5

Proteins and Amino Acids

Proteins and amino acids are critical molecules because of the role they play in the structure and metabolism of all living organisms. Fish and shrimp cannot synthesize all amino acids and must acquire several in their diet, through the consumption of protein or mixtures of amino acids.

François Magendie (1783–1855), in his textbook *Précis Élémentaire de Physiologie* (1817), was the first to report the importance of the type of nitrogenous compounds in the diet of animals. A century later, in 1914, L. B. Mendel and T. B. Osborne studied the protein requirements of rats and demonstrated nutritional requirements for individual amino acids (Carpenter, 2003). Studies in the early 1940s by W. Rose of the University of Illinois found that 10 amino acids were essential for rats. The removal of any one of these essential amino acids from the diet of growing rats led to profound nutritive failure, accompanied by a rapid decline in weight, loss of appetite, and eventually death (Carpenter, 2003). The first definitive studies on protein and amino acid nutrition of fish were conducted by Halver and collaborators in the late 1950s and early 1960s with Chinook salmon (*Oncorhynchus tshawytscha*). Since this seminal work, hundreds of studies involving a very large number of fish and shrimp species have been carried out, and the body of literature is continuously expanding.

This chapter provides an overview of the nutritional biochemistry of proteins and amino acids and discusses the roles and basis of essentiality of amino acids and some of the factors affecting efficiency of utilization of these nutrients. The results of published studies on quantitative amino acid requirements of a large number of commercially important fish and shrimp species are compiled, and methodological approaches, strategies, and challenges associated with defining and meeting essential amino acid requirements of fish and shrimp are also discussed.

PROTEINS AND AMINO ACIDS: BIOCHEMISTRY, ROLES, AND OVERVIEW OF METABOLISM

Proteins and their building blocks, amino acids, are organic compounds that are essential components of all living organisms. Amino acids can link together by a covalent peptide bond between the α-carboxyl end of one amino acid and the α-amino end of the other (Brody, 1999). Any number of amino acids can be joined by successive peptide linkages, forming a peptide chain. An oligomer consisting of two amino acids is called a dipeptide. Peptides of 2 to around 20 amino acid residues are termed polypeptides. Protein typically contains about 300 amino acids. The polypeptide chains that constitute proteins are linear and contain no branching (Brody, 1999). Amino acids can be linked in varying sequences to form a vast variety of proteins. Proteins are defined by their unique sequence of amino acid residues, which is encoded in the genetic material of the organism. The amino acid sequence is the primary structure of protein. Peptide chains are cross-linked by disulfide bridges, hydrogen bonds, and van der Waals forces that result in the formation of the secondary, tertiary, and quaternary structures of proteins (Buxbaum, 2007).

Proteins have numerous structural and metabolic functions. Proteins, such as actin and tubulin, confer stiffness and rigidity to otherwise fluid biological components (Buxbaum, 2007). Collagen and elastin are critical components of connective tissue, such as cartilage. Other proteins, such as myosin, also have a mechanical function and are capable of generating mechanical forces, such as those exerted by contracting muscles (Buxbaum, 2007). Many proteins are enzymes that catalyze biochemical reactions or transporters that allow the entry and exit of molecules through cells. Some proteins are important in cell signaling, immune responses, cell adhesion, and functioning of the cell cycle (Buxbaum, 2007).

Protein is thus an essential component for every type of cell in the body, including muscles, bones, organs, tendons, and ligaments. Body tissues are continuously being formed and broken down. In growing animals, protein synthesis exceeds degradation, and the balance between these two processes results in protein deposition or accretion (Millward, 1989). Protein deposition appears to be the main determinant of live weight (biomass) gain in fish (Dumas et al., 2007). The close association between live weight gain and protein

mass (Figure 5-1) is due to the close association of water with protein (Figure 5-2). Protein hydration is important for their structure and activity, and proteins help maintain the aqueous intracellular milieu in a "gel" state (Chaplin, 2006). Conversely, lipid deposition does not always appear to contribute substantially to live weight gain because a large proportion of triglycerides is stored in tissues by substituting for water (Dumas et al., 2007). However, other experimental evidence suggests that lipid deposits can significantly contribute to live weight gain of fish, and differences exist among species, strains, life stages, and animals with different nutritional history.

Protein deposition in organisms is dictated by specific templates determined by genetic and epigenetic "codes" of the animal and specific targets determined by endogenous (genetic, life stage) and exogenous (environment, diet) factors. Thousands of different proteins are produced by biological organisms, and each one of these different proteins has a specific structure, function and/or a unique amino acid sequence (Buxbaum, 2007; Finn and Fyhn, 2010)

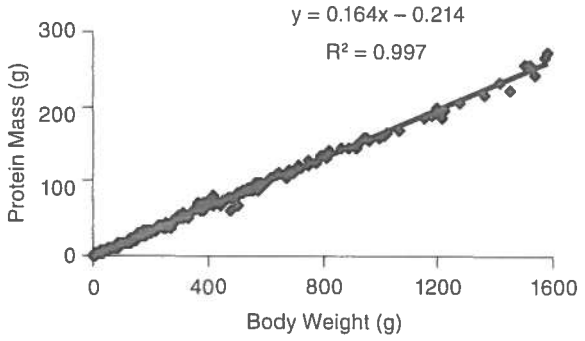

FIGURE 5-1 Relationship between protein mass and live weight of rainbow trout (*Oncorhynchus mykiss*) (Dumas et al., 2007).

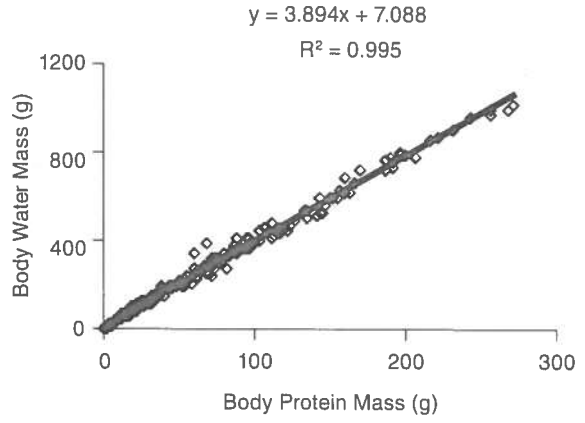

FIGURE 5-2 Relationship between water mass and protein mass of rainbow trout (*Oncorhynchus mykiss*) (Dumas et al., 2007).

(Table 5-1). Different tissues contain different proteins or the same proteins in different proportions. For example, in muscle cells, actin represents about 20% of total protein content, whereas in nonmuscle cells it represents only 5–10%. The amino acid composition of different body proteins also differ significantly. Collagen, a main component of connective tissues, contains only about 3% lysine, whereas myosin and tropomyosin, major components of muscle tissues, contain more than 14% lysine (Pellett and Young, 1984). Interestingly, there are only limited differences in the whole-body amino acid compositions among fish species (Wilson and Cowey, 1985; Kaushik, 1998; Kaushik and Seiliez, 2010). Some differences appear to exist between the whole-body amino acid profiles of shrimp and fish, but overall these differences appear to be minor (Table 5-2). Studies have also shown that the whole-body amino acid composition is minimally affected by body size of fish, at least in juvenile (immature) fish (Kaushik, 1998; Portz and Cyrino, 2003). The relative proportions of the different body components, and proteins these compartments contain, are apparently relatively constant in fish over a wide range of live weight (Dumas et al., 2007).

Amino acids are molecules containing both amine and carboxyl functional groups, with the general formula $H_2NCHRCOOH$, where R is a side chain. The most naturally abundant and metabolically important amino acids are the L-α-amino acids, in which the amino and carboxylate groups are attached to the same carbon atom, called the α-carbon from organic chemistry nomenclature (Brody, 1999). The various α-amino acids differ in which R group, often referred to as the "side-chain," is attached to the α-carbon. The nature and size of the R group can vary from a single hydrogen atom in glycine through a methyl group in alanine to a large heterocyclic group in tryptophan.

The properties of amino acids result from variations in the structures of different R groups, which influence the size, shape, electrical charge, and other characteristics. Amino acids can exist as D- or L-isomers or mixtures of the products. Amino acids are generally found in nature in the L configuration, which are, with a few exceptions, the most biologically active forms. D-amino acids occur in small quantities in certain molecules synthesized by invertebrates and bacteria. Heat-processed ingredients can also contain

TABLE 5-1 Amino Acid Composition of Different Body Proteins of Animals

Protein	Lysine (%)	Leucine (%)	Methionine (%)	Threonine (%)
Collagen	3.0	2.8	0.8	1.8
Myosin	14.6	10.8	3.3	5.4
Actin	7.0	8.2	4.2	7.7
Tropomyosin	18.4	12.1	2.5	3.1
Elastin	0.4	7.2	Traces	0.9

SOURCE: Adapted from Pellett and Young (1984).

TABLE 5-2 Amino Acid Composition (g/16 g N) of Various Fish and Shrimp Species

Amino Acid	Rainbow Trout (*Oncorhynchus mykiss*)	Atlantic Salmon (*Salmo salar*)	Channel Catfish (*Ictalurus punctatus*)	Largemouth Bass (*Micropterus salmoides*)	European Sea Bass (*Dicentrarchus labrax*)	Gilthead Sea Bream (*Sparus auratus*)	Turbot (*Scophthalmus maximus*)	Penaeid Shrimp
Alanine	6.6	6.5	6.3	6.0	6.8	6.8	7.3	5.6
Arginine	6.4	6.6	6.7	8.5	7.5	8.8	7.7	7.4
Asparate	9.9	9.9	9.7	11.8	9.5	9.4	10.3	8.8
Cysteine	0.8	1.0	0.9	0.8	1.0	1.0	1.1	0.8
Glutamate	14.2	14.3	14.4	13.3	15.5	15.1	16.5	16.2
Glycine	7.8	7.4	8.1	7.8	8.1	7.9	9.7	9.0
Histidine	3.0	3.0	2.2	2.1	2.6	2.7	2.5	2.5
Isoleucine	4.3	4.4	4.3	4.0	4.3	4.3	4.3	3.6
Leucine	7.6	7.7	7.4	8.0	7.1	7.3	7.5	6.5
Lysine	8.5	9.3	8.5	8.1	7.9	8.1	8.1	7.8
Methionine	2.9	1.8	2.9	2.6	2.7	3.0	3.4	2.3
Phenylalanine	4.4	4.4	4.1	4.0	4.3	4.7	4.5	3.6
Proline	4.9	4.6	6.0	6.0	5.3	5.3	5.5	8.0
Serine	4.7	4.6	4.9	4.2	4.5	4.5	5.2	3.6
Threonine	4.8	5.0	4.4	4.4	4.4	4.6	4.6	3.8
Tryptophan	1.0	0.9	0.8	0.9	N/A	N/A	N/A	N/A
Tyrosine	3.4	3.5	3.3	2.8	3.9	4.0	4.1	7.5
Valine	5.1	5.1	5.2	4.6	4.7	4.8	4.7	5.1

NOTE: N/A: not available, not reported.
SOURCES: Wilson and Cowey (1985), Kaushik (1998), Portz and Cyrino (2003), Alam (2004), Sara (2007).

D-amino acids because of heat-induced racemization, and chemical synthesis of amino acids result in the production of racemic mixtures of L and D-isomers.

Amino acids are generally represented by three-letter or single-letter abbreviations (Table 5-3) (IUPAC-IUB-JCBN, 1984; Buxbaum, 2007). The three-letter abbreviations are commonly used in animal nutrition, whereas single-letter abbreviations are commonly used in molecular biology and bioinformatics. The convergence of nutritional sciences with molecular genetics and the increasing use of molecular biology techniques and bioinformatics in nutrition studies will likely lead to greater use of single-letter abbreviations in the not too distant future in the animal nutrition literature.

