# Chapter 9
# Biosynthesis of Polyunsaturated Fatty Acids in Aquatic Ecosystems: General Pathways and New Directions

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## 9.1 Introduction

It is now well established that the long-chain, omega-3 (ω3 or n-3) polyunsaturated fatty acids (PUFA) are vitally important in human nutrition, reflecting their particular roles in critical physiological processes (see Chap. 14). In comparison to terrestrial ecosystems, marine or freshwater ecosystems are characterised by relatively high levels of long-chain n-3PUFA and, indeed, fish are the most important source of these vital nutrients in the human food basket. Virtually all PUFA originate from primary producers but can be modified as they pass up the food chain. This is generally termed trophic upgrading, and various aspects of these phenomena have been described in Chaps. 2, 6 and 7 (this volume). However, while qualitative aspects of essential fatty acid production and requirements in aquatic ecosystems are relatively well understood, in order to fully understand and model ecosystems, quantitative information is needed on synthesis and turnover rates of n-3PUFA at different trophic levels in the food web. The present chapter describes the biochemistry and molecular biology involved in the various pathways of PUFA biosynthesis and interconversions in aquatic ecosystems.

To appreciate the biochemical mechanisms involved in their biosynthesis, some understanding of fatty acid chemistry is required. Fatty acids are designated on the basis of their chain lengths, degree of unsaturation (number of ethylenic or 'double' bonds) and the position of their ethylenic bonds. Thus, 18:0 designates a fatty chain with 18 carbon atoms and no ethylenic bonds. In the n- (or omega, ω) nomenclature, the position of double bonds is designated by counting from the methyl terminus, and so 16:1n-7 designates a fatty acid with 16 carbon atoms whose single ethylenic bond is 7 carbon atoms from the methyl end. In the alternative delta (Δ) nomenclature, the position of the ethylenic bond is counted from the carboxyl end, and so 16:1n-7 is written as $16{:}1\Delta^9$. The n- or ω-nomenclatures are

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those now in general use, but the Δ nomenclature remains relevant as it has traditionally been used for characterising fatty acyl desaturase activities. PUFA are defined as fatty acids containing two or more ethylenic bonds and a common example is eicosapentaenoic acid (EPA), 20:5n-3 and 20:5ω3 or, 20:5$^{\Delta 5,8,11,14,17}$ in the Δ nomenclature. Highly unsaturated fatty acid (HUFA) is a term being used increasingly, most often without proper definition, mainly to distinguish the major bioactive long-chain PUFA such as EPA, docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) from the shorter-chain $C_{18}$ PUFA such as linoleic (LIN; 18:2n-6) and ALA (α-linolenic; 18:3n-3) acids. In our laboratory we define HUFA as fatty acids with ≥20 carbons and ≥3 double bonds. A further term requiring careful definition is essential fatty acid (EFA; and see Chap. 13). PUFA are essential dietary components for most animals, and all vertebrates, as they cannot synthesise PUFA de novo from monounsaturated fatty acids. However, which specific PUFA can satisfy the EFA requirements (and prevent EFA deficiency symptoms) in a particular species is entirely dependent upon its endogenous capacity to convert $C_{18}$ PUFA to the biologically active HUFA: ARA, EPA and DHA. Therefore, EFA requirements will vary qualitatively as well as quantitatively among different animal species.

## 9.2 Primary Production of Polyunsaturated Fatty Acids

### *9.2.1 Bacteria*

The presence of n-3PUFA in heterotrophic prokaryotes was first reported by Johns and Perry (1977), who found EPA in the marine bacterium *Flexibacter polymorphus*. Subsequently EPA and DHA were found in a number of bacteria isolated from cold marine habitats, but particularly from intestines of deep-sea fish and invertebrates (Yano et al. 1994). Bacteria producing EPA and DHA were found in the culturable intestinal flora of all species of a selection of ten Arctic and sub-Arctic invertebrates and one of four fish species (Jøstensen and Landfald 1997). In total, 103 out of 330 strains of bacteria tested contained n-3PUFA. The highest prevalences, in >50% of bacterial isolates, were from two species of bivalve *Chlamys islandica* and *Astarte* sp. and in the amphipod *Gammarus wilkitzkii*. PUFA producers clustered into eight groups depending on PUFA profile, six groups had 3.8–18.7% EPA and two groups had 7.1 and 13.5% DHA, both by weight total fatty acid (Jøstensen and Landfald 1997). Interestingly the bacteria contained either EPA or DHA, but not both. Some bacteria associated with coastal Antarctic sea-ice diatom assemblages were found to synthesise DHA (Bowman et al. 1998). Eight strains were identified by 16S rRNA sequence analysis as belonging to the genus *Colwellia*. All exhibited psychrophilic and facultative anaerobic growth and produced 0.7–8.0% of total fatty acids as DHA (Bowman et al. 1998).

*Vibrio* sp. and *Shewanella* sp. comprise the majority of the PUFA-producing bacterial species isolated from the guts of fish and invertebrates. However, there

have been a number of misidentifications, which are discussed in a detailed review of PUFA in marine bacteria by Russell and Nichols (1999). This review also discussed pathways of fatty acid synthesis by bacteria and concluded that conventional aerobic pathways must be used to synthesise PUFA. The discovery of the polyketide pathway allows an anaerobic alternative, which may be more appropriate with respect to the general metabolism of these microorganisms (see later).

### *9.2.2 Photosynthetic Organisms (Microalgae)*

De novo synthesis of n-3PUFA in microalgae involves sequential addition of double bonds to saturated fatty acids via Δ9, Δ12 and Δ15 (or ω3) desaturases to give ALA. A sequence of front-end desaturases (inserting double bonds between the Δ9 bond and the carboxyl terminus) acting with elongases then produces EPA and DHA. Conventionally this sequence is Δ6 desaturase – elongase – Δ5 desaturase – elongase – Δ4 desaturase, but in some species the initial step is elongation to 20:3n-3 followed by Δ8 desaturation. Unlike the situation in vertebrates the last step appears to involve a direct Δ4 desaturation (Meyer et al. 2003; Tonon et al. 2005) rather than the 'Sprecher' shunt operating via 24:5n-3 and 24:6n-3 intermediates.

However, some PUFA that are abundant in some classes of marine microalgae do not fit on the conventional pathway for synthesizing n-3PUFA (e.g. 16:4n-3 and most notably 18:5n-3). Octadecapentaenoic acid ($18{:}5^{\Delta-3,6,9,12,15}$, 18:5n-3) was first reported by Joseph (1975) who found 3.8–22.2% in total lipid from 11 species of marine dinoflagellate. Early studies probably overlooked the presence of 18:5n-3 since on polar GC columns it elutes very close to, or coelutes with, 20:1n-9. It has subsequently been shown to be particularly abundant in prymnesiophytes and dinoflagellates where it may comprise up to 43% of total fatty acid (Volkman et al. 1981; Nichols et al. 1984; Okuyama et al. 1993; Bell et al. 1997) and together with DHA can be the main PUFA. In *Emiliania huxleyi*, eight strains contained 13.7–22.0% 18:5n-3 in the stationary phase, and all cultures showed accumulation of 18:5n-3 during the growth phase (Pond and Harris 1996). This was mainly in digalactosyldiacylglycerol and monogalactosylacylglycerol varying from 33.1–62.1% to 40.9–50.5% of fatty acids, respectively, over the growth cycle (Bell and Pond 1996). Two other algal groups are now known to contain 18:5n-3. Five species of green algae from the *Prasinophyceae* contained 2.2–10.6% of 18:5n-3 in total lipid (Dunstan et al. 1992), while two strains of the raphidophyte *Heterosigma akashiwo* contained 4.6 and 5.2% 18:5n-3 (Nichols et al. 1987).

