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Omega-3 Futures in Aquaculture: Exploring the Supply and Demands for Long-Chain Omega-3 Essential Fatty Acids by Aquaculture Species

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ABSTRACT

Long-chain polyunsaturated fatty acids (LC-PUFA), like 22:6n-3 (Docosahexaenoic acid; DHA) and 20:5n-3 (Eicosapentaenoic acid; EPA), are recognized for a range of important physiological roles in many aquaculture species. While the effects of EPA and DHA on a range of performance attributes and meat qualities are well recognized, an increasing awareness of their role in immune function, reproduction, bone formation and stress response is also emerging. Against this background of demand, global supplies of LC-PUFA are dominated by fish oil production from a diversified range of sources, though news sources are emerging. Among those aquaculture sectors that are the largest users of LC-PUFA resources (salmonids, shrimp, and marine fish), there are varying degrees of capacity by each to endogenously synthesize LC-PUFA and this affects the degree to which they must be obtained *via* the diet. Salmonids, which are the largest user of these nutrients possess some capacity to make EPA and DHA de novo, although evidence supports that salmonids perform better when provided with them preformed. Requirements by shrimp for LC-PUFA are variable, with evidence indicating that some species have capacity to desaturate and elongate fatty acids, whereas others do not. This is consistent with the observation that some species can utilize short-chain polyunsaturated fatty acids, whereas others need pre-formed LC-PUFA in their diet. A third group, marine fish, have limited ability to desaturate and elongate precursor fatty acids and therefore have a critical requirement for LC-PUFA in their diet. Evidence across multiple species indicates that demands for these fatty acids are greater when the animals are young, and this demand decreases as they age. Among the various marine fish species examined estimates of requirements vary substantially and a one-size-fits all approach is clearly not applicable.

KEYWORDS

DHA; EPA; metabolism; production; PUFA; requirement; supply

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) of the omega-3 class (docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA)) are increasingly gaining attention as valuable dietary constituents for both humans and domestic animals (Calder 2015, 2017; Tocher 2015; Simopoulos 2016). Among natural sources of these fatty acids, fish and fish oils remain the largest pool available, but demands for these nutrients for applications in humans, aquaculture, and petfoods are growing, and alternatives to fish oil are emerging. This was the context of a workshop held in Stirling, Scotland in May 2023, where the current state of knowledge of the supply and demand for omega-3 LC-PUFA resources, and in particular how these related to aquaculture, was discussed. Initially, the current state of global production, resource flows and emerging sources of EPA and DHA were discussed. This was followed by an assessment of the roles that EPA and DHA play in both fish and human physiology, and how they are applied to aquaculture feeds to satisfy the nutritional requirements of key species. Finally, gaps in knowledge were considered and paths forward evaluated. This article summarizes of the proceedings of that workshop.

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Current and emerging sources of omega-3 LC-PUFA

The production volume of omega-3 LC-PUFA is dominated by those derived from small pelagic fish (IFFO 2024). Estimates of global fish oil production are around 1,200 ktonnes per annum, though in the past 30 years this has peaked at more than 1,600 ktonnes (in 1996) and been as low as 850 ktonnes (in 1998), but for the more recent past there has generally been stable production averaging around 1,100 ktonnes (Figure 1). While production of fish oils from small pelagic fish in South America and the North Atlantic has dominated global production, increasingly by-products from various fisheries and aquaculture are making a significant contribution to global fish oil production volumes (IFFO 2024).

Based on the levels of the long-chain omega-3 fatty acids, these oils can be divided into different qualities, with EPA and DHA comprising from ~2% to almost 60% of the total fatty acids among them (Table 1). Based on oil type, typical fatty acid profiles and volumes of production, estimates of the total EPA + DHA production from all these resources can be estimated at around 160 ktonnes per annum, though has been impacted in recent years by El Niño events in the southeastern Pacific Ocean (Figure 2). A largely static supply and a growing demand for omega-3 LC-PUFA resources has led to increasing volatility in fish oil prices in the global market (Figure 3). This has encouraged the development of other sources of these fatty acids which are now emerging in the marketplace, including those produced from zooplankton, algae, and genetically modified (GM) plants (Ruiz-Lopez et al. 2014; Tocher et al. 2019; Oliver et al. 2020; Napier and Betancor 2023). So far though, only algal oils contribute any significant volume, with an estimate of 12 ktonnes predicted for 2023 (Figure 2).

Global flows and dynamics of omega-3 LC-PUFA supplies

An analysis of the global flows and dynamics of omega-3 LC-PUFA, based on a systems analysis approach shows various points of substantial losses, both biological and anthropogenic (Hamilton et al. 2020). Notable is the large scale of trophic losses that occur with these nutrients from their production *via* marine net primary productivity (NPP: 1400 Mtonnes) and losses *via* sinking phytoplankton (PP) aggregates (160 Mtonnes), dissolved organic matter (DOM: 220 Mtonnes) and consumption *via* zooplankton (960 Mtonnes) and the associated biological losses therein (840 Mtonnes) (Figure 4). Actual accumulation of omega-3 LC-PUFAs within oceanic marine resources is estimated at 9 Mtonnes, of which 0.671 Mtonnes (671 ktonnes) is captured as fishery resources based on a combination of krill, marine fish, and freshwater sources. Of that omega-3 LC-PUFA resource 369 ktonnes is estimated to flow to fish processing, with 298 ktonnes being rendered through combined fishmeal and fish oil production. Most of the production from fish rendering goes to aquaculture (242 ktonnes), which combined with an additional 75 ktonnes *de novo* production from some aquaculture species (e.g., carps and tilapia), produces a pool of 300 ktonnes of EPA + DHA in the form of farmed fish. The farmed

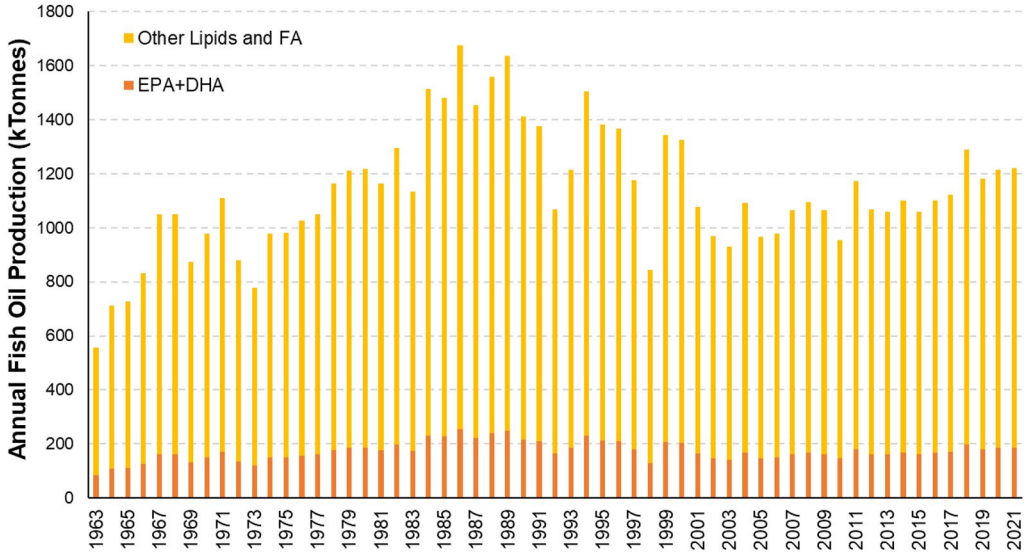


Figure 1. Variability in total volume of annual global fish oil and estimated EPA + DHA production from 1963 to 2021. Data from IFFO (2024).

Table 1. Typical fatty acid profiles (% of total fatty acids) of various marine oils.

	Anchoveta	*Blue whiting*	*Capelin*	*Herring*	*Krill*	*Atlantic mackerel*	*Menhaden*	*Sardine*	*Sandeel*	*Sprat*	*Pangasius by-products*	*Salmon by-products*	[a]*Microalgae*
C14:0	9	5	8	6	9	6	9	8	7	7	2	3	3
C16:0	17	14	10	15	20	15	19	22	17	17	26	12	30
C18:0	4	2	1	2	1	3	3	5	1	3	10	2	2
C20:0	0	0	0	0	0	0	0	1	0	0	0	1	1
C22:0	0	0	0	0	0	0	0	0	0	0	0	1	0
Total SFA	31	21	18	23	31	24	33	38	25	26	37	19	37
C16:1n-7	10	6	8	4	5	5	16	6	9	5	7	3	0
C18:1(n-9 + n-7)	12	14	11	13	16	16	11	10	8	15	42	36	0
C20:1(n-11 + n-9)	1	9	20	10	1	7	1	1	12	6	1	2	0
C22:1(n-11 + n-9)	0	14	24	18	1	13	0	0	17	12	0	0	0
C24:1n-9	0	1	1	1	0	1	0	1	1	1	0	0	0
Total MUFA	23	47	64	46	24	44	28	19	47	41	44	43	0
C18:2n-6	1	1	1	2	2	2	1	3	2	2	12	17	0
C18:3n-6	0	0	0	0	0	0	0	0	0	0	0	1	0
C20:4n-6	2	0	0	0	1	0	2	0	0	0	1	0	2
C22:5n-6	0	0	0	0	0	0	0	0	0	0	0	0	2
Total n-6 PUFA	3	2	1	3	3	3	3	3	2	3	12	19	4
C18:3n-3	1	1	1	2	1	1	1	0	2	1	0	4	0
C18:4n-3	2	2	2	3	4	3	2	0	2	2	0	0	0
C20:3n-3	0	0	0	0	0	0	0	0	0	0	0	0	0
C20:4n-3	1	0	0	0	0	0	0	0	0	0	0	0	1
C20:5n-3 (EPA)	18	7	5	6	23	6	14	14	11	6	0	4	16
C22:5n-3	2	1	0	1	0	1	3	0	1	1	0	3	2
C22:6n-3 (DHA)	10	9	3	9	11	10	8	17	8	10	1	4	40
Total n-3 PUFA	35	21	11	20	39	21	30	30	24	21	2	14	58
EPA + DHA	28	16	8	15	34	16	22	30	19	16	1	8	56

SFA: saturated fatty acids. MUFA: monounsaturated fatty acids. PUFA: polyunsaturated fatty acids.
[a]Veramaris Algal Oil (Tocher et al. 2019). All other data derived from IFFO (2024).

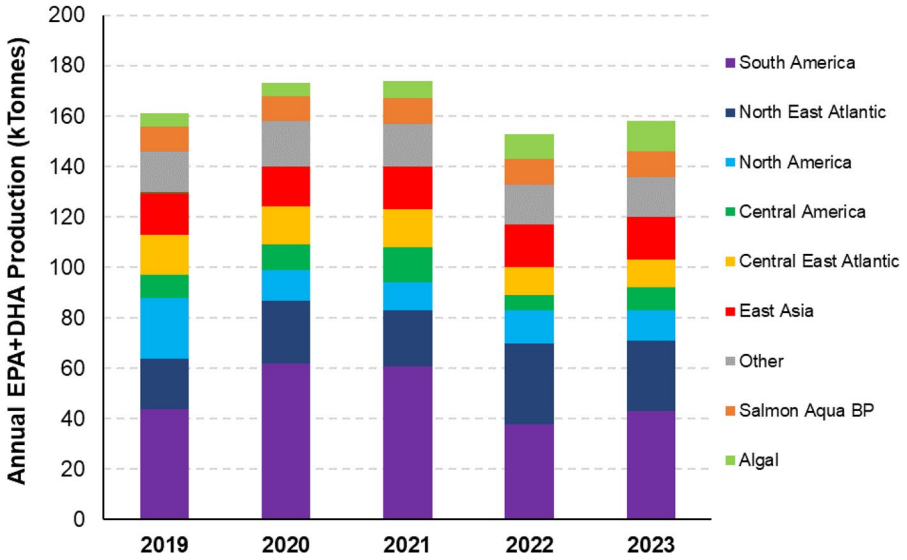


Figure 2. Global commercial production of EPA + DHA by origin. All are fish derived except "Algal". Salmon Aqua BP is derived from salmon aquaculture. Data derived from Holtermann (2023).

fish production is also supplemented by 44 ktonnes as fish oil and 1 ktonne as krill oil for direct human consumption (DHC), as well as 293 ktonnes from whole and processed wild fish. From that combined supply of 638 ktonnes, estimated food losses of 219 ktonnes occur, leaving just over 400 ktonnes available

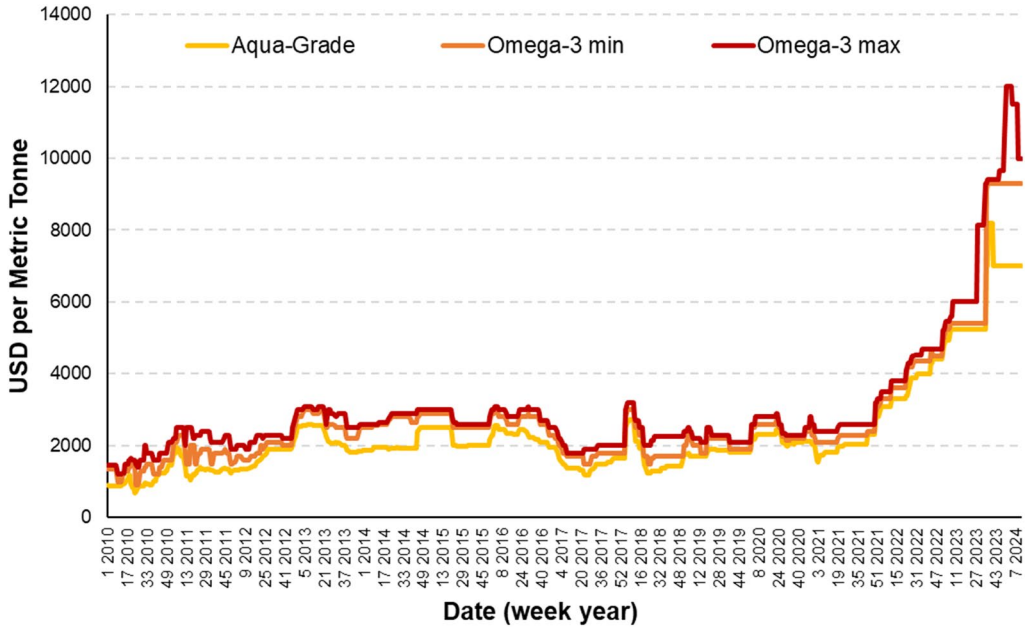


Figure 3. Price variability in three common grades of fish oils between January 2010 and March 2024. Data derived from IFFO (2024).

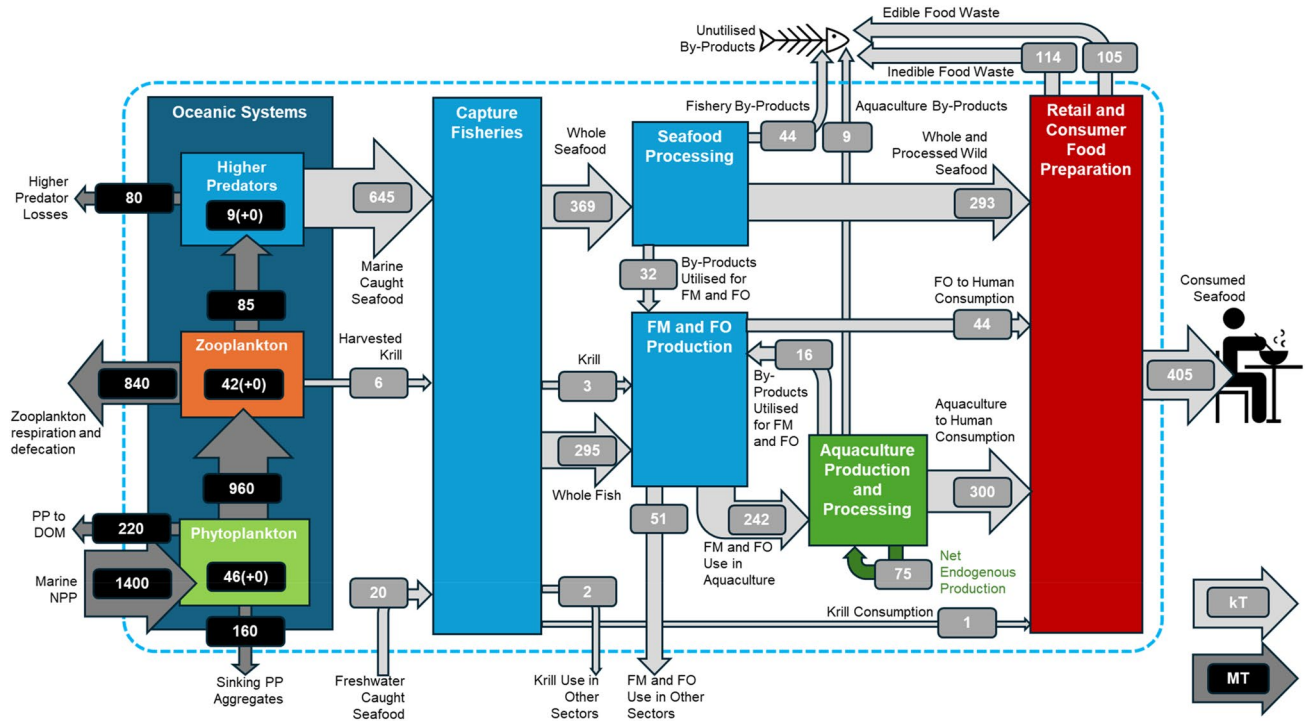


Figure 4. Modeled global flows and pools of EPA and DHA in kTonnes and MTonnes. DOM: dissolved organic matter. FM: fishmeal. FO: fish oil. NPP: net primary production. PP: phytoplankton. Based on Hamilton et al. (2020).

for human consumption (Figure 4). The scale of the losses of the omega-3 LC-PUFA resources through food wastage alone is equivalent to about 50% of that consumed, meaning that considerable scope exists for better nutrient reclamation through improvements in circular-resource thinking. While some reclamation of by-product EPA + DHA from aquaculture (16 ktonnes) and fish processing (32 ktonnes) is included in the model, the comparison between what is reclaimed, and the potential is stark. The model estimates that a total pool of 272 ktonnes of EPA + DHA is available from the combined unutilized by-product resources. The findings from this systems analysis highlight how better management of waste streams

would deliver more EPA + DHA into the combined pool. It was also noted that aquaculture is a greater contributor to human EPA + DHA supplies than the direct consumption of wild fish. In fact, aquaculture is a net producer of EPA + DHA relative to its inputs (300 vs 242 ktonnes of production and input of EPA + DHA respectively) (Hamilton et al. 2020). From a total of 346 ktonnes of EPA + DHA produced through fish rendering, 242 ktonnes went to aquaculture, 44 ktonnes to DHC and 51 ktonnes to other uses (presumably pet, pig and poultry feeds). The change in magnitude of scales between the production end (Mtonnes) and the use end (ktonnes) is a stark reminder of the trophic losses that occur through such nutrient chains and the potential benefits that might be gained by accessing EPA + DHA sources lower in the food-chain. An analysis of changing use of fish oil over time has shown the marked shift from a low-grade industrial oil to a valuable component of aquaculture feeds and more recently as a contributor to DHC (Figure 5). This transition has further accentuated the value of fish oils globally, with increasing competition for the resource.

Searching for new omega-3 LC-PUFA sources

Current production levels of the EPA and DHA are largely based on sustainable yield capacities from global fisheries (Hamilton et al. 2020). As was discussed earlier, current estimates of EPA and DHA production are around 160 ktonnes per annum, with more than 90% of this coming from fishery and aquaculture resources. Current estimates of fish oil obtained from by-products (trimmings) from fishery and aquaculture harvests for human consumption are around the 50% of total production range, more than 680,000 tonnes per annum (IFFO 2024). Wild fishery by-products currently constitute about 25% of global fish oil production, and these are a rich source of EPA and DHA (IFFO 2024). Capacity to expand fish oil production from a by-product resource base has been touted as one option for further development (Stevens et al. 2018). Significant volumes of that by-product fish oil are low in EPA and DHA (e.g., Pangasius oil), or are essentially "second-hand" fish oils, where the EPA and DHA is recovered from aquaculture by-products (e.g., salmon oil). Such oils have been shown to present very favorable sustainability credentials, with life cycle assessment (LCA) estimates showing that they are more sustainable than most crop derived oils (Newton et al. 2023).

In contrast to the recovery of EPA and DHA from fish oils, a range of new omega-3 LC-PUFA sources have been developed, with most attention being given to the production of microalgal (*Schizochytrium*) sources and development of crop plants to include genetically modified (GM) pathways to enable the production of EPA and DHA (Tocher et al. 2019). Microalgal sources of EPA and DHA are of two main forms: algal biomass that is typically 50% lipids, of which about 50% is DHA, and an algal oil which is around 50% to 60% omega-3 LC-PUFA (Kousoulaki et al. 2015; Santigosa et al. 2023a, 2023b). Algal oil is being well accepted across the aquaculture feed sector (Santigosa et al. 2020). In Europe a general negative sentiment against GM crop use has seen low adoption of that technology, though recent legislation changes in the United Kingdom (UK), may be seen as a sign of some future change there (UK Parliament 2023). More broadly, GM technology is well adopted

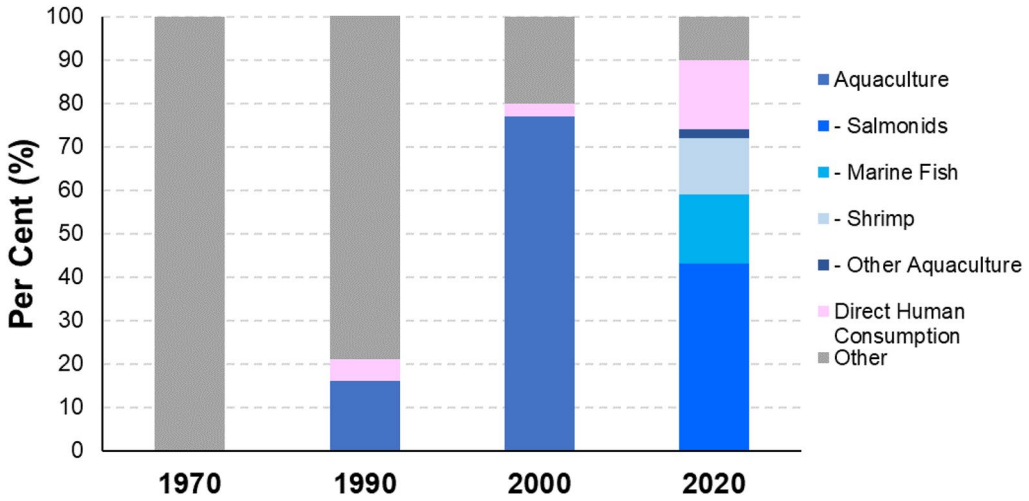


Figure 5. Changing use (% of total) of fish oils between 1970 and 2020. Overlaid on the 2020 aquaculture data is a generalized breakdown of fish oil use by aquaculture major sectors. Data from IFFO (2024).

in North America, South America, and Australia, with a variety of rapeseed (*Brassica napus*) now being commercially available that has ~10% DHA (Napier et al. 2019; Petrie et al. 2020; Zhou et al. 2023). Extensive evaluation of GM rapeseed (canola) has led to its regulatory approval across North America, Australia, Chile and more recently Norway (Ruyter et al. 2019, 2022; Davis and Devine 2023). Concurrent to the developments with rapeseed, other researchers have worked to develop a GM strain of *Camelina sativa* (Petrie et al. 2012, 2014; Ruiz-Lopez et al. 2014). These new varieties of GM-camelina oils have had some remarkable success in terms of the levels of EPA and DHA, with percentages higher than 25% being achieved in some varieties, replicating the omega-3 LC-PUFA levels found in many of the higher quality fish oils (Betancor et al. 2015, 2016). Assessment of both the rapeseed and camelina oils from GM strains in various fish species has shown that both are well accepted and nutritionally show little difference to other marine sources of EPA and DHA (Betancor et al. 2015, 2016; Ruyter et al. 2019, 2022). The commercialization of the GM-Camelina seems to be a step closer with a request for Status Review being submitted in 2023 to USDA-APHIS Biotechnology Regulatory Services under the Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient Rule. If approved this would allow the crop to be grown at large scale in the United States (The Fish Site 2023).

