Highly Unsaturated Fatty Acid Synthesis in Marine Fish: Cloning, Functional Characterization and Nutritional Regulation of Fatty Acyl Δ6 Desaturase of Atlantic Cod (*Gadus morhua* L.)

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Abbreviations: FO, fish oil; HUFA, highly unsaturated fatty acids (carbon chain length ≥ C_{20} with ≥ 3 double bonds); ORF, open reading frame; Q-PCR, quantitative real-time polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; VO, vegetable oil.

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ABSTRACT: This study reports the cloning, functional characterization, tissue expression and nutritional regulation of a Δ6 fatty acyl desaturase of Atlantic cod (*Gadus morhua*). PCR primers were designed based on the sequences of conserved motifs in available fish desaturases and used to isolate a cDNA fragment from cod liver, with full-length cDNA obtained by Rapid Amplification of cDNA Ends. The cDNA for the putative desaturase was shown to comprise 1980bp, including a 261bp 5'-UTR, a 375bp 3'-UTR, and an ORF of 1344 bp that specified a protein of 447 amino acids. The protein sequence included three histidine boxes, two transmembrane regions, and an N-terminal cytochrome b_5 domain containing the haem-binding motif HPGG, all characteristics of microsomal fatty acyl desaturases. The cDNA displayed Δ6 desaturase activity in a yeast expression system. Quantitative real time PCR assay of gene expression in cod showed that the Δ6 desaturase gene was highly expressed in brain, followed by liver, kidney, intestine, red muscle and gill, and at much lower levels in white muscle, spleen and heart. The expression of the Δ6 desaturase gene did not appear to be under significant nutritional regulation, with levels in liver and intestine being barely altered in fish fed a vegetable oil blend by comparison with levels in fish fed fish oil. This was reflected in enzyme activity, as hepatocytes or enterocytes showed very little highly unsaturated fatty acid biosynthesis activity irrespective of diet. Further studies are required to determine why the Δ6 desaturase appears to be barely functional in cod under the conditions tested.

Fish are the only major dietary source for humans of n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3) (1) and, with traditional fisheries declining, aquaculture supplies an increasing proportion of the fish in the human food basket (2). However, the current high use of fish oils, derived from feed-grade marine fisheries, in aquaculture feeds is not sustainable, and will constrain growth of aquaculture activities (3,4). Vegetable oils, a sustainable alternative to fish oil, can be rich in C_{18} polyunsaturated fatty acids (PUFA) such as linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, but lack the n-3HUFA abundant in fish oils (5). The extent to which fish can convert C_{18} PUFA to $C_{20/22}$ HUFA varies with species, and is associated with their capacity for fatty acyl desaturation and elongation (6). Marine fish are unable to produce EPA and DHA from 18:3n-3 at a physiologically significant rate (5) due to apparent deficiencies in one or more enzymes in the desaturation/elongation pathway (7,8). Thus, flesh fatty acid compositions in marine fish fed vegetable oils are characterized by increased levels of C_{18} PUFA and decreased levels of n-3HUFA, compromising their nutritional value to the human consumer (9,10).

Until depletion of the commercial stocks in the 1990's, Atlantic cod (*Gadus morhua* L.) was the most valued food fish obtained from the North Atlantic (11). In recent years significant progress has been made in the culture of cod and the life cycle has been closed, allowing production independently of wild fisheries (12). The large and highly developed market for cod along with the high market price and the decreasing quotas set to preserve wild stocks has greatly increased interest in cod culture in recent years (13-15). However, the establishment of large-scale, sustainable cod culture will require solutions to several nutritional issues including broodstock and larval nutrition and replacement of dietary fish oil with alternatives in ongrowing diets (16-18).

Our overall aim is to determine what regulates HUFA biosynthesis in fish and how it can be optimized to enable fish to make effective use of dietary vegetable oil. Recently, fatty acyl desaturases, critical enzymes in the pathways for the biosynthesis of the long-chain $C_{20/22}$ HUFA from shorter chain C_{18} PUFA, have been cloned from several teleosts (19,20). The cDNAs for Δ6

and Δ5 desaturases have been cloned from Atlantic salmon (*Salmo salar* L.) (21,22) whereas, in contrast, only Δ6 desaturase cDNAs have been cloned from marine fish (23,24). The expression levels of both Δ6 and Δ5 desaturases in salmon were shown to be up regulated in fish fed vegetable oil by comparison with levels in fish fed fish oil (25,26). The specific objectives of the study described here were to investigate fatty acid desaturation and the regulation of the HUFA synthetic pathway in Atlantic cod. Thus, we describe the cDNA cloning, functional characterization, and tissue distribution of a fatty acyl desaturase of cod, and report the effects of nutrition on the expression of fatty acyl desaturase and elongase genes in cod fed diets containing either fish oil or a blend of vegetable oils.

MATERIALS AND METHODS

Cloning of putative fatty acyl desaturase from cod. Liver tissue was obtained from Atlantic cod fed for 3 months with the vegetable oil diet described below. Total RNA was extracted from liver using TRIzol® reagent (GibcoBRL, NY, U.S.A.). 3'-RACE cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, U.S.A) primed by the oligonucleotide, Not1polyT, 5'-GATAGCGCCCGCGTTTTTTTTTTTTTTTV-3'. 5'-RACE-cDNA was synthesized using the SMART™ RACE cDNA amplification kit (Clontech, NJ, U.S.A). Available fish desaturase sequences were aligned to enable the design of the degenerate forward primer, 5'-CARCAYCAYGCNAARCCNAA-3' and reverse primer, 5'-RAANARRTGYTCDATYTG-3'. These were used for PCR isolation of a cod desaturase cDNA fragment. PCR amplification was performed using Thermoprime plus DNA polymerase (ABgene, Surrey, UK) under the following touchdown PCR conditions: initial denaturation at 95 ^{o}C for 1 min, 10 cycles of denaturation at 95 ^{o}C for 15 s, annealing at 62 ^{o}C for 30 s (-1.5 ^{o}C /cycle), and extension at 72 ^{o}C for 90 s, then 6 cycles of denaturation at 95 ^{o}C for 15 s, annealing at 52 ^{o}C for 30 s and extension at 72 ^{o}C for 90 s, followed by 24 cycles of denaturation at 95 ^{o}C for 15 s, annealing at 51 ^{o}C for 30 s and extension at 72 ^{o}C for 90 s. The PCR products were cloned into pBluescript KS II^{+} vector (Stratagene, La Jolla,

CA, U.S.A.). The nucleotide sequences were determined by standard dye terminator chemistry using a Perkin Elmer ABI-377 DNA sequencer following the manufacturer's protocols (Perkin Elmer, Applied Biosystems). Following isolation of a cDNA fragment flanked by the above primers, the specific forward primer 5'-GCCGATGAACATAGACCACG-3' was designed for 3' RACE PCR together with primer Not1PolyT under the following conditions: initial denaturation at 95 ^{o}C for 1 min, 25 cycles of denaturation at 95 ^{o}C for 20 s, annealing at 56 ^{o}C for 30 s, and extension at 72 ^{o}C for 2 min. The reverse primer, 5'-GGTGTTGGTGGTGATAGGGCAT-3', was used in conjunction with forward UPM (universal primer mix, long and short), 5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3' and 5'-CTAATACGACTCACTATAGGGC-3', to perform 5'RACE PCR. Amplification involved an initial step at 95 ^{o}C for 1 min and 70 ^{o}C for 3 min, and 5 cycles of denaturation at 95 ^{o}C for 30s, annealing at 60 ^{o}C (-1^{o}C /cycle) for 30s and extension at 72 ^{o}C for 2 min, followed by 24 cycles of denaturation at 95 ^{o}C for 30s, annealing at 56 ^{o}C for 30s and extension at 72 ^{o}C for 2 min. Nucleotide sequences were determined as above and the 3' and 5' RACE PCR fragment sequences aligned to assemble the full nucleotide sequence of the cod putative desaturase cDNA using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, USA).

