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Study on dietary essential amino acid requirements of red sea bream (*Pagrus major*) and olive flounder (*Paralichthys olivaceus*)

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Study on dietary essential amino acid requirements of red sea bream (*Pagrus major*) and olive flounder (*Paralichthys olivaceus*)

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요약문

첫 번째 실험에서는 (Chapter 2) 12주간의 사양실험을 통해 참돔 (32.04 ± 0.2g)의 valine 요구량을 밝히기 위한 연구를 하였다. 총 6개의 실험사료는 valine을 단계별로 첨가(0.27, 0.79, 1.22, 1.69, 2.04, 2.38%)하여 제조하였으며 사양실험은 3반복구로 진행되었다. Valine이 0.79% 이상 함유된 그룹에서 유의적으로 높은 성장과 사료효율을 보였으며, 참돔의 비특이적 면역반응은 valine함량이 증가함에 따라 2.04% 그룹까지는 유의적으로 향상되었다. 사료 내 valine의 함량이 높을수록 사료섭취 후 암모니아의 배출 수준은 유의적으로 낮았다. 따라서 사료 내 valine의 적정 요구량은 0.9% 로 판단된다. 두 번째 실험에서는 (Chapter 3) 참돔 (13.3 ± 0.2 g)의 arginine 요구량 설정을 위해 진행되었다. Arginine이 각각 1.2, 1.6, 2.0, 2.4, 2.8, 3.2%가 함유된 6개의 실험사료를 3반복구로 하여 9주 동안 사양실험이 진행되었다. Arginine이 2.0% 이상 함유된 그룹에서 arginine이 1.2% 함유된 그룹에 비해 유의적으로 높은 성장률을 보였으며, arginine이 2.4% 함유된 그룹에서 단백질이용효율이 유의적으로 향상되었다. 혈액학적 분석을 통해 arginine 함량이 증가함에 따라 참돔의 선천면역력이 향상되는 것으로 보인다. 따라서 사료 내 arginine의 적정 요구량은 2.54%로 판단된다. 세 번째 실험 (Chapter 4) 은 넙치의 isoleucine 요구량을 밝히기 위해 수행되었으며 각각 다른 양의 isoleucine (0.48, 0.87, 1.43, 1.94, 2.37, 2.78%)을 함유한 6개의 사료를 만들어 3반복구로 하여 9주 동안 사양실험을 진행하였다. 가장 높은 성장을 보인 그룹은 isoleucine을 1.43% 함유한 그룹이

었다. Isoleucine의 함량이 높은 그룹일수록 혈장 내 단백질과 콜레스테롤의 함량이 증가하는 것으로 나타났으며, ALT와 AST에서는 isoleucine의 함량이 증가함에 따라 낮아지는 경향을 보였다. 넙치의 선천면역은 isolecine의 함량이 어느정도 증가함에 따라 향상되는 것으로 나타났다. 따라서 넙치 사료 내 isolecine의 요구량은 1.69%로 판단된다. 네 번째 실험 (Chapter 5)은 넙치 (17.28 ± 0.1 g) 의 methionine 요구량을 밝히기 위해 진행되었다. 6개의 실험사료는 methionine을 사료 내 0.3% - 1.8%가 함유되도록 제작되었으며, 3반복구로 8주 동안 사양실험이 진행되었다. Methionine 함량이 1.5%인 실험구가 0.3% 실험구에 비하여 유의적으로 높은 성장률을 보였다. 사료 내 methionine 함량의 증가는 결과적으로 대부분 전어체 필수 아미노산의 증가를 야기하였다. 사료 내 적정 methionine 함량은 1.63%로 판단된다. 마지막으로 다섯 번째 실험 (Chapter 6)은 넙치(5.41 ± 0.16 g) 사료에 있어 free lysine (FL) 과 dipeptide lysine-glycine (LG)의 이용성을 비교하기 위해 수행되었다. 어분을 통해 lysine을 0.5% 함유한 기초실험사료와 FL와 LG를 각각 0.5% 또는 1.0% 첨가한여 실험사료를 6주간 어체중의 3%로 공급하였다. 기초실험사료에서 다른 실험사료 그룹과 비교 하였을 때 가장 낮은 증체율을 보였다. Two-way ANOVA의 결과로 lysine의 함량과 형태는 넙치의 증체율에 유의적인 영향을 줄 것으로 판단된다. 이러한 결과는 lysine 함량이 증가함에 따라 단백질전환효율 (PER)이 유의적으로 증가하며, LG가 함유된 사료를 먹인 그룹에서 보다 높은 PER 값을 보였다. 전어체 arginine의 함량은 LG를 먹인 그룹에서 유의

적으로 높게 나타났다. 따라서 이번 연구의 결과는 치어기 넙치에서의 단백질 합성이 lysine의 free 형태보다 dipeptide 형태로 공급될 때 더 효율적으로 이용됨을 보여주었다.

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CHAPTER ONE: Literature review

1.1. Global Aquaculture state

World aquaculture production continues to grow, according to the latest available statistics collected globally by FAO, world aquaculture production reached 90.4 million tonnes in 2012. According to the latest information, FAO estimates that world food fish aquaculture production rose by 5.8 percent. The global trend of aquaculture development gaining importance in total fish supply has remained uninterrupted. The mean annual growth rate of aquaculture has been 8.3 (or 6.5 percent except China) percent, higher than the world mean population growth rate, through the years 1970-2008, and its annual per capita supply has increased from 0.7 to 7.8 kg during this period. Farmed food fish contributed a record 42.2 percent of the total 158 million tonnes of fish produced by capture fisheries (including for non-food uses) and aquaculture in 2012. This compares with just 13.4 percent in 1990 and 25.7 percent in 2000. Asia as a whole has been producing more farmed fish than wild catch since 2008, and its aquaculture share in total production reached 54 percent in 2012, with Europe at 18 percent and other continents at less than 15 percent. The expansion and intensification of aquaculture during the past decades have attributed to research, accordance with consumer demand and improved aquaculture policy and governance.

Protein supply from seafood contributes significantly to human needs in several geographic areas, especially in the developing world as well as in the emerging economies of the world. At a global level, about 45% of all fish consumed by humans, totalling about 48 millions tonnes is farm raised (FAO, 2007). Aquaculture thus plays a

vital role in supplying products known to have a high biological value to humans (Bender and Haizelden, 1957) besides providing healthy long-chain ω-3 polyunsaturated fatty acids (Sargent, 1997). Different factors are driving the aquaculture to intensification of which the main limiting factor is the unavailability of sites because of restricted non-agricultural land for exploitation. In this regard novel ways of using environment (land and water) for production are needed. By proceeding of intensification, the need for scientific support, services and skilled forces is in increase.

1.2. Protein and amino acids

Proteins are the most abundant macromolecule in living cells, and they exhibit an amazing diversity in their biological functions. The chemical composition of proteins (Lovell, 1989) consists of carbon (50-55%), hydrogen (6.5-7.5%), nitrogen (15.518%, an average of 16% is usually assumed), oxygen (21 -5-23.5%), and usually sulfur (0.5-2.0%). All proteins, whether from the most ancient lines of bacteria or the most complex forms of life, are constructed from the same ubiquitous set of 20 amino acids (Lehninger et al., 1993). AI1 of the amino acids found in proteins have a carboxyl group and an amino group bonded to an α-carbon atom. They differ from each other in their side chains, which vary in structure, size, and electric charge. This influences the solubility of amino acids in water. In all amino acids, except glycine, the α-carbon is a chiral centre and the tetrahedral arrangement of bonding orbitals gives two sterioisomers of the amino acid, either D or L. The characteristic three dimensional structure of proteins, which dictate their diverse biological activities, require that all

constituent amino acids be of one stereochemical series, usually the L-form (Lehninger et al., 1993).

Proteins are chains of amino acids, each covalently joined to its neighbour by a peptide bond. The bond is formed through a condensation reaction by removal of a hydroxyl group from the α-carboxyl group of one amino acid, and a hydrogen atom from the α-amino group of another. The biological diversity of proteins is due to the distinctive number and sequence of amino acid residues in each protein.

Dietary protein is the major and most expensive component of formulated aquafeeds (Wilson et al., 2002). Fishmeal is generally considered to be the most ideal protein source for aquatic animals, despite its static global production, seasonal/geographical variability in quality and composition, and concern as a vector of contamination (Trushenski et al., 2006). Fish feed constitutes over 60% of the operating cost of aquaculture (Nwanna, 2002). Yang et al. (2002) similarly described that fish feed accounts for 50% or more of the total production cost. Out of this cost, protein takes about 75% (Nwanna and Fashae, 2008) confirming it as the single most expensive component of fish feeds (NRC, 1993; De Silva and Anderson, 1995). Proteins are the major organic material in fish tissue, making up about 65 to 75% of the total on a dry-weight basis. Fish consume protein to obtain amino acids. The protein is digested or hydrolyzed and releases free amino acids, which are absorbed from the intestinal tract and distributed by the blood to the organs and tissues. These amino acids are used by the various tissues to synthesize new protein. A regular intake of protein or amino acids is required because amino acids are used continually by the fish, either to build new proteins (as during growth and reproduction) or to replace existing proteins

(maintenance). Inadequate protein in the diet results in a reduction or cessation of growth and a loss of weight due to withdrawal of protein from less vital tissues to maintain the functions of more vital tissues. On the other hand, if too much protein is supplied in the diet, only part of it will be used to make new proteins, and the remainder will be converted to energy.

Amino acids are building blocks for protein. On the basis of needs from diet for growth, amino acids were traditionally classified as nutritionally essential (indispensable) or nonessential (indispensable) for fish. Essential amino acids are those that either cannot be synthesized or are inadequately synthesized de novo by animals relative to needs. Conditionally essential amino acids must be provided from the diet under conditions where rates of utilization are greater than rates of synthesis. By definition, all nonessential amino acids can be synthesized adequately by aquatic animals.

1.3. The importance of protein and amino acid studies in fish

1.3.1. Comparison of nutrition in fish and other farmed anirnals

Before attempting studies on the requirements for any essential nutrients, factors difierentiating the nutrition of fish from homoeothermic species must be recognized. Firstly, fish are poikilotherrnic animals whose body temperatures change with that of the water in which they live. This factor has nutritionally important implications because, within their species specific temperature tolerance zone, fish do not use energy to maintain body temperature, as do homoeothermic species.

Secondly, because of their aquatic living environrnent, fish continuously expel the

by-products of protein catabolism directly into the ambient water, primarily through passive diffusion of ammonia through the gills. It is estimated that fish excrete 70 to 90 percent of nitrogenous wastes directly into the water, with little or no energy expenditure (Cho and Kaushik, 1990). As a result of this function, ammonia does not accumulate to toxic levels in body tissues, thereby reducing the need for urea or uric acid production prior to nitrogen excretion, as occurs in homeothermic animals. Protein is required for the basic functions of providing essential amino acids and nitrogen for the body. Early work by DeLong et al. (1958) found the protein requirements of fish to be two to four times higher than those for birds and mammals, when expressed as a percentage of the dry diet. Generally fish need two to three times more protein as a percentage of their diet than terrestrial farmed species such as poultry and swine. Tacon and Cowey (1985) attributed this high requirement to the largely omnivorous or carnivorous feeding habits of many aquatic species, and their preferential use of protein for energy needs. Lipids will spare proteins as an energy source, but carbohydrates are poorly utilized by salmonids (Wilson, 1989b). They secrete insufficient enzymes to break down complex carbohydrates, such as raw starch, and their digestive tract is too short, and gut clearance times too rapid, to accommodate prolonged retention for more efficient utilization (Goddard, 1996). Excessive dietary carbohydrate will result in the accumulation of glycogen in the liver.