Omega-3 fatty acid biosynthesis

The biochemical reactions required to produce omega-3 fatty acids, along with all unsaturated fatty acids, are facilitated by a series of enzymes collectively referred to as the desaturase-elongase system. The desaturase enzymes introduce an alkene (unsaturated) bond into fatty acid chains, whereas the elongase enzyme adds an acetyl unit to the chain increasing the chain length by two carbons. The different desaturase reactions are usually defined by the chain position in which the reaction occurs (e.g., delta-9, delta-12, delta 15) (Figure 6). The ability to convert saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA) is a delta-9 desaturase reaction carried out by the enzyme stearoyl-CoA desaturase (SCD), which is found in all eukaryotic organisms (Bloch 1969; Wu et al. 2013). Synthesis of omega-3 or omega-6 polyunsaturated fatty acids (PUFA) is only possible if a delta-12 desaturase enzyme is present, requiring further action by a delta-15 desaturase to produce 18:3n-3 (alpha linolenic acid; ALA), and both

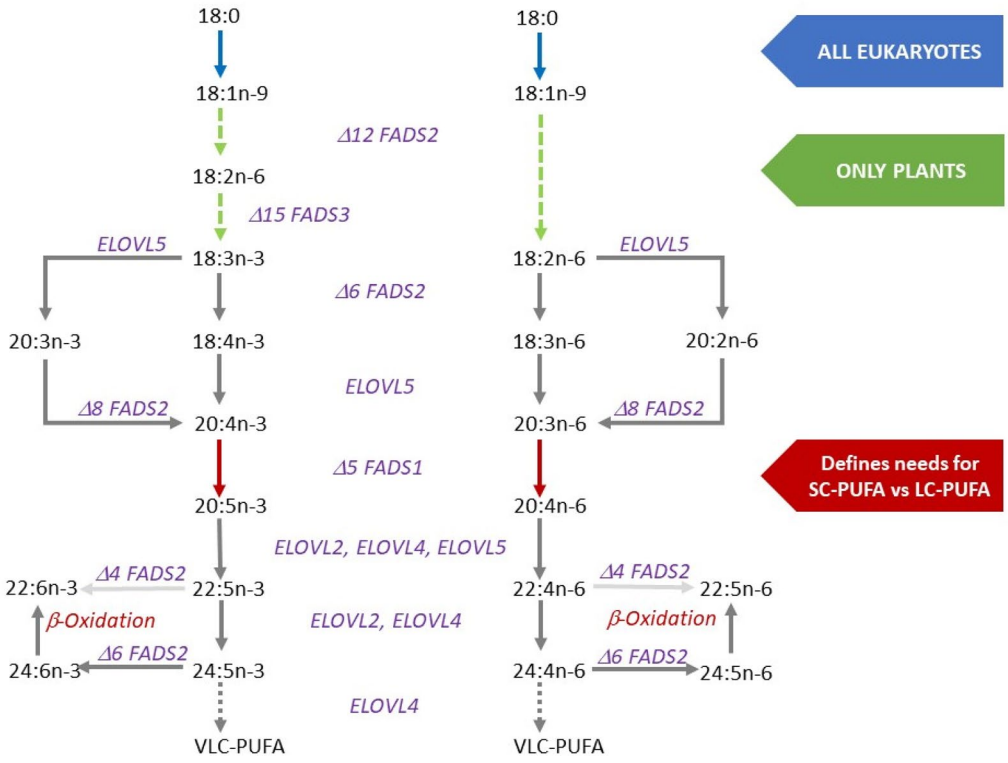


Figure 6. Biosynthesis pathways of long-chain polyunsaturated fatty acids. Shown are the enzymatic points where plants differentiate from animals, and where variation occurs among animals that influences their demands for shorter-chain polyunsaturated fatty acids (SC-PUFA) versus long-chain polyunsaturated fatty acids (LC-PUFA). Enzymes involved are detailed in purple italics.

of these enzymes are largely constrained to plants and some prokaryotes (Figure 6) (Gill and Valivety 1997; López Alonso et al. 2003; He et al. 2020). This step in the synthesis of either omega-3 or omega-6 fatty acids subsequently predestines these fatty acids into their further elongation and desaturation potential, with no switching between omega-3 and omega-6 classes observed from this point onwards (Henderson and Tocher 1987; Teshima et al. 1992; Tocher et al. 1998). The presence of these enzymes differs considerably between and within the prokaryote and eukaryote kingdoms (Henderson and Tocher 1987; Tocher et al. 1998; López Alonso et al. 2003; Castro et al. 2016). Bacteria, algae, and other lower plant forms such as mosses and liverworts have been shown to synthesize both omega-3 and omega-6 LC-PUFAs, with recent studies also indicating that some invertebrates have this capacity as well (Monroig et al. 2016; Kabeya et al. 2018; Monroig and Kabeya 2018). While most higher plants can synthesize short-chain PUFA such as ALA or linoleic acid (18:2n-6; LOA), they are unable to further desaturate these into the LC-PUFA (Ohlrogge and Jaworski 1997; He et al. 2020).

Notably, most chordates cannot synthesize either the omega-3 or omega-6 fatty acids de novo and therefore must obtain these *via* their diet (Tocher et al. 1998; Monroig et al. 2016). This is primarily because chordates lack the delta-12 desaturase and delta-15 desaturase enzymes required to synthesize LOA and ALA from 18:1n-9 and it has been suggested that this is the fundamental basis for dietary essentiality of LOA and ALA by many animal species (Figure 6). The capacity of these SC-PUFA to be further elongated and desaturated to their respective 20-carbon and 22-carbon LC-PUFA derivatives varies greatly between phyla and even within taxonomically similar classes (Monroig et al. 2016; Oboh et al. 2017; Kabeya et al. 2018). An example of this has been with the rabbitfish *Siganus canaliculatus*, a marine species that has been demonstrated to contain the entire pathway required for LC-PUFA biosynthesis (Xie et al. 2021). Included in this capacity have been the recognized roles of various transcription factors, including hepatocyte nuclear factor 4α (HNF4α), liver X receptor alpha (LXRα), sterol regulatory element-binding protein 1 (SREBP-1), peroxisome proliferator-activated receptor gamma (PPARγ) and stimulatory protein 1 (SP1), as well as regulation by various post-transcriptional microRNAs and other fatty acids (Bou et al. 2017c; Østbye et al. 2019).

It is generally considered that both the delta-4 and delta-8 reactions are facilitated by a FADS2 (delta-6 desaturase) enzyme following an additional elongation step (Sprecher et al. 1995). Further evidence was presented that supported this process of delta-6 desaturation in fish producing 22:6n-3 (DHA) from 20:5n-3 (EPA) (Mourente and Tocher 1994). In some fish species a desaturase with dual delta-6 and delta-8 activities has been reported, meaning that desaturation is possible from both 18 and 24 carbon omega-3 PUFA substrates (Tu et al. 2012a). In most vertebrates there are generally considered to be two desaturase enzyme systems (FADS1 and FADS2) (Mourente and Tocher 1994; Vagner and Santigosa 2011). The FADS1 (delta-5 desaturase) enzyme catalyzes the conversion of 20:4n-3 or 20:3n-6 to 20:5n-3 (EPA) or 20:4n-6 (arachidonic acid; ARA) respectively. This reaction is often found absent in those species with an inability to make the LC-PUFA such as EPA or ARA. Among the various FADS1 and FADS2 enzymes, differential substrate affinity has also been reported, with omega-3 fatty acids often outcompeting omega-6 fatty acids (Tu et al. 2012b; Xie et al. 2021).

Essential fatty acid metabolism and physiological function in fish

Essential fatty acids, notably ARA, EPA and DHA contribute to a range of different biological functions. These functions vary considerably among tissues and species (Tocher 2003; Glencross 2009), but include important roles in cell synthesis and replication, neural development, endocrine function and control, ion regulation, immune function, inflammation, and reproduction (Tocher and Glencross 2015).

Limitations to essential fatty acid synthesis by fish

From the 1970s to the 1990s a series of studies identified that there is substantial variability in the capacity of aquatic vertebrates and invertebrates to synthesize the LC-PUFA (primarily ARA, EPA, and DHA) from precursors such as LOA or ALA (Kanazawa et al. 1979; Parker et al. 1980; Tinoco 1982, Takeuchi and Watanabe 1982; Watanabe 1982; Teshima et al. 1992). This capability was observed to vary substantially between phyla and even within taxonomically similar classes. Using [^{14}C] 18:3n-3 (ALA), Parker et al. (1980) traced the uptake, distribution, turnover and elongation and desaturation of this substrate in Coho salmon (*Oncorhynchus kisutch*). They found the highest uptake was by the heart and liver. De novo synthesis of both EPA and DHA was relatively low, and turnover of EPA was observed to be considerably faster than that of DHA.

Other studies have examined the efficiency with which LOA or ALA can be desaturated and elongated

by *Oncorhynchus mykiss* (rainbow trout), and *Cyprinus carpio* (carp) and have reported significant capacity to desaturate and elongate both these C-18 omega-3 and omega-6 fatty acids, whereas *Scophthalamus maximus* (turbot), *Pagrus major* (red seabream), *Trichogaster cosby* (gourami) and *Anguilla japonica* (eel), all had limited ability to produce LC-PUFAs (Tinoco 1982, Watanabe 1982; Millikin 1982). Generally, those fish that cannot elongate and desaturate SC-PUFA are considered to have a dietary requirement for the LC-PUFA whereas those fish that can elongate and desaturate are suggested to only have an "essential" dietary requirement for LOA and ALA (Henderson 1996). Despite this distinction, many of those species that can elongate and desaturate are known to perform better when supplied with the LC-PUFA in their diet preformed (Ruyter et al. 2000a, 2000b; Sprague et al. 2019). Since the LC-PUFAs have known critical metabolic functions, it is not surprising that supplying these nutrients preformed in the diet would be more advantageous to the animal than in the form of their shorter-chain precursors and hence DHA has often been suggested to be the "ultimate" EFA (Masuda 2003). As a generalization, marine fish are considered to have limited ability to desaturate and elongate fatty acids and therefore require the LC-PUFA in their diet, whereas freshwater fish have some capacity to desaturate and elongate fatty acids and therefore can survive with only SC-PUFA in their diets. As pointed out by Xie et al. (2021) though, there are frequent exceptions to this rule.

An example of this was the work by Teshima et al. (1992) who fed [^{14}C]-ALA to a range of species of marine fish (*Pagrus major, Seriola quinqueradiata, Oplengnathus fasciatus, Pseudocaranx dentex, Girella punctata* and *Paralichthys oliviaceaus*) and four species of crustaceans (*Penaeus japonicus, Penaeus orientalis, Paleomon paucidens* and *Macrobrachium rosenbergii*) and examined the distribution of the [^{14}C]. In each of the species only a very limited amount of the [^{14}C] was found in any of the LC-PUFA, indicating that all of these species had little capacity for elongation and desaturation. The greatest amount of elongation and desaturation was observed in the marine fish *P. major*, where elevated levels of both [^{14}C]-20:3n-3 and [^{14}C]-20:5n-3 (EPA) were reported; the only other fish species showing any elevated levels of [^{14}C] in 20:5n-3 (EPA) was *G. punctata*. Each of the four crustacean species had trace amounts of [^{14}C]-20:5n-3, but notably there was a complete absence of [^{14}C]-22:6n-3 (DHA), indicating that none of the species examined had any capacity for de novo synthesis of this fatty acid.

Fatty acid essentiality and deficiency

The inability of most animals to introduce an omega-3 or omega-6 double bond into carbon chains means that for most species these nutrients need to be obtained from the diet (Sargent et al. 1999; Tocher 2003; Glencross 2009). Therefore, when fish are fed diets deficient in such fatty acids there is a risk of them succumbing to a critical nutrient (essential fatty acid; EFA) deficiency (Castell et al. 1972a, 1972b, 1972c). There are various clinical signs exhibited by fish suffering from dietary EFA deficiency. The time taken for such EFA deficiency effects to amortize though varies among species, with marine fish being relatively susceptible in short periods of time (weeks), whereas species like the salmonids can take months and some species appear to apparently not suffer at all (Castell et al. 1972a, 1972b, 1972c; Ruyter et al. 2000a, 2000b; Glencross et al. 2003a, 2014; Rinchard et al. 2007; Salini et al. 2015b). Deficiency symptoms vary, but often include a reduced growth rate and poorer survival, though pathologies observed in fish have included erosion of the fins, myocarditis, and behavioral changes (Castell et al. 1972a, 1972b, 1972c; Watanabe 1982; Sargent et al. 1995; Ruyter et al. 2000a; Glencross et al. 2002b, 2008, 2014; Rinchard et al. 2007; Salini et al. 2015b). Notably, those species suffering from an EFA deficiency often become less resilient to stress, with poorer survival often observed following handling (Castell et al. 1972a, 1972b; Watanabe 1982; Millikin 1982; Salini et al. 2015b; Bou et al. 2017b). Although some fish have the capacity to use ALA as a substrate to make the required LC-PUFAs, many fish do not have this capacity and need to be provided with the pre-formed LC-PUFAs in their diet (Monroig et al. 2016; Oboh et al. 2017; Kabeya et al. 2018). In the absence of dietary ALA or omega-3 LC-PUFA, rainbow trout have been shown to produce elevated levels of eicosatrienoic acid (20:3n-9), with an inverse relationship between the levels of 20:3n-9 and DHA being observed. It has been suggested that the ratio of 20:3n-9 to DHA in liver phospholipids of fish is an indicator of EFA deficiency, with a ratio of 0.4: 1 or greater indicating that the diet is deficient in n-3 PUFA (Castell et al. 1972c).

The cellular processes of beta-oxidation, esterification, desaturation, and elongation of both ALA and LOA have been shown to be affected in Atlantic salmon in response to a level of EFA deficiency (Ruyter and Thomassen 1999). Using a cell culture approach, hepatocytes were shown to incorporate the radiolabel from ALA into lipid fractions at a rate two

to three times faster than from LOA. When cultured under conditions of essential fatty acid deficiency, the rate of radioactivity incorporation doubled. Under those same conditions, beta-oxidation of either LOA or ALA was low and was unaffected by either substrate or diet, whereas beta-oxidation to acid-soluble products was stimulated. Products from elongation and desaturation activity on both ALA and LOA were evident, and this was consistent with other results obtained after intraperitoneal injection of radiolabelled ALA and LOA. Interestingly, in hepatocytes incubated with labeled DHA, the main product was EPA, and the level of this retro-conversion was exacerbated by EFA deficiency, though only 3 to 12% of the DHA was retro-converted (Ruyter and Thomassen 1999).

Retention and partitioning of essential fatty acids

Numerous studies have shown that the fatty acid composition of the whole-body lipids of fish generally mimics that of the diet after prolonged feeding (Kalogeropoulos et al. 1992; Ruyter et al. 2000a; Bell et al. 2003, 2004; Bendiksen and Jobling 2003 Bendiksen et al. 2003; Jobling and Bendiksen 2003; Sales and Glencross 2011; Xu et al. 2020; Glencross et al. 2023a). This effect is influenced by variation in the ratios of neutral to polar lipids within an animal and between tissues, with neutral lipids being highly reflective of diet, whilst polar lipids showing varying degrees of regulation in their fatty acid profiles (Bell et al. 2003; Betancor et al. 2014).

Differences in the partitioning of many fatty acids, but particularly the LC-PUFA into tissues like brain, head kidney, gill, and eyes have been reported by several authors (Castell et al. 1994; Betancor et al. 2014; Sissener et al. 2016; Torrissen et al. 2022). These observations indicate, that for some tissues, the levels of certain fatty acids are quite regulated, whereas other tissues are less regulated and more reflective of the diet. Studies on the partitioning of fatty acids in the turbot identified that DHA has an important role in neural development (Castell et al. 1994). Castell et al. (1994) also showed high levels of ARA partitioned to the gill, heart, and head kidney, implicating that ARA may be important in osmoregulation for that species. Substantial differences in the deposition of DHA into phospholipids of the brain, head kidney, gill, and liver of Atlantic salmon were observed by Betancor et al. (2014). In that study, both the brain and gill phospholipid levels of DHA appeared to be regulated at around 20% (range 19.0% to 21.8%) of total fatty acids, irrespective of the level of DHA intake (range 1 g/kg to 20 g/kg of diet). In contrast, the levels of DHA in the liver and head kidney of fish varied from 21.5% to 32.7%. The observations that certain fatty acids were selectively retained into the phospholipids suggests an important role in cellular membrane structure.

The efficiency of retention and turnover of LC-PUFA varies according to various factors; including the level of dietary intake, the tissue, and the species among others (Koven et al. 1989; Glencross et al. 2003b; Turchini and Francis 2009; Salini et al. 2015b). During starvation, it was noted that Gilthead seabream (*Sparus aurata*) preferentially lost omega-6 fatty acids rather than either omega-9 or omega-3 fatty acids from their body tissues (Koven et al. 1989). In contrast, during this same study the omega-3 fatty acids in the polar lipids were selectively retained in preference to both omega-9 and omega-6 fatty acids. DHA was preferentially conserved compared to EPA (Koven et al. 1989). Salini et al. (2016a) demonstrated in Asian seabass (*Lates calcarifer*) that this loss of fatty acids had an allometric relationship to fish size and that there were significant differences to the rates of loss of specific fatty acids and that this was affected by body size. For most lipids, the relationship of metabolic losses and size has an allometric relationship of ~0.9 (Glencross and Bermudes 2011), but it was noted that among all the fatty acids that this value varied from 0.69 for EPA to 0.95 for 18:1n-9. For ARA, DHA, and EPA the value was <0.80, but for the SC-PUFA and MUFA it was more typically >0.90. Lower values are indicative of a greater effect of size on turnover, potentially indicative of higher demands for the EFA during early stages of development (Salini et al. 2016a). The biosynthesis of saturates and MUFA was also shown to be influenced by metabolism of other non-lipid nutrients (Viegas et al. 2019; Wade et al. 2020).

The uptake and utilization of a range of [^{14}C] MUFA, PUFA, and LC-PUFA, was studied by Oxley et al. (2005). In that work, the authors reported high uptake rates of 18:1n-9 and 18:2n-6 (LOA) by rainbow trout pyloric cecal enterocytes. Esterification into cellular lipids was highest for 16:0 and the 18-carbon fatty acids, which accounted for over a third of the total uptake of the [^{14}C]. Most of the uptake was into triacylglycerols. Incorporation of the fatty acids into phospholipids was highest by 16:0, although incorporation of 20-carbon and 22-carbon fatty acids was also high. About 40% of all fatty acids taken up were catabolized by beta-oxidation, which was highest among the LC-PUFA. Modification of fatty acids by desaturation and elongation was generally low with <10% of fatty acid uptake showing evidence of

transformation. Similar results for Atlantic salmon hepatocytes were reported by Stubhaug et al. (2005). Further studies by Oxley et al. (2007) examining the re-esterification process in Atlantic salmon enterocytes reported that (sn-2) monoacylglycerols were preferentially re-esterified over glycerol-3-phosphate.

The retention efficiency of both PUFA and LC-PUFA appears to be variable and affected more by their level of dietary intake, than the type of oil used in the diet. In a study using the marine fish species *Pagrus auratus* (red seabream) fed diets containing either fish or vegetable oil sources, the retention efficiencies of EPA and DHA were generally unaffected by the source of dietary lipid used or their levels in the diet (Glencross et al. 2003a). In contrast, the retention efficiencies of LOA and ALA were much more variable and clearly affected by their levels in the diet. This suggested that there was selective retention (or beta-oxidation of LC-PUFA to produce these SC-PUFA) of these fatty acids when LOA and ALA were less abundant, such as in diets based on fish oil, but that there was moderate catabolism when they are more abundant such as when fed diets based on vegetable oils. Stubhaug et al. (2007), examined the fatty acid retention efficiency and beta-oxidation capacities of different fatty acids by Atlantic salmon fed either fish or vegetable oils over a full production cycle. In that study, an increased level of beta-oxidation (and reduced retention) was noted in the fish just prior to seawater transfer, where it was also observed that significantly lower levels of beta-oxidation occurred in fish fed vegetable oils, though seasonal effects have also been observed. Following transfer of the fish to seawater there was an increase in the efficiencies of fatty acid retention by fish fed either diet. Differences in the retention of certain fatty acids, between the vegetable oil and fish oil fed fish, were clearly linked to the levels of those specific fatty acids in their diets, and not the types of oils used. This observation was important in demonstrating the influence of the level of a specific fatty acid on its retention efficiency. Notably, those fatty acids that were at particularly low dietary levels generally had higher fatty acid retention efficiencies. Salini et al. (2015b), found that the levels of EPA and DHA in the diet of Asian seabass (*Lates calcarifer*) influenced the efficiency of their own retention. In that study, when a diet used solely fish oils to supply the added lipid (diet EPA + DHA levels = 22 g/kg) the EPA and DHA retention efficiency was about 40%. When the fishoil was diluted with poultry oil there was an increase in the retention efficiency of both EPA and DHA as their respective levels in the diet decreased. This resulted in maximal retention efficiencies of around 75% when no added fish oil was used (diet EPA + DHA levels = 4 g/kg). Importantly, the relationship between intake level and retention was an exponential function indicating that there were lower limits to retention at the upper intake levels, but that with decreasing intake, then efficiency was likely increased further.