Heterologous expression of desaturase ORF in Saccharomyces cerevisiae. Expression primers were designed for PCR cloning of the cod putative desaturase cDNA ORF. The forward primer, 5'-CGGAATTCAAGCTTAAGATGGGAGGTGGAGGGCA-3' contained a *HindIII* site (underlined) and the reverse primer, 5'-GCTCTAGACTCGAGTCACTTATGGAGATAAGCATC-3' contained an *XhoI* site (underlined). PCR was performed using high fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) following the manufacturer's instructions. Amplification involved an initial denaturation step at 95 ^{o}C for 2 min, followed by 30 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 58 ^{o}C for 30 s, and extension at 72 ^{o}C for 90 s followed by a final extension at 72 ^{o}C for 10 min. Following PCR, the DNA fragments were restricted and

ligated into the similarly digested yeast expression vector pYES2 (Invitrogen Ltd, Paisley, UK). Ligation products were then used to transform Top10F' *E. coli* competent cells (Invitrogen Ltd, Paisley, UK) which were screened for the presence of recombinants. Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd, Paisley, UK). Selection of yeast containing the desaturase/pYES2 constructs was on *S. cerevisiae* minimal medium (SCMM) minus uracil. Culture of the recombinant yeast was carried out in $SCMM^{-uracil}$ broth as described previously (19), using galactose induction of gene expression. Each culture was supplemented with one of the following PUFA substrates; α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), dihomo-γ-linolenic acid (20:3n-6), docosapentaenoic acid (22:5n-3) and docosatetraenoic acid (22:4n-6). PUFA were added to the yeast cultures at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1 mM (C_{22}) as uptake efficiency decreases with increasing chain length. Yeast cells were harvested, washed, dried, and lipid extracted by homogenization in chloroform/methanol (2:1, by vol.) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant as described previously (19). Fatty acid methyl esters (FAME) were prepared, extracted, purified by thin layer chromatography (TLC), and analyzed by gas chromatography (GC), all as described below. The proportion of substrate fatty acid converted to the longer chain fatty acid product was calculated from the gas chromatograms as 100 × [product area/(product area + substrate area)]. Unequivocal confirmation of fatty acid products was obtained by GC-mass spectrometry of the picolinyl derivatives as described in detail previously (19).

Fish and diets. Atlantic cod were from a stock held at the Institute of Aquaculture and were fed a commercial fishmeal and oil-based diet prior to the trial. Fish with a mean body weight of 87 ± 5 g, were stocked randomly (at 20 fish/tank) into two 1.5 m tanks of 400-L capacity supplied with recirculated seawater (37 ppt) at a constant temperature of 10 ^{o}C at 400 L/h and were subjected to a photoperiod regime of 12-h light:12-h dark. Fish were fed at a rate of 1.5% biomass/day in two portions (9 am and 2 pm) for three months before sampling. The diets were manufactured in-house

and only differed in the oil added to the basal mix (Table 1). In one diet, fish oil (FO) was the only added oil and in the other fish oil was replaced by a vegetable oil (VO) blend containing rapeseed, linseed and palm oils in a 2 : 2 : 1 ratio (Table 2). The diets were formulated to meet all the known nutritional requirements of marine fish (27). The experiment was conducted in accordance with British Home Office guidelines regarding research on experimental animals.

After 90 days, the fish weighed 258 ± 21g and there was no significant difference between weights of fish on different dietary treatments. Twelve fish per dietary treatment were sampled, with six used for the preparation of hepatocytes and enterocytes from liver and pyloric caeca, respectively. Tissue samples were collected from the other six fish, with liver and caecal tissue collected from all six fish, and brain, heart, kidney, gill, spleen, adipose tissue, white and red muscle collected from three of these fish for RNA extraction and gene expression studies, and liver, caeca and white muscle collected from four fish for lipid and fatty acid analyses. All tissue samples were frozen immediately in liquid nitrogen and stored at –80^{o}C prior to further analyses.

Cod tissue RNA extraction and quantitative real time PCR (Q-PCR). Total RNA extraction was performed as described above. Five µg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega UK, Southampton, UK). Gene expression of the fatty acyl Δ6 desaturase and fatty acid elongase (28) genes in tissues from cod fed the different diets was studied by quantitative real-time PCR (Q-PCR). The PCR primers were designed according to the Δ6 desaturase (accession no. DQ054840), and published elongase (accession no. AY660881) cDNA sequences. For the Δ6 desaturase, the forward and reverse primers were 5'-CCCCAGACGTTTGTGTCAG-3', and 5'-CCTGGATTGTTGCTTTGGAT-3', respectively. For the elongase, the forward and reverse primers were 5'-TGATTTGTGTTCCAAATGGC-3' and 5'-CTCATGACGGGAACCTCAAT-3', respectively. PCR products sizes were 181 and 219 bp, respectively. Amplification of cDNA samples and DNA standards was carried out using SYBR Green PCR Kit (Qiagen, Crowley, West Sussex, UK) and the following conditions: 15 min denaturation at 95^{o}C, 45 cycles of 15 s at 94 ^{o}C, 15 s at 55 ^{o}C and

30 s at 72 °C. This was followed by product melt to confirm single PCR products. Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett Research, Cambridge, UK), with expression of target genes quantified and normalized as described previously (29).

Lipid extraction and fatty acid analyses. Total lipids of livers, pyloric caeca, flesh (white muscle) and diet samples were extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01 % BHT as antioxidant (30). FAME were prepared from total lipid by acid-catalyzed transesterification using 2 ml of 1% H_2SO_4 in methanol plus 1 ml toluene (31) and FAME extracted and purified as described previously (32). FAME were separated and quantified by gas-liquid chromatography (Fisons GC8600, Fisons Ltd., Crawley, U.K.) using a 30 m x 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, U.K). Hydrogen was used as carrier gas and temperature programming was from 50°C to 180°C at 40°C/min and then to 225°C at 2°C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (33).

Activity of the HUFA biosynthesis pathway in hepatocytes and enterocytes. Hepatocytes and enterocytes were prepared from livers and pyloric caeca by collagenase treatment of chopped tissue and sieving through 100 μm nylon gauze essentially as described in detail previously for salmonid and sea bass tissues (26,34). Approximately twice as much cod liver tissue was used for digestion due to the high fat content and consequently lower yield of cells (35). Viability, as assessed by trypan blue exclusion, was > 95% at isolation and decreased by less than 5% over the period of the incubation for both cell types. The enriched enterocyte preparation was predominantly enterocytes although some secretory cells are also present. One hundred μl of the hepatocyte and enterocyte suspensions were retained for protein determination according to the method of Lowry *et al*. (36) following incubation with 0.4 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 °C.

Five ml of each hepatocyte or caecal enterocyte suspension were dispensed into 25 cm^2 tissue culture flasks and incubated at 20 °C for 2 h with 0.3 μCi (~ 1 μM) [1-^{14}C]18:3n-3, added as a BSA

complex (37). After incubation, cells were harvested, washed, and lipid extracted as described previously (26), and total lipid transmethylated and FAME prepared as described above. The FAME were separated by argentation (silver nitrate) thin-layer chromatography (38), located on the plate by autoradiography for 14 days, and quantified by liquid scintillation after being scraped from the plates as described previously (39).

Materials. [1-^{14}C]18:3n-3 (50-55 mCi/mmol) was obtained from NEN (Perkin Elmer LAS (UK) Ltd., Beaconsfield, U.K.). Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3) and docosatetraenoic (22:4n-6) acids (all > 98-99% pure) were purchased from Cayman Chemical Co., Ann Arbor, USA. Linoleic (18:2n-6), α-linolenic (18:3n-3), eicosatrienoic (20:3n-6) acids (all >99% pure), collagenase (type IV), FAF-BSA, BHT and silver nitrate were obtained from Sigma-Aldrich Co. Ltd. (Poole, U.K.). TLC (20 x 20 cm x 0.25 mm) plates pre-coated with silica gel 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany). All solvents were HPLC grade and were from Fisher Scientific (Loughborough, UK).

RESULTS

Sequence analyses. The full length of the putative cod desaturase cDNA (mRNA), as determined by 5' and 3' RACE PCR, was shown to be 1980bp which included a 5'-UTR of 261bp and a 3'-UTR of 375bp. Sequencing revealed that the cDNA included an ORF of 1344 bp, which specified a protein of 447 amino acids (GenBank accession no. DQ054840). The protein sequence included all the characteristic features of microsomal fatty acyl desaturases, including two transmembrane regions, three histidine boxes and an N-terminal cytochrome b_5 domain containing the haem-binding motif, H-P-G-G (Fig.1). Phylogenetic analysis comparing the cod desaturase sequence with desaturases from other fish species and human Δ5 and Δ6 desaturases, clustered the cod most closely with tilapia, sea bream and turbot, and more distantly from carp, zebrafish, and salmonids (Fig.2). A pair-wise comparison between fish and human desaturase sequences showed the amino acid sequence predicted by the cod putative desaturase ORF showed greatest identity to that of

gilthead sea bream Δ6 desaturase (82%), and 64 and 56% identity to the human Δ6 and Δ5 cDNAs, respectively (Table 3).

Functional characterization. The fatty acid composition of the yeast transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0 and 18:1n-9, together with the exogenously derived fatty acids (Fig. 3A and C). The most prominent additional peaks were observed in the profiles of transformed yeast grown in the presence of the Δ6 desaturase substrates, 18:2n-6 and 18:3n-3 (Fig.3A-D). Based on GC retention time and confirmed by GC-MS, the additional peaks associated with the presence of the cod desaturase cDNA were identified as 18:3n-6 (Fig.3B) and 18:4n-3 (Fig.3D), corresponding to the Δ6 desaturation products of 18:2n-6 and 18:3n-3, respectively. Approximately, 33.5% of 18:3n-3 was converted to 18:4n-3, and 17.5% of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the cod desaturase. No additional peaks representing desaturated fatty acid products were observed in the lipids of transformed *S. cerevisiae* incubated with 20:3n-6 or 20:4n-3 (Fig.3E and F), indicating no Δ5 desaturase activity. Similarly, the cod desaturase cDNA did not express any Δ4 desaturase activity as evidenced by the lack of additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data not shown).