TABLE 5-3 Essential and Nonessential Amino Acids

Essential	Abbreviations	
Arginine	Arg	R
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Threonine	Thr	T
Tryptophan	Trp	W
Valine	Val	V
Nonessential		
Alanine	Ala	A
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine[a]	Cys	C
Glycine	Gly	G
Glutamate	Glu	E
Glutamine	Gln	Q
Proline	Pro	P
Serine	Ser	S
Tyrosine[a]	Tyr	Y

[a]Conditionally essential.

Twenty primary amino acids are used by cells in protein biosynthesis (Table 5-3). Aside from the primary amino acids found in proteins, there are a vast number of other amino acids, formed by posttranslational modification. These modifications are often essential for the function or regulation of a protein; for example, the carboxylation of glutamate allows for better binding of calcium cations, and the hydroxylation of proline is critical for maintaining connective tissues.

Beside their role as the building blocks of protein, amino acids also have a variety of roles in metabolism. Amino acids are important in many other biological molecules, such as forming parts of coenzymes, precursors for the biosynthesis of structural molecules (e.g., heme, chitin, and purine bases), metabolic intermediates (e.g., acetate and pyruvate), and neurotransmitters, hormones, biogenic amines, or numerous other molecules (e.g., serotonin, gamma-aminobutyric acid, melamine, nitric oxide, and histamine) important in the response of the organism to different stimuli. However, the conversion of amino acids to these specific compounds is considered to be quantitatively minor compared to that used for protein synthesis or catabolized by organisms (Cowey and Walton, 1989; Moughan, 1999).

Most microorganisms and plants can biosynthesize all 20 primary amino acids, while animals must obtain some of the

amino acids from their diet. The amino acids that an organism cannot synthesize on its own (or is incapable of synthesizing sufficient amounts) are referred to as "essential amino acids" (EAAs) (Table 5-3). In contrast, the nonessential amino acids (NEAAs) can be synthesized from precursors, for example by addition of an amino group to a tricarboxylic acid (TCA)-cycle intermediate, such as α-ketoglutarate or oxaloacetate (Cowey and Walton, 1989; Zubay, 1993).

The essentiality of various amino acids for fish and shrimp has been determined either by feeding trials involving the successive deletion of each amino acid in the diet or by isotopic-labeling studies (Wilson, 1989). In isotopic-labeling studies, a radio-labeled substrate (e.g., ^{14}C glucose) is injected and the radioisotope label is incorporated into those amino acids that the animal is able to synthesize. All amino acids that have not incorporated ^{14}C are considered to be essential (Coloso and Cruz, 1980; Kanazawa and Teshima, 1981).

Distinction between EAAs and NEAAs also can be made in trials where the individual amino acids are deleted from the diet and growth performance analyzed (Wilson, 1989; Cowey, 1994). The deletion of an EAA would significantly reduce animal growth performance, while a NEAA would not affect growth, suggesting that it could be synthesized by the animal. Nose et al. (1974) used this approach to test the essentiality of 18 amino acids for common carp. They identified that 10 of these amino acids resulted in significant reduction in growth performance after 4 weeks.

It is clear from the available evidence published to date that all fish and shrimp require the same 10 EAAs (Table 5-2) required by most other animals (Ketola, 1982; Wilson, 1989; NRC, 1993). These include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Tyrosine is synthesized from phenylalanine, and cysteine is synthesized from methionine. Therefore, these two amino acids are considered semiessential or conditionally essential. These amino acids are frequently included in estimates of requirements (e.g., total sulfur amino acid requirement, or TSAA). There is growing evidence that some amino acids or related compounds, such as taurine, may be essential or conditionally essential for some, but not all, fish species or for certain life stages in certain species, notably the larval stage of marine fish species.

Along with these 10 EAAs, fish have a nonspecific requirement for a source of amino groups (also known as nonspecific nitrogen) for the synthesis of NEAAs. Most NEAAs, except tyrosine, can be synthesized by simple pathways leading from one of four common metabolic intermediates: pyruvate, oxaloacetate, α-ketoglutarate, or 3-phosphoglycerate (Zubay, 1993). Alanine, asparagine, aspartate, glutamate, and glutamine are synthesized by simple one- or two-step amination reactions from their organic precursors, pyruvate, oxaloacetate, or α-ketoglutarate. Cysteine and glycine are derived from serine, which is in turn synthesized from 3-phosphoglycerate.

An Overview of the Dynamic of Amino Acid Utilization

Ingested proteins are broken down during digestion through hydrolysis to free amino acids, dipeptides, and tripeptides by digestive enzymes secreted into the gastrointestinal tract. These products are absorbed by the mucosal cells where intracellular digestion of small peptides occurs; thus only amino acids appear to be released into the portal vein as products of protein digestion (Murai et al., 1987). Some evidence has shown that small amounts of certain whole proteins may be absorbed through the wall of the gastrointestinal tract (McLean et al., 1999). The amounts involved are not quantitatively significant but this process may be a physiologically important mechanism, possibly for modulation of the immune system through antigen sampling (McLean et al., 1999).

Amino acids supplied by the digestion of dietary protein (exogenous source) or breakdown of body protein (endogenous source) enter the free amino acid pool, also called the metabolic pool (Cowey and Walton, 1989; Kaushik and Seiliez, 2010). From this pool, amino acids can be used for body protein synthesis and as precursors for other substances. Utilization of the amino acids is a function of the metabolic demands of the organism, and the efficiency with which other nutrients absorbed by the animal can be utilized to meet those demands. The metabolism of amino acids is complex and highly integrated with continuous flux within and between cells (Wilson and Cowey, 1985; Cowey and Walton, 1989; Kaushik and Seiliez, 2010). Factors, such as nutrient transport across cell membranes, rate of blood flow, organ uptake, and rates of enzyme activity associated with different biochemical pathways, all interact to control metabolism of amino acids, and they are influenced by complex and sensitive hormonal and neural systems (Cowey and Walton, 1989).

Many factors cause amino acid oxidation. However, a common feature is the imbalance between amino acid supply and amino acid utilization for protein synthesis (Weijs, 1993). The principal endproducts of amino acid catabolism are ammonia, carbon dioxide, and bicarbonate. Ammonia is highly toxic and in order to prevent toxicity, higher vertebrates convert ammonia to urea for excretion in the urine. Fish and crustaceans have an extremely efficient transfer mechanism for ammonia across the gills; thus, they have no need to expend energy converting ammonia into urea (Cowey and Walton, 1989). The great majority of fish and shrimp excrete more than 80% of their nitrogenous wastes in the form of ammonia (Kaushik and Cowey, 1991; Mambrini and Guillaume, 1999). Gills are the main excretory organs, accounting for more than 75–90% of total nitrogen excretion (Cowey and Walton, 1989; Kaushik and Cowey, 1991).

The breakdown of amino acids generally occurs in two steps. The first is generally deamination and comprises the removal of the amino group, which is either converted to ammonia or transferred to become the amino group of a

glutamic acid molecule (Cowey and Walton, 1989; Zubay, 1993). The second stage is the conversion of the carbon skeletons (the α-keto acids produced by deamination) to citric acid cycle intermediates (Cowey and Walton, 1989; Zubay, 1993). The carbon backbone of amino acids contain usuable (free) energy that can be harnessed by the TCA cycle or converted into fatty acids and/or glycogen by the animal for future use (Cowey and Walton, 1989). Different amino acids have different carbon skeletons, and their conversions to citric acid intermediates follow correspondingly diverse pathways, which can be grouped according to the intermediate at which they enter the citric acid cycle (Cowey and Walton, 1989). In this respect, amino acids fall into three categories: glucogenic, ketogenic, or glucogenic and ketogenic. Glucogenic amino acids are those that give rise to a net production of pyruvate or TCA-cycle intermediates, such as α-ketoglutarate or oxaloacetate, all of which are precursors of glucose via gluconeogenesis (Guillaume et al., 1999). All amino acids except lysine and leucine are at least partly glucogenic (Cowey and Walton, 1989). Lysine and leucine are the only amino acids that are solely ketogenic, giving rise only to acetyl-CoA or acetoacetyl-CoA, neither of which can bring about net glucose production, although they can be used for ketone body synthesis. Their pathways are similar in the final steps and resemble the steps in the β-oxidation of fatty acids (Cowey and Walton, 1989). Isoleucine, phenylalanine, threonine, tryptophan, and tyrosine give rise to both glucose and fatty acid precursors and are thus characterized as being both glucogenic and ketogenic (Cowey and Walton, 1989).

Evidence suggests that invertebrates and lower vertebrates, including teleost fish, have a better ability to utilize D-isomers of amino acids than do mammals (Cowey and Walton, 1989; Deng et al., 2010). Specific D-amino acid transaminase (D-AAO) may catalyse deamination of D-isomers to α-keto acids, which, in turn, can be reaminated to the natural L-form (Deng et al., 2010). Alternatively, racemases and epimerases can convert D-isomers to racemic mixtures. Significant activity of these enzymatic processes appear to be induced in invertebrates and fish tissues upon feeding D-isomers of amino acids (Deng et al., 2010).

Utilization of amino acids is affected by numerous factors, such as diet composition, chemical form of the amino acids supplied, and a number of biological factors (including species and life stage). In light of the complexity of this issue, the main biological processes that determine efficiency of EAA utilization for body protein deposition (PD) are frequently described (or estimated) in a factorial (or categorized) fashion. Factorial models have been extensively used in poultry and swine nutrition for about four decades and take various forms (D'Mello, 2003; Moughan, 2003).

In the most commonly used framework, EAA utilization is explicitly represented in terms of deposition as body protein, maintenance requirement, inevitable catabolism, preferential catabolism for energy use, and catabolism associated with intakes exceeding requirement (Moughan, 2003). This type of factorial framework has been used to predict EAA requirement of fish (e.g., Hauler and Carter, 2001b; Bodin et al., 2008) and, although it has a number of limitations when applied to fish (reviewed by Bureau and Encarnação, 2006), it provides a relatively straightforward framework of describing some of the determinants of amino acid catabolism and/or retention.

Amino Acid Deposition as Body Protein

A review of the literature indicates that for a large majority of fish and shrimp species fed high-quality diets, deposition of amino acids into body protein represents between 25 and 55% of total amino acids consumed. The deposition of protein is consequently a major determinant of amino acid utilization and requirements of fish and shrimp (Cowey and Walton, 1989).

Because there is a very strong association between live weight gain and PD (Shearer, 1994; Dumas et al., 2007), there also is a very close association between live weight gain and amino acid requirements in absolute terms (g/fish per day). The EAA needs for protein accretion correspond to the amino acid content of tissue protein gain (Kaushik and Seiliez, 2010).

Maintenance Amino Acid Requirements

Maintenance amino acid requirement is defined as the amount of dietary amino acid required to maintain the protein pool of the animal in equilibrium. Amino acid needs for maintenance include a certain amount for loss of endogenous gut proteins, mucins, and other secretions. Amino acids are also required as precursors for various metabolites, neurotransmitters, hormones, cofactors, and the like. Maintenance requirements of certain amino acids may account for a greater proportion of total requirement (maintenance + growth) because they can be involved in a wide variety of other metabolic reactions beside protein synthesis or be subject to significant endogenous losses (Rodehutscord et al., 1997; Nichols and Bertolo, 2008).

Estimate of maintenance requirement for amino acids are generally obtained by linear regression. Diets containing graded levels of protein and essential amino acids are fed, and protein (N) gain is monitored (Rodehutscord et al., 1997; Richard et al., 2010). The intake of amino acid resulting in no net protein (N) gain in the animal is assumed to be the maintenance requirement of the animal.