To the best of our knowledge HUFA synthesis pathways have not been studied in macroalgae/seaweeds. Based on fatty acid compositions, seaweeds can produce ARA and EPA at quite high levels, but generally lack DHA (Sanchez-Machado et al. 2004; Dawczynski et al. 2007). The lack of data precludes any firm conclusions, but the pathways are possibly essentially the same as those found in microalgae of the same group.

### 9.2.3 Protozoans

A protozoan parasite of oysters, *Perkinsus marinus*, has been shown to synthesise LIN and ARA from acetate indicating de novo synthesis of PUFA (Chu et al. 2002). Synthesis of ARA appears to be via a Δ8 pathway, but also involves a $C_{18}$ Δ9-elongating activity catalysed by a FAE1 (*f*atty *a*cid *e*longation *1*)-like 3-ketoacyl-CoA synthase previously only reported in higher plants and algae (Venegas-Caleron et al. 2007). *P. marinus* represents a key organism in the taxonomic separation of the single-celled eukaryotes, the alveolates, and these data imply ancestral endosymbiotic acquisition of plant-like genes. Little is known about PUFA synthesis in other protozoans.

### 9.2.4 Heterotrophic Organisms

The Thraustochytrids are eukaryotic protists in the phylum labyrinthulomycota, the slime nets. They are of interest to the fatty acid biochemist since they produce HUFA-rich oils containing 22:5n-6 and/or DHA. A *Thraustochytrium* sp. was also one of the first organisms in which the presence of a Δ4 desaturase was demonstrated (Qiu et al. 2001). A related *Schizochytrium* sp. has recently been shown to use a polyketide-like pathway for HUFA synthesis (Hauvermale et al. 2006).

### 9.2.5 The Polyketide Pathway and PUFA Synthase

One of the most exciting developments in the field of fatty acid metabolism in recent years was the discovery that PUFA could be synthesized in both prokaryotes and eukaryotes via a completely novel anaerobic pathway using polyketide synthases (Metz et al. 2001). Yazawa (1996) identified five open reading frames[1] (ORFs) from bacterial *Shenawella* sp. strain SCRC2738 found in the intestine of Pacific mackerel that were necessary and sufficient for EPA production in *Escherichia coli.* Metz and colleagues noted that eight of the PUFA-synthesizing domains within the *Shewanella* ORFs were more closely related to polyketide synthase (PKS) than to fatty acid synthase, aerobic desaturases, or elongases (Metz et al. 2001). These included domains of PKS such as acyl carrier protein (ACP), 3-ketoacyl synthase, malonyl-CoA:ACP acyltransferase, 3-ketoacyl-ACP-reductase, chain length factor and acyl transferases. A definitive demonstration of the significance

[1] An open reading frame is the portion of mRNA located between the translation start-code sequence (initiation codon) and the stop-code sequence (termination codon) containing the protein-coding sequence.

of these *Shewanella* ORFs was obtained by heterologous expression in *E. coli* cultured under aerobic and anaerobic conditions. EPA synthesis was found under both conditions ruling out a role for aerobic desaturases and indicating a PKS-like system (Metz et al. 2001).

*Schizochytrium* is a thraustrochytrid-like marine protist that accumulates large amounts of $C_{22}$ HUFA in triacylglycerol (Barclay et al. 1994). Additional support for a PKS-based pathway was provided by sequencing selected clones from a *Schizochytrium* cDNA library. Sequences showing homology to 8 of the 11 domains of the *Shewanella* PKS genes were identified. Further sequencing of cDNA and genomic clones allowed the identification of three ORFs containing domains with homology to those in *Shewanella*. These proteins may constitute a PKS that catalyses DHA and 22:5n-6 synthesis in *Schizochytrium*. The homology between the prokaryotic *Shewanella* and the eukaryotic *Schizochytrium* genes suggests that the PUFA PKS has undergone a lateral gene transfer (Metz et al. 2001). Subsequent work confirmed that the enzyme complex in *Schizochytrium* (renamed PUFA synthase) comprised three genes which account for the production of DHA and 22:5n-6 (Hauvermale et al. 2006). Additionally it was found that the other two abundant fatty acids in *Schizochytrium*, 14:0 and 16:0, were the products of a separate fatty acid synthase which, in terms of sequence homology and domain organisation, resembled those found in fungi (Hauvermale et al. 2006). The presence of this alternative PUFA pathway in *Schizochytrium* was somewhat surprising since a Δ4 desaturase has been cloned from a thraustrochytrid species closely related to *Schizochytrium* showing that the enzymes of the aerobic pathway are also present in these organisms (Qiu et al. 2001).

The polyketide pathway requires six enzyme proteins: 3-ketoacyl synthase (KS), 3-ketoacyl-ACP-reductase (KR), dehydrase (DH), enoyl reductase (ER), dehydratase/2-trans, 3-cis isomerase (DH/2,3I), dehydratase/2-trans and 2-cis isomerase (DH/2,2I). A defined sequence of steps then adds C2 units and double bonds. An important point about the PKS pathway is that it adds double bonds to nascent acyl chains, whereas the desaturase pathway inserts double bonds into intact acyl chains. This makes the PKS pathway more efficient energetically since the ATP used by the desaturase steps in the conventional aerobic pathway is not required. The scheme starts with acetyl-CoA and malonyl-CoA. KS and KR add C2 units while DH and ER or DH/2,3I and DH/2,2I control the positioning of double bonds. Since KS adds two carbon units and double bonds are inserted at three-carbon intervals, two different dehydrase isomerases are required to produce the methylene-interrupted pattern of double bonds characteristic of n-3 and n-6 PUFA (Fig. 9.1).

An extension of the scheme presented by Metz et al. (2001) is shown in Fig. 9.2 illustrating how n-3PUFA could be synthesized by this pathway. The main n-3 pathway goes 16:4n-3, 18:5n-3, 20:6n-3, DHA (Fig. 9.2). The PUFA, ALA, 18:4n-3, 20:4n-3 and EPA do not lie on this pathway (Fig. 9.2). EPA can be produced from 18:5n-3 with a KS, KR, DH, ER step. Omega-6 PUFA are produced by altering the second step in the scheme from KS, KR, DH/2,3I which introduces the Δ3 double bond to give 6:1n-3 to a KS, KR, DH, ER step to give 6:0. The next step then introduces a Δ2 double bond to give 8:1n-6.

