Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish

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Abstract

Fish have an absolute dietary requirement for certain polyunsaturated fatty acids (PUFA) termed "essential fatty acids" (EFA) that include members of both the n-6 and n-3 series typified by linoleic acid, 18:2n-6, and α-linolenic acid, 18:3n-3. However, the biologically active forms of EFA are generally the C_{20} and C_{22} metabolites of 18:2n-6 and 18:3n-3, viz. 20:4n-6, 20:5n-3 and 22:6n-3. Some fish species can convert C_{18} PUFA to the C_{20} and C_{22} PUFA through a series of alternating desaturation and chain elongation reactions mediated by microsomal systems containing elongases and Δ6 and Δ5 fatty acid desaturases. In species that cannot perform these conversions, the C_{20} and C_{22} PUFA themselves are dietary EFA and their C_{18} homologues do not satisfy EFA requirements. The extent to which the foregoing statements apply quantitatively to a given fish species varies widely. Therefore, a vital area in lipid nutrition in fish is the provision of sufficient amounts of the correct EFA to satisfy the requirements for normal growth and development, requirements that can vary quantitatively during the life of the fish and are particularly important factors in larval marine fish. This paper reviews the work on defining and characterising the fatty acid desaturation and elongation pathway in fish. Biochemical studies have been advanced by the use of cell cultures which have elucidated key parts of the pathway. Thus, the presence of the so-called Sprecher shunt, where 22:6n-3 is produced from 20:5n-3 through two successive elongations and a Δ6 desaturase followed by peroxisomal chain shortening, was demonstrated in trout. Similarly, the block in the pathway in marine and/or piscivorous fish could be due to either a deficiency of $C_{18\text{-}20}$ elongase or Δ5 desaturase and this varies between different marine species. Recent work has focussed on the molecular biology of the pathway with the cloning of fatty acid desaturases and elongases from a variety of fish species. Zebrafish have been used as a model species and a unique desaturase possessing both Δ6 and Δ5 activity along with an elongase with very high $C_{18\text{-}20}$ activity have been cloned and characterised. Understanding this pathway is of increased importance due to the current dependence of salmonid and marine fish aquaculture on fish oil, the supply of which is becoming increasingly limited and unsustainable, necessitating the use in fish feeds of sustainable plant oils, rich in C_{18} PUFA, but devoid of C_{20} and C_{22} PUFA.

Introduction

Lipid nutrition of fish is a subject that has received enormous attention in the last 10 years (see Sargent et al. 2002). In particular, much work has focussed on the optimal requirements and functional roles of polyunsaturated fatty acids (PUFA) during larval and early developmental stages of marine fish (Sargent et al. 1999). However, the study of lipid and fatty acid biochemistry of larval fish, especially marine larval fish, is hampered by their very small size. This can place a significant limitation on the amount of material available for study. Of course, the small size of larvae can be compensated, in some instances by numbers, particularly if the enzymes and/or metabolic pathways can be effectively studied in homogenates or some other similar preparation of whole animals. However, it is often far more illuminating to study specific organ, tissue or subcellular fractions and in these cases the considerable practical problems of dissecting large numbers of very small animals through a binocular microscope can be prohibitive. One alternative is to use larger animals. This usually requires the use of older animals such as juveniles and this can be acceptable in some circumstances where the developmental stage of the fish or the ontogeny of the enzyme systems or metabolic pathways is not a major issue. However, a further alternative is to go even smaller, by studying the pathways at a cellular or molecular level.

This paper describes the utilization of both cell culture systems and molecular techniques in the study of the genes, enzymes and metabolic pathways of lipid and fatty acid metabolism in fish. The advantages (and disadvantages) of utilizing cell culture systems in metabolic studies are described and the types of data that can be obtained are illustrated through studies performed in our own laboratory over the last 5-6 years. The aims of these studies were to elucidate the PUFA desaturation and elongation pathway in salmonids, and the nature of the deficiency in the pathway in marine fish, and the metabolic pathway behind the metabolism of 18:5n-3 in fish. Recently, molecular studies have begun to elucidate the genetics of these processes through the cloning and characterisation of the genes involved which will enable further studies of their expression and regulation.

Cell culture studies

Fish cell culture is long-established and many cell lines are available commercially and from various research laboratories around the world. Fish cell culture has mainly been developed

over the years as a diagnostic tool in pathology particularly in the area of virology where the cell lines offer a range of host cells for diagnosis, characterisation and research into therapies. However, cell lines have been used extensively and very successfully in metabolic studies in the mammalian field. Similarly, several years ago, we decided to utilize a variety of cell culture systems, including established cell lines as model systems in our studies investigating lipid and especially fatty acid metabolism in fish.

Advantages of cell cultures

In these studies, cell culture systems offered three main advantages over studies employing whole fish. These can be summarised as control, containment and cost. Firstly, environmental conditions can be controlled easily and very precisely in cell culture systems. Temperature can be controlled simply by adjusting the temperature controller of the incubator and/or by having incubators at different temperatures. Thus, studies investigating both acute and chronic effects of temperature can be performed very easily and in a variety of ways (Tocher and Sargent 1990a). Similarly, the osmolality of the medium can be adjusted easily, at least in the case of increased salinity, by the addition of appropriate amounts of sodium chloride to the medium, as may be required with cell cultures from marine fish (Tocher et al. 1988). We have performed studies in this way to investigate the effects of increasing salinity changes on lipid and fatty acid compositions in an Atlantic salmon (*Salmo salar*) cell line (AS) (Tocher et al. 1994, 1995a). Osmolality below the normal level found in most commercial media preparations (~300 $mOsm.kg^{-1}$) is a little more difficult but possibly of much less interest in any case. However, the medium and associated supplements supply all the nutrition to the cells, and so studies into the effects of nutrients can also be performed with relative ease. There are a considerable number of different media formulations and supplements commercially available from which to choose. As with salinity above, it is easier to look at additional nutrient supplements to the cells and these can be added in high purity and in various forms and concentrations. Removal of specific nutrients may be more difficult if they are normal components of cell culture media formulations although it is entirely possible, albeit slightly more time consuming, to formulate your own medium.