Fatty acyl desaturase and elongase gene expression in cod tissues. The fatty acyl Δ6 desaturase and PUFA elongase genes were expressed in all cod tissues examined. For the desaturase, the abundance of transcript was clearly greatest in brain followed by liver, kidney, intestine, red muscle and gill, and by much lower levels in white muscle, spleen and heart (Fig.4). The abundance of elongase transcript was very high in brain and gill, with lower levels in kidney, spleen, intestine and heart, and a surprisingly low level of expression in liver.

Effect of diet on fatty acid compositions of flesh, liver and pyloric caeca. The FO diet contained around 25% total saturates, predominantly 16:0, approximately 46% total monoenes, with 26% as the long chain monoenes 20:1 and 22:1, 3.7% n-6PUFA predominantly 18:2n-6, and almost 25% n-3PUFA, with almost 20% as the n-3HUFA, 20:5n-3 and 22:6n-3 (Table 2). The VO diet provided

slightly lower levels of total saturates and monoenes and a similar level of n-3PUFA, but was characterized by increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and decreased proportions of n-3HUFA and long chain monoenes. Therefore, the VO diet showed levels of 18:1n-9, 18:2n-6 and 18:3n-3 of around 26%, 10% and 13%, respectively, whereas the combined level of 20:5n-3 and 22:6n-3 was reduced by half to 10% (Table 2).

The total lipid content of liver was greater than 50% of the wet weight consistent with it being the major lipid storage organ in cod, whereas flesh had a low lipid content of 1% with pyloric caeca containing around 3% total lipid (Table 4). Dietary VO had no significant effect on tissue lipid contents. The rank order of the major fatty acids from total lipid of flesh from cod fed FO was 22:6n-3 > 16:0 > 20:5n-3 > 18:1n-9 > 20:1n-9 (Table 4). The changes in fatty acid composition of the diets in response to replacement of FO with VO, described above, were reflected in the flesh fatty acid compositions with increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3, and decreased proportions of n-3HUFA. Pyloric caeca showed a similar fatty acid composition to flesh with dietary VO having a very similar effect. However, consistent with the higher storage (neutral) lipid content, liver fatty acid composition was characterized by higher levels of monounsaturated fatty acids. Thus, the rank order of the major fatty acids from total lipid of liver from fish fed FO was 18:1n-9 > 16:0 > 20:1n-9 > 22:6n-3 > 22:1 > 20:5n-3 > 16:1n-7 (Table 4). However, substitution of FO with VO resulted in similar changes to liver fatty acid composition as in the other tissues, with increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and decreased proportions of n-3HUFA.

Effects of diet on the HUFA biosynthesis pathway in hepatocytes and enterocytes. Total activity of the HUFA biosynthesis pathway as measured by the recovery of radioactivity in the summed desaturated products of [1-^{14}C]18:3n-3 was around 7-fold higher in enterocytes than in hepatocytes in cod (Fig.5). In both cell types, the rate of synthesis of pathway products was unaffected by dietary treatment. The rate of the HUFA biosynthesis pathway in cod hepatocytes was between 40- and 120-fold lower than that observed in hepatocytes of Atlantic salmon smolts of similar size and assayed at the same time of year and fed similar diets (26) (Fig.6). Similarly, the rate of the

pathway in cod enterocytes was between 8- and 17-fold lower than that observed in salmon enterocytes. Furthermore, in contrast to the situation in cod, the rate of the HUFA biosynthesis pathway in salmon was significantly higher in both cell types from fish fed a VO blend compared to fish fed FO.

Effect of diet on expression of fatty acyl desaturase and elongase genes in liver and intestine. The effect of diet on the normalised expression of the Δ6 desaturase and PUFA elongase genes was determined in liver and intestine (pyloric caeca). The expression of both genes tended to be increased in terms of absolute copy number but the data were characterized by a high degree of variation. Therefore, the only statistically significant effect of diet was increased expression of PUFA elongase in the liver (Fig.7).

DISCUSSSION

The study reported here revealed that Atlantic cod express a fatty acyl desaturase and functional analysis in yeast confirmed the cod enzyme as a Δ6 desaturase. Comparing the protein sequence with that of a range of other desaturases of fish and human showed the cod Δ6 sequence to be more similar to the human Δ6 than to the human Δ5, but most similar to the Δ6 desaturases previously cloned from other marine fish, gilthead sea bream (*Sparus aurata*) and turbot (*Psetta maximus*) (20,22). Phylogenetic analysis of the desaturase sequences reflected classical phylogeny, showing the cod (Paracanthopterygii; Gadiformes) branching from the Acanthopterygia (cichlids, perciformes and pleuronectiformes) line, and further separated from both the carp and zebrafish (Ostariophysi; cyprinids), and salmonids (Salmoniformes; salmonidae) (42). Along with the cloning of Δ6 desaturase cDNAs of sea bream and turbot, the work described has confirmed that marine fish have, and express, the gene required for the first step in the HUFA biosynthesis pathway, Δ6 desaturation, suggesting that any deficiencies in this pathway in marine fish would be at a further step such as chain elongation and/or Δ5 desaturation. This is consistent with biochemical data suggesting deficiencies in these steps in turbot and sea bream cell lines (7,8).

However, despite expressing an apparently active Δ6 desaturase, the activity of the HUFA biosynthesis pathway in both hepatocytes and enterocytes in cod was very low, and considerably lower than the activities measured in salmon hepatocytes and enterocytes (26,43). Indeed, the activities were so low that it was not possible to accurately quantify individual products, although in enterocytes, in which the activity was slightly higher, we were able to confirm that only Δ6 desaturated products (18:4n-3, 20:4n-3 and 22:4n-3) were observed on the autoradiographs. Furthermore, the majority product was 18:4, and so very little HUFA (defined as ≥ C_{20} and ≥ 3 double bonds) was actually produced, and neither EPA nor DHA were detected in cod. In contrast, functional expression in the yeast system had shown that the cod Δ6 had substantial enzymatic activity towards 18:3n-3. This perhaps highlights the known limitations of heterologous expression systems that will be using the endogenous yeast transcription system rather than the cod transcription machinery. It is also clear that it is essential that gene expression at the protein level should also be studied to confirm translation of the Δ6 desaturase gene in cod tissues. However, in the same yeast system, the salmon Δ6 gene gave over 60% conversion of 18:3n-3 (22) compared to 33% for the cod Δ6. Therefore, consistent with the salmon expressing higher HUFA synthesis activities, the salmon Δ6 was more active than the cod Δ6 in a comparative system although this comparison is limited by the considerations mentioned above. Conversely, conversion of 18:2n-6 by the cod Δ6 exceeded that of the salmon Δ6 (18% *vs*. 14%) in the yeast expression system (22). This was interesting as it had previously been shown that the cod PUFA elongase was unique among the fish desaturase and elongase genes functionally characterized so far by being more active towards the n-6 substrate than the equivalent n-3 substrates (28). The reason for the cod genes being relatively more active towards n-6 substrates is unclear.

In addition to being considerably lower than in salmon, the rate of the HUFA biosynthesis pathway observed in cod was even lower than that observed in previous studies in other marine fish. The rate of desaturation of ^{14}C-18:3n-3 to all products in sea bass (*Dicentrarchus labrax*) was around 0.2 pmol.h^{-1}.mg protein^{-1} in hepatocytes and between 0.7 and 1.1 pmol.h^{-1}.mg protein^{-1} in

caecal enterocytes, but this latter figure still represented only 2.1% of total radioactivity recovered, with 97.9% of total radioactivity recovered as the substrate [1-^{14}C]18:3n-3 (34). The activities in cod were around 10-fold lower at 0.02 and 0.15 pmol.h^{-1}.mg protein^{-1} in hepatocytes and enterocytes, respectively. It was perhaps noteworthy that the HUFA biosynthesis activities were higher in enterocytes than in hepatocytes in both sea bass and cod. This may be a real biological difference as intestine is now acknowledged as a site of significant fatty acid metabolism, at least in salmonids (44). However, it is possible that part of that difference may be due to the practical problems in isolating cells from such a lipid-rich tissue as cod liver. Despite using twice as much liver as caeca (in terms of wet weight), the cell yield of hepatocytes was very low and may have been a contributing factor. Problems of low yield from lipid rich livers and also analyzing autoradiography data from very low activity samples (see above) were encountered in the previous study in sea bass (34).