Studies, like that of Bell et al. (2003) clearly showed that, with increasing dilution of the EPA and DHA from the diet, there was generally a direct decrease in the levels of EPA and DHA in the body and muscle lipids. It was suggested that on a whole-body basis, these changes in fatty acid composition of the fish over time reflected a dilution model (Jobling 2003, 2004). This dilution model was compared against two sets of independent data with a salmonid and a marine fish and found to be quite accurate with the marine fish, but less so with the salmonid due to its capacity to elongate and desaturate significant amounts of the other PUFA in the diet (Jobling 2004). A variant on this was a whole-body fatty acid balance model approach, developed as a means of estimating the combined effects of elongation, desaturation, and beta-oxidation, on the retention effects in various species (Turchini et al. 2007; Turchini and Francis 2009; Thanuthong et al. 2011; Salini et al. 2017). More recently, it was shown that the subtleties in retention among the various fatty acids, have elements of mathematical predictability with fatty acid type and inclusion (Mock et al. 2020; Glencross et al. 2023a). Combined with elements of the dilution model and a nutrient demand model, these mathematical algorithms allow for the development of a fatty acid assimilation model that can accurately predict the total amount of lipid and the fatty acid profile of that lipid in Atlantic salmon based on known inputs of feed and initial whole-body fatty acid compositions, water temperature, duration of feeding and feed energy density (Glencross et al. 2024).

In a study designed to specifically examine the impact of 18:2n-6 (LOA) on utilization of omega-3 LC-PUFA, three diets containing equal amounts of EPA and DHA (~7·7% of total FA) but different levels of 18:2n-6 (constituting omega-6:omega-3 ratios of about 1, 2 and 6), and another diet with the omega-6:omega-3 ratio at about 1 but twice as much EPA and DHA, were fed to Atlantic salmon (Hundal et al. 2021). It was noted that the increasing levels of dietary 18:2n-6 led to a significant reduction in the level of omega-3 PUFA in the polar lipids of various tissues. Notably, it was EPA levels that were significantly affected while DHA levels were not. Increasing the total level of dietary omega-3 PUFA, whilst

maintaining the same omega-6:omega-3 ratio did increase the level of omega-3 PUFA in polar lipids. Neutral lipid fatty acid contents in various tissues were generally consistent with the absolute dietary intake levels of both omega-3 and omega-6 PUFA.

Role of essential fatty acids in cellular structure and function

The selective partitioning of certain fatty acids, like DHA, to neural, visual, and sperm cells has been reported in many animals, but particularly so in fish (Mourente et al. 1991; Masuda et al. 1999; Masuda 2003; Betancor et al. 2014; Sissener et al. 2016). This partitioning of specific fatty acids to the structural phospholipid membrane components of cells suggests an important functional role in these tissues. The molecular conformation of DHA, with six double bonds provides a structure that is strong but flexible enough to undergo structural transitions (Stillwell and Wassall 2003). It is speculated that this is why DHA is often enriched in cell types (e.g., neural, and visual cells) that are subject to rapid and repeated membrane restructuring. The presence, or indeed the absence, of DHA in retinal cells has been linked to a range of behavioral changes in fish (Masuda 2003; Benítez-Santana et al. 2014). It was noted that yellowtail (*Serioloa quinqueradiata*) fed a DHA-deficient diet did not form schools (Masuda et al. 1999) and barramundi fed a DHA-deficient diet did not develop proactive feeding behavior (Glencross and Rutherford 2011). Fish phospholipids have been reported to contain up to 50% of their total fatty acids as omega-3 LC-PUFA, often predominantly as DHA (Mourente et al. 1991; Betancor et al. 2014). Neural tissue has been shown to be up to 40% lipid, with over 70% of this lipid being phospholipids, in particular a certain type called cephalin (Tocher and Harvie 1988; Bell and Tocher 1989). Of this cephalin, DHA accounts for up to 40% of the fatty acids. It has been observed that DHA can account for more than 40% of the fatty acids in retinal cell cephalin in trout (*Oncorhynchus mykiss*) and over 70% in cod (*Gadus moruha*) (Bell and Dick 1991; Tocher et al. 1992). The high concentrations of DHA and cephalin in the cell membranes of retinal cells are linked to the specialized phospholipid bilayer that has novel liquid crystalline properties (Sargent et al. 1993). In these cells, the abundance of DHA in cephalin helps maintain the cell membrane bilayer in a balance between fluidity and rigidity necessary to accommodate the rapid conformational changes required in the membrane proteins of these cells. DHA has also been reported to affect the abundance of photoreceptor cells and the expression of rhodopsin in larval *Sparus aurata* (Tandler et al. 2023). Importantly, DHA has been reported to be a conformationally stable fatty acid over a relatively wide temperature range and this ability of DHA to withstand conformational change is postulated as enhancing these cell membranes with improved resilience against environmental change (Sargent et al. 1999; Wijesundera et al. 2008).

Consumption of different PUFAs has been linked to a variety of responses in cellular metabolite levels. Menoyo et al. (2006) observed in Atlantic salmon that diets rich in omega-6 PUFAs resulted in a higher plasma glucose concentration compared to diets rich in ALA or omega-3 LC- PUFAs. Concurrently, the activities of specific enzymes such as carnitine palmitoyl transferase and glucose-6-phosphate dehydrogenase were also affected.

Role of essential fatty acids in intercellular signaling

Using a gene sequence expression analysis (GSEA) approach, Glencross et al. (2015) identified that the change of dietary DHA levels led to a broad range of changes in many metabolic pathways, including some beyond those directly connected to lipid metabolism. Notably, some of the pathways most clearly affected (e.g., steroid biosynthesis and N-glycan biosynthesis) had no apparent direct link to fatty acid metabolism. What was useful about that finding was that it provided some indication as to the impact that changes in dietary EPA and DHA levels have on a broader physiological system. This subsequently led to Huyben et al. (2023) focussing specifically on the steroidogenic responses to changes in dietary omega-3 PUFA level, where they found discrete effects on transcriptomic and steroid level responses. Using a similar transcriptomic approach, Atlantic salmon with different body contents of EPA and DHA were found to result in differential gene expression of key carbohydrate/energy metabolism pathways (Horn et al. 2019). The authors observed that higher levels of DHA correlated with increased expression of the glycolytic pathway and production of associated metabolite intermediates (e.g., pyruvate and lactate). By contrast, high levels of EPA were associated with a reduction in expression of genes involved in lipid catabolism, but increased expression of genes regulating the pentose phosphate and glycogenolytic pathways, as well as genes involved in insulin signaling.

LC-PUFAs in phospholipids of cell membranes also play an important role as substrates to produce a

variety of intercellular signaling molecules including eicosanoids, prostaglandins, leukotrienes, and lipoxins (Samuelsson et al. 1987; Bell et al. 1993; Serhan et al. 2000). Notably, those phospholipids with ARA as their sn-2 fatty acid are often the preferential substrate to produce 2-series prostaglandins and 4-series leukotrienes in fish *via* the COX and LOX enzymatic pathways (Tocher and Glencross 2015). In this enzymatic process, the ARA is released from phospholipids by phospholipase A2 following stimulation of the cell (e.g., by immune responses or calcium ionophores) and converted to leukotrienes by the LOX enzyme (Figure 7). A variety of leukotrienes are synthesized, including leukotriene A4, which is considered an unstable epoxide, and is further hydrolyzed to leukotriene B4 or modified by conjugation to glutathione to yield leukotriene C4 and its metabolites, leukotriene D4 and leukotriene E4. Leukotrienes participate in a variety of immune responses including host defence responses, hypersensitivity reactions, and inflammation. Other studies have also suggested a neuroendocrine role for leukotriene C4 in influencing secretion of luteinizing hormone. Lipoxins are also formed by the action of LOX enzymes on ARA. Lipoxin A4 and B4 have been shown to inhibit natural killer cell cytotoxicity.

In addition to prostaglandins and leukotrienes, other signaling molecules (mediators) are also derived from omega-3 LC-PUFA in the form of resolvins, maresins, and protectins (Serhan et al. 2000; Hong et al. 2003, 2005). The biosynthesis of resolvins and protectins from DHA in fish was reported by Hong et al. (2003, 2005). In a study using brain cells from rainbow trout, it was shown that the cells produced bioactive products derived from LOX conversion of DHA, including both 14S-hydroxy-docosahexaenoic acid and 17S-hydroxy-docosahexaenoic acid to subsequently produced neuroprotectin D1, and resolvins D1, D2 and D5.

Essential fatty acids in immune function

It was reported by Thompson et al. (1996) that an elevation of the omega-6 to omega-3 fatty acid ratio in Atlantic salmon post-smolts had a significant effect on the incidence of atherosclerotic lesions, resistance to bacterial infection and the ability of the liver to detoxify xenobiotics. Notably, these effects occurred despite no differences in either the growth or behavior of the fish being observed. Montero et al. (2003) observed that certain pathologies and immunological parameters were elevated in Gilthead seabream in response to an increase in the ratio of omega-6 to omega-3 fatty acids. A suggested mechanism behind the influence of these fatty acids on the immune response is believed to be *via* the production of eicosanoids by macrophages. Notably, the production of leukotrienes by neutrophils has been reported as important to the inflammatory response in fish (Bell

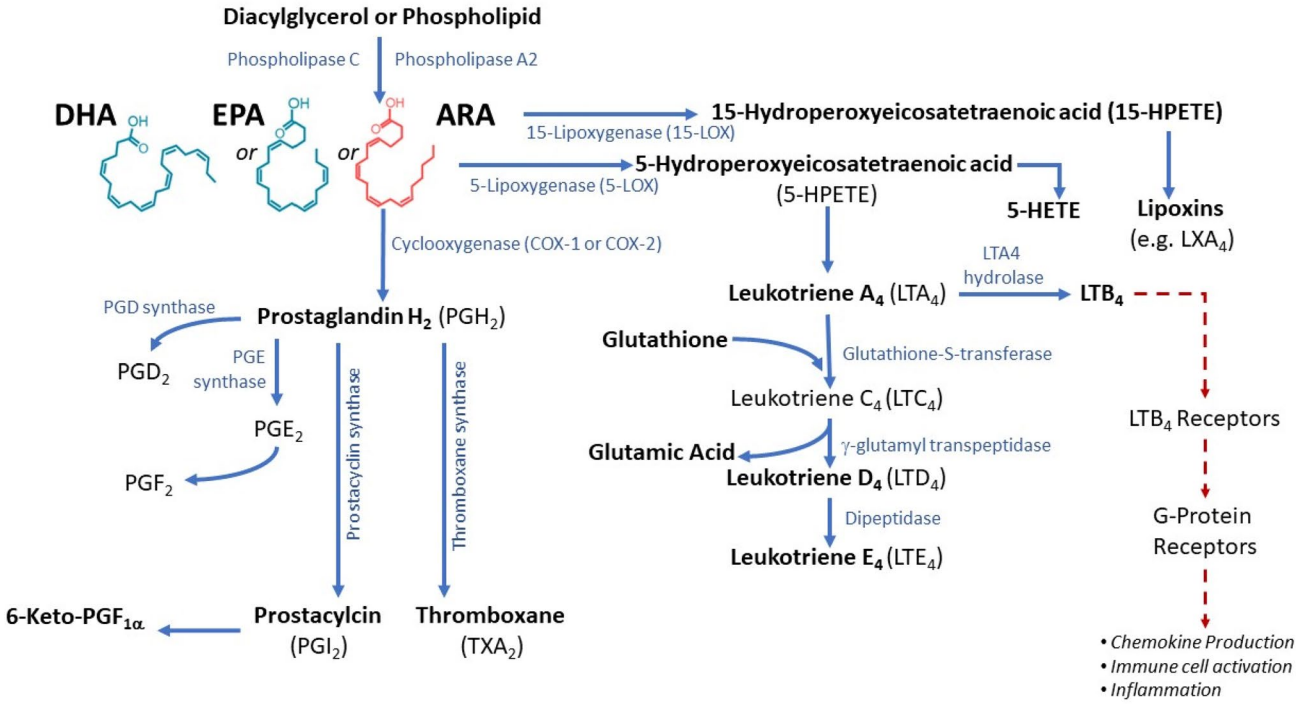


Figure 7. Pathways of biosynthesis of eicosanoids (prostaglandins (PG), leukotrienes (LT), and lipoxins (LX)) from arachidonic acid (ARA).

et al. 1993). Terano et al. (1986) reported that the phospholipid content in Atlantic salmon leukocytes was substantially enriched in omega-6 fatty acids compared to erythrocyte phospholipids and this was implicated as part of the process of substrate regulation of the LOX/COX pathways. Bell et al. (1993) further reported that both EPA and DHA attenuated inflammatory responses in Atlantic salmon from a study examining elevated levels of 18:2n-6, concomitant with reduced levels of EPA and DHA. It was noted that there was a significant increase in inflammatory cardiac necrosis in the fish; however, it was difficult to separate whether it was the increase in 18:2n-6 or decrease in EPA and DHA that was the driver of that response. The ratio of DHA to EPA has further been implicated in a range of immunological responses in fish (Martinez-Rubio et al. 2012, 2013; Zuo et al. 2012). The influence of varying DHA:EPA ratios on the immune response of Atlantic salmon when challenged with a virus (Atlantic salmon reovirus) was examined by Martinez-Rubio et al. (2012, 2013), who found that a lower DHA:EPA ratio resulted in a better response to the virus with a reduced inflammatory response (lower histopathology scores) and lower viral load in the cardiac tissue. The viral infection corresponded with an upregulation in various inflammatory responses, and it was proposed that the higher EPA levels helped to mitigate this inflammatory response (Martinez-Rubio et al. 2012). Analysis of the expression of eicosanoid regulatory genes in the head kidney confirmed this linkage to EPA levels (Martinez-Rubio et al. 2013). The diets leading to these effects were functional diets and also had a lowered lipid content compared to standard commercial diet. It is therefore not certain that this will be directly transferable to a commercial diet, but the results are nonetheless interesting and worth considering with the development of novel sources of omega-3 LC-PUFAs, many of them especially rich in DHA. Other studies have observed that DHA modulates the immune response by certain cell types to the presence of bacterial lipopolysaccharides (Bou et al. 2020). In studies with other species, there have been links between immune function and DHA:EPA ratios. In a study where the DHA:EPA ratio varied from 0.6 to 3.9 and total omega-3 LC-PUFA were kept constant at 10 g/kg, yellow croaker (*Larmichthys crocea*) subjected to a parasite challenge (*Cryptocaryon irritans*) were found to have higher serum lysozyme activity when fed diets with a higher DHA:EPA ratio (Zuo et al. 2012). An interaction between dietary omega-6/omega-3 ratio and stress on the cortisol response in Atlantic salmon after acute stress, has been reported by Hundal et al. (2021). It was suggested that this possibly indicated an altered hypothalamic-pituitary adrenal axis response in the fish fed diets with a high omega-6/omega-3 ratio. It was also noted that the levels of hepatic prostaglandin D2 (PGD2) and leukotriene B4 responded differently to the acute stress depending on the dietary omega-6/omega-3 ratio. Furthermore, higher levels of PGD2 and PGE2 as well as hepatic triacylglyceride levels were also observed when the fish were fed diets with a higher omega-6/omega-3 ratio. The influence of dietary level and ratio of omega-6/omega-3 fatty acids on disease progression and expression of immune and inflammatory markers in Atlantic salmon was examined following challenge with the gill parasite *Paramoeba perurans* (Selvam et al. 2021). Diets with different ratios of omega-6/omega-3 at 1.3, 2.4, and 6.0 and one diet with a ratio of 1.3 but with PUFAs included at a higher overall level in the diet were fed to the fish for a three-month conditioning period. After this period, the fish were subjected to a standardized laboratory challenge and the subsequent development of the disease was monitored. Although the challenge influenced the mRNA expression of various genes involved in immune and inflammatory response (TNF-α, iNOS, IL4-13b, GATA-3, IL-1β, p53, COX2, and PGE2-EP4), no effect of diet was observed on the gene expression. This evidence suggested that the omega6/omega-3 ratio may be less influential in affecting immune responses to parasitic challenges than changes in the DHA:EPA ratio. Recent studies (Løvmo et al. 2022; Sundell et al. 2022) have shown that both DHA and EPA have distinct effects on the hindgut of Atlantic salmon. Each fatty acid was reported to affect gene expression differently, with low levels of omega-3 LC-PUFA causing a reduction in intestinal barrier integrity following stress. There was improved intestinal condition in the fish fed elevated levels of EPA though. Analysis of the gene expression from the study suggested that the fish fed the higher EPA levels had greater upregulation of pathways related to protein turnover compared to fish fed diets with higher DHA levels. The diets high in DHA also did not show the same anti-inflammatory effects in the hindgut, as was seen in the fish fed the high EPA diets.

Essential fatty acids in ionic regulation

It has been reported that the lipids in the gills of Atlantic salmon change in response to varying water salinities (Takeuchi et al. 1989). Notably, the EPA and DHA content of the phospholipids in the gill decreased and the level of ARA increased as salinity increased.

It was suggested that these LC-PUFAs were involved in ion balance regulation by two possible mechanisms. Changes in the LC-PUFA composition of the gill tissue would introduce changes in cell membrane fluidity and as such may influence the permeability of the cell membrane (Leray et al. 1984). Secondly, the LC-PUFAs act as substrates in the production of eicosanoids, which are involved in the regulation of ion metabolism across the gill membrane (Bell et al. 1997). Eicosanoid production in the gill tissue of Atlantic salmon was found to be significantly influenced by the dietary omega-6 fatty acid intake, albeit from LOA, leading into the seawater transfer stage (Bell et al. 1997). This was also consistent with an accumulation of ARA in the liver phospholipids of the animals at the same time. Concurrent to this, the levels of DHA and EPA remained constant, although they increased dramatically following seawater transfer. In studies with the marine species turbot, eicosanoid production from ARA by brain cell cultures, following ionic stimulation, was shown to be differentially affected when either 20:3n-6 or EPA were added to the culture (Bell et al. 1994; Henderson 1996).

Essential fatty acids in reproduction

Essential fatty acids have also been implicated in reproductive viability in many aquaculture species (Tocher et al. 1985; Sargent et al. 1999). Fish in particular deposit a significant portion of their body lipid reserves of omega-3 LC-PUFA into egg phospholipids (Sargent et al. 1995; Sargent et al. 1999). The plasma non-esterified fatty acid (NEFA) content of wild Atlantic salmon showed a marked decline concomitant with a rapid increase in gonadosomatic index, consistent with the partitioning of LC-PUFA to their gonads (Booth et al. 1999). It was reported that high levels of MUFAs are catabolized as energy sources to produce protein and phospholipid constituents of the egg yolk, leading to sparing of the LC-PUFA from similar such catabolism (Sargent et al. 1995). In the egg yolk, LC-PUFA are speculated to be retained as a source for neural and visual development of the larvae. Indeed, there is a large volume of work in the larval nutrition area that focuses specifically on the use of omega-3 LC-PUFA in larval microbound diets and enrichments for live feeds (Izquierdo et al. 2000; Hamre et al. 2013).

LC-PUFA are also important to crustacean reproductive physiology and the subsequent viability of the larvae (Xu et al. 1994; Rotllant et al. 2015). Work by Xu et al. (1994) examined the development of Chinese shrimp (*Penaeus chinensis*) larvae. In that work both dietary ARA and EPA were important to subsequent larval survival and development. A study examining the fatty acid composition of wild and domesticated spawner black tiger shrimp (*Penaeus monodon*) found substantial differences in the EFA content of the gonads. Notably, ARA was relatively conspicuous by its absence in domesticated animals, whereas it was abundant in the gonad tissue of wild animals (Rotllant et al. 2015). It was suggested that this difference explained the variation in reproductive performance of wild versus domesticated animals. This hypothesis was examined by Coman et al. (2011), who fed domesticated *Penaeus monodon* a diet with elevated levels of ARA and found that this improved a variety of reproductive performance measures.

What can we learn from mammalian (human) nutrition?

The original studies determining the nature of fatty acid essentiality were undertaken in mammalian (rat) models (Burr and Burr 1929, 1930). Mammals, like most other vertebrates cannot synthesize ALA de novo and must obtain it from their diet (Holman 1971, 1998). While some tissues in mammals maintain the capacity to convert ALA to EPA and DHA, the rate of this synthesis is generally considered poor and therefore substantiate a need for dietary intake of EPA and (especially) DHA (Holman et al. 1963; Burdge and Calder 2005; Brenna et al. 2009). Dietary intake of these omega-3 LC-PUFA confers a range of cardiovascular and inflammatory regulation benefits, and these have been the subject of considerable research, with the mechanism(s) of action of omega-3 LC-PUFA in eliciting these benefits also being widely studied (Dyerberg et al. 1975; Holman et al. 1992; Nair et al. 1997; Calder 2010, 2012; Simopoulos 2016). The extent of breadth of this work is considerably greater than that done with aquaculture species, and as such presents a clear opportunity to identify additional avenues of investigation (Calder et al. 2020; Calder and Harris 2023).

Many of the studies undertaken on omega-3 LC-PUFA in mammalian systems have focussed on mechanistic aspects of the roles of these nutrients. One of the key observations has been that each of the pathways by which these fatty acids are digested, assimilated, re-esterified, elongated, desaturated, and peroxidized, is in competition with other fatty acids (Garg et al. 1990). There is, in most cases, no substrate specific selection among these enzyme mediated processes, with their actions based largely on the

kinetics of competitive/affinity substrate supply (Klenk and Mohrhauer 1960; Holman et al. 1963). It has been suggested that this competition amongst substrates is a possible basis for the interaction among dietary fatty acids that affect their requirement and utilization (Rahm and Holman 1964; Garg et al. 1990). Studies on mammals have shown that interactions among the omega-3, omega-6, and saturated fatty acids can influence ARA metabolism (Mohrhauer and Holman 1963; Garg et al. 1990). Because of this interaction effect, studies aiming to determine the responses to fatty acids may not be well served by defining the requirement for a single fatty acid, without also considering the potential interactive effects of the other fatty acids.

Other mammalian models (cellular and *in vivo*) have shown that the roles that DHA and EPA contribute to in cells is the modulation of certain metabolic pathways. In particular, the fatty acid composition of cellular membrane phospholipids has been implicated as being an important aspect in the regulation of function of a wide variety of cell types (Mohrhauer and Holman 1963; Holman 1964). This composition of the cell membranes affects not only the membrane structure, but also affects the function of membrane bound proteins (Masoodi et al. 2015). The levels of EPA and DHA have been shown to vary widely among different cell types and from different lipid classes within those cells, and this incorporation even differs according to sex and age (Calder 2008; Walker et al. 2014, 2015). In some cases, the differential deposition of EPA and DHA in certain cell types was so stark as to infer preferential retention/deposition and a discrete function of the fatty acid in certain tissues. The rates of uptake and retention of EPA and DHA by certain cell types has also been notable (Yaqoob et al. 2000). While both fatty acids deposited in the phospholipids of mononuclear cells at about the same rate, once dietary supplementation of both was stopped the EPA levels declined back to baseline levels about eight weeks later, whereas over this same period the DHA levels barely changed. The dose response incorporation of EPA into human mononuclear cells was observed to be almost linear with dietary intake (Rees et al. 2006). Further studies on the time course of incorporation of EPA and DHA into various lipid classes of different tissues have shown that both fatty acids enrich in certain tissues very rapidly (within days; Figure 8) and in a very dose-dependent manner (Browning et al. 2012, 2014).