Cell cultures also offer the advantage of containment. This could include the use of radioisotopes for metabolic tracer studies, potentially hazardous or toxic chemicals such as carcinogens in toxicology studies, and pathogenic or infectious micro-organisms. Containment is primarily achieved through the use of tissue culture flasks that offer sufficient

protection even if used vented, but can be used unvented if an appropriate medium such as Leibovitz L-15, which does not contain bicarbonate buffer and thus does not require exposure to a CO_2 atmosphere, is utilized. To list cost as an advantage of cell culture may be surprising to some but this is certainly a major factor to include. Some capital expenditure is required but this can be tailored somewhat to both specific requirements and budget. Ideally, a dedicated cell culture laboratory with sealed floors and walls, single purpose sink areas, air conditioning and separate areas for media preparation, primary culture preparation and subculture would be desirable but not essential. A vertical laminar air flow cabinet, a cooled incubator, an inverted microscope and a dedicated fridge-freezer set aside in a dedicated area of a larger laboratory are probably the minimum requirements. This represents no more than moderate capital expenditure. Consumables, including media, sera, other reagents and disposable plasticware (flasks, pipettes, centrifuge tubes and vials/containers) are not cheap but save considerable time, a vital factor when man-power is the single most expensive item in the research budget. Perhaps the most important factor in assessing the cost-effectiveness of cell culture is the huge cost of the alternative. Studies with fish require aquaria, with all the associated costs of water supply and purification, fish and feed costs and, of course, husbandry staff. In addition, some studies would be very much more difficult to perform with fish. Studies on temperature effects require aquaria to be maintained at non-ambient temperatures and thus require heating or cooling of the water and/or the room. Work with radioisotopes is extremely difficult with whole fish particularly when ^{14}C is used due to the possibility of production and release of $^{14}CO_2$ into the atmosphere. Containment is similarly a problem when using toxins or pathogens and in all these cases it adds to the costs of performing experiments with fish.

Problems of using fish cell cultures in metabolic studies

The use of cell cultures is not, however, without its own problems. The first of these is temperature. For the majority of fish cell lines the optimum growth temperature is in the 20 - 25 ^{o}C range. These include cell lines from Atlantic salmon (AS), rainbow trout (*Oncorhynchus mykiss*) (RTG-2, RTH) and turbot (*Scophthalmus maximus*) (TF) which are all routinely cultured at 22 ^{o}C. However, the normal ambient temperature in U.K. waters for these species of fish would rarely exceed 15 ^{o}C, a temperature we routinely use as a "holding" temperature, to slow the growth of the cells during periods when they are not being actively used in experiments. Culture at 10 ^{o}C or below usually results in unacceptably low

growth rates even in cell lines from these cooler water fish. Therefore, fish cell lines such as those above are being cultured at a temperature higher than normal, a situation that does not occur in mammalian cell culture. In contrast, other fish species, such as Mediterranean fish including gilthead sea bream (*Sparus aurata*), would normally experience water temperatures in the low 20's and thus cells derived from them (SAF-1) would not be at an unusually high temperature when cultured at 20 – 25 ^{o}C. These are particularly important points to be aware of in relation to temperature adaptation/acclimation studies where the lower temperature, say 10 ^{o}C, actually represents a more normal temperature for some cell lines and 22 ^{o}C could be regarded as a stressed temperature, whereas in other cell lines the opposite would be true.

A second problem with the use of fish cell cultures is one of particular importance in relation to lipid and fatty acid studies. Cell culture media are normally devoid of fatty acids and so cells in culture generally derive all their lipid and fatty acids from the lipid contained in the serum supplement, which is an almost ubiquitous supplement due to its various properties including promotion of attachment, growth and proliferation of the cultured cells. Fetal bovine serum (FBS), the predominant serum supplement used in cell culture including fish cell culture, is relatively rich in PUFA and for mammalian cells, FBS provides a sufficient amount and balance of n-6 and n-3PUFA. In contrast, although the total amount of PUFA is adequate, fish cells grown in FBS display lower percentages of n-3PUFA and are enriched in n-6PUFA in comparison with fish tissues (Tocher et al. 1988). This has important consequences when cultured fish cells are used in studies of fatty acid metabolism. We have used two approaches to solve this problem. Firstly, we investigated the possibility of producing fish cell lines that can grow and proliferate in the absence of serum. To date, we have found one cell line, EPC-EFAD, derived from the carp (*Cyprinus carpio*) epithelial papilloma line, EPC, that can survive and proliferate in essential fatty acid-deficient (EFAD) medium (Tocher et al. 1995b). The EPC-EFAD line has now been growing continually in EFAD medium for over 7 years and 130 passages although the rate of proliferation is lower than the parent EPC line. This cell line is virtually devoid of n-6 and n-3PUFA but contains appreciable amounts of n-9PUFA (Tocher and Dick 2001) and thus does not represent a model system for fish normally although they have been useful in studies on the effects of EFA deficiency on fatty acid metabolism in freshwater fish (Tocher and Dick 1999, 2000, 2001). An alternative solution is to reduce the serum added to the medium and to supplement with a mix of pure fatty acids designed to restore the fatty acid composition of the cells to that of the original tissue in the fish. For instance, primary cultures of turbot brain astroglial

cells established in medium containing FBS contained increased proportions of 18:1(n-9), and total n-9 and n-6 PUFA, and greatly reduced n-3PUFA in comparison with turbot brain. Supplementation with a mixture of 5 μM 20:5n-3 and 25 μM 22:6n-3 acids for 4 days significantly increased the percentages of these acids in total cellular lipid of trout and turbot astrocytes and restored the n-3PUFA composition of the cells to that found in brain (Bell et al. 1994; Tocher et al. 1996).

A final caveat to the use of cell cultures in metabolic studies relates to interpretation and extrapolation of the results. It is obvious that cell cultures are not whole animals. Many factors important in controlling and regulating metabolism are simply not replicated in the cell culture systems. Complex multi-cell type organ structure is difficult to replicate in cell culture and even most tissue specific features such as 3D-structure, orientation and sidedness are lost in culture and, in addition, the cells themselves may be dedifferentiated (as in cell lines) and of changed morphology. Nonetheless, many features of inherent intracellular biochemistry and metabolism will be retained by cells in culture and provided the researcher is aware of the limitations then cell cultures provide a very useful additional experimental tool. Cautious extrapolation to the whole animal is possible particularly when the cell data are entirely consistent with other available data and, particularly, whole animal data, but ultimately whole animal studies are required for final confirmation.

