0.1	7.5 ± 0.2	5.7 ± 0.4	6.5 ± 0.2
18:1(n-7)	3.3 ± 0.2	2.6 ± 0.1	2.4 ± 0.1	2.8 ± 0.0	1.6 ± 0.1	1.3 ± 0.1
18:2(n-6)	6.4 ± 0.1	3.8 ± 0.1	4.4 ± 0.2	1.9 ± 0.1	1.4 ± 0.4	1.2 ± 0.3
18:3(n-3)	0.9 ± 0.2	0.2 ± 0.0	0.7 ± 0.1	1.0 ± 0.2	1.9 ± 0.1	2.0 ± 0.3
18:4(n-3)	1.5 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1
20:1(n-9)	5.7 ± 0.1	2.0 ± 0.1	1.6 ± 0.0	2.5 ± 0.1	1.8 ± 0.2	0.9 ± 0.0
20:1(n-7)	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.8 ± 0.1
20:2(n-6)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.1
20:3(n-6)	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.6 ± 0.1
20:4(n-6)	0.7 ± 0.1	2.1 ± 0.1	1.4 ± 0.0	1.8 ± 0.1	0.6 ± 0.1	7.8 ± 0.5
20:3(n-3)	0.1 ± 0.0	0.1 ± 0.0	tr	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
20:4(n-3)	0.7 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.2	0.2 ± 0.1
20:5(n-3)	5.7 ± 0.1	9.4 ± 0.3	10.7 ± 0.4	6.2 ± 0.2	1.6 ± 0.2	5.4 ± 0.8
22:1(n-11)	4.5 ± 0.2	0.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.2	0.4 ± 0.1	0.1 ± 0.1
22:5(n-6)	0.2 ± 0.0	0.6 ± 0.0	1.2 ± 0.1	1.0 ± 0.0	1.4 ± 0.6	0.6 ± 0.3
22:5(n-3)	2.2 ± 0.1	2.6 ± 0.2	2.0 ± 0.2	3.4 ± 0.1	3.6 ± 0.3	2.9 ± 0.1
22:6(n-3)	11.5 ± 0.9	26.5 ± 0.4	19.6 ± 0.9	33.7 ± 0.9	20.6 ± 2.6	7.8 ± 0.5
Total SFA	23.3 ± 0.9	27.9 ± 0.3	31.8 ± 1.5	21.5 ± 0.5	39.3 ± 0.8	41.0 ± 1.4
Total MUFA	39.4 ± 1.3	19.6 ± 0.3	21.8 ± 0.6	14.0 ± 0.1	10.8 ± 1.0	10.3 ± 0.4
Total PUFA	33.3 ± 0.7	47.8 ± 0.4	42.8 ± 1.5	51.9 ± 1.4	35.6 ± 2.4	31.9 ± 1.6
Total DMA	1.2 ± 0.0	2.7 ± 0.2	nd	8.4 ± 0.2	nd	nd
(n-6)PUFA	9.2 ± 0.2	7.4 ± 0.3	7.7 ± 0.2	5.7 ± 0.2	4.8 ± 0.2	11.3 ± 0.2
(n-3)PUFA	23.6 ± 0.9	40.1 ± 0.4	34.6 ± 1.4	45.9 ± 1.2	29.7 ± 3.2	19.5 ± 1.1

Data are expressed as percentage of weight and represent the mean ± SD (n = 3). SD = 0.0 implies an SD < 0.05. Totals include some other minor components not shown. nd, not detected; tr, trace < 0.1%; CPL, total diradyl glycerophosphocholines; DMA, dimethyl acetal; EPL, total diradyl glycerophosphoethanolamines; MUFA, monounsaturated fatty acid; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; TL, total lipids; TPL, total polar lipids.

and PI contained a high level of saturated fatty acids (Table 2). PUFA comprised 33.3% and 47.8% of the total fatty acids in total lipids and total polar lipids, respectively. The individual glycerophospholipid classes contained between 31.9% and 51.9% PUFA, and were characterized by high levels of DHA, with the exception of PI which contained equal percentages of DHA and AA, each present at 7.8% of the total fatty acids in this class. The percentages of LA exceeded those of LNA in all lipid classes and were particularly high in total lipids and CPL (Table 2). In contrast, the percentages of EPA greatly exceeded those of AA in all lipid classes with the notable exception of PI (Table 2).

