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

2   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 $\Delta 6$ and $\Delta 5$ 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 $\Delta 6$ 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_{18-20} elongase or $\Delta 5$ 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 $\Delta 6$ and $\Delta 5$ activity along with an elongase with very high C_{18-20} 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
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55   Lipid nutrition of fish is a subject that has received enormous attention in the last 10 years

56   (see Sargent et al. 2002). In particular, much work has focussed on the optimal requirements

57   and functional roles of polyunsaturated fatty acids (PUFA) during larval and early

58   developmental stages of marine fish (Sargent et al. 1999). However, the study of lipid and

59   fatty acid biochemistry of larval fish, especially marine larval fish, is hampered by their very

60   small size. This can place a significant limitation on the amount of material available for

61   study. Of course, the small size of larvae can be compensated, in some instances by numbers,

62   particularly if the enzymes and/or metabolic pathways can be effectively studied in

63   homogenates or some other similar preparation of whole animals. However, it is often far

64   more illuminating to study specific organ, tissue or subcellular fractions and in these cases

65   the considerable practical problems of dissecting large numbers of very small animals

66   through a binocular microscope can be prohibitive. One alternative is to use larger animals.

67   This usually requires the use of older animals such as juveniles and this can be acceptable in

68   some circumstances where the developmental stage of the fish or the ontogeny of the enzyme

69   systems or metabolic pathways is not a major issue. However, a further alternative is to go

70   even smaller, by studying the pathways at a cellular or molecular level.

71

72   This paper describes the utilization of both cell culture systems and molecular techniques in

73   the study of the genes, enzymes and metabolic pathways of lipid and fatty acid metabolism in

74   fish. The advantages (and disadvantages) of utilizing cell culture systems in metabolic studies

75   are described and the types of data that can be obtained are illustrated through studies

76   performed in our own laboratory over the last 5-6 years. The aims of these studies were to

77   elucidate the PUFA desaturation and elongation pathway in salmonids, and the nature of the

78   deficiency in the pathway in marine fish, and the metabolic pathway behind the metabolism

79   of 18:5n-3 in fish. Recently, molecular studies have begun to elucidate the genetics of these

80   processes through the cloning and characterisation of the genes involved which will enable

81   further studies of their expression and regulation.

82

83   Cell culture studies

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85   Fish cell culture is long-established and many cell lines are available commercially and from

86   various research laboratories around the world. Fish cell culture has mainly been developed

87 over the years as a diagnostic tool in pathology particularly in the area of virology where the

88 cell lines offer a range of host cells for diagnosis, characterisation and research into therapies.

89 However, cell lines have been used extensively and very successfully in metabolic studies in

90 the mammalian field. Similarly, several years ago, we decided to utilize a variety of cell

91 culture systems, including established cell lines as model systems in our studies investigating

92 lipid and especially fatty acid metabolism in fish.

93

94 <u>Advantages of cell cultures</u>

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96 In these studies, cell culture systems offered three main advantages over studies employing

97 whole fish. These can be summarised as control, containment and cost. Firstly, environmental

98 conditions can be controlled easily and very precisely in cell culture systems. Temperature

99 can be controlled simply by adjusting the temperature controller of the incubator and/or by

100 having incubators at different temperatures. Thus, studies investigating both acute and

101 chronic effects of temperature can be performed very easily and in a variety of ways (Tocher

102 and Sargent 1990a). Similarly, the osmolality of the medium can be adjusted easily, at least in

103 the case of increased salinity, by the addition of appropriate amounts of sodium chloride to

104 the medium, as may be required with cell cultures from marine fish (Tocher et al. 1988). We

105 have performed studies in this way to investigate the effects of increasing salinity changes on

106 lipid and fatty acid compositions in an Atlantic salmon (*Salmo salar*) cell line (AS) (Tocher

107 et al. 1994, 1995a). Osmolality below the normal level found in most commercial media

108 preparations (~300 mOsm.kg^{-1}) is a little more difficult but possibly of much less interest in

109 any case. However, the medium and associated supplements supply all the nutrition to the

110 cells, and so studies into the effects of nutrients can also be performed with relative ease.

111 There are a considerable number of different media formulations and supplements

112 commercially available from which to choose. As with salinity above, it is easier to look at

113 additional nutrient supplements to the cells and these can be added in high purity and in

114 various forms and concentrations. Removal of specific nutrients may be more difficult if they

115 are normal components of cell culture media formulations although it is entirely possible,

116 albeit slightly more time consuming, to formulate your own medium.

117   Cell cultures also offer the advantage of containment. This could include the use of

118 radioisotopes for metabolic tracer studies, potentially hazardous or toxic chemicals such as

119 carcinogens in toxicology studies, and pathogenic or infectious micro-organisms.