Rodehutscord et al. (1997) estimated the maintenance EAA requirement of rainbow trout (live weight = 50 g/fish) to be as follows (in mg per $kg^{0.75}$ per day): lysine, 4; tryptophan, 2; histidine, 2; valine, 5; leucine, 16, and isoleucine, 2. Bodin et al. (2009) obtained a significantly higher estimate

of maintenance lysine requirement (24 mg per $kg^{0.75}$ per day) for rainbow trout. Rollin et al. (2006) and Abboudi et al. (2007) estimated the maintenance Thr requirement of Atlantic salmon fry (live weight = 1–2 g/fish) to be between 5 to 7 mg per $kg^{0.75}$ per day. Richard et al. (2010) estimated maintenance lysine and methionine requirement of *Penaeus monodon* (2 g live weight) to be about 40 and 20 mg per $kg^{0.75}$ per day.

Studies have suggested that the minimum maintenance requirement EAA pattern is quite different from the patterns of EAA in tissue proteins (Rodehutscord et al., 1997). Significant differences between species may also exist. Based on data from Fournier et al. (2003), Kaushik and Seiliez (2010) concluded that there are significant quantitative and qualitative differences in endogenous amino acid losses between rainbow trout and turbot.

Estimates of maintenance requirements should be viewed with a healthy degree of skepticism. The confidence intervals for estimates of maintenance derived from regression analyses are generally not provided but are likely very broad. Amino acid composition of protein gain can also be variable in fish fed diet deficient in EAA (Encarnação et al., 2004), which suggests that N gain may not be an adequate indicator of the maintenance requirement of individual amino acids. In addition, composition of the diet can affect catabolism of some EAA even when the diet is highly deficient in this amino acid (Encarnação et al., 2004, 2006). Finally, there may be significant differences in the nutrient partitioning of animals fed diets highly deficient in EAA compared to fish fed a nutritionally adequate diet. The relevance of "maintenance requirement" to fast-growing animals has often been questioned in the animal nutrition literature (Baldwin and Bywater, 1984; Bureau et al., 2002).

Overall, the maintenance amino acid requirement of domesticated fish and shrimp represents a small proportion (generally between 5 to 20%) of their total amino acid requirement (Rodehutscord et al., 1997; Abboudi et al., 2007, 2009; Richard et al., 2010). The relative contributions of maintenance to the total amino acid needs is likely greater in slow-growing than in fast-growing animals.

Inevitable Amino Acid Catabolism

Inevitable catabolism is defined as the degradation of amino acids through active catabolic pathways, which still occurs when energy supply is not limiting for protein synthesis (Moughan, 1995). This degradation appears to be an "inevitable" consequence of the presence of catabolic systems in cells that cannot be completely inactivated. As a result, a fraction of any absorbed amino acid is catabolized, even when the intake of this amino acid is below requirements for maximum protein deposition (Moughan, 1995; de Lange et al., 2001). This inevitable amino acid catabolism affects the marginal efficiency of utilization of amino acid for PD when amino acid intake is below requirements to achieve maximum protein deposition (PD_{max}). Expressed as a fraction of available amino acid intake, the rate of inevitable amino acid catabolism is assumed to be constant when amino acid intake is between about 70 and 100% of that required to achieve PD_{max} (de Lange et al., 2001; Moehn et al., 2004). Gahl et al. (1995), however, indicated a decrease in efficiency of utilization as limiting EAA intakes increased above maintenance (diminishing returns). Studies in which efficiencies of EAA utilization have been compared suggest that the efficiencies are different for each EAA (Fuller, 1994; Adeola, 1995; Gahl et al., 1996).

Based on a metaanalysis of data from different studies, Hauler and Carter (2001b) observed that lysine utilization for lysine gain (lysine retention/lysine intake) was constant despite differences in dietary lysine concentrations and lysine intakes by the fish. Based on this observation, Hauler and Carter (2001b) suggested that lysine utilization remains constant at marginal lysine intakes over different dietary formulations and life stages. Rodehutscord et al. (2000), Hauler and Carter (2001a), Encarnação et al. (2004, 2006), and Bodin et al. (2009) reported a maximal marginal efficiency of lysine retention of 71 and 78% for rainbow trout and Atlantic salmon, respectively. These observations suggest that inevitable catabolism represents 20 to 30% of digestible lysine consumed above maintenance in these fish species. Efficiency of lysine utilization appears to remain constant at marginal lysine intake over different dietary formulations and life stages in Atlantic salmon (Hauler and Carter, 2001a). There is a general lack of specific data for other amino acids and other fish species. However, based on available evidence, inevitable catabolism can in practice be estimated at about 20 to 40% of digestible amino acid consumed by animals above maintenance requirement.

The results of Encarnação et al. (2004, 2006) and Encarnação (2005), however, provided evidence that, at marginal levels of intake the efficiency of lysine utilization for PD is not constant, but is affected by dietary lysine concentration and intake of digestible energy (DE) supplied as lipids, but not DE supplied by other energy-yielding nutrients, such as amino acids (e.g., mixture of NEAA, leucine). Figure 5-3 illustrates the effect of DE content of the diet on efficiency of lysine utilization in rainbow trout (Encarnação et al., 2004). Studies also showed that the source of dietary amino acids (protein bound vs. free amino acids) (Tantikitti and March, 1995; Zarate and Lovell, 1997; Williams et al., 2001; El Haroun and Bureau, 2006) and the feed ingredient matrix (Nang Thu et al., 2007) can also affect efficiency of EAA utilization for PD. There is still considerable debate about the rate of inevitable catabolism and the factors affecting it, both in terrestrial animals (Moughan, 2003) and fish (Bureau and Encarnação, 2006). Compared to other nonruminant animals, fish seem to have a more "elastic" inevitable EAA catabolism, and the border between "inevitable" and "preferential" catabolism may be blurred in these animals (Bureau and Encarnação, 2006).

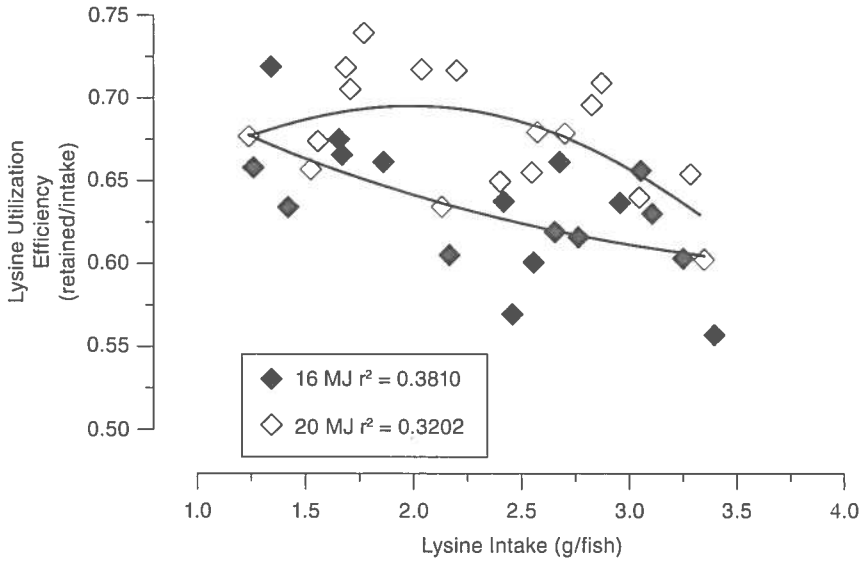

FIGURE 5-3 Effect of lysine and DE content of the diet (3.82 vs. 4.78 Mcal DE or 16 or 20 MJ DE/kg) on efficiency of lysine utilization of rainbow trout (*Oncorhynchus mykiss*) (Encarnação et al., 2004).

Preferential Catabolism

Preferential catabolism refers to catabolism of amino acids to provide energy, when dietary energy intake is limiting PD, which implies that the animal is portioning EAA away from protein synthesis toward catabolism to meet a specific metabolic need (Moughan, 2003). Distinction between preferential and inevitable catabolism can then be derived from the slope of the relationship between metabolizable energy (ME) intake and PD (Mohn and de Lange, 1998).

In fish, where amino acids appear to provide a significant proportion of the total energy (ATP) requirement (Ronnestad et al., 1999, 2003; Finn et al., 2002; Kaushik and Seiliez, 2010), inevitable and preferential catabolism may be difficult to separate. It is not clear to what extent the significant catabolism of amino acids despite adequate ME and net energy (NE) intakes (Encarnação et al., 2006) is related to inevitable losses (maintenance requirement, inevitable catabolism) of amino acids or catabolism of amino acids that are in excess of requirements, or preferential catabolism of amino acid as energy sources. The determinants of amino acid catabolism in fish deserve to be studied more systematically than it has been the case in the past.

Catabolism of Excess Amino Acids

Intake of amino acids in excess of the amounts required for protein deposition, maintenance requirements, inevitable catabolism, and preferential catabolism will result in additional catabolism of these amino acids. Feeding a diet in which the amino acid profile is deficient in one or multiple amino acids compared to dietary requirements will limit protein deposition, limit the retention of the other amino acids, and force their deamination and catabolism.

ESSENTIAL AMINO ACIDS—BIOCHEMISTRY, ROLES, AND DEFICIENCY SIGNS

Lysine

Lysine (abbreviated as Lys or K) is an α-amino acid with the chemical formula $HO_2CCH(NH_2)(CH_2)_4NH_2$. Lysine contains two amino groups with the ε-amino group and is an amino acid that often participates in hydrogen bonding and as a general base in catalysis. Common posttranslational modifications of lysine include methylation of the ε-amino group, giving methyl-, dimethyl-, and trimethyllysine as well as acetylation (Buxbaum, 2007). Collagen contains hydroxylysine, which is derived from lysine by lysyl hydroxylase (Sassi, 2001). Allysine and hydroxyallysine are produced by the actions of the enzyme lysyl oxidase on lysine and hydrolysine in the extracellular matrix. These molecules are essential in the crosslink formation that stabilizes the structure of collagen (Sassi, 2001).

Lysine is abundant in body protein of fish and other animals. Lysine is found in high concentration in ingredients such as fish meal and blood meal and in low concentrations in some plant protein ingredients, notably cereal grain byproducts, such as corn gluten meal and wheat gluten. Due to the reactive nature of its ε-amino group, lysine is sensitive to heat damage and to nonenzymatic glycolyzation reactions resulting in production of Maillard reaction products (Moughan

and Rutherfurd, 1996). These result in irreversible chemical damage to lysine and reduce the amount of available lysine in feedstuffs and feeds (Carpenter, 1960; Moughan and Rutherfurd, 1996). Consequently, lysine is commonly the first limiting amino acid in feeds, particularly those formulated with high levels of plant protein ingredients or with protein ingredients processed under harsh conditions.

Beside reduced growth and feed efficiency, lysine deficiency has been shown to cause some health issues such as dorsal and caudal fin erosion in rainbow trout and common carp (Ketola, 1983; Guillaume et al., 1999).

Sulfur Amino Acids and Related Molecules

Methionine (abbreviated as Met or M) and cysteine (abbreviated Cys or C) are two sulfur-containing amino acids. Methionine is a nonpolar amino acid. Like other hydrophobic amino acids, it can play a role in binding/recognition of hydrophobic ligands such as lipids (Brosnan and Brosnan, 2006b). With a thiol side chain, cysteine is classified as a hydrophilic amino acid. The high reactivity of this thiol means cysteine is an important structural and functional component of many proteins and enzymes, forming disulphide bridges (dimers) in some proteins, which are important in folding the protein chain—RSH + RSH → RS-RS (Brosnan and Brosnan, 2006b; Buxbaum, 2007). Cysteine is readily oxidized to cystine (Cys-Cys) in the environment and is promptly reduced to the two cysteine molecules by the organism.

