Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective

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Abbreviations: ARA, arachidonic acid (20:4n-6); CVD, cardiovascular disease; DHA, docosahexaenoic acid (22:6n-3); DPA, docosapentaenoic acid (22:5n-3); EFA, essential fatty acid; Elovl, fatty acid elongase; EPA, eicosapentaenoic acid (20:5n-3); Fads, fatty acyl desaturase; FM, fishmeal; FO, fish oil; LC-PUFA, long-chain polyunsaturated fatty acids ($\geq$ C20 $\geq$ 3 double bonds); LOA, linoleic acid (18:2n-6); LNA, linolenic acid (18:3n-3); PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; VO, vegetable oil.

ABSTRACT

In the 40 years since the essentiality of polyunsaturated fatty acids (PUFA) in fish was first established by determining quantitative requirements for 18:3n-3 and 18:2n-6 in rainbow trout, essential fatty acid (EFA) research has gone through distinct phases. For 20 years the focus was primarily on determining qualitative and quantitative EFA requirements of fish species. Nutritional and biochemical studies showed major differences between fish species based on whether C_{18} PUFA or long-chain (LC)-PUFA were required to satisfy requirements. In contrast, in the last 20 years, research emphasis shifted to determining "optimal" levels of EFA to support growth of fish fed diets with increased lipid content and where growth expectations were much higher. This required greater knowledge of the roles and functions of EFA in metabolism and physiology, and how these impacted on fish health and disease. Requirement studies were more focused on early life stages, in particular larval marine fish, defining not only levels, but also balances between different EFA. Finally, a major driver in the last 10-15 years has been the unavoidable replacement of fish oil and fishmeal in feeds and the impacts that this can have on n-3 LC-PUFA contents of diets and farmed fish, and the human consumer. Thus, dietary n-3 in fish feeds can be defined by three levels. Firstly, the minimum level required to satisfy EFA requirements and thus prevent nutritional pathologies. This level is relatively small and easy to supply even with today's current high demand for fish oil. The second level is that required to sustain maximum growth and optimum health in fish being fed modern high-energy diets. The balance between different PUFA and LC-PUFA is important and defining them is more challenging, and so ideal levels and balances are still not well understood, particularly in relation to fish health. The third level is currently driving much research; how can we supply sufficient n-3 LC-PUFA to maintain the nutritional quality of farmed fish at the same level as 20 years ago, and similar or better than in wild fish? This level far exceeds the biological requirements of the fish itself and to satisfy it we require entirely new sources of n-3 LC-PUFA. We cannot rely on the finite and limited marine resources that we can sustainably harvest or

efficiently recycle. We need to produce n-3 LC-PUFA *de novo* and all possible options should be considered.

Keywords: Fish oil; essential fatty acids; nutrition; health; metabolism; sustainability

Highlights:

1. Long-chain omega-3 fatty acids are essential nutrients for vertebrates including fish and humans, but they are a finite and limited resource.
2. Dietary levels to satisfy metabolic requirements and sustain optimal growth and health in fish are relatively low, but supra-physiological levels are required to maintain high nutritional quality of farmed products.
3. Aquaculture has an important role to play in the efficient use of existing supplies of long-chain omega-3 and their transfer to human consumers.
4. For aquaculture to continue in this capacity, entirely new sources of long-chain omega-3 fatty acids are required and, currently, transgenic oilseed crops represent the most viable option.

1. Introduction

Fish accounted for 16.7 % of the global population's intake of animal protein and 6.5 % of all protein consumed in 2010 (FAO, 2014). However, in addition to protein, fish are also important dietary sources of minerals including iodine and selenium, and vitamins such as A, D and E (Tacon and Metian, 2013). Arguably, of greatest importance for consumers in the developed world, fish and seafood are unique and rich sources of omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids (Tur et al., 2012), that have well-known and generally accepted beneficial effects in a range of human pathologies including cardiovascular and inflammatory diseases, and an important role in neural development (Gil et al., 2012; Delgado-Lista et al, 2012; Campoy et al., 2012; Calder, 2013). However, marine fisheries worldwide are stagnating with 63 % of assessed fish stocks requiring rebuilding (Worm et al., 2009) and so, consequently, an increasing proportion of fish are farmed, accounting for almost half of all fish for human food in 2012 (FAO, 2014). Feeds for farmed carnivorous species were traditionally based on fishmeal (FM) and fish oil (FO) and, while this practice was good for the supply of n-3 LC-PUFA (Sargent et al., 2002), the reliance on finite marine resources derived from capture fisheries was an unsustainable practice (Naylor et al., 2000; Tacon and Metian, 2009). Therefore, the continued growth of aquaculture was dependent upon the development of more sustainable feeds with alternative ingredients, generally derived from terrestrial agriculture, with important consequences for the supply of n-3 LC-PUFA (Gatlin et al., 2007; Tocher, 2009; Turchini et al., 2011a). This short review summarises the important issues surrounding omega-3 LC-PUFA as key nutrients, particular in the context of aquaculture and its role in the provision of dietary n-3 LC-PUFA for the human consumer. In doing so, it aims to clarify the issues underpinning the use of marine ingredients and their replacement, highlighting the influence and limitations of fish metabolism, and critically assessing options for the future, sustainable supply of n-3 LC-PUFA.

2. Fish oil – Historical perspective, recent trends and current status

The production and use of FO goes back to antiquity when it was the original liquid fuel and burnt in lamps for light, a process that converted n-3 LC-PUFA to CO_2, or "greenhouse" gas. In the 20th century FO, including whale oil, was used extensively in margarine production that required hydrogenation and conversion of n-3 LC-PUFA to saturated and, even worse, trans-fatty acids. Now, in the 21st century the main use of FO is in feeds for aquaculture, primarily as a source of n-3 LC-PUFA that are now prized for their health benefits, not only to the farmed fish itself, but also to the human consumers through the production of high-quality food. Although, FO has also been used in terrestrial animal feeds (pigs and poultry) and small amounts were always used for direct human consumption (particularly cod liver oil), history demonstrates that, in terms of n-3 LC-PUFA, aquaculture represents arguably the best use of bulk FO to date.

However, there are constraints on the use of FO (and FM) in aquaculture. The primary constraint is that these are essentially finite marine resources with production necessarily limited through strict regulation of fishing and catch quotas (Shepherd and Jackson, 2012; Jackson and Shepherd, 2013). Production of FM and FO had been slightly declining in recent years, largely due to increased regulation and reduced quotas in South America, but this situation is appearing more stable (FAO, 2012). The status of FO use in 2012, showed that around 75 % of total global supply was used in aquaculture, with 22% going for direct human consumption (IFFO, 2013) (Fig.1A). Over 80 % of FO used in aquaculture feeds was consumed by salmonids (62 %) and marine fish (19 %) (Fig.1B). Despite the continued growth of aquaculture (FAO, 2014), the use of FO in aquaculture was relatively stable over the last decade with, on average, around 0.8 mt being used (IFFO, 2013) (Fig.1C). Increased use of seafood and aquaculture by-products (including by-catch and trimmings etc.) to produce FM and, to a lesser extent, FO, has partially offset some of the reductions in production from whole fish as a result of increased restrictions on the reduction fisheries (Shepherd and Jackson, 2012; OECD-FAO, 2013). Production is also subject to environmental influences and, whereas the potential impact of climate change is not well understood (Callaway et al., 2012), acute

phenomena such as El Niño have well-known consequences and the next significant event will have major effect on FM and FO supply (FAO/GIEWS, 1997).

Although, the finite and limited nature of FO production and supply is the main constraint to its use in aquaculture, there are other major factors (Bell and Waagbø, 2008). Sustainability issues are, of course, key drivers limiting overall supply, and these will have an increasing impact with the many initiatives currently being developed with respect to both national and international standards and certification of marine ingredients, particularly FM and FO products. In fisheries, International standards are being set by the Marine Stewardship Council (MSC; http://www.msc.org) that operates a certification programme promoting sustainable fishing practices, and similar national schemes also exist such as the UK's Responsible Fishing Scheme (RFS; http://rfs.seafish.org). The Marine Ingredients Organisation (IFFO) also operates the IFFO-RS standard (http://www.iffo.net/iffo-rs), a certification programme to specifically promote responsibly sourced and produced FM and FO. The Global Aquaculture Alliance (GAA) Best Aquaculture Practices (BAP) standards (http://www.gaalliance.org/bap/standards.php) has several certification processes including a Feed Mills BAP Standard that addresses FM and FO conservation with responsible sourcing from approved certified sources. Finally, the Aquaculture Stewardship Council (ASC; http://www.asc-aqua.org) operates a certification and labeling programme to promote responsibly farmed seafood. Increasing adoption and implementation of these standards will further improve sustainability but also likely have impacts both for the supply of FM and FO and their use in aquaculture.