Expression of the cod Δ6 desaturase was greatest in brain followed by liver, kidney and intestine, whereas expression of the PUFA elongase was very high in brain and gill with a low level of expression in liver. In salmon, the expression of both Δ6 and Δ5 desaturases and the PUFA elongase were highest in intestine, liver and brain (22). Mammalian Δ6 and Δ5 desaturases also show relatively high expression in liver and brain, as well as heart and kidney (45,46). A third desaturase gene in humans (FADS3) also shows highest expression in brain, heart and liver (47). In contrast, intestine does not appear to be a site of high desaturase expression in mammals (48). In mouse, a PUFA elongase was expressed to the greatest extent in testis and liver followed by brain and kidney (49). In rat, two elongases were isolated with one, a PUFA elongase (rELO1), being expressed highly in lung and brain whereas the other, responsible for elongating saturated fatty acids (rELO2), was only expressed in liver (50). The tissue expression analysis in cod also showed that expression of PUFA elongase exceeded Δ6 desaturase expression. This was in contrast with the situation in salmon where both Δ6 and Δ5 desaturase expression exceeded expression of PUFA elongase in all tissues (22). This may be real biological difference between cod and salmon, but

there may be other contributing factors. For example, in salmon, there are at least two other desaturase gene sequences in the genome (Zheng, unpublished data). The entire DNA gene sequences for these two desaturases are not known, and the cDNAs have not been isolated, and so the fatty acid specificities, tissue distributions, and hence possible functions, if any, of these desaturases are not known. The function of the third (FADS3) desaturase gene in humans is also unknown at the present time (47).

Mammalian desaturase genes have been demonstrated to be subject to nutritional regulation. The expression of Δ6 desaturase in liver was increased in mice fed triolein (18:1n-9), an EFA-deficient diet, compared to mice fed corn oil, a diet rich in 18:2n-6 (45). Similarly, the expression of both Δ6 and Δ5 desaturases was 4-fold higher in rats fed a fat-free diet or a diet containing triolein compared to that in rats fed either safflower oil (18:2n-6) or menhaden oil (n-3HUFA) (46). Similar results have been obtained in salmonids with dietary linseed oil (rich in 18:3n-3) increasing the expression of liver Δ6 desaturase in rainbow trout, and liver Δ5 desaturase and elongase in Atlantic salmon, compared to levels in fish fed fish oil (20,25). In recent studies, expression levels of both Δ6 and Δ5 desaturases were increased in liver of salmon fed a vegetable oil blend (rich in C_{18} PUFA) compared to levels in fish fed fish oil, but elongase expression was not increased (22,26). In the present study, the expression of Δ6 desaturase in cod liver and intestine was not significantly increased in fish fed vegetable oil compared to that in fish fed fish oil, although expression of the PUFA elongase was increased in liver by dietary vegetable oil. The expression of the genes was generally reflected in the activity of the HUFA biosynthesis pathway and in the fatty acid compositions of the tissues which showed no evidence of any Δ6 desaturase or PUFA elongase activity, with 18:4n-3 and 20:4n-3 levels not maintained, and 18:3n-6 levels only very low and 20:3n-6 not observed at all, in fish fed vegetable oil. Previously, increased desaturase expression was reflected in higher enzyme activities in both mice (45) and salmon (25,26). In the only other study on a marine fish, expression of Δ6 desaturase in sea bream liver was higher in fish fed a

HUFA-free diet compared to that in fish fed a HUFA-rich diet, but activities were not measured in that study (23).

In conclusion, the study described here has demonstrated that Atlantic cod express a fatty acyl desaturase gene, the product of which shows substantial Δ6 desaturase activity in an heterologous yeast expression system. The Δ6 desaturase was highly expressed in brain followed by liver, kidney, intestine and all tissues examined. These data reinforce the impression that the poor ability of marine fish, such as cod, to synthesise HUFA is not due to lack of a Δ6 desaturase, but rather to deficiencies in other parts of the biosynthetic pathway. However, cod hepatocytes and enterocytes showed very little Δ6 desaturase or HUFA biosynthesis activity. The expression of the Δ6 desaturase and PUFA elongase genes did not appear to be under significant nutritional regulation, being generally similar in livers and intestine of fish fed the VO diet compared to fish fed the FO diet, with this reflected in unchanged enzyme activities in hepatocytes and enterocytes. Further studies are required to determine why the Δ6 desaturase appears to be barely functional in cod.

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Legends to Figures

FIG. 1. Comparison of the deduced amino acid sequence of the Δ6 polyunsaturated fatty acyl desaturase from Atlantic cod with that of Δ6 and Δ5 desaturases from Atlantic salmon and human, and the zebrafish Δ6/Δ5 bifunctional desaturase. Deduced amino acid sequences were aligned using ClustalX. Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix and the cut off for shading was 75%. The cytochrome b_5-like domain is dot-underlined, the two transmembrane regions are dash underlined, the three histidine-rich domains are solid underlined and asterisks on the top mark the haem-binding motif, H-P-G-G.

FIG. 2. Phylogenetic tree of cod Δ6 desaturase and desaturases from other fish species (Atlantic salmon, zebrafish, cherry salmon, rainbow trout, seabream, common carp, turbot and tilapia), mammals (mouse and human), fungus (*Mortierella alpina*) and nematode (*Caenorhabditis elegan*s). The tree was constructed using the Neighbour Joining method (40) using *CLUSTALX* and *NJPLOT*. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.

FIG. 3. Functional expression of the Atlantic cod putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*). Panels A and C show the fatty acids extracted from yeast transformed with pYES vector without insert and grown in the presence of 18:2n-6 and 18:3n-3. Panels B, D, E and F show the fatty acid compositions of yeast transformed with pYES vector containing the putative desaturase insert and grown in the presence of 18:2n-6, 18:3n-3, 20:3n-6 and 20:4n-3, respectively. The first four peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are the exogenously added substrate fatty acids, 18:2n-6 and 18:3n-3, respectively. Peaks 6 and 8 in panels B and D were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6, respectively. Peaks 9 and 10 in panels E and F are the exogenously added substrate fatty acids, 20:3n-6 and 20:4n-3, respectively. Vertical axis, FID response; horizontal axis, retention time.

FIG. 4. Tissue distribution of fatty acyl Δ6 desaturase and PUFA elongase genes in Atlantic cod. Transcript (mRNA) copy number was determined by quantitative real-time PCR (Q-PCR) and normalized as described in the Materials and Methods Section. Results are expressed as means ±

SEM (n = 4). L, liver; H, heart; G, gill; WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen; K, kidney.

FIG. 5. Effect of diet on highly unsaturated fatty acid (HUFA) synthesis in hepatocytes and caecal enterocytes after feeding the experimental diets for 90 days. Results are means ± S.D. (n = 4) and represent the rate of conversion ($pmol.h^{-1}.mg\ protein^{-1}$) by desaturation/elongation of [1-^{14}C]18:3n-3 to all desaturated products. There was no significant effect of diet as determined by the Student t-test (41).

FIG. 6. Comparison of highly unsaturated fatty acid (HUFA) synthesis in Atlantic salmon hepatocytes and cod hepatocytes and enterocytes. Presentation of results as described in legend to Fig.5. The salmon data were obtained in salmon smolts of similar size (220g) to the cod in the present trial and assayed at the same time of year (July). The salmon were fed similar FO and VO blend (26). An asterisk denotes a significant effect of diet as determined by the Student t-test ($P < 0.05$) (41).

FIG. 7. Effect of dietary vegetable oil on the expression of fatty acyl Δ6 desaturase and elongase genes in liver and intestine (pyloric caeca) from Atlantic cod. Transcript (mRNA) copy number was determined by quantitative real-time PCR (Q-PCR) and normalized as described in the Materials and Methods Section. Results are expressed as means ± SEM (n = 4). An asterisk denotes a significant effect of diet as determined by the Student t-test ($P < 0.05$) (41).

TABLE 1

Formulations and proximate compositions of experimental diets[a]

	Fish oil	Vegetable oil
Fishmeal[b]	670	670
Soyabean meal[c]	86	86
Wheat[d]	90	90
Vitamin mix[e]	10	10
Mineral mix[f]	24	24
Carboxymethylcellulose	15	15
Fish oil[g]	100	-
Rapeseed oil[h]	-	40
Linseed oil[i]	-	40
Palm oil[j]	-	20
Choline chloride	4	4
Antioxidant mix[k]	1	1
Proximate composition		
Protein	50.0 ± 0.1	50.0 ± 0.2
Lipid	15.7 ± 0.1	15.3 ± 0.1
Ash	10.4 ± 0.1	10.4 ± 0.1
Fibre	1.6 ± 0.2	1.6 ± 0.3
Moisture	12.1 ± 0.0	13.3 ± 0.1

[a]Formulation in $g.Kg^{-1}$. Proximate compositions are percentages of total diet and are means ± S.D. (n=3).

[b]LT94, Norsemeal Ltd., London, UK.

[c]Cargill, Swinderbury, UK.

[d]J.D. Martin, Tranent, UK.

[e]Supplied (per kg diet): KH_2PO_4, 22g; $FeSO_4.7H_2O$, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg.

[f]Supplied (mg/kg diet): ascorbic acid, 1000; myo-inositol, 400; nicotinic acid, 150; calcium pantothenate, 44; all-rac-a-tocopheryl acetate, 40; riboflavin, 20; pyridoxine hydrochloride, 12; menadione, 10; thiamine hydrochloride, 10; retinyl acetate, 7.3; folic acid, 5; biotin, 1; cholecalciferol, 0.06; cyanocobalamin, 0.02.