Amino acid oxidation makes a significant contribution to the generation of metabolic energy (ME) (Lehninger et al., 1993). The fraction of ME derived from amino acids varies greatly with the type of organism and the metabolic situation, but this contribution must be recognized when studying amino acid requirements since

more than 50% of ingested protein is likely used as a primary energy source (Cho, 1992). Immediately following a meal, carnivores may obtain up to 90% of their energy requirement from amino acid oxidation (Lehninger et al., 1993). When a protein-rich diet provides a surplus of amino acid that cannot be utilized for protein synthesis the excess is catabolized because it cannot be stored. The carbon skeletons of histidine and methionine are channelled into the chic acid cycle via α-ketoglutarate and succinyl-CoA respectively and then, like all glucogenic amino acids, they are used in the liver to produce glucose for immediate energy or glycogen, the storage form of carbohydrate in animals. Tissues which rely on glucose as a fuel produce energy largely by gluconeogenesis, with protein providing the principle source of gluconeogenic precursors (NaIton and Cowey, 1982).

Fourthly, the difference between aquatic and terrestrial rearing environments provides practical difficulties in quantifying feed intake, and hence feed conversion, in fish. Unlike terrestrial species, when fish are fed there is an immediate loss and deterioration of unconsumed feed that makes accurate determination of feed consumption very difficult. These factors give an indication of the challenges associated with nutrient requirement studies in fish that are not usually implicated in studies with terrestrial, homoeothermic species. Nutritionists have, nonetheless, established guidelines for the nutrient requirements of a remarkable number of the species that are presently cultured on a commercial basis, worldwide.

1.3.2. Protein for the growth and maintenance of fish

Proteins are probably the most important nutritional contributors to growth,

because they provide the necessary amino acids for maintenance and increase of muscle mass, which takes place during growth and development (Peragon et al., 1994). Dietary amino acids are necessary for both growth and maintenance, with growth being quantitatively more important in Young, rapidly growing fish. Growth is largely a process of protein deposition, and this is determined by the protein or amino acid supply in the diet (Cowey, 1993; Cowey, 1994), as well as the post-prandial availability of these dietary components (Garzon et al., 1994). Protein deposition is the sum of two continuous processes, synthesis and degradation. Increased rates of growth from protein deposition result when there is an acceleration of synthesis faster than degradation (Cowey, 1993). Much of the protein being laid down as tissue protein during growth is composed of relatively large amounts of a small number of different proteins such as actin, myosin, collagens, etc. The amino acids deposited during growth are also basically the same in different species (Cowey, 1994). Cho et al. (1992) noted that phylogenetically distant species exhibit relatively similar amino acid requirements, despite widely divergent protein requirements.

Cells are in a dynamic state, with their components undergoing constant degradation and resynthesis. Amino acids are withdrawn from body pools for this maintenance, which includes synthesis of proteins, nucleic acids, and lesser components of cells. Maintenance includes replacing losses from the intestine and integument, oxidation of amino acids, conversion of amino acids to other nitrogen containing molecules, and protein turnover. Also a part of the maintenance aspect of protein nutrition is the fact that comparatively large amounts of ingested amino acids are deaminated and the carbon skeletons used as an energy source (Cowey, 1994). It is

important to note that protein turnover itself does not necessarily result in a net loss of amino acids from the body. Rates of protein turnover in fish muscle are very low, and as muscle accounts for about 60% of the weight of fish, whole body protein turnover is not likely to be a major factor in amino acid losses (Cowey, 1994).

The types of protein found in the body of fish are generally based on function or solubility (Lovell, 1989). Fibrous proteins are insoluble proteins including collagen, elastin, and keratin. Collagen is a component of connective tissues, bone, skin, fins, gill operculum, and blood vessels. Elastins are found in arteries, tendons, and other tissues that stretch. Keratins are found only in small amounts in fish. Contractile proteins are those found in muscle. Actin, myosin, and tropomyosin B are the three proteins involved in muscle contraction. Globular proteins are water extractable from tissue. They include enzymes, protein hormones, and proteins of the soluble fraction of blood (serum) (Lovell, 1989).

Although, like other animals, fish do not have a protein requirement per se, they need a well-balanced mixture of essential amino acids and a source of non-essential amino acids, and must consume protein to obtain these amino acids (Wilson and Halver, 1986). Cowey and Sargent (1972) determined that of the twenty amino acids found in protein, fish require the following ten essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. An absolute requirement for these ten essential amino acids has been found in all fish species studied to date. An essential amino acid is one that cannot be synthesized in the body, from materials ordinarily available to the cells, at a rate to meet the requirements for optimum protein synthesis, it must, therefore, be included in the diet (Maynard et al.,

1979; Cho et al., 1984; Bender, 1985). Nonessential amino acids can be eliminated from the diet as long as there is an adequate supply of organic nitrogen, since they can be synthesized in adequate amounts from common metabolic intermediates (Bender, 1985).

1.4. Amino acid metabolism

Amino acids have numerous functions in fish. The most familiar role amino acids play is as the building blocks of proteins. Protein synthesis can account for a substantial portion (20 – 42%) of the energy expenditures of growing fish (Houlihan et al., 1993, 1995). Most (85%) fish species are carnivorous (Love, 1980) and optimal growth requires very high dietary protein levels (30 –55% crude protein) (Wilson, 1989). Fish display an efficiency of conversion of dietary protein to tissue protein that is up to 20-fold higher than that observed for chickens, pigs, and cattle (Tacon and Cowey, 1985). The efficiency of conversion of dietary protein into tissue protein is somewhat paradoxical given that fish rely extensively on amino acids as energy sources and thus burn the precursors for proteins. Several hypotheses have been put forward to explain this. Certainly, the direct excretion of ammonia as the end product of nitrogen metabolism is more energetically favorable than expending energy to convert it to less toxic forms such as urea or uric acid. The direct oxidation of dietary amino acids also avoids the energetic expense of synthesizing storage molecules such as glucose and lipids for subsequent use.

In energetic terms, a major function of amino acids is as catabolic substrates to provide ATP for biomechanical, synthetic, and transport processes. Amino acids provide

14 –85% of the energy requirements of teleost fish (van Waarde, 1983) and 10 – 40% in nonteleost species such as sturgeon (Dabrowski et al., 1987). In rainbow trout, 35– 40% of a representative amino acid such as leucine is oxidized while the remainder is converted to protein. This is a substantially higher rate of catabolism than in mammals (20%) (Fauconneau and Arnal, 1985).

The role of one amino acid in particular, glutamine, differs in fish compared to mammals. Glutamine does not serve as a nitrogen store for ammonium under normal conditions in fish as it does in mammals, and circulating levels of this amino acid are therefore lower in fish. Synthesis of glutamine in tissues, such as muscle, for export to other tissues also does not occur in fish. These differences in glutamine metabolism have a substantial impact on the metabolism of other amino acids in fish and are discussed in more detail in subsequent sections. Despite many years of research on the metabolism of fishes, little is known of the factors regulating the flow of amino acids into catabolic or anabolic pathways. It has been suggested that some of the partitioning of amino acids toward growth rather than catabolism in fish may be due to the 10-fold lower Michaelis constant (Km) for amino acids of aminoacyl tRNA synthetases compared to the Km of the transaminases that funnel amino acids into oxidative pathways (Walton, 1985). This hypothesis has not been tested and the Km for any tRNA synthetases has not been measured in fish. In spite of this, the pool size of free amino acids has been suggested to play an important role in regulating protein synthesis (Houlihan et al., 1993).

Most previous reviews of amino acid metabolism in fish have focused primarily on the applied nutritional aspects as they relate to aquaculture (Tacon and Cowey, 1985;

Walton, 1985; Cowey and Walton, 1989; Wilson, 1989; Cowey, 1993). More recently Jurss and Bastrop (1995) reviewed other aspects of fish amino acids metabolism. This current review examines some aspects of the intermediary metabolism of amino acids in different tissues of fish with particular reference to the factors influencing and controlling the pathways involved.

1.4.1. Digestion and uptake of amino acids

Proteolysis is initiated in the stomach by pepsin, with further hydrolysis of peptides and aminopeptides taking place in the lumen of the intestine by the action of trypsin, chymotrypsin, and leucine aminopeptidase. In the rabbitfish all of these enzymes have alkaline pH optima, as in mammals (Sabapathy and Teo, 1995).

Amino acids are taken up from the gut as free amino acids or as dipeptides. Dipeptides are transported independent of cation gradients with hydrolysis to their component amino acids taking place immediately after transport (Reshkin and Ahearn, 1991). Plasma free amino acid levels peak about 12 h after feeding and then return to prefeeding values (Murai et al., 1987; Navarro et al., 1997).

Adaptation of the intestinal transport system to different dietary levels of amino acids is known to occur in fish. For example, the Km for the transporters of amino acids in the guts of herbivorous and omnivorous fishes is lower than those for carnivores, presumably an adaptation to the lower gut levels of amino acids (Ferraris and Ahearn, 1984). Four sodium-dependent amino acid carrier systems have been identified in the intestinal brush-border membrane of the eel: (1) a carrier for the cationic amino acids, lysine and arginine; (2) an anionic carrier for glutamate and aspartate; (3) a carrier for

proline and N-methylated amino acids; and (4) a neutral amino acid carrier for alanine, glycine, serine, cysteine, and others (Storelli et al., 1989).

Transfer of absorbed amino acids to the blood side occurs at the basolateral surface of intestinal cells. The basolateral membrane has at least three amino acid carriers (Reshkin et al., 1988; Collie and Ferraris, 1995). Alanine, lysine, and phenylalanine are transported by sodium-independent carriers (Reshkin et al., 1988), proline and glutamate transport occur by the same sodium-dependent carrier, and glycine transport uses the outward potassium gradient and inward sodium gradient (Reshkin et al., 1988). Due to the direction of the electrochemical gradients for sodium and potassium, these carriers likely function to bring amino acids into the cell rather than the reverse (Reshkin et al., 1988). This may be necessary to provide intestinal cells with synthetic precursors and oxidative substrates between periods of feeding. Future research should be directed toward understanding the regulation and coupling of amino acid transport and metabolism in the intestine.

1.5. Impacts of aquaculture expansion on aquafeed industry

The expansion of aquaculture production has been accompanied by rapid growth of aquafeed production. The challenge facing the aquaculture industry is to identify economically viable and environmentally-friendly alternatives to fish meal and fish oil on which many present aquafeeds are largely based. While the supply of fish meal and oil is arguably sustainable, the anticipated growth in demand internationally for use in aquafeeds is expected to exceed the supply in the next decade. Thus, the aquafeeds industry has recognized for many years that viable utilization of plant feedstuffs

formulated in aquafeeds for the production of cold, cool and warmwater aquatic species is an essential requirement for future development of aquaculture. Such plant feedstuffs must provide nutritious diets that will effectively grow aquatic species with minimal environmental impact and produce high quality fish flesh to confer human health benefits in a cost-effective manner. As the aquaculture industry continues to expand on a global scale, access to key feedstuffs, such as fish meal and oil, will become increasingly limited because of a finite wild-harvest resource. In addition to concerns about the sustainability of fisheries resources, other issues including the potential presence of organic and inorganic contaminants in fish meal and the net effect of demand-and-supply economics in the global market require enhanced efforts to thoroughly evaluate reasonable alternatives such as various plant feedstuffs.

1.6. Developing low or non-fish meal diets

One of the major issues affecting the aquaculture industry is the availability of protein-rich feedstuffs. Under intensive fish farming conditions, fish meal and fish oil are the most common feedstuffs supplying the essential nutrients (amino acids, fatty acids, minerals and trace elements) vital for growth, health, reproduction and physiological well-being of farmed fish. While the marine capture fisheries remains constant, the demand for such feedstuffs derived from capture fisheries is on the increase and the costs are rocketing. In this context, replacement of fishmeal by alternative protein sources remains a major thrust area of research and much has been accomplished in reducing the level of fishmeal in all species (Gatlin et al., 2007; Lim et al., 2008). Fishmeal is unique in that it is not only an excellent source of high quality

protein having an ideal amino acid profile for fish and shrimp. Fish meal is also a good source of essential fatty acids, minerals and trace elements. In choosing alternatives to fish meal, it is then necessary to look at the amino acid profile, but also at other macro and micronutrients.

From a quantitative point of view, efficiency of protein utilization and muscle protein growth are the most crucial issues. Although fish are generally considered better converters of dietary protein, compared with terrestrial vertebrates, given the global context of rapid development of aquaculture and the increasing costs and dearth of protein-rich feedstuffs, there is an impending necessity for improvements in dietary protein utilization, achievable only by optimising dietary supply in tune with the different physiological needs of organisms. This then necessitates a full understanding of the physiological basis for the requirements and efficient exploitation of available sources to meet such needs.