The presence of contaminants/undesirables such as persistent organic pollutants (POPS) including dioxins and PCBs is another potential constraint, at least with some marine ingredients (Tocher, 2009; FAO/WHO, 2011). In general, this is an issue that had been diminishing to some extent with the increasing replacement of dietary FM and FO that meant levels of these undesirable marine environmental contaminants in feeds were decreasing and unlikely to increase in the future (Berntssen et al., 2010). However, this issue has been raised again with the recent announcement

(http://www.marineharvest.com/about/news-and-media/news/marine-harvest-salmon-even-healthier/) indicating that at least one international fish farming company is planning to utilise only feed formulated with "cleaned" FO (Olli et al., 2010). It remains to be seen whether or not this is an overall beneficial development for aquaculture and, in particular, salmon farming.

3. Roles of n-3 LC-PUFA in vertebrates

3.1 Metabolic pathways for the biosynthesis LC-PUFA

The LC-PUFA are most commonly defined as PUFA with ≥ C20 and ≥ 3 double bonds and, although not a perfect definition, it includes all the main biologically active LC-PUFA such as EPA, DHA and arachidonic acid (ARA, 20:4n-6) and also includes other important n-3 fatty acids such as docosapentaenoic acid (DPA, 22:5n-3) and 20:4n-3 (Tocher, 2003). As PUFA (all fatty acids with 2 or more double bonds) cannot be synthesised *de novo* in vertebrates due to the lack of Δ12 (or ω6) and Δ15 (or ω3) desaturases responsible for the production of PUFA from 18:1n-9, they are required to be supplied in the diet (Sargent et al., 2002). However, C_{18} PUFA such as α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LOA, 18:2n-6) can be converted to the biologically active LC-PUFA in vertebrates although this varies with species dependent upon the presence and expression of genes of fatty acid desaturation and elongation (Tocher, 2003) (Fig. 2). In consequence, which PUFA can satisfy essential fatty acid (EFA) requirements varies similarly with species (Tocher 2010).

3.2 Metabolic roles of LC-PUFA

Like all fatty acids, LC-PUFA have important structural roles as constituents of phospholipids that are the major components of cellular biomembranes, and confer various functional properties by affecting both physico-chemical properties of the membrane (e.g. fluidity) as well as influencing membrane protein (e.g. receptors, carriers and enzymes) functions (Tocher, 1995). Similarly, as components of triacylglycerols (TAG) and other storage lipids such as wax esters, LC-PUFA can

function as an energy reserve, with energy recovered through fatty acid β-oxidation in mitochondria (Tocher, 2003). In addition, however, underpinning the essentiality of PUFA, LC-PUFA have more specific functional roles as important regulators of metabolism either as themselves or their derivatives. Regulation can be extracellular at the level of tissues through LC-PUFA derivatives including eicosanoids such as prostaglandins, leukotrienes, lipoxins, resolvins and protectins, or intracellular as ligands for transcription factors that control gene expression (Tocher, 2010). These regulatory roles underpin the importance of LC-PUFA as belonging to either the n-6 or n-3 series, the concept of balance in dietary intake of the two PUFA series that cannot be interconverted in vertebrates (see Fig.2), and the impact that dietary LC-PUFA can have on both health and disease (Lands, 2014).

3.3 Roles of n-3 LC-PUFA in human health

It has long been appreciated that dietary n-3 LC-PUFA can have important, generally beneficial effects on human health largely based on two main lines of evidence, epidemiological studies and randomised controlled (intervention) trials, although laboratory studies investigating biochemical and molecular mechanisms can also support these approaches (Gil et al., 2012).

3.3.1 Cardiac and cardiovascular disease (CVD)

Epidemiological studies, that are normally large and long, looking at n-3 LC-PUFA intake (fish/fish oil) and/or status (blood/tissue fatty acid compositions) almost uniformly show a protective effect, decreasing the risk of developing CVD (Delgado-Lista et al., 2012). When all confounders are controlled the data are robust albeit they show only an "association" and not "cause and effect". However, randomised controlled trials, normally designed for patients with some disease already (i.e. in at-risk patients), have also been generally positive and show that patients benefit from dietary n-3 LC-PUFA therapy (Calder and Yaqoob, 2012; Delgado-Lista et al., 2012). Until recently, meta-analyses were also largely positive although there have been some recent studies showing less effect (Rizos et al., 2012;

Chowdhury et al., 2014). The biochemical mechanisms underlying the beneficial effects of n-3 LC-PUFA are primarily based on their lowering of known risk factors for CVD (blood cholesterol and TAG, hypertension) and effects on platelet function (Uauy and Valenzuela, 2000). Molecular mechanisms are less well understood but include effects on hepatic lipid metabolism such as decreasing TAG synthesis, that involve, among other mechanisms, the important role of PUFA in transcriptional regulation of genes of lipid metabolism (Deckelbaum et al., 2006; Georgiadi and Kersten, 2012). Based on their effects on CVD, many health agencies worldwide recommend up to 500 mg/d of EPA and DHA for reducing CVD risk or 1 g/d for secondary prevention in existing CVD patients, with a dietary strategy for achieving 500 mg/d being to consume 2 fish meals per week with at least one of oily fish (ISSFAL, 2004; Gebauer et al., 2006; Aranceta and Perez-Rodrigo, 2012).

3.3.2 Inflammatory disease

The most robust evidence for a beneficial effect of dietary n-3 LC-PUFA in inflammatory disease has been obtained in rheumatoid arthritis (Miles and Calder, 2012). The doses required to gain benefit are much higher than for CVD, at around 3g per day and so dietary n-3 LC-PUFA are still not part of classical treatment plans. There is also increasing evidence for beneficial effects of dietary n-3 LC-PUFA in Inflammatory Bowel Disease (IBD) such as Crohn's disease and ulcerative colitis (Cabré et al., 2012). Although there are studies showing some beneficial effects of n-3 LC-PUFA in various other inflammatory diseases there are too few studies to be conclusive. In contrast, there is a very large body of research into the mechanisms of action of n-3 LC-PUFA on inflammatory pathways (Rangel-Huerta et al., 2012). Until recently, the major mechanism was thought to be the anti-ARA effect of n-3 LC-PUFA. In brief, inflammatory responses are largely driven by eicosanoids produced from the major n-6 LC-PUFA, ARA, but other LC-PUFA, particularly EPA, compete with ARA at level of the cyclooxygenase (COX) and lipoxygenase enzymes

responsible for the production of eicosanoids, both reducing the production of pro-inflammatory ARA derivatives and producing less inflammatory EPA-derived mediators (Calder, 2007). However, there is considerable research showing a wide range of mechanisms potentially underpinning the effects of n-3 LC-PUFA on inflammatory responses and immune pathways (Chapkin et al., 2009; Calder, 2013). In particular in recent years, it has been established that the n-3 LC-PUFA, EPA and DHA, are also precursors of other COX-2 derived non-classical eicosanoid derivatives called resolvins, maresins and protectins, which are specialized pro-resolving mediators (PSM) that bring about resolution of the inflammatory response and a return to homeostasis (Serhan and Petasis, 2011; Weylandt et al., 2012). In addition, as ligands of various transcription factors, n-3 LC-PUFA and their derivatives can also have affects on expression of genes in inflammatory and immune pathways (Chapkin et al., 2009; Schmuth et al., 2014). Therefore, n-3 LC-PUFA have a combination of effects acting to reduce the respiratory burst and increase resolution (Calder, 2013). While ARA-EPA competition and the effects of n-3 LC-PUFA on eicosanoid production (Montero and Izquierdo, 2011; Martinez-Rubio et al., 2013) and inflammatory and immune gene expression have also been studied in fish (Montero and Izquierdo, 2011; Martinez-Rubio et al., 2012, 2014), the role of EPA and, especially, DHA in the production of SPM has not been studied in fish.

3.3.3 Neural development

There is good robust evidence that decreased DHA status can lead to cognitive and visual impairment and that DHA supplements have positive beneficial outcomes in pre-term infants (Carlson et al., 1993). In term infants, DHA supplementation appears less effective suggesting that it is rectifying low DHA status that is effective rather than increasing "normal" DHA status (Campoy et al., 2012). There have also been several reports of potential beneficial effects of dietary DHA supplementation in a number of

psychological/behavioural/ psychiatric disorders including attention deficit hyperactivity disorder (ADHD) and depression and, although there are some reports of benefits, there are insufficient studies and data to draw firm conclusions (Ortega et al., 2012). However, it is becoming generally recognized that n-3 LC-PUFA are potential key nutrients to prevent various pathological conditions associated with the normal aging process (Ubeda et al., 2012). This has prompted research into the effects of n-3 LC-PUFA on dementia, including Alzheimer's disease (AD) and other age-related cognitive impairments (Dangour et al., 2012). In general, there is a lack of agreement in findings from intervention studies to support a benefit of n-3 LC-PUFA on cognitive function but this may also reflect intrinsic limitations in the design of published studies (Dangour et al., 2012). However, DHA supplementation trials in patients with some pre-diagnosed cognitive impairment indicated that this appeared to slow AD progression (Quin et al., 2010). In long-term animal AD model trials, n-3 LC-PUFA improved cognitive function and diminished the amount of neuronal loss (Hooijmans et al., 2012). As chronic inflammation is observed in AD, the mechanism of n-3 LC-PUFA is postulated to be due to down-regulation of inflammation and promotion of resolution of the inflammatory response (Hjorth et al., 2013).