120 Containment is primarily achieved through the use of tissue culture flasks that offer sufficient

121   protection even if used vented, but can be used unvented if an appropriate medium such as

122   Leibovitz L-15, which does not contain bicarbonate buffer and thus does not require exposure

123   to a CO_2 atmosphere, is utilized. To list cost as an advantage of cell culture may be surprising

124   to some but this is certainly a major factor to include. Some capital expenditure is required

125   but this can be tailored somewhat to both specific requirements and budget. Ideally, a

126   dedicated cell culture laboratory with sealed floors and walls, single purpose sink areas, air

127   conditioning and separate areas for media preparation, primary culture preparation and

128   subculture would be desirable but not essential. A vertical laminar air flow cabinet, a cooled

129   incubator, an inverted microscope and a dedicated fridge-freezer set aside in a dedicated area

130   of a larger laboratory are probably the minimum requirements. This represents no more than

131   moderate capital expenditure. Consumables, including media, sera, other reagents and

132   disposable plasticware (flasks, pipettes, centrifuge tubes and vials/containers) are not cheap

133   but save considerable time, a vital factor when man-power is the single most expensive item

134   in the research budget. Perhaps the most important factor in assessing the cost-effectiveness

135   of cell culture is the huge cost of the alternative. Studies with fish require aquaria, with all the

136   associated costs of water supply and purification, fish and feed costs and, of course,

137   husbandry staff. In addition, some studies would be very much more difficult to perform with

138   fish. Studies on temperature effects require aquaria to be maintained at non-ambient

139   temperatures and thus require heating or cooling of the water and/or the room. Work with

140   radioisotopes is extremely difficult with whole fish particularly when ^{14}C is used due to the

141   possibility of production and release of $^{14}CO_2$ into the atmosphere. Containment is similarly a

142   problem when using toxins or pathogens and in all these cases it adds to the costs of

143   performing experiments with fish.

144

145   <u>Problems of using fish cell cultures in metabolic studies</u>

146

147   The use of cell cultures is not, however, without its own problems. The first of these is

148   temperature. For the majority of fish cell lines the optimum growth temperature is in the 20 -

149   25 ^{o}C range. These include cell lines from Atlantic salmon (AS), rainbow trout

150   (*Oncorhynchus mykiss*) (RTG-2, RTH) and turbot (*Scophthalmus maximus*) (TF) which are

151   all routinely cultured at 22 ^{o}C. However, the normal ambient temperature in U.K. waters for

152   these species of fish would rarely exceed 15 ^{o}C, a temperature we routinely use as a

153   "holding" temperature, to slow the growth of the cells during periods when they are not being

154   actively used in experiments. Culture at 10 ^{o}C or below usually results in unacceptably low

155 growth rates even in cell lines from these cooler water fish. Therefore, fish cell lines such as
156 those above are being cultured at a temperature higher than normal, a situation that does not
157 occur in mammalian cell culture. In contrast, other fish species, such as Mediterranean fish
158 including gilthead sea bream (*Sparus aurata*), would normally experience water temperatures
159 in the low 20's and thus cells derived from them (SAF-1) would not be at an unusually high
160 temperature when cultured at $20 - 25\ ^oC$. These are particularly important points to be aware
161 of in relation to temperature adaptation/acclimation studies where the lower temperature, say
162 $10\ ^oC$, actually represents a more normal temperature for some cell lines and $22\ ^oC$ could be
163 regarded as a stressed temperature, whereas in other cell lines the opposite would be true.
164
165 A second problem with the use of fish cell cultures is one of particular importance in relation
166 to lipid and fatty acid studies. Cell culture media are normally devoid of fatty acids and so
167 cells in culture generally derive all their lipid and fatty acids from the lipid contained in the
168 serum supplement, which is an almost ubiquitous supplement due to its various properties
169 including promotion of attachment, growth and proliferation of the cultured cells. Fetal
170 bovine serum (FBS), the predominant serum supplement used in cell culture including fish
171 cell culture, is relatively rich in PUFA and for mammalian cells, FBS provides a sufficient
172 amount and balance of n-6 and n-3PUFA. In contrast, although the total amount of PUFA is
173 adequate, fish cells grown in FBS display lower percentages of n-3PUFA and are enriched in
174 n-6PUFA in comparison with fish tissues (Tocher et al. 1988). This has important
175 consequences when cultured fish cells are used in studies of fatty acid metabolism. We have
176 used two approaches to solve this problem. Firstly, we investigated the possibility of
177 producing fish cell lines that can grow and proliferate in the absence of serum. To date, we
178 have found one cell line, EPC-EFAD, derived from the carp (*Cyprinus carpio*) epithelial
179 papilloma line, EPC, that can survive and proliferate in essential fatty acid-deficient (EFAD)
180 medium (Tocher et al. 1995b). The EPC-EFAD line has now been growing continually in
181 EFAD medium for over 7 years and 130 passages although the rate of proliferation is lower
182 than the parent EPC line. This cell line is virtually devoid of n-6 and n-3PUFA but contains
183 appreciable amounts of n-9PUFA (Tocher and Dick 2001) and thus does not represent a
184 model system for fish normally although they have been useful in studies on the effects of
185 EFA deficiency on fatty acid metabolism in freshwater fish (Tocher and Dick 1999, 2000,
186 2001). An alternative solution is to reduce the serum added to the medium and to supplement
187 with a mix of pure fatty acids designed to restore the fatty acid composition of the cells to
188 that of the original tissue in the fish. For instance, primary cultures of turbot brain astroglial

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189   cells established in medium containing FBS contained increased proportions of 18:1(n-9),

190   and total n-9 and n-6 PUFA, and greatly reduced n-3PUFA in comparison with turbot brain.