1.7. Plant proteins as potential protein alternatives

Fish meal has been the protein source of choice in aquafeeds for many reasons, including its high protein content, excellent amino acid profile, high nutrient digestibility, general lack of antinutrients, relative low price (until recently) and its wide availability. To reach a sustainable aquaculture, new alternative protein sources are needed to be introduced for stable aquafeed production (Higgs et al., 1995). It is suggested that the increased use of plant proteins in fish diets can reduce the cost of fish feeds (Lim and Lee, 2009). Plant-derived feedstuffs all have some characteristics that place them at a disadvantage to fish meal in terms of their suitability for use in

aquafeeds. However, demand for protein ingredients is expected to exceed the annual world supply of fish meal in the next decade, and this increased demand will change the economic and nutritional paradigms that up to now have resulted in high use levels of fish meal in aquafeeds.

To be a viable alternative feedstuff to fish meal in aquafeeds, a candidate ingredient must possess certain characteristics, including wide availability, competitive price, plus ease of handling, shipping, storage and use in feed production. Furthermore, it must possess certain nutritional characteristics, such as low levels of fiber, starch, especially non-soluble carbohydrates and antinutrients, plus have a relatively high protein content, favourable amino acid profile, high nutrient digestibility and reasonable palatability. Although some plant-derived ingredients, such as soy protein concentrate or wheat gluten, possess most of these characteristics, they have been too expensive relative to the price of fish meal to be used in most aquafeeds. It is likely that a combination of plant-derived feed ingredients will be required to replace fish meal, and that supplements, such as amino acids, flavourings and possibly exogenous enzymes, will be needed to produce aquafeeds without fish meal that support growth rates necessary for the economic production of farmed fish.

1.8. Importance of amino acid requirement studies

To reach a sustainable aquaculture, new alternative protein sources including cheaper plant or animal origin proteins are needed to be introduced for stable aquafeed production (Higgs et al., 1995). It is suggested that the increased use of plant proteins in fish diets can reduce the cost of fish feeds (Lim and Lee, 2009). However, most plant

proteins have a secondary AA profile compared to fish meal and do not meet the EAA requirement of fish, hence crystalline amino acids are being supplemented to the diet to cover the EAA requirements (Fournier et al., 2003; NRC, 2011). Regarding this issue, knowledge of dietary EAA requirement of fishes is necessary for the formulation of diets with higher protein utilization efficiency and reduced nitrogen excretion.

1.9. Qualitative Amino Acid Requirements

The first successful amino acid test diet for fish was developed by Halver (1957). He developed his initial test diet based on previous amino acid test diets used in determining the amino acid requirements of young albino rats. Halver (1957) compared test diets containing 70% crystalline L-amino acids formulated based on the amino acid patterns of whole chicken egg protein, chinook salmon egg protein, and chinook yolk sac fry protein. The test diet based on whole chicken egg protein gave the best growth and feed efficiency for chinook salmon for a 12-week period. Therefore, this test diet was used to determine the qualitative amino acid needs of Chinook salmon (Halver et al., 1957). These workers determined the essentiality of the 18 common protein amino acids by comparing the relative growth rates of chinook salmon fed the basal and the specific amino acid-deficient diets over a 10-week period. The results indicated that the following 10 amino acids were indispensable for chinook salmon: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. All other species that have been studied to date have been shown to require the same 10 amino acids.

1.10. Quantitative Amino Acid Requirements

Most investigators have used the method developed by Halver and co-workers (Mertz, 1972) to determine the quantitative amino acid requirements of fish. This procedure involves feeding graded levels of one amino acid at a time in a test diet containing either all crystalline amino acids or a mixture of casein, gelatin, and amino acids formulated so that the amino acid profile is identical to that of whole chicken egg protein except for the amino acid being tested. This procedure has been used successfully with several species, however, the amino acid test diets must be neutralized with sodium hydroxide for utilization by carp (Nose et al., 1974) and channel catfish (Wilson et al., 1977).

Other investigators have used semipurified and practical diets supplementedwith crystalline amino acids to estimate the amino acid requirements of certain fish. The semipurified diets have usually included an imbalanced protein as the major source of the dietary amino acids, e.g., zein (Kaushik, 1979) or corn gluten (Halver et al., 1958; Ketola, 1983), which are deficient in certain amino acids. Practical-type diets utilize normal feed ingredients to furnish the bulk of the amino acids. These may be formulated with a fixed amount of intact protein, and the remaining protein equivalent is made up of crystalline amino acids (Luquet and Sabaut, 1974; Jackson and Capper, 1982;Walton et al., 1984a). The various problems inherent in using these types of diets to assess the amino acid requirements of fish have been discussed elsewhere (Wilson, 1985).

1.10.1. Growth Studies

Most of the amino acid requirement values have been estimated based on the

conventional growth response curve or Almquist plot. Replicate groups of fish are fed diets containing graded levels of the test amino acid until measurable differences appear in the weight gain of the test fish. A linear increase in weight gain is normally observed with increasing amino acid intake up to a break point corresponding to the requirement of the specific amino acid, at which the weight gain levels off or plateaus.

Various methods have been used to estimate or calculate the break point corresponding to the requirement value based on the weight gain data. The requirement values for chinook salmon (reviewed byMertz, 1972), common carp, and Japanese eel (Nose, 1979) were estimated using an Almquist plot without the aid of any statistical analysis, whereas others have used regression analysis to generate the Almquist plot (Harding et al., 1977; Akiyama et al., 1985a). Wilson et al. (1980) used the continuous broken-line model developed by Robbins et al. (1979) to estimate the requirement values. Santiago and Lovell (1988) used both the broken-line model and quadratic regression analysis to estimate the requirement values for Nile tilapia based on weight gain data. Quadratic regression analysis resulted in the lowest error term for estimating the requirement values, whereas the broken-line model yielded the lowest error term for only three requirement values.Most of the requirement values that have been reported within the last 10 years have been estimated based on the broken-line model.

1.10.2. Serum or Tissue Amino Acid Studies

Some investigators have found a high correlation of either serum or blood and muscle free amino acid levels with dietary amino acid intake in fish. The hypothesis is that the serum or tissue content of the amino acid should remain low until the

requirement for the amino acid is met and then increase to high levels when excessive amounts of the amino acid are fed.

This technique has proven useful in confirming the amino acid requirements in only a few cases. For example, of the 10 indispensable amino acid requirement studies in the channel catfish, only the serum lysine (Wilson et al., 1977), threonine (Wilson et al., 1978), histidine (Wilson et al., 1980), and methionine (Harding et al., 1977) data were useful in confirming the requirement values estimated based on weight gain data. Serum methionine data on sea bass (Thebault et al., 1985) and serum lysine of hybrid striped bass (Griffin et al., 1992) have been used to confirm the requirement values for these species. Blood and muscle arginine concentrations were found to increase gradually in rainbow trout fed increasing levels of arginine and were not useful for assessing the arginine requirement of this species (Kaushik, 1979). Walton et al. (1984b) were unable to use blood tryptophan levels to confirm the tryptophan requirement of rainbow trout. Of the 10 amino acids required by Nile tilapia, Santiago and Lovell (1988) were able to use only the muscle free lysine, threonine, and isoleucine concentrations to confirm the requirement values for these amino acids based on growth studies.

1.11. Classification of amino acids

Amino acids may be grouped according to their transport affinities or essential role in animal nutrition or on the basis of catabolic fate of the carbon skeleton. Additional subsets are also recognized, based on common structural features. Thus, leucine, isoleucine and valine are referred to as the branched-chain amino acids (BCAA),

whereas phenylalanine and tyrosine are categorized as the aromatic amino acids. Assessments of requirements for sulphur amino acids (SAA) are invariably based on a summation of methionine and cyst(e)ine needs, but it will be recalled that homocysteine is another member of this group. Another subset, the excitatory amino acids, includes glutamate and aspartate.

1.11.1. Branched-chain amino acids

Branched-chain amino acids (BCAAs) are essential amino acids whose carbon structure is marked by a branch point. The BCAAs are valine, isoleucine and leucine having aliphatic side chains that are nonlinear.

These three EAAs play important structural roles and are primarily deposited in body protein, notably in skeletal muscles (Cowey and Walton, 1989). 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. 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 signaling 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). 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. 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, 2006).

1.11.2. Arginine

Arginine has been established as an essential amino acid in diets of many fish species (NRC, 1993; Cowey, 1994). It is most limiting amino acid in plant protein sources such as corn, sesame and Zein meal (Mai et al., 1994; Berge et al., 1997; Luo et al., 2007). It is precursor of several biologically important metabolites, including nitric oxide (NO), polyamines and creatine (Wu and Morris, 1998; Galli, 2007; Grillo and Colombatto, 2007). Also, it participates in several metabolic pathways including protein synthesis, urea production and metabolism of glutamic acid and proline (Hird, 1986).

1.11.3. Methionine

Methionine is the sulphur-containing amino acid required by terrestrial vertebrates as well as various fish species for normal growth and metabolic functions. Methionine takes part in protein synthesis and other important physiological functions (Lovell, 1989). The essential amino acid methionine is important because it is used for protein synthesis, converted to cystine for incorporation into protein, and in the form of S-adenosyl-methionine, it is the principal donor of methyl groups in the body. As S-adenosyl methionine, methionine is the precursor of the polyamines spermine and spermidine which have diverse physiological role related to cell proliferation and growth (Murray et al., 1996). Methionine is also a precursor of choline and Kasper et al. (2000) reported that when methionine is not in excess in the diet of Nile tilapia, Oreochromis niloticus, choline is required for growth. Methionine is usually the first limiting amino acid in many fish diets, especially those containing higher levels of plant protein sources such as soybean meal, peanut meal and copra meal (Tacon and Jackson, 1985; Dabrowski et al., 1989). The study of sulphur amino acid nutrition in fish has become increasingly important as dietary formulations incorporate lower levels of fish meal and higher levels of plant feedstuffs (Twibell et al., 2000). Studies have demonstrated that supplementation of methionine to plant protein diets is able to improve growth response of many fish species (Mukhopadhyay and Ray, 2001; Takagi et al., 2001; Opstvedt et al., 2003). In fish both excess and restricted dietary methionine content have been reported to affect growth performance, feed intake and carcass quality (Jackson and Capper, 1982; Rumsey et al., 1983; Mambrini et al., 2001; Sveier et al., 2001). Also, cataracts were observed in several salmonid species fed with

methionine-deficient diets (Walton et al. 1982; Rumsey et al. 1983; Keembiyehetty and Gatlin, 1993). Thus, to maximize the utilization of cheap plant and animal protein sources, especially those that have low methionine levels, the determination of methionine requirement in semipurified diets and its application to practical diets are important for the development of cost-effective feed.

1.12. Utilization of peptides in formulated diets in fish

Young teleosts can obtain dietary amino acid in the form of protein-bound, free amino acids (FAA) or peptides. When peptides or FAA are the major amino acid sources in the diets, absorption can be completed in the intestine bypassing the digestion by proteases secreted in the stomach and from pancreas to the intestine. In mammals, amino acids are mainly taken up and released from the gut by the Na+-dependent amino acid transport system or the Na+-independent amino acid transport system located in the intestine mucosal cells. Peptides have different transport systems from free amino acids. The mammalian peptide transporter, oligopeptide transporter (Pept-1), is also abundantly expressed in larval zebrafish *(Danio rerio)* (Verri *et al.* 2003). Studies with larvae of several fish species showed faster absorption of small peptides and FAA compared with protein if injected into the digestive tract prior to their metamorphosis (Rust *et al.* 1993; Ronnestad *et al.* 2003). Therefore, it seems that free amino acids and small peptides are promising dietary amino acids sources in the formulated diets for teleost fish at their early life stages.