Types of cell culture systems

Different types of cultured cell systems can be utilized to fit the particular requirements of the studies. In our own studies we have used three types, the first of which is short-term cultures, where the cells are attached to the substrate (plastic), but there is no growth or division over the time-course of the experiment, around 2 – 24 h (Buzzi et al 1996, 1997). The major benefit of these cultures is that the cells retain their differentiated phenotype. The retention of differentiated phenotype is also the aim with primary cultures that are attached, and grow and divide over a much longer period of time, ranging from days to weeks (Tocher and Sargent 1990b). Depending upon the cell type, some limited subculture of primary cultures may be possible but not always. Established cell lines are immortal, growing and dividing at infinitum with routine subculture necessary to maintain the cells in optimum condition (Tocher et al. 1988). The down side of cell lines being that they are usually de-differentiated,

possessing either fibroblast or epithelial morphology. The following sections describe the use of some of these cell cultures as model systems to investigate specific aspects of fatty acid metabolism in fish.

Determining the PUFA desaturation/elongation pathway in trout

All vertebrates, including fish, lack Δ12 and Δ15 (ω3) desaturases and so cannot form 18:2n-6 and 18:3n-3 from 18:1n-9. Therefore, 18:2n-6 and 18:3n-3 are essential fatty acids in the diets of vertebrates. These dietary essential fatty acids can be further desaturated and elongated to form the physiologically essential C_{20} and C_{22} PUFA, 20:4n-6, 20:5n-3 and 22:6n-3 (Fig.1). With one exception the reactions occur in the microsomal fraction of the liver and the same enzymes act on the n-3 and the n-6 fatty acid series. Originally the insertion of the last, Δ4, double bond in 22:6n-3 was assumed to occur through direct Δ4 desaturation of its immediate precursor 22:5n-3. However, Howard Sprecher and coworkers showed that in rat liver, the 22:5n-3, is further chain elongated to 24:5n-3 which is then converted by Δ6 desaturation to 24:6n-3 which is then converted, by a chain shortening reaction in the peroxisomes, to 22:6n-3 (Sprecher 1992; Sprecher et al. 1995).

Whether the production of 22:6n-3 in fish involved Δ4 desaturation of 22:5n-3 or Δ6 desaturation of 24:5n-3 with chain shortening of the resultant 24:6n-3 to 22:6n-3 was investigated in our laboratory by Buzzi et al. (1996, 1997). The cell system chosen was primary hepatocytes prepared by collagenase perfusion of intact, isolated liver from rainbow trout fed a n-3PUFA-deficient (olive oil) diet to stimulate the PUFA desaturation pathway. These cells were maintained in short-term culture for up to 24h. Incubation of hepatocytes for 3h with [1-^{14}C]18:3n-3 or [1-^{14}C]20:5n-3, added as complexes with fatty acid-free bovine serum albumin, resulted in the recovery of large amounts of radioactivity as 22:6n-3 with only traces of radioactivity recovered in C_{24} PUFA (Table 1). In contrast, when liver microsomes were incubated for 3h with the same radioactive fatty acids, no radioactivity was recovered in 22:6n-3, but substantial amounts of radioactivity were recovered in 24:5n-3 and 24:6n-3 (Table 1). These data suggested that the pathway as proposed by Sprecher for rat liver also occurred in trout liver. Incubation of the trout hepatocytes with [1-^{14}C]24:5n-3 resulted in radioactivity being recovered in both 22:6n-3 and 24:6n-3 (Table 2). Similarly, incubation of trout hepatocytes with [1-^{14}C]24:6n-3 resulted in the recovery of radioactivity

in 22:6n-3 (Table 2). Thus, the experiments with primary hepatocytes prepared from rainbow trout had provided data consistent with the fact that the production of 22:6n-3 in trout occurred through the so-called "Sprecher shunt". Thus, 20:5n-3 is elongated by two sequential steps to 24:5n-3 which is then desaturated by a Δ6 desaturase to 24:6n-3, all in the microsomes, and that this intermediate is then chain shortened to 22:6n-3 at an extra-microsomal site, presumably peroxisomes (Buzzi et al. 1996,1997). While all the steps in the pathway from 18:3n-3 to 22:6n-3 in Fig.1. have so far been established for fish only in rainbow trout hepatocytes, there is accumulating evidence that the same pathway occurs in primary hepatocytes from Atlantic salmon (Tocher et al. 1997), Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*) (Tocher et al. 2001a), zebrafish (*Danio rerio*), tilapia (*Oreochromis niloticus*) (Tocher et al. 2001b), and carp cells in culture (Tocher and Dick 1999). Cell studies were unable to resolve whether the same Δ6 fatty acid desaturase catalysed each of these steps or whether different Δ6 desaturases (isoenzymes) were involved for the C_{18} and C_{24} PUFA (see later).

Determining the deficiency in the PUFA desaturation/elongation pathway in marine fish

It had been known for some time that the EFA requirements of freshwater and marine fish are qualitatively different, as in rainbow trout 18:3n-3 alone can satisfy the EFA requirement, with 18:2n-6 only required for optimal growth, whereas in the most studied marine species, turbot, the longer chain PUFA 20:5n-3 and 22:6n-3 are required. This suggested a difference in the fatty acid desaturase/elongase activities, and it was subsequently shown that this *in vivo* difference was also present in cultured cell lines (Tocher et al. 1989). Initial studies involving supplementation of turbot cells (TF) in culture, compared to both rainbow trout cells (RTG-2) and Atlantic salmon cells (AS), with various n-3 and n-6 PUFA had shown that the apparent deficiency in the desaturase/elongase pathway in turbot was either in the C_{18} to C_{20} elongase (C_{18-20} elongase) multi-enzyme complex or the the fatty acyl Δ5 desaturase step (Tocher et al. 1989). Defective C_{18-20} elongase appeared the more likely of the two alternative based on (i) the ability of turbot cells to produce 20:4n-6 when supplemented with 20:3n-6, which bypasses the elongase and indicated the presence of some Δ5 desaturase activity, (ii) the accumulation of 18:4n-3 and 18:3n-6 in cells supplemented with 18:3n-3 and 18:2n-6, respectively, and (iii) the accumulation of 18:2n-9, and not 20:2n-9 or 20:3n-9, in cells cultivated in the absence of EFA. However, results from *in vivo* injection studies with other marine fish species such as gilthead sea bream were more consistent with a deficiency in Δ5