Greater percentages of radioactivity from injected ^{14}C-labelled LA, LNA and AA were recovered in body total lipid (all approximately 25%) compared with the percentage of radioactivity recovered from injected ^{14}C-EPA (11.6%) (Table 3). In all cases, less than 0.6% of the total radioactivity recovered in the body lipids was present as free fatty

Table 3. Incorporation of radioactivity from injected ^{14}C-polyunsaturated fatty acids into lipid classes in juvenile gilthead sea bream

	^{14}C-polyunsaturated fatty acid			
	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
Incorporation into total lipid (dpm × 10^{-6}/fish)	1.7 ± 0.5^{a}	1.6 ± 0.1^{a}	1.7 ± 0.1^{a}	0.8 ± 0.2^{b}
Recovery in total lipid	25.1 ± 8.1^{a}	23.9 ± 1.8^{ab}	25.4 ± 2.1^{a}	11.6 ± 4.0^{b}
Recovery in lipid classes				
total polar lipids	36.1 ± 2.3^{a}	28.9 ± 1.0^{a}	64.2 ± 4.2^{b}	51.8 ± 5.8^{b}
glycerophosphocholines	26.4 ± 1.2^{ab}	21.7 ± 0.9^{a}	26.4 ± 2.4^{ab}	29.5 ± 3.7^{b}
glycerophosphoethanolamines	5.1 ± 0.7^{a}	3.6 ± 0.2^{a}	12.8 ± 0.9^{b}	8.2 ± 0.8^{c}
phosphatidylserine	0.9 ± 0.1^{a}	0.7 ± 0.1^{a}	2.4 ± 0.2^{b}	1.8 ± 0.0^{c}
phosphatidylinositol	1.1 ± 0.2^{a}	0.6 ± 0.0^{a}	16.3 ± 1.1^{b}	2.7 ± 0.2^{c}
phosphatidic acid/cardiolipin	1.9 ± 0.3^{a}	1.4 ± 0.4^{ab}	1.4 ± 0.4^{ab}	1.0 ± 0.1^{b}
glycosylglycerides	0.5 ± 0.1^{a}	0.8 ± 0.1^{a}	4.1 ± 0.3^{b}	7.9 ± 1.7^{c}
sphingomyelin	0.3 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.6 ± 0.1^{bc}	0.9 ± 0.1^{c}
total neutral lipids	63.8 ± 2.3^{a}	70.9 ± 1.3^{b}	35.8 ± 4.9^{c}	48.0 ± 4.9^{c}
monoacylglycerol	2.0 ± 0.5^{a}	1.6 ± 0.2^{ab}	4.3 ± 0.1^{bc}	2.9 ± 1.0^{c}
diacylglycerol	1.5 ± 0.1^{a}	2.6 ± 0.6^{ab}	2.5 ± 0.8^{a}	5.7 ± 2.5^{b}
free fatty acids	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.5 ± 0.2^{b}	0.6 ± 0.1^{b}
triacylglycerol	59.2 ± 2.9^{a}	65.7 ± 1.7^{a}	27.7 ± 1.3^{b}	36.5 ± 4.8^{c}
steryl ester	0.9 ± 0.1	0.9 ± 0.2	0.7 ± 0.2	0.9 ± 0.4

Recovery in total lipid is presented as the percentage of injected radioactivity. Recoveries in lipid classes are percentages of the total radioactivity recovered in lipid. All data are means ± SD (n = 3). SD = 0.0 implies an SD < 0.05. Values within a given row not bearing the same superscript letter are significantly different at $p < 0.05$. If no superscript appears, values are not different.