In addition to its role as a precursor in protein synthesis, L-methionine participates in a wide range of other metabolic reactions including the production of S-adenosylmethionine (SAM) (Baker, 2006), L-cysteine, glutathione, taurine, sulphate, phosphatidylcholine, and other phospholipids (Brosnan and Brosnan, 2006b). SAM itself is involved in the synthesis of creatine, epinephrine, melatonin, and the polyamines spermine and spermidine, among several other substances (Brosnan and Brosnan, 2006b). L-methionine is also a glycogenic amino acid and may participate in the formation of D-glucose and glycogen.

Methionine is converted to SAM by methionine adenosyltransferase. SAM serves as a methyl-donor in many methyltransferase reactions and is converted to S-adenosylhomocysteine (SAH). SAM, a remarkably versatile molecule, is said to be second, only to ATP, in the number of enzymes that require it (Brosnan and Brosnan, 2006b). Adenosylhomocysteinase converts SAH to homocysteine, which can be used to regenerate methionine or to form cysteine. Methionine can be regenerated from homocysteine via methionine synthase (Brosnan and Brosnan, 2006b). It can also be remethylated using glycine betaine (NNN-trimethyl glycine) to methionine via the enzyme betaine-homocysteine methyltransferase (BHMT). To a certain extent, betaine and choline can both help spare methionine in terms of the methyl donor function (Baker, 2006). Homocysteine can also be converted to cysteine. Cystathionine-β-synthase combines homocysteine and serine to produce cystathionine. Instead of degrading cystathionine via cystathionine-β-lyase, as in the biosynthetic pathway, cystathionine is broken down to cysteine and α-ketobutyrate via cystathionine-γ-lyase (Brosnan and Brosnan, 2006b). The enzyme α-ketoacid dehydrogenase converts α-ketobutyrate to propionyl-CoA, which is metabolized to succinyl-CoA (Brosnan and Brosnan, 2006b).

Walton et al. (1982) demonstrated a reduction in the methionine requirement in rainbow trout as dietary cysteine increased from 0 to 2% of the diet. These results have subsequently been supported by observations by Rumsey et al. (1983) and Cowey et al. (1992) and with numerous other fish species (NRC, 1993). When cystine is included in the diet, the need for methionine is reduced because cystine replaces methionine in the synthesis of cysteine and its derivatives. Cysteine is incapable of meeting the entire methionine requirement because the irreversibility of the cystathionine synthase reaction prevents cysteine from being converted back to homocysteine and serine (Brosnan and Brosnan, 2006b).

The conversion of methionine into cysteine complicates the estimation of a precise methionine requirement as the level of dietary cysteine will vary the amount of dietary methionine required by the fish. Some researchers have therefore expressed the requirement as a single total sulfur amino acid (TSAA) requirement, or as a "Met + Cys" requirement. Estimates of the TSAA requirement of rainbow trout are between 0.8 and 1.1% of the diet (Rumsey et al., 1983; Cowey et al., 1992). Dietary cysteine is estimated to have replacement values for methionine of 40 to 60% for various species of fish (Wilson, 2002). Estimates include 60% replacement value for channel catfish (Harding et al., 1977), 44% for blue tilapia (Liou, 1989), 42% for rainbow trout (Kim et al., 1992a), and 40% for red drum and hybrid striped bass (Moon and Gatlin, 1991; Griffin et al., 1992). Available evidence suggests that cysteine can provide almost half of the TSAA needs for protein accretion in several fish species that have been evaluated (NRC, 1993; Goff and Gatlin, 2004). Some studies have suggested that no more than 3 g/kg of dietary cysteine (0.3%) is an effective source of sulfur amino acids in fish, meaning that dietary cysteine in excess of this amount has no methionine sparing effect (Kim et al., 1992a; Pack et al., 1995). It is recommended that diets be formulated with separate methionine and cysteine requirements to ensure that the animal needs are effectively met (Rodehutscord et al., 1995a). In practice, many feedstuffs contain an excess of cysteine to methionine, which allow nutritionists to focus mainly on meeting the methionine requirement.

As in the case for other essential nutrients, feeding diets with low methionine content results in poor growth and feed efficiency. In addition, salmonids, including rainbow trout, Atlantic salmon (*Salmo salar*), and lake trout (*Salvelinus namaycush*), also suffer from cataracts when given a diet deficient in methionine (Poston et al., 1977). The lens begins

to become opaque after 2 to 3 months, depending on the extent of the deficiency. Riboflavin, thiamine, vitamin A, zinc, tryptophan, and, more recently, histidine deficiencies have also been implicated in the development of ocular pathologies in fish (Poston et al., 1977; Simmons et al., 1999; Waagbo, 2010).

The mechanism linking methionine deficiency to the development of cataract is not completely understood. However, it is likely related to the role of methionine in the formation of glutathione. Oxidative damage is known to occur to protein-bound thiol groups in the lens of older mammals. Glutathione, which requires methionine or N-acetylcysteine for synthesis (Ferrer et al., 1990), probably has a role in protecting the lens from such damage (Cowey et al., 1992). Low dietary methionine supply might prevent the turnover of oxidized methionine molecules in the lens, leading to the formation of disulfide bonds and the development of ocular opacity (Simmons et al., 1999).

Methionine is the most toxic of the primary amino acids. Dietary levels in excess of two or three times the requirement level has been shown to affect growth in mammals (Edmonds and Baker, 1987; Baker, 2006). Growth of rainbow trout also seems to be negatively affected by very high methionine intake (Poppi et al., 2010). Methionine toxicity is thought to be due to hepatic accumulation of SAM (Regina et al., 1993). Alleviation of the effects of methionine toxicity can be achieved in rats with supplemental glycine and serine. Glycine facilitates the catabolism of excess SAM by the glycine-N-methyltransferase enzyme (Baker, 2006). Serine is combined with homecysteine in the transulfuration pathway to form cystathionine and then cysteine. This pathway can also eventually produce taurine, which can be easily excreted by the animal. Yokoyama and Nakazoa (1992) found elevated hepatic concentrations of cystathionine and reduced hepatic levels of serine when diets containing excess methionine were fed to rainbow trout. They postulated that cystathionine synthesis via the transulfuration pathway proceeds more rapidly than cysteine biosynthesis from cystathionine in this species.

Methionine Isomers

Large quantities of methionine are produced industrially by chemical synthesis for use in animal feeds. The chemical synthesis process produces a racemic mixture of D- and L-isomers of methionine (DL-Met). Most animals utilize D- and L-isomers of methionine with similar efficiencies (Baker, 2006). Utilization of D-methionine requires its deamination by D-amino acid oxidase and subsequently reamination to L-methionine. Rainbow trout can use D-methionine to replace L-methionine on an equimolar basis (Kim et al., 1992a). This metabolic capacity is probably also characteristic of other fish as well as crustacean species (Guillaume et al., 1999).

Methionine Hydroxy Analog

An hydroxy analog of methionine, 2-hydroxy-4-(methylthio)butanoic acid (HMB or OH-Met), also known as DL-methionine-hydroxyanalog (MHA), is commercially available and has found widespread use in animal feeds (Baker, 2006). The HMB differs from methionine by having a hydroxyl group on the alpha carbon rather than an amino group (Dibner, 2003). Like synthetic DL-met, HMB has one asymmetrical carbon atom and therefore occurs as a racemic mixture of L-isomer and D-isomer (Baker, 2006). Because HMB bears a hydroxyl group instead of an amino group, it is an organic acid (Dibner, 2003). Uptake of DL-met and HMB across the brush border membrane takes place by two different transport systems (Brachet and Puigserver, 1987; Richards et al., 2005). Methionine is transported by the system B amino acid transporter, whereas MHA-FA is transported via an H^+-dependent transporter, Monocarboxylate Transporter 1, which is also involved in the transport of lactic acid (Martın-Venegas et al., 2007). Upon absorption by the animal, HMB is rapidly converted to L-met in the liver using two different enzymes for D- and L-isomers, a dehydrogenase for the D-isomer and an oxidase for the L-isomer (Baker, 2006).

The biological efficiency of various forms of HMB, including liquid free acid form and crystalline calcium salt of HMB has been the focus of numerous trials with different terrestrial livestock species. There has been considerable debate over the biological efficacity of HMB in comparison to DL-met (Jansman et al., 2003). Various metaanalyses of data from a large number of trials with poultry have suggested that the relative biological efficiency of HMB in comparison with DL-met is about 75 to 80% on an equimolar basis (Jansman et al., 2003; Baker, 2006; Sauer et al., 2008). The results of studies carried out with different fish species (Robinson et al., 1978; Keembiyehetty and Gatlin, 1995, 1997a; Cheng et al., 2003a; Goff and Gatlin, 2004; Kelly et al., 2006) also support the notion that HMB can be used as a source of methionine by fish but that its relative biological efficiency is lower than that of DL-met on an equimolar basis. On the basis of available experimental evidence, the committee considers it reasonable to assume that the biological efficacy of HMB for fish is about 75 to 80% that of DL-met on an equimolar basis. The difference in biological efficiency between HMB and DL-met may be related to the differences in the absorption dynamic of these two compounds, a phenomenon that has been well documented in poultry (Drew et al., 2003) but has yet to be examined in fish and crustaceans.

Taurine

Taurine, or 2-aminoethanesulfonic acid, is an organic acid. It is also a major constituent of bile and can be found in the lower intestine and in small amounts in the tissues of animals. Taurine is a derivative of cysteine, and it is synthe-

sized by the transsulfuration pathway (Goto et al., 2002). Taurine is often referred to as an amino acid in the literature although strictly speaking, it is not an amino acid because it lacks a carboxyl group.

Functional roles of taurine include conjugation of bile acids, osmoregulation, putative neurotransmitter function, cell membrane stabilization, and antioxidant effects (Lombardini et al., 1979; Gaull and Wright, 1986; Huxtable, 1992). Small polypeptides have been identified that contain taurine, but to date no aminoacyl tRNA synthetase has been identified as specifically recognizing taurine and capable of incorporating it into a protein.

Taurine appears to be a major contributor to osmotic pressure balance in some animals. It can be found at levels as high as the total pool of free amino acids in marine fish and invertebrates. Studies suggest significant interspecific differences in both the pathway and capacity of taurine biosynthesis in fish (Goto et al., 2002; Kim et al., 2008). Taurine biosynthesis appears to be very low in some species (Tagaki et al., 2005). A relatively large number of studies suggest that taurine may be conditionally essential for some juvenile marine fish and shrimp species, such as Japanese flounder (*Paralichtys olivaceus*) (Park et al., 2002; Kim et al., 2003, 2005a,b), European sea bass (Martinez et al., 2004), red sea bream (*Pagrus major*) (Goto et al., 2001), yellowtail (*Seriola quinqueradiata*) (Matsunari et al., 2005; Tagaki et al., 2005; Kim et al., 2008), cobia (*Rachycentron canadum*) (Lunger et al., 2007), and black tiger shrimp (*Penaeus monodon*) (Shiau and Chou, 1994), but not for common carp (*Cyprinus carpio*) and Atlantic salmon (Espe et al., 2008). One study reported a positive effect of taurine supplementation on growth of rainbow trout (Gaylord et al., 2006). Evidence suggests that taurine may be conditionally essential in freshwater fish during early life stages (e.g., larval stage) (Zhang et al., 2006). Taurine supplementation effectively reduced the severity of green liver disease in yellowtail fed a diet devoid of fish meal and containing less than 0.1% taurine (Tagaki et al., 2005). This suggests that taurine deficiency might be at the origin of this nutritional disease. Differences in the rate of synthesis of taurine from cysteine and metabolic and physiological demands for taurine may explain differences observed between species, life stages, and studies. Relatively few studies have examined the effect of graded levels of taurine on growth and health of different fish species reared in different environmental conditions and fed diets of different compositions. This makes it difficult to determine the taurine requirement of different species.