desaturase activity (Mourente and Tocher 1994). Therefore, as the situation in marine fish was unclear, and as a deficiency in the fatty acid elongase activity responsible for the conversion of C_{18} to C_{20} PUFA had not been reported in any other animal or cell line, we aimed to establish unequivocally the location of the defect in the desaturase/elongase pathway in marine fish using the established cell lines, AS, TF and SAF-1. Each of these cell lines was incubated for 4 days with various ^{14}C-labelled n-3PUFA that were the direct substrates for individual enzymic steps in the desaturation/elongation pathway (Ghioni et al. 1999; Tocher and Ghioni 1999). Thus, 18:3n-3 was the direct substrate for Δ6 desaturase, 18:4n-3 was the direct substrate for C_{18-20} elongase, 20:4n-3 was the substrate for Δ5 desaturase and 20:5n-3 was the substrate for C_{20-22} elongase (Table 3). The data in Table 3 show the percentage of radioactivity recovered as the products of each enzymic step. Thus, the results showed that all three cell lines had substantial Δ6 activity as 76%, 82% and 66% of radioactivity from
[1-^{14}C]18:3n-3 was recovered as Δ6 desaturated products in AS, TF and SAF-1 cells, respectively. However, both marine cell lines showed very reduced C_{18-20} elongase activity compared with AS cells. However, whereas the SAF-1 cell line showed virtually no Δ5 desaturase activity, the TF cell line showed considerable Δ5 activity (Table 3). All cell lines showed similar levels of C_{20-22} activity. Thus the primary deficiency in the PUFA desaturation/elongation pathway in gilthead sea bream cells was established to be at the level of Δ5 desaturase whereas the only deficiency observed in the TF cells was at the C_{18-20} elongase. The SAF-1 cell line may also show a deficiency in C_{18-20} elongase but it is possible that the virtual absence of Δ5 activity results in the accumulation of 20:4n-3 which inhibits C_{18-20} elongase through a feedback mechanism. Irrespective of which enzyme step was deficient, the cell line data was entirely consistent with earlier feeding studies and in vivo studies indicating that marine fish were unable to produce significant amounts of 20:5n-3 and 22:6n-3 from 18:3n-3.

Determining the metabolism of 18:5n-3 in fish

Octadecapentaenoic acid (all-*cis* 18:5n-3) is a fatty acid characteristically present in certain algal groups in marine phytoplankton, including dinoflagellates, haptophytes and prasinophytes, all of which have important roles in the marine ecosystem (Sargent et al. 1995). 18:5n-3 is usually co-associated in these organisms with 22:6n-3. Given that

biosynthesis of 22:6n-3 involves peroxisomal chain shortening of its precursor 24:6n-3, it is possible that 18:5n-3 is biosynthesized by chain shortening of 20:5n-3. However, marine zooplankton and fish ingesting phytoplankton contain little or no 18:5n-3 demonstrating that this fatty acid is readily metabolized by marine animals. It could be completely catabolized by marine animals by β-oxidation but it may also be directly chain elongated to 20:5n-3.

In this study, [U-^{14}C]18:4n-3 and [U-^{14}C]18:5n-3 were prepared from the haptophycean alga *Isochrysis galbana* cultured in sodium ^{14}C-bicarbonate, and their metabolism studied in cultured cells from turbot (TF), sea bream (SAF-1) and Atlantic salmon (AS) that differ in their abilities to perform C_{18} to C_{20} elongation reactions. The rationale being that the TF cell line's deficiency in C_{18} to C_{20} fatty acid elongase would perhaps help to differentiate between the two possible pathways for the metabolism of 18:5n-3 in fish as suggested above. Incubation of the cell lines with both labelled 18:4 and 18:5 showed two remarkable features (Table 4). Firstly, no radiolabelled 18:5 was ever detected in any of the three cell lines, even when labelled 18:5n-3 was incubated with the cells and even in short incubations of less than 1h. Secondly, the pattern of distribution of radioactivity was identical for both fatty acids, that is the recovery of radioactivity in different fatty acid fractions after incubation with [U-^{14}C]18:5 was identical to the distribution of radioactivity after incubation with [U-^{14}C]18:4 (Table 4). Indeed, the pattern only varied between the cell lines based upon the differences in their PUFA desaturation/ elongation pathways. The one difference between incubation with 18:4 and 18:5 was that the quantitative recovery of radioactivity was significantly lower with 18:5n-3. These results showed that 18:5n-3 was not metabolised in fish cells by chain elongation to 20:5n-3. In retrospect, this was perhaps unsurprising as, unlike 18:4n-3, 18:5n-3 is not a normal intermediate in the desaturation/elongation pathway (Fig.1). However, 18:5n-3 is a normal intermediate in the pathway for the β-oxidation of 20:5n-3 (Fig.2). In contrast, 18:4n-3 is not an intermediate in the PUFA β-oxidation pathway although the first step in the β-oxidation of 18:4n-3, dehydrogenation, results in the formation of *trans* Δ2, all-*cis* Δ6,9,12,15-18:5 (2-*trans* 18:5n-3) (Fig.2). The 2-*trans* 18:5n-3 intermediate is also produced by the action of a Δ^3, Δ^2-enoyl-CoA-isomerase acting on 18:5n-3, this enzyme being the next step in the β-oxidation pathway after the production of 18:5n-3. Thus, 2-*trans* 18:5n-3 is a common intermediate in the β-oxidation of both 18:4n-3 and 18:5n-3. It appeared therefore that 18:5n-3 incorporated into the fish cells was treated as a β-oxidation intermediate by the fish cell lines resulting in the production of 2-*trans* 18:5n-3 in amounts which probably

exceeded the capacity of the β-oxidation pathway. This resulted in the reversal of the dehydrogenase step and production of labelled 18:4n-3 (Fig.2) which was then metabolised as normal via the desaturation/elongation pathway producing labelled 20:4n-3 and 20:5n-3 (Fig.1). A proportion of the 2-*trans* 18:5n-3 proceeded down the β-oxidation pathway resulting in the overall lower recovery of radioactivity when the cells were incubated with 18:5 compared to cells incubated with 18:4. To further test this hypothesis, cells were also incubated with either 18:5n-3 or 2-*trans* 18:5n-3, and similar mass increases of 18:4n-3 and its elongation and further desaturation products occurred in cells incubated with 18:5n-3 or 2-*trans* 18:5n-3. We therefore concluded that 18:5n-3 was readily converted biochemically to 18:4n-3 via a 2-*trans* 18:5n-3 intermediate generated by a Δ^3, Δ^2-enoyl-CoA-isomerase acting on 18:5n-3 and, therefore, that 2-*trans* 18:5n-3 was implicated as a common intermediate in the β-oxidation of both 18:5n-3 and 18:4n-3 (Ghioni et al. 2001).

