

## REVIEW ARTICLE

# Fatty acid requirements in ontogeny of marine and freshwater fish

**Douglas R. Tocher**

Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

**Correspondence:** DR Tocher, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK. E-mail: d.r.tocher@stir.ac.uk

### Abstract

Essential fatty acid (EFA) requirements vary qualitatively and quantitatively with both species and during ontogeny of fish, with early developmental stages and broodstock being critical periods. Environment and/or trophic level are major factors, with freshwater/diadromous species generally requiring C<sub>18</sub> polyunsaturated fatty acids (PUFA) whereas marine fish have a strict requirement for long-chain PUFA, eicosapentaenoic, docosahexaenoic and arachidonic acids. Other than marine fish larvae, defining precise quantitative or semi-quantitative EFA requirements in fish have received less attention in recent years. However, the changes to feed formulations being forced upon the aquaculture industry by the pressing need for sustainable development, namely the replacement of marine fish meal and oils with plant-derived products, have reintroduced EFA into the research agenda. It is particularly important to note that the physiological requirements of the fish to prevent deficiency pathologies and produce optimal growth may not parallel the requirements for maintaining nutritional quality. For instance, salmonids can be successfully cultured on vegetable oils devoid of long-chain n-3 PUFA but not without potentially compromising their health benefits to the human consumer. Solving this problem will require detailed knowledge of the biochemical and molecular basis of EFA requirements and metabolism.

**Keywords:** essential fatty acid, fish, ontogeny, marine, freshwater

### Introduction

All vertebrates have an absolute dietary requirement for certain, specific polyunsaturated fatty acids

(PUFA). If a dietary deficiency occurs, the animal stops growing and reproducing, it develops various pathologies and eventually dies (Das 2006). The PUFA in question are termed 'essential fatty acids' (EFA) and they include members of both the n-6 and n-3 series typified by linoleic acid, 18:2n-6, and  $\alpha$ -linolenic acid, 18:3n-3 (Das 2006). All vertebrate species probably require both n-6 and n-3 PUFA, but the biologically active forms of EFA are generally the C<sub>20</sub> and C<sub>22</sub> metabolites of 18:2n-6 and 18:3n-3, specifically 20:4n-6 [arachidonic acid (ARA)], 20:5n-3 [eicosapentaenoic acid (EPA)] and 22:6n-3 [docosahexaenoic acid (DHA)], which in aquaculture are often termed highly unsaturated fatty acids (HUFA, PUFA with  $\geq$  C<sub>20</sub> and  $\geq$  3 double bonds). Vertebrate species have varying abilities to convert C<sub>18</sub> PUFA to the C<sub>20</sub> and C<sub>22</sub> HUFA and so in species that cannot perform these conversions, the C<sub>20</sub> and C<sub>22</sub> HUFA themselves are dietary EFA and their C<sub>18</sub> homologues do not satisfy EFA requirements. In species that can perform the conversions, C<sub>18</sub>, C<sub>20</sub> and C<sub>22</sub> HUFA can all be termed EFA with the C<sub>20</sub> and C<sub>22</sub> HUFA often being more effective nutritionally than their C<sub>18</sub> counterparts. Definition of the optimal amounts of the correct EFA to satisfy the requirements for normal growth and development has, in the past, been one of the most studied areas of lipid metabolism in fish. This has been driven by the needs of the rapidly developing aquaculture industry. Of particular importance, these requirements can vary quantitatively during the ontogeny of fish. Therefore, accurate definition of EFA requirements for a given fish species involves determining not only the absolute requirements of specific PUFA, but also the optimal balance between different PUFA and how these requirements vary at different life stages. Essential fatty acid

requirements, particularly in marine larval fish, have been the subject of several earlier reviews (Sargent, Bell, Bell, Henderson & Tocher 1995a; Izquierdo 1996; Sargent, McEvoy & Bell 1997; Sargent, Bell, McEvoy, Tocher & Estevez 1999; Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher 1999) and a very comprehensive review of EFA in aquacultured species has very recently been published (Glencross 2009).

### Roles of EFA and deficiency symptoms

Like all fatty acids, PUFA can serve as important sources of cellular energy and this is independent of any role they may have as EFA, and so in high dietary concentration all fatty acids are used as energy sources (Tocher 2003). Thus, the extent to which any fatty acid is utilized for energy is largely dependent upon its dietary concentration with possibly two exceptions, the major C<sub>22</sub> fatty acids, 22:1n-11 and DHA. Irrespective of dietary concentration, 22:1n-11 tends to be a highly oxidized, whereas DHA tends to be conserved, primarily due to it being a relatively poor substrate for  $\beta$ -oxidation (Sargent, Tocher & Bell 2002). However, it was recently shown that even DHA can have relatively low retention when fed in high concentrations (Stubhaug, Lie & Torstensen 2007). Large amounts of PUFA are also required for cellular membrane structure and function, as they are integral elements of phospholipids that are the fundamental components of lipid bilayers. This is particularly the case with DHA that has important structural and functional roles in all membranes, but especially neural membranes (Feller 2008; Wassell & Stillwell 2008). However, small but important amounts of PUFA, particularly C<sub>20</sub> PUFA, have unique roles in controlling and regulating cellular metabolism and animal physiology. Central to this role is the regulated, dioxygenase-catalysed oxidation of ARA and EPA to produce highly bioactive eicosanoids, autocrine hormones with a short half-life produced by cells to act in their immediate vicinity (Schmitz & Ecker 2008). Almost all tissues produce eicosanoids, and they have a wide range of physiological actions in blood clotting, immune and inflammatory responses, cardiovascular tone, renal and neural functions, and reproduction (Schmitz & Ecker 2008).

Long-term absence of EFA from the diet leads to deficiency symptoms that, in fish, most often include reduced growth and increased mortality (Glencross 2009). Other pathologies that have been noted

include myocarditis, pale/swollen (fatty) liver, intestinal steatosis, fin erosion, bleeding from gills, lordosis, reduced reproductive potential and shock syndrome (Sargent *et al.* 2002; Glencross 2009). Only certain PUFA can prevent the occurrence of these deficiency symptoms, and so satisfy the criteria for essential nutrients and thus be classified as EFA. Therefore, prevention of deficiency pathologies can be regarded as the fundamental test of whether a fatty acid is an EFA for a given species.