191   Supplementation with a mixture of 5 μM 20:5n-3 and 25 μM 22:6n-3 acids for 4 days

192   significantly increased the percentages of these acids in total cellular lipid of trout and turbot

193   astrocytes and restored the n-3PUFA composition of the cells to that found in brain (Bell et

194   al. 1994; Tocher et al. 1996).

195

196   A final caveat to the use of cell cultures in metabolic studies relates to interpretation and

197   extrapolation of the results. It is obvious that cell cultures are not whole animals. Many

198   factors important in controlling and regulating metabolism are simply not replicated in the

199   cell culture systems. Complex multi-cell type organ structure is difficult to replicate in cell

200   culture and even most tissue specific features such as 3D-structure, orientation and sidedness

201   are lost in culture and, in addition, the cells themselves may be dedifferentiated (as in cell

202   lines) and of changed morphology. Nonetheless, many features of inherent intracellular

203   biochemistry and metabolism will be retained by cells in culture and provided the researcher

204   is aware of the limitations then cell cultures provide a very useful additional experimental

205   tool. Cautious extrapolation to the whole animal is possible particularly when the cell data are

206   entirely consistent with other available data and, particularly, whole animal data, but

207   ultimately whole animal studies are required for final confirmation.

208

209   Types of cell culture systems

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211   Different types of cultured cell systems can be utilized to fit the particular requirements of the

212   studies. In our own studies we have used three types, the first of which is short-term cultures,

213   where the cells are attached to the substrate (plastic), but there is no growth or division over

214   the time-course of the experiment, around 2 – 24 h (Buzzi et al 1996, 1997). The major

215   benefit of these cultures is that the cells retain their differentiated phenotype. The retention of

216   differentiated phenotype is also the aim with primary cultures that are attached, and grow and

217   divide over a much longer period of time, ranging from days to weeks (Tocher and Sargent

218   1990b). Depending upon the cell type, some limited subculture of primary cultures may be

219   possible but not always. Established cell lines are immortal, growing and dividing at

220   infinitum with routine subculture necessary to maintain the cells in optimum condition

221   (Tocher et al. 1988). The down side of cell lines being that they are usually de-differentiated,

222   possessing either fibroblast or epithelial morphology. The following sections describe the use

223   of some of these cell cultures as model systems to investigate specific aspects of fatty acid

224   metabolism in fish.

225

226   <u>Determining the PUFA desaturation/elongation pathway in trout</u>

227

228   All vertebrates, including fish, lack $\Delta 12$ and $\Delta 15$ ($\omega 3$) desaturases and so cannot form 18:2n-6

229   and 18:3n-3 from 18:1n-9. Therefore, 18:2n-6 and 18:3n-3 are essential fatty acids in the

230   diets of vertebrates. These dietary essential fatty acids can be further desaturated and

231   elongated to form the physiologically essential C_{20} and C_{22} PUFA, 20:4n-6, 20:5n-3 and

232   22:6n-3 (Fig.1). With one exception the reactions occur in the microsomal fraction of the

233   liver and the same enzymes act on the n-3 and the n-6 fatty acid series. Originally the

234   insertion of the last, $\Delta 4$, double bond in 22:6n-3 was assumed to occur through direct $\Delta 4$

235   desaturation of its immediate precursor 22:5n-3. However, Howard Sprecher and coworkers

236   showed that in rat liver, the 22:5n-3, is further chain elongated to 24:5n-3 which is then

237   converted by $\Delta 6$ desaturation to 24:6n-3 which is then converted, by a chain shortening

238   reaction in the peroxisomes, to 22:6n-3 (Sprecher 1992; Sprecher et al. 1995).

239

240   Whether the production of 22:6n-3 in fish involved $\Delta 4$ desaturation of 22:5n-3 or $\Delta 6$

241   desaturation of 24:5n-3 with chain shortening of the resultant 24:6n-3 to 22:6n-3 was

242   investigated in our laboratory by Buzzi et al. (1996, 1997). The cell system chosen was

243   primary hepatocytes prepared by collagenase perfusion of intact, isolated liver from rainbow

244   trout fed a n-3PUFA-deficient (olive oil) diet to stimulate the PUFA desaturation pathway.

