6

Lipids

Defining dietary lipid requirements is complicated by the varied chemical nature and functional roles of lipids. Unlike proteins, lipids are a diverse range of very different compounds that are grouped together simply on the basis of their solubility in organic solvents. Most lipids are "complex," meaning that they contain fatty acids usually esterified to alcohol groups in the case of acylglycerols (glycerides) and to amino groups in the case of sphingolipids. In terms of function, lipids can be divided into two groups: (1) polar lipids, including phospholipids that play predominantly structural roles, and (2) neutral lipids, including triacylglycerols (TAG or triglycerides), whose role is storage, primarily of energy but also cellular components. Simple lipids do not contain fatty acids, with the most important one in animals, including fish, being cholesterol that can be unesterified as a functional component of cell membranes or in a storage form esterified to a fatty acid.

As most lipids are complex, fatty acids usually comprise the bulk of dietary lipid intake. The requirement for specific fatty acids depends upon their differing functional roles and whether they can be synthesized endogenously. All fatty acids can serve as energy sources, but some specific long-chain polyunsaturated fatty acids (LC-PUFA) also have a number of essential roles in metabolism. Lipid requirements therefore encompass a gross requirement for energy and more specific requirements for functional lipid classes such as cholesterol, intact phospholipid, and essential fatty acids. In addition, individual fatty acids can be delivered in a variety of chemical forms including TAG, phospholipids, steryl and wax esters, free fatty acids, or synthetic concentrates such as methyl- or ethyl-esters.

FATTY ACID STRUCTURE AND NOMENCLATURE

In the n- or "omega" nomenclature, fatty acids are described by the general formula, X:Yn-z, where X is the chain length, Y is the number of ethylenic/double bonds, and n-z (or ωz) denotes the position of the first double bond relative to the methyl end of the aliphatic chain. Thus, 16:0 denotes a saturated fatty acid containing 16 carbons and no double bonds (all carbons saturated with hydrogen), and 18:1n-9 (18:1ω9) designates a monounsaturated fatty acid with 18 carbon atoms with a single, normally *cis*, double bond 9 carbon atoms from the methyl end (Figure 6-1). Polyunsaturated fatty acids (PUFA) contain two or more double bonds, generally separated by a single methylene (CH_2) group. Thus, 20:4n-6 (20:4ω6) is a 20-carbon chain with four methylene-interrupted double bonds with the first double bond situated 6 carbon atoms from the methyl end of the molecule. Similarly, 22:6n-3 (22:6ω3) is a 22-carbon chain with 6 double bonds with the first double bond situated 3 carbon atoms from the methyl end (Figure 6-2).

In the alternative Δ (delta) nomenclature, 22:6n-3 is written as 22:6Δ4,7,10,13,16,19 with Δ signifying the positions of the double bonds from the carboxyl end of the molecule. The n-nomenclature is more convenient and commonly used, although the Δ nomenclature is often used for specifying fatty acyl desaturase (Fad) activities. Thus, a desaturase that introduces an ethylenic (double) bond 6 carbons from the carboxyl end of the molecule is termed Δ6 Fad. Fatty acids also have trivial names often reflecting their sources such as palmitic acid (16:0) from palm oil, oleic acid (18:1n-9) from olive oil, and α-linolenic acid (18:3n-3) from linseed oil. Slightly more useful are their chemical names, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), that reflect both the numbers of carbon atoms and double bonds they contain.

LIPID CLASS STRUCTURES

Sterols, tetracyclic hydrocarbon alcohol compounds are the most important simple lipids (i.e., not containing fatty acids) with cholesterol being the predominant sterol in animals including fish (Figure 6-3). Unesterified cholesterol is an essential component of all cell membranes, but can also be found in steroidogenic tissues esterified to fatty acid in the form of neutral lipid droplets as a store of hormone precursor.

The major neutral lipid is triacylglycerol (TAG), which

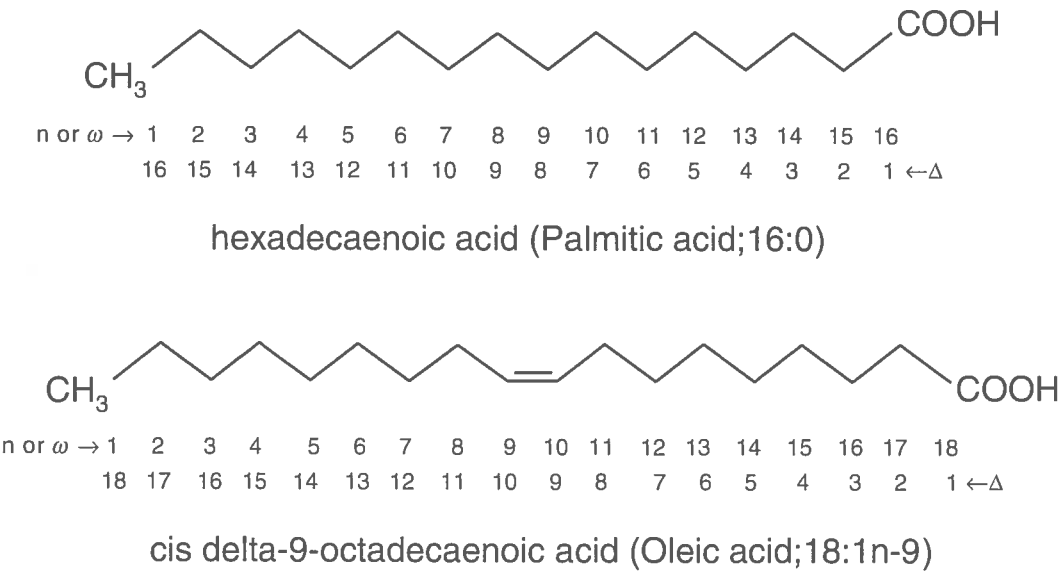


FIGURE 6-1 Palmitic (16:0) and oleic (18:1n-9) acids showing the *n* carbon numbering system.

consists of three fatty acids esterified to the alcohol groups of glycerol (Figure 6-3). Generally saturated and monounsaturated fatty acids are preferentially located in the *sn*1 and *sn*3 positions, whereas PUFA are preferentially located in the *sn*2 (middle) position, although, as with phospholipids, there are many exceptions (Tocher, 2003). The main role of TAG is for energy storage, and another form of lipid store is wax ester that consists of a fatty acid esterified to a fatty alcohol. Wax ester is abundant in marine zooplankton, particularly calanoid copepods and euphausiids, which are natural foods for many marine fish. The fatty alcohols are generally saturated or monounsaturated and, in the case of high-latitude marine zooplankton, can be rich in $C_{20/22}$ monounsaturated alcohols. The large amounts of 20:1n-9 and 22:1n-11 fatty acids in northern hemisphere fish oils are derived from the oxidation of the corresponding fatty alcohols during digestion and absorption of wax esters in zooplanktonivorous fish (Tocher, 2003).