Molecular studies

Very recently, molecular biological and genetic techniques have begun to be applied to lipid and fatty acid metabolism in fish in order to elucidate the genetics of the above pathways through the cloning and characterisation of the genes involved enabling further studies on the expression and regulation of the genes. These techniques have particular advantages when applied to larvae. Firstly, the small size of fish larvae presents no problem in the preparation of RNA and/or cDNA even if tissue-specific RNA is required as relatively little tissue is required. The larval RNA/cDNA can not only be used in routine gene expression studies through conventional Northern blotting or real-time PCR but can also be used for cloning genes expressed specifically in larvae. In addition, modern in-situ hybridisation techniques can also be used to locate organ- and tissue-specific gene expression and are equally, or indeed more, able to be applied to larvae as to larger fish. The above cell culture studies have demonstrated the great significance of PUFA desaturase and elongase enzymes in fish. Several questions still remained though including a) was there one or two different Δ6 desaturases (isoenzymes) for the desaturation of C_{18} and C_{24} PUFA, and b) what were the precise defects in Δ5 desaturase and $C_{18\text{-}20}$ elongase in marine fish (Tocher et al. 1998). The following sections describe our current studies aimed at cloning and characterising PUFA desaturase and elongase genes in fish.

Cloning and characterisation of PUFA desaturase genes in fish

A zebrafish EST sequence (Genbank accession no. AI497337) was identified that displayed high homology to mammalian Δ5 and Δ6 desaturase genes. Thus, cDNA was synthesized from zebrafish liver total RNA using reverse transcriptase and a portion of this cDNA was then subjected to PCR amplification with appropriate primers predicted from the zebrafish EST sequence. The products were cloned into the pYES2 plasmid, and nucleotide sequences determined. The 1590 bp open reading frame of the zebrafish cDNA encoded a protein with substantial similarity to vertebrate Δ6 desaturases. Overall amino acid identities were 64% to human Δ6 desaturase and 58% to human Δ5 desaturase (Hastings et al. 2001). In addition, the zebrafish protein contained a similar N-terminal cytochrome b_5-like domain and the three catalytically important histidine boxes conserved in all members of the desaturase gene family and believed to be involved in catalysis. When the zebrafish cDNA was expressed in the non PUFA-producing yeast *Saccharomyces cerevisae* it conferred the ability to convert linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) to their corresponding Δ6 desaturated products, 18:3n-6 and 18:4n-3 (Table 5). However, in addition, it conferred on the yeast the ability to convert di-homo-γ-linoleic acid (20:3n-6) and eicosatetraenoic acid (20:4n-3) to arachidonic acid (20:4n-6) and eicosapentanoic acid (20:5n-3), respectively, indicating that the zebrafish gene encoded an enzyme having both Δ6 and Δ5 desaturase activities (Table 5). The enzyme was more active towards n-3 and Δ6 substrates compared to n-6 and Δ5 substrates. This was the first report of a functionally characterized polyunsaturated fatty acid desaturase enzyme of fish, and the first report of a fatty acid desaturase in any species with both Δ6 and Δ5 activities. Recently, we have shown that the zebrafish desaturase has no Δ4 desaturase activity but was able to desaturate 24:5n-3 to 24:6n-3 suggesting that a single Δ6 desaturase may be responsible for the desaturation of both C_{18} and C_{24} substrates (Table 5).

Further PUFA desaturase genes with homology to the zebrafish desaturase and vertebrate Δ6 desaturase genes in general have been cloned from fish. Genes from carp, Atlantic salmon and cod have been cloned in our own laboratory and other putative desaturase genes have been cloned from cherry salmon (*Oncorhynchus masou*), tilapia, sea bream and rainbow trout (Seilez et al., 2001). Most of these genes remain to be functionally characterised but preliminary data has suggested that the Atlantic salmon gene also has both Δ6 and Δ5 activities with the latter being greater. Phylogenetic analysis indicated that, with respect to

other functionally characterized genes, the zebrafish sequence had highest homology with mammalian Δ6 desaturases, with human Δ5 desaturase appearing to be distinct from the Δ6 desaturase sequences (Fig.3). All the fish genes clustered together. Although more fatty acid desaturase genes may be found in zebrafish, salmon and mammals, it is conceivable that the bi-functional desaturase described here is a component of a prototypic vertebrate PUFA biosynthetic pathway that has persisted in freshwater fish species. That humans and other mammals have two distinct enzymes for Δ5 and Δ6 desaturation may be an adaptation to a terrestrial diet providing lower amounts of pre-formed C_{20} and C_{22} PUFA than the diets of a vertebrate ancestor that they share with freshwater fish. Functional divergence of the products of a putative ancient gene duplication event is a possible mechanism underlying adaptation to such a dietary change.

Cloning and characterisation of PUFA elongase genes in fish

Fatty acid elongation, the addition of 2-carbon units, is effected in four steps each catalysed by a specific enzyme. The first step is a condensation reaction of the precursor fatty acyl chain with malonyl-CoA to produce a β-ketoacyl chain that is then hydrogenated in three successive steps. The condensation step is widely regarded as the "elongase", and the one that determines the substrate specificity and is rate limiting. *Mortierella alpina* elongase (GLELO) amino acid sequence cDNA encoding a PUFA elongase was used to probe *in silico* for related sequences in the Genbank EST database. This identified mammalian, chicken, Xenopus and zebrafish ESTs. Consensus PCR primers were designed in conserved motifs and used to isolate full length cDNA from livers of several fish species using the rapid amplification of cDNA ends (3" and 5"RACE) strategies to clone full length elongase cDNAs of zebrafish, carp, salmon and turbot (AF465520). The amplified cDNAs encoded putative open reading frames (ORFs) of 291-295 amino acids whose sequences were highly conserved among the fish species and with other vertebrate elongases. The fish elongase polypeptides have up to 7 predicted transmembrane (TM) domains, a canonical endoplasmic reticulaum retention signal, and several potential phosphorylation sites which may be important in regulation of enzyme function. Expression of the zebrafish gene in the yeast *S. cerevisiae* demonstrated that the ORFs encoded a fatty acid elongase with substrate specificity ranging from the monounsaturated fatty acid palmitoleic acid (16:1n-7) to the long chain highly unsaturated fatty acid, 22:5n-3. The zebrafish elongase activity was in the rank