### Quantitative requirements during ontogeny

Well over 40 years ago it was appreciated that n-3 PUFA, including both 18:3n-3 and HUFA, were required for optimal growth and prevention of signs of EFA deficiency in rainbow trout, *Ocorhynchus mykiss* (Walbaum) (Higashi, Kaneko, Ushiyama & Sugihashi 1964). However, it was John Castell working on rainbow trout in Lee's group in the early 1970s who first provided detailed descriptions of the effects and symptoms of EFA deficiency, and the first accurate estimation of quantitative requirements (Castell, Sinnhuber, Wales & Lee 1972a, b; Castell, Lee & Sinnhuber 1972). Since then there have been around 50 publications that have contributed to defining quantitative and semi-quantitative requirements in about 30 species of fish (see Tables 1–3). Very few of these have been in the last 10 years when work has focussed more on larval marine fish and the relative requirements of ARA, EPA and DHA (Lund, Steenfeldt & Hansen 2007; Hamre & Harboe 2008a, b; Lund, Steenfeldt, Banta & Hansen 2008). Therefore, there appears less desire to determine absolute EFA requirements, particularly in juvenile and sub-adult fish. There are probably two main reasons. Firstly, the experiments are not easy and costly. Diets completely devoid of EFA are required and these have to be formulated with lipid-free ingredients and fish generally find these less palatable, and a regression structure should be used requiring a reasonable number of tanks. Secondly however, there is now probably sufficient information on a wide enough range of species to enable us to be quite confident in predicting both qualitative and quantitative requirements for any new species of interest (Sargent *et al.* 2002; Tocher 2003). One factor important to note when discussing quantitative requirements is that there is possibly more than one requirement level. The minimal requirement level is the amount of EFA required

**Table 1** Quantitative essential fatty acid (EFA) requirements of juvenile and sub-adult freshwater and diadromous fish species

Species	EFA	Requirement (% dry diet)	Reference
Rainbow trout	<i>Oncorhynchus mykiss</i> (Walbaum)	18:3n-3 n-3HUFA	Castell <i>et al.</i> (1972a) Takeuchi & Watanabe (1976)
Chum salmon	<i>O. keta</i> (Walbaum)	18:2n-6 & 18:3n-3	1.0 of each Takeuchi <i>et al.</i> (1979)
Coho salmon	<i>O. kisutch</i> (Walbaum)	18:2n-6 & 18:3n-3	1.0 of each Yu & Sinnhuber (1979)
Cherry salmon	<i>O. masou</i> (Brevoort)	18:3n-3 or n-3HUFA	1.0 Thongrod <i>et al.</i> (1990)
Atlantic salmon	<i>Salmo salar</i> L.	18:3n-3 n-3HUFA	1.0 Ruyter <i>et al.</i> (2000a) Ruyter <i>et al.</i> (2000b)
Arctic charr	<i>Salvelinus alpinus</i> (L.)	18:3n-3	1.0–2.0 Yang <i>et al.</i> (1994)
Common carp	<i>Cyprinus carpio</i> L.	18:2n-6 18:3n-3	1.0 Takeuchi & Watanabe (1977) Takeuchi & Watanabe (1977)
Grass carp	<i>Ctenopharyngodon idella</i> Valenciennes	18:2n-6 18:3n-3	1.0 Takeuchi <i>et al.</i> (1991) Takeuchi <i>et al.</i> (1991)
Tilapia	<i>Tilapia zilli</i> (Gervais) <i>Oreochromis nilotica</i> L.	18:2n-6 18:2n-6	1.0 Takeuchi <i>et al.</i> (1983) Takeuchi <i>et al.</i> (1983)
Eel	<i>Anguilla japonicus</i> Temminck & Schlegel	18:2n-6 & 18:3n-3	0.5 of each Takeuchi <i>et al.</i> (1980)
Ayu	<i>Plecoglossus altivelis</i> Temminck & Schlegel	18:3n-3 or EPA	1.0 Kanazawa <i>et al.</i> (1982)
Milkfish	<i>Chanos chanos</i> (Forsskål)	18:2n-6 & 18:3n-3	0.5 of each Bautista & de la Cruz (1988)
Channel catfish	<i>Ictalurus punctatus</i> (Rafinesque)	18:3n-3 n-3HUFA	1.0–2.0 Sato <i>et al.</i> (1989) Sato <i>et al.</i> (1989)
Whitefish	<i>Coregonus laveratus</i> (L.)	18:3n-3 n-3HUFA	> 1.0 Watanabe <i>et al.</i> (1989) Watanabe <i>et al.</i> (1989)
Sheatfish	<i>Silurus glanis</i> L.	18:3n-3	1.0 Borgut <i>et al.</i> (1998)
Striped bass	<i>Morone chrysops</i> (Rafinesque) x <i>M. saxatilis</i> (Walbaum)	n-3HUFA	1.0 Gatlin <i>et al.</i> (1994)

HUFA, highly unsaturated fatty acids; EPA, eicosapentaenoic acid.