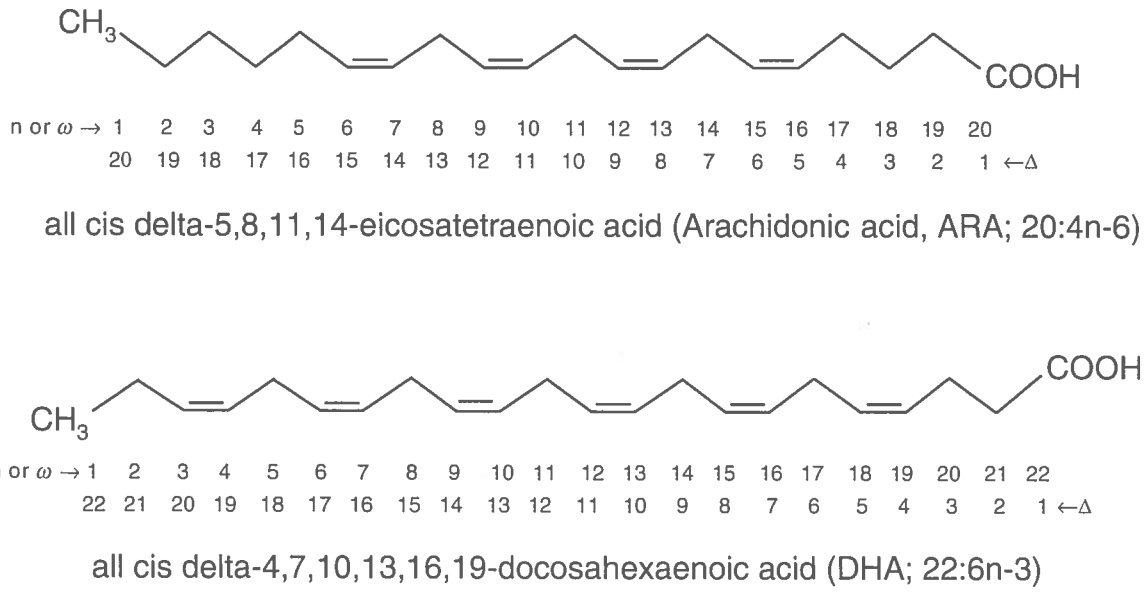
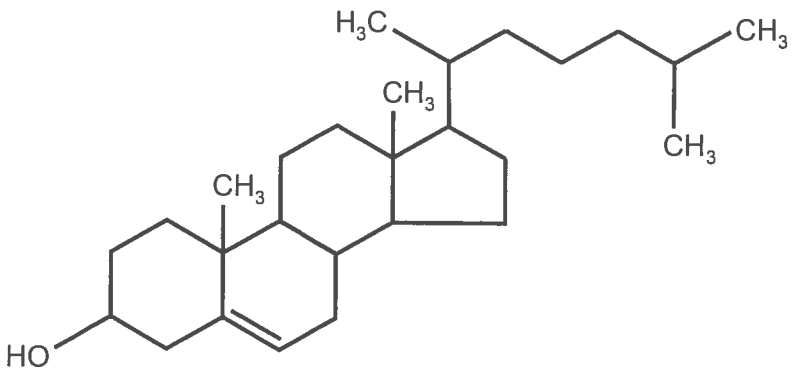
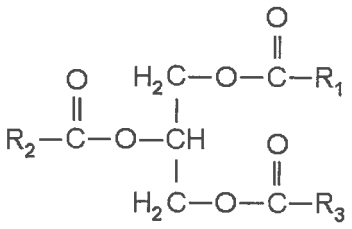


FIGURE 6-2 Arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids showing the *n* and Δ carbon numbering systems.



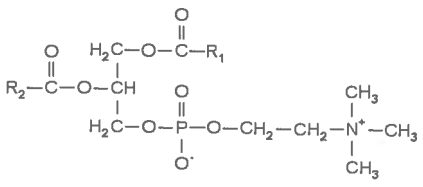
Cholesterol



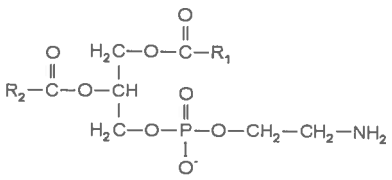
Triacylglycerol

FIGURE 6-3 The structures of cholesterol and triacylglycerol.

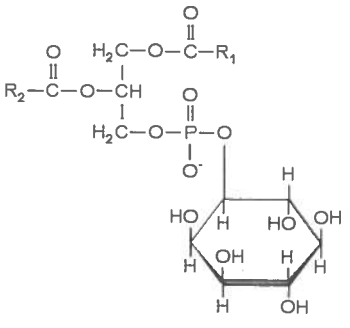
Phospholipid is a general term comprising all lipids containing phosphorus including sphingomyelin, although the term is commonly used to describe phosphoglycerides, which are the predominant polar lipids. Phosphoglycerides are all derived from phosphatidic acid (PA), which is L-glycerol 3-phosphate esterified with two fatty acids. Esterification of the "bases" choline, ethanolamine, serine, and inositol to the phosphate group of PA results in the major phosphoglycerides, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Figure 6-4). Generally, saturated and monounsaturated fatty acids are preferentially esterified at *sn*-1 with PUFA preferentially esterified on position *sn*-2, although there are many exceptions (Tocher, 1995, 2003). Sphingolipids are a group of complex polar lipids based on the long-chain amino alcohol sphingosine, or a related base, with a long-chain saturated or monounsaturated fatty acid linked to the amino group to form a ceramide, and different polar head groups are attached to the primary alcohol group. Further groups can be esterified to the alcohol group of sphingosine such as phosphocholine to form sphingomyelin (Figure 6-4) or sugar moieties to form the cerebrosides.



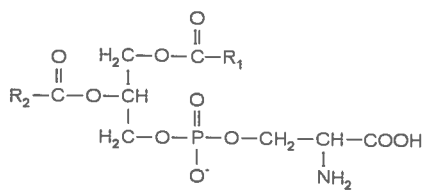
Phosphatidylcholine



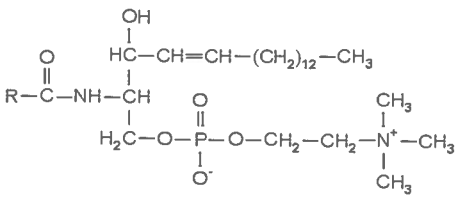
Phosphatidylethanolamine



Phosphatidylinositol



Phosphatidylserine



Sphingomyelin

FIGURE 6-4 The structures of the main phospholipid classes.

GENERAL LIPID METABOLISM

Most of the basic pathways of lipid metabolism including digestion and absorption, lipid transport, lipogenesis, and β-oxidation are essentially the same in fish as they are in mammals. The following summary is based on several reviews where more detailed accounts can be found (Sargent et al., 1989, 2002; Olsen and Ringø, 1997; Tocher, 2003; Mourente et al., 2007; Tocher et al., 2008).