order $C_{18-20} > C_{20-22} > C_{22-24}$ and was more active towards n-3 substrates than n-6 substrates (Table 6). Recently, functional characterisation of the salmon and turbot elongases has revealed that they have similar specificities to the zebrafish enzyme with the rank order for overall activity being zebrafish > salmon > turbot. The turbot enzyme was relatively more active towards the C_{20} substrates than C_{18} substrates compared to the zebrafish and salmon enzymes. However, it was particularly interesting that the turbot gene coded for a functionally active protein. This was not contradictory to the cell culture data as, although the deficiency in the desaturation/elongation pathway appeared to be at the C_{18-20} elongase step in TF cells, there was activity present. The sequence data suggested another possibility for low C_{18-20} elongase activity in TF cells as the Kozak sequence (which marks the following methionine codon as the start codon) in the turbot cDNA is a poor signal for initiation of translation and turbot elongase was less efficient than zebrafish and salmon elongases particularly for C_{18} substrates.

Conclusions

The use of a variety of cell culture systems has greatly advanced biochemical studies which have in turn elucidated key parts of the PUFA desaturation and elongation pathway in fish. The presence of the so-called Sprecher shunt, where 22:6n-3 is produced from 20:5n-3 through two successive elongations and a Δ6 desaturase followed by peroxisomal chain shortening, was demonstrated in primary hepatocytes isolated from trout. Similarly, studies on established cell lines revealed that the block in the pathway in marine and/or piscivorous fish was due to either a deficiency of C_{18-20} elongase or Δ5 desaturase and this varied between different marine species. Current work is focussing on the molecular biology of the pathway with the cloning of fatty acid desaturases and elongases from a variety of fish species. Zebrafish have been used as a model species and a unique desaturase possessing both Δ6 and Δ5 activity and an elongase with very high C_{18-20} activity have been cloned and characterised. The zebrafish desaturase was capable of desaturating both C_{18} and C_{24} Δ6 substrates. Understanding this pathway is of increased importance due to the current dependence of salmonid and marine fish aquaculture on fish oil, the supply of which is becoming increasingly limited and unsustainable, necessitating the use in fish feeds of sustainable plant oils, rich in C_{18} PUFA, but devoid of C_{20} and C_{22} PUFA (Sargent et al. 2002).

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Table 1. Desaturation of [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 by hepatocytes and liver microsomes from rainbow trout fed an (n-3)-deficient diet. Results are expressed as a percentage of total radioactivity recovered in specific fatty acids in polar lipids and are means ± SD (n=3). Based on data taken from Buzzi et al. (1996).

Fatty acid	Hepatocytes	Microsomes
[1-^{14}C]18:3n-3		
18:3	22.5 ± 0.8	52.7 ± 4.5
20:3	3.7 ± 0.5	19.0 ± 4.9
22:3	1.1 ± 0.1	3.0 ± 1.0
24:3	1.2 ± 0.1	1.9 ± 0.2
18:4	8.9 ± 0.7	9.0 ± 0.3
20:4	4.6 ± 1.7	0.3 ± 0.0
22:4	1.5 ± 0.2	1.6 ± 0.2
20:5	15.3 ± 3.6	6.0 ± 1.1
22:5	4.8 ± 1.0	1.5 ± 0.1
24:5	trace	2.7 ± 0.4
22:6	36.4 ± 7.5	trace
24:6	trace	2.4 ± 0.6
[1-^{14}C]20:5n-3		
20:5	39.7 ± 0.7	57.8 ± 1.0
22:5	10.1 ± 0.5	13.8 ± 2.1
24:5	4.3 ± 0.4	4.7 ± 0.1
22:6	45.9 ± 0.7	trace
24:6	trace	23.6 ± 1.2

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Table 2. Metabolism of [1-^{14}C]24:5n-3 and [1-^{14}C]24:6n-3 by hepatocytes from rainbow trout fed an (n-3)-deficient diet. Results are means ± SD (n=3). Based on data taken from Buzzi et al. (1997).

Fatty acid	Radioactivity recovered in specific fatty acid fractions in total polar lipid (percentage)
[1-^{14}C]24:5n-3	
20:5	trace
22:5	1.4 ± 0.1
24:5	56.6 ± 9.9
22:6	23.1 ± 6.2
24:6	18.9 ± 5.2
[1-^{14}C]24:6n-3	
20:5/22:5	11.5 ± 1.5
22:6	28.1 ± 4.8
24:6	60.4 ± 3.6

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Table 3. Apparent activities of enzymes of the PUFA desaturation and elongation pathway in Atlantic salmon (AS), turbot (TF) and sea bream (SAF-1) cell lines. Data represents the percentage of total radioactivity recovered as products of each enzymic step. n.d., not detected. Based on data recalculated from Ghioni et al. (1999) and Tocher and Ghioni (1999).

	Δ6 desaturase			C_{18-20} elongase			Δ5 desaturase			C_{20-22} elongase		
Substrate	AS	TF	SAF-1	AS	TF	SAF-1	AS	TF	SAF-1	AS	TF	SAF-1
[1-^{14}C]18:3n-3	76.0	81.9	66.1	60.3	18.5	25.2	38.7	11.2	n.d.	4.9	3.2	n.d.
[U-^{14}C]18:4n-3	-	-	-	81.2	25.9	19.0	56.4	19.5	0.7	9.2	5.1	n.d
[U-^{14}C]20:4n-3	-	-	-	-	-	-	38.8	62.3	0.7	7.8	17.8	n.d
[1-^{14}C]20:5n-3	-	-	-	-	-	-	-	-	-	12.1	12.8	10.9

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Table 4. Recovery of radioactivity in specific fatty acids after incubation of Atlantic salmon (AS), turbot (TF) and gilthead sea bream (SAF-1) cell lines with [U-^{14}C]18:4n-3 and [U-^{14}C]18:5n-3. Data represent the percentage of total radioactivity recovered. n.d., not detected. Based on data taken from Ghioni et al. (2001).