**Table 2** Quantitative essential fatty acid (EFA) requirements of juvenile and sub-adult marine fish species

Species	EFA	Requirement (% dry diet)	Reference
Turbot	<i>Psetta maxima</i> (L.)	n-3HUFA ARA	Gatesoupe <i>et al.</i> (1977) Castell <i>et al.</i> (1994)
Red seabream	<i>Pagrus major</i> (Temminck & Schlegel)	n-3HUFA or EPA EPA DHA	0.5 1 0.5 Takeuchi <i>et al.</i> (1990) Takeuchi <i>et al.</i> (1990)
Gilthead seabream	<i>Sparus aurata</i> L.	n-3HUFA n-3HUFA DHA:EPA DHA	0.9 (DHA:EPA = 1) 1.9 (DHA:EPA = 0.5) 0.5 1.7 Takeuchi <i>et al.</i> (1992)
Striped jack	<i>Pseudocaranx dentex</i> (Bloch & Schneider)	n-3HUFA n-3HUFA	1.0 2.5 Takeuchi (1997)
Sea bass	<i>Dicentrarchus labrax</i> L.	n-3HUFA	1.0 Coutteau <i>et al.</i> (1996)
Yellowtail flounder	<i>Pleuronectes ferrugineus</i> (Storer)	n-3HUFA	2.5 Whalen <i>et al.</i> (1999)
Japanese flounder	<i>Paralichthys olivaceus</i> (Temminck & Schlegel)	n-3HUFA	1.4 Takeuchi (1997)
Starry flounder	<i>Platichthys stellatus</i> (Pallas)	n-3HUFA	0.9 Lee <i>et al.</i> (2003)
Silver bream	<i>Rhabdosargus sarba</i> (Forsskål)	n-3HUFA	1.3 Leu <i>et al.</i> (1994)
Korean rockfish	<i>Sebastes schlegelii</i> Hilgendorf	n-3HUFA EPA or DHA	0.9 1.0 Lee <i>et al.</i> (1993) Lee <i>et al.</i> (1994)
Red drum	<i>Sciaenops ocellatus</i> (L.)	n-3HUFA EPA+DHA	0.5–1.0 0.3–0.6 Lochmann & Gatlin (1993) Lochmann & Gatlin (1993)

HUFA, highly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

**Table 3** Quantitative essential fatty acid (EFA) requirements of larval and early juvenile fish

Species		EFA	Requirement (% dry diet)	Reference
<i>Freshwater</i>				
Common carp	<i>C. carpio</i>	n-6PUFA	1.0 (0.25% 18:2n-6)	Radunzneto <i>et al.</i> (1996)
		n-3PUFA	~ 0.05	Radunzneto <i>et al.</i> (1996)
Rainbow trout	<i>O. mykiss</i>	DHA essential	?	Wirth <i>et al.</i> (1997)
Striped bass	<i>M. chrysops</i> x <i>M. saxatilis</i>	18:3n-3	?	Webster & Lovell (1990)
		n-3HUFA	< 0.5%	Webster & Lovell (1990)
<i>Marine</i>				
Atlantic cod	<i>Gadus morhua</i> L.	EPA	?	Zheng <i>et al.</i> (1996)
		DHA	~ 1.0	Takeuchi <i>et al.</i> (1994)
Gilthead seabream	<i>S. aurata</i>	n-3HUFA	5.5 (DHA:EPA = 0.3)	Rodriguez <i>et al.</i> (1994a)
		n-3HUFA	1.5 (DHA:EPA = 2.0)	Rodriguez <i>et al.</i> (1998a)
		n-3HUFA	1.5 (in phospholipid)	Salhi <i>et al.</i> (1999)
		DHA:EPA	~ 2	Rodriguez <i>et al.</i> (1994b)
Red seabream	<i>P. major</i>	n-3HUFA	2.1 (with 1.0 DHA)	Furuita <i>et al.</i> (1996a)
		DHA	1.0–1.6	Furuita <i>et al.</i> (1996a)
		EPA	2.3	Furuita <i>et al.</i> (1996a)
Striped jack	<i>P. dentex</i>	DHA	1.6–2.2	Takeuchi <i>et al.</i> (1996)
		EPA	< 3.1	Takeuchi <i>et al.</i> (1996)
Yellowtail	<i>Seriola quinqueradiata</i> Temminck & Schlegel	n-3HUFA	3.9 (DHA:EPA = 0.5)	Furuita <i>et al.</i> (1996b)
		DHA	1.4–2.6	Furuita <i>et al.</i> (1996b)
		EPA	3.7	Furuita <i>et al.</i> (1996b)
Mahimahi	<i>Coryphaena hippurus</i> L.	n-3HUFA	0.6–1.0	Ostrowski & Kim (1993)
Turbot	<i>P. maxima</i>	DHA required	?	Reitan <i>et al.</i> (1994)

PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

in the diet to prevent deficiency pathologies. However, further increments in dietary EFA level may improve growth and survival and so optimal levels could then be defined. Few studies have reported minimal and optimal levels and, as growth and survival have been the predominant criteria used, it could be argued that many quantitative and semi-quantitative requirements reported may be more optimal than true minimal requirements. Requirements also vary with developmental and, possibly physiological, stage, of the fish and probably also with lipid content of the diet further complicating the definition of absolute quantitative requirements (Sargent *et al.* 2002).

#### Juveniles and sub-adults of freshwater and diadromous species

The EFA requirements estimated for juveniles and sub-adults of the freshwater and diadromous fish species so far studied indicate that the EFA require-

ments can generally be satisfied by the C<sub>18</sub> PUFA, 18:3n-3 and/or 18:2n-6, at around 1% of the diet dry weight. Table 1 indicates that freshwater species can fall into three main categories, cold water species including salmonids that require mainly 18:3n-3, warm water species such as tilapia that mainly require mainly 18:2n-6 and species that require significant amounts of both such as channel catfish *Ictalurus punctatus* (Rafinesque) and common carp *Cyprinus carpio* L. Although the C<sub>18</sub> PUFA are usually effective in satisfying the EFA requirements of freshwater fish, with some species, including salmonids, n-3HUFA can satisfy the EFA requirements at lower levels than 18:3n-3 and can increase growth over that obtained with 18:3n-3 alone (Sargent, Henderson & Tocher 1989). A similar effect was observed in channel catfish with growth significantly improved by inclusion of dietary n-3 HUFA (Santha and Gatlin 1991). Surprisingly, requirements of freshwater fish for n-6 HUFA, specifically ARA, have received little attention (Bell & Sargent 2003).