acids, the form in which the precursors were injected. Other than slightly greater recovery of radioactivity from ^{14}C-labelled LNA in total neutral lipids, there were no significant differences between the distribution among the lipid classes of radioactivity from LA and LNA. However, the incorporation of radioactivity from the ^{14}C-labelled C_{20} PUFA into polar lipids was significantly greater than the incorporation of radioactivity from the ^{14}C-labelled C_{18} PUFA (64.2% and 51.8% for AA and EPA, respectively, versus 36.1% and 28.9% for LA and LNA, respectively) (Table 3). In polar lipids, the radioactivity from all four isotopes was predominantly incorporated into CPL (21.7% to 29.5% of total radioactivity incorporated), generally followed by the incorporation into EPL (3.6% to 12.8%) (Table 3). However with ^{14}C-AA, the second-most highly labelled glycerophospholipid class was PI (16.3%). The incorporation of radioactivity from both of the ^{14}C-labelled C_{20} PUFA into EPL was significantly greater than the incorporation of radioactivity from either ^{14}C-labelled C_{18} PUFA into that class (Table 3).

Table 4 shows the percentage distribution of radioactivity from injected ^{14}C-PUFA in specific fatty acid fractions from total body lipids from gilthead sea bream 48 hours after injection. With ^{14}C-LA, 94.4% of the radioactivity present in total lipid fatty acids was recovered as LA itself. Only 5.6% was recovered in all other fatty acid fractions. In comparison, significantly less of the injected ^{14}C-LNA was recovered unmetabolized (88.9%), although the percentage metabolized via the desaturase/elongase pathway was still small with 1.2% recovered in the EPA fraction and only 1% recovered as DHA (Table 4). With ^{14}C-AA, 96.1% of the recovered radioactivity was located in the AA fraction, with only 1.3% in 22:4n-6 and 2.6% in 22:5n-6 (Table 4). As with the C_{18} PUFA, significantly less of the radioactivity from the injected ^{14}C-labelled EPA was recovered unmetabolized (74.6%), with 9.2% and 2.3% recovered in 22:5n-3 and 24:5n-3, respectively, and 9.6% and 4.3% recovered in DHA and 24:6n-3, respectively (Table 4).

Table 4. Metabolism of injected ^{14}C-polyunsaturated fatty acids *via* the desaturase/elongase pathway in juvenile gilthead sea bream

Fatty acid fraction	Polyunsaturated fatty acid injected			
	^{14}C-18:2(n-6)	^{14}C-18:3(n-3)	^{14}C-20:4(n-6)	^{14}C-20:5(n-3)
18:2(n-6)	94.4 ± 0.8	–	–	–
18:3(n-6)	1.8 ± 0.1	–	–	–
18:3(n-3)	–	88.9 ± 0.1	–	–
18:4(n-3)	–	3.5 ± 0.8	–	–
20:3(n-6)	1.9 ± 0.6	–	–	–
20:3(n-3)	–	2.1 ± 0.5	–	–
20:4(n-6)	1.2 ± 0.0	–	96.1 ± 0.2	–
20:4(n-3)	–	2.9 ± 0.1	–	–
20:5(n-3)	–	1.2 ± 0.1	–	74.6 ± 0.6
22:4(n-6)	0.7 ± 0.1	–	1.3 ± 0.1	–
22:5(n-6)	–	–	2.6 ± 0.1	–
22:5(n-3)	–	0.4 ± 0.1	–	9.2 ± 1.0
22:6(n-3)	–	1.0 ± 0.0	–	9.6 ± 0.1
24:5(n-3)	–	tr	–	2.3 ± 0.0
24:6(n-3)	–	tr	–	4.3 ± 0.3

Values represent the radioactivity found in each fatty acid fraction expressed as a percentage of total radioactivity recovered and are means ± SD (n = 3). SD = 0.0 implies an SD < 0.05. tr, trace (<0.1%).