	AS		TF		SAF-1	
Fatty acid	18:4	18:5	18:4	18:5	18:4	18:5
18:4n-3	18.8	24.0	74.1	76.7	81.0	82.6
20:4n-3	23.6	23.2	4.4	4.5	13.2	10.3
22:4n-3	1.2	1.1	0.8	1.1	5.1	6.0
18:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5n-3	48.4	46.1	16.4	14.8	0.7	1.1
22:5n-3	4.5	3.3	1.6	1.2	n.d.	n.d.
22:6n-3	1.7	n.d.	n.d.	n.d.	n.d.	n.d.

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Table 5. Desaturase activities associated with the zebrafish PUFA desaturase gene expressed in the yeast *Saccharomyces cerevisiae*. Results are expressed as the percentage of substrate fatty acid converted to the product fatty acid. . n.d. not detected.

Substrate fatty acid	Product fatty acid	Substrate desaturated (percentage)	Desaturase activity
18:3n-3	18:4n-3	29.4	Δ6
18:2n-6	18:3n-6	11.7	Δ6
20:4n-3	20:5n-3	20.4	Δ5
20:3n-6	20:4n-6	8.3	Δ5
22:5n-3	22:6n-3	n.d.	Δ4
22:4n-6	22:5n-6	n.d.	Δ4
24:5n-3	24:6n-3	~5-10%	Δ6
24:4n-6	24:5n-6	2-5%	Δ6

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Table 6. Elongase activities associated with the zebrafish PUFA elongase gene expressed in the yeast *Saccharomyces cerevisiae*. Results are expressed as the percentage of substrate fatty acid converted to the product fatty acid. n.d. not detected.

Substrate fatty acid	Product fatty acid	Substrate elongated (percentage)	Elongase activity
18:4n-3	20:4n-3	85.4	C_{18-20}
18:3n-6	20:3n-6	70.7	C_{18-20}
20:5n-3	22:5n-3	46.4	C_{20-22}
20:4n-6	22:4n-6	25.6	C_{20-22}
22:5n-3	24:5n-3	4.9	C_{22-24}
22:4n-6	24:4n-6	trace	C_{22-24}

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631 Figure Legend

632

633 Figure 1. Pathways for the biosynthesis of C_{20} and C_{22} PUFA from 18:3n-3 and 18:2n-6
634 showing the two possible routes for the production of 22:6n-3 from 20:5n-3 (and
635 22:5n-6 from 20:4n-6). Δ6, Δ5 and Δ4 represent microsomal fatty acyl desaturase
636 activities, E1, E2 and E3 denote microsomal fatty acyl elongase activities and SC
637 denotes peroxisomal chain shortening. The dotted lines indicate pathways for
638 which there is no direct evidence in fish.

639

640 Figure 2. Section of the β-oxidation pathway for n-3PUFA showing the position of 2-
641 *trans* 18:5n-3 as a common intermediate in the β-oxidation of 18:5n-3 and 18:4n-3.

642

643 Figure 3. Phylogeny of desaturase deduced amino acid sequences. Sequences marked with
644 an asterisk are not functionally characterized. Data base accession numbers for the
645 nucleic acid sequences are indicated. Deduced amino acid sequences were aligned
646 using ClustalX and sequence phylogenies were predicted using the Neighbour Joining
647 method of Saitou and Nei (1987). Confidence in the resulting phylogenetic tree
648 branch topology was measured by bootstrapping the data through 1000 iterations with
649 the numbers representing the frequencies with which the tree topology presented here
650 was replicated after the iterations. Horizontal branch lengths are proportional to the
651 number of amino acid replacements per position, the scale bar indicating this value.

652

653

Fig.1.