Compared with FAA-based or protein based diets, peptide-based diets may play a positive role in the development of the brush border enzyme expression and gut

differentiation in young fish (Zambonino *et al.* 1997, Cahu *et al.* 2001). Aragao et al. (2004) showed that retention of amino acids was improved when *Artemia*-fed postlarval Senegal sole was supplemented with dipeptides through direct meal intubation. More advantages of peptide inclusion in common carp diets were earlier found by Carvalho *et al.* (1997) who reported that protein hydrolyzates (peptides) can be effectively utilized in this species for growth. A study conducted by Dabrowski et al. (2003) showed that synthetic dipeptides-based diet sustained the high growth rate of rainbow trout alevins and juveniles. Espe *et al.* (1999) showed that muscle protein synthesis in Atlantic salmon decreased 1.5-2.0 folds and the growth rate significantly decreased with an increase in proportion of small peptides. In contract, more recent studies conducted by Aragao *et al*. (2004) showed that dipeptides could improve dietary amino acid balance in comparison to free AA in juvenile Senegal sole. Terjesen et al. (2006) reported that a diet with 1:1 ratio of protein to synthetic dipeptides resulted in better performance by rainbow trout alevins than an entirely dipeptide-based diet, and comparable to a protein-based diet in terms of growth rate, survival and muscle indispensible free amino acid levels.

1.13. Chapter Justification

The aims of this dissettation are to (1) determine the essential amino acid requirements of juvenile red sea bream (chapters 2 and 3) and olive flounder (chapters 4 and 5) (2) to compared the utilization efficiency of two different forms of amino acids including free and dipeptide forms in terms of growth performance and the concentrations of whole-body amino acids (chapter 6).

The justification of each chapter is as follows;

Chapter 2 is about the evaluation of dietary valine requirement of juvenile red sea bream. In this study the valine requirement level was estimated based on fish growth performance and the effects of dietary valine level was examined on fish hematology, innate immunity and protein utilization.

Chapter 3 was conducted to determine dietary arginine requirement of juvenile red sea bream and to assess the arginine effects on hematological and innate immune parameters.

Chapter 4 focuses on the effects of varying dietary isoleucine levels on growth performance, hematology and non-specific immune response of juvenile olive flounder.

Chapter 5 focuses on dietary methionine requirement of juvenile olive flounder. In this study, fish growth performance and whole-body amino acid composition were examined.

Finally, chapter 6 was carried out to compare the growth performance and whole-body amino acid composition of juvenile olive flounder fed free or dipeptide form of lysine.

CHAPTER TWO

Dietary valine requirement of juvenile red sea bream *Pagrus major*

2.1. Introduction

Protein is the most expensive dietary component and nutrient affecting fish growth performance. The knowledge of fish protein requirement is of limited value without essential amino acid (EAA) requirement data. Amino acids (AA) are not only the building blocks of proteins but also they serve as key regulators of major metabolic pathways (Meijer, 2003; Jobgen et al., 2006). Determination of EAA requirement of cultured fish is extremely important for formulation of well-balanced, low-cost and environmental-friendly diets due to their significant effects on muscle growth in fish and nitrogen pollution to water (Small and Soares, 1999). Deficiency of most EAA results in a reduced weight gain, poor feed conversion, loss of appetite and lower disease resistance (Cowey, 1979). Formulation of diets that meet the EAA requirement of fish but do not exceed an optimum level is important, as excessive levels of EAA lead to higher ammonia excretion (Cai et al., 1996; Yang et al., 2002).

To reach a sustainable aquaculture, new alternative protein sources including cheaper plant or animal origin proteins are needed to be introduced for stable aquafeed production (Higgs et al., 1995). It is suggested that the increased use of plant proteins in fish diets can reduce the cost of fish feeds (Lim and Lee, 2009). However, most plant proteins have a secondary AA profile compared to fish meal and do not meet the EAA

requirement of fish, hence crystalline amino acids are being supplemented to the diet to cover the EAA requirements (Fournier et al., 2003; NRC, 2011). Knowledge of dietary EAA requirement of fishes is necessary for the formulation of diets with higher protein utilization efficiency and reduced nitrogen excretion.

Branched-chain amino acids (BCAA) (valine, isoleucine, leucine) play important roles in protein synthesis and inhibiting protein degradation (Holecek et al., 2001; Shimomura et al., 2006). Valine has been identified as an EAA for fish (Nose, 1979). It is involved in different metabolic pathways and is necessary for growth, tissue repair and maintenance of nitrogen balance in the body. Valine deficiency has resulted in a reduced growth performance of common carp (Nose, 1979) and Mrigal carp (*Cirrhinus mrigala*) (Ahmed and Khan, 2006). The valine requirements have been determined for common carp (*Cyprinus carpio*) (Nose, 1979), channel catfish (*Ictalurus punctatus*) (Wilson et al., 1980), lake trout (*Salvelinus namaycush*) (Hughes et al., 1983), Nile tilapia (*Oreochromis niloticus*) (Santiago and Lovell, 1988), rainbow trout (*Oncorhynchus mikiss*) (Rodehutscord et al., 1997), rohu carp (*Labeo rohita*) (Abidi and Khan, 2004a), Mrigal carp (*Cirrhinus mrigala*) (Ahmed and Khan, 2006) and Jian carp (*Cyprinus carpio* var. Jian) (Dong et al., 2012).

Red sea bream is a high value commercial marine cultured fish in Korea and Japan. This species is strictly carnivorous and is generally fed with trash fish or formulated diets with high fish meal content (Huang et al., 2007). The demand for red sea bream has been grown tremendously for the last decade because it is a high-quality sashimi grade fish with high market value. Extensive research has been conducted to determine the basic nutritional and husbandry requirements for this species. However, quantitative

estimate of EAA requirement of the fish is limited to lysine (Forster and Ogata, 1998). To our knowledge, there is no available data on dietary valine requirement of red sea bream. Therefore, the present study was conducted to determine valine requirement of red sea bream. EAA requirement is generally determined on the basis of growth rates using diets with graded levels of the EAA under study (Wilson et al., 1978; Twibell et al., 2000; Abidi and Khan 2004a,b; Ahmed et al., 2004). Hematological parameters have been regarded as valuable means of determining the health status of fish, as they are supposed to be more sensitive to dietary manipulations. Accordingly, in the present study the changes in blood parameters were examined to confirm the optimum dietary valine requirement level for red sea bream.

2.2. Materials and methods

2.2.1. Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 2-1. Six isonitrogenous (45% protein) and isocaloric (4.38 kcal g^{-1} gross energy) diets were formulated using fish meal and a mixture of crystalline AA to contain graded levels of L-valine (0.27 – 2.38% dry diet). Fish meal was included to increase palatability of the semi-purified diets. The mixture of crystalline AA without valine was prepared according to Dabrowski et al. (2003) and used as the main protein source. The basal diet contained a minimum level of valine from fish meal and supplemented with incremental levels (0.4%) of L-valine. Targeted dietary valine concentrations were 0.27, 0.79, 1.22, 1.69, 2.04 and 2.38%. The experimental diets were kept isonitrogenous by decreasing the level of glycine as the valine level was increased. The energy value of each diet was estimated on the basis of physiological fuel value, i.e., 3.99 kcal g^{-1} proteins or carbohydrates and 9.01 kcal g^{-1} lipids (Lee and Putnam, 1973). The pH of the experimental diets was adjusted to 7.0 – 7.5 by adding 4 N NaOH solution. Amino acid composition of fish meal and the experimental diets were determined using an automated amino acid analyzer (Beckman 7300, Beckman Instruments, Palo Alto, CA). Concentrations of the essential and non-essential amino acids in the experimental diets are shown in Table 2-2. All dry ingredients were thoroughly mixed and after addition of squid liver oil and double distilled water extruded through a meat chopper machine (SMC-12, Kuposlice, Korea) in 3 mm diameter. Then, the diets were freeze-dried for 24 h, crushed into desirable particle sizes, sealed in bags and stored at –20 ^{o}C until used.

Table 2-1. Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	**1**	**2**	**3**	**4**	**5**	**6**
White fish meal	13.0	13.0	13.0	13.0	13.0	13.0
Free AA mix[1]	34.5	34.5	34.5	34.5	34.5	34.5
Valine	0.0	0.4	0.8	1.2	1.6	2.0
Glycine	2.0	1.6	1.2	0.8	0.4	0.0
Dextrin	32.0	32.0	32.0	32.0	32.0	32.0
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[2]	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix[3]	2.0	2.0	2.0	2.0	2.0	2.0
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Dry matter (DM)	94.0	94.0	94.0	92.7	93.0	94.0
In DM:						
Protein	45.9	45.6	46.0	45.6	46.0	46.1
Lipid	8.7	8.5	8.7	8.9	8.9	8.9
Ash	3.8	3.5	3.7	3.6	3.4	3.5

[1]Free amino acid mixture (g kg^{-1} of mixture): arginine hydrochloride, 33.6; lysine hydrochloride, 40.4; methionine, 22.4; histidine, 15.7; isoleucine, 20.2; leucine, 31.4; phenylalanine, 40.4; threonine, 17.9; tryptophan, 4.5; alanine, 248.9; glutamic acid, 248.9; glycine, 275.8.

[2]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3]Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin,

36.4; Ca-$_{D}$-pantothenate, 12.7; myo-inositol, 181.8; $_{D}$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

Table 2-2. Analyzed amino acid composition of the experimental diets (% dry diet).

	1	2	3	4	5	6
EAA[1]						
Arg	1.34	1.36	1.40	1.32	1.41	1.36
His	0.74	0.71	0.67	0.76	0.70	0.69
Ile	0.87	0.82	0.79	0.80	0.84	0.78
Leu	1.22	1.20	1.19	1.22	1.18	1.21
Lys	1.61	1.64	1.70	1.65	1.64	1.68
Met	0.89	0.80	0.82	0.84	0.78	0.84
Phe	1.64	1.62	1.66	1.64	1.71	1.70
Thr	0.87	0.84	0.84	0.82	0.90	0.86
Val	0.27	0.79	1.22	1.69	2.04	2.38
NEAA[2]						
Ala	9.20	9.32	9.40	9.12	9.28	9.24
Asp	1.20	0.96	1.10	1.13	1.18	0.97
Cys	0.13	0.09	0.11	0.10	0.14	0.11
Glu	10.30	10.25	10.21	10.34	10.26	10.30
Gly	12.81	12.32	11.91	11.45	11.07	10.65
Pro	0.62	0.61	0.69	0.60	0.59	0.68
Ser	0.76	0.69	0.74	0.72	0.68	0.70
Tyr	0.34	0.32	0.30	0.31	0.34	0.32

[1]Essential amino acids

[2]Non-essential amino acids

2.2.2. Fish and feeding trial

Juvenile red sea bream were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). The health status of fish was checked upon arrival, and they were quarantine bathed with 100 mg l^{-1} formalin for 20 min. All the fish were fed the basal diet for one week to be acclimated to the semi-purified diet and the experimental conditions. At the end of the acclimation period, 30 randomly selected fish (averaging at 32.04 ± 0.2 g) were stocked into each polyvinyl circular tanks of 150 L capacity and supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed with one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 12 weeks. Uneaten food was siphoned out 30 min after feeding and weighed to determine the feed intake. Growth of fish was measured with three-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish. The water temperature during the feeding trial ranged from 19 to 23 °C and the photoperiod was maintained on a 12:12 light:dark schedule.

2.2.3. Sample collection and analyses

At the beginning of the experiment 10 fish were sampled and stored at –20 °C for analysis of initial whole-body proximate composition. At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Three intact fish per tank (9 fish per dietary treatment) were selected and kept at −20 °C for whole-body proximate composition analysis. Three another fish per tank were randomly captured, anesthetized with 2-phenoxyethanol (200

mg l^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for determination of hematocrit, hemoglobin and respiratory burst activity. After the above mentioned measurements with whole blood, plasma were separated by centrifugation at 5000 × g for 10 min and stored at −70 °C for determination of immunoglobulin (Ig) level and blood biochemical parameters including plasma total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose and cholesterol. Another set of blood samples (3 fish per tank) were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation for 10 min at 5000 × g and stored at −70 °C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase (MPO), superoxide dismutase (SOD) and antiprotease activities.