In general, dietary lipids are digested mainly in the proximal intestine and pyloric caeca, if present, with the pancreas or hepatopancreas generally assumed to be the major source of digestive enzymes, TAG lipases, and phospholipases. The main products of digestion of the major classes of lipids are free fatty acids along with partial acylglycerols, predominantly 2-monoacylglycerols, lyso-phospholipids, cholesterol, and fatty alcohols. The basic physical processes of digestion and absorption, including bile-enhanced emulsification and transport of the hydrolysed products, are assumed to be generally similar to that in mammals. Thus, the main hydrolytic products are solubilized or emulsified in bile salt micelles, followed by diffusion to the intestinal mucosa where uptake into the enterocytes occurs, probably mainly by passive diffusion. In the intestinal mucosal cells, the predominant fate of the absorbed free fatty acids is reesterification with glycerol, partial acylglycerols, and lyso-phospholipids to reform TAG and phospholipids. Steryl and wax esters may also reform, although free cholesterol is easily transported from the mucosal cells, and fatty alcohols are oxidized to the corresponding fatty acid in the epithelial cells.

Although some free fatty acids can be transported bound to albumin-like proteins, the majority of lipids are transported in the blood in the form of lipoproteins that are qualitatively similar to those found in mammals. The relative proportions of lipid and protein in these lipoproteins vary from chylomicrons (which transport the majority of absorbed dietary lipid away from the intestine) and very low density lipoproteins (VLDL) that have a high lipid:protein ratio, through low density lipoprotein (LDL), to high density lipoproteins (HDL) that have a low lipid:protein ratio. The proportions of the different lipoprotein classes vary with species. Female fish also produce vitellogenin, another lipoprotein, specifically for transporting lipid to the developing oocytes during the process of vitellogenesis. Intracellular transport of fatty acids is facilitated by specific low molecular weight, highly conserved tissue-specific cytoplasmic fatty acid binding proteins (FABP) that bind both long-chain fatty acids and other hydrophobic ligands. Excess dietary lipid is deposited in adipose cells although the precise tissue location can vary between fish species. Most species have intraperitoneal (intestinal) adipose tissue, and some deposit lipid as a layer between the skin and flesh. However, the so-called oily fish, such as herring and salmon, also deposit lipid in the flesh, and others, such as cod and halibut store lipid predominantly in the liver.

Lipogenesis describes the biosynthetic reactions for the endogenous formation of new lipid. The carbon source for the biosynthesis of new lipids is acetyl-CoA formed in mitochondria from the oxidative decarboxylation of pyruvate (carbohydrate source) or the oxidative degradation of some amino acids (protein source). The key lipogenesis pathway is catalyzed by the cytosolic fatty acid synthetase (FAS) multienzyme complex, which produces the saturated fatty acids 16:0 and 18:0. Monounsaturated fatty acids are produced by microsomal stearoyl CoA desaturase (SCD) or Δ9 desaturase, producing 18:1n-9 and, to a lesser extent, 16:1n-7. Fatty acid elongases produce longer chain saturated and monounsaturated fatty acids, such as 20:0 and 20:1n-9. However, PUFA cannot be synthesized de novo by any vertebrate and must be obtained in the diet. Fatty acids are esterified into complex lipids, including membrane phospholipids and TAG, by essentially the same pathways as in mammals. Fish lipids, and specifically membrane phospholipids, are rich in LC-PUFA that must be protected from peroxidation by endogenous systems including a suite of enzymes such as catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase, and antioxidant compounds including glutathione, vitamin E, and vitamin C.

Fatty acid catabolism is the major source of energy in many species of fish. Whereas the biosynthesis of fatty acids occurs in the cytosol, the catabolism of fatty acids occurs in the cellular organelles, mitochondria, and peroxisomes. The process is termed β-oxidation and involves the sequential cleavage of two-carbon units, released as acetyl-CoA, through a cyclic series of reactions catalyzed by several distinct enzyme activities rather than a multienzyme complex. Briefly, activated fatty acids are transported into the mitochondrion in the form of fatty acylcarnitine esters formed through the action of carnitine acyltransferase, converted back into fatty acyl-CoA derivatives that then undergo a round of dehydrogenation, hydration, second hydrogenation, and cleavage steps to produce acetyl-CoA and NADH. Acetyl-CoA can then be metabolized via the tricarboxylic cycle to produce more NADH, which can then provide metabolic energy in the form of ATP through the process of oxidative phosphorylation. In vitro studies comparing relative oxidation rates of fatty acids have generally shown the following orders of preference: saturated/monounsaturated > PUFA > LC-PUFA, with shorter chain > longer chain and n-6 > n-3. However, in vivo studies investigating fatty acid deposition show that, generally, the higher the concentration of a fatty acid in the diet, the lower its relative deposition (retention), implying increased concentration leads to increased oxidation. Therefore, oxidation of a fatty acid is a balance between enzyme specificities and substrate fatty acid concentrations (competition). One possible exception to this is DHA that is resistant to β-oxidation, as the Δ4 double bond requires peroxisomal oxidation to be removed, and so is poorly oxidized in mi-

tochondria resulting in DHA appearing to be retained in tissues, independent of dietary concentration.

DIETARY LIPID LEVEL

A true lipid requirement for any species of fish or shrimp cannot be specifically defined because it is influenced by a variety of nutritional factors. As a macronutrient, lipid is principally a source of energy.The amount of dietary lipid required is influenced by the contents of dietary protein and carbohydrate, which can also serve as sources of energy. As previously described, protein and carbohydrate can also be sources of lipid through lipogenesis with amino acids and pyruvate serving as the main carbon sources. As protein sources are the most costly ingredients in diets formulated for commercial use, the goal is to minimize dietary protein that might be used as a source of energy. Therefore, with an appropriate amount of energy supplied by lipid, protein requirements can be reduced or "spared." In turn, the level of lipid required to satisfy the energy requirement could be reduced through the provision of sources of carbohydrates in species that can effectively utilize these nutrients. However, carbohydrates are more efficiently digested by some species (often herbivorous/omnivorous) than others. Thus, some species may have limited capabilities of digesting carbohydrate, thereby restricting its use as an effective source of energy. It may be that species that have evolved on high-lipid food sources are more likely to have a poor utilization of dietary carbohydrate. Environmental temperature may be another factor for the difference as most investigated carnivores are coldwater species and most herbivorous species are warmwater fish. The amount of dietary lipid is also affected by its source relative to the satisfaction of requirements for essential fatty acids (EFA). The relative amount of lipid to satisfy the EFA requirements is dependent upon lipid source(s) and corresponding fatty acid profiles. For commercial diet formulation, generally TAG-rich oils/fats are provided as ingredients of diets to ensure that specific requirements for PUFA and/or LC-PUFA are effectively satisfied.