245   These cells were maintained in short-term culture for up to 24h. Incubation of hepatocytes for

246   3h with [1-^{14}C]18:3n-3 or [1-^{14}C]20:5n-3, added as complexes with fatty acid-free bovine

247   serum albumin, resulted in the recovery of large amounts of radioactivity as 22:6n-3 with

248   only traces of radioactivity recovered in C_{24} PUFA (Table 1). In contrast, when liver

249   microsomes were incubated for 3h with the same radioactive fatty acids, no radioactivity was

250   recovered in 22:6n-3, but substantial amounts of radioactivity were recovered in 24:5n-3 and

251   24:6n-3 (Table 1). These data suggested that the pathway as proposed by Sprecher for rat

252   liver also occurred in trout liver. Incubation of the trout hepatocytes with [1-^{14}C]24:5n-3

253   resulted in radioactivity being recovered in both 22:6n-3 and 24:6n-3 (Table 2). Similarly,

254   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

289 desaturase activity (Mourente and Tocher 1994). Therefore, as the situation in marine fish

290 was unclear, and as a deficiency in the fatty acid elongase activity responsible for the

291 conversion of C_{18} to C_{20} PUFA had not been reported in any other animal or cell line, we

292 aimed to establish unequivocally the location of the defect in the desaturase/elongase

293 pathway in marine fish using the established cell lines, AS, TF and SAF-1. Each of these cell

294 lines was incubated for 4 days with various ^{14}C-labelled n-3PUFA that were the direct

295 substrates for individual enzymic steps in the desaturation/elongation pathway (Ghioni et al.

296 1999; Tocher and Ghioni 1999). Thus, 18:3n-3 was the direct substrate for Δ6 desaturase,

297 18:4n-3 was the direct substrate for C_{18-20} elongase, 20:4n-3 was the substrate for Δ5

298 desaturase and 20:5n-3 was the substrate for C_{20-22} elongase (Table 3). The data in Table 3

299 show the percentage of radioactivity recovered as the products of each enzymic step. Thus,

300 the results showed that all three cell lines had substantial Δ6 activity as 76%, 82% and 66%

301 of radioactivity from

302 [1-^{14}C]18:3n-3 was recovered as Δ6 desaturated products in AS, TF and SAF-1 cells,

303 respectively. However, both marine cell lines showed very reduced C_{18-20} elongase activity

304 compared with AS cells. However, whereas the SAF-1 cell line showed virtually no Δ5

305 desaturase activity, the TF cell line showed considerable Δ5 activity (Table 3). All cell lines

306 showed similar levels of C_{20-22} activity. Thus the primary deficiency in the PUFA

307 desaturation/elongation pathway in gilthead sea bream cells was established to be at the level

308 of Δ5 desaturase whereas the only deficiency observed in the TF cells was at the C_{18-20}

309 elongase. The SAF-1 cell line may also show a deficiency in C_{18-20} elongase but it is possible

310 that the virtual absence of Δ5 activity results in the accumulation of 20:4n-3 which inhibits

311 C_{18-20} elongase through a feedback mechanism. Irrespective of which enzyme step was

312 deficient, the cell line data was entirely consistent with earlier feeding studies and in vivo

313 studies indicating that marine fish were unable to produce significant amounts of 20:5n-3 and

314 22:6n-3 from 18:3n-3.

315

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

317

318 Octadecapentaenoic acid (all-*cis* 18:5n-3) is a fatty acid characteristically present in certain

319 algal groups in marine phytoplankton, including dinoflagellates, haptophytes and

320 prasinophytes, all of which have important roles in the marine ecosystem (Sargent et al.

321 1995). 18:5n-3 is usually co-associated in these organisms with 22:6n-3. Given that

322   biosynthesis of 22:6n-3 involves peroxisomal chain shortening of its precursor 24:6n-3, it is

323   possible that 18:5n-3 is biosynthesized by chain shortening of 20:5n-3. However, marine

324   zooplankton and fish ingesting phytoplankton contain little or no 18:5n-3 demonstrating that

325   this fatty acid is readily metabolized by marine animals. It could be completely catabolized

326   by marine animals by β-oxidation but it may also be directly chain elongated to 20:5n-3.

327

328   In this study, [U-^{14}C]18:4n-3 and [U-^{14}C]18:5n-3 were prepared from the haptophycean alga

329   *Isochrysis galbana* cultured in sodium ^{14}C-bicarbonate, and their metabolism studied in

330   cultured cells from turbot (TF), sea bream (SAF-1) and Atlantic salmon (AS) that differ in

331   their abilities to perform C_{18} to C_{20} elongation reactions. The rationale being that the TF cell

332   line's deficiency in C_{18} to C_{20} fatty acid elongase would perhaps help to differentiate between

333   the two possible pathways for the metabolism of 18:5n-3 in fish as suggested above.