Selenocysteine and Selenomethionine

Selenocysteine (abbreviated as Sec or U) is an amino acid that is present in several enzymes, notably glutathione peroxidases, tetraiodothyronine 5' deiodinases, thioredoxin reductases, formate dehydrogenases, glycine reductases, and some hydrogenases. It is unique among amino acids because it is the only one synthesized directly on a tRNA (Ganichkin et al., 2008; Castellano et al., 2009). Selenocysteine is structurally similar to cysteine except the sulfur in cysteine is replaced with selenium, making the side chain-CH2-SeH. However, selenocysteine is not synthesized from Cys, but rather from serine (sidechain-CH2-OH), covalently linked to tRNA, by the replacement of oxygen with Se. Selenium is converted to selenophosphate, a high-energy molecule, at the cost of one ATP molecule, which then reacts with serine to form selenocysteinyl-tRNA (Ganichkin et al., 2008). Overall, Sec and Cys residues do not seem to be functionally exchangeable in proteins, suggesting that selenoproteins have important specific roles in the metabolism of vertebrates (Castellano et al., 2009). Proteins containing Sec (selenoproteins) apparently account for the essentiality of Se to vertebrates and have an important role in several pathologies associated with Se deficiency (Behne and Kyriakopoulos, 2001; Castellano et al., 2009). In mammals, there are 19 selenoproteins with known functions, and all of them are enzymes (Behne and Kyriakopoulos, 2001). The glutathione peroxidase (GPx) family of selenoproteins catalyzes the reduction of lipid and hydrogen peroxides to lipid alcohols and water, respectively, with glutathione as a reductant (Miranda et al., 2009). The GPx family is an essential component of the antioxidative system protecting membrane lipids and macromolecules from oxidative damage (Miranda et al., 2009).

Selenomethionine (Se-met and Sem) is another L-amino acid containing selenium. L-Se-met is synthesized by plants, marine algae, and yeast along with Met in quantities depending on the amount of Se available (Schrauzer, 2000). Selenomethionine was detected in organs in their intact form, suggesting that selenoamino acids are absorbed and delivered to organs in their intact forms, at least in mammals (Suzuki et al., 2006). There is no available evidence suggesting that Se-met is an essential nutrient. However, it seems to be a highly bioavailable source of Se. In vivo, Sem is randomly incorporated instead of methionine in protein The replacement of Met by Se-met does not significantly alter protein structure, but may influence the activity of enzymes if Se-met replaces Met in the vicinity of the active site (Scrauzer, 2000). Selenomethionine in protein is readily oxidized to Se-met oxide, which is easily reduced back to Se-met by glutathione (GSH). For Se-met to stimulate synthesis of selenoproteins such as GPx, the Se must be released by enzymatic degradation and converted to selenophosphate, which is the substrate for cotranslational Sec synthesis (Allmang and Krol, 2006; Miranda et al., 2009).

Branched-Chain Amino Acids: Leucine, Isoleucine, and Valine

Branched-chain amino acids (BCAAs) refer to the three amino acids (leucine, isoleucine, and valine) having aliphatic

side chains that are nonlinear. Leucine (abbreviated as Leu or L) is an α-amino acid with an isobutyl R group. Isoleucine (abbreviated as Ile or I) has a sec-butyl side chain that is a large aliphatic hydrophobic chiral side chain. Four stereoisomers of isoleucine are possible although, in nature, only one enantiomeric form, (2S,3S)-2-amino-3-methylpentanoic acid, exists. Valine (abbreviated as Val or V) has an isopropyl side chain.

The key property of the three BCAAs is their hydrophobicity. Therefore, in proteins, these amino acids are largely excluded from aqueous environments, but they interact well with other hydrophobic molecules (Brosnan and Brosnan, 2006a). They are largely found in the hydrophobic interior core of globular proteins where their interactions with other similar amino acids play a key role in determining the three-dimensional shapes of these proteins and, hence, their functions (Brosnan and Brosnan, 2006a). These three EAAs play important structural roles and are primarily deposited in body protein, notably in skeletal muscles (Cowey and Walton, 1989; Brosnan and Brosnan, 2006a). Valine is also involved in the synthesis of the myelin covering of the nerves, and valine deficiency can cause degenerative neurological conditions in mammals. Because of their critical roles in the protein structure, most proteins have a relatively high proportion of BCAAs, and these represent a significant proportion of amino acids consumed by animals.

The increase in circulating BCAAs that occurs after a protein-containing meal is "sensed" by a number of different tissues and has important effects in these tissues (Yang et al., 2008). Thus, the BCAAs serve as important signals to other tissues; among the tissues that respond to BCAA concentrations are brain and skeletal muscle (Brosnan and Brosnan, 2006a). Leucine is increasingly recognized as an anabolic nutrient signal, communicating the presence of an ingested protein-containing meal to peripheral tissues, and stimulating insulin secretion by the β-cells of the pancreas and protein synthesis in muscle and adipose tissue through the target of rapamycin signalling pathway (Yang et al., 2008).

The metabolism of BCAAs differs from that of the other amino acids in three important respects. First, rather than being restricted to the liver as for most EAAs, the catabolic enzymes for BCAAs are distributed widely in body tissues, including the kidney, muscle, and even the central nervous system (Cowey and Walton, 1989; Brosnan and Brosnan, 2006a). Second, all three BCAAs share the same common transporter for intestinal absorption. Finally, the first steps in the oxidation of each of these three amino acids are catalyzed by two common enzymes, and so the organism metabolizes these three amino acids using the same enzymatic system (Cowey and Walton, 1989; Brosnan and Brosnan, 2006a). The first step in BCAA catabolism is transamination catalyzed by BCAT (branched-chain aminotransferase) isozymes. In this reaction, the amino group is transferred from a BCAA to α-ketoglutarate to form glutamate and the respective branched-chain α-keto acid (BCKA). The keto acid products are irreversibly oxidized by the second enzyme in the catabolic pathway, the mitochondrial BCKA dehydrogenase enzyme complex (Brosnan and Brosnan, 2006a).

Antagonisms Among Branched-Chain Amino Acids

Interactions between the BCAAs, leucine, isoleucine, and valine are known to produce antagonistic effects in chicks, pigs, rats, and humans (D'Mello, 1994). Reduction of plasma isoleucine and valine concentration after consumption of an excessive amount of leucine has been reported in rats, chicks, pigs, and humans (Block and Harper, 1991; Langer et al., 2000). Leucine-induced changes in plasma levels of isoleucine and valine have mainly been attributed to competitive inhibition during intestinal absorption and increased oxidation through BCKA dehydrogenase activation (Block and Harper, 1991; D'Mello, 1994; Langer et al., 2000).

In fish, antagonism involving BCAAs have not been fully assessed, and the results obtained have shown some inconsistencies. No effect of excess leucine on the other BCAAs was observed by Robinson et al. (1984), Choo et al. (1991), and Rodehutscord et al. (1997). However, Chance et al. (1964) observed that the isoleucine requirement of Chinook salmon (*Oncorhynchus tshawytscha*) increased slightly with increasing concentrations of dietary leucine. Hughes et al. (1983) observed changes in concentrations of BCAAs in lake trout (*Salvelinus namaychus*) given diets containing increasing amounts of valine. Plasma isoleucine and leucine were both elevated in valine-deficient fish, and their concentrations decreased as dietary valine was increased. An antagonist effect of excess leucine on plasma and muscle levels of other BCAAs has also been reported by Hughes et al. (1984) in lake trout and Yamamoto et al. (2004) in rainbow trout. Yamamoto et al. (2004) reported a negative effect of feeding diets formulated to low Ile:Leu and Val:Leu ratios. Choo et al. (1991) and Encarnação (2005) did not observe any effect of excess dietary leucine on plasma valine and isoleucine concentrations. Rainbow trout showed a high tolerance for dietary leucine; no growth depression occurred with concentrations as high as 9.2% of diet (Choo et al., 1991). Even with excessive dietary leucine concentrations (13.4% of diet), which were overtly toxic, the concentrations of free valine and isoleucine in plasma, liver, and muscle were not depressed (Choo et al., 1991).

Arginine

Arginine (abbreviated as Arg or R) is an α-amino acid with a side chain consisting of a 3-carbon aliphatic straight chain, the distal end of which is capped by a complex guanidinium group. Arginine is in zwitterionic form at neutral pH. The guanidinium group is positively charged in neutral, acidic, and even most basic environments and thus imparts basic chemical properties to arginine. Because of the conjugation between the double bond and the nitrogen lone pairs,

the positive charge is delocalized, enabling the formation of multiple H-bonds. The distributing basics of the moderate structure found in geometry, charge distribution, and ability to form multiple H-bonds make arginine ideal for binding negatively charged groups. In mammals, arginine is classified as a semiessential or conditionally essential amino acid, depending on the developmental stage and health status of the individual (Baker, 2007). Arginine is an intermediate of the urea cycle and can be synthesized from citrulline (Wan et al., 2006). However, in fish and shrimp, arginine has been shown to be an EAA due to the very poor activity of the urea cycle. Huggins et al. (1969) demonstrated that all five enzymes of the urea cycle exist in teleosts. The urea cycle enzymes appear to be expressed during embryogenesis in a number of teleosts, but their activity is highly down-regulated during later life stages (Chadwick and Wright, 1999). There is evidence that teleosts can utilize ornithine and citrulline to synthesize arginine (Chiu et al., 1986). Some teleosts, such as *Heteropneustes fossilis* and *Oreochromis alcalicus grahami*, also upregulate the activity of the urea cycle during certain environmental conditions (Randall et al., 1989). The ability of these animals to produce arginine from the urea cycle to meet their arginine requirement has not been investigated.

Arginine is a precursor for creatine and nitric oxide synthesis and serves as a potent stimulant of insulin and growth hormone so that it may play an important role in anabolic processes (Wan et al., 2006).

Antagonism of arginine by excess dietary lysine is a phenomenon that has been characterized in a number of animal species, including chicks, rats, guinea pigs, and dogs (Baker, 2007). Excessive levels of dietary lysine have been shown to cause growth depression, which can be alleviated with additional dietary arginine (Austic and Scott, 1975). Lysine and arginine are transported on the same dibasic amino acid carrier, and competitive inhibition between these two amino acids can affect their absorption, transport, and metabolism (Kaushik and Fauconneau, 1984). In birds, excess lysine enhances arginine catabolism and increases the arginine requirement by inducing renal arginase (Jones et al., 1967; D'Mello and Lewis, 1970). In mammals, competitive inhibition of arginase by L-Lys has been observed and this can result in reduced arginine catabolism and urea production (Statter et al., 1978; Fico et al., 1982).

Studies with different fish species have yielded no convincing evidence of antagonism between lysine and arginine. Robinson et al. (1981), Tibaldi et al. (1994), and Alam et al. (2002a) have found no negative effect of feeding excess lysine on growth and/or plasma arginine levels in channel catfish, European sea bass, and Japanese flounder. Conversely, Kaushik and Fauconneau (1994) observed a significant decrease in plasma arginine in rainbow trout in response to increasing the dietary lysine level from 1.8 to 3.0% of the diet. However, the results of Kaushik and Fauconneau (1994) can probably be explained by the fact that the control diet (1.8% lysine) was marginally deficient in lysine (Encarnação et al., 2004). Increasing dietary lysine levels may have improved protein deposition and increased demand for other EAA, including arginine, thereby reducing plasma levels and oxidation of arginine.

Threonine

Threonine (abbreviated as Thr or T), together with serine and tyrosine, is one of three primary amino acids bearing an alcohol group. The threonine residue is susceptible to numerous posttranslational modifications. The hydroxy side chain can undergo O-linked glycosylation. In addition, threonine residues undergo phosphorylation through the action of a threonine kinase. In its phosphorylated form, it can be referred to as phosphothreonine.