Although an "optimum" level of dietary lipid cannot be truly defined for any species, there is a range within which dietary lipid should be supplied. The lower limit will be defined as the amount of lipid required to supply the requirements for EFA (and cholesterol and phospholipid in some species at specific life stages), which will depend upon the precise lipid source(s) and their corresponding fatty acid profiles. However, higher dietary levels may be necessary to satisfy obligatory lipid deposition required to successfully fulfill or realize certain physiological stages often associated with reproduction (migration/spawning). Increasing dietary lipid above the minimum level will support higher growth rates, possibly partly based on protein sparing, toward an upper limit where excess lipid leads to unwanted deposition of lipid in the peritoneal cavity, liver, or other tissues (Company et al., 1999; Craig et al., 1999; Gaylord and Gatlin, 2000). This represents wasted energy as there is little point in supplying an energy-yielding nutrient that is simply deposited unused in tissue stores. Of course, deposited lipid contributes to increased weight, but as it is not flesh (muscle), it is not contributing to yield. This is highlighted in species such as Atlantic cod (*Gadus morhua*) that deposit lipid in the liver or other species with large perivisceral storage. So-called "oily" fish such as Atlantic salmon (*Salmo salar*), which deposit significant amounts of lipid in the flesh, are able to tolerate and utilize higher dietary lipid levels.

Fish

Not withstanding the above caveats, various studies have investigated the relationships between dietary lipid contents, growth, and lipid deposition in fish. Weight gain was increased in rainbow trout (*Oncorhynchus mykiss*) in fish fed dietary lipid at 21% compared to 8–11% (Luzzana et al., 1994), and growth was higher in brown trout (*Salmo trutta*) fed dietary lipid at 29% compared to 21% (Arzel et al., 1993). Furthermore, weight gain in Atlantic salmon was higher in fish fed diets containing 38–47% lipid compared to fish fed 31% lipid (Hemre and Sandnes, 1999). However, high dietary lipid increases flesh lipid levels in freshwater fish and salmonids including rainbow trout (Dias et al., 1999) and Atlantic salmon (Bell et al., 1998; Hemre and Sandnes, 1999). Despite this, the upper level for dietary lipid in salmon diets doubled between the 1970s and late 1990s, when an optimal dietary lipid level of 35% was suggested (Einen and Roem, 1997). However, deposition of excess dietary lipid in the flesh can impact carcass and product quality, causing problems of oily texture and pigmentation that lead to consumer and processor resistance (Bell et al., 1998; Hillestad et al., 1998) and may influence early sexual maturation in males (Shearer and Swanson, 2000). Some problems may be alleviated by feeding a low-fat "finishing" diet prior to slaughter (Rasmussen et al., 2000).

However, in contrast to the above situation with salmonids, it should be noted that > 85% of all farmed finfish production is of freshwater, predominantly low trophic level fish species including carps and tilapia (Tacon et al., 2010), which generally cannot tolerate such high levels of dietary lipid (often < 10%). This may be associated with these species having natural diets that generally contain lower levels of lipid and, perhaps, higher levels of carbohydrate that they are thus adapted to utilize more effectively and efficiently (see Chapter 7). As a result these species seem to have a lower ability to utilize high dietary lipid and so commercial feeds.

Weight gain of European sea bass (*Dicentrarchus labrax*) was increased in fish fed diets containing lipid at 15% compared to 9% lipid (Manuel Vergara et al., 1996), and 19% compared to 11 and 15% (Lanari et al., 1999), but a lower limit to the growth-promoting effect of high-fat diets in marine fish was indicated because growth rate was higher in sea bass fed 24% lipid compared to fish fed 30% lipid (Peres

and Oliva-Teles, 1999). Flesh, organ, and visceral lipid increases as dietary lipid increases in marine fish including turbot (*Psetta maximus*) (Saether and Jobling, 2001) and sea bass (Catacutan and Coloso, 1995). High-fat diets may also promote the development of fatty liver pathology (Caballero et al., 1999).

Shrimp

In shrimp and other crustaceans, weight gain responses to different levels of dietary oils, either alone or in combination, indicate that highest gains are generally achieved at dietary levels of 5–6% inclusion. Higher levels (> 10%) often retard growth (Kanazawa et al., 1977a; Davis and Robinson, 1986; Sheen and D'Abramo, 1991), most probably due to a reduction in consumption caused by high caloric content and/or an inability to metabolize high levels efficiently (reduced digestibility). Reduced growth has been shown to be associated with accumulation of lipid in tissue (Castell and Covey, 1976; Ponat and Adelung, 1983; González-Félix et al., 2002a). These conclusions on dietary lipid levels were drawn from experiments in which marine-derived sources containing good profiles of n-3 LC-PUFA, including cod liver oil, menhaden fish oil, pollock liver oil, and short-neck clam oil, were used. Some studies have included plant oils that are good sources of n-6 PUFA.

In summary, because of the complex metabolic interactions between protein, lipid, and carbohydrate mentioned at the beginning of the section, definition of precise dietary lipid requirements in fish and shrimp are not particularly useful or meaningful. Although lipid up to 20% of the dry weight of the diet allows protein to be effectively utilized for growth in many fish species without depositing excessive lipid in the tissues (Sargent et al., 2002), lipid can have a protein-sparing effect in many species that has driven the use of so-called "high-energy" (high-lipid) diets to become increasingly widespread in aquaculture. High-energy diets can have consequences by altering lipid and fatty acid metabolism with health and welfare implications for the fish and product quality for the consumer (Sargent and Tacon, 1999). More detailed accounts of nutritional energetics and the role of lipid as an energy source and its interaction with other dietary components, including protein and carbohydrate, are provided in Chapter 4.

SPECIFIC REQUIREMENTS

Essential Fatty Acids

As vertebrate and crustacean species cannot synthesize any PUFA from monounsaturated fatty acids de novo (see Figure 6-5), they therefore have an absolute dietary requirement for certain specific n-3 and n-6 PUFA. Dietary deficiency of these "essential fatty acids" results in various pathologies, the animal stops growing and reproducing, and eventually dies (Das, 2006). The biologically active PUFA required for many essential metabolic and physiological processes are the LC-PUFA, 20:4n-6 (ARA, arachidonic acid), 20:5n-3 (EPA) and 22:6n-3 (DHA) (Das, 2006). In contrast, the shorter chain C_{18} PUFA, typified by linoleic acid 18:2n-6 and α-linolenic acid 18:3n-3, have no specific metabolic roles in themselves, although they can serve as precursors for the corresponding n-6 and n-3 LC-PUFA (Sargent et al., 1995a). Note that vertebrates and crustaceans are unable to interconvert the n-6 and n-3 PUFA families (Figure 6-5). Species vary in their capacity to convert C_{18} PUFA to LC-PUFA. In species that cannot perform these conversions, dietary C_{20} and C_{22} LC-PUFA are essential, and their C_{18} homologues do not satisfy EFA requirements. In species that can perform the conversions, C_{18} PUFA, and C_{20-22} LC-PUFA can all be termed EFA with the LC-PUFA often being more effective nutritionally than their C_{18} counterparts. Definition of the optimal amounts of EFA to satisfy the requirements for normal growth and development has been a well-studied area of lipid metabolism in fish, driven by the needs of the aquaculture industry. Of particular importance, the requirements can vary quantitatively during ontogenesis and, therefore, accurate definition of EFA requirements for a given species involves determining not only the absolute requirements of specific PUFA and the optimal balance between different PUFA, but also how these requirements vary at different life stages (Tocher, 2010).