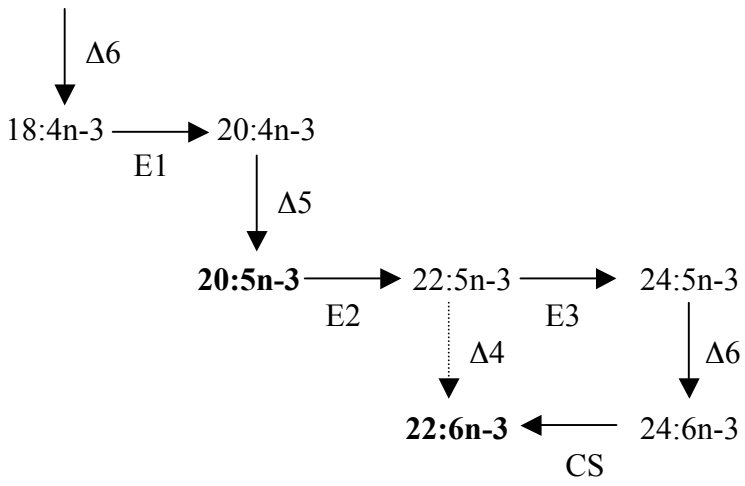
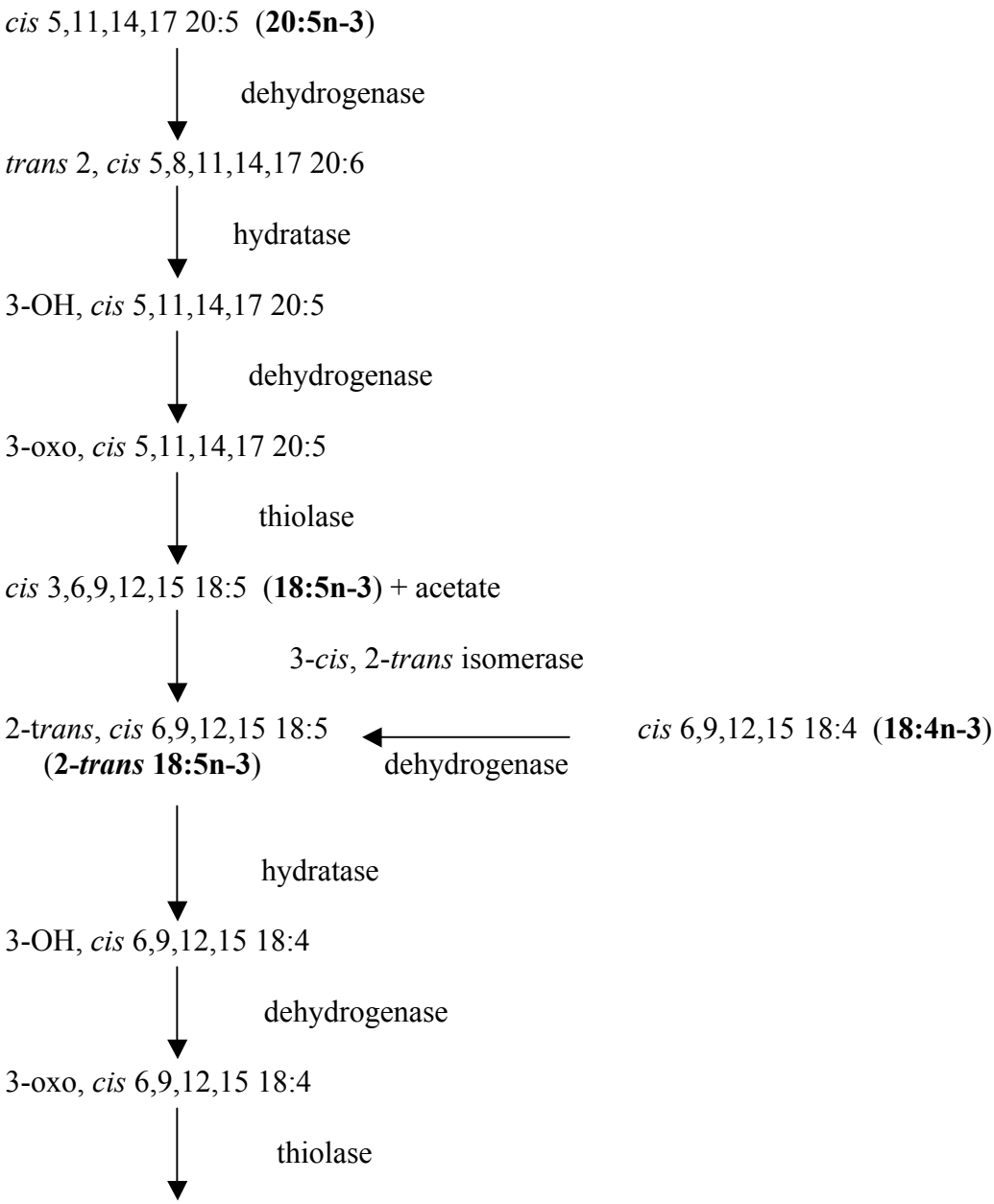
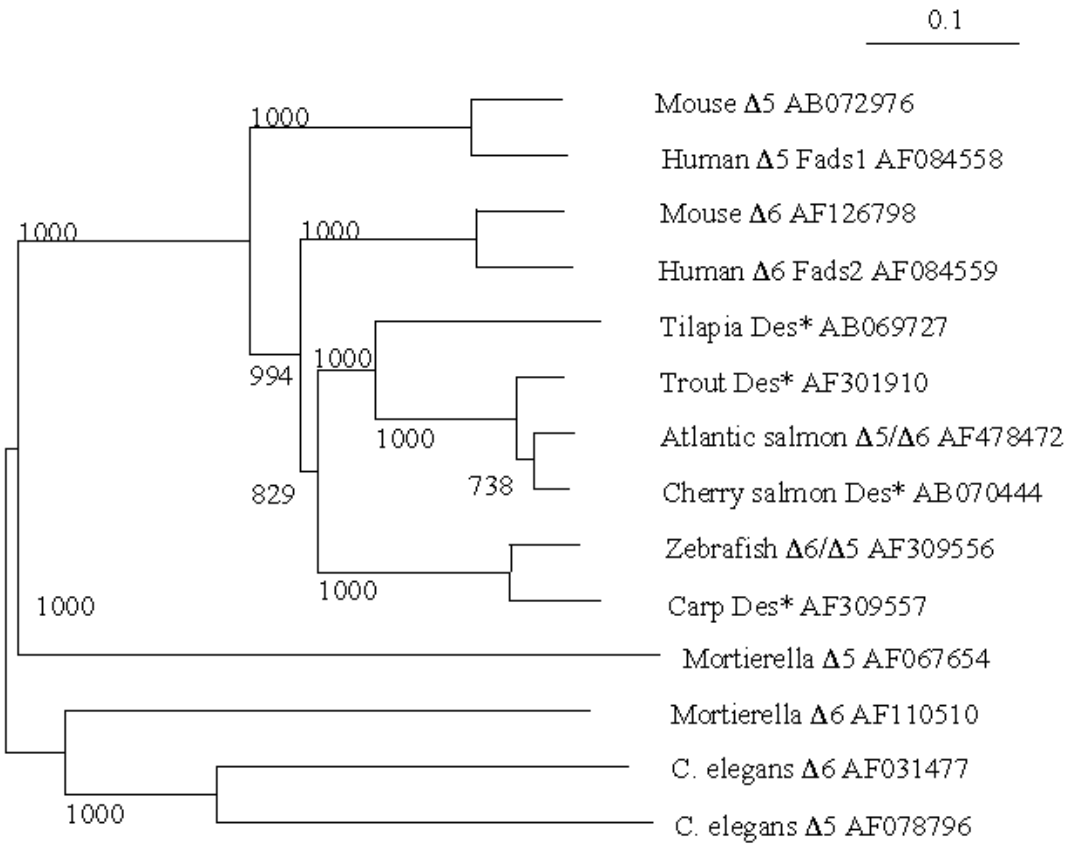

18:3n-3
Δ6
18:4n-3
E1
20:4n-3
Δ5
20:5n-3
E2
22:5n-3
E3
24:5n-3
Δ4
Δ6
22:6n-3
24:6n-3
CS
18:2n-6
Δ6
18:3n-6
E1
20:3n-6
Δ5
20:4n-6
E2
22:4n-6
E3
24:4n-6
Δ4
Δ6
22:5n-6
24:5n-6
CS

Fig.2.


cis 5,11,14,17 20:5 (**20:5n-3**)
dehydrogenase
trans 2, *cis* 5,8,11,14,17 20:6
hydratase
3-OH, *cis* 5,11,14,17 20:5
dehydrogenase
3-oxo, *cis* 5,11,14,17 20:5
thiolase
cis 3,6,9,12,15 18:5 (**18:5n-3**) + acetate
3-*cis*, 2-*trans* isomerase
2-t*rans*, *cis* 6,9,12,15 18:5
(**2-*trans* 18:5n-3**)
cis 6,9,12,15 18:4 (**18:4n-3**)
dehydrogenase
hydratase
3-OH, *cis* 6,9,12,15 18:4
dehydrogenase
3-oxo, *cis* 6,9,12,15 18:4
thiolase
cis 4,7,10,13 16:4 (**16:4n-3**) + acetate

735

736 Fig.3.



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