Omega-3 PUFAs are also known to affect metabolic pathways as allosteric effectors on the activities of other enzymes and as substrates (Kang and Weylandt 2008). As an example, it is known that the ratio of dietary LOA to ALA has a significant effect on the activity of adenylate cyclase, the enzyme responsible for the synthesis of the signaling molecule cAMP from ATP (Morson and Clandinin 1986). Therefore, *via* this route alone omega-3 PUFA have a clear regulatory role in the production of chemical messengers, or effectors of secondary messengers. These cellular messaging systems have been shown to affect gene expression in various cell types. Additional evidence has implicated that omega-3 LC-PUFA have a role in inhibiting inflammation by affecting the activity of the NLRP3 inflammasome, a key regulator of metabolic inflammation (Ralston et al. 2017). PPARs have also been identified as being important to the regulation of the immune response, with many of these responses observed at the gene transcriptional level (Diep et al. 2000; Zúñiga et al. 2011).

It has been suggested that the EPA:DHA ratio is important in modulating ARA levels, and this was indicated as a possible locus for modulating those interactive effects among PUFA, especially in some white cell types (Garg and Li 1994; Healy et al. 2000; Walker et al. 2015). Studies on model membranes have shown that EPA and DHA differentially influence lipid oxidation, signal transduction, fluidity, and cholesterol domain formation, due in part to distinct membrane interactions, but notably because they have distinct regionalization within the membrane (Sherratt

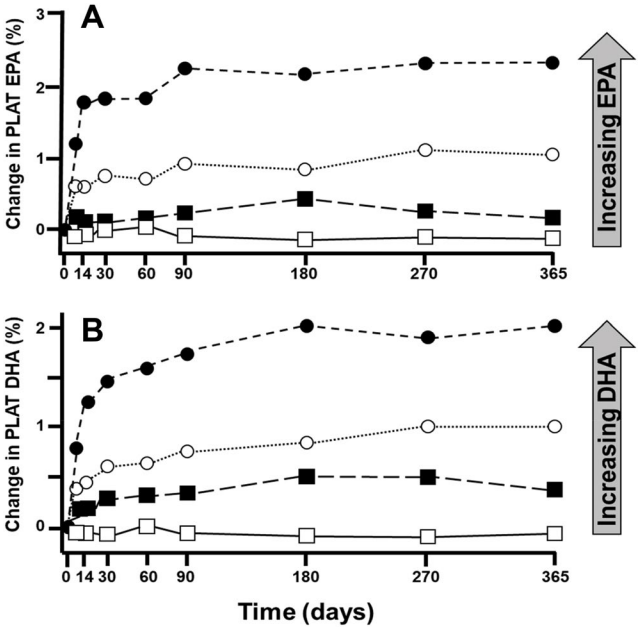


Figure 8. Temporal uptake of (A) EPA and (B) DHA into platelets of human participants fed different doses of fish oil. Increasing EPA and DHA doses related to zero □, one ■, two ○, or four ● meals weekly of oily fish. Figure derived from Browning et al. (2012).

and Mason 2018). Work by Hung et al. (1999) on diets fed to rats based on either fish oil or safflower oil found that IgG and IgM production were accentuated in those rats fed fish oil. Additionally, lower amounts of LTB_4 (from ARA) were observed, but higher amounts of LTB_5 (from EPA) were produced, which was indicated to be reflective of more EPA and less ARA entering the LOX pathway. It was also observed that EPA exerted a greater impact on immune functions than DHA. Similar effects were also reported by Volker et al. (2000), who found that feeding rats diets biased toward EPA more than DHA reduced the inflammation associated with arthritis. These beneficial effects of EPA were further reinforced by Martins (2009) based on a meta-analysis of published data, finding that EPA but not DHA was responsible for alleviating depression.

It was only with DHA:EPA ratios of 1:1 and 1:2 that there was a significant reduction in the levels of inflammation markers and oxidative stress (Molinar-Toribio et al. 2015). Similar results were shown by both Dasilva et al. (2016) and Shang et al. (2017), where a lower DHA:EPA ratio reduced steatosis damage in the liver of mice fed a high-fat diet, improved markers of cardiovascular disease and reduced inflammatory risks. A recent comparison of omega-3 SC-PUFA against omega-3 LC-PUFA on inflammatory responses of an endothelial cell line have shown that whilst all omega-3 PUFA exert some effects, only EPA and DHA had "potent" anti-inflammatory effects on the cultured cells; there was a hierarchy of anti-inflammatory effects of DHA → EPA → stearidonic acid > ALA (Baker et al. 2020).

Essential fatty acids were also shown from mammalian studies to have an important role as precursors of oxylipins, such as the eicosanoids, which have been implicated as an additional avenue by which LC-PUFA affect various physiological and cellular mechanisms (Bergström et al. 1964; Silver et al. 1973; Kinsella et al. 1990; Ostermann et al. 2019; Von Gerichten et al. 2021, 2023). Eicosanoids regulate a range of functions such as steroid biosynthesis, gastric secretions, and smooth muscle contraction, among others (Fischer 1989; Calder 2006). ARA is considered the primary substrate for the LOX and COX enzyme systems and associated pathways that produce the 2-series prostanoids and 4-series leukotrienes. Both EPA and DHA have a modulation role in the production of these eicosanoids, principally by competitive interactions with ARA for access to the LOX and COX enzymes (Culp et al. 1979; Bruckner et al. 1984; Kinsella et al. 1990).

In addition to prostaglandins and leukotrienes, other signaling molecules (mediators) also derived from LC-PUFA in the form of specialized pro-resolving mediators (SPM), are gaining increasing attention (Serhan et al. 2000, 2002, 2008, 2009; Hong et al. 2005; Mas et al. 2012; Barden et al. 2016; Kuda 2017). These SPM, such as the resolvins, protectins and maresins, were initially identified using mammalian cell models (Serhan et al. 2000, 2002). Resolvins are derived principally from EPA and DHA and have autocrine/paracrine functions in tissues where they are involved in mediation of the recovery from an inflammatory response (Serhan et al. 2000, 2002; Mas et al. 2012; Barden et al. 2016). These molecules have been classified into either resolvin Ds or resolvin Es based on whether they are synthesized from DHA or EPA respectively (Figure 9). The SPMs are derived from both DHA as 17 R-hydroxy-containing di- and tri-hydroxy-docosanoid molecules termed D-series resolvins, and from EPA as 18 R-hydroxyeicosapentaenoic acid (HEPE) and 15 R-HEPE molecules to produce E-series resolvins. Each of these molecule classes are used by leukocytes to form additional classes of signaling molecules. EPA is enzymatically converted by COX-2 or cytochrome P450, whereas DHA is converted by COX-2 or either of a 12- or 15-lipoxygenases (Serhan et al. 2002). The mediators act as inhibitors of leukocyte trans-endothelial migration and cellular infiltration of vascular tissue. Notably, this observation identified that the control of acute inflammation is an active process, involving a variety of lipid derived mediators generated in inflammatory exudates during the resolution phase (Schwab et al. 2007; Serhan et al. 2008). The lipid mediators act as agonists of anti-inflammation, with multiple mechanisms of action identified in various tissues. The mediators influence a range of white blood cell classes. Infiltration of both neutrophils and eosinophils into tissues is reduced, they also stimulate recruitment of monocytes and enhance macrophage phagocytosis of dying neutrophils. The removal of phagocytes from the site of inflammation to the lymphatic system and stimulation of mucosal antimicrobial defence systems are also stimulated. During resolution of inflammation, both EPA and DHA derived mediators are generated within the resolving exudates, including resolvin E1 (RvE1) and protectin D1 (Schwab et al. 2007; Mas et al. 2012). RvE1 and PD1 have specific actions in regulating tissue inflammation resolution, where they promote phagocyte removal by regulating leukocyte infiltration.

Overall, a distinct difference between much of the mammalian work and that seen in aquaculture, has been the focus in the former on mechanistic elements

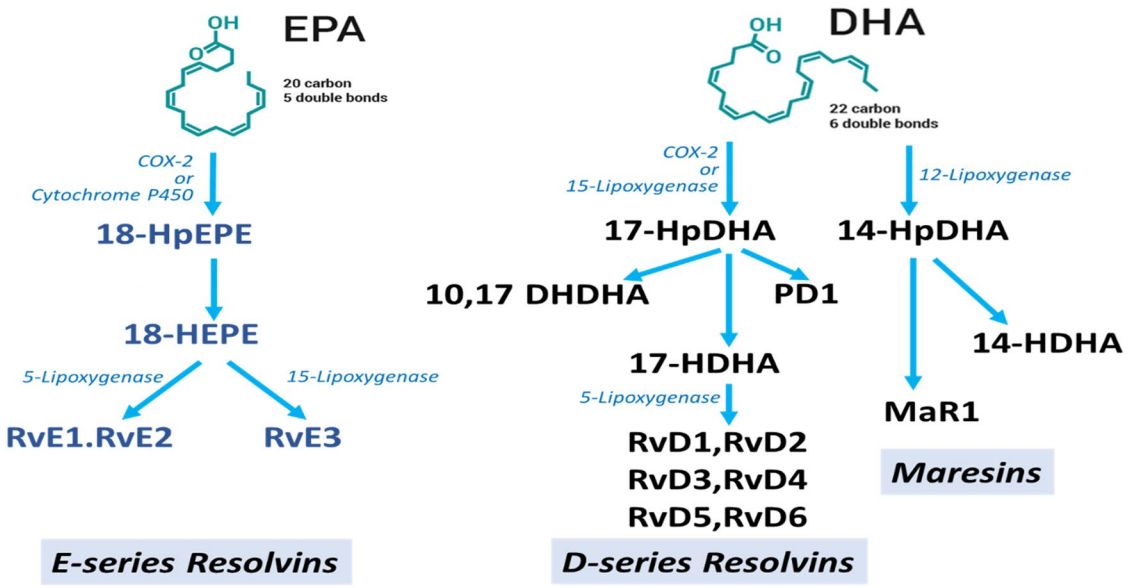


Figure 9. Biosynthesis pathways for the various specialized pro-resolving mediators (SPMs) including both the E- and D-series resolvins and maresins.

of influence of the nutrients on animal and cellular function. There has been a strong focus on impacts of EPA and DHA on immune inflammatory function. In contrast to this, the work in aquatic species has had a much stronger focus on dose-response effects and the determination of optimal supply/intake strategies. Given the differences in imperatives between the two domains, the health allied work of the former and the commercial direction of the later, this difference is quite understandable. As aquaculture science moves forward, it is likely to align with those directions of other vertebrate animal and cellular systems more closely.

Factors affecting the nutritional requirements for essential fatty acids

Defining what is meant by "requirement"

It is important to recognize that nutritional requirements have both qualitative and quantitative dimensions to them (Millikin 1982; NRC 2011; Hardy and Kaushik 2021). Assessment of a qualitative aspect of requirement must be undertaken with caution in the absence of some level of quantitative assessment. As such, it is important to note that the design constraints of many studies used to define these requirements have substantial bearing on their findings (Glencross 2009). There are multiple design constraints applied in nutritional experimentation that impact on the outcomes arrived at in animal experiments; among these are diet specification choice, feeding regime, animal size and duration of the study. It is also important to recognize that requirements and specifications are not the same thing. Diets are (usually) formulated to specifications approximating an interpretation of satisfying an animal's requirements for a certain nutrient(s) and energy, within the constraints of working with a limited range of ingredients (Glencross et al. 2020, 2023b).

In terms of a quantitative requirement, the functional "end-point" needs to be considered in terms of defining the response and requirement level (Kaushik 1995; King 1996). Such end-points can be set at basal, maintenance, and optimal levels of intake. While basal requirements are defined as the losses observed over a defined period of time during starvation, and maintenance as the intake level required to achieve net zero gain or loss, optimal requirements are a bit more circumstantial (Figure 10). Such requirements can be perceived as optimal for growth (mass gain), nutrient deposition (compositional growth), or for animal health (Morgan et al. 1975; Campbell et al. 1988; Fournier et al. 2002). While sometimes these might be overlapping in terms the defined intake level, often these are different levels of intake as well (Shearer 2000; Applegate and Angel 2014).

Beyond qualitative and quantitative dimensions, whether the requirement for EFA occurs as a function of absolute dietary intake or as a function of the total proportion of the fatty acids that they represent, has also been assessed in several studies (Watanabe 1982; Glencross et al. 2002b; Huyben et al. 2021).

The first, by Watanabe (1982), was in a study with rainbow trout, using a series of diets with 50, 100 or 150 g/kg of total lipid, to which purified ALA was added in diets that otherwise contained only purified 18:0. The results of that study implied that a requirement of 10% of total fatty acids (TFA) in dietary lipid 100 g/kg would be required at 10 g/kg, but that if the dietary lipid was only 50 g/kg then the fatty acid would be required at only 5 g/kg. These results demonstrated that EFA requirements are a function of the proportion of lipid/total fatty acids present in the diet (though this may also be construed as a function of diet energy density, though in that design it was not possible to isolate that aspect). Similarly, the basis of the requirement for EFA by shrimp was examined (Glencross et al. 2002b). In that study, the authors examined whether the requirement was a function of absolute dietary level or a function of the total proportion of the fatty acids that they represented. Again, it was demonstrated clearly that EFA requirements are a function of the proportion of lipid/total fatty acids present in the diet. Both the experiments of Watanabe (1982) and Glencross et al. (2002b) were unable to isolate energy intake effects from their designs. Huyben et al. (2021) however examined this absolute versus relative story with Atlantic salmon but formulated the diets to be isoenergetic on a digestible basis. Using diets with low (180 g/kg) and high (230 g/kg) levels of lipids, and low (7 g/kg) and high (14 g/kg) levels of omega-3 LC-PUFA the authors were able to demonstrate a clear interaction effect between omega-3 LC-PUFA level and lipid level, thereby confirming that even when digestible energy levels are balanced, the requirement for omega-3 LC-PUFA is relative to the total proportion of the fatty acids that they represent. An interpretation of what this means in terms of the application of existing requirement knowledge for Atlantic salmon across a range of dietary lipid levels is presented in Figure 11.

The impact of the form of a fatty acid on its utilization by fish is another consideration. In many studies, purified fatty acids in methyl-ester or ethyl-ester form have been used. Neither of these forms of fatty acids are naturally occurring, with fatty acids usually found in free fatty acid, mono-, di- or triglyceride forms, and as part of phospholipids or cholesteryl esters. Several studies have examined this issue in aquaculture species. Sigurgisladottir et al. (1992) examined the digestibility of different chemical forms of omega-3 LC-PUFA, as triacylglycerols (TAG), free fatty acids (FFA) or ethyl esters (EE), when fed to Atlantic salmon. The omega-3 LC-PUFA content of the lipids was well absorbed, with the digestibility being 90–98% when fed as TAG or FFA. When fed as EE, the fatty acids were significantly less well digested than when fed as FFA or TAG forms. Glencross and Smith (1997) similarly examined the utilization of methyl-ester (ME), EE, and sodium-salt FFA forms synthesized from the same TAG source of fish oil. In that study the authors fed the different lipid forms to *Penaeus monodon* shrimp and examined growth, feed intake, digestibility, and retention of fatty acids and lipid classes. Both ME and EE forms were poorly utilized by the shrimp, with reduced growth and perturbed retention of both fatty acids and lipid classes compared to the TAG fed shrimp. While FFA were not used as well as TAG, most effects were not significantly different from the responses seen in

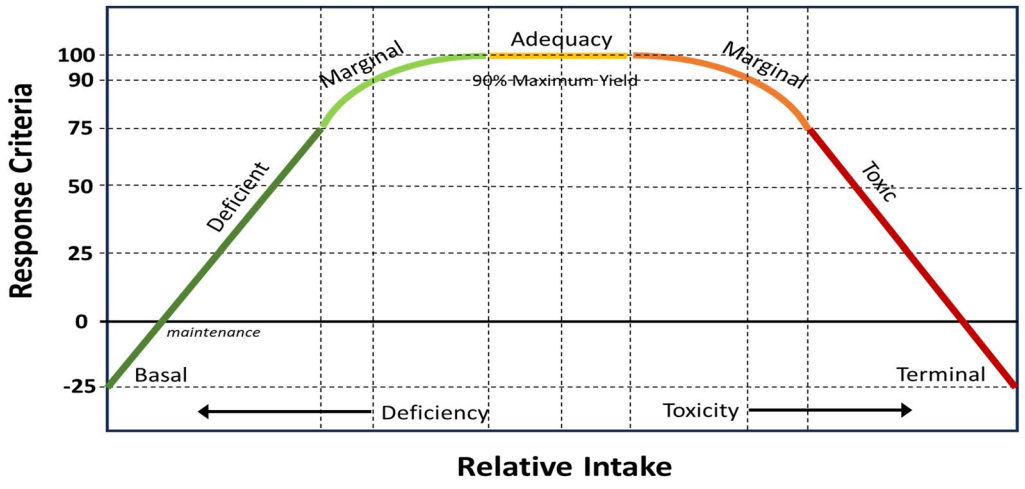
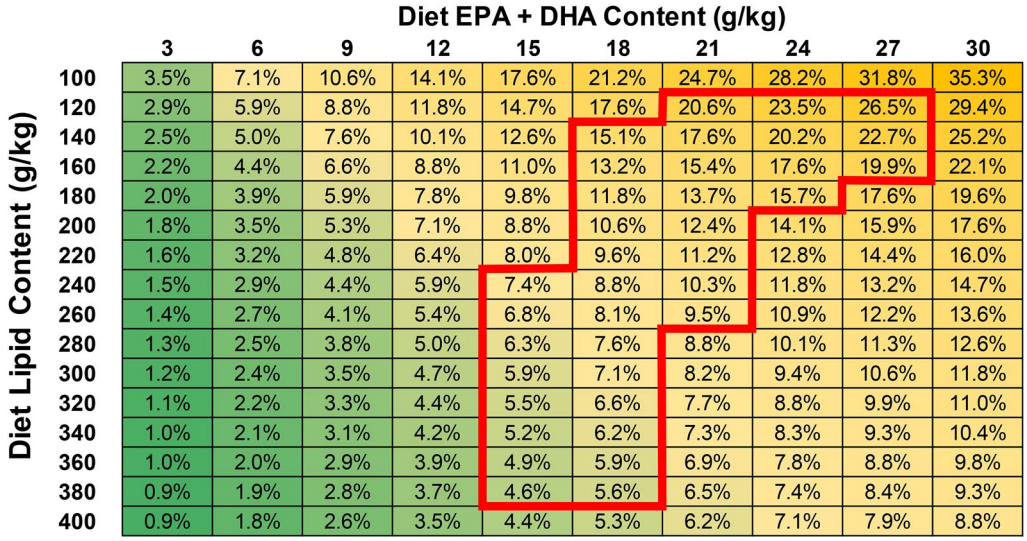


Figure 10. Critical zones of response by animals to nutrient supply. Relative intake levels required to illicit indicated responses vary markedly between nutrients and species.



Diet Lipid Content (g/kg)	3	6	9	12	15	18	21	24	27	30
100	3.5%	7.1%	10.6%	14.1%	17.6%	21.2%	24.7%	28.2%	31.8%	35.3%
120	2.9%	5.9%	8.8%	11.8%	14.7%	17.6%	20.6%	23.5%	26.5%	29.4%
140	2.5%	5.0%	7.6%	10.1%	12.6%	15.1%	17.6%	20.2%	22.7%	25.2%
160	2.2%	4.4%	6.6%	8.8%	11.0%	13.2%	15.4%	17.6%	19.9%	22.1%
180	2.0%	3.9%	5.9%	7.8%	9.8%	11.8%	13.7%	15.7%	17.6%	19.6%
200	1.8%	3.5%	5.3%	7.1%	8.8%	10.6%	12.4%	14.1%	15.9%	17.6%
220	1.6%	3.2%	4.8%	6.4%	8.0%	9.6%	11.2%	12.8%	14.4%	16.0%
240	1.5%	2.9%	4.4%	5.9%	7.4%	8.8%	10.3%	11.8%	13.2%	14.7%
260	1.4%	2.7%	4.1%	5.4%	6.8%	8.1%	9.5%	10.9%	12.2%	13.6%
280	1.3%	2.5%	3.8%	5.0%	6.3%	7.6%	8.8%	10.1%	11.3%	12.6%
300	1.2%	2.4%	3.5%	4.7%	5.9%	7.1%	8.2%	9.4%	10.6%	11.8%
320	1.1%	2.2%	3.3%	4.4%	5.5%	6.6%	7.7%	8.8%	9.9%	11.0%
340	1.0%	2.1%	3.1%	4.2%	5.2%	6.2%	7.3%	8.3%	9.3%	10.4%
360	1.0%	2.0%	2.9%	3.9%	4.9%	5.9%	6.9%	7.8%	8.8%	9.8%
380	0.9%	1.9%	2.8%	3.7%	4.6%	5.6%	6.5%	7.4%	8.4%	9.3%
400	0.9%	1.8%	2.6%	3.5%	4.4%	5.3%	6.2%	7.1%	7.9%	8.8%

Figure 11. An interpretation of the influence of the absolute amount of EPA + DHA (horizontal axis) required at different dietary lipid levels (vertical axis) on a relativity basis (% total fatty acids; color range from green [low] to orange [high]) of essential fatty acid levels. Shown within the red area is the indicative marginal (90%–100%) requirement for growth by Atlantic salmon as the animal grows and progresses from lower fat to higher fat diets. Data derived from Huyben et al. 2021.

animals fed the TAG diets. Similar differences in the efficiency of uptake of EPA and DHA by humans were reported by Lawson and Hughes (1988), who noted that FFA were better absorbed than TAG, but that EE were substantially poorer absorbed than both. Combined, these observations would suggest that the use of either EE or ME as means to manipulate the fatty acid profile of a diet is likely to impact the outcome of the experiment and as such would potentially void any assessment of the response to dietary EFA manipulation.