Methodological Challenges

Appreciation of quantitative EFA data requires some considerations of the methodology used for determining these requirements. The methodology is difficult because an EFA-deficient feed has to be produced, which requires an essentially lipid-free diet. This is hard to achieve without affecting other important aspects of the diet, such as attraction and palatability. The consequence of these difficulties is that EFA requirements were generally measured in small fish fed diets with much lower lipid levels than are commonly used today, with consequently lower growth rates. Therefore, the quoted estimates of EFA requirements probably represent the levels that were sufficient to (1) prevent appearance of deficiency signs and (2) to maintain growth at that particular, albeit low, dietary lipid level. The EFA requirements can be expressed as a percentage of the total lipid, percentage of diet, or percentage of total fatty acids. It is apparent that the quantitative requirement for EFA may vary with the total dietary lipid level, and this may also vary with the stage of development (Izquierdo, 1996). For instance, the requirement for n-3 LC-PUFA appeared to increase as the level of lipid in the diet increased in red sea bream (*Pagrus major*) fingerlings (Takeuchi et al., 1992a), yellowtail (*Seriola quinqueradiata*) fingerlings (Takeuchi et al., 1992b), and *Penaeus monodon* (Glencross et al., 2002a), although there was no apparent variation in the requirement for n-3 LC-PUFA as

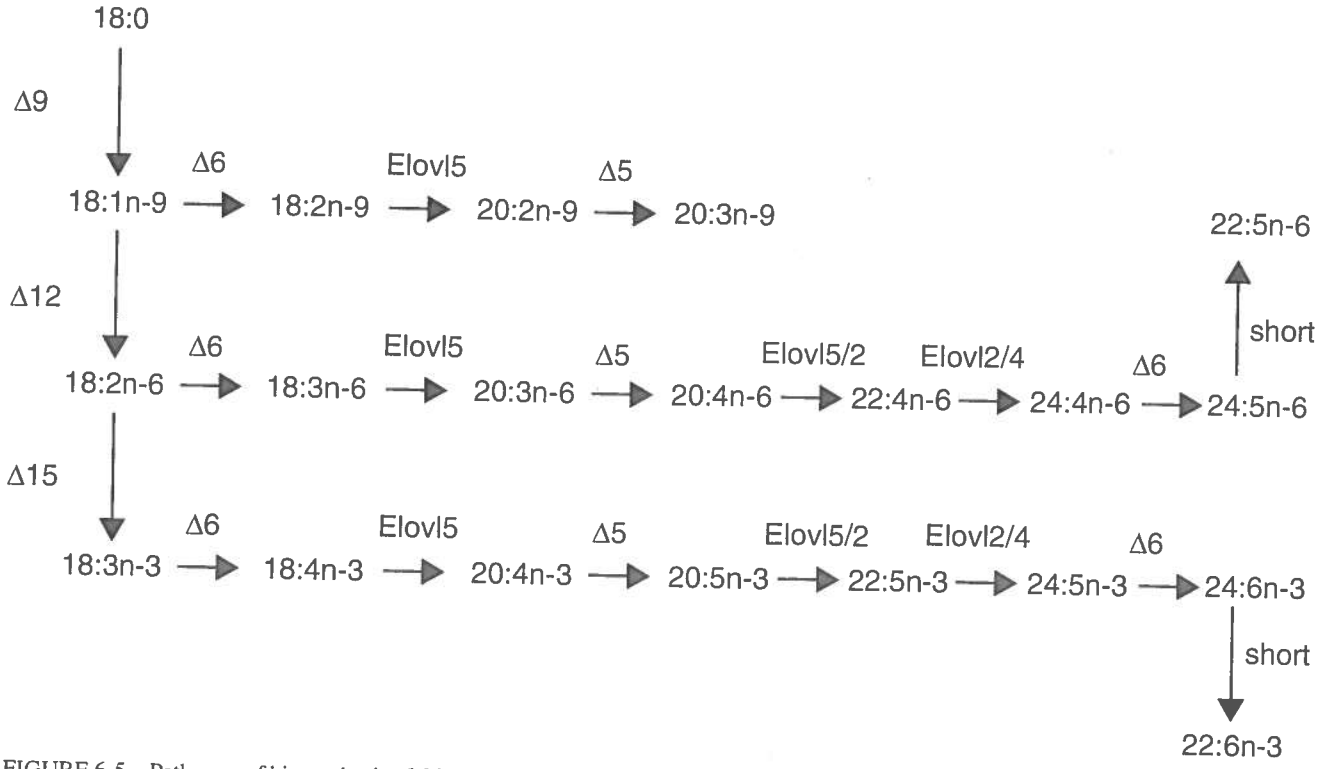


FIGURE 6-5 Pathways of biosynthesis of C20 and C22 long-chain polyunsaturated fatty acids (LC-PUFA) from n-3, n-6, and n-9 C_{18} PUFA. Δ9, stearoyl CoA desaturase (SCD); Δ5 and Δ6, front-end fatty acyl desaturases (Fad). Evidence suggests that the same Δ6 Fad operates on both C_{18} and C_{24} fatty acyl substrates; Δ12 and Δ15, Fads found only in plants and some invertebrates, and hence 18:2n-6 and 18:3n-3 cannot be formed in any vertebrate; Elovl2, Elovl4, and Elovl5, PUFA elongases; short, peroxisomal chain shortening.

the dietary lipid level increased in larval gilthead sea bream (*Sparus aurata*) (Salhi et al., 1994) or *Litopenaeus vannamei* (González-Félix et al., 2002a, 2003). However, in later studies, growth retardation has been observed in sea bream and turbot fed diets with high levels of dietary fish oil substituted with vegetable oils (devoid of LC-PUFA), despite the diets being formulated to supply EPA and DHA above the estimated EFA requirements (Caballero et al., 2003; Regost et al., 2003). These later studies used higher lipid levels (16–20%) supporting higher growth rates than in previous EFA requirement studies (8–12%) (Kalegeropoulos et al., 1992; Ibeas et al., 1994, 1997). One explanation may be that the higher growth rates supported by diets with increased lipid can only be achieved with similarly increased EFA. Therefore when EFA levels are reduced, albeit still above the "EFA requirement," by substituting vegetable oil, decreased growth can be observed (Caballero et al., 2003; Regost et al., 2003). Growth retardation was not apparent in salmonids fed similar vegetable oil diets, suggesting that endogenous production of EPA and DHA from dietary 18:3n-3 may be sufficient to maintain the physiological requirements for these fatty acids and prevent growth suppression (Bell et al., 2004; Torstensen et al., 2005). Therefore, increments in dietary EFA level above the reported "requirement" may improve growth and survival, suggesting that there may also be "optimal" EFA levels. Despite the suggestion that EFA requirements can vary based on diet formulation, it is likely, for the reasons argued at the beginning of this section, that the quoted EFA requirement levels are good indicators of minimum levels that should be provided to prevent pathology.