[g]FOSOL, Seven Seas Ltd., Hull, UK.

[h]Tesco, Cheshunt, UK.

[i]Croda, Hull, UK.

[j]United Plantations Bhd, Jenderata Estate, Teluk Intan, Malaysia.

[k]Dissolved in propylene glycol and contained (g/L): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40.

All the other ingredients were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK.

TABLE 2
Fatty acid composition of experimental diets for Atlantic cod (*Gadus morhua*)[a]

	Fish oil	Vegetable oil
14:0	6.5	2.6
16:0	15.4	14.3
18:0	1.9	2.5
Total saturated[b]	24.7	20.2
16:1n-7[c]	6.0	3.1
18:1n-9	11.7	26.4
18:1n-7	2.6	2.5
20:1n-9[d]	11.1	5.7
22:1n-11[e]	14.9	6.2
Total monoenes	46.4	43.9
18:2n-6	2.7	10.3
20:4n-6	0.5	0.2
Total n-6 PUFA[f]	3.7	10.7
18:3n-3	1.3	13.6
18:4n-3	2.8	0.9
20:4n-3	0.5	0.2
20:5n-3	8.4	4.0
22:5n-3	0.9	0.3
22:6n-3	10.8	6.1
Total n-3 PUFA[g]	24.8	25.1
Total PUFA[h]	28.9	35.9

[a]Results are presented as percentages of total fatty acids by weight and are means of analyses of two samples.
[b]Totals contain 15:0, 17:0, 20:0 and 22:0 at up to 0.4%.
[c]Contains n-9 isomer present at up to 0.1%.
[d]Contains n-11 and n-7 isomers present at up to 0.4%.
[e]Contains n-9 and n-7 isomers at up to 1.0%.
[f]Totals contain 20:2n-6, 20:3n-6 and 22:5n-6 at up to 0.2%.
[g]Totals contain 20:3n-3 present at up to 0.1%.
[h]Contains C_{16} PUFA present at up to 0.4%.

TABLE 3

Identity matrix showing the results of a pair-wise comparison between the amino acid sequences of fish and human fatty acyl desaturases[a]

	Gilthead seabream Δ6	Turbot Δ6	Rainbow trout Δ6	Atlantic salmon Δ6	Atlantic salmon Δ5	Zebrafish Δ6/Δ5	Human Δ6	Human Δ5
Atlantic cod Δ6	82	77	76	75	76	70	64	56
Gilthead seabream Δ6		84	76	76	77	68	65	57
Turbot Δ6			72	72	73	68	62	56
Rainbow trout Δ6				94	92	65	65	57
Atlantic salmon Δ6					91	65	65	58
Atlantic salmon Δ5						64	63	57
Zebrafish Δ6/Δ5							65	56
Human Δ6								61

[a] Percentage of identical amino acid residues

TABLE 4

Total lipid content and fatty acid composition of total lipid from liver, pyloric caeca and flesh (white muscle) of Atlantic cod fed diets containing fish oil (FO) or a vegetable oil blend (VO)[a]

	Liver		Pyloric caeca		Flesh	
Fatty acid	FO	VO	FO	VO	FO	VO
Lipid content	58.3 ± 3.1	52.7 ± 5.2	3.1 ± 0.4	3.2 ± 0.5	1.0 ± 0.2	1.0 ± 0.2
14:0	4.5 ± 0.4	2.2 ± 0.2 *	2.9 ± 0.8	1.6 ± 0.2 *	2.0 ± 0.4	1.0 ± 0.1 *
16:0	14.6 ± 0.7	12.1 ± 0.5 *	17.7 ± 0.6	15.2 ± 0.3 *	18.2 ± 0.8	16.1 ± 0.9 *
18:0	2.6 ± 0.5	2.9 ± 0.1	3.4 ± 0.4	4.0 ± 0.6	3.0 ± 0.2	3.5 ± 0.1 *
Total saturated[b]	22.2 ± 0.8	17.5 ± 0.6 *	24.4 ± 0.5	21.2 ± 0.6 *	23.5 ± 0.7	20.8 ± 0.9 *
16:1n-7[c]	6.6 ± 0.5	3.9 ± 0.4 *	3.2 ± 0.8	2.3 ± 0.4	2.9 ± 0.6	1.6 ± 0.3 *
18:1n-9	18.7 ± 0.7	31.8 ± 1.3 *	11.5 ± 0.6	22.5 ± 2.3 *	11.4 ± 1.8	17.7 ± 1.7 *
18:1n-7	4.1 ± 0.4	3.5 ± 0.1	3.0 ± 0.1	2.7 ± 0.1	2.5 ± 0.2	2.3 ± 0.2
20:1n-9[d]	12.1 ± 0.9	7.1 ± 0.7 *	6.7 ± 1.1	4.6 ± 0.2 *	4.7 ± 1.2	2.9 ± 0.4 *
22:1	8.4 ± 0.5	3.7 ± 0.4 *	5.7 ± 2.0	3.1 ± 0.6	2.6 ± 1.3	1.4 ± 0.2
24:1n-9	0.6 ± 0.1	0.4 ± 0.0	1.9 ± 0.2	1.4 ± 0.3	0.7 ± 0.1	0.6 ± 0.0
Total monoenes	50.5 ± 0.6	50.4 ± 0.7	32.2 ± 4.3	36.7 ± 2.9	24.8 ± 5.0	26.5 ± 2.2
18:2n-6	3.7 ± 1.0	10.7 ± 0.2 *	2.3 ± 0.3	8.6 ± 1.1 *	2.9 ± 0.2	8.8 ± 0.6 *
20:3n-6	0.5 ± 0.3	0.4 ± 0.4	0.2 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.4 ± 0.0	0.2 ± 0.0 *	1.7 ± 0.3	0.9 ± 0.2 *	1.2 ± 0.2	0.8 ± 0.1 *
Total n-6 PUFA[e]	5.1 ± 1.2	11.5 ± 0.5 *	4.7 ± 0.2	10.4 ± 0.9 *	4.7 ± 0.3	10.1 ± 0.6 *
18:3n-3	1.3 ± 0.1	10.9 ± 0.9 *	0.8 ± 0.2	7.4 ± 1.6 *	1.0 ± 0.4	7.3 ± 1.2 *
18:4n-3	2.4 ± 0.1	0.9 ± 0.1 *	1.1 ± 0.3	0.5 ± 0.1 *	1.2 ± 0.3	0.4 ± 0.0 *
20:4n-3	0.6 ± 0.0	0.2 ± 0.0 *	0.5 ± 0.0	0.2 ± 0.0 *	0.5 ± 0.1	0.3 ± 0.0
20:5n-3	7.7 ± 0.6	3.7 ± 0.2 *	11.8 ± 1.0	7.1 ± 0.9 *	13.7 ± 1.9	9.8 ± 0.6 *
22:5n-3	0.9 ± 0.0	0.4 ± 0.0 *	1.2 ± 0.2	0.6 ± 0.1 *	1.5 ± 0.1	1.0 ± 0.1 *
22:6n-3	9.3 ± 1.1	4.5 ± 0.3 *	22.5 ± 3.2	15.2 ± 3.6 *	28.7 ± 3.9	23.0 ± 2.4 *
Total n-3 PUFA[f]	22.2 ± 1.8	20.6 ± 0.9	38.1 ± 3.8	31.2 ± 3.1 *	46.5 ± 4.7	41.8 ± 2.1
Total PUFA	27.3 ± 1.1	32.2 ± 1.1 *	42.8 ± 3.9	41.7 ± 2.2	51.2 ± 4.8	52.0 ± 1.8

[a]Results are expressed as percentage of wet weight (lipid content) and percentage of total fatty acids (fatty acid composition) and are means ± S.D. (n=4). Asterisks denote a significant effect of diet on the fatty acid composition of each tissue as determined by the Student's t-test ($P < 0.05$) (41).

[b]Totals include 15:0 and 20:0 present at at up to 0.3%.

[c]Includes 16:1n-9 at up to 0.2%.

[d]Includes 20:1n-7 at up to 0.4%.

[e]Totals include 18:3n-6, 20:2n-6 and 22:5n-6 present at up to 0.4%.

[f]Totals include 20:n-3 present at up to 0.2%.