Analyses of moisture and ash contents of samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea). Hematocrit was determined by microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of total protein, glucose and cholesterol and activities of ALT and AST were determined by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

Oxidative radical production by phagocytes during respiratory burst was measured through NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Briefly, blood and NBT (0.2%) (Sigma, St. Louis, MO, USA) were mixed in equal proportion (1:1) and incubated for 30 min at room temperature. Then 50 µl was taken

out and dispensed into glass tubes. One ml of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as the blank.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein concentration was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules using a 12% solution of polyethylene glycol (Sigma). The difference in protein concentration represents the Ig content.

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200 µl of suspension was placed in each well of 96-well plates, and 20 µl serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340, Biochrom, Cambridge, UK). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

MPO activity was measured according to Quade and Roth (1997). Briefly, 20 µl of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma, USA) in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 µl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in a microplate reader.

SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Serum antiprotease activity was measured according to the method described by Ellis et al. (1990), with slight modifications (Magnadóttir et al., 1999). Briefly, 20 µl of serum was incubated with 20 µl of standard trypsin solution (Type II-S, from porcine pancreas, 5 mg ml^{-1}, Sigma-Aldrich) for 10 min at 22 °C. Then, 200 µl of phosphate buffer (0.1 M, pH 7.0) and 250 µl azocasein (2%) (Sigma) were added and incubated for 1 h at 22 °C. Five hundred microliters of trichloro acetic acid (10%) was added and further incubated for 30 min at 22 °C. The mixture was centrifuged at 6000×*g* for 5 min and 100 µl of the supernatant was transferred to the wells of a 96 well flat bottomed microplate containing 100 µl of 1 N NaOH. Optical density was read at 430 nm. For a 100% positive control, buffer replaced the serum, while for the negative control buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

Trypsin inhibition (%) = $(A_1 – A_2/A_1) \times 100$

where A_1 = control trypsin activity (without serum); A_2 = trypsin activity remained after adding serum.

2.2.4. Ammonia excretion

At the end of the experiment, 10 fish per tank were randomly captured and stocked into 18 plastic rectangular tanks of 64 L capacity. The fish were acclimated to the tanks for 2 days and were fed the corresponding diets to apparent satiation. On the third day, the remaining diets and fecal materials were removed after the last feeding, water flow was stopped and ammonia excretion was measured after 2 and 24 h. The sampling times were considered as characteristic for postprandial and basal levels (Kaushik and Dabrowski, 1984; Dabrowski, 1986). The ammonia concentration was determined with a water analyzer (RQflex 10, Merck, Germany) using kits. The following equation was used for calculation of ammonia excretion:

Ammonia excretion = $\Delta N\text{-}NH_3 \times v / (m \times t)$

where the $\Delta N\text{-}NH_3$ is the change in total ammonia concentration during each test period; v is the water volume (l); m is the fish biomass (kg); and t is the test period (h).

2.2.5. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$. A broken-line regression model was used for determination of optimum dietary valine level based on fish weight gain and protein deposition. Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

2.3. Results

Fish growth performance was significantly ($P < 0.05$) affected by dietary valine levels (Table 2-3). Significantly higher weight gain (WG) and specific growth rate (SGR) were obtained by increment of valine level from 0.27 to 0.79%. Although higher growth rates were obtained at higher valine levels than 0.79%, the differences were not significant ($P > 0.05$). The lowest feed intake was recorded at minimum dietary valine level and significantly differed from that of the other groups. Feed conversion ratio (FCR) was significantly improved by the increment of dietary valine level ≥0.79%, but no further significance was found beyond the level. Protein efficiency ratio (PER) and protein deposition (PD) were significantly increased in fish fed ≥0.79% valine compared to those of fish fed the basal valine level. The optimal dietary valine requirement level was estimated at 0.90 and 0.99% in diet by the broken-line regression analysis on the basis of WG and PD, respectively (Figs. 2-1 and 2-2, respectively). Survival rate varied from 86 to 95% without significant differences among dietary treatments (Table 2-3).

Whole-body composition varied significantly among dietary treatments (Table 2-4). Moisture content significantly decreased as dietary valine level increased from 0.27 to 0.79%. Fish fed dietary valine levels of ≥0.79% exhibited significantly higher protein contents than fish fed the minimum valine level. Lipid content was increased by increment of valine level and the highest significant level was observed in fish fed 2.04% valine. Ash content did not significantly differ among treatments.

Hematocrit, hemoglobin and plasma glucose concentration as well as ALT activity were not significantly influenced by dietary valine levels (Table 2-5). Plasma total

protein level was increased in fish fed dietary valine levels of 1.22 - 2.04% compared to the fish fed 0.27% valine and thereafter decreased significantly with the additional supplementation (Table 2-5). Significantly lower AST and higher cholesterol levels were found in fish fed 2.04% valine compared to those fed the diet containing 0.27% valine.

Non-specific immune responses were positively affected by dietary valine levels (Table 2-6). Respiratory burst, MPO and lysozyme activities in fish fed the diet containing 2.04% valine were significantly higher than those of the fish fed the basal valine level. Plasma Ig level significantly increased in fish fed dietary valine levels of ≥1.22%. However, SOD and antiprotease activities were not significantly different among all the treatments.

Significantly lower postprandial ammonia excretion levels were obtained in the groups fed dietary valine levels of 1.69 and 2.04% in comparison to fish fed 0.27% valine (Fig. 2-3).

Table 2-3. Growth performance of red sea bream (initial body weight, 32.04±0.2) fed different levels of dietary valine for 12 weeks.

Valine level	FBW[1](g)	WG[2] (%)	SGR (%)[3]	FI[4](g/fish)	FCR[5]	PER[6]	PD (g/fish)[7]	Survival (%)
0.27	47.09±2.80^{b}	46.38±8.07^{b}	0.45±0.07^{b}	55.43±3.47^{b}	3.77±0.45^{a}	0.55±0.06^{b}	0.09±0.02^{c}	87.78±12.62
0.79	68.47±1.98^{a}	113.75±8.56^{a}	0.90±0.05^{a}	69.11±1.86^{a}	1.90±0.16^{b}	1.09±0.09^{a}	0.20±0.02^{b}	95.56±5.09
1.22	70.24±2.69^{a}	119.73±9.20^{a}	0.94±0.05^{a}	69.03±2.91^{a}	1.81±0.15^{b}	1.14±0.09^{a}	0.22±0.01^{ab}	95.56±3.85
1.69	71.06±2.80^{a}	121.89±7.33^{a}	0.95±0.04^{a}	65.11±4.41^{a}	1.67±0.05^{b}	1.23±0.03^{a}	0.25±0.00^{a}	87.78±1.92
2.04	74.17±1.53^{a}	131.67±3.80^{a}	1.00±0.02^{a}	66.63±4.73^{a}	1.58±0.13^{b}	1.29±0.11^{a}	0.26±0.01^{a}	88.89±5.09
2.38	69.43±2.97^{a}	116.83±9.71^{a}	0.92±0.05^{a}	67.90±1.24^{a}	1.82±0.18^{b}	1.12±0.11^{a}	0.22±0.02^{ab}	86.67±3.33

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight

[2]Weight gain= [(final body weight – initial body weight) / initial body weight × 100]

[3]Specific growth rate = [(ln final body weight - ln initial body weight) / days] × 100

[4]Feed intake (g/fish) = dry feed consumed (g)/fish

[5]Feed conversion ratio= dry feed fed/wet weight gain

[6]Protein efficiency ratio= wet weight gain/total protein given

[7] Protein deposition = Protein gain/protein intake

Table 2-4. Whole-body proximate composition of red sea bream fed different levels of dietary valine for 12 weeks.

Valine level	Moisture	Protein	Lipid	Ash
0.27	72.54±0.49^{a}	15.99±0.65^{b}	6.14±0.46^{b}	5.16±0.20
0.79	68.88±0.94^{b}	17.01±0.27^{a}	8.38±1.09^{ab}	4.54±0.45
1.22	68.91±1.32^{b}	17.28±0.31^{a}	8.42±1.29^{ab}	4.37±0.22
1.69	69.83±0.50^{b}	17.77±0.17^{a}	8.58±1.26^{ab}	4.69±0.28
2.04	68.29±0.98^{b}	17.66±0.37^{a}	9.53±0.27^{a}	4.68±0.16
2.38	68.56±0.46^{b}	17.41±0.12^{a}	8.34±0.27^{ab}	5.23±0.60

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P < 0.05). The lack of superscript letter indicates no significant differences among treatments.

Table 2-5. Hematological parameters of red sea bream fed different levels of dietary valine for 12 weeks.

Valine level	Ht[1]	Hb[1]	Total protein[3]	ALT[4]	AST[5]	Glucose[6]	Cholesterol[7]
0.27	30.72±1.78	4.42±0.39	2.88±0.16^{c}	13.04±2.82	24.44±5.54^{a}	35.14±3.03	122.54±11.62^{b}
0.79	34.33±3.72	5.22±0.39	3.44±0.36^{bc}	11.50±3.59	19.79±3.32^{ab}	38.39±6.20	147.02±8.45^{ab}
1.22	33.87±1.30	5.11±0.43	3.81±0.24^{ab}	9.01±0.97	19.23±0.79^{ab}	36.85±2.72	146.87±9.32^{ab}
1.69	32.90±0.46	5.00±0.59	3.93±0.06^{ab}	8.47±1.67	16.10±1.80^{ab}	38.69±2.29	137.85±10.76^{ab}
2.04	34.07±0.50	5.42±0.51	4.35±0.43^{a}	8.46±0.91	14.90±0.69^{b}	36.53±1.94	157.70±8.83^{a}
2.38	32.80±2.43	4.76±0.08	3.34±0.16^{bc}	8.24±1.60	20.78±3.86^{ab}	35.06±1.90	130.17±15.64^{ab}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Hematocrit

[2]Hemoglobin

[3]Total protein (g dl^{-1})

[4] Alanine aminotransferase activity (U l^{-1})

[5] Aspartate aminotransferase (U l^{-1})

[6]Glucose (mg dl^{-1})

[7]Total cholesterol (mg dl^{-1})

Table 2-6. Innate immune parameters of red sea bream fed different levels of dietary valine for 12 weeks.

Valine level	Lysozyme[1]	NBT[2]	MPO[3]	Ig[4]	SOD[5]	Antiprotease[6]
0.27	9.13±2.91^{b}	0.75±0.07^{b}	0.88±0.11^{b}	3.00±0.40^{c}	67.46±3.24	29.18±3.57
0.79	10.73±1.07^{b}	0.82±0.16^{ab}	1.01±0.12^{ab}	4.04±0.42^{c}	74.05±5.09	32.60±2.91
1.22	10.40±0.67^{b}	0.89±0.07^{ab}	1.12±0.13^{ab}	7.04±1.50^{b}	75.19±2.78	30.04±3.13
1.69	12.47±1.09^{ab}	0.86±0.06^{ab}	1.10±0.03^{ab}	8.59±0.55^{ab}	75.40±3.27	31.74±4.44
2.04	14.79±0.57^{a}	1.00±0.08^{a}	1.21±0.13^{a}	9.97±1.00^{a}	76.81±2.62	29.59±2.11
2.38	12.64±0.23^{ab}	0.87±0.03^{ab}	1.03±0.13^{ab}	6.55±0.76^{b}	72.40±7.71	29.70±0.78

Values are mean of triplicate groups and presented as mean ± S.D. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1] Lysozyme (U ml^{-1})

[2]Nitro blue tetrazolium activity (Absorbance)

[3]Myeloperoxidase (Absorbance)

[4]Immunoglobulin (mg ml^{-1})

[5]Superoxide dismutase (% inhibition)

[6]Anti-protease activity (% trypsin inhibition)