### Juveniles and sub-adults of marine species

In contrast to freshwater species, studies on juvenile and sub-adult marine fish indicate that the EFA requirements cannot be met by C<sub>18</sub> PUFA and that the n-3 HUFA, EPA and DHA, are required (Table 2). For juveniles of several species including turbot *Psetta maxima* (L.), red sea bream *Pagrus major* (Temminck & Schlegel), European sea bass *Dicentrarchus labrax* L., red drum *Sciaenops ocellatus* (L.) and Korean rockfish *Sebastes schlegelii* Hilgendorf, the EFA requirements can be met by levels of n-3 HUFA of less than or up to 1% of the dry weight of the diet. Other species including silver bream *Rhabdosargus sarba* (Forsskal), striped jack *Pseudocaranx dentex* (Bloch & Schneider) and yellowtail flounder *Pleuronectes ferrugineus* (Storer) appear to require levels of n-3 HUFA > 1%. Similar to freshwater fish, the quantitative requirement for n-6 HUFA, specifically ARA, has not been fully determined in marine fish (Bell & Sargent 2003). However, trials with turbot were consistent with the hypothesis that ARA was essential and a value of around 0.3% of the dry weight of the diet was estimated in weaned fish (Castell, Bell, Tocher & Sargent 1994; Bell, Castell, Tocher, MacDonald & Sargent 1995). Quantitative EFA requirements of juvenile marine fish have been shown to vary with dietary lipid levels and the dietary DHA:EPA ratio (Kalogeropoulos, Alexis & Henderson 1992; Ibeas, Izquierdo & Lorenzo 1994; Ibeas, Cejas, Fores, Badia, Gomez, Lorenzo & Hernandez 1997). Thus, with a dietary ratio of 0.5 for DHA:EPA the requirement for total n-3 HUFA was around 1.9% of the diet, whereas at a DHA:EPA ratio of 1 the requirement was only 0.9% of the diet, consistent with DHA generally having a higher EFA value for fish than EPA (Kalogeropoulos *et al.* 1992; Watanabe 1993; Ibeas *et al.* 1994).

### Early life stages – larvae and fry

There are few data on the EFA requirements of early life stages of freshwater fish species (Table 3). This is because feeding newly hatched larvae or fry is not normally a problem with freshwater fish as they are generally large enough to accept formulated feeds whose composition can be defined to ensure maximal growth and survival and, consequently, the freshwater aquaculture industries have been very successful in rearing high-quality fry. However, there is evidence that n-3 HUFA and DHA may be more important and, possibly, essential in larvae of some species of freshwater fish compared with adults

or juveniles (Webster and Lovell 1990; Wirth, Steffens, Meinelt & Steinberg 1997). In contrast, the small size and often poorly developed digestive system of marine fish larvae has had a major consequence with respect to defining their precise EFA requirements (Izquierdo, Socorro, Arantzamendi & Hernandez-Cruz 2000; Conceição, Morais & Rønnestad 2007; Yufera & Darias 2007). Formulated first feeds such as defined micro-diets have been difficult and slow to develop necessitating the use of live feeds (Cahu & Zambonino-Infante 2001; Koven, Kolkovski, Hadas, Gamsiz & Tandler 2001; Robin & Vincent 2003; Kvale, Yufera, Nygard, Aursland, Harboe & Hamre 2006). However, the preferred live feeds such as rotifers and *Artemia*, although convenient, are nutritionally inadequate for marine fish being relatively poor in HUFA and so enrichment processes are required. Despite much work it is still difficult to enrich live feeds to provide adequately balanced levels of HUFA and, in particular, sufficient DHA. The details of marine larval fish nutrition, live feeds and enrichments is covered elsewhere in this volume and will not be discussed further here (Conceição, Yufera, Makridis, Morais & Dinis 2009).

Using a combination of enriched live feeds and fabricated microdiets the quantitative and semi-quantitative EFA requirements of the larval and very early juvenile stages of various marine fish species have been determined (Table 3). As noted above, the absolute values reported can vary dependent upon the criteria measured, such as survival, growth and vitality, as well as dietary lipid level (Salhi, Izquierdo, Hernandez-Cruz, Gonzalez & Fernandez-Palacios 1994; Furuita, Takeuchi, Watanabe, Fujimoto, Sekiya & Imaizumi 1996b). In general, larvae are characterized by having a greater requirement for n-3 HUFA than juveniles and pre-adult fish, although there are relatively few species where the requirements at larval and juvenile stages can be directly compared (Tables 1–3). Furthermore, the relative proportions of different EFA are more important in larval marine fish with the absolute requirement for n-3 HUFA varying with DHA:EPA ratio such that the requirement decreases with increasing DHA:EPA ratio (Rodriguez, Perez, Izquierdo, Mora, Lorenzo & Fernandez-Palacios 1994; Rodriguez, Perez, Badia, Izquierdo, Fernandez-Palacios & Hernandez 1998). Thus EFA requirements are often satisfied by a lower level of DHA than can be achieved with EPA (Watanabe 1993), with the higher efficacy of DHA related to its role in the rapidly developing visual and neural tissues, which account for a relatively greater propor-

tion of total body mass in larval stages (Sargent *et al.* 2002). As for n-6 HUFA, ARA has been shown to influence growth in larval gilthead sea bream *Sparus aurata* L. (Rodriguez, Perez, Izquierdo, Mora *et al.* 1994) and, at a fixed dietary n-3 HUFA level and DHA:EPA ratio, ARA up to 1.5% and 1% of the dry weight of the diet was found to improve growth in larval sea bream (Bessonart, Izquierdo, Salhi, Hernandez-Cruz, Gonzalez & Fernandez-Palacios 1999) and Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel) (Estevez, Ishikawa & Kanazawa 1997). Dietary ARA also improved survival after handling stress in sea bream larvae, particularly when fed before the stress rather than when fed after (Koven, Barr, Lutzky, Ben-Atia, Weiss, Harel, Behrens & Tandler 2001). In contrast, yellowtail flounder larvae required diets highly enriched in DHA whereas high dietary ARA inhibited growth, increased mortality and had negative effects on pigmentation (Ishizaki, Takeuchi, Watanabe, Arimoto & Shimizu 1998).