FIG.1

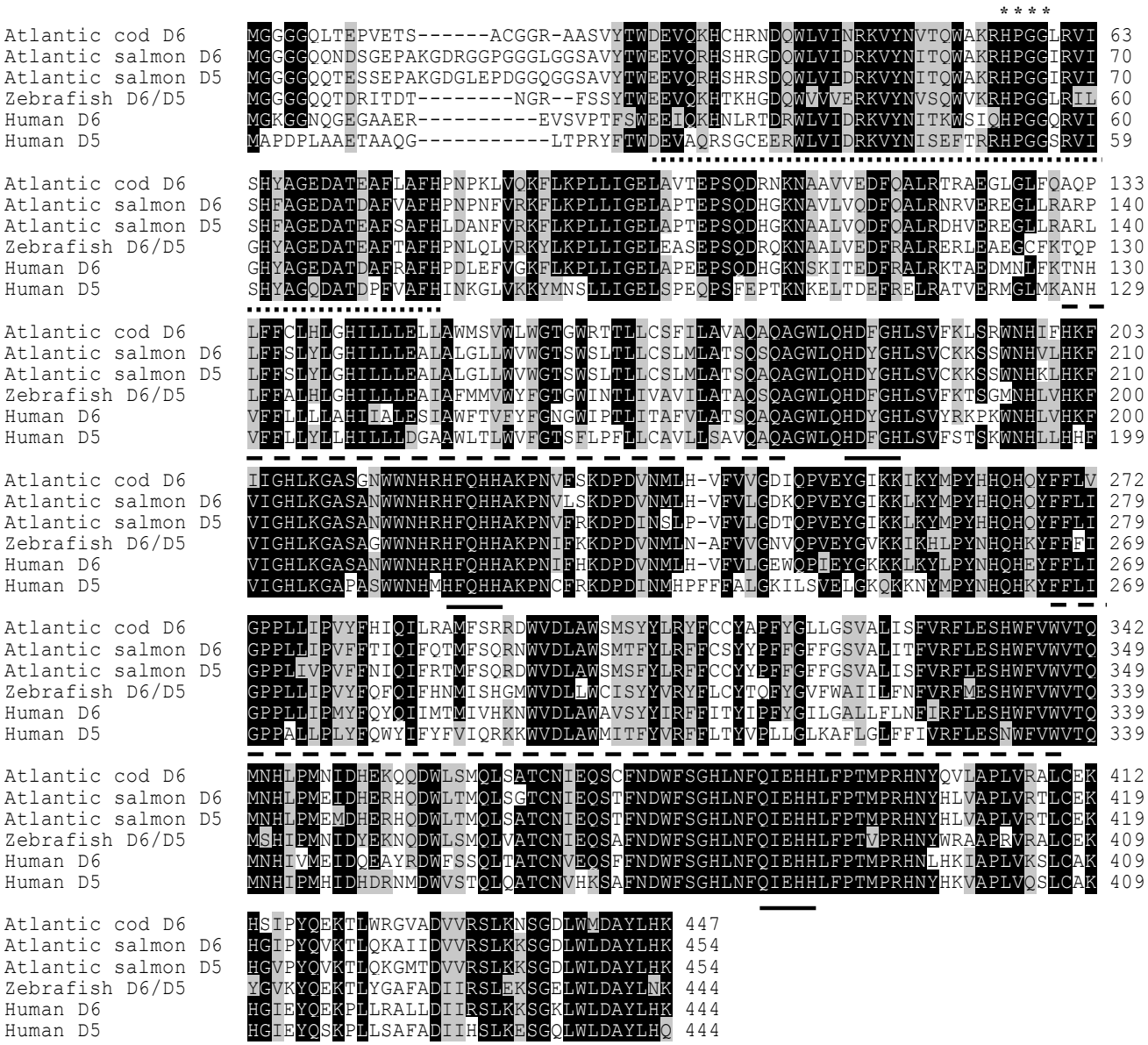

Atlantic cod D6
Atlantic salmon D6
Atlantic salmon D5
Zebrafish D6/D5
Human D6
Human D5

FIG.2

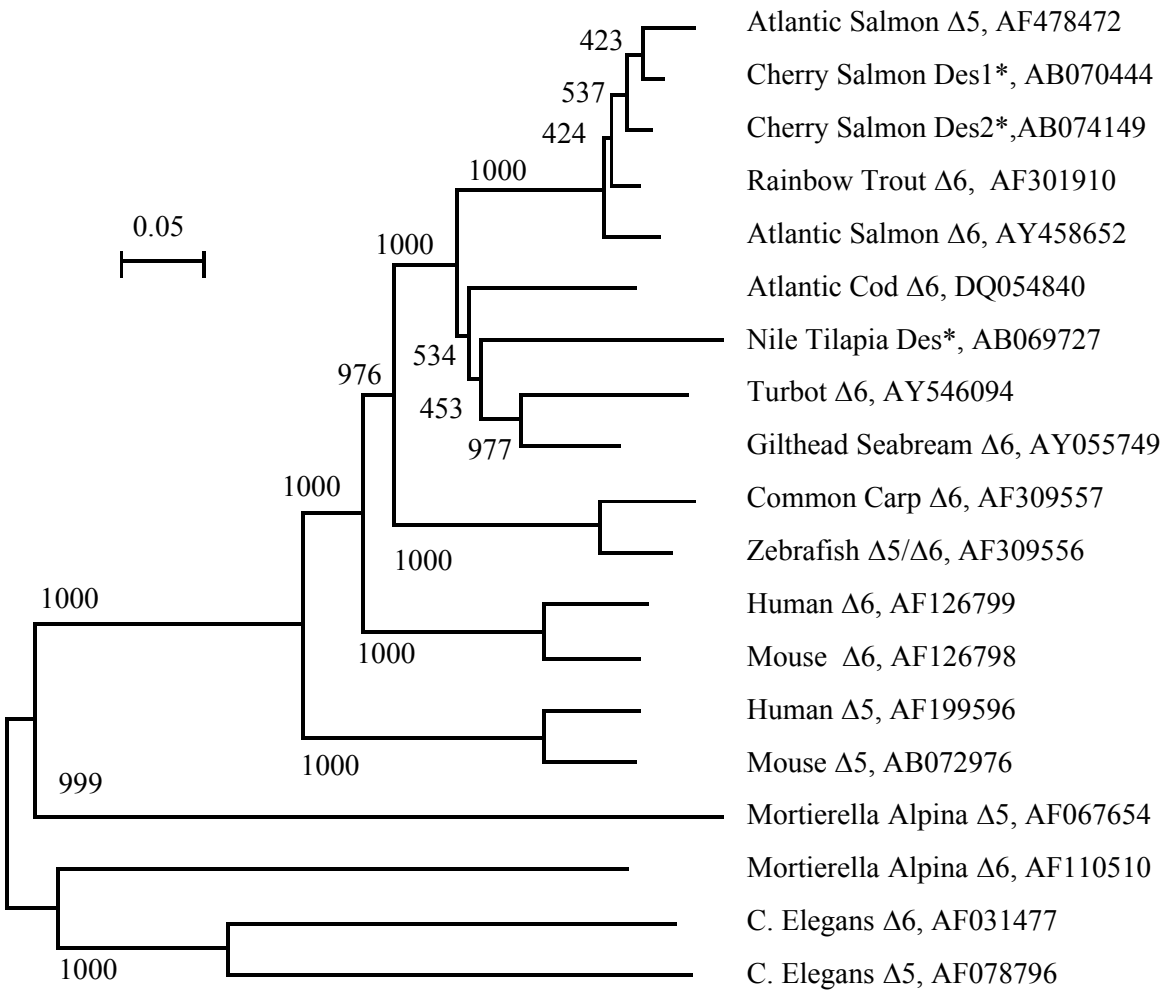
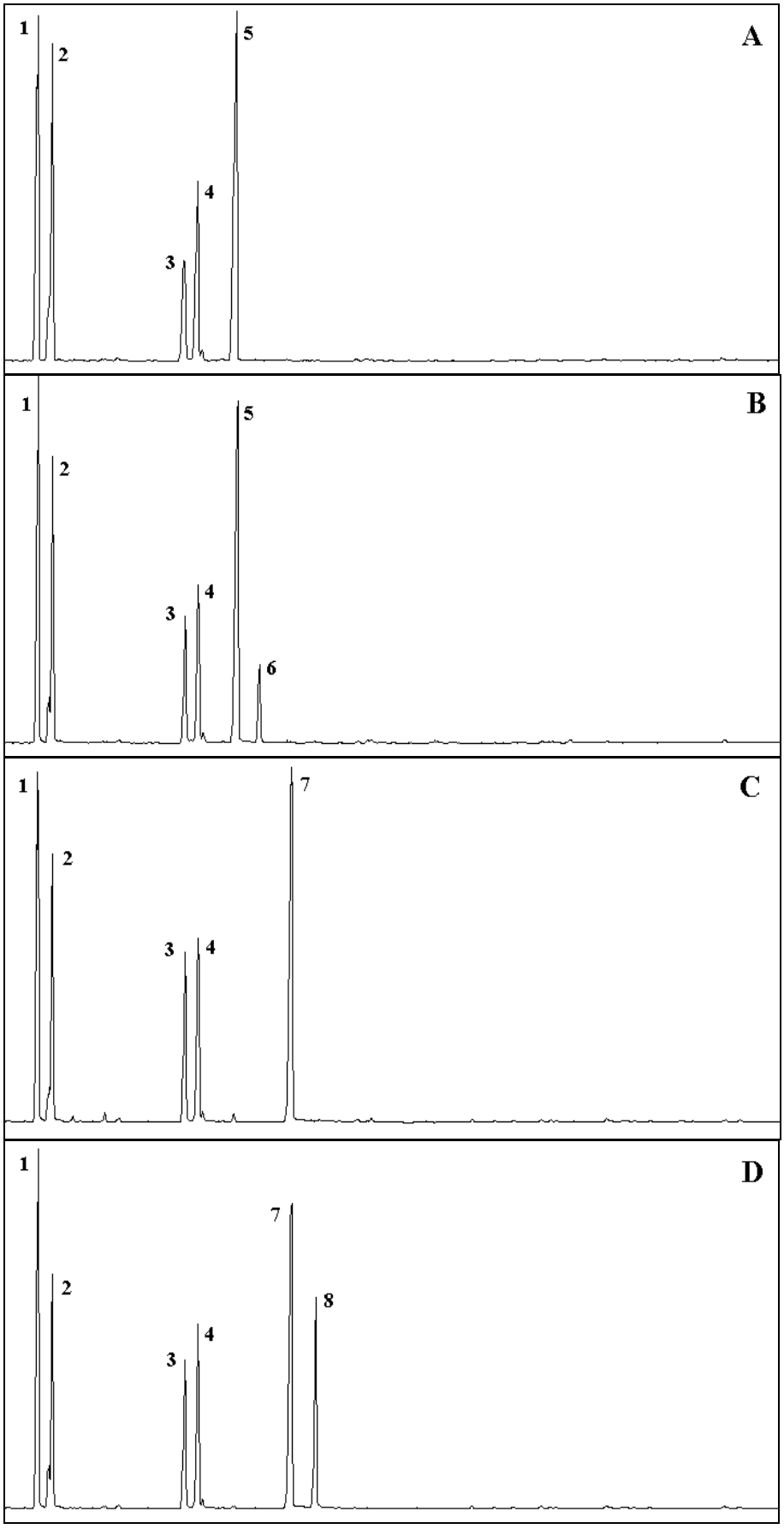