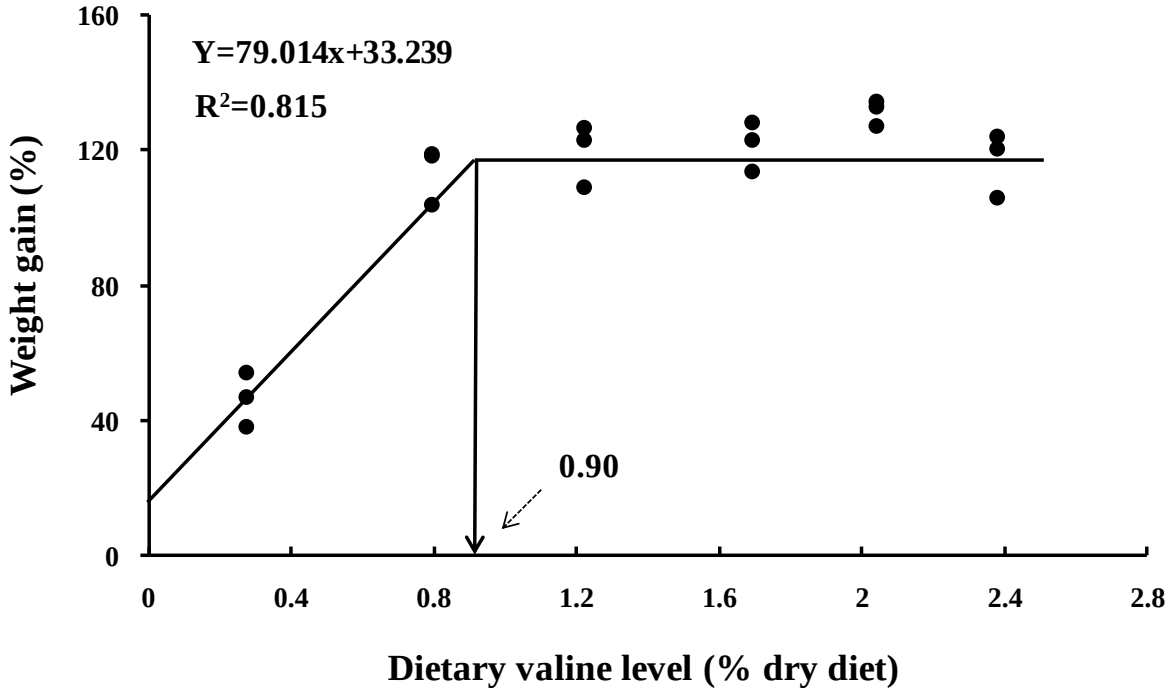

Fig. 2-1. Relationship between weight gain and dietary valine level for red sea bream as described by broken-line regression.

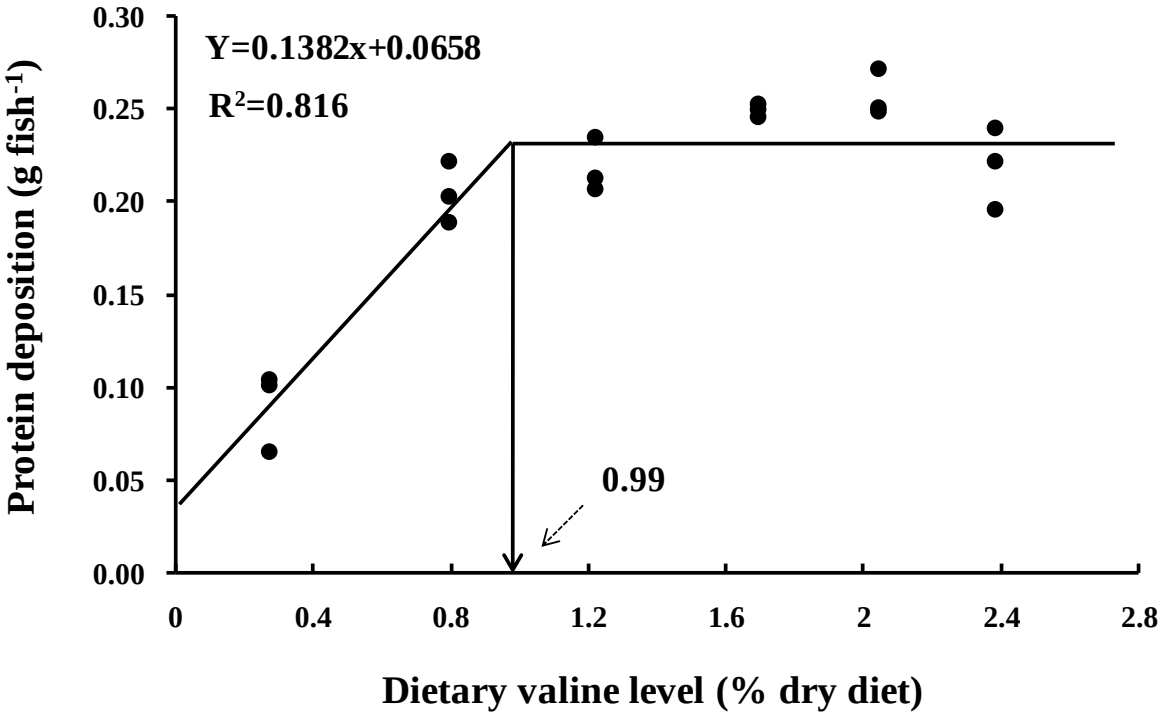

Fig. 2-2. Relationship between protein retention and dietary valine level for red sea bream as described by broken-line regression.

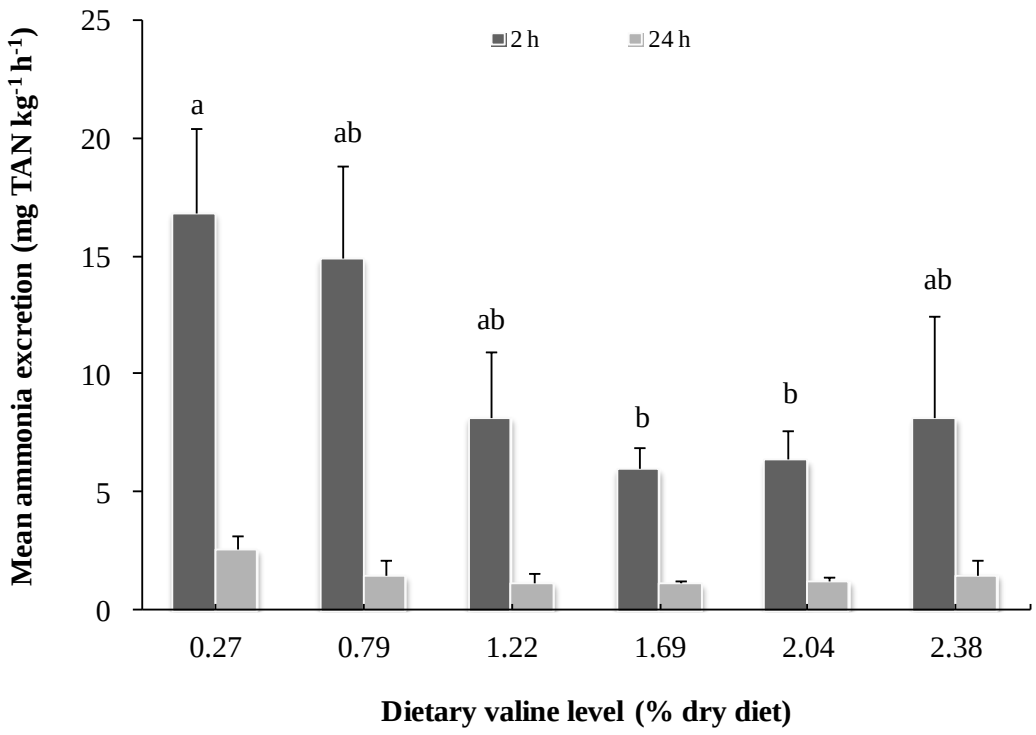

Fig. 2-3. Ammonia excretion of red sea bream fed diets with different levels of dietary valine. Bars with different letters are significantly different ($P < 0.05$).

2.4. Discussion

Results of the present study showed that growth performance of juvenile red sea bream was affected by dietary valine levels. The reduced growth performance of fish fed valine deficient diets has been demonstrated to be primarily due to the loss of appetite and poor feed utilization efficiency (Ahmed and Khan, 2006; Rollin et al., 2006; Dong et al., 2012). In the current study fish fed the minimum valine level exhibited significantly lower feed intake than other groups and confirmed the previous findings. The notion that dietary valine level affects fish feed utilization further confirmed by the results of the present study as significantly higher PER and PD and lower FCR values were recorded in fish fed the diets containing ≥0.79% valine. In the present study, a depressed growth performance was observed when fish were fed the diet containing 2.38% valine. Similarly, the results of earlier studies showed the reduced growth performance of rohu carp (Abidi and Khan 2004a) and Jian carp (Dong et al., 2012) when they were provided with excessive dietary valine. It has been demonstrated that excessive intake of one AA results in toxic effects and negatively influences the utilization of other AAs (Coloso et al., 1999).

In the present study the highest growth performance and feed utilization efficiency were observed in fish fed 2.04% dietary valine. The optimum valine requirement level was determined by the broken-line regression analysis on the basis of WG and PD and estimated to be approximately 0.9% diet. This value is similar to the reported requirements for channel catfish, 0.7% (Wilson et al., 1980) lake trout, 0.8-1.0% (Hughes et al., 1983) and rainbow trout, 0.8-1.6% diet (Rodehutscord et al., 1997). However, it is lower than the requirements of other fish species including common carp,

1.4% (Nose, 1979); Nile tilapia, 1.6% (Santiago and Lovell, 1988); rohu carp, 1.5% (Abidi and Khan, 2004a); Mrigal carp, 1.52% (Ahmed and Khan, 2006) and Jian carp, 1.37% (Dong et al., 2012). The variations in valine requirement level have been attributed to differences in species, experimental conditions, composition of basal diet and sources of supplemental amino acids along with the used mathematical models (Abidi and Khan, 2004a).

Fish whole-body composition was significantly influenced by dietary valine levels. Whole-body protein and lipid contents showed a similar trend with fish growth performance and moisture content decreased significantly. These are in agreement with the results in rohu carp (Abidi and Khan, 2004a) and Mrigal carp (Ahmed and Khan, 2006). Also, Dong et al. (2012) reported the significant increase of whole-body protein in Jian carp by increment of dietary valine level, but the fat content showed a decreasing trend. The significant role of dietary valine in protein synthesis has been earlier reported in fishes (Ahmed and Khan, 2006). The significant improvement of PD by increment of dietary valine level in this study further confirmed that supplementation of appropriate level of valine in diets for red sea bream would enhance protein deposition, which is the major part of fish weight gain (Cowey, 1994).

Hematological parameters are used as valuable biological indicators in response to dietary manipulations (Adhikari et al., 2004; Shah and Altindag, 2005; Maheswaran et al., 2008). To our knowledge, there is little available information on the effects of dietary valine on hematological parameters of fish species. In the present study, hematological parameters of red sea bream were taken into account and significant differences were found among dietary treatments. Higher hematocrit and hemoglobin

values were found in fish fed valine levels of ≥0.79%, although the differences were not significant. Among the different component of plasma, the levels of total protein, glucose, total cholesterol, ALT and AST are regarded as useful indices for assessment of health status in fish. In this study, plasma total protein level was significantly influenced by valine levels and higher values were found by increment of dietary valine up to2.04%. Earlier, Zhou et al. (2010) reported the significant increase of serum total protein level in black sea bream (*Sparus macrocephalus*) when they were fed incremental levels of lysine. The results of another study by Cao et al. (2012) showed the significant increase of plasma total protein concentration in juvenile yellow catfish (*Pelteobagrus fulvidraco*) fed optimum dietary lysine level. AST and ALT are transaminase enzymes and generally are used as indices of liver function in vertebrates. Increment of AST and ALT levels generally indicate the liver abnormal function (Bain, 2003). They are also used as valuable diagnostic means of stress responses in several fish species (De Smet and Blust, 2001; Almeida et al., 2002; Choi et al., 2007). Their concentration in plasma increases in response to several factors such as pollution and ammonia and nitrite toxications (Das et al., 2004). In the present study plasma AST level decreased by increment of dietary valine up to 2.04% thereafter increased by further increment. Also, plasma ALT levels decreased by increment of valine level up to 2.04%, however, the differences were not significant. It has been demonstrated that plasma cholesterol level is affected by the type of dietary proteins (Carroll and Hamilton, 1975; Kritchevsky, 1979; Terpstra et al., 1983). In this concept, the main influencing factor has been suggested to be the difference in the AA composition of dietary proteins. Sugiyama et al. (1996) investigated the relationship between the AA

composition of dietary proteins and plasma cholesterol concentration in rats fed different dietary proteins. Their results showed a significant positive correlation between the plasma total cholesterol level and plasma concentration of valine. In the present study plasma cholesterol concentration was affected by dietary valine levels and significantly increased in fish fed 2.04% valine compared with those fed 0.27% valine. This finding may provide more evidence for hypercholesterolemic effect of valine.