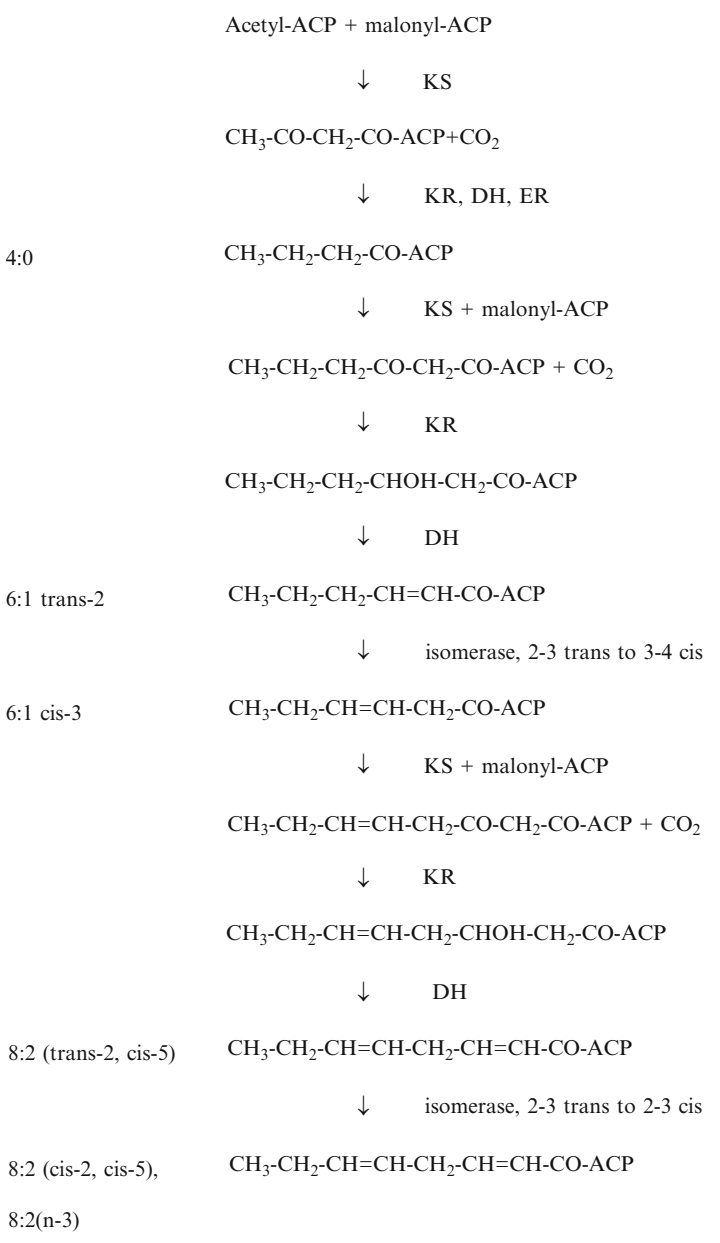


**Fig. 9.1** Fatty acid synthesis using the polyketide pathway (from Metz et al. 2001). *DH* dehydrase; *ER* enoyl reductase; *KR* 3-ketoacyl-ACP-reductase; *KS* 3-ketoacyl synthase

A major question following on from this work is to what extent other marine microorganisms might be synthesizing PUFA via a PKS pathway rather than a conventional desaturase-elongase pathway. Since a Δ3 desaturase was not known, initial suggestions were that 18:5n-3 could be produced by chain-shortening EPA

| | | | |
|---|---|---|---|
| C2 | | | KS = 3-ketoacyl synthase |
| ↓ | KS, KR, DH, ER | | KR = 3-ketoacyl-ACP-reductase |
| C4 | | | DH = dehydrase |
| ↓ | KS, KR, DH/2,3i | | ER = enoyl reductase |
| C6 | 6:1n-3 | Δ3 | DH/2,3i = dehydrase 2-trans, 3-cis isomerase |
| ↓ | KS, KR, DH/2,2i | | DH/2,2i = dehydrase 2-trans, 2-cis isomerase |
| C8 | 8:2n-3 | Δ2, 5 | |
| ↓ | KS, KR, DH, ER | | |
| C10 | 10:2n-3 | Δ4, 7 | |
| ↓ | KS, KR, DH/2,3i | | |
| C12 | 12:3n-3 | Δ3, 6, 9 | |
| ↓ | KS, KR, DH/2,2i | | |
| C14 | 14:4n-3 | Δ2, 5, 8, 11 | |
| ↓ | KS, KR, DH, ER | | |
| C16 | 16:4n-3 | Δ4, 7, 10, 13 | |
| ↓ | KS, KR, DH/2,3i | | |
| C18 | 18:5n-3 | Δ3, 6, 9, 12, 15 | |
| ↓ | KS, KR, DH/2,2i | | |
| C20 | 20:6n-3 | Δ2, 5, 8, 11, 14, 17 | |
| ↓ | KS, KR, DH, ER | | |
| C22 | 22:6n-3 | Δ4, 7, 10, 13, 16, 19 | |

**Fig. 9.2** A scheme showing the synthesis of 22:6n-3 using PKS (adapted from Metz et al. 2001). *DH* dehydrase; *DH/2,2i* dehydratase/2-trans, 2-cis isomerase; *DH/2,3i* dehydratase/2-trans, 3-cis isomerase; *ER* enoyl reductase; *KR* 3-ketoacyl-ACP-reductase; *KS* 3-ketoacyl synthase

(Joseph 1975; Volkman et al. 1981). This hypothesis became more plausible with the discovery that the last step in the synthesis of DHA in mammals is a chain shortening of 24:6n-3 to give DHA (Sprecher 2000). However, 16:4n-3 and 18:5n-3 are mainstream fatty acids on the PKS pathway.

This scheme also answers another problem with algal fatty acid composition, that of PUFA with >22 carbon atoms. These PUFA are under-reported in the literature because most workers stop their GC runs after the elution of DHA on polar-type columns.

However, workers who have used longer time programs have found $C_{26}$ and $C_{28}$ PUFA (e.g. 28:7n-6 and 28:8n-3, Mansour et al. 1999) in some species of marine dinoflagellates. These two fatty acids are on the PKS pathway (Fig. 9.3). Such fatty acids can be derived via conventional elongase, desaturase and chain-shortening steps but this is a cumbersome pathway.

The PKS pathway could thus offer an alternative solution to PUFA synthesis in some species of microalgae to the conventional pathway, but at present there is no direct evidence to support the presence of this pathway in microalgae. However, there have also been difficulties in demonstrating the conventional pathway. The heterotrophic dinoflagellate *Crypthecodinium cohnii* did not convert ALA to DHA, but de novo synthesis from acetate was rapid (Henderson and Mackinlay 1991). The dinoflagellate *Amphidinium carterae* did not convert the deuterated tracer $D_5$-17,17,18,18,18-linolenic acid ($D_5$-ALA) to longer-chain n-3PUFA (M.V. Bell, unpublished). Polyketide-derived metabolites are widespread in bacteria, fungi, microalgae and plants (O'Hagan 1995), and the dinoflagellates produce a wide variety of very complex toxins (e.g. brevetoxins) that are believed to be synthesised by a polyketide pathway (reviewed by Shimizu 1996; see Chap. 4). Thus, some of the PKS pathway enzymes are present in phytoplankton and involved in the synthesis of other metabolites.

Some parts of this pathway are speculative and experiments are needed to confirm the pathway in different organisms, especially those marine flagellates containing 18:5n-3. These could take the form of identifying the full array of necessary genes or identifying fatty acid intermediates. The latter may be difficult since intermediates may not be released from the enzyme protein into the fatty acid pool and would therefore be present in very small amounts.

| | | |
|---|---|---|
| C22 | 22:6n-3 | Δ4, 7, 10, 13, 16, 19 |
| ↓ | KS, KR, DH/2,3i | |
| C24 | 24:7n-3 | Δ3, 6, 9, 12, 15, 18, 21 |
| ↓ | KS, KR, DH/2,2i | |
| C26 | 26:8n-3 | Δ2, 5, 8, 11, 14, 17, 20, 23 |
| ↓ | KS, KR, DH, ER | |
| C28 | 28:8n-3 | Δ4, 7, 10, 13, 16, 19, 22, 25 |

**Fig. 9.3** A scheme showing the synthesis of 28:8n-3 from 22:6n-3 using PKS. *DH* dehydrase; *DH/2,2i* dehydratase/2-trans, 2-cis isomerase; *DH/2,3i* dehydratase/2-trans, 3-cis isomerase; *ER* enoyl reductase; *KR* 3-ketoacyl-ACP-reductase; *KS* 3-ketoacyl synthase