3.3.4 Cancer

Epidemiological studies have indicated that, in general, consumption of oily fish or taking n-3 LC-PUFA supplements may have a protective effect (i.e. decreasing risk) in colo-rectal, breast and prostate cancers (Gerber, 2012). Further evidence is difficult to obtain as randomised, controlled trials similar to those carried out in CVD are not possible in cancer, but there are studies indicating some beneficial effects of n-3 LC-PUFA supplementation in chemotherapy (Bougnoux et al., 2009). In small lung carcinoma, n-3 LC-PUFA appear to sensitise cancerous cells to chemotherapeutants thus increasing efficiency and perhaps decreasing side effects, enabling the patient to undergo more cycles (Murphy et al., 2011a).

In addition, n-3 LC-PUFA may protect against the muscle mass loss likely due to hyper-metabolism promoting proteolysis that is often associated with cancer (Murphy et al., 2011b). The mechanisms underlying the latter two beneficial effects are not understood, but are possibly based on the effects of n-3 LC-PUFA on inflammatory processes.

3.3.5 Negative reports

Two recent studies have associated n-3 LC-PUFA status with prostate cancer (Brasky et al., 2011, 2013). The interpretation of these data has been criticized on a number of grounds including the fact that n-3 LC-PUFA intake was not monitored in these studies and, as n-3 LC-PUFA supplementation is not uncommon in cancer patients (see above), the reason for the observed association between n-3 LC-PUFA and prostate cancer cannot be established. However, these negative reports have impacted on sales of FO-derived n-3 LC-PUFA products for direct human consumption and halted the ever-increasing trend over the last few years that peaked at 25 % of total FO in 2010-11, but that slipped back to 22 % in 2012 (IFFO, 2013). More generally, negative effects of dietary n-3 LC-PUFA have been attributed with the pro-oxidant effect of LC-PUFA (Garrido et al., 1989; Tsuduki et al., 2011). This can occur through the use of dietary sources that already contain oxidized lipids if not prepared/stored properly with appropriate anti-oxidant protection, but also through pro-oxidant effects *in vivo* (Albert et al., 2013; Garcia-Hernandez et al., 2013). Increased lipid oxidation products and anti-oxidant defence mechanisms (enzymes etc.) are both generally observed in n-3 LC-PUFA supplementation trials, and the need to also increase anti-oxidant intake when on supplements is well known. However, a certain level of "peroxide tone" is required for eicosanoid biosynthesis (Smith, 2005), and there is also evidence that n-3 LC-PUFA can have anti-oxidant actions (Giordano and Visoli, 2014).

4. Fish, aquaculture and n-3 LC-PUFA

The above summary highlights the roles of n-3 LC-PUFA in health and in preventing and/or mitigating disease. However, whereas the public now have a good appreciation of the problems with human diets today in terms of too high levels of fat, saturated fat and cholesterol, there is far less understanding of fatty acids and "omega-3". Evidence suggests that human beings evolved on a diet with a ratio of n-6 to n-3 PUFA of about 1:1, whereas modern so-called "Western" diets have a ratio of up to 20-25:1, indicating that diets are deficient in n-3 PUFA compared with the diet on which humans evolved and their genetic patterns were established (Simopoulous, 2011). Considering the key roles of LC-PUFA in controlling and regulating lipid metabolism and, especially, inflammatory/immune responses, the dietary imbalance n-6 and n-3 PUFA is critical. Therefore, there is a need to rebalance our dietary intakes. The n-6/n-3 PUFA ratio in global oil and fat supply can be calculated from the data for individual oil/fat production (Gunstone, 2011) and oil fatty acid compositions (Gunstone and Harwood, 2007) to be around 24:1 post-hydrogenation (Gunstone, personal communication), Therefore, it is difficult to greatly reduce dietary intake of n-6 PUFA, although this a key element of the Mediterranean diet rich in olive oil. Increasing n-3 PUFA intake is entirely feasible, but LNA has relatively little functional role, other than as a precursor of EPA and DHA, and the LC-PUFA biosynthesis pathway is not very active in humans (Brenna et al., 2009). Therefore, increasing n-3 LC-PUFA intake is the most efficient strategy to redress dietary PUFA imbalance as the biological effects largely reside with them. Fish and seafood traditionally supplied our dietary n-3 LC-PUFA, not because fish themselves produce them, but because aquatic food webs are n-3 LC-PUFA rich and, as 96 % of global water is ocean, EPA and DHA are predominantly of marine origin and fish simply accumulate them (Bell and Tocher, 2009a; Tocher, 2009). By formulating feeds with FM and FO, aquaculture simply replicated this diet and so farmed fish were also rich in n-3 LC-PUFA. However, from 2000-2012 aquaculture has grown, on average, by 6.2 % annually and has been the fastest growing animal protein production sector for many years and, indeed, is outpacing population growth (FAO, 2014). In contrast, as described above, FM and FO are finite resources and FO supply in particular would have limited this growth in aquaculture if

these marine products were not increasingly replaced in aquafeeds by alternative ingredients, predominantly terrestrial crop-derived plant meals and vegetable oils (Gatlin et al., 2007; Hardy, 2010; Turchini et al., 2011a). The necessity to reduce the level of FO use prompted considerable research into the development of sustainable feeds and feeding strategies to maintain n-3 LC-PUFA levels in farmed fish despite the lower level of dietary n-3 LC-PUFA (Turchini et al., 2009, 2011a; Sales and Glencross, 2011). As a result, although modern aquafeeds contain far lower levels of both FM and FO, a recent study showed farmed salmon remain one of the best sources of EPA + DHA for human consumers (Henriques at al., 2014). Although the relative proportions of EPA and DHA were lower in farmed salmon products than in wild salmon products (Fig. 3A, C), in absolute terms the farmed salmon delivered, on average, double the dose of EPA and DHA than the wild salmon (Fig. 3B, C). Therefore, the recommended dose (500 mg/d or 3.5 g/week) of EPA and DHA for lowering risk of CVD could be achieved by eating two 150 g portions of farmed salmon, whereas 4-5 portions of wild salmon would be required (Henriques et al., 2014).

5. Fish nutrition and n-3 LC-PUFA requirements

As mentioned above, fish like all vertebrates require a dietary source of PUFA. The qualitative and quantitative EFA requirements of fish are reported in detail in earlier reviews (Glencross, 2009; Tocher, 2010) and are discussed in detail in the recently updated NRC volume on the Nutrient Requirements of Fish and Shrimp (NRC, 2011). Briefly, EFA requirements can be satisfied generally by C_{18} PUFA in freshwater and salmonid species whereas marine species require LC-PUFA, EPA, DHA and ARA (Tocher, 2010). In species where requirements can be satisfied by C_{18} PUFA, LC-PUFA can satisfy at a lower level, and requirements tends to be greater in early life stages (larvae) when the relative proportions of LC-PUFA (EPA, DHA and ARA) are particularly important (Tocher, 2010). Quantitative EFA requirements can be described on three levels. The physiological EFA requirement is the level to prevent classical nutritional pathology (EFA deficiency signs) and this is low, often around 1 % of the diet, or possibly lower if supplied by LC-

PUFA. The published EFA requirement data for fish species probably represent good estimates of this requirement level (Glencross, 2009; Tocher, 2010). However, many early EFA studies were performed in small fish fed diets with relatively low lipid level for relatively short durations at a time when growth expectations were lower. Now, modern high-energy (lipid) diets and the consequent higher growth rates suggest that EFA requirements should perhaps be reassessed (NRC, 2011). Therefore, there may be a higher level of EFA requirement to support optimum growth and health, although this is currently not well defined for any species and would likely vary dependent upon other dietary factors and fish metabolism (Tocher, 2003). The final requirement level is that to maintain nutritional quality based on n-3 LC-PUFA content of the flesh (Tocher, 2009). As such, this is not a requirement of the fish at all, but rather that of the human consumers (Simopoulos, 2000). To satisfy this level, EPA and DHA need to be supplied well in excess of the requirements for optimal health and growth of the fish so that they are deposited and stored in fish lipid.

6. Influence of endogenous metabolism on n-3 LC-PUFA levels in fish

The fatty acid composition of fish is dependent upon the interaction of diet (prey or feed fatty acid compositions) with endogenous metabolism. The above discussion focussed on diet and this section now describes the potential influences that metabolism can have on n-3 LC-PUFA composition and content in farmed fish.

6.1.Digestion and absorption

The efficiency of digestion and absorption of nutrients is quantified by measuring Apparent Digestibility Coefficients (ADC), which are estimated by comparing dietary and faecal nutrients in comparison to an indigestible marker (Bell and Koppe, 2011). The ADCs of all PUFA, including LC-PUFA, are usually high for most fish species, but they can vary dependent upon dietary lipid content, fatty acid composition, chemical form (TAG, phospholipid), and temperature (Bell and

Koppe, 2011). However, in general, this means that dietary PUFA are efficiently digested and absorbed and therefore these processes do not greatly influence dietary fatty acid compositions.