Threonine is abundant in mucins. Evidence with mammals suggests that gut mucin synthesis may comprise a significant proportion of the whole-body threonine requirement (Nichols and Bertolo, 2008). Fish produce significant amounts of mucus, notably during stressful conditions, transfer to seawater, or exposure to heavy metals, ammonia, and pollutants (Eddy and Fraser, 1982). Mucus production may represent a non-negligible portion of the threonine requirement under certain conditions.

Threonine is metabolized to pyruvate via threonine dehydrogenase. An intermediate in this pathway can undergo thiolysis with CoA to produce acetyl-CoA and glycine. Beside a depression of growth and feed efficiency, threonine deficiency does not appear to cause specific deficiency signs (Ahmed, 2007).

Tryptophan

Tryptophan (abbreviated as Trp or W) is an α-amino acid containing an indole functional group. Only the L-stereoisomer of tryptophan is used in structural or enzyme proteins, but the D-stereoisomer is occasionally found in naturally produced peptides. Tryptophan functions as a biochemical precursor for several compounds, including 5-HT (serotonin), a neurotransmitter, which is synthesized via tryptophan hydroxylase. Serotonin, in turn, can be converted to melatonin (a neurohormone), via N-acetyltransferase and 5-hydroxyindole-O-methyltransferase activities.

Beside reduced growth and feed efficiency, tryptophan deficiency leads to scoliosis (lateral curvature of the vertebral column) and to a derangement of mineral metabolism in certain salmonids, including rainbow trout (Walton et al., 1984a), sockeye salmon (*Oncorhynchus nerka*) (Halver and Shanks, 1960), and chum salmon (*Oncorhynchus keta*) (Akiyama et al., 1986). Significantly greater concentrations of calcium (Ca) (a fourfold increase over control), sodium (Na), and potassium (K) were found in the kidneys of tryptophan-deficient trout (Walton et al., 1984a). Concentrations of

Ca, magnesium (Mg), Na, and K in the livers of tryptophan-deficient trout were also significantly greater than in normal trout. The underlying mechanisms for this accumulation of minerals in liver and kidney have not been resolved.

Scoliosis induced by Trp deficiency may be reversed by restoring tryptophan to an adequate concentration or by inclusion of serotonin in the diet (Akiyama et al., 1986). Thus, the experimental evidence suggests that this condition may be related to a decline in circulating level of serotonin (Akiyama et al., 1986).

The synthesis of serotonin in the brain is dependent on availability of tryptophan. This occurs because the rate-limiting enzyme in the biosynthetic pathway of 5-HT, tryptophan hydroxylase, is unsaturated with Trp under normal physiological conditions (Hoglund et al., 2005). Feeding high dietary levels of Trp resulted in elevated body and brain Trp content, increased brain 5-HTergic activity, affected the behavioral response to stress, and reduced aggressiveness in different fish species (Winberg et al., 2001; Lepage et al., 2002; Hseu et al., 2003; Hoglund et al., 2005, 2007).

Niacin can also be synthesized from tryptophan via kynurenine and quinolinic acids as key biosynthetic intermediates, although there is evidence that fish are unable to convert any significant amount of tryptophan into niacin (Ng et al., 1997).

Histidine

Like arginine and lysine, histidine (abbreviated as His or H) is classified as a basic amino acid. However, histidine has a positively charged imidazole functional group that can act as both an acid and a base, i.e., it can both donate and accept protons under some conditions. This amino acid side chain has important roles as a coordinating ligand in metalloproteins, and also as a catalytic site in certain enzymes, such as aiding the catalytic functions of chymotrypsin (digestive enzyme) and those enzymes involved with metabolism of proteins and carbohydrates. The residue can also serve a role in stabilizing the folded structures of proteins. Histidine is also found abundantly in hemoglobin, is the direct precursor of histamine, and is an important source of carbon atoms in the synthesis of purines.

The enzyme histidine ammonia-lyase converts histidine into ammonia and urocanic acid (Cowey and Walton, 1989) in the liver. In the liver, urocanate is further processed into ammonia, glutamate, and a 1-C fragment that it used in the folate coenzyme system. Decarboxylation into histamine plays important roles in immune function and as a paracrine agent acting on the stomach.

Following removal of blood meal from feeds in the mid-1990s, a high incidence of cataracts was observed in Atlantic salmon smolts in Northern Europe (Breck et al., 2003; Waagbo et al., 2010). Studies confirmed the mitigating effect of blood meal on development of cataract in these fish (Breck et al., 2003). Blood meal is considerably richer in histidine (4–6% histidine) than fish meal (1–2% histidine) and other common fish-feed ingredients. A series of studies published by Bjerkas and Sveier (2004), Breck et al. (2003; 2005a,b), and Tröße et al., (2009) present evidence suggesting that feeding diets containing 0.9 to 1.0% total histidine support maximal growth but could result in high incidence and severity of cataract in fast-growing Atlantic salmon smolts. These studies report a significant interaction among genotype, salinity variation/saltwater transition, and water temperature and dietary histidine level on the development of cataract in salmon. The exact mechanism by which histidine mitigates cataract development is not completely understood (Waagbo et al., 2010). Histidine and related compounds (imidazoles) may play important biochemical roles, such as osmoregulation, muscle pH buffering, and detoxification of reactive carbonyl species (Waagbo et al., 2010). Breck et al. (2005b) found that increasing dietary histidine inclusion at levels from 0.9 to 1.4% of diet in salmon undergoing smoltification had a significantly positive effect on eye lens protein turnover and n-acetyl histidine (NAH) content of the lens. NAH may play a role in protecting the lens against variation in osmotic pressure and oxidative stress associated with seawater transfer (Breck et al., 2005b). Protein turnover is likely an essential mechanism for repairing damage to eye lens proteins and maintaining clarity of the eye lens (Breck et al., 2005b). The "metabolic" requirement for histidine may increase during the smoltification process and the subsequent period of fast growth in seawater. Histidine requirement for optimal ocular health appears to be significantly higher than that for maximal growth in fast-growing Atlantic salmon undergoing transition to seawater (Waagbo et al., 2010). A higher requirement for optimal ocular health compared to that for maximizing growth was also reported for methionine in Arctic charr (*Salvelinus alpinus*) (Simmons et al., 1999).

Phenylalanine and Tyrosine

Phenylalanine (abbreviated as Phe or F) is a nonpolar α-amino acid because of the hydrophobic nature of the benzyl side chain. Tyrosine (abbreviated as Tyr or Y) or 4-hydroxyphenylalanine is synthesized in the body from phenylalanine and is considered a semiessential, or conditionally essential, amino acid. Fish readily convert phenylalanine to tyrosine so that phenylalanine alone can meet requirements for aromatic amino acids (Wilson, 1989; Guillaume et al., 1999). However, the presence of tyrosine in the diet will reduce some of the requirement for phenylalanine. Phenylalanine sparing by tyrosine is believed to be between 40–60% in the species studied to date (e.g., NRC, 1993; Guillaume et al., 1999).

Aside from being a proteinogenic amino acid, tyrosine has a special role by virtue of the phenol functionality. It occurs in proteins that are part of signal transduction processes and functions as a receiver of phosphate groups that are transferred to the hydroxyl group by protein kinases

(so-called receptor tyrosine kinases), with phosphorylation changing the activity of the target protein. L-tyrosine also can be converted into the catecholamines, norepinephrine, and epinephrine, dopamine (a neurotransmitter), and thyroxin (Cowey and Walton, 1989).

The catabolism of L-tyrosine involves a series of reactions that yield fumarate and acetoacetate (3-ketobutyroate). Acetoacetate is a ketone body that can be converted into acetyl-CoA, which in turn can be oxidized by the TCA cycle or be used for fatty acid synthesis (Cowey and Walton, 1989).

QUANTITATIVE PROTEIN AND ESSENTIAL AMINO ACID REQUIREMENTS

Protein Requirements

The protein requirement of animals corresponds to the increasingly well-understood requirements for specific EAA and to a nonspecific need for amino groups (nonspecific nitrogen) for the synthesis of NEAA, as well as the contribution of amino acids to meeting the energy and other metabolic needs of the animal.

Protein generally refers to crude protein (CP); that is, N × 6.25, a definition based on the assumption that proteins contain 16% N. This assumption is simplistic and rarely appropriate because the percentage of N in pure proteins is known to vary significantly, depending on their amino acid composition (Mariotti et al., 2008). Using a fixed conversion factor (e.g., 6.25) can lead to a 10–20% error in estimation of the true protein content of certain protein-rich ingredients (Mariotti et al., 2008). Nitrogenous compounds in feeds do not only comprise protein and amino acids; they also include numerous compounds such as nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids, and nitrogenous glycosides (Mariotti et al., 2008). The contribution of these different compounds to the total N content of ingredients is highly variable. Consequently, crude protein (as well as digestible protein) refers to a mixture of very different substances differing in terms of their biochemical nature and nutritive value (Mariotti et al., 2008). The sum of individual amino acids in ingredients (as analyzed by reference laboratories using rigorous methodological approaches and modern equipment) only seems to account for 80 to 90% of the "protein" content of feed ingredients (Helland et al., 2010). This suggests that N-containing compounds other than amino acids may account for as much as 10–20% of the crude protein content of feed ingredients.

All dietary proteins are not identical in their nutritive value, which is a function of their digestibility and amino acid profile. The amino acid composition of proteins in different ingredients differs markedly (NRC, 1993). Digestibility of protein and availability of individual amino acids also vary considerably among ingredients. The capacity of different dietary protein sources to meet the EAA and other metabolic needs of animals is expected to differ considerably.

Amino acids also play an important role in meeting the energy (metabolic) requirements of fish and shrimp and serve as metabolic fuels for most fish and crustacean species (Kaushik and Seiliez, 2010). The persistent use of the dietary crude protein concept by most nutritionists and feed manufacturers is understandable because it is a simple and practical parameter but it is also to some extent perplexing.

The protein requirements have been examined for a very large number of fish and shrimp species at different life

TABLE 5-4 Recommended Dietary Protein Levels (%) for Various Fish Species of Commercial Importance (As-Fed Basis)

	Weight Range				
Species	< 20 g	20–200 g	200–600 g	600–1,500 g	> 1,500 g
Atlantic salmon (*Salmo salar*)	48	44	40	38	34
Channel catfish (*Ictalurus punctatus*)	44	36	32	32	28
Common carp (*Cyprinus carpio*)	45	38	32	28	28
Nile tilapia (*Oreochromis niloticus*)	40	34	30	28	26
Pacific salmon (*Oncorhynchus* spp.)	55	45	40	38	38
Rainbow trout (*Oncorhynchus mykiss*)	48	40	38	38	36
European sea bass[a] (*Dicentrarchus labrax*)	55	50	45	45	—
Gilthead sea bream[b] (*Sparus auratus*)	50	45	40	40	—

[a]This recommendation applies to other sea bass species.
[b]This recommendation applies to other sea bream species.

TABLE 5-5 Recommended Dietary Protein Levels (%) of Different Shrimp Species

Species	Weight Range		
	0.1–5 g	5–20 g	> 30 g
Tiger shrimp (*Penaeus monodon*)	45	40	40
Pacific white shrimp (*Litopenaeus vannamei*)	40	35–40	35
Kuruma prawn (*Marsupenaeus japonicus*)	50	45	40

stages. Tables 5-4 and 5-5 provide practical estimates of dietary protein requirements of fish and shrimp as a percentage of the diet. Understanding the nutritional constraints and limitations used in arriving at the protein requirement is important for their proper application. This information provides a simple basis for formulation of practical feeds for different species, notably when limited information is available on the EAA requirements and optimal levels (or the effectiveness) of lipid and carbohydrate as energy sources are not well defined for a given species. Estimates of protein requirements should be considered highly approximate, especially in the current context in which an increasingly wide variety of feedstuffs is used in the formulation of feeds for different fish and shrimp species.