Fish

The quantitative and semiquantitative requirements for EFA have been reported for around 30 species of fish (Tables 6-1 through 6-3). In the past 10 years studies focused more on larval marine fish and the relative requirements of ARA, EPA, and DHA rather than defining absolute EFA requirements in juveniles and subadults of more species (Lund et al., 2007, 2008; Hamre and Harboe, 2008a,b). This is probably because the experiments are difficult and expensive, because, in addition to the problems of diet formulation discussed above, a regression protocol should be used requiring significant numbers of experimental units. However, there is probably sufficient information on a wide enough range of species to predict qualitative and semiquantitative EFA requirements for new species of interest (Tocher, 2003, 2010). Requirements for EFA also vary with developmental and possibly physiological stage, further complicating the definition of absolute quantitative requirements (Sargent et al., 2002).

TABLE 6-1 Reported Quantitative Essential Fatty Acid (EFA) Requirements of Juvenile and Subadult Freshwater and Diadromous Species of Finfish[a]

Species	Scientific Name	EFA	Requirement (% Dry Diet)	Reference
Arctic charr	*Salvelinus alpinus*	18:3n-3	1.0–2.0	Yang et al. (1994)
Atlantic salmon	*Salmo salar*	18:3n-3	1.0	Ruyter et al. (2000a)
		n-3 LC-PUFA	0.5–1.0	Ruyter et al. (2000b)
Ayu	*Plecoglossus altivelus*	18:3n-3 or EPA	1.0	Kanazawa et al. (1982)
Channel catfish	*Ictalurus punctatus*	18:3n-3	1.0–2.0	Satoh et al. (1989)
Cherry salmon	*Oncorhynchus masou*	18:3n-3 or n-3 LC-PUFA	1.0	Thongrod et al. (1990)
Chum salmon	*Oncorhynchus keta*	18:2n-6 and 18:3n-3	1.0 of each	Takeuchi et al. (1979)
Coho salmon	*Oncorhynchus kisutch*	18:2n-6 and 18:3n-3	1.0 of each	Yu and Sinnhuber (1979)
Common carp	*Cyprinus carpio*	18:2n-6	1.0	Takeuchi and Watanabe (1977)
		18:3n-3	0.5–1.0	Takeuchi and Watanabe (1977)
Grass carp	*Ctenopharyngodon idella*	18:2n-6	1.0	Takeuchi et al. (1991)
		18:3n-3	0.5	Takeuchi et al. (1991)
Japanese eel	*Anguilla japonicus*	18:2n-6 and 18:3n-3	0.5 of each	Takeuchi et al. (1980)
Milkfish	*Chanos chanos*	18:2n-6 and 18:3n-3	0.5 of each	Bautista and de la Cruz (1988)
Rainbow trout	*Oncorhynchus mykiss*	18:3n-3	0.7–1.0	Castell et al. (1972)
		n-3 LC-PUFA	0.4–0.5	Takeuchi and Watanabe (1976)
Sheatfish	*Silurus glanis*	18:3n-3	1.0	Borgut et al. (1998)
Striped bass	*Morone chrysop* × *Morone saxatilis*	n-3 LC-PUFA	1.0	Gatlin et al. (1994)
Tilapia	*Tilapia zilli*	18:2n-6	1.0	Kanazawa et al. (1980)
	Oreochromis nilotica	18:2n-6	0.5	Takeuchi et al. (1983)
	Oreochromis niloticus × *Oreochromis aureus*	n-3 required	?	Chou and Shiau (1999)
Whitefish	*Coregonus laveratus*	18:3n-3	> 1.0	Thongrod et al. (1989)
		n-3 LC-PUFA	0.5–1.0	Watanabe et al. (1989)

[a]Based on Tocher (2010).

Freshwater and Diadromous Species

Reported estimates for juveniles and subadults of freshwater and diadromous fish species indicate that EFA requirements can be satisfied by the C_{18} PUFA, 18:3n-3, and 18:2n-6, at around 1% of the diet dry weight (Table 6-1). In terms of EFA, freshwater/diadromous species were traditionally subdivided into three groups: coldwater species including salmonids that have a higher requirement for 18:3n-3 (compared to 18:2n-6), warmwater species such as tilapia (*Oreochromis* spp.) that have a higher requirement for 18:2n-6, and species that require significant amounts of both such as common carp (*Cyprinus carpio*). However, growth of hybrid tilapia (*O. niloticus* × *O. aureus*) was significantly improved by feeding cod liver oil compared to corn oil and so, although not quantitatively defined, tilapia also require n-3 fatty acids, or at least n-3 LC-PUFA, for maximal growth (Chou and Shiau, 1999). Therefore, it is likely that all freshwater/diadromous fish require both n-3 and n-6 PUFA, with coldwater fish possibly having a requirement for higher levels of n-3 compared to n-6. Although the C_{18} PUFA are usually effective in satisfying the EFA requirements of freshwater fish, for some species, including salmonids, n-3 LC-PUFA can satisfy the EFA requirements at lower levels than 18:3n-3 and increase growth over that obtained with 18:3n-3 alone (Ruyter et al., 2000b). Similarly, growth was significantly improved in channel catfish (*Ictalurus punctatus*) by inclusion of dietary n-3 LC-PUFA (Santha and Gatlin, 1991). There are few data on the requirements of freshwater fish for the main n-6 LC-PUFA, ARA (Bell and Sargent, 2003).

The early life stages of freshwater fish species have received little attention, and so there are few data reports providing estimated EFA requirements (Table 6-2). Newly hatched larvae or fry of many freshwater fish are large enough to accept formulated feeds whose composition can be defined to ensure maximal growth and survival such that feeds are not a problem in rearing high-quality fry. However, there is evidence that n-3 LC-PUFA and DHA may be more important and, possibly, essential in larvae of some species of freshwater fish compared to adults or juveniles (Webster and Lovell, 1990; Wirth et al., 1997). Broodstock nutrition is also critical to produce high-quality eggs and larvae with

TABLE 6-2 Reported Quantitative Essential Fatty Acid (EFA) Requirements of Larvae and Early Juveniles of Finfish[a]

Species	Scientific Name	EFA	Requirement (% Dry Diet)	Reference
Freshwater				
Common carp	*Cyprinus carpio*	n-6 PUFA	1.0	Radunzneto et al. (1996)
		n-3 PUFA	~ 0.05	Radunzneto et al. (1996)
Rainbow trout	*Oncorhynchus mykiss*	DHA essential	?	Wirth et al. (1997)
Marine				
Atlantic cod	*Gadus morhua*	EPA required	?	Zheng et al. (1996)
		DHA	~ 1.0	Takeuchi et al. (1994)
Gilthead sea bream	*Sparus aurata*	n-3 LC-PUFA	5.5 (DHA:EPA = 0.3)	Rodriguez et al. (1994a)
		n-3 LC-PUFA	1.5 (DHA:EPA = 2.0)	Rodriguez et al. (1998a)
		n-3 LC-PUFA	1.5 (in phospholipid)	Salhi et al. (1999)
		DHA:EPA	~ 2	Rodriguez et al. (1994b)
Mahi mahi	*Coryphaena hippurus*	n-3 LC-PUFA	0.6–1.0	Ostrowski and Kim (1993)
Red sea bream	*Pagrus major*	n-3 LC-PUFA	2.1 (with 1.0 DHA)	Furuita et al. (1996a)
		DHA	1.0–1.6	Furuita et al. (1996a)
		EPA	2.3	Furuita et al. (1996a)
Striped bass	*Morone chrysops* × *Morone saxatilis*	18:3n-3	?	Webster and Lovell (1990)
		n-3 LC-PUFA	< 0.5%	Webster and Lovell (1990)
Striped jack	*Pseudocaranx dentex*	DHA	1.6–2.2	Takeuchi et al. (1996)
		EPA	< 3.1	Takeuchi et al. (1996)
Turbot	*Psetta maxima*	DHA required	?	Reitan et al. (1994)
Yellowtail	*Seriola quinqueradiata*	n-3 LC-PUFA	3.9 (DHA:EPA = 0.5)	Furuita et al. (1996b)
		DHA	1.4–2.6	Furuita et al. (1996b)
		EPA	3.7	Furuita et al. (1996b)

[a]Based on Tocher (2010).