6.2 Oxidation

In vitro studies comparing relative oxidation rates of fatty acids have suggested a rank order for oxidation of saturated/monounsaturated fatty acid > PUFA > LC-PUFA, with n-6 > n-3, and shorter chain > longer chain within saturated fatty acids at least (Henderson, 1996). However, *in vivo* studies investigating fatty acid deposition in comparison to dietary intake show that the higher the concentration of a fatty acid in the diet, generally the lower its relative deposition, which implies increased (dietary) concentrations result in increased oxidation (Stubhaug et al., 2007). These studies indicate that oxidation of a fatty acid is a balance between enzyme specificities and substrate fatty acid concentrations (competition) with the latter appearing to dominate. This is generally the case for most PUFA including EPA, and so oxidation increases with increased dietary concentration, with generally no preferential retention (Stubhaug et al., 2007). The only general exception is DHA that is consistently shown to be deposited in tissues such as flesh and liver at higher levels than dietary inclusion showing it is preferentially retained (Brodtkord et al., 1997; Glencross, 2009). This is likely simply due the fact that the Δ4 double bond in DHA is resistant to mitochondrial β-oxidation, requiring peroxisomal oxidation to be removed, and this means it is not as easily oxidised as other fatty acids (Madsen et al., 1999).

6.3 LC-PUFA biosynthesis

As alluded to earlier, although LNA and LOA must be obtained in the diet, there are pathways for the endogenous production of LC-PUFA from C_{18} PUFA in fish, although the activity of these pathways is dependent upon the genes (enzymes) present (Fig. 1). The same enzymes operate on both n-3 and n-6 fatty acids and so there must be competition between the two PUFA series although, recently, it was shown that LOA had little effect on LNA bioconversion in rainbow trout,

Oncorhynchus mykiss (Emery et al., 2013). The biosynthesis of LC-PUFA should be looked upon as two separate pathways with EPA being the common component. The EPA pathway, production of EPA from LNA, requires two desaturations via a Δ6/Δ8 desaturase (depending upon whether LNA is first elongated or not) and a Δ5 desaturase (Cook and McMaster, 2004). The DHA pathway, production of DHA from EPA, can proceed directly through elongation and Δ4 desaturation or via the "Sprecher shunt" that involves elongation to 24:5n-3, Δ6 desaturation (by the same Δ6 as in the EPA pathway), and peroxisomal chain shortening (Sprecher, 2000). The molecular components, fatty acyl desaturase (Fads) and elongase (Elovl) genes, transcripts and enzymes, involved in the pathways, their fatty acyl specificities, activities, species and tissue distributions have been described and discussed in detail in several recent reviews (Bell and Tocher, 2009a; Tocher, 2010; Torstensen and Tocher, 2011; Monroig et al., 2011a, 2013; NRC, 2011) and so the present review will only focus on the most relevant aspects.

The EPA pathway in teleost fish is often incomplete, primarily due to a lack of Δ5 desaturase, and so synthesis of EPA from LNA is not possible in many, particularly marine, carnivorous species (Tocher, 2010). However, the DHA pathway from EPA is probably functional in most teleost fish, including marine species, at least in some tissues such as brain (Tocher et al., 2006; Zheng et al., 2009; Monroig et al., 2011a). Salmonids and many freshwater species have complete pathways and can produce both EPA and DHA from LNA (Tocher, 2010; Monroig et al., 2011a). The expression of Δ6 and Δ5 desaturase and some elongase genes are subject to nutritional regulation and are up-regulated, generally by up to 2-3–fold, in fish fed VO-based diets compared to fish fed FO-based diets (Leaver et al., 2008a; Torstensen and Tocher, 2011; Vagner and Santigosa, 2011) and this has been shown to produce a similar increase in LC-PUFA biosynthesis in liver and intestinal tissues in salmonids (Zheng et al., 2004, 2005; Torstensen and Tocher, 2011). The up-regulation in fish fed VO is likely due mainly to relieving the suppression of gene expression exerted by dietary LC-PUFA, especially DHA (Leaver et al., 2008b; Thomassen et al., 2012), although increased substrate (e.g. LNA) concentration may also stimulate expression

(Cleveland et al., 2012). Either way, dietary PUFA are likely to exert their effects through molecular mechanism(s) involving their role as ligands of key transcription factors such as sterol regulatory element binding proteins (SREBP) (Minghetti et al., 2011; Carmona-Antoñanzas et al., 2014). Irrespective of mechanism, the up-regulation of LC-PUFA biosynthesis is not sufficient to maintain tissue EPA and DHA in fish fed VO-fed at the same level as in fish fed FO (Bell and Tocher, 2009b; Tocher et al., 2011). This is entirely expected as metabolic pathways such as LC-PUFA have evolved to satisfy the requirements of the fish, not of human beings. Therefore, the differences between freshwater/salmonid and marine species in the EPA pathway are probably a consequence of the differing levels of LC-PUFA in the respective environments (higher in marine) leading to different evolutionary pressures, and are reflected in their qualitative EFA requirements (Leaver et al., 2008b; Carmona-Antoñanzas et al., 2013). DHA production from EPA is more conserved although there are few data, but this pathway is likely important particularly for brain development and function and, again, an evolutionary adaptation (Zheng et al., 2009). In species with complete LC-PUFA pathways such as salmonids, endogenous biosynthesis is sufficient to satisfy the relatively low levels of their normal physiological requirements, and there has been no biochemical/metabolic driver or evolutionary pressure to synthesise excess EPA and DHA just to be deposited in TAG and stored (Leaver et al., 2008b; Castro et al., 2012).

6.4 Practical applications

Therefore, fatty acid metabolism appears to impose more limitations than opportunities for enhancing n-3 LC-PUFA levels in fish. Can anything practical be done within the limitations imposed?

6.4.1. Enhancing endogenous LC-PUFA production and retention

There are two main options for enhancing endogenous LC-PUFA production and/or retention in fish using either a nutritional/biochemical/pharmacological approach or a

transgenic approach. The former alterantive was based on finding a nutritional strategy (careful formulation, fatty acid ratios etc.) or a specific dietary supplement (chemical or biochemical) that would increase LC-PUFA biosynthesis and/or retention by mechanisms such as influencing gene expression or enzyme activities (Torstensen and Tocher, 2011). Nutritional strategies such as carefully formulating diets with "optimal" C_{18} PUFA or micronutrient levels have been researched, particularly in trout, and have been successful in altering the efficiency of LC-PUFA production (Turchini et al., 2011b; Lewis et al., 2013). Dietary supplementation has been investigated with bioactive fatty acids (conjugated linoleic acid, 3-thia acids, petroselinic acid), fibrates and other botanicals, such as the lignin sesamin, with some limited success including increasing LC-PUFA biosynthesis (Ruyter et al., 1997; Moya-Falcón et al., 2004; Kennedy et al., 2006; Trattner et al., 2008a,b; Randall et al., 2013). However, any uplift in biosynthetic activity observed in the above studies has been relatively small and insufficient to compensate for the lack of dietary LC-PUFA.

Genetic modification (GM) is another potential option for increasing endogenous production or retention of n-3 LC-PUFA in farmed fish (Maclean and Laight, 2000). Proof of concept of GM to enhance production characteristics in farmed fish already exists as transgenic lines transformed with growth hormone/promoter genes have been produced that demonstrate growth enhancement in several species, including Atlantic salmon (Devlin et al., 2004). There have also been research studies in fish specifically targeting LC-PUFA metabolism. Transgenic zebrafish (*Danio rerio*) have been produced by transformation with Δ6 and Δ5 fatty acyl desaturases of masu salmon (*Oncorhynchus masou*) (Alimuddin et al., 2005, 2007), and both zebrafish (Alimuddin et al., 2008) and the marine teleost, nibe croaker *Nibea mitsukurii* (Kabeya et al., 2014), have been transformed with fatty acid elongase from masu salmon. Recently, the production of n-3 PUFA, specifically LNA from 18:1n-9, was demonstrated in GM-zebrafish transformed with fat-1 and fat-2 genes (Pang et al., 2014). However, in all these studies the increments in EPA and DHA levels have been

small and so this enhancement, by a combination of adding function and/or overexpression of biosynthetic genes, may have limitations based on the metabolic and physiological controls of the biochemical pathways. Furthermore, the production and use of GM fish is likely to face many socio-political, ecological and environmental challenges before it could become commercially viable.

6.4.2 Genetic Selection

It was recently shown that flesh n-3 LC-PUFA level is a heritable trait in salmon (Leaver et al., 2011) and therefore it could be beneficial to introduce this trait into selective breeding programmes (Gjedrem, 2000). The differences in levels of n-3 LC-PUFA in flesh between different families/strains reported so far are relatively small (2-3 % of total fatty acids) (Morais et al., 2012a), but it demonstrates that there is scope for this strategy and the variability that may exist in wild populations is simply not known. Therefore, although the current knowledge of the variability in this trait would suggest this could not be a solution to the issue of reduced dietary n-3 LC-PUFA in farmed fish, it is nonetheless a sensible approach to select for this trait to optimise the efficiency of n-3 LC-PUFA metabolism and flesh levels, irrespective of likely dietary levels in the future.