334   Incubation of the cell lines with both labelled 18:4 and 18:5 showed two remarkable features

335   (Table 4). Firstly, no radiolabelled 18:5 was ever detected in any of the three cell lines, even

336   when labelled 18:5n-3 was incubated with the cells and even in short incubations of less than

337   1h. Secondly, the pattern of distribution of radioactivity was identical for both fatty acids,

338   that is the recovery of radioactivity in different fatty acid fractions after incubation with [U-

339   ^{14}C]18:5 was identical to the distribution of radioactivity after incubation with [U-^{14}C]18:4

340   (Table 4). Indeed, the pattern only varied between the cell lines based upon the differences in

341   their PUFA desaturation/ elongation pathways. The one difference between incubation with

342   18:4 and 18:5 was that the quantitative recovery of radioactivity was significantly lower with

343   18:5n-3. These results showed that 18:5n-3 was not metabolised in fish cells by chain

344   elongation to 20:5n-3. In retrospect, this was perhaps unsurprising as, unlike 18:4n-3, 18:5n-3

345   is not a normal intermediate in the desaturation/elongation pathway (Fig.1). However, 18:5n-

346   3 is a normal intermediate in the pathway for the β-oxidation of 20:5n-3 (Fig.2). In contrast,

347   18:4n-3 is not an intermediate in the PUFA β-oxidation pathway although the first step in the

348   β-oxidation of 18:4n-3, dehydrogenation, results in the formation of *trans* Δ2, all-*cis*

349   Δ6,9,12,15-18:5 (2-*trans* 18:5n-3) (Fig.2). The 2-*trans* 18:5n-3 intermediate is also produced

350   by the action of a Δ^3, Δ^2-enoyl-CoA-isomerase acting on 18:5n-3, this enzyme being the next

351   step in the β-oxidation pathway after the production of 18:5n-3. Thus, 2-*trans* 18:5n-3 is a

352   common intermediate in the β-oxidation of both 18:4n-3 and 18:5n-3. It appeared therefore

353   that 18:5n-3 incorporated into the fish cells was treated as a β-oxidation intermediate by the

354   fish cell lines resulting in the production of 2-*trans* 18:5n-3 in amounts which probably

355 exceeded the capacity of the β-oxidation pathway. This resulted in the reversal of the

356 dehydrogenase step and production of labelled 18:4n-3 (Fig.2) which was then metabolised

357 as normal via the desaturation/elongation pathway producing labelled 20:4n-3 and 20:5n-3

358 (Fig.1). A proportion of the 2-*trans* 18:5n-3 proceeded down the β-oxidation pathway

359 resulting in the overall lower recovery of radioactivity when the cells were incubated with

360 18:5 compared to cells incubated with 18:4. To further test this hypothesis, cells were also

361 incubated with either 18:5n-3 or 2-*trans* 18:5n-3, and similar mass increases of 18:4n-3 and

362 its elongation and further desaturation products occurred in cells incubated with 18:5n-3 or 2-

363 *trans* 18:5n-3. We therefore concluded that 18:5n-3 was readily converted biochemically to

364 18:4n-3 via a 2-*trans* 18:5n-3 intermediate generated by a Δ^3, Δ^2-enoyl-CoA-isomerase acting

365 on 18:5n-3 and, therefore, that 2-*trans* 18:5n-3 was implicated as a common intermediate in

366 the β-oxidation of both 18:5n-3 and 18:4n-3 (Ghioni et al. 2001).

367

368 <u>Molecular studies</u>

369

370 Very recently, molecular biological and genetic techniques have begun to be applied to lipid

371 and fatty acid metabolism in fish in order to elucidate the genetics of the above pathways

372 through the cloning and characterisation of the genes involved enabling further studies on the

373 expression and regulation of the genes. These techniques have particular advantages when

374 applied to larvae. Firstly, the small size of fish larvae presents no problem in the preparation

375 of RNA and/or cDNA even if tissue-specific RNA is required as relatively little tissue is

376 required. The larval RNA/cDNA can not only be used in routine gene expression studies

377 through conventional Northern blotting or real-time PCR but can also be used for cloning

378 genes expressed specifically in larvae. In addition, modern in-situ hybridisation techniques

379 can also be used to locate organ- and tissue-specific gene expression and are equally, or

380 indeed more, able to be applied to larvae as to larger fish. The above cell culture studies have

381 demonstrated the great significance of PUFA desaturase and elongase enzymes in fish.

382 Several questions still remained though including a) was there one or two different $\Delta 6$

383 desaturases (isoenzymes) for the desaturation of C_{18} and C_{24} PUFA, and b) what were the

384 precise defects in $\Delta 5$ desaturase and C_{18-20} elongase in marine fish (Tocher et al. 1998). The

385 following sections describe our current studies aimed at cloning and characterising PUFA

386 desaturase and elongase genes in fish.

387

<u>Cloning and characterisation of PUFA desaturase genes in fish</u>

A zebrafish EST sequence (Genbank accession no. AI497337) was identified that displayed high homology to mammalian $\Delta 5$ and $\Delta 6$ 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 $\Delta 6$ desaturases. Overall amino acid identities were 64% to human $\Delta 6$ desaturase and 58% to human $\Delta 5$ 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 $\Delta 6$ 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 $\Delta 6$ and $\Delta 5$ desaturase activities (Table 5). The enzyme was more active towards n-3 and $\Delta 6$ substrates compared to n-6 and $\Delta 5$ 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 $\Delta 6$ and $\Delta 5$ activities. Recently, we have shown that the zebrafish desaturase has no $\Delta 4$ desaturase activity but was able to desaturate 24:5n-3 to 24:6n-3 suggesting that a single $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ and $\Delta 5$ activities with the latter being greater. Phylogenetic analysis indicated that, with respect to