Diet design and management

In defining commercial feed specifications for EFA, like other nutrients, the study designs need to consider commercial comparative approaches. By contrast, in nutritional research, the approaches to the diet may not necessarily approximate commercial diets, but rather focus on maximizing control over nutrient presentation using highly refined ingredients and "purified diets" to minimize covariates usually encountered in the use of typical commercial ingredients (Glencross 2009). When considering the overall specifications of a diet, it is critical that the levels of all other nutrients are at or above recognized requirement levels. Otherwise, it is likely that other unintended nutrient constraints may impact on the animal responses and result in confounding effects. In this regard, it could be argued that the diet should be as analogous in its general specifications to a modern commercial diet as possible. Additionally, it needs to be recognized that these specifications change with animal size and as such the use of a single diet formulation strategy across large ranges in animal size is inappropriate. For example, in diet specifications for Atlantic salmon, it is notable that the diets vary in their lipid levels from about 150 g/kg when fish are <1 g up to almost 400 g/kg when fish are >3000 g (Einen and Roem 1997; Hillestad et al. 1998; Torstensen et al. 2004). Because of these changes in diet specifications with fish sizes, usually concomitant with changes in pellet size, it arguably makes more sense to examine fish performance within specific size stanzas, as is done in pig and poultry nutrition research (NRC 1994, 2012).

There are arguments for and against the use of acclimation periods with different species. Some species clearly need time to adapt to conditions and diets and their responses to diets can be seen to change over time. For example, Glencross et al. (2014, 2015) found that across a nine-week experiment with Atlantic salmon the responses to graded levels of DHA changed and it was not until the week-6 to week-9 period that a clear dose-response effect was seen. Other species, such as Asian seabass adapt to their conditions and diets much quicker and clear effects can be seen in as little as two weeks (Salini et al. 2015b). It was somewhat linked to this adaptation time issue that some researchers advocated a "wash-out" phase prior to conducting EFA requirement trials. In these wash-out periods the intention was to deplete the endogenous reserves of EFA and therefore hopefully clarify the animal's response to the different levels in a subsequent dose-response study (Sargent et al. 1995). Use of a wash-out period was originally

adopted from a similar strategy used in determining requirements for vitamins (Christensen 1980). Attempts to use the wash-out strategy in EFA studies have not been very successful though. In most cases a limited reduction in EFAs was achieved, the animals do not grow well, further undermining acclimation and the wash-out effect and they often become physiologically compromized and then don't perform well in the subsequent dose-response study (Ruyter et al. 2000b; González-Félix et al. 2002b; Tu et al. 2013). This strategy also provokes potential compensatory responses to nutrient supply, which would further complicate interpretation of any requirement level. An alternative strategy is simply to conduct the dose-response study over an extended period and/or biological turnover. A turnover, or weight change of the order of 300% has been suggested, though no specific biological basis for this value has been defined (NRC 2011). In some cases, such an increment is clearly non-sensical (e.g., growing a fish from 2 kg to 6 kg), but in others it is arguably insufficient (e.g., growing a fish from 1 g to 3 g). It is suggested that a more logical strategy would be to simply base the assessment period on the size-range for the pellet size used, which would equate to a standard range of fish sizes depending on whatever pellet size was used, and that this would vary depending on fish species as well. This strategy is more like the approach used in porcine nutrition, where typically trials are conducted over defined growth size ranges (NRC 2012). The other advantage of this, is that it means we can increasingly focus the specifications on a more defined animal size class, while holding all other nutrients in the diet constant, rather than introducing multiple variables each time we extend a trial and change additional parameters like protein and lipid level as well as the EFA profile.

Finally, when clinically defining an animal's requirements there is a clear argument for ensuring the animal's environment is optimized and therefore the animal's response is unencumbered by other factors. Because commercial aquaculture often undertakes production in systems that are far from optimized, there needs to be some consideration of the effect of environmental constraints on the nutritional demands of the species being studied. For example, there is a growing list of studies examining requirements for EFA being undertaken using challenging environmental conditions, including hypoxia and high-water temperatures (Norambuena et al. 2015; Bou et al. 2017b; Huyben et al. 2020, 2021). Examining responses under more industrially applicable conditions like these increase the utility of the data on requirements but do not undermine the need for the baseline understanding of what the animal needs biologically when there are no environmental constraints.

Impacts of other fatty acids

One of the critical factors in evaluating the responses of animals to fatty acid supply, is the issue of complementarity. In this regard, the variable proportional supply of one fatty acid must by nature affect the proportional supply of another. As noted earlier, EFA requirements are a function of the total proportion of the fatty acids that they represent, and this means that due to this complementarity there can arguably (and in reality) be impacts of other fatty acids on the response to omega-3 fatty acids.

Studies on various aquaculture species have shown differential values of SC-PUFA versus LC-PUFA (reviewed by Glencross 2009). Initially it was considered that there might be some potential in using omega-3 SC-PUFA as an alternative means to enrich the omega-3 content of fish. Despite some evidence of capacity by some species to elongate and desaturate ALA to the omega-3 LC-PUFA, in most cases when preformed LC-PUFAs were provided there were greater nutritional benefits to the animal (Kanazawa et al. 1979).

Studies on aquatic animals examining the influence of the dietary omega-3 to omega-6 fatty acid ratios on growth have generally focussed on diets modulating either the ALA to LOA ratio or the DHA to ARA ratio, with few studies examining an EPA to ARA ratio (Yu and Sinnhuber 1979; Glencross and Smith 1999; Glencross et al. 2002a; Senadheera et al. 2010; Norambuena et al. 2015; Sissener et al. 2017). Studies on the requirements for ALA and LOA by Coho salmon (*Oncorhynchus kisutch*) identified that these fish predominantly had an optimal omega-3 (ALA) requirement at an ALA inclusion of ~12%TFA (10 g/kg diet). It was further noted that an excess of ALA added to diet (> 30%TFA; > 25 g/kg) had a negative impact (Yu and Sinnhuber 1979). The addition of LOA to the diet, in the absence of ALA, had no impact on performance. In contrast though, the addition of LOA to the diet when there was 10 g/kg of ALA resulted in a progressive deterioration in performance with each increasing inclusion of LOA from 0 g/kg upwards. These observations indicate that there is no ideal ratio of omega-3: omega-6 PUFAs in *O. kisutch*, as any inclusion of LOA had a negative impact.

In a factorial study on the LOA and ALA requirements of Tiger shrimp (*Penaeus monodon*), both LOA and ALA promoted growth well, though the

combination of the two together at the right ratio resulted in an enhanced level of growth (Glencross and Smith 1999). Notably, the optimal level of either LOA or ALA when combined was different to the optimal level observed when each fatty acid was provided in isolation. This effect was like that reported by Greenberg et al. (1950) in studies with rats, who termed this a "sparking" effect. Glencross et al. (2002a) went on to further examine the relationship with the omega-3: omega-6 fatty acid ratio in tiger shrimp, when multiple SC-PUFAs and LC-PUFAs were used to vary the fatty acid ratio and a statistical response surface modeling approach was applied to identify several potential interactions (Figure 12). From that work the authors proposed there was an ideal ratio between the omega-3 and omega-6 fatty acids of ~2.5: 1 for that shrimp species. The authors also implicated a mechanism for the interaction based on substrate competition for the *FADS2* (delta-6 desaturase) enzyme, or *via* the fatty acids acting as allosteric effectors to other physiological processes. In that work (Glencross et al. 2002a), it was also noted that there were several interaction peaks present in the modeled response surface. It was hypothesized that these were due to a differential affinity hierarchy among the various dietary fatty acids and that there may be elements of an omega-3 to omega-6 ratio basis to this. It was likely to be an even more complex function than this, as there were clearly effects related to fatty acid chain length as well as fatty acid class. The authors went on to propose an affinity hierarchy for the *FADS2* enzyme system based on 18:1n-9 < 18:2n-6 < 18:3n-3 < 20:4n-6 < 20:5n-3 < 22:6n-3.

ALA is the most common omega-3 SC-PUFA found in plant oils such as those from flaxseed and camelina, and to a lesser degree in rapeseed (canola) and soybean oils. Various researchers have examined the use of oils like those from flaxseed and camelina in particular as a means of examining the potential for these oil sources to contribute to the overall omega-3 PUFA content in fish (Hixson et al. 2014, 2017; Betancor et al. 2015; Mock et al. 2019). Studies by Hixson et al. (2014, 2017) examined the complete replacement of fish oil with a camelina oil and found limited differences in performance, though the flesh adiposity of fish fed fish oil was higher than that of fish fed camelina oil. A notable change in the fatty acid profiles of the fish was observed consistent with that of the diets, with an increase in SC-PUFA content in those fish fed camelina and rapeseed oils. The use of these oils also had a significant effect on the expression of genes for enzymes involved in lipid and cholesterol synthesis. More recently Mock et al. (2019) examined the inclusion of camelina oil in diets for Atlantic salmon in two separate trials and found that there was poorer performance of the fish as well as a reduction in the levels of omega-3 LC-PUFA in the fillet, though this did not affect the perceived sensory qualities. The added ALA in the diet did support additional endogenous synthesis of LC-PUFAs by the salmon, but irrespective of this, the level of synthesis was inadequate compared to the levels of LC-PUFA obtained from salmon fed a diet where 20% of the added oils was a fish oil, highlighting a critical difference in the roles played by SC-PUFA and LC-PUFA in this species.

Requirements by salmonids

The dietary requirement of salmonids for omega-3 LC-PUFA (in most cases meaning DHA and EPA) has been reported to range from 5%TFA to 14%TFA (10 g/kg to 25 g/kg of the diet) depending on species, fish size, and experimental conditions (Ruyter et al. 2000b; Glencross et al. 2014; Bou et al. 2017a; Table 2). Interestingly, levels of omega-6 fatty acids in excess of 12%TFA (10 g/kg) have been shown to depress growth in some studies but not others (Watanabe 1982; Millikin 1982; Ruyter et al. 2000b; Glencross et al. 2014). Among the growing collection of studies examining the requirements for omega-3 LC-PUFA in salmonids, a range of DHA:EPA and EPA:ARA ratios have been demonstrated to have various effects on performance and metabolism (Codabaccus et al. 2012; Glencross et al. 2014; Norambuena et al. 2015; Bou et al. 2017a). A comparison among the results of Ruyter et al. (2000b) and those of Glencross et al.

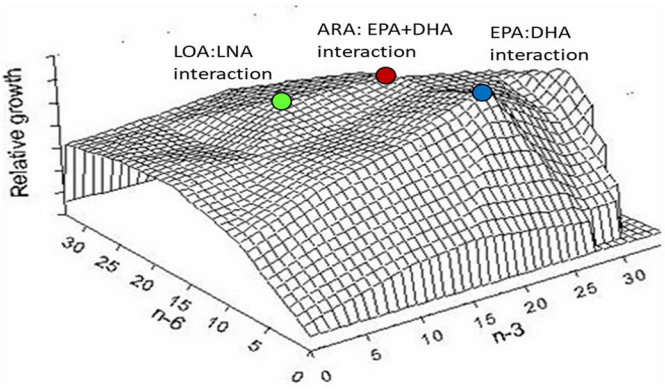


Figure 12. Interactions between omega-3 and omega-6 fatty acids as demonstrated by a generalized additive model (GAM) based on meta-analysis for separate statistical analysis of studies on LOA x LNA (ALA) requirements, EPA x DHA requirements, and ARA requirements of *Penaeus monodon*. Figure derived from Glencross et al. (2002a).

(2014) and/or Bou et al. (2017a) suggest that there is only a subtle, if any, apparent effect of life stage or fresh/saltwater on the absolute quantitative requirements of LC-PUFA by Atlantic salmon. In each of these three studies the peak responses were observed with LC-PUFA levels between 10 and 15 g/kg of the diet (varying from 5% TFA to 14% TFA), showing differences in terms of the relative quantitative requirements (Figure 13). In larger fish experiencing challenging/stressful conditions, Bou et al. (2017b) argued that this requirement increased to 17 g/kg (~6% TFA). Notably, the optimal inclusion level for growth appeared to be independent of the type of omega-3 LC-PUFA (or SC-PUFA) used in each of these studies, with similar requirement levels observed for ALA, EPA, DHA, and EPA + DHA. Additionally, there does not appear any strong evidence for an optimal ratio of DHA:EPA in the diet with no effect of variable ratios observed on the various parameters reported (Codabaccus et al. 2012; Glencross et al. 2014; Betancor et al. 2016; Emery et al. 2016; Bou et al. 2017a).

The nutritional responses to variable LC-PUFA supply do however appear to be affected by how long the fish have been fed the experimental diets, with salmonids appearing to be less responsive to changes in dietary fatty acids than many other aquaculture species. Importantly, there does appear to be a critical period of duration required to see effects in such studies but increasing them beyond certain time-frames (i.e., to using a full production cycle) does not appear to affect the outcome. Notably, the responses observed by Glencross et al. (2014) after nine-weeks were no different to those reported by Rosenlund et al. (2016) after nine-months in terms of an estimate of the quantitative requirements.

An important observation has been that of the whole-body fatty acids when salmon have been fed diets with varying ratios of DHA:EPA. Notably, there appears to be some level of conservation around the ratio of 3:1 (DHA:EPA) when salmon are given a range of dietary options (Brodtkorb et al. 1997; Ruyter and Thomassen 1999; Ruyter et al. 2000a). The whole-body retention data also provide important details in terms of net retention efficiencies of the various fatty acids. It appears for salmon that in most cases the retention of EPA is nominal, and the retention of DHA is dose-dependent, being preferentially retained when deficient, but retained in-line with that of other fatty acids at high levels of intake (Glencross et al. 2014; Bou et al. 2017b). In terms of tissue specific deposition of DHA and EPA it has been suggested that DHA plays a more prominent and arguably more important role (Betancor et al. 2014). Regulation of DHA levels in the brain for example has been reported by several authors (Brodtkorb et al. 1997; Betancor et al. 2014). Importantly, DHA inclusion at low levels, provided that there was either/or ALA or EPA, demonstrated that there is still considerable endogenous synthesis of DHA suggesting that salmon regulate their DHA subject to there being precursors present and that EPA is often sacrificed to make this DHA (Betancor et al. 2016; Mock et al. 2019). An elegant study published by Mock et al. (2019) quantified the extent of omega-3 LC-PUFA biosynthesis from ALA and EPA and the impact that dietary level of DHA had on the biosynthesis and subsequent effects on fillet fatty acid profiles. Although no differences in phenomic parameters were observed, an analysis of fatty acid composition and calculated *in vivo* metabolism indicated that the endogenous production of omega-3 LC-PUFA in fish fed a diet with no added fish oil was high since the fillet levels of omega-3 LC-PUFA were similar to those from fish fed a diet with added fish oil. The most significant finding of this study though was the observation that provision of an abundant dietary omega-3 substrate (ALA or EPA), when combined with the end product of metabolism (DHA) served to increase the overall final fillet levels of omega-3 LC-PUFA. In particular, it was noted that beta-oxidation of dietary ALA spared EPA from catabolism, highlighting the potential for enhancing endogenous synthesis.

Among the earliest studies examining quantitative EFA requirements of Atlantic salmon (*Salmo salar*) was that by Brodtkorb et al. (1997). In this study four diets (protein: 520 g/kg; lipid: 200 g/kg) with different amounts of LC-PUFA (18.4% to 43.5% of total fatty acids), but with only marginally varying DHA: EPA ratios (0.63:1, 0.67:1, 0.75:1, 0.80:1), were fed to small fish (initial weight 0.15 g; final size ~1.76 g) over a 93-day period in freshwater. Each of the diets was fed to excess, however no specific feed intake data were recorded. At the end of the study no significantly different effects were observed among treatments on fish growth. Analysis of the levels of DHA in the brain tissue demonstrated that the levels of this fatty acid were maintained at ~35%TFA and were not responsive to dietary treatment. The levels of EPA in the brain were much lower (~7%TFA) but did respond to the levels of dietary intake. Notably, the levels of both DHA and EPA in the eyes were more responsive to the diet than those in the brain, but there were still large differences between the levels of DHA (21.8–31.8%TFA) and EPA (5.7–10.8%TFA), despite

Table 2. A summary of the indicative requirements for EPA, DHA, or EPA + DHA by the various aquaculture species reported in this review.

		Relative (% TFA)			Absolute (g/kg diet)			
Species	Fish size	EPA	DHA	EPA + DHA[a]	Lipid (range)	EPA	DHA	EPA + DHA[a]
Atlantic salmon	< 0.5	5%	15%	20%	120–160	6	18	24
(*Salmo salar*)	0.5–2	5%	11%	16%	140–180	7	15	22
	2–5	5%	9%	14%	160–200	8	14	21
	5–15	5%	7%	12%	180–220	9	12	20
	15–50	5%	6%	10%	200–260	9	11	20
	50–200	4%	4%	8%	240–300	9	9	18
	200–500	4%	4%	7%	260–320	9	9	17
	500–1000	3%	3%	7%	280–340	9	9	17
	1000–2000	3%	3%	6%	300–360	8	8	17
	2000–4000	3%	3%	5%	320–380	7	7	15
Asian seabass	< 0.5	13%	13%	26%	80–100	10	10	20
(*Lates calcarifer*)	0.5–2	11%	11%	21%	80–100	9	9	18
	2–10	7%	7%	14%	100–120	7	7	14
	10–50	5%	5%	10%	120–160	6	6	12
	50–200	4%	4%	7%	140–180	5	5	10
	200–500	3%	3%	6%	160–200	5	5	9
	500–1000	2%	2%	4%	180–220	4	4	8
	1000–2000	2%	2%	3%	200–260	4	4	7
	2000–4000	1%	1%	3%	240–300	3	3	6
European seabass	< 0.5	10%	10%	20%	80–100	9	9	17
(*Dicentrarchus labrax*)	0.5–2	8%	8%	16%	100–120	8	8	16
	2–10	6%	6%	12%	120–160	7	7	14
	10–50	4%	4%	9%	140–180	6	6	12
	50–200	4%	4%	7%	160–200	6	6	11
	200–500	3%	3%	6%	180–220	5	5	10
	500–1000	2%	2%	5%	200–260	5	5	9
Gilthead seabream	< 0.5	8%	16%	24%	80–100	7	14	20
(*Sparus aurata*)	0.5–2	8%	12%	20%	100–120	8	12	20
	2–10	8%	8%	16%	120–160	9	10	19
	10–50	7%	5%	12%	140–180	10	7	16
	50–200	7%	3%	10%	160–200	10	5	15
	200–500	6%	3%	9%	180–220	10	5	15
	500–1000	5%	3%	8%	200–260	10	5	15
Yellowtail kingfish	< 0.5	16%	16%	30%	80–100	14	14	26
(*Seriola lalandi*)	0.5–2	12%	12%	24%	100–120	12	12	24
	2–10	10%	10%	20%	120–160	12	12	24
	10–50	9%	9%	18%	140–180	12	12	24
	50–200	8%	8%	16%	160–220	13	13	26
	200–500	7%	7%	14%	200–240	13	13	26
	500–1000	6%	6%	12%	200–260	11	11	23
	1000–2000	5%	5%	11%	220–280	11	11	22
	2000–4000	5%	5%	10%	240–300	11	11	22
White shrimp	PL15	5%	5%	10%	120–180	6	6	13
(*Penaeus vannamei*)	PL30	4%	4%	8%	80–120	3	3	7
	0.1–0.5	3%	3%	6%	60–100	2	2	4
	0.5–1.0	3%	3%	5%	60–100	2	2	3
	1–5	3%	3%	5%	50–80	1	1	3
	5–20	3%	3%	5%	50–80	1	1	3
Black tiger shrimp	PL15	10%	10%	20%	120–180	13	13	26
(*Penaeus monodon*)	PL30	8%	8%	16%	80–120	7	7	14
	0.1–0.5	6%	6%	12%	70–110	5	5	9
	0.5–1.0	5%	5%	10%	70–110	4	4	8
	1–5	4%	4%	8%	60–90	2	2	5
	5–20	4%	4%	7%	60–90	2	2	4

[a]EPA + DHA assumed to be at a ratio of 1:1. Unless contrasting evidence was available the EPA and DHA requirements were assumed to be equal to 50% each of the total EPA + DHA requirements.

that the levels in the diet were not as disparate. Like the observations of the omega-3 LC-PUFA levels in the eye, both the liver and muscle omega-3 LC-PUFA levels were responsive to intake dose, though the levels of EPA remained 2 to 3 times lower than DHA in each tissue despite being marginally higher in the diet. A key observation from this study was that above a gross dietary level of 30 g/kg of omega-3 LC-PUFA there were no effects on performance of salmon. At this level of omega-3 LC-PUFA there was also no effect of DHA:EPA ratio. The results of Brodtkorb et al. (1997) suggested a relative insensitivity of Atlantic salmon to changes in dietary EFA supply. It should be noted though that the levels used were comparatively high for what is now known about requirements for most fish species.

Ruyter et al. (2000a, 2000b) sought to challenge this notion of insensitivity to EFA intake by Atlantic

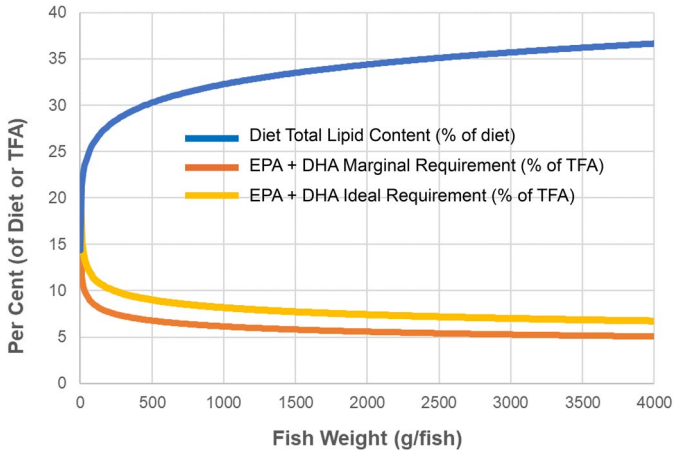


Figure 13. An interpretation of the marginal requirement for growth of EPA + DHA by Atlantic salmon as a function of fish size, based on the findings of this review is shown as an orange line ($y = 16.167x^{-0.14}$). Additionally shown as a blue line is the typical total lipid density of feeds used for fish of those sizes in modern commercial diets ($y = 17.104x^{0.092}$). For fish reared under challenging conditions the proposed solution would be an upward shift of the EPA+DHA as represented by the gold-colored line as an Ideal Requirement ($y = 21.502x^{-0.14}$).

salmon by conducting a study with graded levels 0%, 1.4%, 3%, 7%, 15% and 29%TFA (0, 1, 2, 5, 10 and 20 g/kg) of either LOA, ALA or EPA + DHA (at a ratio 1:1) in a diet with 80 g/kg of lipid. Each of the EFA, included as methyl esters in purified diets, were fed to 4 g fingerlings in freshwater (7-8 °C) over a 120-day period. The authors reported that problems with the initial acceptance of the diets by the fish in the study had potentially compromized them in the first month. Following this early period there was a subsequent recovery by the animals in the latter 90 days of the study. By the end of this 90-day period there was no response of the fish to the LOA, but fish did respond to both the ALA and EPA + DHA, with a peak response at an inclusion level of 15%TFA (10 g/kg diet), although the response was greater to the EPA + DHA inclusion than to that of the ALA. Above the optimal response level for both ALA and EPA + DHA, further increasing the inclusion of either fatty acid resulted in poorer performance of the fish. Based on the results of this study, it could be argued that the requirement for omega-3 LC-PUFA by young fish in freshwater is close 15%TFA (10 g/kg). It could also be suggested that ALA can satisfy this requirement as the fish grew well, but that EPA + DHA is more effective in meeting the requirements (Figure 14).