7. The supply of n-3 LC-PUFA for aquaculture

The above discussion has concluded that lipid and fatty acid metabolism in fish is fundamentally limited by biochemistry and physiological control mechanisms such that a solution to maintaining high n-3 LC-PUFA levels in tissues when dietary levels are low is unlikely be found within the fish itself. This returns us full circle to dietary supply as the only viable option for maintaining high levels of n-3 LC-PUFA in farmed fish.

7.1 The real problem of FO as a source of n-3 LC-PUFA for aquaculture

The use of FO (and FM) as sources of n-3 LC-PUFA was a perfectly sensible approach 30-40 years ago during the initial the development of intensive aquaculture. Both marine ingredients were readily accepted and digested by fish, had favourable nutrient compositions and, simply put, they represented the natural food for the fish (NRC, 2011). Furthermore, FM and FO are just commodities similar to other raw materials or primary agricultural products that have long-since been globally produced and marketed, and aquaculture simply grew to become the biggest customer for these marine resources (Jackson and Shepherd, 2013). However, the danger of stagnating fisheries and growing aquaculture with an over-dependence on a limited range of feed ingredients, and the issues and difficulties of replacement, especially of FO, have been appreciated for many years (Sargent and Tacon, 1999; Naylor et al., 2000). Analyses of fish-in fish-out (FIFO) ratios of aquaculture production was developed as a potentially useful tool in assessing the use of marine ingredients. Although this analyses showed that FIFO for the aquaculture of all fed species combined had decreased from 1.0 in 1995 to about 0.7 in 2006 with a further projected decrease towards 0.2 by 2020 (Tacon and Metian, 2008), the ratio was embraced by environmental and anti-fish farming groups to wrongly imply that aquaculture, particularly salmon farming, was an inefficient practice wasting precious marine resources. The methodology for calculating FIFO (Kaushik and Troell, 2010) and the initial high values of 4-5:1 for salmon farming (Tacon and Metian, 2008) have been challenged (Jackson, 2009), and it has been suggested that FIFO should be replaced by marine nutrient dependency ratios for which the amount of each marine nutrient used in feed is divided by the amount of that nutrient in the farmed product, producing separate ratios for protein (MPDR) and oil (MODR) (Crampton et al., 2010). However, the fundamental flaw with FIFO ratio is that it has no nutritional basis and is in no way a measure of production efficiency. It is well established that aquaculture is generally very efficient and top performing species like Atlantic salmon (Torrissen et al., 2011) have a lower feed conversion ratio (FCR) than any terrestrial animal production with commercial values of 1.1 to 1.2 compared to ~2 for poultry, ~3 for pigs, and > 6 for lamb and beef (Shepherd and Little, 2014). In addition, salmon show higher

protein and energy retentions, and harvest and edible yields than terrestrial meat production (Shepherd and Little, 2014). The only practical use that a FIFO can serve is perhaps as a monitor of how different aquaculture sectors are developing because, overall, with expanding production and finite supplies of FO (and FM), the only way is down for FIFO. Indeed, it has been recently demonstrated that Atlantic salmon can be a net producer of both marine protein (Crampton et al., 2010; Bendiksen et al., 2011) and n-3 LC-PUFA (Sanden et al., 2011; Turchini et al., 2011b), although for the latter it should be stressed that the levels of n-3 LC-PUFA in the products are far lower than those in fish fed FO.

The real problem with FM and, especially, FO, as with many commodities, is supply and demand. What was not sustainable was the use of high volumes of finite resources, which is not suggesting that FO and FM are themselves unsustainable resources (Jackson and Shepherd, 2013). The sustainability of the industrial/reduction fisheries is no different to any other fishery in the world. To be sustainable, all fisheries have to be managed and regulated (FAO, 2005), as they are in for many species in several areas including S. America and Europe (Jackson and Shepherd, 2012). Application of the IFFO-RS standard to the whole marine ingredient/resource sector, including Asia, although challenging, is continuing and represents real improvement (IFFO, 2012). However, FO and FM supplies will always be finite and limiting. As FO (and FM) are the primary sources, this implies that the current global supply of n-3 LC-PUFA is similarly finite and limited, and the gap between supply and demand can be estimated. Based on the recommended dose for cardiac health, the total demand for n-3 LC-PUFA is over $1^{1}/_{4}$ million metric tonnes (mt) whereas total supply is optimistically estimated at just over 0.8 million mt indicating a shortfall of over 0.4 million mts (Table 1). The majority of supply (almost 90 %) is from capture fisheries, whether as food fish or via FO and FM, with relatively small additional amounts realistically estimated from seafood by-products and recycling, unfed aquaculture and algal sources. While it is acknowledged that the calculations in Table 1 contain some assumptions and estimates, and the precise extent of the difference can be the argued, the fact that the gap exists is not in question irrespective of how it

is calculated (Naylor et al., 2009). There is a fundamental, global lack of n-3 LC-PUFA to supply all human needs, whether by direct consumption or via aquaculture.

7.2 Alternative sources of n-3 LC-PUFA

7.2.1 Other oils of marine origin

Possible alternative marine sources for oils include utilising lower trophic levels, specifically zooplankton such as krill and calanoid copepods in the southern and northern hemispheres, respectively, and mesopelagic fish. The biomass at lower trophic levels is large, but there are inherent dangers associated with fishing down the marine food web (Pauly et al., 1998).

The utilisation of krill and copepods has been studied and, in general, zooplankton can be potentially good lipid sources (Olsen et al., 2011). However the harvesting of krill and copepods poses significant technological challenges and cost. For most species, lack of schooling behaviour makes harvest by traditional trawling technology an expensive economic option (Olsen et al., 2011). Antarctic krill, which do form schools, is the only species being targeted for commercial harvest, apart from a small scientific quota (~1000 mt) of the calanoid copepod, *Calanus finmachicus* (Croxall and Nicol, 2004). MSC-certified krill meal up to 10% is currently being used in some salmon feeds during the seawater phase in both Norway and Scotland as the higher growth rate and consequent shorter cycle compensates for the increased cost. Other feeds can have krill meal at even higher levels, but these are premium feeds focussed on health benefits and are used sparingly. Therefore these krill products are not being used as primary sources of n-3 LC-PUFA. Currently, krill lipid products are used almost exclusively for the human nutraceutical market. Although there may be evidence that harvesting krill, and potentially copepods, could be sustainable, there are still significant environmental and ecological concerns (Olsen et al., 2011). For instance, Antarctic krill are near the base of a food chain that includes whales and penguins that would suggest there could be opposition to greatly increased exploitation (Hill et al., 2006).

Copepods have an additional problem, as the oil is rich in wax esters rather than TAG, which may limit its widespread use (Olsen et al., 2004; Bogevik et al., 2010).

Mesopelagic fish that inhabit the intermediate pelagic water masses between the euphotic zone at 100 m depth and the deep bathypelagic zone at 1000 m are available in potentially large quantities (1 - 6 billion mt), with lantern fish, myctophids, constituting about 60 % of biomass (Irigoien, 2014). Different species can contain between 16 and 60 % of dry weight (Falk-Petersen, 1986; El-Mowafi et al., 2010) as lipid that can be in the form of TAG or, in some species, wax esters and, regardless of species, they are all good sources of n-3 LC-PUFA (Olsen et al., 2011). On the positive side, they are resources that, so far, have not been the subject of commercial exploitation, and they do not compete with existing or potential human feed production. Negative points include biological (seasonal variation), ecological (mixed fishery difficult to manage), technical (capture methods and on-board processing), and nutritional (wax esters) issues.

7.2.2.By-catch and seafood processing by-products

In almost all fisheries there are non-target catch and/or discarded target catch that, together, make up the by-catch. However, both the precise definition and resultant estimates of by-catch can be controversial (FAO, 2009). In 2005, the discard rate was estimated at around 7 million mt/year or 8 % of global catch (Kelleher, 2005). By its nature, by-catch is a diffuse resource (Batista, 2007) and this imposes a major limitation to its usefulness as a source of FO, although processing of by-products, including oil production, at sea is an increasing trend (Falch et al., 2007). Another limitation is that by-catch includes a multitude of species, not necessarily “oily”, which limits the quantity and quality (lipid class and fatty acid compositions, lipid soluble nutrients and contaminants) of the oils produced (Batista, 2007).