421 other functionally characterized genes, the zebrafish sequence had highest homology with

422 mammalian Δ6 desaturases, with human Δ5 desaturase appearing to be distinct from the Δ6

423 desaturase sequences (Fig.3). All the fish genes clustered together. Although more fatty acid

424 desaturase genes may be found in zebrafish, salmon and mammals, it is conceivable that the

425 bi-functional desaturase described here is a component of a prototypic vertebrate PUFA

426 biosynthetic pathway that has persisted in freshwater fish species. That humans and other

427 mammals have two distinct enzymes for Δ5 and Δ6 desaturation may be an adaptation to a

428 terrestrial diet providing lower amounts of pre-formed C_{20} and C_{22} PUFA than the diets of a

429 vertebrate ancestor that they share with freshwater fish. Functional divergence of the products

430 of a putative ancient gene duplication event is a possible mechanism underlying adaptation to

431 such a dietary change.

432

433 <u>Cloning and characterisation of PUFA elongase genes in fish</u>

434

435 Fatty acid elongation, the addition of 2-carbon units, is effected in four steps each catalysed

436 by a specific enzyme. The first step is a condensation reaction of the precursor fatty acyl

437 chain with malonyl-CoA to produce a β-ketoacyl chain that is then hydrogenated in three

438 successive steps. The condensation step is widely regarded as the "elongase", and the one

439 that determines the substrate specificity and is rate limiting. *Mortierella alpina* elongase

440 (GLELO) amino acid sequence cDNA encoding a PUFA elongase was used to probe *in silico*

441 for related sequences in the Genbank EST database. This identified mammalian, chicken,

442 Xenopus and zebrafish ESTs. Consensus PCR primers were designed in conserved motifs

443 and used to isolate full length cDNA from livers of several fish species using the rapid

444 amplification of cDNA ends (3" and 5"RACE) strategies to clone full length elongase

445 cDNAs of zebrafish, carp, salmon and turbot (AF465520). The amplified cDNAs encoded

446 putative open reading frames (ORFs) of 291-295 amino acids whose sequences were highly

447 conserved among the fish species and with other vertebrate elongases. The fish elongase

448 polypeptides have up to 7 predicted transmembrane (TM) domains, a canonical endoplasmic

449 reticulaum retention signal, and several potential phosphorylation sites which may be

450 important in regulation of enzyme function. Expression of the zebrafish gene in the yeast *S.*

451 *cerevisiae* demonstrated that the ORFs encoded a fatty acid elongase with substrate

452 specificity ranging from the monounsaturated fatty acid palmitoleic acid (16:1n-7) to the long

453 chain highly unsaturated fatty acid, 22:5n-3. The zebrafish elongase activity was in the rank

14

454 order $C_{18-20} > C_{20-22} > C_{22-24}$ and was more active towards n-3 substrates than n-6 substrates

455 (Table 6). Recently, functional characterisation of the salmon and turbot elongases has

456 revealed that they have similar specificities to the zebrafish enzyme with the rank order for

457 overall activity being zebrafish > salmon > turbot. The turbot enzyme was relatively more

458 active towards the C_{20} substrates than C_{18} substrates compared to the zebrafish and salmon

459 enzymes. However, it was particularly interesting that the turbot gene coded for a

460 functionally active protein. This was not contradictory to the cell culture data as, although the

461 deficiency in the desaturation/elongation pathway appeared to be at the C_{18-20} elongase step in

462 TF cells, there was activity present. The sequence data suggested another possibility for low

463 C_{18-20} elongase activity in TF cells as the Kozak sequence (which marks the following

464 methionine codon as the start codon) in the turbot cDNA is a poor signal for initiation of

465 translation and turbot elongase was less efficient than zebrafish and salmon elongases

466 particularly for C_{18} substrates.

467

468 **Conclusions**

469

470 The use of a variety of cell culture systems has greatly advanced biochemical studies which

471 have in turn elucidated key parts of the PUFA desaturation and elongation pathway in fish.

472 The presence of the so-called Sprecher shunt, where 22:6n-3 is produced from 20:5n-3

473 through two successive elongations and a Δ6 desaturase followed by peroxisomal chain

474 shortening, was demonstrated in primary hepatocytes isolated from trout. Similarly, studies

475 on established cell lines revealed that the block in the pathway in marine and/or piscivorous

476 fish was due to either a deficiency of C_{18-20} elongase or Δ5 desaturase and this varied between

477 different marine species. Current work is focussing on the molecular biology of the pathway

478 with the cloning of fatty acid desaturases and elongases from a variety of fish species.

479 Zebrafish have been used as a model species and a unique desaturase possessing both Δ6 and

480 Δ5 activity and an elongase with very high C_{18-20} activity have been cloned and characterised.

481 The zebrafish desaturase was capable of desaturating both C_{18} and C_{24} Δ6 substrates.

482 Understanding this pathway is of increased importance due to the current dependence of

483 salmonid and marine fish aquaculture on fish oil, the supply of which is becoming

484 increasingly limited and unsustainable, necessitating the use in fish feeds of sustainable plant

485 oils, rich in C_{18} PUFA, but devoid of C_{20} and C_{22} PUFA (Sargent et al. 2002).