## 9.3 Polyunsaturated Fatty Acid Metabolism in Invertebrates

### *9.3.1 Zooplankton*

Marine copepods are thought to be unable to synthesise HUFA, and growth rates and reproductive success have been linked to the availability of these fatty acids in the phytoplankton (e.g. Pond and Harris 1996). The ability of four species of marine zooplankton to synthesise HUFA from ALA was tested directly using liposomes containing $D_5$-ALA. Female *Calanus finmarchicus, Calanoides acutus, Dropanopus forcipatus* and calyptopus larvae of *Euphausia superba* readily ingested the liposomes and incorporated $D_5$-ALA into their somatic lipid pool, but only negligible amounts of desaturation products were detected after 96-h incubation in *C. finmarchicus, D. Forcipatus and E. superba* with none in *C. acutus* (Bell et al. 2007). It was concluded that these four species were indeed unable to synthesise PUFA at ecologically significant rates under the conditions of this experiment. However, feeding studies have suggested that some other species of copepod may be able to synthesise HUFA from ALA. The harpacticoid copepods *Tisbe holothuriae* and *Tisbe* sp. accumulated substantial amounts of EPA and DHA when fed algae containing small amounts of these fatty acids implying synthesis (Nanton and Castell 1998). The freshwater copepod *Eucyclpos serrulatus* (Desvilettes et al. 1997) accumulated DHA and *Daphnia pulex* (Schlechtriem et al. 2006) accumulated EPA when fed green algae lacking this fatty acid.

### *9.3.2 Other Invertebrates*

There is little information available on PUFA synthesis across the wide range of other aquatic invertebrate genera. The freshwater rotifer *Brachionus plicatilis* was able to synthesise n-3PUFA de novo when fed diets lacking these fatty acids (Lubzens et al. 1985). The sea urchin *Psammechinus miliaris* was able to convert dietary $D_5$-ALA to $D_5$-EPA, but the rate of conversion was very slow, 0.09 μg g tissue$^{-1}$ mg$^{-1}$ ALA eaten over 14 days (Bell et al. 2001a).

There is rather more information available, especially in the older literature, concerning PUFA biosynthesis in terrestrial invertebrates. Twelve species of insect have been identified which can synthesise LIN de novo (e.g. Cripps et al. 1986). *Caenorhabditis elegans* can synthesise ARA and EPA using the pathway as described later for vertebrates, and thus has been used as a model organism in studies of HUFA synthesis in animals (Watts and Browse 1999). It seems likely, therefore, that the ability to synthesise PUFA is widespread in the invertebrate kingdom. Studies are required to determine which pathway is used and the contribution of invertebrates to the aquatic PUFA pool.

## 9.4 Production of Highly Unsaturated Fatty Acids in Fish

### *9.4.1 Pathways for Biosynthesis of Highly Unsaturated Fatty Acids*

Vertebrates, including fish, lack the Δ12 and ω3 (Δ15) desaturases and so cannot form LIN and ALA, respectively, from 18:1n-9 and, therefore, PUFA are essential dietary components. However, dietary LIN and ALA can, with varying efficiencies, be further desaturated and elongated in vertebrates to form HUFA, including ARA, EPA and DHA (Fig. 9.4). Fatty acyl desaturation is an aerobic reaction catalysed by a terminal oxygenase (the 'desaturase') requiring reducing equivalents, derived from NADPH, delivered via an electron transport chain including cytochrome $b_5$ and a reductase. Elongation 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 subsequently hydrogenated in three successive steps. The condensation step determines the substrate specificity and is the rate-limiting step of the process and is therefore regarded as being the 'elongase' enzyme. The main features of the HUFA synthesis pathway, most fully studied in rats (Sprecher 2000), are summarised later.

With one exception, the reactions occur in the smooth endoplasmic reticulum with the same enzymes acting on both n-3 and n-6 fatty acids, although the affinity of the enzymes is generally higher for the n-3 series. DHA rather than EPA is the main end product of desaturation and elongation of ALA, whereas ARA rather than 22:5n-6 is the primary end product of desaturation and elongation of LIN.

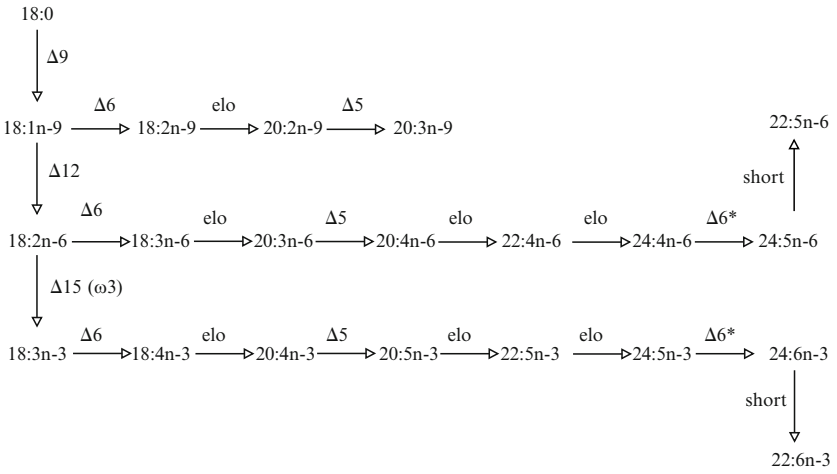


**Fig. 9.4** Pathways of biosynthesis of $C_{20}$ and $C_{22}$ highly unsaturated fatty acids from n-3, n-6 and n-9 $C_{18}$ precursors. Δ5, Δ6, Δ6*, Δ9, Δ12, Δ15 (ω3), fatty acyl desaturases; elo, fatty acyl elongases; short, chain shortening

The insertion of the last, Δ4, ethylenic bond in DHA and 22:5n-6 does not occur through direct Δ4 desaturation of their immediate precursors 22:5n-3 and 22:4n-6. Rather, these intermediates are chain elongated to $C_{24}$ fatty acids, 24:5n-3 and 24:4n-6, which are then converted by Δ6 desaturation to 24:6n-3 and 24:5n-6, respectively. The Δ6-desaturated $C_{24}$ fatty acids are then chain shortened in the peroxisomes to DHA and 22:5n-6. Thus, Δ5 fatty acid desaturation occurs at one step in the pathway, involving 20:3n-6 or 20:4n-3, whereas Δ6 fatty acid desaturation occurs at two steps, first involving LIN or ALA and second involving 24:4n-6 or 24:5n-3. Heterologous expression studies of human and rat Δ6 desaturases showed that the same enzymes are active on $C_{18}$ and $C_{24}$ fatty acids (De Antueno et al. 2001; D'Andrea et al. 2002).

In common with all vertebrates, PUFA are essential dietary components for fish. However, the biologically active and physiologically important PUFA in fish are the HUFA, ARA, EPA and DHA, as LIN and ALA have no specific or unique metabolic role in themselves (Sargent et al. 2002; Tocher 2003). Indeed, the great majority of dietary ALA is catabolised in fish (Bell et al. 2001; Bell and Dick 2004) as it is in mammals. Therefore, the major role of dietary $C_{18}$ PUFA in fish is to function as precursors for $C_{20}$ and $C_{22}$ HUFA, highlighting the importance of the HUFA synthesis pathway. Early nutritional studies suggested that ALA and/or LIN could satisfy the EFA requirements of freshwater fish, whereas the n-3HUFA, EPA and DHA were required to satisfy the EFA requirements of marine fish (see Sargent et al. 2002). Dietary conversion studies performed in turbot using radioactive substrates in vivo strongly suggested that this marine species was unable to produce EPA and ARA from ALA and LIN, respectively, although these experiments were unable to determine precisely the deficiency in the HUFA synthesis pathway (see Tocher 2003).