Another potential source of fish/marine oils is seafood industry by-products including viscera, heads, carcasses and trimmings, particularly those produced from pelagic fisheries and aquaculture. Whereas these by-products can produce significant amounts of FM (Lekang and

Gutierrez, 2007), the production of oil is largely dictated by species. Thus, by-products from oily species including salmon, herring and mackerel can be a source of substantial FO whereas by-products from other pelagic (white fish) fisheries have generally lower lipid contents (Lekang and Gutierrez, 2007). Liver from species like cod and halibut have traditionally been used for FO production, but production is now relatively small (~ 40,000 mt) and goes mostly for direct human consumption as vitamin A and D supplements as much as sources of n-3 LC-PUFA (Hertrampf and Piedad-Pascual, 2000). Oil is now being actively recovered from aquaculture species waste, particularly salmon farming, with around 20,000 mt reportedly recovered in Norway (Rubin, 2009), and 50,000 mt in Chile in 2006 (Tacon and Metian, 2008).

Other limitations to the use of oils from aquaculture by-products include regulatory issues preventing intra-species use to prevent recycling of contaminants and disease transfer, and so oils from aquaculture are currently used either for human nutrition or other farmed species depending on quality (Skåra et al., 2004). In addition, recycled oils from freshwater aquaculture species including carps and tilapia can be of limited value due to the generally low levels of n-3 LC-PUFA (Borghesi et al., 2008). Overall, by-catch and seafood by-products are potential sources of FO and, although there is currently some production, the contribution of these sources to overall FO supply is not well quantified (Jackson and Shepherd, 2012; Shepherd and Jackson, 2013).

7.2.3.Microalgae

Potentially, culture of the main primary producers, marine microalgae, could offer the ideal long-term, sustainable solution to the problem of n-3-LC-PUFA supply (Apt and Behrens, 1999). Various photosynthetic microalgae are already commonly used in hatcheries to supply both EPA (e.g. diatoms) and DHA (e.g. flagellates) to live feeds, rotifers (*Brachionus* spp.) and *Artemia* nauplii, for the rearing of larval marine crustacean and fish species (Muller-Feuga, 2004). Production usually employs medium- to high-density batch, semi-continuous or continuous culture in relatively small volumes (Stottrup and McEvoy, 2003). Up-scaling of production to the volumes

required for algal oil and/or algal biomass to supply the amount of n-3 LC-PUFA required to replace FO in commercial aquafeed production has very significant biological and technological challenges. Economic production of n-3 LC-PUFA would require algae to demonstrate simultaneous high growth and high lipid content with a high proportion of EPA and DHA. These can be almost exclusive traits as lipid deposition is often associated with conditions when growth is limited (e.g. N limitation) (Richmond, 2008). Technical challenges include efficient capture of light energy in high-density culture with effective temperature control. These issues remain to be solved but this is not to say that algal species with favourable biological characteristics cannot be found, and photo-bioreactor technologies may improve considerably in the future.

In contrast, heterotrophic microalgal species including *Crypthecodinium* and thraustochytrids such as *Schizochytrium* are already being utilised for the commercial production of DHA using large-scale biofermentor technology (Raghukumar, 2008). Even so, the high production costs are generally limiting the use of these products to direct human consumption mainly in the form of DHA supplementation of infant formulae (Ward and Singh, 2005). Research showed that substituting FO with thraustochytrid oil in the diet of Atlantic salmon parr significantly increased muscle DHA without any detriment to growth (Miller et al., 2007). Similarly, biomass from *Schizochytrium* and *C. cohnii* have been used to substitute for FO with no deleterious effects in larval microdiets and starter feeds for gilthead seabream (*Sparus aurata*) (Atalah et al., 2007; Ganuza et al., 2008). Currently, a thraustrochytrid algal biomass product (DHAgold, DSM, Maryland) containing around 18 % DHA (by weight) is commercially available and apparently being used in aquaculture (Miller et al., 2011). Therefore, these DHA-rich products from heterotrophic microalgae may have niche markets in marine hatcheries, particularly for high-value marine species. Production volumes would have to be increased and costs reduced before these products could be viable for wider application in aquaculture. Biochemically, *Schizochytrium* sp. are particularly interesting as they appear to have two alternative pathways for n-3 LC-PUFA biosynthesis (Lippmeier et al., 2009). Primary production of DHA and 22:5n-6 in *Schizochytrium* is

via a PUFA synthase (Metz et al., 2001), a series of 3 genes similar to those found in LC-PUFA-producing marine bacteria (Yazawa, 1996; Morita et al., 2000). In addition, a series of aerobic PUFA desaturase and elongase genes are also present in S*chizochytrium* (Lippmeier et al., 2009).

7.2.4 Transgenics

As discussed above, it is unlikely that microalgal biomass can be produced on the scale necessary and at an economic cost to satisfy the demands of aquaculture for n-3 LC-PUFA, at least in the short- to medium-term (Miller et al., 2011). However, microalgae represent a highly valuable source of genes encoding for the biosynthetic enzymes required for n-3 LC-PUFA production (Venegas-Caleron et al., 2010). The overall strategy being to genetically modify existing organisms that have oil deposition as a major trait and thus combine this with the n-3 LC-PUFA biosynthesis trait. Potential candidates include other oleaginous microorganisms or conventional oilseed crops to produce entirely novel sources of *de novo* n-3 LC-PUFA (Zhu et al., 2010; Sayanova and Napier, 2011).

Metabolic engineering of the oleaginous yeast *Yarrowia lipolytica* has resulted in a strain that produced EPA at 15 % of dry weight (Xue et al., 2013). It was shown that the EPA-*Yarrowia* cell mass was suitable as a feed ingredient for Atlantic salmon (Hatland et al., 2012) although disruption was required to increase the bioavailability of lipid and EPA (Berge et al., 2013). To the best of the author's knowledge, the transgenic yeast oil or cell biomass are not yet fully commercially available although they appear to be used by aDuPont/AquaChile venture (Verlasso®) to produce a niche salmon product (http://futurefood2050.com/turning-yeast-into-sustainable-fish-food/). However, as the production of the transgenic yeast uses biofermentor technology similar to that used for *Schizochytrium*, it appears unlikely that it could be produced in volumes and at a cost that would make it viable as a large-scale alternative to FO in aquaculture, at least in the short- to medium-term.

Oilseed crops dominate world oil production and there is a highly organised and well-established infrastructure for the cultivation, harvest, processing, distribution, marketing and utilisation of vegetable oils (Salunkhe et al., 1992). Therefore, oilseed crops are highly practical platforms from which to develop a novel, renewable supply of n-3 LC-PUFA. However, conventional plant breeding strategies cannot be used as the genes required for LC-PUFA synthesis are simply not present in higher plants, leaving transgenesis as the only option for modification of oilseeds to contain LC-PUFA. Therefore, the only currently viable approach to developing a novel, renewable supply of EPA and DHA is the metabolic engineering of oilseed crops with the capacity to synthesise n-3 LC-PUFA in seeds (Haslam et al., 2013). Production of n-3 LC-PUFA in terrestrial plant seeds was demonstrated in the model plant Arabidopsis (Petrie et al., 2012; Ruiz-Lopez et al., 2013), and very recently reported in an oilseed crop, *Camelina sativa* (Petrie et al., 2014; Ruiz-Lopez et al., 2014). *C. sativa* or false flax, is a member of the Brassicaceae family and an ancient crop that, in the wild-type, produces an oil with LNA at up to 45 % of total fatty acids (Gunstone and Harwood, 2007). Transgenic *C. sativa* lines have now been developed by transformation with algal genes encoding the n-3 LC-PUFA biosynthetic pathway and expression restricted to the seeds via seed-specific promoters to produce oils with over 20 % of total fatty acids as n-3 LC-PUFA, either as EPA alone or as EPA + DHA (12 % + 8 %) (Ruiz-Lopez et al., 2014).

As well as being easily transformable by Agrobacterium floral infiltration, *Camelina* has additional desirable traits including modest input requirements (water and pesticides) and ability to thrive in semi-arid conditions (Tocher et al., 2011). In the US, several states are actively growing *Camelina* as a biofuels crop, indicating the wide acceptance of this crop platform. Furthermore, wild-type Camelina oil has already been shown to be suitable for inclusion in fish feeds and contains no anti-nutritional factors detrimental to fish growth (Petropoulos et al., 2009; Morais et al., 2012b; Hixson et al., 2014). Ultimately, all animal production will depend on terrestrial plants/agriculture and this requires land. However, it is pertinent to emphasise that the production of n-3 LC-PUFA in terrestrial oilseed crops should not require additional arable land as the ideal

solution would be to switch some VO production from n-6 PUFA-rich crops to the new n-3 LC-PUFA crops. However, irrespective of the advances in science, one of the greatest challenges, at least in Europe, will be to change public opinion towards acceptance of genetically modified products before these transgenic oils can be used commercially on a global scale.