It has been shown that deficiency of dietary protein or AAs leads to impaired immune function. AAs are involved in synthesis of a variety of specific proteins, including cytokines and antibodies, and play key roles in metabolic pathways of the immune response. BCAA are involved in biosynthesis of glutamine, as part of the immune system, by providing α-amino group (Newsholme and Calder, 1997). Also, the carbon skeletons of BCAA are required by leucocytes for protein synthesis (Waithe et al., 1975). The results of cell culture studies have demonstrated the essentiality of BCAA for synthesis of protein, RNA and DNA by lymphocytes (Calder, 2006). A number of animal studies have shown that deficiency of BCAA results in impairment of immune function. In mice, deficiency of dietary BCAA disrupts several immune functions leading to increased susceptibility to disease (Petro and Bhattacharjee, 1981). Dietary supplementation of BCAA resulted in greater number of liver-associated lymphocytes, cytotoxic T lymphocytes and natural killer cells in rats (Tsukishiro et al., 2000). There is limited available information regarding the effects of BCAA on immune function in fish species. In the present study the effects of varying levels of dietary valine were investigated on some aspects of humoral non-specific immune response and the results showed positive effect of valine on immune function. Lysozyme, respiratory

burst and MPO activities as well as plasma Ig level significantly increased by increment of valine level up to 2.04% and further increment resulted in immunosuppressive effects. It has been suggested that as BCAA share the same transporter on the cell membrane the imbalances in their dietary composition can result in impairment of immune function (Aschkenasy, 1979).

Formulation of diets with balanced AA profile is necessary for optimization of dietary protein utilization (Boisen et al., 2000; Green and Hardy, 2002; Rollin et al., 2003). Imbalances in AA composition of diet results in reduced feed intake and protein utilization (D'Mello, 1994; Yamamoto et al., 2000; Berge et al., 2002; Green and Hardy, 2002; Gómez-Requeni et al., 2003). Deficiency of even an individual AA in the diet limits protein synthesis (Cole and Van Lunen, 1994) and leads to increased deamination and oxidation of AA and consequently increased nitrogenous excretion (Cho and Kaushik, 1985). In the present study fish fed 0.27% valine showed higher postprandial ammonia excretion level than those fed 1.69 and 2.04% valine probably indicating imbalances in dietary AA composition.

In conclusion, the results showed the essentiality of valine for optimal growth of juvenile red sea bream. The optimum valine requirement level was estimated to be approximately 0.9% diet (2% of dietary protein) by broken-line regression analysis on WG and PD. Fish immune response and hematological parameters were influenced by dietary valine levels and the results confirmed the requirement level determined based on fish growth. The findings in this study can aid in formulation of well-balanced practical diets for juvenile red sea bream.

CHAPTER THREE

Dietary arginine requirement of juvenile red sea bream *Pagrus major*

3.1. Introduction

Quantitative estimation of the essential amino acid (EAA) requirements in diets for fishes is extremely important due to their critical impacts on muscle deposition, feed costs and nitrogen wastes (Small and Soares, 1999; Nguyen and Davis, 2009). Arginine has been established as an EAA in diets of many fish species (NRC, 1993; Cowey, 1994). It is the most limiting amino acid in plant protein sources such as corn, sesame and zein meal (Mai et al., 1994; Berge et al., 1997; Luo et al., 2007). It is a precursor of several biologically important metabolites including nitric oxide (NO), polyamines and creatine (Wu and Morris, 1998; Galli, 2007; Grillo and Colombatto, 2007). Also, it participates in several metabolic pathways including protein synthesis, urea production and metabolism of glutamic acid and proline (Hird, 1986).

Arginine has been established as an immunonutrient in higher animals such as humans, rodents, swine and poultry (Evoy et al., 1998; Li et al., 2007; Roth, 2007; Wu, 2010). There have been accumulating evidences that arginine influences immune function in fish as well. Buentello and Gatlin (1999, 2001) showed that supplementation of arginine in diets for channel catfish (*Ictalurus punctatus*) influences the NO production which is a strong antioxidant molecule used to combat a variety of invading pathogens. Also, dietary supplementation of arginine enhanced the innate immunity of

channel catfish *in vivo*, and a moderate inclusion level of arginine in culture media augmented phagocytosis *in vitro* (Buentello et al., 2007). A significant increase in neutrophil oxidative radical production was found in red drum (*Sciaenops ocellatus*) when they were fed supplemental dietary arginine (Cheng et al., 2011). Recently the changes in immune response of fish are taken into account in arginine requirement studies (Zhou et al., 2012; Ren et al., 2013; Yue et al., 2013; Pohlenz et al., 2014; Ren et al., 2014).

The dietary arginine requirement has been estimated for many fish species and literature review shows the high variations in requirement level among species. Therefore, it is critical to determine the quantitative arginine requirement for each species. Red sea bream is one of the most important fish in Korea and Japan. Total amount of 2,755 tons red sea bream was produced in Korea in 2013 (Ministry of Maritime Affairs and Fisheries of Korea, 2013). However, to the best of our knowledge, quantitative requirements of the species for EAAs have just been reported for lysine (Forster and Ogata, 1998) and valine (Rahimnejad and Lee, 2013) and there is no available data on its arginine requirement. Therefore, the present study was carried out to evaluate the dietary arginine requirement of red sea bream and the effects of varying levels of arginine on hematological and innate immune parameters.

3.2. Materials and methods

3.2.1. Experimental diets

Formulation and proximate composition of the experimental diets are provided in Table 3-1. Six isonitrogenous (50% crude protein) and isocaloric (4.23 kcal g^{-1} gross energy) diets were formulated to contain graded levels of arginine ranging from 1.2 to 3.2% of dry diet. The basal diet contained 1.2% of arginine from fish meal and soybean protein concentrate and five other diets were prepared by supplementation of L-arginine at 0.4% increments. The diets were kept isonitrogenous using glycine. A mixture of crystalline amino acids without arginine was used to simulate the amino acid composition of juvenile red sea bream whole-body protein (Alam et al., 2005) except for arginine. The energy content of each diet was estimated on the basis of physiological fuel value, i.e., 3.99 kcal g^{-1} proteins or carbohydrates and 9.01 kcal g^{-1} lipids (Lee and Putnam, 1973). All dry ingredients were thoroughly mixed and after addition of squid liver oil and double distilled water extruded through a meat chopper machine (SMC-12, Kuposlice, Busan, Korea) in 3 mm diameter. Then, the diets were freeze-dried for 24 h, crushed into desirable particle sizes, sealed in bags and stored at –20 ^{o}C until used.

Table 3-1. Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	1	2	3	4	5	6
White fish meal	25.0	25.0	25.0	25.0	25.0	25.0
SPC[a]	8.0	8.0	8.0	8.0	8.0	8.0
FAA mix[b]	25.6	25.6	25.6	25.6	25.6	25.6
Arginine	0.0	0.4	0.8	1.2	1.6	2.0
Glycine	2.0	1.6	1.2	0.8	0.4	0.0
Dextrin	20.9	20.9	20.9	20.9	20.9	20.9
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[c]	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix[d]	2.0	2.0	2.0	2.0	2.0	2.0
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Dry matter (DM)	90.5	90.0	89.7	89.4	90.0	89.7
In DM:						
Protein	50.1	50.1	51.0	51.2	51.6	51.4
Lipid	15.5	15.1	15.0	15.1	15.2	15.4
Ash	4.42	4.40	4.35	4.32	4.31	4.38

[a]Soybean protein concentrate

[b]Free amino acid mixture (g kg^{-1} of mixture): arginine hydrochloride, 33.6; lysine hydrochloride, 40.4; methionine, 22.4; histidine, 15.7; isoleucine, 20.2; leucine, 31.4; phenylalanine, 40.4; threonine, 17.9; tryptophan, 4.5; alanine, 248.9; glutamic acid, 248.9; glycine, 275.8.

[c]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[d]Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

3.2.2. Fish and feeding trial

Juvenile red sea bream were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). The health status of fish was checked upon arrival, and they were quarantine bathed with 100 mg l^{-1} formalin for 20 min. All the fish were fed the basal diet for 2 weeks to become acclimatized to the experimental conditions and facilities. At the end of the acclimation period, 20 randomly captured fish (averaging at 13.3 ± 0.2 g) were distributed into each polyvinyl circular tanks of 120 L capacity and supplied with sand filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed with one of the test diets to apparent satiation twice daily (09:00 and 17:00 h) for 9 weeks. Uneaten food was siphoned out 30 min after feeding and weighed to determine the feed intake. Growth of fish was measured with three-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish. The water temperature during the feeding trial ranged from 21 to 27 °C and the photoperiod was maintained on a 12:12 light:dark schedule.

3.2.3. Sample collection and analyses

At the beginning of the experiment 10 fish were sampled and stored at –20 °C for analysis of initial whole-body proximate composition. At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Six intact fish per tank (18 fish per treatment) were selected and kept at −20 °C for whole-body and muscle composition analysis. Three fish per

tank were randomly captured, anesthetized with 2-phenoxyethanol (200 mg l^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for determination of hematocrit, hemoglobin and respiratory burst activity. After the above mentioned measurements with whole blood, plasma were separated by centrifugation at 5000 × *g* for 10 min and stored at −70 °C for determination of total immunoglobulin (Ig) level and blood biochemical parameters including plasma total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cholesterol and triglyceride. Another set of blood samples (3 fish per tank) were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation for 10 min at 5000 × *g* and stored at −70 °C for the analysis of total nitric oxide synthase (T-NOS), lysozyme, myeloperoxidase (MPO), superoxide dismutase (SOD) and antiprotease activities.

Analyses of moisture and ash contents of samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Seoul, Korea). Hematocrit was determined by microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of total protein, glucose, cholesterol and triglyceride and activities of ALT and AST were determined by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

Oxidative radical production by phagocytes during respiratory burst was measured through NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Briefly, blood and NBT (0.2%) (Sigma, St. Louis, MO, USA) were mixed in equal

proportion (1:1) and incubated for 30 min at room temperature. Then 50 µl was taken out and dispensed into glass tubes. One ml of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as the blank.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein concentration was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules using a 12% solution of polyethylene glycol (Sigma). The difference in protein concentration represents the Ig content.

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200 µl of suspension was placed in each well of 96-well plates, and 20 µl serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (*UVM 340, Biochrom, Cambridge, UK*). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

MPO activity was measured according to Quade and Roth (1997). Briefly, 20 µl of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma, USA) in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 µl of 4 M sulfuric acid. Finally,

the optical density was read at 450 nm in a microplate reader.

SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Serum antiprotease activity was measured according to the method described by Ellis et al. (1990), with slight modifications (Magnadóttir et al., 1999). Briefly, 20 µl of serum was incubated with 20 µl of standard trypsin solution (Type II-S, from porcine pancreas, 5 mg ml^{-1}, Sigma-Aldrich) for 10 min at 22 °C. Then, 200 µl of phosphate buffer (0.1 M, pH 7.0) and 250 µl azocasein (2%) (Sigma) were added and incubated for 1 h at 22 °C. Five hundred microliters of trichloro acetic acid (10%) was added and further incubated for 30 min at 22 °C. The mixture was centrifuged at 6000×g for 5 min and 100 µl of the supernatant was transferred to the wells of a 96 well flat bottomed microplate containing 100 µl of 1 N NaOH. Optical density was read at 430 nm. For a 100% positive control, buffer replaced the serum, while for the negative control buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

Trypsin inhibition (%) = $(A_1 – A_2/A_1) \times 100$

where A_1 = control trypsin activity (without serum); A_2 = trypsin activity remained after adding serum.