The HUFA pathway has been most extensively studied in hepatocytes from the salmonids, rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Biochemical studies established the presence of the entire HUFA synthesis pathway from ALA to DHA, including the role of $C_{24}$ intermediates, in rainbow trout hepatocytes (Buzzi et al. 1996, 1997). There is also extensive evidence, based on the conversion of radioisotopes, that EPA and DHA are produced from ALA in hepatocytes from Atlantic salmon (Tocher et al. 1997), and several other species of freshwater fish including Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*) (Tocher et al. 2001a), tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*) (Tocher et al. 2001b). In contrast, studies in hepatocytes from marine fish have consistently shown very little desaturation of ALA occurs with no production of EPA or DHA (Sargent et al. 2002; Tocher 2003). Early studies with cell lines established that the inability of marine fish cells to produce EPA and DHA was due either to limited activities of $C_{18}$ to $C_{20}$ elongase, or fatty acyl Δ5 desaturase (Tocher et al. 1989; Tocher and Sargent 1990). Subsequent studies utilising isotopically labelled substrates for the $C_{18-20}$ elongase ([U-$^{14}$C]18:4n-3 and (D5)18:4n-3), and Δ5 desaturase ([U-$^{14}$C]20:4n-3 and (D5)20:4n-3) showed that a turbot cell line had low $C_{18-20}$ elongase activity (Ghioni et al. 1999), whereas a gilthead sea bream cell line had very low Δ5 desaturase activity (Tocher and Ghioni 1999).

Although results from the cell line studies are entirely consistent with data from feeding studies, they require confirmation by in vivo studies as it is not certain that desaturase and elongase enzymes continue to be expressed in cultured cells exactly as they are in vivo. It is also difficult to extrapolate whole body nutritional and physiological requirements from such in vitro studies. The degree of HUFA synthesis from $C_{18}$ PUFA is dependent upon the activities of fatty acyl desaturases and elongases, and in turn these may be dependent on the extent to which HUFA are readily available in natural diets. For instance, carnivores such as cats, which can obtain abundant preformed HUFA from their natural prey, have a very poor ability to form HUFA and appear to show very low Δ6 and Δ5 desaturase activities (see Sargent et al. 2002). Similarly, marine fish have large amounts of EPA and DHA in their natural diets, whereas the natural prey of many freshwater fish, particularly their invertebrate prey, may be much less rich in n-3HUFA, especially DHA (see Chapter 6 for detailed discussion). Thus, although freshwater fish originally evolved in the ocean, they moved to the terrestrial freshwater ecosystem where conversion of ALA to DHA may be more necessary, whereas marine fish remained in an environment where such conversion is less advantageous.

### *9.4.2 Molecular Biology of Fatty Acyl Desaturases and Elongases*

The biochemistry of HUFA synthesis, including pathways and reaction mechanisms, has been well described for a number of years but, until recently, little was known of the genes and gene products involved and of the factors affecting their expression and function(s), respectively. Significant progress has now been made in characterising the desaturases and elongases involved in HUFA synthesis in animals (Tocher et al. 1998). Full-length cDNAs for Δ6 desaturases have been isolated from the nematode worm *Caenorhabditis elegans* (Napier et al. 1998), rat (Aki et al. 1999), mouse and human (Cho et al. 1999a). Fatty acyl Δ5 desaturase genes have been isolated from *C. elegans* (Michaelson et al. 1998; Watts and Browse 1999) and human (Cho et al. 1999b; Leonard et al. 2000). Genes involved in the elongation of PUFA have been cloned and characterised from *C. elegans* (Beaudoin et al. 2000), human (Leonard et al. 2000), mouse (Leonard et al. 2002) and rat (Inagaki et al. 2002).

Desaturase cDNAs from fish were first cloned from zebrafish and rainbow trout (Hastings et al. 2001; Seiliez et al. 2001). Functional characterisation of the zebrafish desaturase cDNA by heterologous expression in the yeast *Saccharomyces cerevisiae*, which lacks the ability to synthesise HUFA, showed the enzyme to have both Δ6 and Δ5 desaturase activity. Desaturases cloned from other freshwater fish, including rainbow trout and common carp (*Cyprinus carpio*), were shown to be unifunctional Δ6 desaturases (Zheng et al. 2004a). In contrast, separate cDNAs for Δ6 and Δ5 desaturases have been cloned from Atlantic salmon (Hastings et al. 2004; Zheng et al. 2005a), whereas only Δ6 desaturase cDNAs have been cloned from marine fish, including gilthead sea bream (*Sparus aurata*), turbot (*Psetta*

*maximus*) and cod (*Gadus morhua*) (Seiliez et al. 2003; Zheng et al. 2004a; Tocher et al. 2006). Thus, the zebrafish desaturase is unique, being the only bifunctional desaturase involved in HUFA synthesis so far isolated, not only in fish, but, in general, in vertebrates studied to date. Similar to human and rat Δ6 desaturases, zebrafish desaturase also showed a low level of activity towards $C_{24}$ fatty acids indicating that it could be responsible for all three desaturation steps required for the production of DHA. All of the fish desaturases were more active towards the n-3 fatty acid than the equivalent n-6 substrate (Table 9.1).

The fish desaturase cDNAs encode proteins of between 444 and 454 amino acids with the zebrafish and carp desaturase cDNAs encoding proteins of 444 amino acids similar to mammalian desaturases, whereas marine fish desaturase proteins contain 1–3 additional amino acids and salmonids, an additional 8–10 amino acids. The protein sequences of fish desaturases possess all the characteristic features of microsomal fatty acid desaturases, including three histidine boxes, two transmembrane regions and an N-terminal cytochrome $b_5$ domain containing the haem-binding motif, HPGG. Thus, the fish desaturases are fusion proteins presumably containing both desaturase and cytochrome $b_5$ functions. The phylogenetic sequence analyses grouped the fish desaturases largely as expected based on classical phylogeny with the carp and zebrafish (Ostariophysi; cyprinids), trout and salmon (Salmoniformes; salmonidae), and tilapia, sea bream and turbot (Acanthopterygia; cichlids, perciformes and pleuronectiformes) appearing in three distinct clusters with the cod (Paracanthopterygii; Gadiformes) branching from the Acanthopterygia line (Fig. 9.5) (Nelson 1994). The function of Δ6 desaturase in species that do not readily convert ALA to EPA is not known. In evolutionary terms the fact that a functional Δ6 desaturase gene has been retained implies a biologically meaningful function. As the same Δ6 desaturase likely also operates in the pathway from EPA to DHA, it is possible that a functional Δ6 has been retained to enable manipulation and 'fine tuning' of membrane EPA/DHA ratios in fish, such as marine species, that can obtain both fatty acids from the diet, but not necessarily always at optimal ratios.

Although the Δ6 desaturases cloned from marine fish show similar activities in the yeast expression system to the Δ6 desaturases cloned from salmonids and freshwater fish (Zheng et al. 2004a), the Δ6 activities measured in hepatocytes and enterocytes from cod and sea bass are very low (Mourente et al. 2005; Tocher et al. 2006). Gene expression studies investigating tissue distributions may offer an explanation. In Atlantic salmon, the enzymes of HUFA synthesis, Δ6 and Δ5 desaturases and PUFA elongase, are all expressed most highly in liver > intestine > brain, with much lower levels in other tissues (Zheng et al. 2005a). In contrast, in cod, the highest expression of Δ6 desaturase and elongase was in the brain, where expression exceeded that in liver by over 50-fold (Tocher et al. 2006). Thus, the major role for Δ6 desaturase in marine fish may be the maintenance of optimal brain and neural tissue DHA levels by conversion of dietary EPA.