As a dietary concentration, the dietary protein requirements of fish and shrimp seem to be appreciably higher than those of terrestrial warm-blooded animals. However, although the protein requirement in terms of dietary concentration (percentage of the diet) is high, the absolute requirement (grams of protein intake per kilogram of body weight gain) is highly comparable, although significant differences seem to exist among species (Figure 5-4). This is because fish have a lower maintenance energy requirement than warm-blooded animals, which results in a similar amount of body weight gain per unit of protein ingested as in warm-blooded animals but better feed efficiency (gain:feed). Some fish species, such as the Atlantic salmon, are typically more efficient converters of protein than domesticated warm-blooded animals and "omnivorous" fish species, such as tilapia and common carp (Figure 5-4). Direct or indirect comparisons of species indicate that carnivorous species often have higher protein retention efficiencies (N gain/N intake) than do omnivorous fish (e.g., Pei et al., 2004). These observations cast doubt on the oft-voiced "opinion" that "carnivorous" fish species rely more heavily on amino acids to meet their energy/metabolic requirements than do omnivorous fish species.

Digestible Protein to Digestible Energy Ratio

Amino acids play an important role in meeting the energy (metabolic) requirements of fish and shrimp and are generally efficient metabolic fuels for most fish and crustacean species. The use of protein as a dietary source of energy by animals is considered undesirable because of the relatively high cost of protein compared to the cost of other energy-yielding nutrients (starch and lipids) and because of the release of ammonia associated with the catabolism of amino

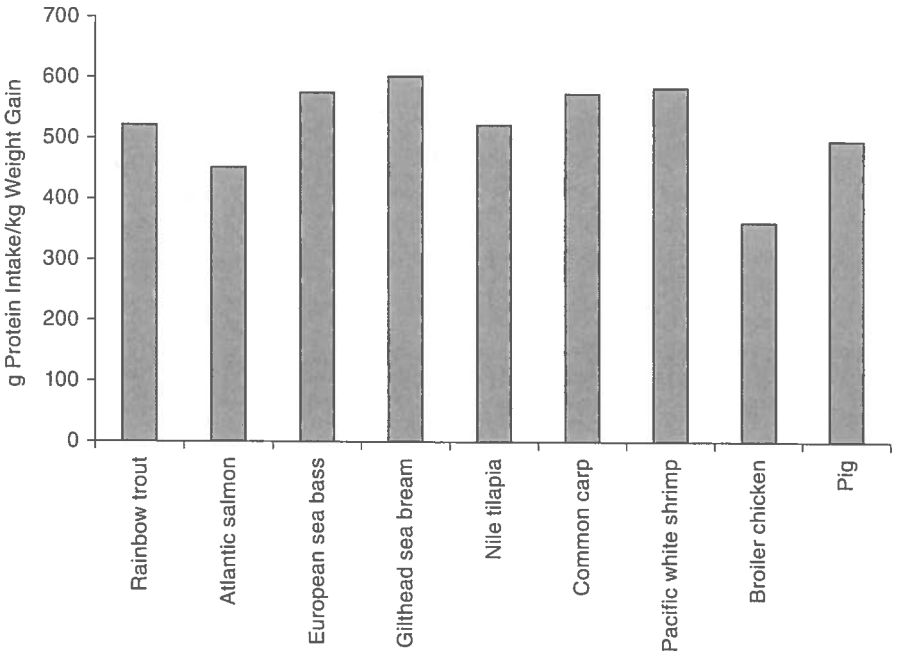

FIGURE 5-4 Protein intake per kilogram of live weight gain in different fish and shrimp species, chicken, and swine.

acids. In general, some economy can be made if other dietary energy-yielding nutrients are present in adequate amounts to reduce dietary amino acid catabolism, an effect commonly referred to as "protein-sparing." There is a strong argument that digestible protein (DP) to DE ratio is a more rational way of expressing protein requirement than the dietary "crude protein" requirement (percentage CP of diet).

A decrease in digestible protein to digestible energy ratio (DP:DE) achieved by reducing the dietary DP levels with or without concomitant increase in the dietary non-protein DE supply has proven to be extremely efficient in improving protein (nitrogen) utilization and decreasing nitrogenous loses in numerous farmed fish species (Lee and Putnam, 1973; Cho and Kaushik, 1985; Kaushik and Oliva-Teles, 1985; Cho and Woodward, 1989; Hillestad and Johnsen, 1994; Vergara et al., 1996; Einen and Roem, 1997; Grisdale-Helland and Helland, 1997, Helland and Grisdale-Helland, 1998; Hillestad et al., 1998; Steffens et al., 1999; Azevedo et al., 2004a,b; Satoh et al., 2004). Variation in the DE content of the diet also involves other energy-yielding nutrients that may not have the same ability to be utilized by fish and spare dietary amino acids (Encarnação et al., 2006). In general, increasing the lipid content of the diet can help reduce dietary protein (amino acid) catabolism in a number of species. A number of studies also have shown that extra DE provided as lipid can have a limited impact on efficiency of protein utilization under certain conditions (Azevedo et al., 2004a,b). Many fish species have a limited ability to utilize carbohydrate (e.g., starch) efficiently even when gelatinized. In excess of certain levels, the metabolic utilization of absorbed glucose is limited in most fish, and the amount of net energy that can be derived from digestible carbohydrate is limited (Bureau, 1997), although there are differences between species (Kaushik and Seiliez, 2010).

Overall, differences seem to exist among species and life stages in terms of optimal DP/DE. For most species studied so far, optimal DP/DE seems to range from about 84 to 105 g DP/Mcal DE (20 to 25 g DP/MJ DE) (Winfree and Stickney, 1981; Cho and Woodward, 1989; Azevedo et al., 2004a,b; Wang et al., 2006). Optimal DP/DE for species that show high capacity to utilize high dietary levels of lipids (e.g., Atlantic salmon) can be below 84 g DP/Mcal DE (20 g DP/MJ DE) (Einen and Roem, 1997). The optimal DP/DE of carnivorous fish species that are relatively poorly tolerant of dietary lipids (or "lean" species), such as Asian sea bass, haddock, and cobia, seems to be closer to 117 to 134 g DP/Mcal DE (28 to 32 g DP/MJ DE) (Catacutan and Coloso, 1995; Kim et al., 2004; Tibbett et al., 2005; Webb et al., 2010). In some species, feeding a low DP/DE diet (e.g., high-lipid diets) can improve protein utilization but result in undesirable levels of lipid deposition (Tibbett et al., 2005), which may have long-term implications in terms of final product quality or health of the animal.

Evidence suggests that the protein requirements, as a proportion of the mass of the diet, decrease as fish increase in size. For example, 25% protein was adequate in the diet of channel catfish of 114 to 500 g, but 35% protein produced faster gains than did 25% protein in fish weighing between 14 to 100 g (Page and Andrews, 1973). Similar results have been obtained with salmonids, common carp, and tilapia (Wilson and Halver, 1986). The optimal DP:DE ratio for young Atlantic salmon has been estimated to be between 84–100 g DP/Mcal DE (20–24 g DP/MJ DE), whereas evidence suggest that for large salmon (> 2.5 kg) it may decrease to 67–71 g DP/Mcal DE (16–17 g DP/MJ DE) (Einen and Roem, 1997). Results from a study conducted on gilthead seabream (*Sparus aurata* L.) predicts that optimum DP:DE ratio could decrease from 119 down to 82 g DP/Mcal DE (28.5 down to 19.5 g DP/MJ DE) as the animal grows from 10 to 250 g (Lupatsch et al., 2001b).

Despite requiring (and/or tolerating) lower dietary protein concentrations, larger fish frequently show lower protein retention efficiency (N gain/N intake) than do smaller fish. Studies in rainbow trout indicated that protein retention efficiency decreased very significantly and the ratio of lipid to protein deposited (LD:PD) increased dramatically with an increase in live body weight of these animals (Azevedo et al., 2004a,b; Peña-Ortega and Bureau, 2004; Azevedo et al., 2005; Dumas et al., 2007). A higher absolute amount of protein (grams of protein per kilogram of biomass gain) can therefore be required by some species as the fish grow larger despite a significant decrease in their protein concentration or optimal DP:DE ratio for their diet.

Effect of Environmental Factors

Because the metabolism of fish and shrimp is affected by temperature and other environmental factors (Bureau et al., 2002), it is often assumed that environmental conditions may have a significant effect on nutritional requirement of fish and crustaceans. However, so far no convincing evidence exists to show that protein requirement or optimal DP:DE ratio is affected by water temperature or other environmental factors (e.g., salinity) (NRC, 1981; Lupatsch and Kissil, 2005), at least within a "normal" range of conditions (range specific to each species and/or strain). A small decrease in protein and energy digestibility is observed with decreasing water temperature but the efficiency of DP and DE utilization of rainbow trout and grouper fed various rations at different water temperatures does not seem to be affected (Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999; Lupatsch et al., 2001; Lupatsch and Kissil, 2005). In general, all feeding and growth functions increase in parallel as water temperature rises. A significant increase in protein requirement may occur in Asian sea bass (Barramundi) reared at temperatures that are significantly above the thermal optima, possibly as a means for compensating for lower feed intake of the animal, a reduction in the efficiency of utilization of protein, and an increase in the rate of protein losses (Bermudes et al., 2010).

Factorial models have been proposed as straightforward

means to estimate protein requirements of fish and shrimp to account for the potential impacts of diet composition, life stage, growth rate, and environmental factors (Lupatsch et al., 1998, 2001a, 2003, 2008, 2010; Glencross, 2008, Glencross et al., 2010; Richard et al., 2010). Using factorial approaches, the requirement is generally estimated as an absolute amount of protein (or digestible protein) required per kilogram of body weight per day for explicitly expressed levels of performance (e.g., expected or achievable level of performance) and life stages. Information on feed intake and diet composition (e.g., digestible energy levels) allow the backcalculation of "optimal" dietary protein concentration. In a number of studies, an absolute protein requirement (grams of DP required per kilogram of body weight per day) has been estimated for "maximal production" (Gatlin et al., 1986; Watanabe et al., 2000; Richard et al., 2010). Maximal production of an animal is highly dependent on genetics, diet composition, environmental conditions (e.g., temperature), husbandry practices, health status, and other variables and the absolute protein requirements for maximum production calculated consequently can only be valid for the specific conditions encountered in the study. Consequently, estimates of protein (or amino acid) requirements expressed in absolute terms (grams of DP required per kilogram of body weight per day) need to be presented within a production or growth modeling context as opposed to stand alone estimates (Lupatsch et al., 1998; Bureau et al., 2002; Glencross et al., 2010).

QUANTIFYING ESSENTIAL AMINO ACID REQUIREMENTS

Methodological Approaches

The EAA requirements of fish have been a topic of investigation for more than 50 years. Initial studies with Chinook salmon were conducted by Halver and coworkers in the late 1950s, when they evaluated various amino acid test diets. Most successful diets were based on the profile of whole hen egg, and this diet was used to determine qualitative amino acid requirements of Chinook salmon (Halver, 1957). Subsequently, quantitative requirements for the 10 EAA were investigated by the same group, providing a model for much of the work on quantitative EAA requirements of other fish species (Wilson, 1989). Most quantitative estimates of EAA requirements have been established by dose-response studies, although a number of other methods have also been applied. These methodological approaches are based on analyses such as plasma or tissue amino acid concentration and rates of amino acid oxidation. However, these methods have always been subsidiary to growth studies (Kim et al., 1992c; Cowey, 1994).