Metamorphosis in marine flatfish including pigmentation and eye migration is an area that has received much attention in recent years in relation to EFA and, in particular the role of ARA (Lund *et al.* 2007, 2008). Decreased n-3 HUFA, and increased ARA and ARA:EPA have been associated with malpigmentation (and impaired eye migration) and so there has been increasing focus on dietary DHA:EPA:ARA ratios (Villalta, Estevez & Bransden 2005; Hamre & Harboe 2008a, b). There are critical periods during the pre-metamorphic stages when EFA are particularly important in absolute and relative terms as well as duration of feeding although these are all species dependent. Thus, the early supply of DHA was essential for correct pigmentation in turbot (Reitan, Rainuzzo & Olsen 1994) and pigmentation success was related to dietary ARA in sole *Solea solea* (L.) (Lund *et al.* 2008), and HUFA levels, including ARA, in neural tissues in Japanese flounder (Estevez and Kanazawa 1996; Estevez *et al.* 1997). Further studies in turbot showed that ARA levels in neural tissue lipids were negatively correlated with pigmentation and that the optimum dietary EPA level was more dependent on dietary ARA than DHA level, emphasizing the importance of dietary DHA:EPA:ARA ratios (Estevez, McEvoy, Bell & Sargent 1999). Therefore, there is increasing evidence for the essentiality of dietary ARA for optimal growth and development of marine fish larvae although quantitative requirements have not been fully established and excess can cause problems at metamorphosis (Rodriguez, Perez, Izquierdo, Mora *et al.* 1994; Ishizaki *et al.* 1998;

Bessonart *et al.* 1999; Estevez *et al.* 1999; Hamre, Holen & Moren 2007; Lund *et al.* 2007, 2008).

### Broodstock

The above has shown that quantitative and qualitative EFA requirements are important for marine fish larvae from first feeding. Studies have also shown that egg quality criteria, including hatching and fertilization rates, and early survival, were positively correlated with increased levels of n-3HUFA and ARA in gilthead sea bream (Harel, Tandler, Kissil & Applebaum 1992; Fernandez-Palacios, Izquierdo, Robaina, Valencia, Salhi & Vergara 1995; Rodriguez, Cejas, Martin, Badia, Samper & Lorenzo 1998), Atlantic cod *Gadus morhua* L. (Pickova, Dutta, Larsson & Kiessling 1997; Salze, Tocher, Roy & Robertson 2005) and European sea bass (Bruce, Oyen, Bell, Asturiano, Farndale, Carrillo, Zanuy, Ramos & Bromage 1999). Furthermore, the DHA:EPA ratio in eggs was positively correlated with quality criteria (Pickova *et al.* 1997). The fatty acid compositions of eggs are generally more conserved and relatively less influenced by diet than other fish tissues, reflecting the importance of specific compositions in the gametes (Sargent *et al.* 2002). However, many studies have shown that egg fatty acid compositions can be affected by broodstock diets in various species, including gilthead sea bream (Fernandez-Palacios *et al.* 1995; Almansa, Perez, Cejas, Badia, Villamandos & Lorenzo 1999), European sea bass (Bell, Farndale, Bruce, Navas & Carillo 1997), striped jack (Vassallo Agius, Watanabe, Mushiake, Kawano & Satoh 1998), Atlantic cod (Silversand, Norberg, Holm, Lie & Haux 1995), yellowtail (Verakunpiriya, Watanabe, Mushiake, Kiron, Satoh & Takeuchi 1996), Eurasian perch *Perca fluviatilis* L. (Abi-ayad, Melard & Kestemont 1997) and Nile tilapia *Oreochromis nilotica* L. (Santiago & Reyes 1993). This clearly indicates that broodstock nutrition is vital in both marine and freshwater fish to producing high-quality eggs and larvae with EFA contents optimized to give the developing embryos and larvae the best chance of success at a time of increased EFA requirement (Tandler, Harel, Koven & Kolkovski 1995; Izquierdo, Fernandez-Palacios & Tacon 2001).

### Qualitative requirements – evolutionary, biochemical and molecular mechanisms

Although trophic level (piscivorous/carnivorous vs. herbivorous) may also play a role, the above section