486

487

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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

621

622

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

623

624

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).

Substrate	$\Delta 6$ desaturase			C_{18-20} elongase			$\Delta 5$ desaturase			C_{20-22} elongase		
	AS	TF	SAF-1	AS	TF	SAF-1	AS	TF	SAF-1	AS	TF	SAF-1
$[1\text{-}^{14}C]18\text{:}3n\text{-}3$	76.0	81.9	66.1	60.3	18.5	25.2	38.7	11.2	n.d.	4.9	3.2	n.d.
$[U\text{-}^{14}C]18\text{:}4n\text{-}3$	-	-	-	81.2	25.9	19.0	56.4	19.5	0.7	9.2	5.1	n.d
$[U\text{-}^{14}C]20\text{:}4n\text{-}3$	-	-	-	-	-	-	38.8	62.3	0.7	7.8	17.8	n.d
$[1\text{-}^{14}C]20\text{:}5n\text{-}3$	-	-	-	-	-	-	-	-	-	12.1	12.8	10.9

625

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).

Fatty acid	AS		TF		SAF-1	
	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.

626

627

627

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	$\Delta6$
18:2n-6	18:3n-6	11.7	$\Delta6$
20:4n-3	20:5n-3	20.4	$\Delta5$
20:3n-6	20:4n-6	8.3	$\Delta5$
22:5n-3	22:6n-3	n.d.	$\Delta4$
22:4n-6	22:5n-6	n.d.	$\Delta4$
24:5n-3	24:6n-3	~5-10%	$\Delta6$
24:4n-6	24:5n-6	2-5%	$\Delta6$

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629

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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}

630

631

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). $\Delta6$, $\Delta5$ and $\Delta4$ 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.
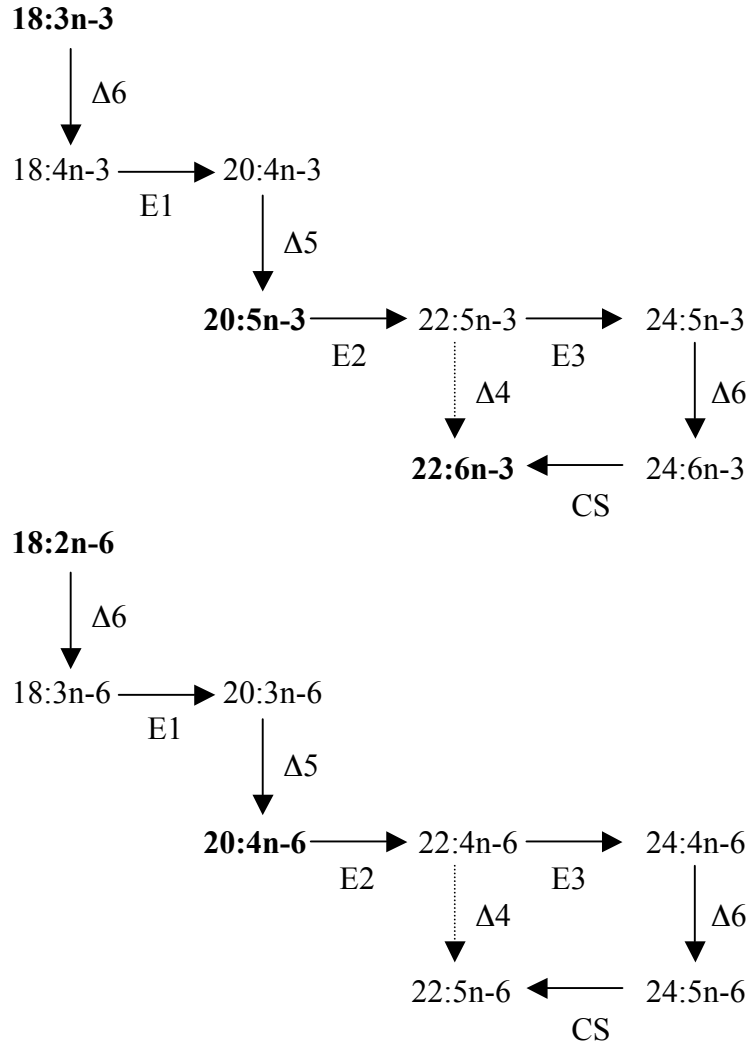
642

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

653
654
655 Fig.1.
656
657
658
659 **18:3n-3**
660
661 ↓ Δ6
662
663 18:4n-3 ——————► 20:4n-3
664 E1
665 ↓ Δ5
666
667 **20:5n-3** ——————► 22:5n-3 ——————► 24:5n-3
668 E2　　　　　　　 E3
669 ⋮ Δ4　　　　　　　 ↓ Δ6
670
671 **22:6n-3** ◄—————— 24:6n-3
672 CS
673 **18:2n-6**
674
675 ↓ Δ6
676
677 18:3n-6 ——————► 20:3n-6
678 E1
679 ↓ Δ5
680
681 **20:4n-6** ——————► 22:4n-6 ——————► 24:4n-6
682 E2　　　　　　　 E3
683 ⋮ Δ4　　　　　　　 ↓ Δ6
684
685 22:5n-6 ◄—————— 24:5n-6
686 CS
687
688
689