0.05
423
537
424
1000
1000
534
976
453
977
1000
1000
1000
1000
1000
999
1000
Atlantic Salmon Δ5, AF478472
Cherry Salmon Des1*, AB070444
Cherry Salmon Des2*,AB074149
Rainbow Trout Δ6, AF301910
Atlantic Salmon Δ6, AY458652
Atlantic Cod Δ6, DQ054840
Nile Tilapia Des*, AB069727
Turbot Δ6, AY546094
Gilthead Seabream Δ6, AY055749
Common Carp Δ6, AF309557
Zebrafish Δ5/Δ6, AF309556
Human Δ6, AF126799
Mouse Δ6, AF126798
Human Δ5, AF199596
Mouse Δ5, AB072976
Mortierella Alpina Δ5, AF067654
Mortierella Alpina Δ6, AF110510
C. Elegans Δ6, AF031477
C. Elegans Δ5, AF078796

FIG.3



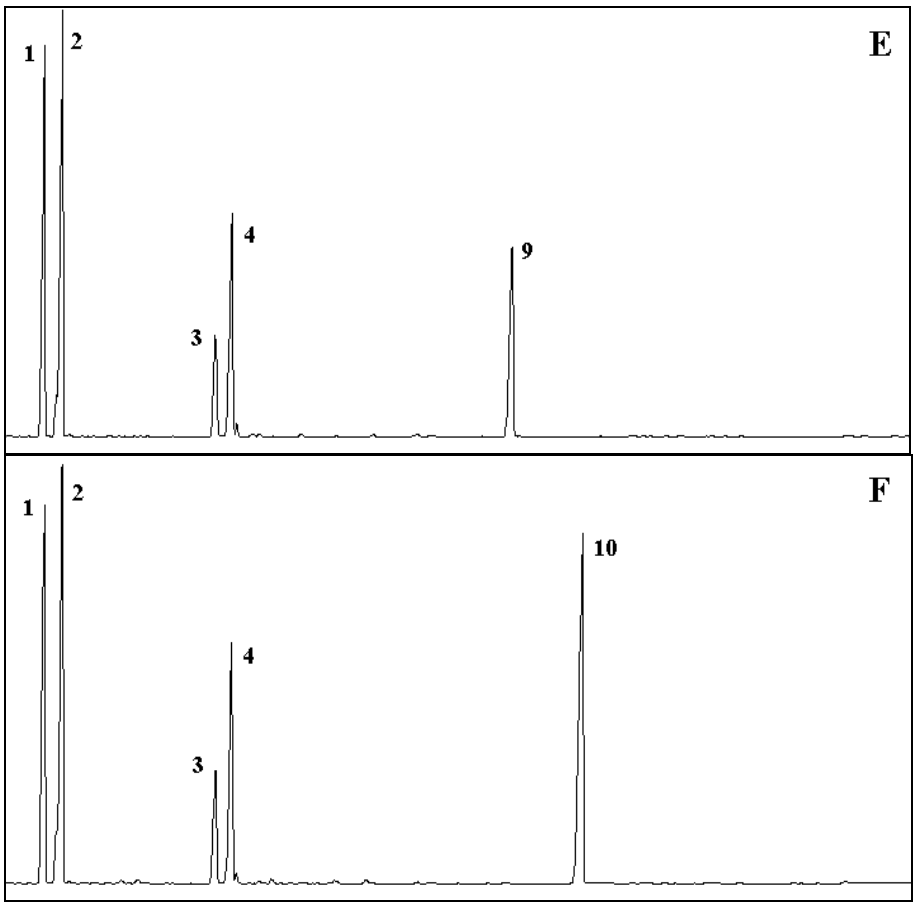

E
1
2
3
4
9
F
1
2
3
4
10

FIG. 4

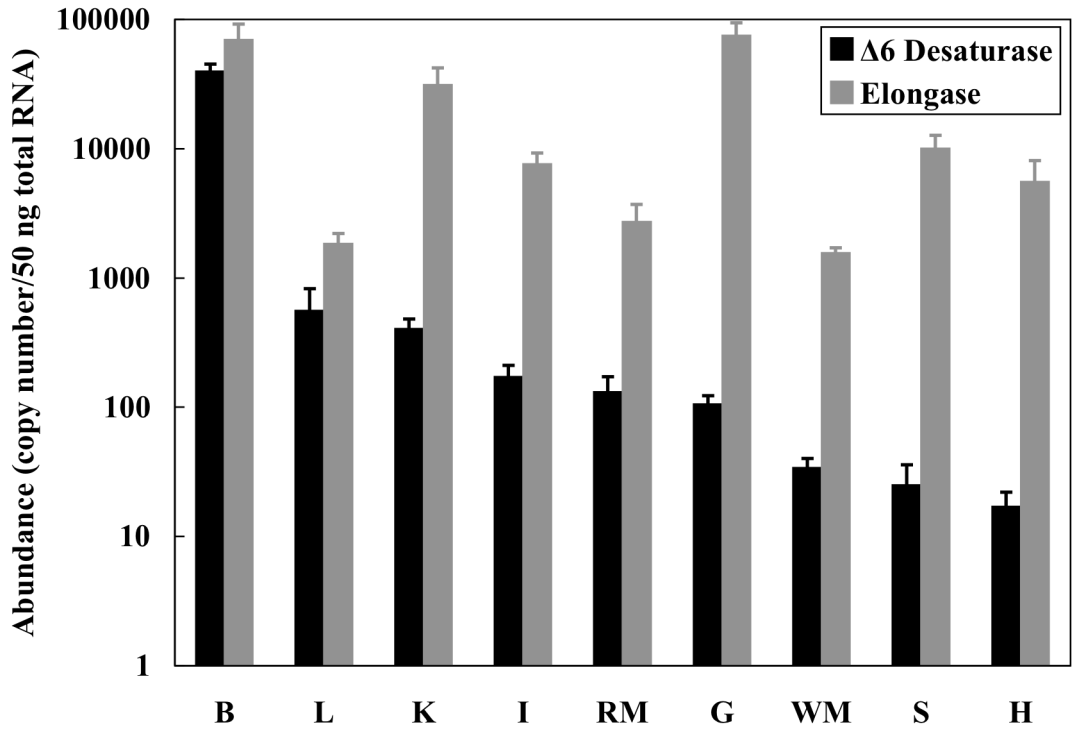

Abundance (copy number/50 ng total RNA)
100000
10000
1000
100
10
1
Δ6 Desaturase
Elongase
B
L
K
I
RM
G
WM
S
H