During the early 2000s substitution of fish oil with vegetable oils in salmon feeds became common place (Bell et al. 2003; Torstensen et al. 2004, 2005, 2008). The vegetable oils were typically high in omega − 6 fatty acids, and although many studies reported good growth results (summarized by Turchini et al. 2009; and Sales and Glencross, 2011), there were major changes in fatty acid composition of the diets and subsequently the fish (Sales and Glencross 2011; Nichols et al. 2014; Tocher 2015). Concerns were also raised regarding the effect of the higher dietary omega − 6: omega − 3 fatty acid ratio caused by vegetable oil on growth, biochemical composition, bone development, and eicosanoid production by the fish (Berge et al. 2009). To assess the impact of variation in the omega − 6 to omega − 3 fatty acid ratio, a full-cycle experiment was carried out starting with small (1.3 g) fingerlings of Atlantic salmon during their freshwater-stage, where the fish were fed diets with either fish oil or soybean oil as the main lipid sources. The experiment was run for 174 days, up until smoltification, and following smoltification the performance of the fish was observed for a further 18 months in the sea-water stage. At the end of the freshwater experimental period, performance of the fish was significantly better if they were fed the fish oil (95.4 g) diet than the soybean oil (87.4 g) diet. An analysis of the whole-body mineral levels at this point showed significant variation of both Ca (2400 and 3500 mg/kg) and P (3200 and 3900 mg/kg). This led to a corresponding Ca:P ratio which ranged from 0.74 to 0.91. Radiographic analysis revealed distinctive patterns of spinal pathologies developing over time but, these were considered similar with both treatments.

The impact of DHA:EPA ratio on performance of Atlantic salmon was examined by Codabaccus et al. (2012). In that study the authors used four diets (protein: 38%; lipid: 24%) with varying DHA: EPA ratios (ranging from 0.5:1 to 2.8:1), and an overall omega-3 LC-PUFA level that varied from 14%TFA to 30% TFA

(28 to 58 g/kg) to study the impact on small post-smolt salmon (initial fish size 72 g → final fish size ~153 g) reared in seawater. The diets were pair-fed over a 75-day period to avoid intake-mediated effects and at the end of the study no significant differences in weight gain or feed conversion ratio (FCR) were observed. As with the study in Brodtkorb et al. (1997), the ratios of DHA:EPA in the liver and muscle tended to be 1.3 to 3.4 times higher than that in the diet, suggesting that DHA was selectively retained and/or synthesized from EPA in this species.

A dose-response study with Atlantic salmon post-smolts fed diets (protein: 52%; lipid: 18%) where DHA was the only omega-3 LC-PUFA included in the diet at 1%, 3%, 6%, 8%, and 12%TFA (~1, 5, 10, 15 and 20 g/kg) was reported by Glencross et al. (2014, 2015). Three additional treatments were included to examine the interactions of DHA with either EPA or ARA. This included treatments where the DHA was present at 6%TFA (10 g/kg) with ARA also included at 6%TFA (10 g/kg) (D10A), and another diet (D10E) with both DHA and EPA included at 6%TFA (10 g/kg). The third additional treatment also similarly included both DHA and EPA, but with a slight reduction in the total amount of LC-PUFA to be only 6%TFA (10 g/kg) (D5E). Each of the diets was pair-fed to avoid intake-mediated effects and although it was only a short-term study (62 days) in seawater, the fish showed relatively good growth rates (initial fish size 111 g → final fish size ~231 g). Of the diets containing EPA, a DHA:EPA ratio of 1.25: 1 (D10E) and 1.1: 1 (D5E) was present, compared to the corresponding DHA diets that had ratios of 19: 1 and 7: 1. When the diets containing the lower levels of EPA (D5E) were compared to those with DHA alone (D5), the performance (weight gain and FCR) of the fish was better. When compared on an equivalent total LC-PUFA basis against treatment D10, there was no difference in performance. This suggests that when the total LC-PUFA level was accounted for, the inclusion of EPA at this lower threshold was somewhat irrelevant. When the diets with a higher level of EPA (D10E) were compared to those with an equivalent amount of DHA alone (D10) or total LC-PUFA (D20), the performance was marginally better in both cases. It was speculated by the authors that any requirement for EPA might be small, and this suggested that EPA could possibly be omitted from the diet of Atlantic salmon based on these observations. Analysis of the composition of the whole-body fatty acids of the fish indicated that the DHA:EPA ratio was generally regulated at around 2:1 across all treatments, though this varied from 1.78 to 2.74 depending on dietary DHA level. Retention efficiency of EPA, DPA and DHA showed distinct contrasts across the different treatments, with DHA retention being >100% when it was acutely deficient in the diet. This efficiency dramatically declined though as dietary levels increased. The addition of EPA to the diet increased DHA retention, whereas inclusion of ARA had no effect on DHA retention at all. EPA retention was largely unaffected by DHA, EPA or even ARA inclusion to the diet. ARA retention was unaffected by DHA. Subsequent analysis of the gene expression from that study by microarray found that the peak transcriptomic responses for key metabolic pathways also occurred at similar DHA inclusion levels as the phenomic responses (Glencross et al. 2015). It was suggested that the responses of those and various other pathways, including those directly and indirectly linked to LC-PUFA metabolism, provided a good

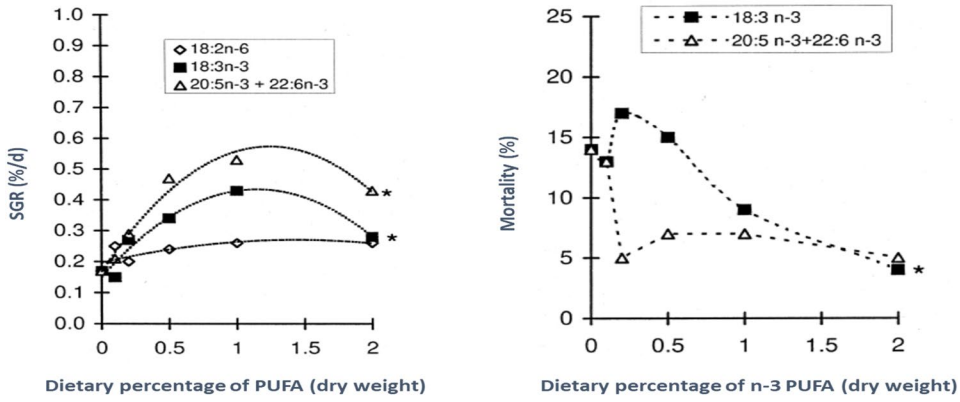


Figure 14. Response of specific growth rate (SGR) and mortality by Atlantic salmon parr to varying levels of dietary LOA (18:2n-6), ALA (18:3n-3), and EPA + DHA (20:5n-3 + 22:6n-3). Notable are the absence of any response to LOA, the similar peak intake level for ALA and EPA + DHA, and that the response to EPA + DHA is significantly greater than that to ALA. Figures from Ruyter et al. (2000b).

corroboration of the observed biological responses to the varying inclusion levels.

Huguet et al. (2015) reported a study examining the effects of ARA to EPA ratios (0.2, 0.9, 2.5:1), on nutrient digestibility by Atlantic salmon when fed at either of two temperatures. The authors reported that the temperature effects were generally greater than the effects of diet on most parameters. This study concluded that the dietary treatments and time effects had only minor impacts on digestibility, whereas environmental temperature resulted in significantly modified digestibility values, which increased with increasing temperature. Varying the EPA:ARA ratio in the diet had only minor direct effects on digestibility, with no direct effect on overall nutrient digestibility, and fundamentally only statistically significant effects on the digestibility of EPA and ARA themselves. In this study the authors varied the amount of ARA (0.8% to 5.6%TFA; 1.2 to 8.7 g/kg) and EPA (2.4% to 7.1%TFA; 3.7 to 10.9 g/kg) as the key variables, but with the dietary levels of DHA held constant (2.0%TFA; ~3 g/kg). The diets (protein:49%; lipid:18%) were fed to the fish (initial size 161 g) to apparent satiety twice a day, over a 100-day period in recirculated freshwater. After the initial 100-day period, the population was then split across two temperatures (10 °C and 20 °C) for a further 50 days. The phenomic performance effects from that work were reported by Norambuena et al. (2015) in a subsequent publication. Fish growth in this study was quite poor (e.g., the best 10 °C treatment grew from 162 g to 245 g over the 150-day period, a gain of only 83 g). Feed intake increased numerically with increasing inclusion of EPA, though not significantly so (range 1.2 to 1.4 g/fish/d). FCR was not reported. No significant growth differences were noted among any of the treatments. Fish in the high ARA treatment group were numerically lower in weight than those in the best performing treatment (212 g vs. 245 g). An assessment of the bioconversion of EPA showed that this fatty acid was mostly bioconverted to DHA in each of the treatment groups, but with some of the EPA deposited unconverted and a little less beta-oxidized. Generally, as EPA inclusion in the diet increased, the proportional bioconversion of EPA to DHA decreased. These observations were consistent with some gene transcription data, which showed that at low EPA levels that the expression of the gene FADS1(delta-5 desaturase) was upregulated.

Emery et al. (2016) undertook an experiment in freshwater where EPA and DHA were added singularly to a low LC-PUFA diet at both 50% and 100% of the levels of a fish oil (FO) control. Additional treatments in the study included diets where both EPA and DHA were included at 50% and 100% of their levels in the fish oil treatment, and a negative control where all the added lipid in the diet was provided as tallow (predominantly saturates and monounsatrates). Over the 98-day duration of the study, the fish (initial fish size 53 g → final fish size ~186 g) were fed the diets to apparent satiety. A range of significant effects were observed in performance criteria among the treatments. Unusually fish on the negative control (tallow) diet performed the best. Those fish fed the 100% EPA and DHA treatment did not grow to the same extent as the FO control, achieving only 85% of the FO growth, implicating that factors other than LC-PUFAs in the dietary treatments were impacting on performance. Unusually, the addition of both EPA and DHA to the diet at a level ~50% of that in the FO, performed better than addition of both EPA and DHA at 100%. Fish fed the EPA-100% treatment performed equivalent to those fed the DHA-100% treatment, though fish fed the DHA-50% treatment grew slightly better than both. Notably, fish on all three of these treatments grew equivalent to those fish fed fish oil. A positive dose-response effect to DHA was observed in deposition in the muscle, supporting that DHA was selectively retained. As with other studies, EPA was retained at lower efficiencies than DHA (Glencross et al. 2014; Betancor et al. 2015), suggesting that it was either converted to other products or metabolized, with increases in the levels of DPA (22:5n-3) suggesting the former. The authors proposed that the results demonstrated that dietary omega-3 LC-PUFA demand was effectively met by DHA alone. The authors did notably mention that EPA bioconversion to DHA was largely influenced by substrate availability, and that the presence of preformed DHA had little inhibitory effect, which are observations consistent with those reported by Betancor et al. (2016).

The dose-response to DHA and EPA (across the range of 1.4% to 7.4%TFA), equivalent to 5, 9, 11, 14, and 18 g/kg in high-fat diets (Pro:39% Lip:35%) by Atlantic salmon was examined by Rosenlund et al. (2016) in a series of two studies. In the first of the studies, the DHA:EPA ratios ranged from 0.8 to 1.17. Diet characteristics for only some of the diets used in the study were shown, so it is difficult to reconcile all dietary treatments in the study. In the first study, the initial fish size was ~161 g → final fish size ~1433 g over a 142-day period in seawater. The authors then split the population from the first trial to run for another 151 days at either 12 °C or 6 °C. Continuing the trial for longer, or at different temperatures, did not appear to affect the outcome, with

a similar optimal LC-PUFA level defined at ~5%TFA (~10 g/kg). In the second trial (initial fish size ~1437 g → final fish size ~3380 g), run over a 151-day period in seawater, a dose-response effect to omega-3 LC-PUFA was also observed. This second study gave a contrasting result to the first one, suggesting a size-dependent requirement. In the first trial with the smaller fish (161 g → 3000 g), the best growth was observed with omega-3 LC-PUFA levels between 2.7%TFA and 3.4%TFA (9 and 11 g/kg). In the second study, with the larger fish, no plateau to the dose-response was observed suggesting that the optimum had not been reached at even the highest LC-PUFA inclusion level of 7.4%TFA (~25 g/kg). No response to DHA:EPA ratio was observed in either trial. As with earlier reported studies, irrespective of DHA level in the diet, a 2-to-5-fold increase in the level of DHA in the whole-body fatty acids was seen relative to the diet (Glencross et al. 2014; Betancor et al. 2015). The relative increase between diet and whole-body levels was subject to dietary LC-PUFA inclusion level, with greater retention efficiency being observed at lower inclusion levels.

The influence of the dietary ALA:LOA ratio on performance by Atlantic salmon (80 g/fish) was examined by Sissener et al. (2017). Sissener et al. (2017) manipulated the SFA, MUFA and LOA contents of a series of diets, whilst keeping the EPA + DHA content constant at 4.5%TFA (~11 g/kg) and found that there was no impact on the growth or feed utilization by the fish. Furthermore, it was observed that there was net synthesis of DHA with all treatments and that notably, varying the ALA:LOA ratio did not impact elongation and desaturation of ALA to EPA or DHA. These authors also noted a clear negative relationship between DHA in the diet and the efficiency of DHA retention. A positive relationship between omega-6 fatty acid content in the diet and DHA retention was also observed.

The dose response of Atlantic salmon to EPA, DHA or EPA + DHA (ratio 1:1), when included in diets at 0, 5, 10, 15, and 20 g/kg (equivalent to 0.1%TFA → 9.3%TFA) was reported by Bou et al. (2017a). Various DHA:EPA ratios were used in the study, ranging from 0.25 to 5.70. Fish (initial size ~53 g → final fish size ~380 g) were fed diets approximating typical commercial specification diets (protein:46%; lipid:25%), for a duration of 182 days in seawater. A curvilinear dose-response effect was seen for each of the series of diets (EPA, DHA, and EPA + DHA). In the EPA diet series, the best growth was seen at an inclusion level of 6.9%TFA (15 g/kg), while in the DHA diet series, the best growth was seen at an inclusion level of 9.3%TFA (20 g/kg). For the EPA + DHA diets, the best growth was seen at an inclusion level of 2.3%TFA (5 g/kg). When examined across all diets in each of the series, the weight gain responses plateaued at ~ 4.6%TFA (~10 g/kg) of omega-3 LC-PUFAs in the diet. Feed efficiency reflected the growth observed in each series of diets, consistent with little variation in feed intake being reported across the treatments. A clear decline in the retention efficiency of EPA, DPA, and DHA was seen by increasing the dietary level from 0%TFA to 2.3%TFA (5 g/kg). Irrespective of the type of LC-PUFA supplied (diet series), the level of retention of EPA was about the same. Marked differences were noted in both DPA (22:5n-3) and DHA retention, subject to the diets used. This observation implies significant potential elongation, desaturation and/or beta-oxidation of the various omega-3 LC-PUFAs by the fish. Retention of DHA was also highly variable among the various tissues. Some tissues (e.g., brain) highly regulated their levels (~25%TFA), whilst others (e.g., intestine) closely reflected the levels from the diet, albeit usually at slightly elevated levels by comparison. Overall, an important finding from this study was that irrespective of whether omega-3 LC-PUFAs were supplied as EPA, DHA, or a combination the optimal performance of the fish was relatively consistent at about 4.6%TFA (10 g/kg) and the performance characteristics (growth and feed utilization) were about the same. Notably, there was no evidence from this study of any DHA:EPA ratio effect on phenomic performance parameters. The variable responses to dietary DHA levels in terms of deposition and gene expression supported that Atlantic salmon regulate DHA levels in some tissues, but not all.

In a follow-up to their previous study, the impact to Atlantic salmon of a dose-response to LC-PUFA of 2, 10 and 17 g/kg in high-fat diets (protein:36%; lipid:33%) across two different life-stages was examined by Bou et al. (2017b). In this study the dietary DHA:EPA ratios were not manipulated but did vary from 0.5 to 0.8, and a range of diet sizes (and varying specifications) were used (4, 5 and 7 mm) across the study. In the first study the initial fish size was ~355 g → ~1200 g. In the second part of the study the fish size was from ~1201 g → 3460 g. It was unclear how long each trial was. Feed intake was not reported. Notably, the second trial was run in three sea cages (1 per treatment) experiencing "challenging" conditions. During these challenging conditions high mortality rates were observed (63%, 53% and 16%, for the 2 (<1%TFAs), 10 (3.5%TFA) and 17 (6%TFA) g/kg treatments respectively). In both

parts of the study a dose-response effect to the dietary LC-PUFA level was observed. In the first trial, with smaller fish (355 g → 1200 g), weight gain increased with increasing LC-PUFA inclusion, with no plateau observed. In the second part of the study with the larger fish (~1201 g → 3460 g) a peak in performance was observed at the 10 g/kg level and ironically, at the highest LC-PUFA inclusion level (17 g/kg; 6%TFA), weight gain was poorer than in the deficient (2 g/kg; < 1%TFA) diet. As was previously reported, that treatment suffered considerable mortalities and it is likely the fish were physiologically struggling on various fronts. Various etiologies were observed with a deterioration in intestinal health score reported with decreasing inclusion of LC-PUFA. There were also problems with increased levels of lipid in the liver, intestine, and viscera, reduced intervertebral spacing and incidences of mid-intestinal hyper-vacuolization observed in fish fed the lowest LC-PUFA levels. Levels of DHA in the liver phospholipids where about 10-fold higher than in the diet, whereas the levels of DHA in the neutral lipids were 3-fold higher than that in the diet.

It was suggested that a higher dietary level of LOA may also increase the requirement for EPA and DHA by Atlantic salmon (Hundal et al. 2021). To assess this, a trial using three diets containing equal amounts of EPA + DHA (8% TFAs) and different levels of LOA producing a range of omega-6: omega-3 FA ratios (about 1, 2, and 6), as well an additional diet with the omega-6 to omega-3 fatty acid ratio at about 1:1, but with twice as much EPA + DHA, were fed to Atlantic salmon. It was observed that increasing the level of dietary LOA led to significantly reduced level of EPA in tissue polar lipids, but no impact on DHA levels. It was found that maintaining a stable omega-3 LC-PUFA in the polar lipids could only be achieved by increasing the dietary omega-3 LC-PUFA content and maintaining the same omega-6 to omega-3 fatty acid ratio. The authors concluded that a better use of dietary EPA could be achieved by minimizing the levels of dietary LOA.

In a follow-up to the earlier study by Bou et al. (2017b), the effects of increasing the dietary levels of EPA + DHA in Atlantic salmon reared in sea cages were examined (Lutfi et al. (2023)). As with the earlier study, a range of diet sizes (4, 6, and 9 mm) and specifications (4 mm: protein:44%; lipid:28%; 7 mm: protein:39%; lipid:30%; 9 mm: protein:36%; lipid:35% transitioning to protein:33%; lipid:38%) were also used across the study, which ran for about 15-months. Within those varying diets, a series of four treatments were employed containing on average across all diet sizes 10, 13, 16, and 31 g/kg of EPA + DHA (corresponding to 3.3%, 4.2%, 5.0%, and 10.0%TFA as EPA + DHA). The experiment commenced with fish of a mean starting weight of 275 g which were fed one of the four dietary treatments until they reached approximately 5000 g. As with the first study (Bou et al. 2017b), the second trial was run in sea cages (3 per treatment) experiencing conditions similar to farm operations with variable temperatures, salinities, and the occurrence of an outbreak of cardiomyopathy syndrome (CMS) toward the end of the study. No significant effects on growth were seen over the first six months of the study, where all fish grew from 275 g to ~990 g. With an additional nine months, those fish fed the diet with 10.0% EPA + DHA showed a significantly better growth performance and fillet quality compared to all treatments. This growth difference was largely due to a significantly higher feed intake, with no difference in feed conversion observed among any of the treatments. Those fish fed the diets with 3.3% to 5.0% EPA + DHA diet were not significantly different among each other in terms of growth, feed intake, or feed conversion. Fish fed the diet containing 10% EPA + DHA had lower levels of lipid in their liver than all three lower EPA + DHA treatments. Improvements in fillet quality parameters, including levels of EPA and DHA in the meat (mg/g), a higher visual color score and a reduced incidence of melanin spots were seen as the EPA + DHA was increased in the diet (Ytrestøyl et al. 2023). Additionally, those fish fed the 10% EPA + DHA diet showed lower mortality during the CMS outbreak, although the effects were not statistically significant. Overall, the findings by Lutfi et al. (2023) highlighted the importance of high levels (>5.0%) of dietary EPA and DHA to Atlantic salmon reared in sea cages to optimize a range of performance attributes including the robustness of the animal and a range of fillet qualities.

Requirements by marine fish

Marine fish differ from salmonids in that they are generally considered not capable of the conversion of ALA to LC-PUFA (Sargent et al. 1999; Glencross 2009). This deficiency, coupled with the fact that marine fish, in particular, deposit a significant portion of their body stores of triacylglycerol as omega-3 LC-PUFA provides a clear insight to the importance of dietary LC-PUFA to these species (Glencross 2009). This combination of a significant demand coupled with an inability for endogenous production increases the sensitivities to supply impacting these animals. Among the various marine fish studied, there are

several groups of fish that predominate: the *Centropomidae* (Asian seabasses), the *Moronidae* (European seabasses), the *Sparidae* (Seabreams), and the *Seriolas* (Kingfish) (Table 2).

Asian seabass (Barramundi)

Asian seabass (*Lates calcarifer*), also known as Barramundi, have been shown to have a clear requirement for omega-3 LC-PUFAs (Borlongan and Parazo 1991; Boonyaratpalin 1997; Glencross and Rutherford 2011; Salini et al. 2015b, 2017). Studies by Buranapanidgit et al. (1988, 1989), demonstrated that a total omega-3 LC-PUFA (as combined EPA and DHA) supply of 10 to 17 g/kg of the diet (10%–17% TFAs) was optimal for growth of Asian seabass. Williams et al. (2006) explored the demands for omega-3 LC-PUFA (as combined EPA and DHA) in a study examining the overall inclusion levels of LC-PUFA through the addition of various blends of fish oil and soybean oil and found an optimal inclusion level of a total omega-3 LC-PUFA content of 19 g/kg (~10% TFA). That study also reported the effect of manipulating the omega-3 to omega-6 fatty acid ratio on fish performance, with optimal performance observed with an omega-3 to omega-6 fatty acid ratio of 1.5-1.8:1 (Williams et al. 2006).