Elongases of HUFA biosynthesis have been cloned from a number of fish species including the freshwater fish zebrafish, carp and tilapia, the salmonids, rainbow trout and anadromous Atlantic salmon, and marine fish, cod, turbot and sea bream (Hastings et al. 2004; Agaba et al. 2004, 2005). The elongase cDNAs encode

**Table 9.1** Functional characterisation of fish fatty acyl desaturase cDNAs

| | Zebrafish Δ6/Δ5 | | Carp Δ6 | | Rainbow trout Δ6 | | Atlantic salmon Δ5 | | Atlantic salmon Δ6 | | Sea bream Δ6 | | Turbot Δ6 | | Atlantic cod Δ6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 |
| Δ6 | 29.4 | 11.7 | 7.1 | 1.5 | 31.5 | 3.6 | 0.6 | 0.4 | 60.1 | 14.4 | 23.1 | 12.2 | 59.5 | 31.2 | 59.5 | 31.2 |
| Δ5 | 20.4 | 8.3 | 0.5 | 0.5 | 0.6 | 0.0 | 10.2 | 0.9 | 2.3 | 0.0 | 1.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| Δ4 | 0.0 | 0.0 | 0.0 | 0.0 | nd | nd | 0.0 | 0.0 | 0.0 | 0.0 | nd | nd | 0.0 | 0.0 | 0.0 | 0.0 |
| Δ6* | ~5 | ~2 | nd | nd | nd | nd | 0.0 | 0.0 | tr | 0.0 | nd | nd | nd | nd | nd | nd |

Results are presented as percentage of substrate fatty acid converted to desaturated product. Fatty acid substrates were Δ6 activity, 18:3n-3 and 18:2n-6; Δ5 activity, 20:4n-3 and 20:3n-6; Δ4 activity, 22:5n-3 and 22:4n-6; Δ6*activity, 24:5n-3 and 24:4n-6

*nd* not determined; *tr* trace

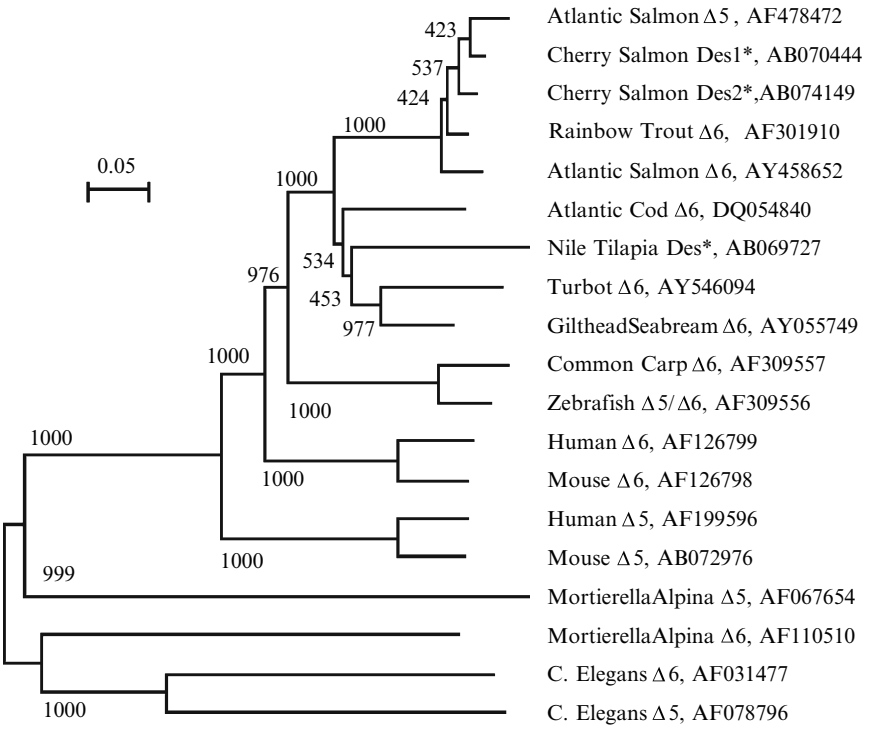


**Fig. 9.5** Phylogenetic tree comparing amino acid sequences of fatty acyl desaturases from fish, mammals, fungus (*Mortierella alpina*) and nematode (*Caenorhabditis elegans*). The tree was constructed using the Neighbour Joining method using *CLUSTALX* and *NJPLOT* (Perrière and Gouy 1996). The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1,000 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized

proteins of 288–294 amino acids that are highly conserved among the fish species. The predicted polypeptides included characteristic features of microsomal elongases, including a single histidine box redox centre motif, a canonical ER retention signal (carboxyl-terminal dilysine targeting signal), multiple transmembrane regions, and KXXEXXDT. QXXFLHXYHH (which contains the histidine box), NXXXHXXNYXYY and TXXQXXQ motifs, which are highly conserved in all PUFA elongases cloned to date (Meyer et al. 2004). Phylogenetic analysis comparing all the elongase sequences cloned from fish, along with a range of elongases from mammals, bird, insect, fungus and nematode, grouped the fish elongases into four clusters largely as expected based on the main groups of modern teleosts identified in classical phylogeny and similar to the grouping observed with desaturases, with the clusters being catfish and zebrafish, cod, trout and salmon, and tilapia, sea bream and turbot (Fig. 9.6). The fish elongases clustered most closely with the mammalian ELOVL5/elovl5 and ELOVL2/elovl2 elongases that have been functionally

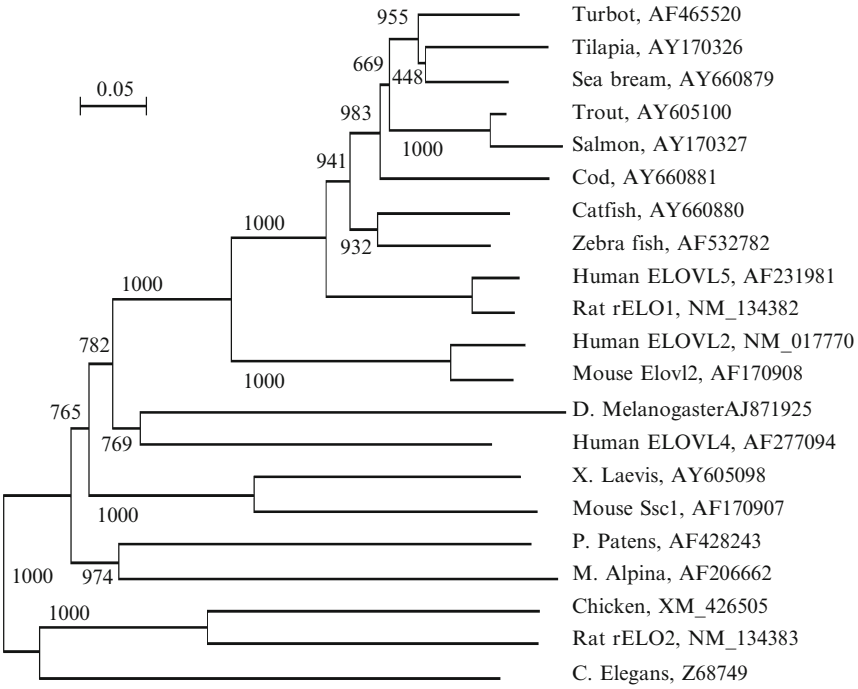


**Fig. 9.6** Phylogenetic tree comparing amino acid sequences of fatty acyl elongases from fish with those of elongases from mammals, bird (chicken, *Gallus gallus*), amphibian (*Xenopus laevis*), insect (*Drosophila melanogaster*), fungus (*Mortierella alpina*), nematode (*Caenorhabditis elegans*) and plant (moss, *Physcomitrella patens*). Tree construction and description as in Fig. 9.5