EFA contents optimized for the specific requirements of the developing embryos and larvae (Tandler et al., 1995; Izquierdo et al., 2001; Quintero et al., 2010), and broodstock diets have been shown to affect egg fatty acid compositions such as in Eurasian perch (*Perca fluviatilis*) (Abi-ayad et al., 1997) and Nile tilapia (*Oreochromis niloticus*) (Santiago and Reyes, 1993).

Marine Species

The reported EFA requirements of juvenile and subadult marine fish suggest that C_{18} PUFA cannot satisfy the requirement, and so the n-3 LC-PUFA, EPA, and DHA are required (Table 6-3). Levels of n-3 LC-PUFA of less than or up to 1% of diet dry weight can meet the requirements for juvenile turbot, red sea bream, European sea bass, red drum (*Sciaenops ocellatus*), and Korean rockfish (*Sebastes schlegeli*), whereas levels above 1% appear to be required by silver bream (*Rhabdosargus sarba*), striped jack (*Pseudocaranx dentex*), and yellowtail flounder (*Pleuronectes ferrugineus*). The quantitative requirement for n-6 LC-PUFA has not been fully determined (Bell and Sargent, 2003), but studies suggested ARA was essential in turbot with a requirement of around 0.3% of diet (dry weight) estimated in weaned fish (Castell et al., 1994; Bell et al., 1995a). Quantitative EFA requirements of juvenile sea bream varied with the dietary DHA:EPA ratio, with requirements for total n-3 LC-PUFA of around 1.9 and 0.9% of diet with dietary ratios of 0.5 and 1.0% of DHA:EPA, respectively, consistent with DHA generally having a higher EFA value for fish than EPA (Kalogeropoulos et al., 1992; Watanabe, 1993; Ibeas et al., 1994; Brinkmeyer and Holt, 1998; Wu et al., 2002).

The definition of precise EFA requirements of larval marine fish is complicated by their small size and generally poorly developed digestive system, difficulties of preparing microdiets, and the use of live feeds (Izquierdo et al., 2000; Cahu and Zambonino-Infante, 2001; Koven et al., 2001a; Robin and Vincent, 2003; Kvale et al., 2006; Conceição et al.,

TABLE 6-3 Reported Quantitative Essential Fatty Acid (EFA) Requirements of Juvenile and Subadult Marine Species of Finfish[a]

Species	Scientific Name	EFA	Requirement (% Dry Diet)	Reference
European sea bass	*Dicentrarchus labrax*	n-3 LC-PUFA	1.0	Coutteau et al. (1996a)
Gilthead sea bream	*Sparus aurata*	n-3 LC-PUFA	0.9 (DHA:EPA = 1)	Kalegeropoulos et al. (1992)
		n-3 LC-PUFA	1.9 (DHA:EPA = 0.5)	Ibeas et al. (1994)
		DHA:EPA	0.5	Ibeas et al. (1997)
Grouper	*Epinephelus malabaricus*	n-3 LC-PUFA, DHA > EPA	1.0	Wu et al. (2002)
Japanese flounder	*Paralicthys olivaceus*	n-3 LC-PUFA	1.4	Takeuchi (1997)
Korean rockfish	*Sebastes schlegeli*	n-3 LC-PUFA	0.9	Lee et al. (1993)
		EPA or DHA	1.0	Lee et al. (1994)
Red drum	*Sciaenops ocellatus*	n-3 LC-PUFA	0.5–1.0	Lochman and Gatlin (1993)
		EPA + DHA	0.3–0.6	Lochman and Gatlin (1993)
Red sea bream	*Pagrus major*	n-3 LC-PUFA or EPA	0.5	Yone (1978)
		EPA	1	Takeuchi et al. (1990)
		DHA	0.5	Takeuchi et al. (1990)
Silver bream	*Rhabdosargus sarba*	n-3 LC-PUFA	1.3	Leu et al. (1994)
Starry flounder	*Paralichthys stellatus*	n-3 LC-PUFA	0.9	Lee et al. (2003)
Striped bass	*Morone chrysop* × *Morone saxatilis*	n-3 LC-PUFA	1.0	Gatlin et al. (1994)
Striped jack	*Pseudocaranx dentex*	DHA	1.7	Takeuchi et al. (1992c)
Turbot	*Psetta maxima*	n-3 LC-PUFA	0.8	Gatesoupe et al. (1977)
		ARA	~ 0.3	Castell et al. (1994)
Yellowtail flounder	*Pleuronectes ferrugineus*	n-3 LC-PUFA	2.5	Whalen et al. (1999)
Yellowtail/Kingfish	*Seriola* spp.	n-3 LC-PUFA	2.0–2.4	Deshimaru et al. (1982)

[a]Based on Tocher (2010).

2007, 2010; Yufera and Darias, 2007). However, the quantitative and semiquantitative EFA requirements of larvae of various marine species have been estimated using a combination of enriched live feeds and fabricated microdiets (Table 6-2). The reported values can vary dependent upon the criteria measured, such as survival, growth, and vitality, as well as dietary lipid level (Salhi et al., 1994; Furuita et al., 1996b). Although there are few species where the requirements at larval and juvenile stages can be directly compared, larvae are generally characterized by having a higher requirement than juveniles and preadult fish for n-3 LC-PUFA (Tables 6-2 and 6-3). As with juveniles, EFA requirements in larval marine fish can often be satisfied by a lower level of DHA than can be achieved with EPA (Watanabe, 1993), with the higher efficacy of DHA related to its role in the rapidly developing visual and neural tissues, which account for a relatively greater proportion of total body mass in larval stages (Sargent et al., 2002). Thus, the relative proportions of the different EFA are important in larval marine fish with the absolute requirement for n-3 LC-PUFA decreasing with increasing DHA:EPA ratio (Rodriguez et al., 1994a, 1998a). Growth in larval gilthead sea bream was influenced by ARA (Rodriguez et al., 1994a), and, at a fixed level of dietary n-3 LC-PUFA and DHA:EPA ratio, ARA up to 1.5% and 1% of diet dry weight improved growth in larval sea bream (Bessonart et al., 1999) and Japanese flounder (*Paralichthys olivaceus*), respectively (Estevez et al., 1997). Dietary ARA also improved survival after handling stress in sea bream larvae, particularly when fed prior to the stress (Koven et al., 2001b), whereas high dietary ARA inhibited growth, increased mortality, and had negative effects on pigmentation in yellowtail flounder larvae (Ishizaki et al., 1998).