8. Conclusions

Although PUFA in general are essential nutrients in vertebrates, it is the highly bioactive LC-PUFA that have the major critical metabolic functions including maintaining physiological homeostasis, cardiovascular health, inflammatory and immune responses and neural development. The qualitative EFA requirements of fish depend on the extent of endogenous LC-PUFA biosynthesis and so LNA can satisfy requirements in some species whereas EPA and DHA are required in others. Irrespective, the quantitative EFA requirements for optimal growth and health of fish are relatively low. In contrast, the level of dietary n-3 LC-PUFA required to maintain farmed fish as good sources of these nutrients for human consumers is high, and the finite and limited supply of FO, the principle source of EPA and DHA, and the lack of sustainable alternatives containing n-3 LC-PUFA has posed a major challenge. Optimising n-3 LC-PUFA biosynthesis and retention in fish through nutritional strategies and genetic selection are sensible approaches for the efficient use of limited resources. However, it is unlikely that metabolism or genetics of fish can be modulated or manipulated sufficiently to compensate for reduced dietary EPA and DHA. Therefore, alternative sources of dietary n-3 LC-PUFA are required. While novel marine sources and improved use of fisheries and seafood by-products can contribute in the short to medium term, the only long-term solution is an entirely new supply of d*e novo* EPA and DHA. While mass culture of the primary producers, marine phototropic microalgae, would arguably represent the most natural solution, major biological and technological challenges remain to be solved. In the meantime, GM solutions, expressing algal genes in oleaginous platforms, are already developed with transgenic oilseed crops currently presenting the most effective solution for the large-scale production of EPA and DHA.

The science has been successful and the agricultural and industrial infrastructure is already in place and, although there are significant socio-political challenges to the acceptance of GM, particularly in Europe, other parts of the world will embrace this solution to the problem of n-3 LC-PUFA supply that transcends aquaculture and is important to everyone.

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Legends to Figures

Fig.1. Current global use of fish oil. A. Major fates of fish oil in 2012; B. Consumption of fish oil by the various aquaculture sectors in 2012; C. Trend in volumes (million tonnes) of fish oil used by aquaculture and other sectors from 2005-2012.

Fig.2. Pathways of long-chain polyunsaturated fatty acid biosynthesis in fish. All activities have been demonstrated in teleost fish species but not all species express all activities. The Δ8 desaturation activity is an inherent characteristic of the Δ6 Fad of most teleosts, especially marine species (Monroig et al., 2011b). A discrete Δ5 Fad has only been isolated from Atlantic salmon although bifunctional Δ6Δ5 Fads have been characterised in zebrafish, rabbitfish, *Siganus canaliculatus* and Mexican silverside, *Chirostoma estor* (Hastings et al., 2001; Li et al., 2010; Monroig et al., 2014). The presence of Δ4 Fad enabling direct production of 22:6n-3 from 22:5n-3 has been demonstrated in rabbitfish, Senegalese sole, *Solea senegalensis* and Mexican silverside, *Chirostoma estor* (Li et al., 2010; Morais et al., 2012c; Monroig, et al., 2014). The Sprecher shunt pathway via C24 fatty acid intermediates has been shown in trout (Buzzi et al., 1997) and the Δ6 Fad of Atlantic salmon and zebrafish can operate on both C18 and C24 fatty acids (Tocher et al., 2003). Δ4 Fad, Δ5 Fad and Δ6 Fad, fatty acyl desaturases; Elovl2, Elovl4 and Elovl5, fatty acid elongases.

Fig.3. The relative proportions (percentage of total fatty acids, panel A), absolute contents (g / 100g flesh, panel B) and consolidated data (panel C) of EPA + DHA in farmed (grey bars) and wild (black bars) salmon products obtained from major UK retailers. On the x-axis, each letter represents a retailer and the following number denotes a specific product. Data are means ± S.D. (n = 2-4 depending upon product for panels A and B; n = 34 and 12 for farmed and wild products, respectively, in panel C). Superscript letters in panel C represent significant differences between

farmed and wild products as determined by t-tests. Reprinted with permission (Henriques et al., 2014).

Table 1. Potential annual demand for n-3 LC-PUFA based on recommended intake for secondary prevention of CVD compared with global annual supply from major sources.

	Million tonnes	Notes
Demand (3.5g/week x 52 weeks x 7 billion)	**1.274**	Based on 500 mg/day (ISSFAL, 2004)
Supply (see below)	**0.84**	Less than $^2/_3$. See below for breakdown
Gap (Demand - Supply)	**0.434**	
Capture fisheries (food)		
Landed	75.0	FAO, 2014.
Edible yield (50%)	37.5	
Lipid content (5%)	1.875	
n-3 LC-PUFA content (20%)	**0.375**	
Capture fisheries (feed)		
Landed	20.0	FAO, 2014.
Fishmeal produced (22%)	4.4	Fishmeal yield generally constant at 22%
Lipid content (10%)	0.44	
n-3 LC-PUFA content (25%)	**0.11**	
Fish oil produced	1.0	Oil yield is variable
n-3 LC-PUFA content (25%)	**0.25**	
Seafood by-products (incl. aquaculture)		About 50 % of by-products are produced
Fishmeal produced	2.0	from aquaculture and, therefore, the
Lipid content (5%)	0.1	n-3 LC-PUFA is mainly derived from
n-3 LC-PUFA content (10%)	**0.01**	fishmeal and fish oil and , as such,
Fish oil produced	0.2	already counted above.
n-3 LC-PUFA content (10%)	**0.02**	
Aquaculture (unfed)		Mainly filter-feeding carps (7.1 mt) and
Produced	20.5	bivalves (13.4 mt) (FAO, 2014)
Edible yield (50%)	10.25	
Lipid content (5%)	0.525	
n-3 LC-PUFA content (10%)	**0.05**	
Aquaculture (endogenous production)	NA	Salmonids and fed freshwater species[1]
Macroalgae		Including species used for carageenin,
Produced	23.8	algin, agar and iodine (~16 mt)
Food or feed use	7.0	Human consumption & aquaculture
Lipid content (2%)	0.14	
n-3 LC-PUFA content (15%)	**0.02**	

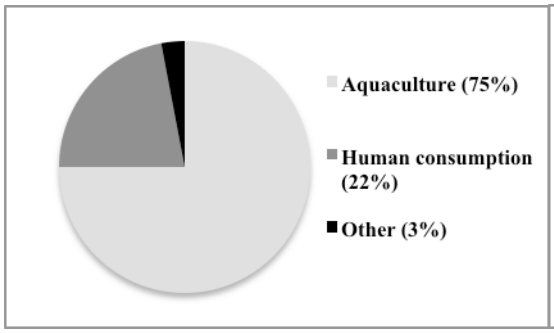
Production figures are from 2012 (FAO, 2014). All lipid and n-3 LC-PUFA contents/yields are estimated averages over the wide range of species in each category.

[1],Salmonids include salmon and trout spp. Freshwater species includes eels, tilapia and other freshwater species that still utilise some fishmeal and fish oil (13% of total fish oil used in aquaculture) in feeds.

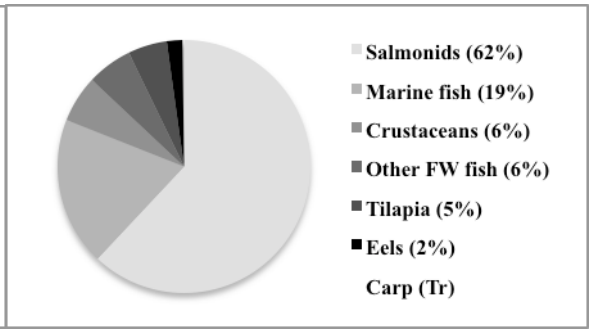
NA, not available. Although there will be some endogenous production of n-3 LC-PUFA inversely related to fishmeal and oil contents of feeds, this cannot be accurately estimated, but will likely be minor based on volume of fish oil consumed by these sectors (75 % of all fish oil used in aquaculture).

Fig. 1.

A


Aquaculture (75%)
Human consumption (22%)
Other (3%)

B


Salmonids (62%)
Marine fish (19%)
Crustaceans (6%)
Other FW fish (6%)
Tilapia (5%)
Eels (2%)
Carp (Tr)