characterised and shown to be PUFA elongases (Leonard et al. 2000, 2002). They were more distant from other elongases including rat rELO1 (also known as rat Elovl6), that has been shown to be predominantly a 16:0 to 18:0 elongase (Inagaki et al. 2002), and uncharacterised chicken (an elovl6 homologue), human ELOVL4 and mouse Ssc1 elongases that are presumed to be involved in sphingolipid metabolism and the synthesis of very long-chain fatty acids such as 26:0. Heterologous expression in yeast, *S. cerevisiae*, demonstrated that the zebrafish elongase had the ability to lengthen PUFA with chain lengths from $C_{18}$ to $C_{22}$ and also monounsaturated, but not saturated fatty acids. Most of the fish elongases showed a pattern of activity towards PUFA in the rank order $C_{18} > C_{20} > C_{22}$, although the tilapia and turbot elongases had similar activity towards 18:4n-3 and 20:5n-3 (Table 9.2). The fish elongases also generally showed greater or similar activities with n-3 than with n-6 homologues, with the exception of the cod enzyme, which was more active towards n-6 fatty acids. Thus, in fish, a single PUFA elongase gene is all that is required to perform all the elongations required for the full functioning of the HUFA synthesis pathway from ALA to DHA. Whether this will actually be the case can only be speculated when dealing with species with unsequenced genomes. However,

**Table 9.2** Functional characterisation of fish fatty acyl elongase cDNAs

| | Zebrafish | | Catfish | | Tilapia | | Atlantic salmon | | Sea bream | | Turbot | | Atlantic cod | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 | n-3 | n-6 |
| $C_{18–20}$ | 85.4 | 70.7 | 87.5 | 78.3 | 82.6 | 71.2 | 71.8 | 42.1 | 85.9 | 84.3 | 49.0 | 22.8 | 7.4 | 15.7 |
| $C_{20–22}$ | 49.4 | 25.6 | 48.1 | 40.8 | 86.6 | 61.8 | 38.9 | 22.6 | 66.8 | 43.9 | 39.3 | 21.3 | 0.8 | 1.5 |
| $C_{22–24}$ | 4.9 | 0.5 | 2.8 | 0.9 | 1.0 | tr | 0.7 | 0.3 | 4.7 | 0.8 | 0.6 | 0.6 | nd | nd |

Results are presented as percentage of substrate fatty acid converted to elongated product. Fatty acid substrates were $C_{18–20}$ elongase activity, 18:4n-3 and 18:3n-6; $C_{20–22}$ elongase activity, 20:5n-3 and 20:4n-6; $C_{22–24}$ elongase activity, 22:5n-3 and 22:4n-6
*nd* not detected; *tr* trace

for zebrafish, whose genome is largely sequenced, it does appear that only two genes, the bifunctional Δ6/Δ5 desaturase and the single PUFA elongase, are involved in the production of DHA from dietary ALA as no other PUFA desaturases or elongases appear to be present.

### *9.4.3 Regulation of Highly Unsaturated Fatty Acid Biosynthesis in Fish*

Both Δ6 and Δ5 desaturase activities are reported to be under nutritional and/or endocrine control in mammals, although Δ6 desaturase is regarded as the rate-limiting step in the biosynthesis of ARA. Regulation of desaturase activity in mammals may involve transcriptional control of gene expression. Thus, the expression of Δ6 desaturase in liver was increased in mice fed triolein (18:1n-9), an EFA-deficient diet, compared to mice fed corn oil, a diet rich in LIN (Cho et al. 1999a). Similarly, the expression of both Δ6 and Δ5 desaturases was fourfold higher in rats fed a fat-free diet or a diet containing triolein compared to that in rats fed either safflower oil (LIN) or menhaden oil (n-3HUFA) (Cho et al. 1999b).

The expression of HUFA biosynthesis genes in fish has been investigated using a range of different techniques including Northern blotting, quantitative real-time polymerase chain reaction (Q-PCR) and cDNA microarrays. The expression of Δ5 desaturase and elongase genes in liver of salmon, as determined by Q-PCR, could be increased in a graded manner by increasing dietary linseed oil (Zheng et al. 2004b). Expression of both Δ5 desaturase and elongase genes was positively and negatively correlated with dietary ALA and n-3 HUFA, respectively. In salmon fed rapeseed oil (RO), the expression of the Δ5 desaturase and PUFA elongase in liver was determined using a cDNA mini microarray containing around 70 genes involved in lipid metabolism (Jordal et al. 2005). Of the genes in the array, the Δ5 desaturase gene showed the greatest degree of regulation and was one of only three genes that were up-regulated in fish fed RO compared to fish fed fish oil (FO). The up-regulation of the expression of the Δ5 desaturase gene in liver of fish fed RO was confirmed by Q-PCR. Although around 25 other genes were down-regulated in fish fed RO compared to fish fed FO, the expression of the PUFA elongase was unaffected by dietary oil. Recently, the expression of all the genes of HUFA synthesis in salmon fed FO and vegetable oil (VO) was investigated using an Atlantic salmon 17K cDNA microarray. Of the fifteen gene features that showed the strongest regulation, seven were due to fatty acyl desaturases including both Δ6 and Δ5 desaturase sequences included on the array as candidate genes along with three other ESTs that were annotated as Δ6/Δ5 desaturase. The expression of the PUFA elongase was not affected significantly by dietary VO diet in the Jordal et al. (2005) study.

The expression of both Δ6 and Δ5 desaturase genes and the PUFA elongase gene has also been determined by Q-PCR at various points during the entire 2-year production cycle in salmon fed diets containing either FO or a VO blend (Zheng et al.

2005b). Gene expression of Δ6 desaturase was highest around the point of seawater transfer and lowest during the seawater phase. In addition, the expression of both Δ6 and Δ5 desaturase genes was generally higher in fish fed VO compared to fish fed FO, particularly in the seawater phase. The results were consistent with HUFA biosynthesis activity, which peaked around seawater transfer, was lower in seawater, and higher in fish fed VO compared to fish fed FO. The expression of PUFA elongase was also investigated, but no up-regulation was observed. In all the studies of gene expression, the expression of the desaturases was increased by up to twofold in fish fed VO compared with fish fed FO (Zheng et al. 2004b, 2005a, b; Jordal et al. 2005). This compares well with biochemical studies of enzyme activities and HUFA synthesis, which show generally a twofold to threefold increase in activity in fish fed VO at the highest inclusion levels (see Tocher 2003). Taken together, these studies show that both nutritional and environmental modulation of HUFA biosynthesis in Atlantic salmon involves regulation of fatty acyl desaturase gene expression.

### *9.4.4 Use of Stable Isotopes for In Vivo Studies of HUFA Biosynthesis*

Although DHA is a functional EFA in all vertebrates, including fish, there is little quantitative information available on rates of formation of DHA in any species or on the bioequivalence of ALA and DHA. The use of stable isotope tracers and GC–MS has allowed these problems to be addressed at the whole animal level, and has given vital information on nutritional and physiological requirements for PUFA at the organismal level which can be difficult to obtain from in vitro studies on tissues or cells where large extrapolations are necessary. The principle of using stable isotope tracers to investigate n-3PUFA metabolism in fish is straightforward though a number of practical difficulties have to be overcome in relation to diet preparation and presentation to the fish. Administering tracer via the diet is the most natural way of delivery and minimises stress due to handling which may alter lipid metabolism if fish are given tracer by injection.