In recent years, increasing attention has been paid to the role of EFA, particularly ARA, in metamorphosis of marine flatfish including pigmentation and eye migration (Lund et al., 2007, 2008). Decreased n-3 LC-PUFA and increased ARA and ARA:EPA were associated with malpigmentation and impaired eye migration, increasing the focus on dietary DHA:EPA:ARA ratios (Villalta et al., 2005; Hamre and Harboe, 2008a,b). During the premetamorphic stages, there are critical periods when the absolute and relative amounts of EFA and the duration of feeding are particularly important, although these vary among species. In turbot, the early supply of DHA was essential for correct pigmentation (Reitan et al., 1994), and ARA levels in neural tissues were negatively correlated with pigmentation, with the optimum

dietary EPA level being more dependent on dietary ARA than DHA level, emphasizing the importance of dietary DHA:EPA:ARA ratios (Estevez et al., 1999). Pigmentation success was related to dietary levels of ARA and LC-PUFA in neural tissues of Japanese flounder (Estevez and Kanazawa, 1996; Estevez et al., 1997) and dietary ARA in common sole (*Solea solea*) (Lund et al., 2008). Therefore, although there is increasing evidence for the essentiality of dietary ARA for optimal growth and development of marine fish larvae and although precise requirements are not defined, excess can cause problems at metamorphosis in flatfish (Rodriguez et al., 1994a; Ishizaki et al., 1998; Bessonart et al., 1999; Estevez et al., 1999; Hamre et al., 2007; Lund et al., 2007, 2008).

As with freshwater fish, broodstock nutrition is vital in marine fish to produce high-quality eggs and larvae with EFA contents optimized to give the developing embryos and larvae the best chance of success at a time of increased EFA requirement (Tandler et al., 1995; Izquierdo et al., 2001). Many studies have demonstrated that egg fatty acid compositions are affected by broodstock diets in various species including sea bream (Fernandez-Palacios et al., 1995; Almansa et al., 1999), sea bass (Bell et al., 1997), striped jack (Vassallo Agius et al., 1998), Atlantic cod (Silversand et al., 1995), and yellowtail (Verakunpiriya et al., 1996). Egg quality criteria, such as hatching, fertilization rates, and early survival, were positively correlated with increased levels of n-3 LC-PUFA and ARA in eggs of sea bream (Harel et al., 1992; Fernandez-Palacios et al., 1995; Rodriguez et al., 1998b), Atlantic cod (Pickova et al., 1997; Salze et al., 2005), and sea bass (Bruce et al., 1999), and with DHA:EPA ratio in cod (Pickova et al., 1997).

Shrimp

Kanazawa and Teshima (1977) conducted studies with the Kuruma prawn/shrimp *Marsupenaeus japonicus* and demonstrated the inability to synthesize n-3 and n-6 PUFA and LC-PUFA. Over the past 30 years, all investigations with shrimp and other crustaceans have supported these initial studies. Therefore, all crustaceans are reported to have an absolute requirement for specific PUFA and/or LC-PUFA (Table 6-4). Different studies have expressed the EFA requirements of shrimp in different ways, including as a percentage of diet weight, total dietary lipid, or total dietary fatty acids. Some dietary experiments have used different sources of oils with various fatty acid profiles to study responses to various fatty acids in the diet. Other approaches to understanding nutrition and nutritional requirements were approached through the use of pure TAG, or fatty acid methyl or ethyl ester concentrates. For most of the studies conducted with species of juvenile shrimp, TAG sources have been used and the dietary lipid content has commonly ranged between 30 and 75 g/kg diet (3.0 and 7.5%).

Early investigations by Kanazawa et al. (1979a,b) were based upon the use of pure fatty acids in the form of methyl esters in diets containing 18:1n-9 (40 g/kg) and 10 g/kg of either 18:2n-6, 18:3n-3, EPA, and DHA, or 18:1n-9 (50 g/kg) and fed to *M. japonicus*. Weight gains of prawns fed the diets containing either PUFA or LC-PUFA were higher than those fed 18:1n-9 alone. A hierarchy of effectiveness of fatty acids relative to LC-PUFA and PUFA was shown according to the following order: EPA > DHA > 18:3n-3 > 18:2n-6. This work has been supported by other investigations with marine shrimp demonstrating that LC-PUFA, particularly EPA, were more biologically active and elicited significantly higher growth rates than PUFA. Merican and Shim (1997) found that DHA had the highest EFA activity measured as weight gain in the marine tiger shrimp *P. monodon*.

An array of studies by Glencross and coworkers with *P. monodon* supported EFA requirements for n-3 and n-6 PUFA and LC-PUFA (Glencross and Smith, 1999, 2001a,b; Glencross et al., 2002a,b). Glencross and Smith (1999) found that the addition of either 18:2n-6 or 18:3n-3 yielded maximum growth when included at a concentration of 12 g/kg with the overall lipid level being 75 g/kg. They also found that the requirements differed when both EFA were included. Single additions of either EPA or DHA at about 9 g/kg also enhanced weight gain (Glencross and Smith, 2001a). Additional studies confirmed the interactive effects of EFA with requirements for both fatty acids being about 1/3 of what was observed when they were added as exclusive sources. Additional investigations led to an estimate of an ideal n-3 to n-6 ratio of 2.5:1 (Glencross et al., 2002a). This observation supported the results of early experiments that found that best growth responses were elicited by a combination of marine and plant oils, sources of n-3 and n-6 fatty acids, respectively (Deshimaru et al., 1979). Glencross et al. (2002b) also demonstrated that requirements for EFA are based upon the total amount of dietary lipid. Therefore, the proportion of the EFA in the lipid is key to the satisfaction of EFA requirements rather than the absolute level. However, other studies with *L. vannamei* did not indicate a change in the absolute requirement of LC-PUFA with increasing levels of dietary lipid (González-Félix et al., 2002a, 2003). It was also shown that addition of dietary PUFA and LC-PUFA increased weight gain and that the requirement for 18:3n-3 was between 7 and 10 g/kg, and for DHA it was 10 g/kg (Xu et al., 1993, 1994). Kanazawa et al. (1979b,c) also observed the best growth response using a combination of dietary PUFA and LC-PUFA, with the best growth in juvenile *M. japonicus* achieved with either 18:2n-6 or 18:3n-3 added at 10 g/kg in combination with n-3 LC-PUFA derived from pollock residual liver oil and short-necked clam lipids.

D'Abramo and Sheen (1993) examined the qualitative EFA requirements of the caridean shrimp *Macrobrachium rosenbergii*, which spend most of their life cycle in freshwater, by feeding juveniles diets containing pure sources of 18:2n-6, 18:3n-3, ARA, and DHA. Relative to the control diet that contained 60 g/kg of lipid composed of a mixture of