C

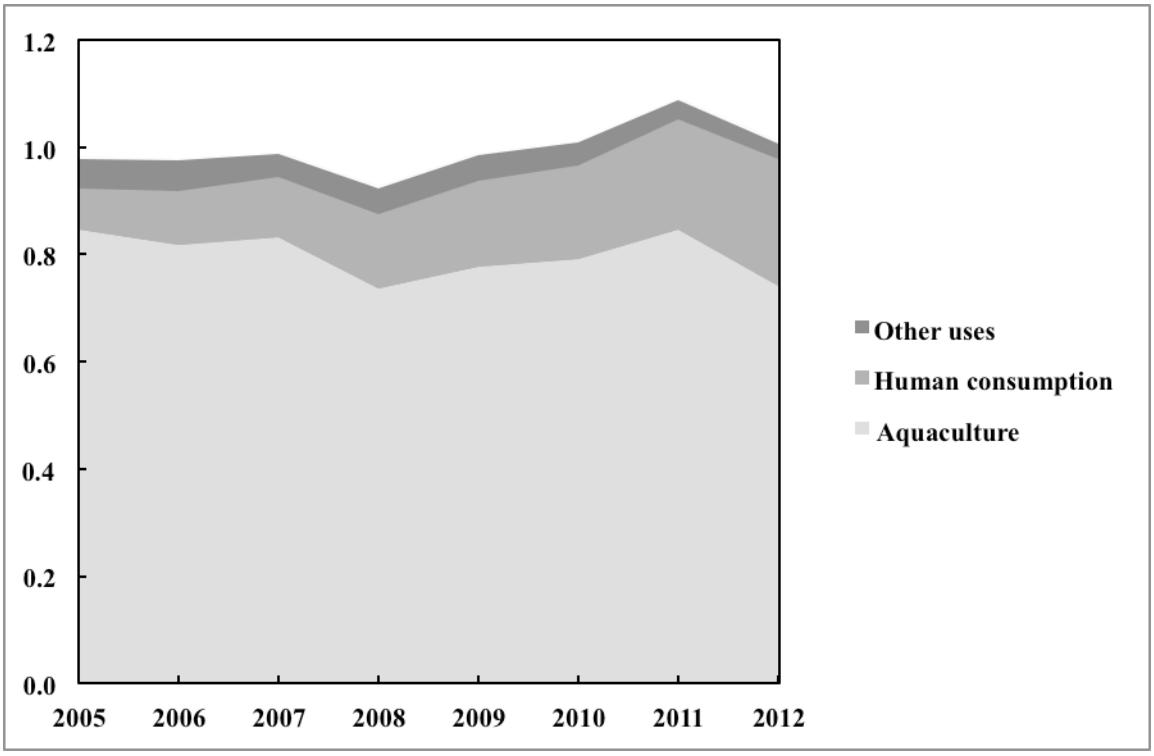

1.2
1.0
0.8
0.6
0.4
0.2
0.0
2005
2006
2007
2008
2009
2010
2011
2012
Other uses
Human consumption
Aquaculture

Fig. 2.

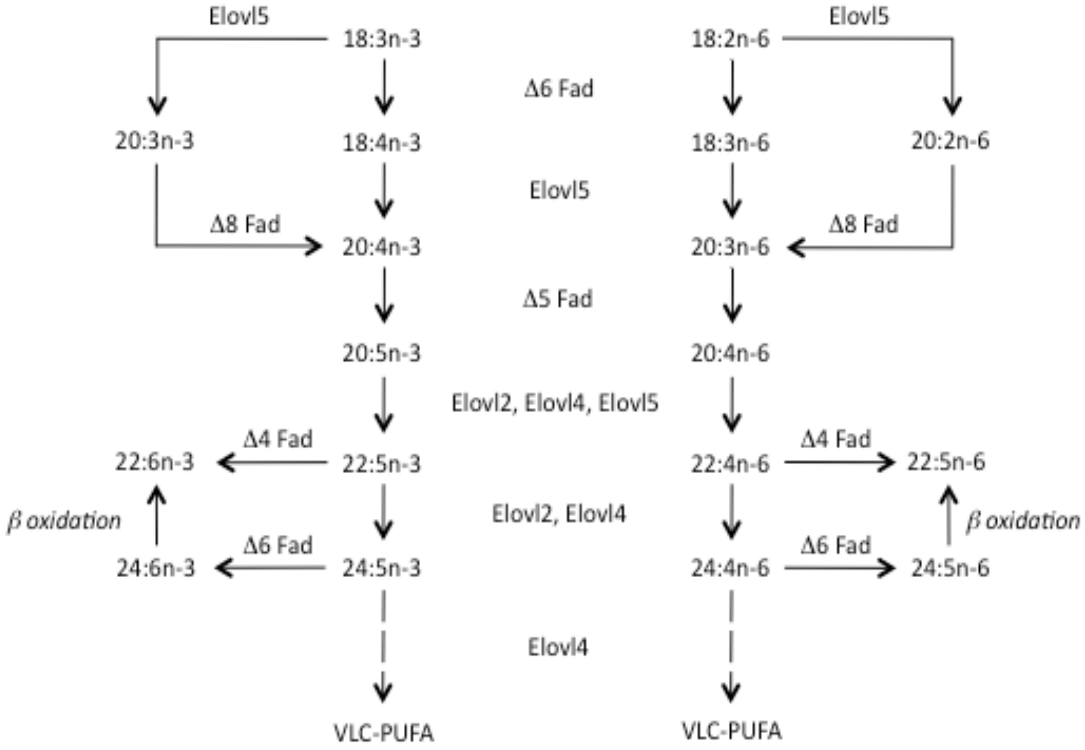
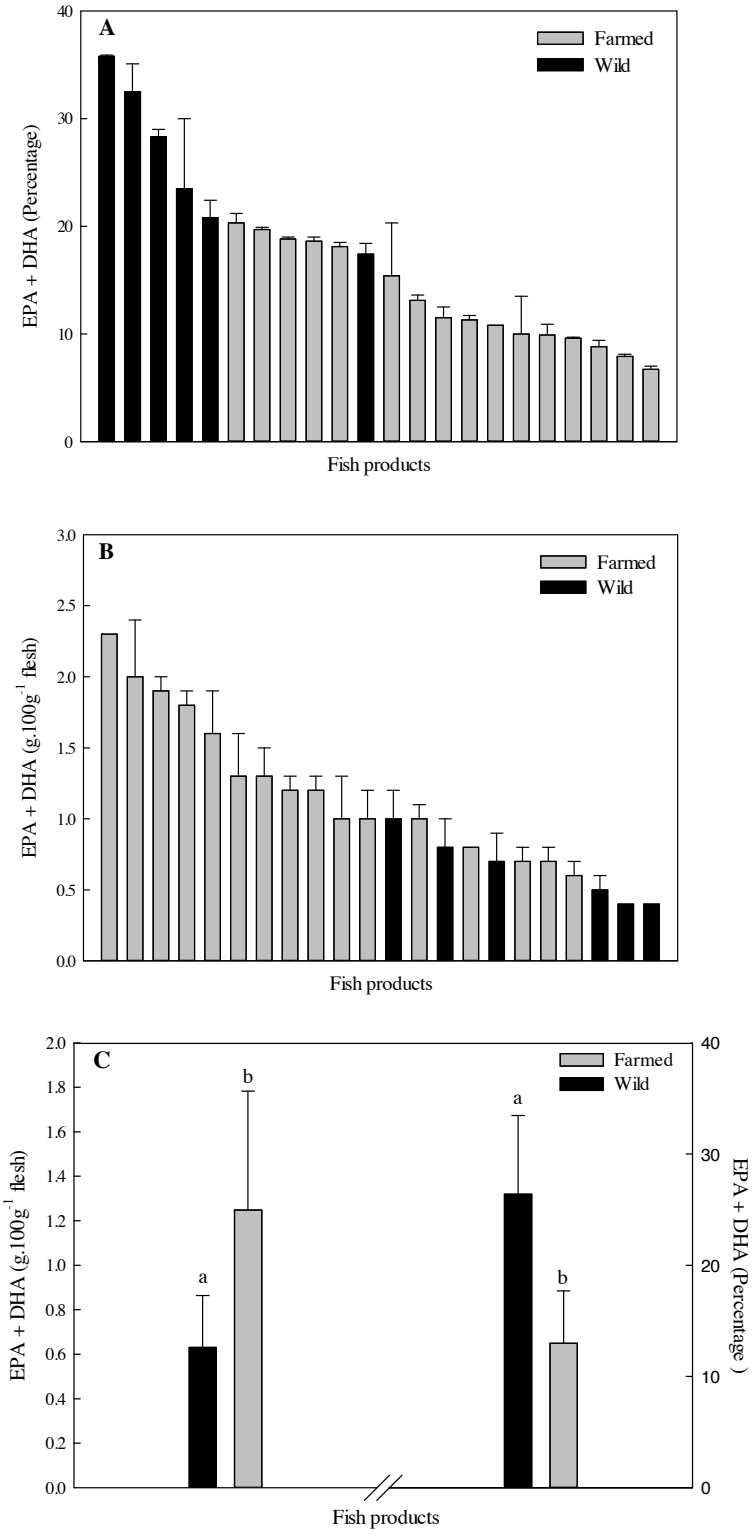

Elovl5
18:3n-3
Δ6 Fad
20:3n-3
18:4n-3
Elovl5
Δ8 Fad
20:4n-3
Δ5 Fad
20:5n-3
Elovl2, Elovl4, Elovl5
Δ4 Fad
22:6n-3
22:5n-3
β oxidation
Elovl2, Elovl4
Δ6 Fad
24:6n-3
24:5n-3
Elovl4
VLC-PUFA
Elovl5
18:2n-6
18:3n-6
20:2n-6
Δ8 Fad
20:3n-6
20:4n-6
Δ4 Fad
22:4n-6
22:5n-6
β oxidation
Δ6 Fad
24:4n-6
24:5n-6
VLC-PUFA

Fig. 3.


A
Farmed
Wild
EPA + DHA (Percentage)
Fish products
B
Farmed
Wild
EPA + DHA (g.100g-1 flesh)
Fish products
C
Farmed
Wild
a
b
a
b
EPA + DHA (g.100g-1 flesh)
EPA + DHA (Percentage)
Fish products