Discussion

The juvenile gilthead sea bream used in the present study were rich in neutral lipids, particularly triacylglycerols, despite starvation for one week, suggesting that the fish were in good condition, at least in respect to general nutrition, prior to the experiment (Fraser *et al.* 1988). The lipid class composition was also very similar to that observed in juvenile turbot (Linares and Henderson 1991). The phospholipid composition of total lipid from the sea bream was very similar to compositions previously reported for fish with CPL and EPL accounting for approximately 45% and 29% of total polar lipids, respectively, with between 8% and 5% of PS, PI, cardiolipin (includes phosphatidic acid) and sphingomyelin (Henderson and Tocher 1987); Linares and Henderson 1991). The distribution of the individual fatty acids among the separate glycerophospholipid classes in total lipid from whole juvenile sea bream, with high 16:0 and relatively low PUFA in CPL, high PUFA in EPL, high 18:0 and PUFA, predominantly C_{22} PUFA, in PS and high 18:0 and C_{20} PUFA, particularly AA, in PI, was similar to that noted previously for sea bream brain lipids (Mourente *et al.* 1992; Mourente and Tocher 1993), and in several previous studies using a variety of fish tissues or cells (Bell *et al.* 1983; Tocher and Sargent 1984; Tocher and Harvie 1988; Linares and Henderson 1991; Mourente *et al.* 1991; Mourente and Tocher 1992). Therefore, based on previous data from many fish species, the lipid class and fatty acid compositions of juvenile gilthead sea bream were not unpredictable.

Both labelled C_{18} and C_{20} PUFA were incorporated into polar lipid in greater proportions (29%–64%) than the proportion of polar lipids (<24%) in total lipid. However, the data clearly show that the selectivity for C_{20} incorporation into polar lipid classes was far greater than that for C_{18} PUFA. The selectivity of the incorporation of C_{20} PUFA over C_{18} PUFA into glycerophospholipids has been observed in fish before both *in vivo* (Linares and Henderson 1991) and *in vitro* with fish cells in culture (Tocher and Sargent 1990a,b). The selective incorporation of specific PUFA into particular glycerophospholipid classes implies specificity in the enzyme mechanisms involved in *de novo*

synthesis and/or the deacylation-reacylation reactions of glycerophospholipid turnover, including CDP-diacylglycerol phosphotransferases and lysophospholipid acyltransferases (Bell *et al.* 1986; Greene and Selivonchick 1987).

The specific incorporation and retention of AA in PI in juvenile sea bream is in agreement with previous analytical studies in both marine (Bell *et al.* 1983; Tocher and Sargent 1984) and freshwater fish tissues (Henderson and Tocher 1987), and incorporation studies including injection of turbot *in vivo* (Linares and Henderson 1991) and *in vitro* studies on plaice, *Pleuronectes platessa,* neutrophils (Tocher and Sargent 1986), rainbow trout, *Oncorhynchus mykiss*, astrocytes (Tocher and Sargent 1990a) and mixed brain cells (Tocher *et al.* 1991), and various fish cell lines (Tocher 1990; Tocher and Dick 1990; Tocher and Mackinlay 1990). In mammals, AA is the precursor of a wide range of highly biologically active derivatives collectively termed eicosaniods (Smith 1989). The weight of evidence indicates that AA is the predominant eicosanoid precursor in both marine and freshwater fish, despite the preponderance of EPA, and n-3 PUFA in general, in fish tissues (Henderson *et al.* 1985; Tocher and Sargent 1987; Henderson and Tocher 1987; Tocher *et al.* 1991). Although no direct evidence exists, it has long been proposed that PI may play a role in the provision of eicosanoid precursor in n-3 PUFA-rich tissues or species (Bell *et al.* 1983; Tocher and Sargent 1984).

Values for the percentage of incorporation of radioactivity in juvenile gilthead sea bream are similar to that found for eel *Anguilla japonica* (Kanazawa *et al.* 1979) and turbot (Linares and Henderson 1991). Similarly, the values obtained for the incorporation of ^{14}C-PUFA into total polar and neutral lipids in the present study with gilthead sea bream were comparable with the values reported in the turbot study (Linares and Henderson 1991). However, in the marine fish species studied by Kanazawa *et al.* (1979), there was no consistent pattern for the distribution of radioactivity between neutral and polar lipids, whereas, in the freshwater species studied, the incorporation into neutral lipids always exceeded that into polar lipids (Kanazawa *et al.* 1979). It was shown recently that the distribution of injected ^{14}C-PUFA between neutral and polar lipids was dependent on the specific tissue analyzed (Olsen *et al.* 1990).