Using a factorial design, Alhazzaa et al. (2011) examined the effects of freshwater or seawater on barramundi fed on diets containing different oil sources: fish oil; stearidonic acid (SDA, 18:4n-3) rich Echium oil (EO), or rapeseed oil (RO), rich in both LOA and ALA. Fish fed the EO grew slower than those fed either the FO and RO diets and water salinity had no impact on the response to the diets. Using a mass balance analysis, the authors were able to show that the barramundi were able to use the SDA to bypass the first rate-limiting step in omega-3 LC-PUFA biosynthesis. Despite this, the fish did not accumulate significantly more EPA or DHA. It was suggested that barramundi had limited efficiency for LC-PUFA biosynthesis from their 18-carbon dietary precursors. These observations were further confirmed by Tu et al. (2012a, 2012b), who demonstrated the presence of a desaturase with low delta-6 desaturase activity but noted that the enzyme also possessed delta-8 desaturase ability that utilized 20-carbon fatty acids. This observation suggested an alternative desaturation pathway potentially existing in the barramundi. These authors went on to further examine the capacity of barramundi to elongate and desaturate ALA in a feeding study with various levels of ALA (Tu et al. 2013). The study showed that although the levels of ALA in the liver and fillet increased with increasing dietary ALA intake, there was no corresponding increase in the levels of either EPA or DHA, and notably the fish grew slower than those fed on a diet with higher EPA and DHA levels. Expression of the delta-6 desaturase (FADS2) and elongase (ELOVL5/2) genes increased 10-fold and 3-fold respectively. Interestingly, the level of expression of the two genes was not affected by the dietary level of ALA. A significant amount of variation between individual fish in their tissue DHA levels was observed, suggesting a significant heterogeneity in the capacity of this species for conversion of ALA and/or retention of omega-3 LC-PUFA.

The etiology of omega-3 LC-PUFA deficiency was studied by Salini et al. (2015b) using three diets with a deficient (1 g/kg; 1.4%TFA), low (4 g/kg; 3.7%TFA) and supra-optimal (22 g/kg; 21.5%TFA) supply of LC-PUFA. Effects of EFA deficiency occurred rapidly in the small fish (~10 g) used in the study, with significant effects on performance observed within two weeks and which continued to exacerbate over time (Salini et al. 2015b). Increased health abnormalities were seen from those fish fed the deficient diet, though not from the diet with low levels of LC-PUFA. Taking an allometric study approach, Salini et al. (2016a) examined the requirements for each of the LC-PUFAs based on their basal requirements during starvation. From this study the authors found that the basal requirements of some LC-PUFA was more sensitive to fish size than others, with notably EPA turnover being more influenced by fish size than that of either ARA or DHA. Glencross and Rutherford (2011) examined the quantitative requirements for DHA from 1 g/kg (4%TFA) to 19 g/kg (20%TFA). Increasing the level of DHA had a profound effect on fish health with a high degree of ionic dysfunction occurring with high-levels of DHA when in the absence of EPA. This dysfunction was ameliorated with the addition of EPA in the diet, but not by the addition of ARA. Known roles of these 20-carbon fatty acids in the production of prostaglandins and leukotrienes were implicated in terms of this impact on the ionic regulation in this species (Glencross and Rutherford 2011). In a follow-up study, it was observed that DHA had limited effect on growth of barramundi and that the effect was independent of whether the diets were pair-fed or fed to apparent satiety, so apparently not linked to intake dependent effects (Morton et al. 2014). It was further noted that as DHA was increased in the diet, the incidence of proactive feeding behavior also increased, suggesting a positive link between DHA and either brain and/or visual development (Glencross and Rutherford 2011). Increasing the inclusion of DHA in the diet resulted in an increased retention efficacy (from ~150% to ~300%) of EPA, suggesting capacity for

retro-conversion of DHA to make EPA, when EPA was present in the diet at low inclusion levels. Addition of ARA to the diet exacerbated the retention of EPA. Addition of EPA to the diet reduced this retention efficiency to ~75%. Each of these three responses suggest an important requirement for EPA by this species. With increasing inclusion of DHA in the diet the retention of DHA declined from ~130% to ~60%. Interestingly, the retention of ARA was also accentuated by the increasing inclusion of DHA, but not to the same extent as seen for the impact of DHA inclusion on EPA retention (Glencross and Rutherford 2011). In contrast to those observations, a series of dose-response studies by Salini et al. (2016b) found that the addition of either ARA (1 to 13 g/kg; 1.1%–15.3%TFA) or EPA (1 to 15 g/kg; 1.1%–17.6%TFA) had nominal impact on any of the performance metrics of barramundi provided that DHA was included at a low level (4 g/kg; 4.7%TFA) in all diets. Salini et al. (2016b) also assessed the expression of the lipoxygenase (ALOX-5) and cyclooxygenase (COX-1) genes involved in regulation of eicosanoid synthesis and concluded that consideration should be given to the balance between ARA and EPA in terms of the inducible nature of those enzymes. In another study (Salini et al. 2015a), replaced the fish oil content of a diet with poultry oil and found that the fish (~200 g) did not respond rapidly as was seen in earlier studies with smaller fish, and that no significant effects on performance were seen after a 12-week feeding period. A similar lack of response to replacement of the fish oil was also seen in another study examining the progressive dilution of fish oil from the diet of juvenile (~150 g) barramundi with a vegetable oil (ricebran oil) (Glencross et al. 2016). These observations raise some questions on the responsiveness of larger barramundi to an omega-3 LC-PUFA deficiency. It should be noted though that in both cases (Salini et al. 2015a; Glencross et al. 2016), these were relatively large fish (initial weight ~200 g and 150 g respectively) and their requirements of LC-PUFA were postulated to have been adequately satisfied during their earlier development. These observations suggested that provided the requirements for LC-PUFA are met earlier in life, the fish may be relatively resilient to changes in supply at later stages.

European seabass

Studies on European seabass initially focussed on the replacement of fish oil with different vegetable oil sources (Izquierdo et al. 2003). With the replacement of fish oil, the levels of the omega-3 LC-PUFA in the diets decreased to 35 g/kg (~14%TFA) relative to the reference diet which had 67 g/kg (~25%TFA) of LC-PUFA. No impact on any of the performance metrics of the fish was noted with this level of reduction in the LC-PUFA. The replacement of fish oil was also concomitant with a change in the omega-3 to omega-6 ratio from 3.3: 1 with the fish oil, to 0.8: 1 with the high level of vegetable oil inclusion. The authors suggested that the requirement for omega-3 LC-PUFA for this species had to be less than 35 g/kg and that above this level of LC-PUFA that the European seabass was relatively ambivalent to changes in dietary fatty acids. In a subsequent study with six levels of omega-3 LC-PUFA ranging from 2 g/kg to 19 g/kg (1.5%–12.3%TFA), Skalli and Robin (2004) examined the requirements of European seabass in a 12-week study. In this study the DHA: EPA ratio was kept constant at 1: 1.5 in each diet and after 12 weeks the fish fed the 2 g/kg diet had significantly poorer performance than those on all other treatments. Improvements in performance were seen with increasing levels of omega-3 LC-PUFAs up to the 7 g/kg treatment, and above this level no further improvements were observed. The inclusion of variable levels (8, 31, and 59 g/kg; 5%TFA, 21%TFA, 40%TFA) of DHA was studied by Betancor et al. (2011) in larval European seabass. These authors found that above the 8 g/kg inclusion of DHA, further increases in DHA induced higher levels of mortality as well as poorer growth. These negative effects of higher DHA levels were suggested to be linked to an increased peroxidation risk. Further studies with juvenile European seabass examined constraints to LC-PUFA use based on four replacement levels of fishmeal and three replacement levels of fish oil (Torrecillas et al. 2017). A combination of rapeseed, flaxseed and palm oil was used to replace the fish oil, whilst maintaining the overall lipid levels. The variation in omega-3 LC-PUFA, because of this replacement of the fish oil (and changes in fishmeal), ranged from 1 to 50 g/kg (0.7%–28.2%TFA). Notably, fish on the diets with only 10% added fishmeal and 3% added fish oil did not perform significantly poorer than those on the control diet. Below that level of inclusion though, there was a clear decline in performance, implicating that a critical threshold for omega-3 LC-PUFA in European seabass was between 11 and 17 g/kg (5%–9%TFA). In diets where there was no added fishmeal or fish oil, the supplementation of the diet with LC-PUFAs from algal and fungal sources to 10 g/kg (5%TFA) restored the performance to equivalent to that in those fed the diet with 3% fish oil. Gisbert et al. (2005) reported that European sea bass larvae used the LC-PUFAs contained within the phospholipid (PL) fraction more efficiently than those from

the neutral lipid (NL) fraction of the diet. In a study comparing the effects of dietary lipid class (PL vs. NL) and the level of omega-3 LC-PUFA with five diets assessing different levels of EPA and DHA being fed to the larvae from first feeding, the best results in terms of phenomic traits, and development (maturation of the digestive system and histological development of the liver and intestine) were obtained in the treatment fed with 25%TFA (23 g/kg) of EPA and DHA in the PL fraction of the diet. Those fish fed high levels of neutral lipids (110 g/kg), containing 28%TFA (26 g/kg) of EPA and DHA showed large levels of intracellular and intercellular lipid deposition in the anterior intestine. In contrast, lower levels of lipid accumulation were not observed when fish were fed with low or moderate levels of EPA and DHA in either PL or NL forms in the diet.

Seabreams

Requirements for seabreams were initially evaluated with studies on red seabream (*Pagrus major* and *Pagrus auratus*) in the 1970s (Kanazawa et al. 1979). Among the earliest studies reported was one with diets containing 30 to 40 g/kg of ALA. The fish grew poorly, but when fed diets with 20 g/kg of omega-3 LC-PUFA, they had much better growth. Following those early studies, it was found that a dietary supply of DHA sustained superior seabream performance to EPA when fed to larvae (Watanabe 1982). Takeuchi (1997) suggested that the efficacy of DHA was virtually twice that of EPA, with the inclusion for EPA needing to be around 10 g/kg compared to 5 g/kg for DHA. In another study with larvae fed with microdiets, Liu et al. (2004) found that larval red seabream performed optimally when fed a ratio of DHA:EPA of >2.3: 1. Requirements for omega-3 LC-PUFA by juvenile red seabream were later studied by Takeuchi et al. (1992), who examined their inclusion at each four dietary lipid levels of 50, 100, 150 and 200 g/kg. In that study, inclusion of the omega-3 LC-PUFA (as an equal balance of EPA and DHA) ranged from 12 g/kg (7%TFA) to 42 g/kg (almost 99%TFA) depending on diet lipid level. The best performance was observed when fish were fed diets with 150 g/kg lipid. With fish fed either the 150 g/kg or 200 g/kg lipid diets, the best performance was observed from fish fed diets with an omega-3 LC-PUFA level of 37 g/kg (25%TFA and 19%TFA respectively). Whereas in the 100 g/kg lipid diets the best growth was seen with 12 g/kg omega-3 LC-PUFA (10%TFA). The authors suggested that an omega-3 LC-PUFA inclusion level of 20% of TFA was appropriate for this species that the omega-3 LC-PUFA level needed to be increased in proportion with increasing dietary lipid levels. More recently studies have focused on fish oil replacement with red seabream, where the fish were fed diets where all the added oil was supplied as either rapeseed oil or soybean oil (Glencross et al. 2003a, 2003b). In these studies, no decline in growth was seen even when either rapeseed oil or soybean oil completely replaced all added fish oil. The diets contained a high inclusion of fishmeal (600 g/kg). It was estimated that these diets had a total omega-3 LC-PUFA level of 16 g/kg (18%TFA). As such, the observation that performance of the fish was not impacted confirms those earlier observations of Takeuchi et al. (1992). Other recent studies have focussed on the ratio between DHA and EPA (Mozanzadeh et al. 2016; Jin et al. 2017). In those studies, the findings indicate little impact of changes to the DHA:EPA ratio on performance attributes, though a range of tissue composition, haematological and transcriptomic effects were reported.

Gilthead seabream (*Sparus aurata*) has been the focus of much of the work on requirements for omega-3 LC-PUFA, primarily EPA and DHA, which have been extensively studied in larval fish (Koven et al. 1989, 1993; Salhi et al. 1994). Studies by Koven et al. (1989) examining the total lipid and omega-3 LC-PUFA requirements of larval Gilthead seabream both during starvation and when fed enriched live feeds (rotifers). During this work, the authors noted that it was mostly the neutral lipids which were depleted during starvation with little change in the level of polar lipids. Of those fatty acids that were lost from the neutral lipids, there was a preferential depletion in order of omega-6 > omega-9 > omega-3, but this order was reversed to omega-3 > omega-9 > omega-6 in the polar lipids. Notably, the loss of DHA was observed to be less than that of EPA during starvation (Koven et al. 1993). From these results, the authors concluded that the physiological strategy of the larvae was to conserve the more valuable omega-3 fatty acids during starvation, and that this provided an indication of the importance of these nutrients to this species. Studies on requirements for omega-3 LC-PUFA by larval gilthead seabream were examined using a 2 × 2 factorial design with omega-3 LC-PUFA ranging from 6.3%TFA to 18.2%TFAs (based on 7 g/kg and 20 g/kg) and lipid levels (~130 g/kg and ~200 g/kg) (Salhi et al. 1994). In this study, the authors found that increasing the lipid level improved larvae performance. Indeed, performance was more responsive to the higher lipid diets, than the lower lipid diets, irrespective of the omega-3 LC-PUFA levels. From these results, the

authors concluded that omega-3 LC-PUFA requirements were not necessarily related to lipid content of the diet but were perhaps more influenced by a factor such as the dietary EPA to DHA ratio. The importance of the ratio between EPA and DHA to Gilthead seabream has been explored by several researchers (Kalogeropoulos et al. 1992; Ibeas et al. 1994, 1996, 1997; Rodriguez et al. 1994). In the earliest studies, the best performance of the fish was observed with a DHA to EPA ratio around 1: 1 (Kalogeropoulos et al. 1992). In later work, it was suggested that the optimal ratio appeared to vary depending on the size of the fish, with an ideal ratio of DHA to EPA for larvae being around 1.5: 1 for which then decreased to 1: 1 or 0.5: 1 for juvenile or larger fish (Ibeas et al. 1994, 1996, 1997; Rodriguez et al. 1994). In the initial study by Ibeas et al. (1994), four different levels of omega-3 LC-PUFA ranging from 8 g/kg to 29 g/kg of the diet were examined. In that study, the fish fed the lowest omega-3 LC-PUFA diet (8 g/kg) grew significantly less well than those fed the higher omega-3 LC-PUFA levels, with growth improving up to an inclusion level of 19 g/kg, suggesting that the optimal omega-3 LC-PUFA level for this species, in diets with about 80 to 100 g/kg of lipid was about 19%–23%TFA, similar to the 20%TFA proposed by Takeuchi et al. (1992). In further work, Ibeas et al. (1996) examined the dietary level for omega-3 LC-PUFA required by juvenile seabream. Using a series of diets containing 109 g/kg lipid with omega-3 LC-PUFA levels ranging from 2.1%TFA to 16.1%TFA (2 g/kg to 15 g/kg of the diet), performance improvements were seen with increasing levels of omega-3 LC-PUFA up to an inclusion of 11%TFA (10 g/kg). Above this level, further increases caused a deterioration in the performance of the fish. More recently, the response of Gilthead seabream to a gradient of omega-3 LC-PUFA (DHA + EPA) was reported by Houston et al. (2022) (Figure 15). These authors used a series of six diets with LC-PUFA levels ranging from 14 g/kg to 54 g/kg and observed significant effects on growth at each of two fish sizes. Notably, the authors reported that requirements were higher when the fish were smaller (25 g → 80 g) than when they were larger (80 g → 220 g), with the estimated requirement changing from 1.5% of the diet to 1.1% of the diet respectively. The distinction was even more notable when examined based on feed conversion ratio (2.5% c.f. 1.6%). Notably, the data show that when the overall assessment is used to define the requirement based on a long-term approach, that the requirements for the smaller animals are underestimated and those for the larger animals are overestimated.

The addition of dietary ARA (3.9%TFA, 7.6%TFA, and 11.2%TFA) to Gilthead seabream diets, with 150 g/kg of lipid, was examined by Fountoulaki et al. (2003). DHA and EPA contents of the diets were kept constant at around 15%TFA (20 g/kg), with an additional treatment having a fortified level of DHA and EPA at 30%TFA (40 g/kg). Interestingly there were no differences in fish performance observed among any of the treatments. A key observation from that work was that ARA was preferentially found in the polar lipid fatty acids. Studies on the replacement of fish oil with different vegetable oil sources when fed to Gilthead seabream have been reported by Izquierdo et al. (2003, 2008). These authors found that replacement of the fish oil had no effect on the growth of the fish. Notably though, in those studies, the replacement of fish oil resulted in the levels of the omega-3 LC-PUFAs declining to 35 g/kg compared to 67 g/kg (this equates to ~16% TFA from ~30% TFA in a diet with 250 g/kg of lipid). This change in LC-PUFA levels was concomitant with a change in the omega-3 to omega-6 ratio from 3.3: 1 with the fish oil treatment, to 0.8: 1 with the addition of vegetable oils. The reduction of omega-3 LC-PUFA in the diet through the replacement of marine oils with vegetable oils was also mirrored by an increase in the expression of the FADS2 (delta-6 desaturase) gene (Izquierdo et al. 2008). A decrease in DHA + EPA in the diet was found to impair both visual and brain function in larval seabream (Benítez-Santana et al. 2014). It was DHA alone that was found to play an important role in behavior development though its impact on particular (Mauthner) cells in the brain.

Seriolas

Seriolas (kingfish) are a group of pelagic marine fish that have shown little capacity for tolerance of variation in salinity and are usually associated with highly carnivorous/planktivorous dietary habits. They are also notable in that they are highly dependent on omega-3 LC-PUFA intake from the diet. Studies on the omega-3 LC-PUFA requirements and metabolism of these species have indicated a generally high dependence on the omega-3 LC-PUFA, with most of the species unable to convert ALA into either EPA or DHA (Yone, 1978). While a requirement has been reported for the omega-3 fatty acids, none has been reported for the omega-6 fatty acids (Furukawa 1966; Tsukuhara et al. 1967). In a study with juvenile yellowtail, the best growth was obtained using a lipid level at 90 g/kg inclusion, which included 20 g/kg of omega-3 LC-PUFA (~27%TFA). Similar performance was also obtained with a lipid inclusion of 150 g/kg but using pollack

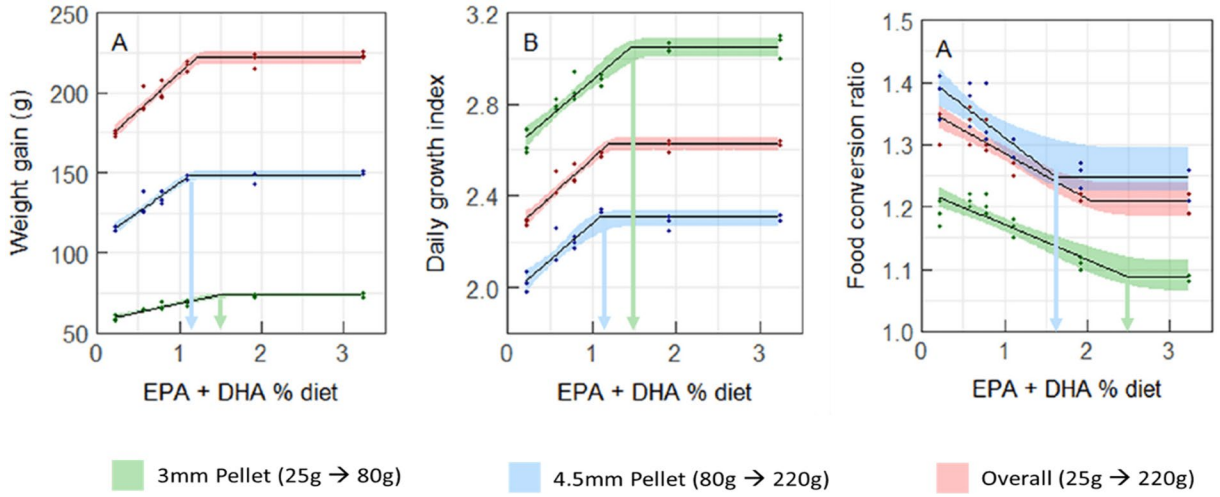


Figure 15. Effects of fish (Gilthead seabream) size class on responses to varying levels of dietary EPA and DHA. Notable is how the requirements are higher when the fish were smaller. Figures from Houston et al. (2022).

liver oil which also provided omega-3 LC-PUFA at ~20 g/kg (~13%TFA). This suggested that the requirements for omega-3 LC-PUFA by this species were more reliant on gross level than the proportional amount (Deshimaru et al. 1982). Another study in longfin yellow tail (*Seriola rivoliana*) larvae reported the best survival after air exposure with an inclusion of dietary DHA up to 4.1%TFA, although it did not significantly affect fish final growth (Mesa-Rodriguez et al. 2018). Studies examining the comparative value of the omega-3 LC-PUFA to *Seriolas* have shown DHA to be superior to EPA in promoting survival and growth (Furuita et al. 1996). Another study by Rombenso et al. (2016) on the pelagic species California Yellowtail (*Seriola dorsalis*) found that EPA was relatively dispensable to that species, with greater importance being given to DHA and ARA. Barreto-Curiel et al. (2017) examined the basal demands of juvenile amberjack (*Seriola lalandi*) through monitoring losses during starvation. A fed control was included in the study. Results showed that lipid was used as the main source of energy. Both SC-PUFA and LC-PUFA were highly conserved.

The complete replacement of added fish oil by poultry oil or rapeseed (canola) oil in yellowtail kingfish (*Seriola lalandi*) diets was examined by Bowyer et al. (2012), as well as a blend between rapeseed and fish oil. As an additional factor the authors included treatments at optimal and suboptimal temperatures. Fish fed the rapeseed oil diet had poorer growth performance, feed conversion, and nutrient retention, and showed green liver and lower plasma cholesterol levels than those fed the other diets. The content of 18:1n-9, LOA, and 18:3n-6 all increased in the fillet lipids which correlated well with the poultry oil or rapeseed oil inclusion. Levels of EPA, DHA, and ARA in the fillet lipids were all significantly reduced with increasing contents of dietary poultry oil or rapeseed oil. That the complete replacement of fish oil by rapeseed oil resulted in poorer fish growth, but a similar reduction was not seen with the use of poultry oil suggested an interaction effect with the higher LOA and/or ALA present in the rapeseed oil diet.