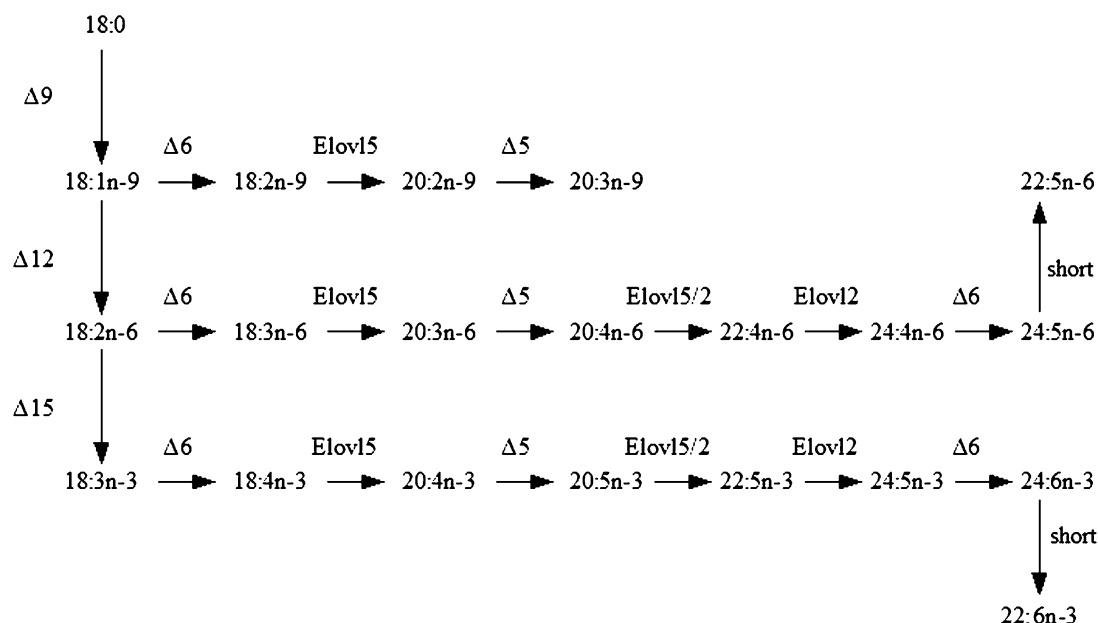
generally showed that qualitative EFA requirements vary with environment (freshwater vs. marine) (Sargent *et al.* 2002). It is likely that this is an evolutionary adaptation to the availability of fatty acids in the different environments. Polyunsaturated fatty acids originate in aquatic food webs, as in all ecosystems, in the primary producers, essentially the plants. Marine phytoplankton produce high levels of the n-3 HUFA, EPA and DHA, whereas in freshwater, phytoplankton are characterized by higher levels of 18:2n-6 and 18:3n-3, with reasonable EPA, but generally low DHA (Sargent, Bell, Bell, Henderson & Tocher 1995b). Thus, marine fish inhabit an environment rich in HUFA and so have had no evolutionary pressure to retain the ability to endogenously produce them, whereas the lower level of HUFA, particularly DHA, has maintained this pressure in freshwater fish. Biosynthesis of HUFA in vertebrates, including fish, involves sequential desaturation and elongation of 18:2n-6 and 18:3n-3 (Cook 1996). Synthesis of ARA is achieved by  $\Delta 6$  desaturation of 18:2n-6 to produce 18:3n-6 that is elongated to 20:3n-6 followed by  $\Delta 5$  desaturation (Fig. 1). Synthesis of EPA from 18:3n-3 uses the same enzymes and pathway as for ARA, but DHA synthesis requires two further elongation steps, a second  $\Delta 6$  desaturation and a chain shortening step (Sprecher 2000). Thus, the ability of any species to convert  $C_{18}$  PUFA to HUFA is associated with their complement of fatty acyl desaturase and elongase enzymes. Essential fatty acid requirements indicate that freshwater fish are capable of producing the biologically active HUFA from  $C_{18}$  PUFA, and so must express all the biosynthetic activities necessary, whereas marine fish cannot (Tocher 2003).

There is biochemical and molecular evidence to support the above hypothesis. Early *in vivo* studies using radioactive fatty acids suggested that EPA and ARA were produced from 18:3n-3 and 18:2n-6, respectively, in the freshwater rainbow trout, but not in the marine turbot (Owen, Adron, Middleton & Cowey 1975). More recent studies with fish cell lines indicated that a turbot cell line (TF) had low  $C_{18}$ – $C_{20}$  fatty acyl elongase activity, whereas a gilthead sea bream cell line (SAF-1) had very low  $\Delta 5$  fatty acyl desaturase activity (Ghioni, Tocher, Bell, Dick & Sargent 1999; Tocher & Ghioni 1999). These biochemical studies established that deficiencies in the HUFA synthesis pathway likely account for the differences in EFA requirements between marine and freshwater fish.

Fish fatty acyl desaturases were first cloned from zebrafish *Danio rerio* (Hamilton) and rainbow trout (Hastings, Agaba, Tocher, Leaver, Dick, Sargent &

Teale 2001; Seiliez, Panserat, Kaushik & Bergot 2001). The zebrafish desaturase was shown to be a unique enzyme, possessing both  $\Delta 6$  and  $\Delta 5$  desaturase activities and is so far the only such bifunctional desaturase isolated from any vertebrate. In contrast, mammals possess separate genes for  $\Delta 5$  and  $\Delta 6$  desaturases (Marquardt, Stohr, White & Weber 2000), and separate cDNAs for unifunctional  $\Delta 6$  and  $\Delta 5$  desaturases have been cloned from Atlantic salmon *Salmo salar* L. indicating the presence of multiple desaturase genes in some fish species (Hastings, Agaba, Tocher, Zheng, Dickson, Dick & Teale 2005; Zheng, Tocher, Dickson, Bell & Teale 2005). However, the zebrafish desaturase also showed low activity towards  $C_{24}$  PUFA indicating that it could function at two steps in the HUFA synthesis pathway, consistent with it being the only PUFA desaturase represented in the zebrafish genome (Fig. 1). Desaturase cDNAs from other freshwater fish, including rainbow trout and common carp encode unifunctional  $\Delta 6$  desaturases (Zheng, Seiliez, Hastings, Tocher, Panserat, Dickson, Bergot & Teale 2004). Despite considerable efforts in our own, and other, laboratories, to date only  $\Delta 6$  desaturase cDNAs have been cloned from marine fish, including gilthead sea bream, turbot and Atlantic cod (Seiliez, Panserat, Corraze, Kaushik & Bergot 2003; Zheng, Seiliez *et al.* 2004; Tocher, Zheng, Schlechtriem, Hastings, Dick & Teale 2006). The presence of genes for  $\Delta 5$  and  $\Delta 6$  desaturase in Atlantic salmon, and of a bifunctional  $\Delta 5/\Delta 6$  desaturase in the zebrafish, is consistent with the biochemical evidence for the production of DHA from 18:3n-3 in these species. Similarly, the inability of marine species to produce DHA could be explained by the lack of a  $\Delta 5$  desaturase gene, a deficiency that has little consequence in the HUFA-rich marine ecosystem. In contrast, the bi-functional zebrafish gene and multiple sub-functionalized salmon genes have enabled these species, which spend all or a significant part of their lifecycles in freshwater environments, to endogenously produce essential HUFA. The retention of the  $\Delta 6$  desaturase gene in marine fish may be related to the production of DHA from EPA rather than desaturation of 18:3n-3. It may be that the activity is required to maintain membrane DHA levels, particularly in sensitive tissues such as neural tissues. The highest level of expression of  $\Delta 6$  desaturase in cod was found in the brain whereas, in salmon, liver and intestine were the tissues of highest expression, which is consistent with this hypothesis (Tocher *et al.* 2006).