In the present study, LA and LNA were extensively utilized as substrates for esterification enzymes and incorporated mainly into CPL, EPL and triacylglycerol in juvenile gilthead sea bream, although these two fatty acids were not major components of any lipid class. Injected labelled LA and LNA were also extensively incorporated into lipids in juvenile turbot (Linares and Henderson 1991). The percentages of radioactivity recovered in longer chain and/or more unsaturated fatty acids in total lipids of juvenile gilthead sea bream injected with ^{14}C-LA or ^{14}C-LNA were only 5.6% and 11.1%, respectively. This clearly indicates that the conversion of dietary C_{18} PUFA to HUFA in gilthead sea bream is low, or at best very slow. All the desaturases in gilthead sea bream showed greater activity towards the n-3 precursors compared with the equivalent n-6 precursors. The Δ6-desaturase showed reasonable levels of activity with 5.6% of ^{14}C-LA and 9% of ^{14}C-LNA being desaturated. In the case of the Δ4-desaturase, 2.6% of ^{14}C-AA was desaturated to pentaenes, whereas a total of 13.9% of ^{14}C-EPA was desaturated to hexaenes. The Δ5-desaturase activities were the lowest, with only 1.7% and 2.6% of Δ5 products from ^{14}C-LA and ^{14}C-LNA, respectively, obtained. These data indicate that gilthead sea bream fit the pattern suggested for marine fish with low levels of Δ5-desaturase activity necessitating the provision of n-3 HUFA in the diet. The detection of significant amounts of radioactivity associated with 24:5n-3 and 24:6n-3 was consistent with the hypothesis, postulated recently, that apparent Δ4-desaturation of EPA may occur via two elongation steps followed by Δ6-desaturation of the C_{24} pentaene and limited β-oxidation to DHA (Voss *et al.* 1991).

Fish have to be starved for a very considerable period of time in order to deplete the neutral lipid levels. In the present experiment, the sea bream were starved for one week prior to the experiments, and so the tissue reserves of C_{20} and C_{22} PUFA, such as EPA and DHA, will still be significant. Therefore, the sea bream were not deficient in long-chain PUFA for phospholipid synthesis. This could

possible affect the bioconversion of the injected PUFA. However, it is unlikely that neutral lipid reserved of C_{20} and C_{22} PUFA would significantly repress Δ6, Δ5 and Δ4 desaturase activities after a 7 day fast (Brenner 1981). The rates of bioconversion of C_{18} and C_{20} PUFA *via* the desaturase/elongase pathway may be even lower if measured in sea bream on a normal feeding schedule, as was demonstrated in the Arctic charr (*Salvelinus alpinus* L.) (Olsen and Ringo 1992).

The impairment in Δ5-desaturase activity was similar to that found in turbot both *in vivo* (Henderson and Linares 1991) and *in vitro* (Tocher *et al.* 1989; Tocher and Mackinley 1990). Deficiency in the desaturase/elongase pathway enzymes, particulary Δ5-desaturase, has been postulated to be a charcteristic of marine fish (Henderson and Tocher 1987; Sargent *et al.* 1989). Gilthead sea bream display relatively great thermotolerance (10–36°C) and halotolerance (5–60 ppt) capacity (Arias *et al.* 1984; Chervinski 1984), and so it is noteworthy that two quite different species, turbot and gilthead sea bream, displayed the same defect in PUFA metabolism. However, dietary characteristics (carnivorous, omnivorous or herbivorous) may be a major factor determining the capacity of bioconversion of PUFA in different fish species. Unfortunately, the majority of marine fish, or certainly the species studied, are predominantly carnivorous. Similar studies of marine fish species that are more herbivorous, and of freshwater fish that are extreme carnivores, are required to further elucidate this area.

In conclusion, the results of the present study, investigating the metabolism of labelled PUFA *in vivo*, indicate that juvenile gilthead sea bream have only limited ability to convert C_{18} PUFA to HUFA. Therefore, the results also confirm that gilthead sea bream require the provision of preformed EPA, and probably DHA, in the diet. This is consistent with the results from nutritional studies on larvae, post-larvae and fingerling gilthead sea bream. The impairment in the desaturase/elongase pathway was at the level of the Δ5 desaturase. The low activity of Δ5 desaturase combined with the consistent finding that AA is selectively retained in membrane PI suggests that, in addition to EPA and DHA, gilthead sea bream may also have a requirement for preformed AA in the diet.

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