Growth Response Assays

Most estimates of EAA requirements have been determined based on conventional growth response assays. In such studies, a basal diet, deficient in a single EAA but meeting all other known nutrient requirements of the animal, is supplemented with graded levels of the EAA studied. Various types of chemically defined, purified, and natural ingredients have been used to provide graded increments of the amino acid under test. Most studies have used test diets in which the nitrogen component consisted of either amino acids or a mixture of amino acids, casein, and gelatin formulated to provide an indispensable amino acid composition identical with some reference protein (such as whole hen's egg protein or fish body protein) minus the amino acid under test (Cowey, 1994). These diets are generally fed to young, fast-growing fish, although the method can also be applied to other life stages depending on the objective of the investigation.

Quantification of EAA requirements is generally based on analysis of dose-response curves with weight gain used as a response criterion. The lowest level of EAA maximizing live weight gain is then identified as the minimum dietary requirement. Protein and EAA depositions are also increasingly used as response parameters along with weight gain (e.g., Pfeffer et al., 1992; Rodehutscord et al., 1995a, 1997; Hauler and Carter, 2001b; Encarnação et al., 2004). Evidence suggests that protein and EAA depositions may be a more robust and rational criteria. Estimates of requirements based on protein deposition have been shown to be higher than those obtained based on weight gain in some studies (Encarnação et al., 2004).

Alternative dietary and experimental designs have been used to estimate protein and EAA requirements in some studies (Gatlin et al., 1986; Gurure, 1997; Hung et al., 2004; Abboudi et al., 2006, 2007; Liebert and Benkendorff, 2007; Helland et al., 2010). These approaches include the requirement at ration level (RRL) method, in which a diet that may or may not be nutritionally complete is fed at graded levels, thus achieving graded intake of protein and/or amino acids to estimate requirements for maintenance and maximum growth or protein gain (a type of factorial method). The diet dilution technique, in which serial dilution of a "summit diet" also known to be first limiting in a specific EAA are fed to animals is another approach to achieve intake of incremental amino acid levels. The main advantage of these approaches is that the amino acid balance of the diet does not change in the different diets with graded levels of EAA fed to the animal (Gous and Morris, 1985; D'Mello, 2003). However, the supply of several nutrients varies at once and/or there is a substitution of energy-yielding nutrients that arguably are more important confounding factors than small imbalances in the profile of dietary amino acids (D'Mello, 2003). The estimate of requirements obtained with the RRL method is generally expressed as absolute values (e.g., g/fish BW per day) and may be applicable only to the specific conditions

(performance level, growth rate and protein deposition) observed in the study.

Whole-Body Amino Acid Profile

A review of the literature indicates that for a large majority of fish species fed high-quality diets, deposition of amino acids into body protein represents between 25 to 55% of total amino acids consumed. The deposition of protein, consequently, is one of the determinants of amino acid requirements by fish, and composition of body protein deposited can be used to provide an indication of the diet EAA profile required by the animal. Early estimates of amino acid requirements of fish were based on the amino acid profiles of fish, egg, and whole-body proteins (Wilson, 1989; NRC, 1993). This approach is still popular to develop initial estimates of amino acid requirements of fish and shrimp species for which limited information is available on their nutritional requirements (Kaushik, 1998; Kaushik and Seiliez, 2010). However, because a minor proportion (typically less than 50%) of the digested amino acids is generally retained, the profile of retained EAA may not fully correspond to the profile of EAA required by the animal. Certain amino acids are known to be preferentially retained in tissues, whereas others appear to play more active metabolic roles and may be less efficiently retained.

In most cases, a reasonably good agreement between body EAA profile and EAA requirement (% protein) has been observed. Wilson and Poe (1987) observed very good agreement between EAA requirement pattern and whole-body EAA profile of channel catfish and between EAA requirement pattern and catfish egg EAA profile. Nose (1979) also found good agreement between body EAA and EAA requirements of common carp.

Amino acid profile of whole body or muscle protein (Ogino, 1980; Mambrini and Kaushik, 1995) or whole body A:E ratios (EAA content/total EAA × 1,000) (Arai, 1981; Moon and Gatlin, 1991; Wilson, 1994; Brown, 1995) have also been employed to generate simple quantitative estimates of EAA requirements of fish. These are at the basis of many of the estimates of the "ideal protein" pattern of amino acids published for many studies (as discussed later in this chapter). However, it is generally recognized that EAA requirement estimates based on body protein overestimate the requirement for amino acid preferentially deposited in body protein, such as leucine and lysine, and underestimate the requirements of amino acids playing important metabolic roles, such as methionine, threonine, histidine, and arginine.

Factorial Models of Amino Acid Requirements

The mathematical representation of the main biological processes that determine EAA utilization for body protein deposition has been an increasingly used methodological approach to estimate EAA requirements of fish and shrimp (Hauler and Carter, 2001b; Teshima et al., 2002; Bodin et al., 2008, 2009; Richard et al., 2010). These models are often extensions of protein and energy requirement models discussed earlier (Lupatsch et al., 1998; Bureau et al., 2002; Glencross et al., 2010). Factorial EAA requirement models are generally based on integration of information derived from different methodological approaches (e.g., whole-body EAA profile, estimates of maintenance requirements and inevitable catabolism of amino acids obtained by experimentation [growth assays] or metaanalyses of published studies, growth, and bioenergetic modeling).

Factorial models are practical because they enable the estimation of EAA requirements as a function of diet composition, life stage, and growth rate. The main limitations of current factorial models are that they estimate EAA requirement by generating independent estimates for each EAA, they assume that there are no interactions among EAA and other nutrients (e.g., fatty acids and glucose), and assume constant efficiency of utilization of amino acids regardless of physiological state. They also assume that "feed intake" or "energy requirement" or "feed conversion ratio" are "determinant"; that is, they are "independent" from diet composition, nutrient intake, interaction between nutrients, changes in target protein, and lipid deposition or physiological state. More efforts need to be invested in developing a more rational, yet practical, framework of EAA and nutrient requirements of fish and shrimp (Bureau and Encarnação, 2006; Hua and Bureau, 2010).

Blood and Muscle Amino Acid Levels

At subrequirement intake levels, the serum or tissue content of the tested EAA should remain low until the requirement for the EAA is met and then increase to high levels when excessive amounts of the amino acid are fed (Wilson, 1989). This technique has proven to be useful in corroborating the EAA requirements, but only in a few cases. In channel catfish, serum lysine data (Wilson et al., 1977) were useful in confirming the requirement values estimated by weight gain data. However, this technique has not always been reliable for assessing EAA requirements (Kaushik and Luquet, 1979; Hughes et al., 1983; Walton et al., 1986; Were, 1989; Kim et al., 1992b). Its validity seems to be linked to (1) the nature of the EAA tested, (2) interactions between the different amino acids, and (3) time elapsed between meal and blood sampling (Mambrini and Kaushik, 1995)

Amino Acid Oxidation Studies

Direct and indirect oxidation studies are based on tissue-free amino acid concentrations and give a measure of the partitioning of the EAA between protein synthesis and oxidation (Kim et al., 1983). The direct oxidation method is based on the principles that, at limiting levels, rates of oxidation of the EAA under study should be low because concentration in

the free amino acid pool should be small, the major portion being utilized for protein synthesis, and little would be oxidized (Kim et al., 1983). Thus, the oxidation rate of the tested amino acid should remain low until the requirement level is reached and then it would increase sharply. The intake level that produces a marked increase in amino acid oxidation should then be a direct indicator of the requirement value for that specific amino acid (Kim et al., 1983).

Indirect oxidation studies measure the oxidation of an EAA other than the one under study. In this instance, incorporation of this other amino acid into tissue protein is limited by the intake level of the amino acid under study; consequently, high rates of oxidation of this other amino acid will occur. As dietary concentration of the amino acid under study increases, tissue protein synthesis will increase progressively, and the amounts of other amino acids being oxidized will decrease as proportionally larger amounts are used for protein synthesis (Kim et al., 1983; Cowey, 1994).

These techniques have been evaluated in rainbow trout with limited success. Compared to growth studies, oxidation studies gave similar estimates for lysine (Walton et al., 1984b) and tryptophan (Were, 1989) requirements, but apparently resulted in unreliable estimates of arginine requirements (Fauconneau et al., 1992; Lall et al., 1994). These discrepancies could be explained by the variable rates of oxidation of the different EAA, differences in the techniques of administration of the labeled amino acids, and the inherent variability associated with the technical complexity of the method (Mambrini and Kaushik, 1995).

SUMMARY OF PUBLISHED ESTIMATES OF ESSENTIAL AMINO ACID REQUIREMENTS OF FISH AND SHRIMP

Estimation of EAA requirements of fish and shrimp has been the objective of a large number of published studies (> 200 papers). These studies have been very diverse in terms of scope and methodological approaches used. Quantitative estimates have been generated for all 10 essential amino acids in a number of species (or highly related group of species), including channel catfish, common carp, Indian major carp (rohu and mrigal), Nile tilapia, Pacific salmon (Chinook, chum, coho), and rainbow trout. Increasing information is available on marine fish species (cobia, croaker, drum, sea bass, sea bream, turbot, flounder, and others). However, the significant numbers (10) of EAA and the very large number of fish and shrimp species cultivated around the world result in significant dilution of research efforts. In addition, the experimental design of many studies is deficient, and few studies can be considered sufficiently robust to generate reliable estimates of EAA requirements. An improvement of the quality of efforts invested in the estimation of EAA requirements of different fish and shrimp species would be a valuable contribution to the aquaculture nutrition community.

Tables 5-6 to 5-15 provide a summary of the experimental conditions and conclusion of studies on EAA requirements of fish. Table 5-16 summarizes information from studies on penaeid shrimp. Estimates of EAA requirements vary significantly among studies. Part of this variability can be attributed to differences among species but very significant variability exists within species. This variability may be attributable to experimental design and conditions (including weight of fish, composition and physical characteristics of the experimental diets, number of treatment and replicates, feeding method, and growth rate achieved) but also to the wide range of mathematical and statistical approaches used to estimate requirements, which may have a significant effect on estimate of EAA requirements (Rodehutscord and Pack, 1999; Encarnação et al., 2004; Wang et al., 2010).

ESSENTIAL AMINO ACID REQUIREMENTS IN THE CONTEXT OF FEED FORMULATION

Nutrient requirements find their usefulness in their translation into nutritional recommendations for feed formulations. Formulating cost-effective feeds meeting essential amino acid requirements of fish and shrimp can represent a challenge. Aquaculture feeds are different from other livestock feeds due to the wide variability of their composition in terms of digestible protein, lipid, carbohydrate, and DE contents (Encarnação et al., 2004; Bureau and Encarnação, 2006). The impact of diet composition on EAA utilization and requirements of fish and shrimp has been the focus of a limited number of studies and thus remains poorly understood and controversial (Encarnação et al., 2004, 2006; Bureau and Encarnação, 2006). Significant differences in opinion exist as to how EAA requirement data should be expressed and EAA levels deemed adequate in feed formulations should be calculated (Bureau and Encarnação, 2006). This situation limits the ability to review and interpret information on EAA requirements of fish and make recommendations that are widely applicable to practical conditions, for example, the wide variability in protein, and digestible energy (or energy-yielding nutrient) levels to which commercial feeds for a given species are formulated (Encarnação et al., 2004; Bureau and Encarnação, 2006).

Basic Approaches and Considerations

When formulating feeds, an appropriate safety margin is allocated on top of established EAA requirement values to compensate for putative processing and storage losses, variation in composition, digestibility, and bioavailability of nutrients in feed ingredients, as well as account for variations in requirements caused by environmental and biotic factors (NRC, 1993). Part of this safety margin is to account for lower digestibility of nutrients in feed ingredients compared to the high-quality ingredients used in laboratory diets (NRC, 1993). Most estimates of EAA requirements have been determined with diets in which EAA supplied were near 100%