In summary, the method is to add a known amount of labelled fatty acid to the diet, feed a known amount of diet to the fish, and determine quantitatively the fatty acid profile and tissue distribution of labelled product fatty acids at various times post-dose. The commercially available deuterated fatty acid, $D_5$-ALA, is an ideal tracer for this type of work. The mass increment of five is readily detected and quantitated by GC-negative chemical ionisation MS of the pentafluorobenzyl ester. This methodology was deployed in experiments with trout and gave information on rates of synthesis, which tissues are important in HUFA biosynthesis, changes during development, and tissue distribution of intermediate and product fatty acids. A time course of DHA synthesis from 3 to 35 days showed that synthesis was slow. Whole body accumulation of $D_5$-DHA was linear over the first 7 days corresponding to a rate of 0.54 ± 0.12 μg $D_5$-DHA g wet weight of fish$^{-1}$ mg $D_5$-ALA consumed$^{-1}$ day$^{-1}$ (Bell et al. 2001b). Maximum accretion of $D_5$-DHA was 4.3 ± 1.2 μg g fish$^{-1}$ mg

$D_5$-ALA consumed$^{-1}$ after 14 days. The great majority of the $D_5$-tracer was catabolised with the combined recovery of $D_5$-ALA plus $D_5$-DHA being 2.6%. One of the most significant findings was that the concentration of DHA in the fish decreased during the 13-week period on the experimental diet and the amount of DHA synthesised from ALA was only 5% of that obtained directly from the fish meal in the diet (Bell et al. 2001b), even though DHA synthesis was fully induced under these conditions.

Experiments over a shorter time course showed that pyloric ceca were more active than liver in DHA synthesis, and that the great majority of $D_5$-ALA was catabolised very rapidly (Bell et al. 2003a). Subsequent studies with intestinal enterocytes confirmed directly that they are active in DHA synthesis (Tocher et al. 2002). Deuterated intermediate pathway fatty acids, including 24:5n-3 and 24:6n-3, were identified in liver, ceca, brain and eyes, but while $D_5$-DHA was the main product in liver and ceca, in neural tissue over a longer time course there was a build up of pentaene intermediates $D_5$-EPA and $D_5$-22:5n-3 (Bell et al. 2003b). The results showed that the kinetics of accumulation and depletion of the various n-3 PUFA differ among tissues. The presence of pathway intermediate fatty acids provided further evidence that liver and ceca possess the full metabolic pathway for synthesis of DHA, whereas brain and eyes are less active with an accumulation of pentaene intermediate fatty acids.

During these studies it was noted that smaller fish within each cohort always gave the highest rate of DHA synthesis. A study was undertaken to measure the rate of DHA synthesis in trout from first feeding up to 10 g weight in fish fed either FO or a VO diet consisting of soybean lecithin, linseed oil and high oleic acid sunflower oil providing sufficient LIN and ALA to satisfy EFA requirements (Bell and Dick 2004). It was found that trout fed a VO diet from first feeding (~0.2 g weight) gave an initial rate of 5.4 μg $D_5$-DHA g fish$^{-1}$ mg $D_5$-ALA consumed$^{-1}$ 7 days$^{-1}$ which increased rapidly to a peak activity of ~50 μg $D_5$-DHA g fish$^{-1}$ mg $D_5$-ALA consumed$^{-1}$ 7 days$^{-1}$ at around 1 g weight then declined rapidly to ~12 μg $D_5$-DHA g fish$^{-1}$ mg $D_5$-ALA consumed$^{-1}$ 7 days$^{-1}$ at 2 g weight and continued to fall thereafter. Fish fed a FO diet showed the same pattern, but DHA synthesis was repressed and the rate was approximately ten times lower. Fish fed the VO diet were unable to maintain their DHA concentration, the tissue content falling from 6.2 mg DHA g fish$^{-1}$ to 2.4 by ~5 g weight (Bell and Dick 2004). These results have important implications for the replacement of FO and fish meal in diets by VO.

## 9.5 Concluding Remarks

Aquatic ecosystems produce the majority of long-chain n-3HUFA, and therefore studies of the molecular mechanisms involved in their biosynthesis have not been conducted for purely scientific interest alone. Such studies are of considerable importance to human health, as there is simply not enough n-3HUFA available to meet the human population's dietary requirements (see Chap. 8). Man evolved with

a diet having an n-6:n-3 PUFA ratio of about 1-2:1, but this ratio is now as high as 15–25:1 in the west (Simopoulos 2000). This imbalance is implicated in many pathological conditions, and it is widely accepted that we must reduce the n-6:n-3 ratio in our diet (see Chap. 14), with n-3HUFA being more effective than ALA in balancing excess n-6PUFA (Simopoulos 2000). Fish is the major source of n-3HUFA in our diet but, with a burgeoning human population and declining capture fisheries, we will be increasingly dependent on aquaculture for our supplies of fish and n-3HUFA. The paradox is that, traditionally, aquaculture feeds have used marine raw ingredients, fish meal and oil, themselves rendered from reduction fisheries (Arts et al. 2001). VOs may be sustainable alternatives, but they do not contain n-3HUFA, leaving ALA as the only source of n-3PUFA in plant-based diets (Sargent et al. 2002). Hence the considerable interest in determining pathways for the synthesis of n-3HUFA from ALA in fish with the aim being to develop diets to optimally switch on the genes necessary for EPA and DHA production (Tocher 2003). Transgenics are also an option, and a study has already shown that overexpression of Δ6 activity in zebrafish, transformed with a salmon desaturase, led to modestly increased tissue levels of EPA and DHA (Alimuddin et al. 2005). The transgenic approach may be the only option in marine fish with an apparently incomplete HUFA synthesis pathway. Even if successful, these approaches alone cannot solve our n-3HUFA supply problems as VOs rich in ALA, such as perilla, linseed, camelina and hemp, are currently limiting, with LIN supply in total global fat and oil production exceeding that of ALA by about 24-fold.

In the wild, fish naturally obtain n-3HUFA from the food chain, with microalgae and other unicellular organisms being the major primary producers. Therefore, this is also where we must look for provision of the n-3HUFA in the future. The cloning of genes from marine microalgae and the discovery of the role of the PKS pathway-like PUFA synthase are perhaps the truly exciting advances in this area. However, the marine heterotrophs, *Crypthecodinium cohnii*, a dinoflagellate, and *Schizochytrium sp.*, thraustochytrids, are currently being used to produce DHA for supplementing infant formulae and short shelf-life food products, respectively, through traditional fermentor technology. However, whether fermentation and direct algal culture can be a solution is debatable, and many see the production of EPA and DHA in transgenic oil seed crops as more promising. The molecular tools, the biosynthetic genes, are becoming available and initial transgenic trials have demonstrated the viability of the approach (Abbadi et al. 2004; Robert et al. 2005). However, fluidity and oxidation issues remain significant scientific problems in developing oil seeds capable of storing high levels of HUFA, with consumer acceptance of transgenics a further hurdle. Providing these problems can be overcome, our view is that fish will remain a major source of n-3HUFA in the human food basket, but that fish will be farmed using diets containing transgenic seed oils. In conclusion, studies of the molecular mechanisms of HUFA synthesis in aquatic environments have produced not only a great deal of scientifically highly interesting information, but also a suite of potentially very valuable molecular tools in the form of genes involved in HUFA biosynthesis.

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