In mammals, fatty acyl elongase (ELOVL; Elongation of Very Long chain fatty acids) genes are termed



**Figure 1** Pathways of biosynthesis of C<sub>20</sub> and C<sub>22</sub> highly unsaturated fatty acids (HUFA) from n-3, n-6 and n-9 C<sub>18</sub> polyunsaturated fatty acids (PUFA).  $\Delta 9$ , stearoyl CoA desaturase (SCD);  $\Delta 5$  and  $\Delta 6$ , front-end fatty acyl desaturases. Evidence suggests that the same  $\Delta 6$  desaturase operates on both C<sub>18</sub> and C<sub>24</sub> fatty acid substrates;  $\Delta 12$  and  $\Delta 15$ , fatty acyl desaturases found only in plants and some invertebrates, and hence 18:2n-6 and 18:3n-3 cannot be formed in any vertebrate; Elov12 and Elov15, PUFA elongases; Short, peroxisomal chain shortening.

ELOVL1–7 to indicate different fatty acid specificities and, of these, at least ELOVL2 and ELOVL5 are known to participate in HUFA biosynthesis (Leonard, Bobik, Dorado, Kroeger, Chuang, Thurmond, Parker-Barnes, Das, Huang & Mukerji 2000; Leonard, Kelder, Bobik, Chuang, Lewis, Kopchick, Mukerji & Huang 2002; Jakobsson, Westerberg & Jacobsson 2006). ELOVL cDNAs have been cloned from a number of fish including the freshwater species zebrafish, common carp and tilapia, the salmonids, Atlantic salmon and rainbow trout, and the marine species cod, turbot and gilthead sea bream (Agaba, Tocher, Dickson, Dick & Teale 2004; Agaba, Tocher, Zheng, Dickson, Dick & Teale 2005; Hastings *et al.* 2005). Phylogenetic analysis grouped the fish elongase cDNAs into a closely related cluster with greatest similarity to mammalian ELOVL5. Consistent with this, fish ELOVLs all elongated n-3 and n-6 PUFA and monounsaturated fatty acids with chain lengths of C<sub>18</sub> and C<sub>20</sub>, similar to mammalian ELOVL5 (Leonard *et al.* 2000). However, the zebrafish genome and the Atlantic salmon EST database (<http://www.tigr.org>) both contain a second fatty acyl elongase gene that is clearly related to ELOVL2. Recently, we cloned the cDNA for this second elongase from Atlan-

tic salmon and functional expression in yeast showed it was predominantly active towards C<sub>20</sub> and C<sub>22</sub> HUFA, similar to mammalian ELOVL2 (Leonard *et al.* 2002). It was recently reported that the pufferfish *Fugu rubripes* (Temminck & Schlegel), stickleback *Gasterosteus aculeatus* L. and medaka *Oryzias latipes* (Temminck & Schlegel) and possibly all other Acanthopterygii do not possess ELOVL2 homologues and so it is likely that the characterized ELOVL5 cDNAs of sea bream and turbot are the sole PUFA elongase genes in these species (Leaver, Bautista, Björnsson, Jönsson, Krey, Tocher & Torstensen 2008). Therefore, varying competences of different fish species to biosynthesize HUFA probably not only depend on their genome complement of desaturase genes but also elongases.

The expression of desaturase and elongase genes can be regulated in response to dietary fatty acid composition and this is correlated with HUFA synthesis activity. The expression of  $\Delta 5$  desaturase and ELOVL5 genes was positively correlated with dietary 18:3n-3, and negatively correlated with n-3 HUFA content, in Atlantic salmon fed a linseed oil diet rich in 18:3n-3 (Zheng, Tocher, Dickson, Bell & Teale 2004). The activity of the HUFA biosynthetic pathway



was positively correlated with desaturase and elongase expression. Similarly,  $\Delta 5$  desaturase gene expression was up-regulated in salmon fed a high level of rapeseed oil compared with fish fed fish oil (Jordal, Torstensen, Tsoi, Tocher, Lall & Douglas 2005), and the expression of  $\Delta 6$  desaturase was up-regulated in sea bream and sea bass liver in fish fed diets containing vegetable oils (Leaver, Bautista *et al.* 2008). Throughout a 2-year growth trial in salmon, the expression of both  $\Delta 6$  and  $\Delta 5$  desaturases was generally higher in fish fed a vegetable oil blend compared with fish fed fish oil (Zheng, Torstensen, Tocher, Dick, Henderson & Bell 2005). Expression of  $\Delta 6$  desaturase was consistently highest around the point of seawater transfer and lowest during the seawater phase. Consistent with the expression data, HUFA biosynthesis also varied during the growth cycle with peak activity around seawater transfer, and was higher in fish fed vegetable oil compared with fish fed fish oil (Zheng, Torstensen *et al.* 2005). These studies showed nutritional and environmental modulation of HUFA biosynthesis in salmon that involved regulation of desaturase gene expression, with higher expression at parr-smolt transformation and when fed diets containing reduced HUFA. Recent studies using microarray technology to determine effects on the transcriptome have confirmed that among other changes, salmon are capable of up-regulating HUFA biosynthetic genes in response to reductions of dietary HUFA (Leaver, Villeneuve, Obach, Jensen, Bron, Tocher & Taggart 2008; Taggart, Bron, Martin, Seear, Hoyheim, Talbot, Villeneuve, Sweeney, Houlihan, Secombes, Tocher & Teale 2008).