28

689 Fig.2.
690
691 *cis* 5,11,14,17 20:5 (**20:5n-3**)
692
693   dehydrogenase
694
695 *trans* 2, *cis* 5,8,11,14,17 20:6
696
697   hydratase
698
699 3-OH, *cis* 5,11,14,17 20:5
700
701   dehydrogenase
702
703 3-oxo, *cis* 5,11,14,17 20:5
704
705   thiolase
706
707 *cis* 3,6,9,12,15 18:5 (**18:5n-3**) + acetate
708
709   3-*cis*, 2-*trans* isomerase
710
711 2-t*rans*, *cis* 6,9,12,15 18:5 ⟵   *cis* 6,9,12,15 18:4 (**18:4n-3**)
712   (**2-*trans* 18:5n-3**)   dehydrogenase
713
714
715   hydratase
716
717 3-OH, *cis* 6,9,12,15 18:4
718
719   dehydrogenase
720
721 3-oxo, *cis* 6,9,12,15 18:4
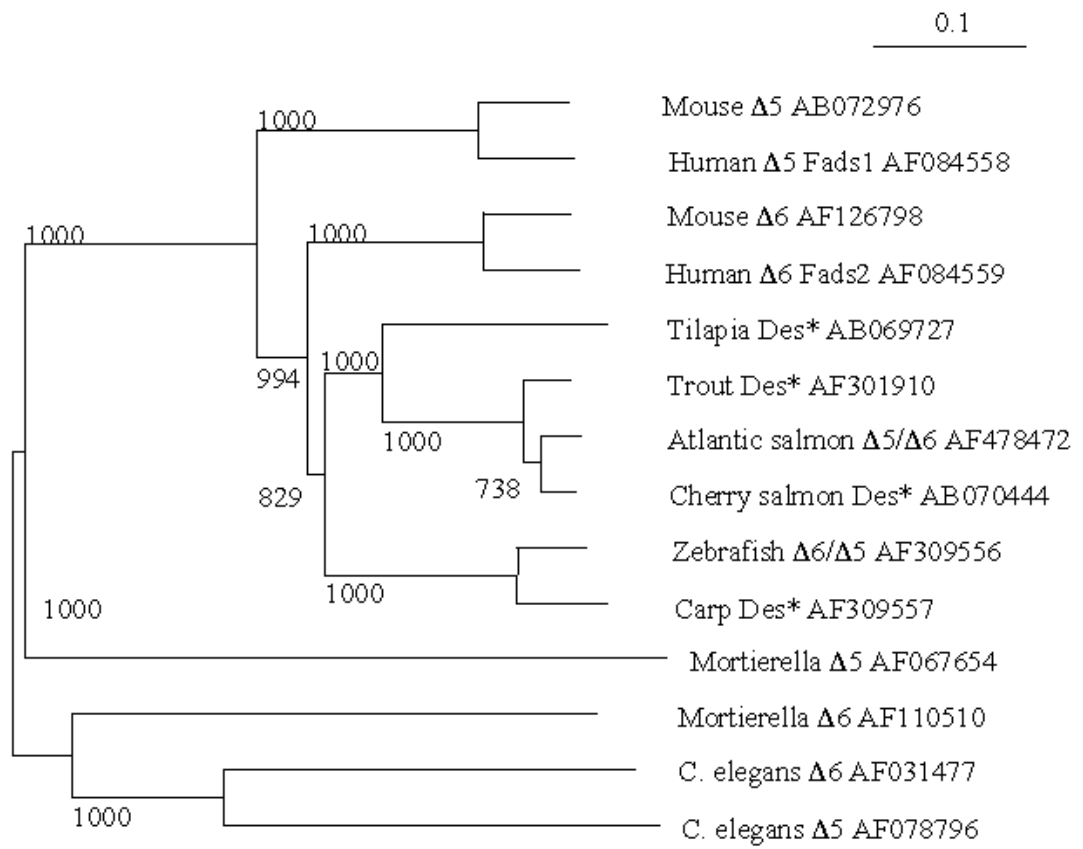722
723   thiolase
724
725 *cis* 4,7,10,13 16:4 (**16:4n-3**) + acetate
726
727
728
729
730
731
732
733
734

735

736   Fig.3.

0.1

Mouse Δ5 AB072976

Human Δ5 Fads1 AF084558

Mouse Δ6 AF126798

Human Δ6 Fads2 AF084559

Tilapia Des* AB069727

Trout Des* AF301910

Atlantic salmon Δ5/Δ6 AF478472

Cherry salmon Des* AB070444

Zebrafish Δ6/Δ5 AF309556

Carp Des* AF309557

Mortierella Δ5 AF067654

Mortierella Δ6 AF110510

C. elegans Δ6 AF031477

C. elegans Δ5 AF078796

737