FIG. 5

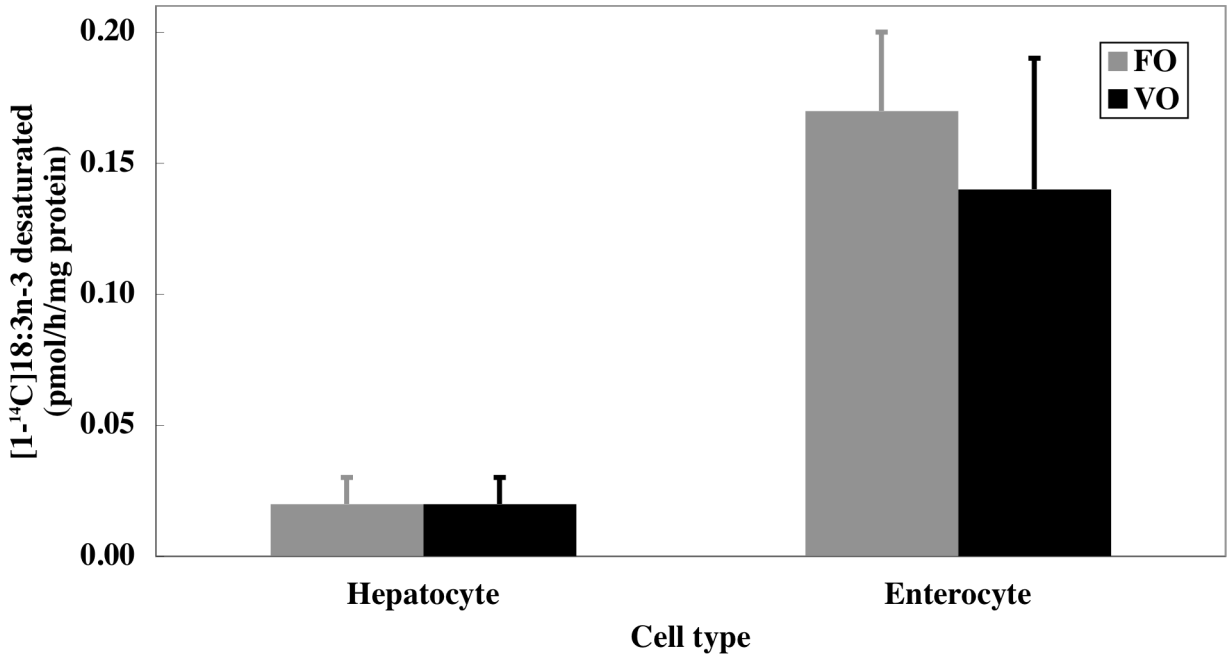

0.20
0.15
0.10
0.05
0.00
[1-14C]18:3n-3 desaturated (pmol/h/mg protein)
FO
VO
Hepatocyte
Enterocyte
Cell type

FIG.6

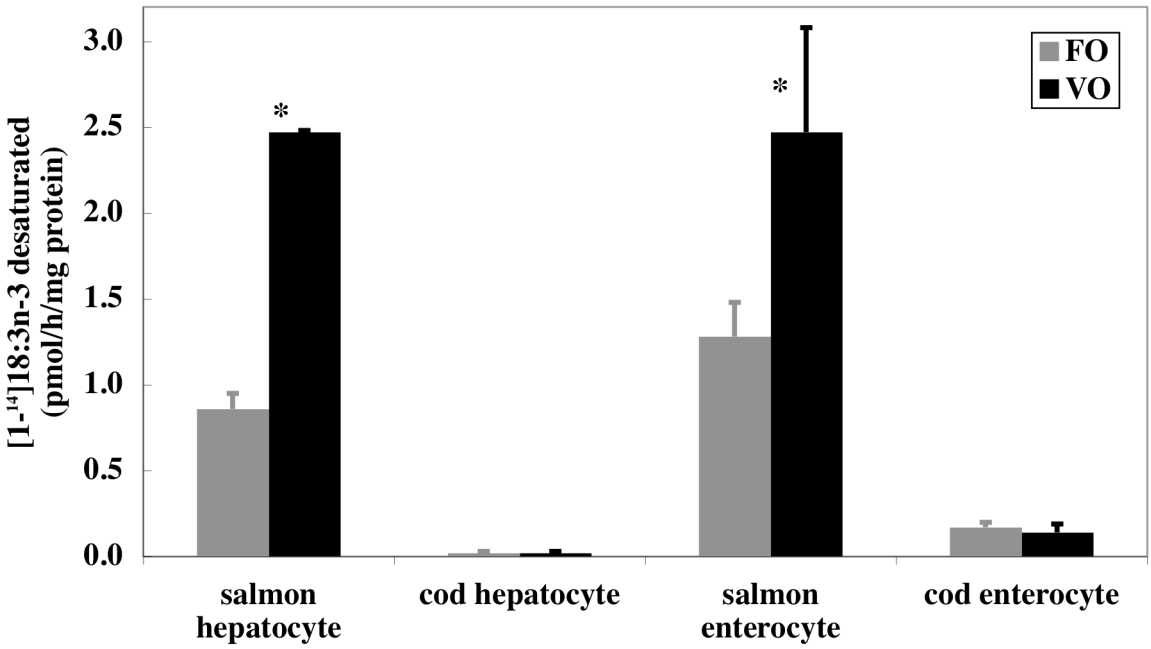
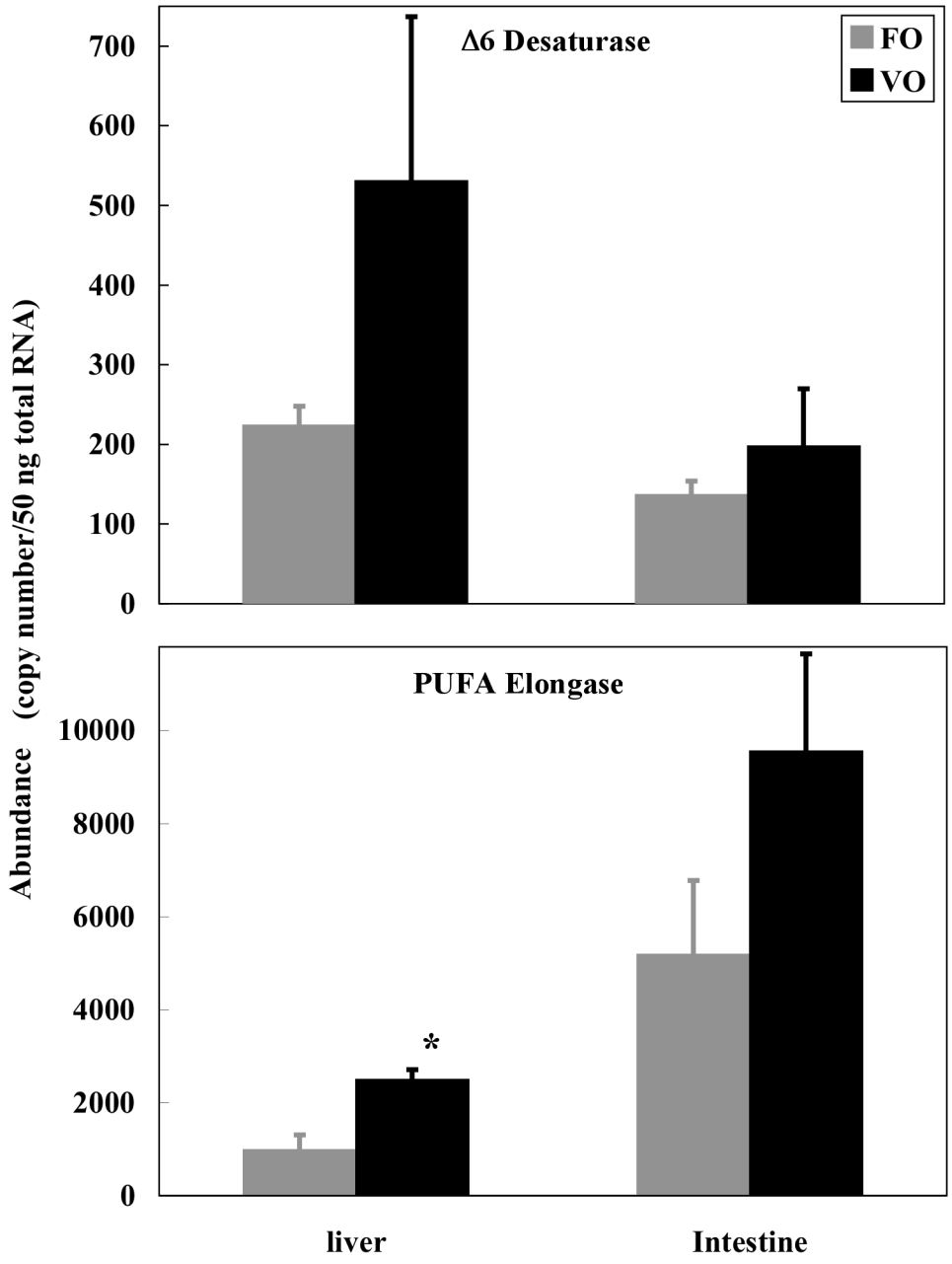

[1-14]18:3n-3 desaturated (pmol/h/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
FO
VO
*
*
salmon hepatocyte
cod hepatocyte
salmon enterocyte
cod enterocyte

FIG.7


Δ6 Desaturase
FO
VO
700
600
500
400
300
200
100
0
PUFA Elongase
10000
8000
6000
4000
2000
0
*
Abundance (copy number/50 ng total RNA)
liver
Intestine