Rombenso et al. (2016) examined whether ARA, EPA, and DHA were each required or whether, as in other fish, EPA was comparatively unimportant in maintaining performance of Seriola spp. The authors assessed growth performance and tissue fatty acid composition of juvenile California Yellowtail (*Seriola dorsalis*) fed diets where the oil components included a fish oil-based positive control, a soybean oil-based negative control, or a series of experimental diets based on soybean oil but supplemented with ARA, EPA, DHA, ARA, and DHA, or all three fatty acids combined to achieve 50% or 100% of the concentrations observed in the fish oil diet. Fish fed the negative control soybean oil diet had significantly reduced performance relative to those fed the fish oil diet. The addition of ARA alone to the diet had no effect on performance, and inclusion of either EPA or DHA did not produce the expected positive effects on growth. When fed the diet supplemented with both ARA and DHA, the fish grew as well as those fed the fish oil diet. Those fish fed diets where all three fatty acids were added at 50% or 100% of the level in the fish oil diets, outperformed all treatments. Based on their results the authors suggested that soybean oil could completely replace fish oil in Seriola diets, provided adequate levels of DHA and ARA were added. Though the authors did also mention that

there was value in diets that had all three fatty acids (ARA, EPA, and DHA), they suggest that ARA and DHA were the primary drivers of LC-PUFA essentiality in this species.

The impact of variable levels of dietary fish oil, when using poultry oil as the replacement, was studied to determine the optimal dietary omega-3 LC-PUFA levels in diets of large (2.67 kg) yellowtail Kingfish (*Seriola lalandi*) (Stone et al. 2020). Eight diets were assessed that contained variable omega-3 LC-PUFA levels that ranged from 7.5 (3.3%TFA) to 29.5 g/kg of diet (13.2%TFA). A positive quadratic response between dietary omega-3 LC-PUFA level and growth rate was observed. Based on this response it was suggested that the optimal level of omega-3 LC-PUFA was 21.2 g/kg (9.3%TFA). The authors reported that this equated to a daily intake of 191 mg/kg/d. This observation by the authors was one of the few reports for any species where the requirement was provided as a weight-based daily intake. Above a dietary level of 23.9 g/kg of LC-PUFA no improvement in growth was observed. There was a similar negative quadratic relationship between diet omega-3 LC-PUFA level and FCR. Based on the FCR, the optimal level of omega-3 LC-PUFA was 22.6 g/kg, equating to a daily intake of 203 mg/kg/d.

Requirements by shrimp

Identification of requirements for fatty acids by shrimp presents a range of constraints compared to those of fish. Shrimp have a comparatively poor ability to utilize large amounts of lipid in their diet due primarily to a weaker lipid emulsification capacity of their digestive systems (Glencross et al. 2002c). Among aquaculture species, the assessments of the EFA requirements for penaeid shrimp remain among the more comprehensive, with requirements, both qualitative and quantitative, established for several species (Table 2). Among the earliest studies examining the requirements for omega-3 LC-PUFAs were those with Kuruma shrimp (*Penaeus japonicus*), in a series of studies undertaken from the 1970s to the 1990s. This was subsequently followed by work in the 1980s through to the 2000s on the requirements by Tiger shrimp (*Penaeus monodon*), and more recently the White-leg shrimp (*Penaeus vannamei*).

Early studies on the dietary inclusion of purified forms of LOA, ALA, EPA, and DHA in diets for the *P. japonicus* were undertaken by Kanazawa et al. (1979). In these studies, with the lipid content of each of the diets based on purified 18:1n-9 methyl esters, and each of the EFAs added as methyl esters at an inclusion level of 10 g/kg (20% TFA) to a total lipid content of 50 g/kg. Growth of the shrimp improved with addition of each of the EFA relative to those fed the lipid free diet or the diet with only 18:1n-9. Best performance was seen with the addition of EPA, followed by the addition of DHA. Although performance of shrimp fed the LOA and ALA was better than that of the controls, it was still poorer than that observed with EPA or DHA. Consequently, the authors concluded that the omega-3 LC-PUFA were the most important EFAs for shrimp, with only nominal EFA value being attributed to the SC-PUFA LOA and ALA (Kanazawa et al. 1979). Optimal levels of EPA were subsequently determined as 20%TFA (10 g/kg total dietary content) (Kanazawa 1992). Requirement for DHA was found to be higher, at an inclusion level of 20 g/kg (40% TFA), though diets with higher level of DHA than 20 g/kg in that experiment were not included. Kanazawa (1992) went on to propose a nutritional value ranking for EFA in diets for shrimp based on: EPA > DHA > ALA > LOA > 18:1n-9.

Those early studies were followed by Teshima et al. (1992), who fed four species of crustaceans (*Penaeus japonicus, Penaeus orientalis, Paleomon paucidens,* and *Macrobrachium rosenbergii*), diets containing the radiolabelled SC-PUFA and examined the distribution of the [^{14}C]. In each of the species only a very limited amount of the radiolabel was found in any of the LC-PUFAs, indicating that little to no elongation and desaturation occurred in any of these species. Each of the four crustacean species had trace amounts of radiolabelled EPA, but notably there was a complete absence of radiolabelled DHA, indicating that none of the species examined had any capacity for *de novo* synthesis of this fatty acid. In a subsequent study, the differences between larval and juvenile *P. japonicus* were examined and it was noted that there was a significant amount of radiolabel found in both EPA and DHA in the larvae, supporting that during the larval phase these animals had significantly greater capacity to elongate and desaturate ALA to produce LC-PUFA than in the juvenile phase (Teshima et al. 1992).

Studies with *P. monodon* initially commenced using a nutrient deletion approach (Merican and Shim 1996). In that study, growth was only negatively impacted by the omission of ALA and DHA from the diet supporting that these two fatty acids clearly had greater nutritional value than the other EFA. The authors suggested that this species had no specific requirement for LOA, ARA, or EPA. In the absence of DHA, EPA could not necessarily substitute for DHA to satisfy the EFA requirements for this species

(Merican and Shim 1996). The quantitative requirements for both ALA and DHA by *P. monodon* were subsequently studied by Merican and Shim (1997) using a dose-response approach with graded levels of each of the fatty acids. Estimates from that study suggested that the optimal requirement (based on best growth) was around 60%TFA (25 g/kg) for ALA and 33%TFA (15 g/kg) for DHA. Of the two fatty acids evaluated, the gain in growth was significantly better in response to the inclusion of DHA, supporting that this fatty acid has the greatest EFA potential within this species.

A series of studies demonstrated that *P. monodon* has a distinct requirement for both the omega-3 and omega-6 fatty acids (Glencross and Smith 1999, 2001a, 2001b; Glencross et al. 2002a, 2002b, 2002c). It was noted though that the inter-relationship between these two families of fatty acids can have confounding effects. This species clearly responded to the addition of either LOA or ALA to the diet. In the absence of the other, the addition of either LOA or ALA resulted in improved growth over that seen in the absence of any other EFA (Glencross and Smith 1999). When added singularly, both LOA and ALA resulted in maximal growth when included at 16%TFAs (12 g/kg). The factorial design used to examine the requirements for LOA and ALA clearly identified that an interaction effect was present. Notable was that the optimal requirements for each fatty acid differed from that observed when each was the sole EFA in the diet (Glencross and Smith 1999). The requirements for EPA and DHA by *P. monodon* were examined by Glencross and Smith (2001a), using another factorial design approach (Figure 16) (Glencross and Smith 1999). In that study, the best performance with the sole inclusion of either EPA or DHA was achieved at about 12%TFA (equivalent to about 9 g/kg of diet). These requirements were substantially lower than the 33%TFA (15 g/kg) reported by Merican and Shim (1997) as the requirement for DHA; however, the notable feature of the Glencross and Smith (2001a) study was that the EPA and DHA requirements were examined on top of pre-optimized LOA and ALA requirements. The factorial nature of the design also allowed the examination of the combined requirements of EPA and DHA, which were observed to both be considerably lower than their sole requirement levels. In the combined state, the optimal performance was achieved with only 4%TFA as EPA + DHA in the diet (equivalent to about 3 g/kg). As observed with the earlier LOA x ALA study, the EPA x DHA study demonstrated that interactive effects could be synergistic in terms of EFA nutrition in shrimp. The requirements for the omega-6 LC-PUFA ARA were further examined by Glencross and Smith (2001b). In that study ARA was included into diets prior optimized for LOA, ALA, EPA and DHA or just LOA and ALA. The resulting growth of shrimp from that study demonstrated that the inclusion of ARA in either series of diets had a negative impact on growth (Glencross and Smith 1999). These findings corroborated those reported earlier by Merican and Shim (1996), who also found that ARA did not confer any growth promoting benefit to *P. monodon*. Using a meta-analysis approach, Glencross et al. (2002a) examined the relationship between growth and the omega-3: omega-6 fatty acid ratio in the diet, where various combinations of fatty acids had been changed to alter the fatty acid ratio. Response surface modeling techniques identified several interactions among the fatty acid classes, with three sub-level interactions among various fatty acid combinations observed. From this work the authors proposed ideal ratio between the omega-3 and omega-6 fatty acids of around 2.5: 1 for *P. monodon*.

The requirements of *P. vannamei* were reported in a series of studies by González-Félix et al. 2003). These authors used a factorial design to examine the responses to three dietary lipid levels (30 g/kg, 60 g/kg, and 90 g/kg) and three dietary omega-3 LC-PUFA levels equivalent to 5 g/kg, 10 g/kg and 20 g/kg (ranging from 5%TFA to 66%TFA). The authors concluded from that study that *P. vannamei* was able to satisfy its dietary requirements for omega-3 LC-PUFAs with a very low dietary inclusion level of 5%TFA (5 g/kg) and suggested that their actual requirement may in fact be even lower (González-Félix et al. 2002a). In responses to the increasing the level of dietary lipid, an increase in lipid deposition was observed in the digestive gland and muscle of the shrimp. No differences in growth were observed among any of the lipid levels examined. In a subsequent study, González-Félix et al. (2002b) examined the influence of dietary phospholipids on the requirements for DHA. Using a factorial design, three levels of phosphatidylcholine (0 g/kg, 15 g/kg or 30 g/kg) were matched with three levels of DHA (0.1%TFA, 5%TFA, and 10%TFA, equivalent to 0 g/kg, 2.5 g/kg or 5 g/kg) or EPA + DHA (0.2%TFA, 3%TFA, and 7%TFA, equivalent to 0 g/kg, 2 g/kg or 4 g/kg). A control treatment was included based on a diet containing 5%TFA (2.5 g/kg) of omega-3 LC-PUFAs (i.e., EPA and DHA). The results from that study indicated that there were no interactive effects between DHA and dietary phospholipid. Growth was significantly enhanced by the inclusion of DHA or omega-3 LC-PUFA in the diet. The inclusion of the

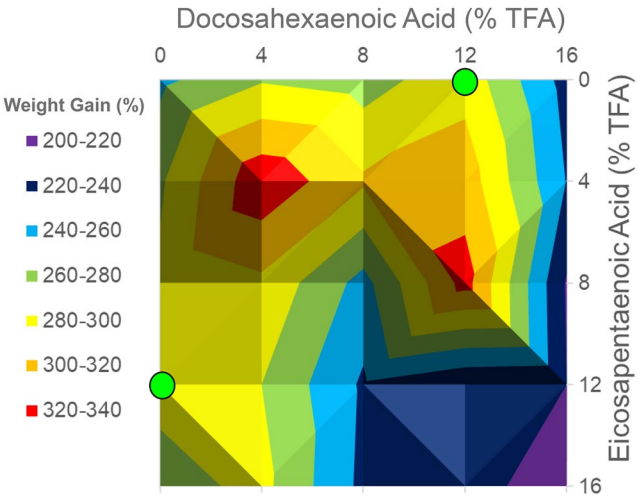


Figure 16. Interactive requirements for EPA and DHA by *Penaeus monodon*. Shown as green circles on the vertical and horizontal axes are the optimal sole requirement levels for EPA and DHA. Figure derived from Glencross and Smith (2001b).

phospholipid at 30 g/kg in the diet, so provided significant benefits. These same authors went on to subsequently evaluate the effect of varying dietary levels of LOA and ALA (González-Félix et al. 2003). Using a purified ethyl ester fatty acid base of 16:0 and 18:0, different levels of LOA and ALA were then added either singly at one of three levels (5%TFA, 10%TFA, and 20%TFA, equating to 2.5 g/kg, 5 g/kg and 10 g/kg) or in combination with each other at total inclusion of 5 g/kg of both LOA and ALA, but with the ratio (ALA:LOA) varying from 1: 1 to 9: 1. A reference diet containing omega-3 LC-PUFA was also included. The results showed that there was no significant response of the shrimp to either LOA or ALA inclusion in the diets and accordingly the authors suggested that they could not demonstrate any requirement for either of the SC-PUFA for this shrimp species. The response to the reference diet in that study also demonstrated that the nutritional value of the omega-3 LC-PUFA was significantly greater than for either LOA or ALA. Following these earlier studies González-Félix et al. (2009) examined the requirements for DHA and ARA in 3 × 3 factorial study, also allowing some exploration of the omega-3: omega-6 ratio on shrimp performance. Diets were formulated with about 90 g/kg of total lipid, and different levels of DHA and ARA were included from algal meals (*Schizochytrium* (DHA) or *Mortierella* (ARA)) to provide three levels of DHA (1%TFA, 2%TFA and 3%TFA, equating to diet levels of 0.8 g/kg, 1.4 g/kg and 2.1 g/kg) and three levels of ARA (0.5%TFA, 0.75%TFA and 1.0%TFA, equating to diet levels of 0.38 g/kg, 0.53 g/kg and 0.67 g/kg) but among all the combinations the omega-3: omega-6 ratio varied from 0.21: 1 to 1.07: 1. After six weeks feeding, no significant effects of either DHA, ARA or the omega-3:omega-6 ratio were evident on any of the shrimp performance criteria. Shrimp fed the fish oil reference feed grew best, but not significantly better than any of the test treatments. More recently, studies by Feng et al. (2021) have shown that DHA, EPA, and ALA all confer significant benefits on phenomic performance parameters of *P. vannamei*. It was evident from the results of that work that the inclusion level of the specific fatty acid was important in terms of the response achieved. The best growth was seen with ALA at 3.9%TFA, when DHA and EPA were included at 2.7%TFA and 1.7%TFA, respectively. A similar level of performance was observed when EPA was included at 3.1%TFA, and DHA and ALA were included at 2.8%TFA and 2.6%TFA, respectively, and when DHA was included at 5.8%TFA, and EPA and ALA were included at 1.8%TFA and 2.6%TFA, respectively. The findings provided a contrast to the earlier results of González-Félix et al. (2003), who reported no benefits of dietary ALA inclusion.

Limitations to current knowledge on the need for essential fatty acids

Gaps in knowledge on omega-3 LC-PUFA function

The omega-3 LC-PUFA have been implicated in a broad range of physiological roles ranging from cellular synthesis and proliferation, signaling molecule (hormones and cytokines) production and regulation, and regulation of endogenous synthesis of lipids among others. Despite substantial progress over the past decade, the precise molecular and biochemical pathways that cause an animal to either grow faster or slower according to PUFA intake have still not been clearly elucidated. This may be because it is not a simple cause-and-effect process, but rather a cascade of effects involving multiple interacting metabolic and signaling pathways. The gene-sequence-expression-analysis (GSEA) results of Glencross et al. (2015) and Horn et al. (2019) seem to support this. Additional recent observations, like those from studies on various salmonid species, have shown that dietary omega-3 LC-PUFA trigger a clear feed intake response, leading to improved growth but usually no improvements in feed efficiency (Geurden et al. 2007; Roy et al. 2020; Huyben et al. 2021). These observations raise the question as to whether the effects of omega-3 LC-PUFA are on improvements in dietary utilization or merely intake responses, with recent evidence suggesting the latter.

While omega-3 LC-PUFA may have significant effects on various developmental, metabolic, and immunological responses, it has been difficult to separate growth from intake effects in many studies (Hundal et al. 2022; Huyben et al. 2023; Carr et al. 2023).

To better unravel the processes by which omega-3 LC-PUFA work, and how and by what pathways they affect the various performance attributes of any particular fish species, we need to encompass a different approach than the traditional "omics" technologies have offered so far. The analysis of simple end-point transcriptomic responses has only progressed understanding of how omega-3 LC-PUFA regulate performance to demonstrate that some genes are up- or down-regulated when omega-3 LC-PUFA are deficient, even though sometimes no differences in phenomic responses are apparent. Perhaps the more useful strategies have been those encompassing a more holistic overview of pathway analysis responses (e.g., GSEA) based on microarray analysis as reported by Glencross et al. (2015) and Horn et al. (2019). It is also important to recognize the comment from Page et al. (2003), that if the fundamental nutritional strategy underlying an experiment from which samples are collected is flawed, then no molecular technique is going to "rescue" the usefulness of the data.

The development of better models of omega-3 LC-PUFA demand will provide an improved mechanistic basis with which to understand the multi-faceted role that it is likely that these nutrients play. The complex nature of the many different roles and loci that omega-3 LC-PUFAs seem to exert their influence on lends itself to the application of modeling to provide some means of simultaneously examining such complex relationships. While mechanistic and empirical models have proven useful in determining energy, protein, and even amino acid demands by fish and other animals, they are yet to be applied to the nutritional demand for EFA (Dumas et al. 2010; Glencross and Bermudes 2012; Hua and Bureau 2019). Coupling the development of such models with traditional dose-response nutrient requirement studies would be the logical point to begin examining this possibility, though studies on aspects of LC-PUFA retention efficiencies and maintenance demands will also no doubt be required (Salini et al. 2016a).

Understanding of the role of some omega-3 LC-PUFA (e.g., EPA) in regulation of eicosanoid synthesis has progressed somewhat, identifying key roles of many regulatory elements in the regulation of the immune response (Martinez-Rubio et al. 2013). How this response is affected by other LC-PUFA such as ARA and DHA, remains to be explored. Indeed, this whole area of LC-PUFA influence over inflammation in fish will be a critical area of research moving forward (Ralston et al. 2017). While it has been shown that EPA has a clear role in regulation of inflammation, specific intake thresholds are unknown, as is whether retro-conversion of DHA can adequately supply the EPA required for this process. Studies with mammalian models have also shown that many of SPM are synthesized not just from EPA, but that a broader array appear to be derived from DHA with some from DPA omega-3 too. Although a higher DHA:EPA ratio has been shown to stimulate some innate immune response factors such as serum lysozyme activity, further innate responses or even pathogen challenge responses in response to DHA levels and DHA to EPA ratios remain to be explored (Martinez-Rubio et al. 2012, 2013). In this regard, cellular models as a biological basis on which to examine aspects of these interactions would be worth revisiting (Bou et al. 2016; Estévez et al. 2018). Cellular models could be particularly useful in studying the mechanistic role of inflammation and immune function mediation of EPA and other LC-PUFAs and would be a logical place to begin such work exploring the roles of DHA and ARA and interactions among the different PUFAs. Furthermore, the structure of the lipids in emerging sources such as microalgal or GM-derived oils may differ from those produced natively (Broughton et al. 2022), and their impacts on fish health, immune response, smoltification or reproduction requires further study.

Gaps in knowledge of LC-PUFA requirements

Despite the growing volume of work on the requirements (both qualitative and quantitative) for LC-PUFA by fish, there remains a further need for studies on key aquaculture species. Clear, concise studies examining the quantitative requirements for different LC-PUFA, particularly in combination, such as DHA:EPA:ARA remain to be explored. Importantly, we now know that many of these requirements change with animal size/age and life-stage, and environmental conditions, and these features need further consideration for most species. Experiments need to move toward examining effects over discrete growth periods and avoid long-term over-arching studies, though remain mindful of long-term deficiency constraints. Substantial capacity exists for the reanalysis of past studies to focus in on such responses in discrete growth (feed size) periods, within studies. While the amount of work on salmonid species is extensive, further research on the requirements of marine species

needs to be undertaken, as these species are more sensitive to perturbations in omega-3 LC-PUFA supply.

The prior nutritional history of an animal and how this affects subsequent requirement responses has not been examined. For example, whether a fingerling seabass can be fed a high LC-PUFA diet and thereby reduce the need for LC-PUFA in subsequent stages of production needs to be explored. If such a "pre-loading" is feasible, it may save considerable amounts of marine oil use in the more feed intensive later grow-out stages. Additionally, many of the dietary specifications used for various species have changed considerably since the early studies on EFAs were undertaken and it would be practical to revisit some of these works in the context of those changes in dietary formulation strategies seen since those early days. For example, the energy density of diets for salmonids is now some 10% to 20% greater (e.g., 20.6 MJ DE vs 17.8 MJ DE) at the same life stage than it was 20 to 30 years ago, meaning that fish now eat 10% to 20% less feed to achieve the same growth. This reduction in intake will also affect the overall intake levels of many other nutrients, which anecdotally formulators are now having to increase above previously estimated requirement levels as they are proving inadequate in these modern formulations (e.g., phosphorus specifications are now higher at 12–18 g/kg compared to the 7 to 10 g/kg used 20 to 30 years ago) (Ketola 1975; Åsgård and Shearer 1997).

Most estimates of the requirement levels for omega-3 LC-PUFA for marine fish, salmonid and shrimp species appear to be somewhere in the range of 5% to 10%TFA (5 g/kg to 20 g/kg, subject to dietary lipid level; Table 2), though this varies substantially with animal size and health status (Glencross 2009; Carr et al. 2023). This level of variability represents almost a four-fold difference in the need for inclusion of EPA + DHA source depending on which level is chosen and species considered. Further refinement of the actual requirement level by production stage (feed pellet size) would provide some basis from which to make the use of omega-3 LC-PUFA more efficient and allow a targeted use for those stages of production where the role of these nutrients is greater. Studies looking at finer-scale gradations of LC-PUFA need to be undertaken.

Conclusions

It is evident that there remains much to be done to both improve our understanding of the physiological roles of omega-3 LC-PUFA in aquaculture species and better define their requirement for the myriad of species now being farmed in aquaculture (Table 2). As these resources further increase in value, the importance of this refinement will likely grow. Capacity for additional growth in EPA and DHA volumes from fishery resources appears limited, though the significant volumes now coming from by-products from fisheries and aquaculture bode well for attempts to create a greater circular economy for these nutrients. New sources whether from algal or GM crop options are emerging and will become increasingly important resources into the future.

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Disclosure statement

BG has both a university affiliation and a commercial affiliation with a member-based organization representing the international marine ingredient sector. EB has a commercial affiliation with a member-based organization representing the international marine ingredient sector. All other authors have solely a university affiliation and no declared interests.

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