### Biochemical markers of EFA deficiency in fish

In mammals, the absence of dietary EFA can lead to the production of 20:3n-9 (Mead acid) in the tissues. Mead acid is therefore a biochemical marker, with a ratio of 20:3n-9:ARA in tissue phospholipids of 0.4 or higher indicating a state of EFA deficiency. The biochemical mechanism of 20:3n-9 production is based on the expression and fatty acid specificity of  $\Delta 6$  desaturases that are in the rank order 18:3n-3 > 18:2n-6 > 18:1n-9 (Tocher, Leaver & Hodgson 1998). Firstly, in the absence of HUFA, desaturase expression is increased or, more accurately, is not suppressed (Tocher 2003). Secondly, it is only in the absence of 18:2n-6 and 18:3n-3 that 18:1n-9 can serve as a substrate for  $\Delta 6$  desaturase and be desaturated to 18:2n-9, elongated to 20:2n-9 and desaturated by  $\Delta 5$  to 20:3n-9 (Fig. 1). Thus, it is only

with a dietary lack of both PUFA and HUFA that Mead acid can be produced. In freshwater fish and salmonids, 20:3n-9 is also a marker of EFA deficiency as they contain all the activities necessary for its production from 18:1n-9. However, as n-3 PUFA dominate in fish, Castell *et al.* (1972a, b) suggested that 20:3n-9:DHA in tissue phospholipids would be a better indicator ratio in rainbow trout, with 0.4 still being the value indicating EFA deficiency and this was supported in subsequent studies (Watanabe, Ogino, Koshiishi & Matsunaga 1974; Watanabe, Thongrod, Takeuchi, Satoh, Kubota, Fujimaki & Cho 1989). In common whitefish or powan *Coregonus lavaretus maraena* (L.) fed an EFA deficient diet, liver polar lipids showed levels of 20:3n-9 of over 10% and a 20:3n-9:DHA ratio up to 2.6, whereas neutral lipids showed much lower levels of n-9 PUFA (Watanabe *et al.* 1989). Supplementing with dietary 18:2n-6/18:3n-3 or, especially n-3HUFA, decreased n-9 PUFA levels. In marine fish that cannot biosynthesise EPA or ARA, production of Mead acid is not possible and so 20:3n-9 cannot be a marker of EFA deficiency. However, 18:2n-9 and 20:2n-9 were reported in tissues of gilthead sea bream fed diets with low levels of EFA (Kalogeropoulos *et al.* 1992). This was consistent with the aforementioned failure to clone  $\Delta 5$  desaturase from gilthead sea bream and the sea bream cell line displaying very low  $\Delta 5$  desaturase activity (Tocher & Ghioni 1999). In contrast, in the turbot cell line that was shown to have very low  $C_{18-20}$  elongase activity (Ghioni *et al.* 1999), 18:2n-9 accumulated in the absence of PUFA in the medium (Tocher, Sargent & Frerichs 1988). Irrespective of whether cells were of marine or freshwater species origin, n-9 PUFA accumulated in fish cell lines grown in the absence of PUFA and were reduced by supplementation of the growth media with PUFA (Tocher, Carr & Sargent 1989).

### Relevance of EFA requirements and metabolism to aquaculture and human nutrition

Global fisheries are in decline and consequently aquaculture is providing an increasing proportion of fish in the human food basket, up to 43% in 2005 (Food & Agriculture Organisation 2006). Paradoxically, traditional diets in aquaculture have been based on fishmeal and fish oil, themselves derived from fisheries that, at best, have reached their sustainable limit (Tacon 2004; Pike 2005). To continue to expand and supply the burgeoning world population with fish, aquaculture must find suitable, sustainable alternatives. Plant products are the obvious choice,

and considerable research has investigated the use of plant meals and vegetable oils as replacements for fishmeal and oil in the diets of farmed fish (De Francesco, Parisi, Medale, Kaushik & Poli 2004; Kaushik, Coves, Dutto & Blanc 2004; Izquierdo, Montero, Robaina, Caballero, Rosenlund & Ginés 2005; Torstensen, Bell, Rosenlund, Henderson, Graff, Tocher, Lie & Sargent 2005; Espe, Lemme, Petri & El-Mowafi 2006; Bell & Waagbø 2008). These studies have shown that fish can be grown successfully on diets with substantial amounts of fishmeal replaced by carefully formulated mixtures of plant protein sources (De Francesco *et al.* 2004; Gomez-Requeni, Mingarro, Caldach-Giner, Medale, Martin, Houlihan, Kaushik & Perez-Sanchez 2004; Espe *et al.* 2006). Other studies have shown that salmonids can be grown with 100% of dietary fish oil replaced by blended vegetable oils, and marine fish can be grown with up to 60% replacement of fish oil, without any serious effect on growth rates (Bell, Torstensen & Sargent 2005; Izquierdo *et al.* 2005; Torstensen *et al.* 2005). Current research in Europe is focussed on defining maximal replacement levels of both fishmeal and fish oil in dual-substituted diets in major aquaculture species including Atlantic salmon, rainbow trout, gilthead sea bream and common carp (<http://www.aquamaxip.eu>).

However, fish represent a virtually unique source of n-3 HUFA ('omega-3') in our diet and the health benefits of consuming increased levels of n-3 HUFA are now widely accepted, and the range of human diseases and conditions that respond to n-3 HUFA continues to grow (Brouwer, Geelen & Katan 2006; Calder 2006, 2007; Ruxton, Reed, Simpson & Millington 2007; Torrejon, Jung & Deckelbaum 2007; Nagao & Yanagita 2008). High levels of n-3 HUFA were assured in farmed fish by the use of fishmeal and, particularly, fish oil in the diets, so the increasing use of vegetable oils, that are devoid of HUFA, is not without consequences (Torstensen, Frøyland, Ørnsrud & Lie 2004). Unfortunately, irrespective of their complement of desaturase and elongase genes and increased expression and activity when fed diets with low HUFA, all fish fed high levels of vegetable oils are characterized by reduced levels of EPA and DHA in their flesh, potentially compromising their nutritional benefit to the human consumer (Izquierdo, Obach, Arantzamendi, Montero, Robaina & Rosenlund 2003; Regost, Arzel, Robin, Rosenlund & Kaushik 2003; Torstensen *et al.* 2004; Bell & Waagbø 2008).

These issues of n-3 HUFA supply for the human population have emphasized the need for further

knowledge of EFA requirements and PUFA metabolism in fish. In our own laboratory, the primary objectives in applying genomic and molecular technologies to the area of EFA metabolism in fish have been to identify the genes encoding HUFA biosynthetic enzymes, to determine how they are regulated and how their expression can be optimized to enable fish to make effective use of dietary vegetable oils. Recent studies have focussed on investigating the variation in n-3 HUFA synthesis and retention in different strains/families as important genetic traits in farmed fish (<http://www.aquamaxip.eu>).

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