Serum total nitric oxide synthase (T-NOS) activity was measured using a commercial kit (Calbiochem-Novabiochem Corporation, USA).

3.2.4. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$. A polynomial regression model was used for determination of optimum dietary arginine level based on weight gain (WG). Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

3.3. Results

Growth performance and feed utilization results are shown in Table 3-2. Fish fed ≥ 2.0% arginine exhibited significantly ($P < 0.05$) higher growth performance than those fed 1.2% arginine. Slight improvements in feed conversion ratio (FCR) and protein efficiency ratio (PER) were obtained by increment of dietary arginine level but the differences were not significant. However, fish fed 2.4% arginine showed significantly higher protein productive value (PPV) than fish fed 1.2% arginine. Feed intake and survival did not significantly differ among dietary treatments. The optimal dietary arginine requirement level was estimated to be 2.54% in diet by the polynomial regression analysis on WG (Fig. 3-1).

Whole-body protein content increased in fish fed 2-2.4% arginine and differed significantly from that of the fish fed 1.2% arginine (Table 3-3). A similar trend was observed for muscle protein content, however, the differences were not significant. Whole-body lipid content decreased significantly in groups of fish fed 2-2.4% arginine compared to those fed 1.2% arginine and muscle lipid did so in groups fed 2-2.8% arginine. The group of fish fed 1.2% arginine exhibited the lowest muscle moisture content even though the differences were not significant.

Plasma total protein level increased significantly at arginine levels of 2.4-2.8% in comparison to fish groups fed ≤ 1.6 arginine (Table 3-4). Significantly lower ALT and glucose levels were recorded at dietary arginine levels of 2-2.4% and ≥1.6%, respectively. However, hematocrit, hemoglobin, cholesterol and triglyceride levels and AST activity were not significantly influenced.

Significantly higher lysozyme activities were found at over 2.4% arginine compared to the fish offered 1.2% arginine and MPO activity was significantly elevated at arginine levels of 2.4-2.8% (Table 3-5). The groups of fish fed 2-2.8% arginine showed significantly higher plasma Ig levels. Serum T-NOS activity was enhanced by increment of dietary arginine level up to 2.8% and significant differences were found between the groups fed 1.2 and 2.8% arginine (Fig. 3-2). However, respiratory burst, SOD and antiprotease activities were not significantly influenced by dietary arginine levels.

Table 3-2. Growth performance of red sea bream (initial body weight, 13.25 ± 0.2) fed different levels of dietary arginine for 9 weeks.

Arg level	FBW[1] (g)	WG[2] (%)	SGR[3] (%)	FI[4](g/fish)	FCR[5]	PER[6]	PPV[7]	Survival (%)
1.2	48.90±0.85^{c}	269.21±8.72^{c}	2.11±0.04^{c}	72.98±7.45	1.95±0.14	1.03±0.07	17.38±2.28^{b}	88.33±11.55
1.6	51.76±1.13^{bc}	289.05±7.56^{bc}	2.19±0.03^{bc}	78.71±2.18	1.92±0.07	1.04±0.04	17.87±0.76^{b}	86.67±10.41
2.0	54.38±1.01^{ab}	308.63±6.18^{ab}	2.27±0.02^{ab}	75.73±7.52	1.78±0.17	1.11±0.10	20.44±2.29^{ab}	91.67±7.64
2.4	56.62±2.83^{a}	328.20±20.42^{a}	2.34±0.08^{a}	74.74±2.10	1.68±0.10	1.22±0.17	23.10±1.90^{a}	93.33±5.77
2.8	55.71±1.21^{ab}	319.91±17.42^{ab}	2.31±0.07^{ab}	72.75±3.89	1.69±0.01	1.15±0.01	20.35±0.16^{ab}	95.00±8.66
3.2	53.42±1.05^{ab}	305.83±10.83^{ab}	2.26±0.04^{ab}	76.85±6.52	1.81±0.13	1.08±0.08	18.60±2.54^{ab}	85.00±8.66

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05).

[1]Final body weight

[2]Weight gain= [(final body weight – initial body weight)/initial body weight × 100]

[3]Specific growth rate = [(ln final body weight - ln initial body weight) / days] × 100

[4]Feed intake (g/fish) = dry feed consumed (g)/fish

[5]Feed conversion ratio= dry feed fed/wet weight gain

[6]Protein efficiency ratio= wet weight gain/total protein given

[7]Protein productive value= protein gain / protein intake

Table 3-3. Proximate composition of whole body and muscle of red sea bream fed different levels of dietary arginine for 9 weeks.

	Arg level					
	1.2	**1.6**	**2.0**	**2.4**	**2.8**	**3.2**
Whole-body (%)						
Protein	17.38±0.37^{b}	17.87±0.31^{ab}	18.52±0.20^{a}	18.65±0.52^{a}	17.69±0.31^{ab}	17.74±0.37^{ab}
Lipid	14.76±0.48^{a}	13.61±0.92^{a}	11.11±0.64^{b}	11.03±0.55^{b}	13.17±1.22^{ab}	12.69±1.20^{ab}
Ash	4.44±0.82	4.01±0.23	4.02±0.46	3.57±0.30	4.13±0.36	3.85±0.41
Moisture	65.66±1.95	65.57±0.13	66.47±1.04	66.44±0.73	65.60±0.74	64.69±0.62
Muscle (%)						
Protein	21.82±1.27	22.91±0.47	23.34±0.95	23.25±0.47	22.41±0.76	22.50±0.67
Lipid	2.47±0.30^{a}	2.16±0.19^{ab}	1.66±0.37^{bc}	1.34±0.12^{c}	1.50±0.26^{bc}	2.05±0.22^{ab}
Ash	1.45±0.14	1.46±0.15	1.46±0.06	1.53±0.04	1.45±0.04	1.43±0.09
Moisture	73.77±0.45	75.13±0.45	75.16±0.76	75.05±0.44	75.35±1.09	75.21±0.42

Values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

Table 3-4. Hematological parameters of red sea bream fed different levels of dietary arginine for 9 weeks.

Arg level	Ht[1]	Hg[1]	Total protein[3]	ALT[4]	AST[5]	Glucose[6]	Cholesterol[7]	TG[8]
1.2	45.9±1.7	7.03±0.71	4.76±0.07^{b}	19.64±1.65^{a}	32.24±2.28	4.99±0.20^{a}	256±21.6	284±25.1
1.6	43.7±6.1	7.20±0.39	4.78±0.50^{b}	15.86±1.02^{ab}	30.50±3.11	3.63±0.41^{b}	242±33.2	303±31.8
2.0	42.4±0.9	6.75±0.37	5.58±0.06^{ab}	14.54±1.60^{b}	29.00±2.91	2.15±0.25^{c}	247±35.7	288±14.2
2.4	45.0±4.0	7.23±0.57	6.22±0.13^{a}	14.80±2.92^{b}	28.91±3.24	2.47±0.24^{c}	264±16.5	310±34.0
2.8	45.9±3.4	7.16±0.56	5.97±0.50^{a}	15.60±0.31^{ab}	30.92±3.59	2.71±0.29^{c}	264±19.7	306±28.6
3.2	42.5±2.8	6.65±0.52	5.80±0.64^{ab}	15.13±1.83^{ab}	31.17±3.17	2.84±0.34^{bc}	260±15.9	289±29.9

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1]Haematocrit

[2]Haemoglobin

[3]Total protein (g/dL)

[4]Alanine aminotransferase activity (U/L)

[5]Aspartate aminotransferase (U/L)

[6]Glucose (mg/dL)

[7]Total cholesterol (mg/dL)

[8]Triglyceride

Table 3-5. Non-specific immune response parameters of red sea bream fed different levels of dietary arginine for 9 weeks.

Arg level	Lysozyme[1]	NBT[2]	MPO[3]	Ig[4]	SOD[5]	Antiprotease[6]
1.2	14.19±1.22^{b}	0.96±0.03	1.13±0.12^{c}	14.88±2.56^{c}	66.03±6.91	29.11±1.55
1.6	18.02±1.45^{ab}	1.00±0.10	1.25±0.12^{bc}	18.67±1.36^{bc}	62.50±4.45	32.52±3.79
2.0	19.63±1.79^{ab}	1.06±0.06	1.31±0.04^{bc}	23.33±4.99^{ab}	75.29±3.41	32.46±3.00
2.4	22.56±1.90^{a}	0.95±0.11	1.64±0.09^{a}	28.49±1.96^{a}	74.73±1.42	32.38±2.04
2.8	20.42±2.91^{a}	1.01±0.06	1.53±0.15^{ab}	28.31±3.73^{a}	74.64±7.60	30.28±3.68
3.2	21.42±2.53^{a}	1.08±0.06	1.41±0.13^{abc}	21.65±1.19^{abc}	75.35±7.90	30.22±4.37

Values are mean of triplicate groups and presented as mean ± S.D. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1] Lysozyme (U/ml)

[2]Nitro blue tetrazolium activity (Absorbance)

[3]Myeloperoxidase (Absorbance)

[4]Immunoglobulin (mg/ml)

[5]Superoxide dismutase (% inhibition)

[6]Anti-protease activity (% trypsin inhibition)

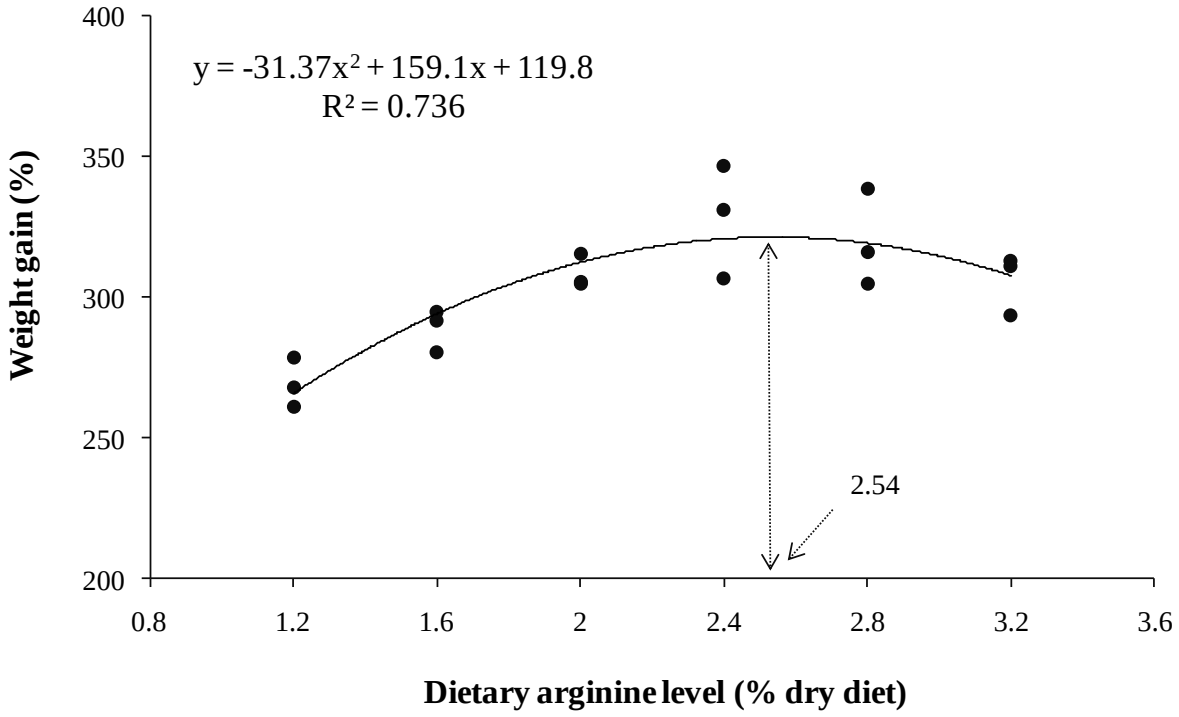

Fig. 3-1. Second-order polynomial relationship of weight gain to dietary arginine levels.

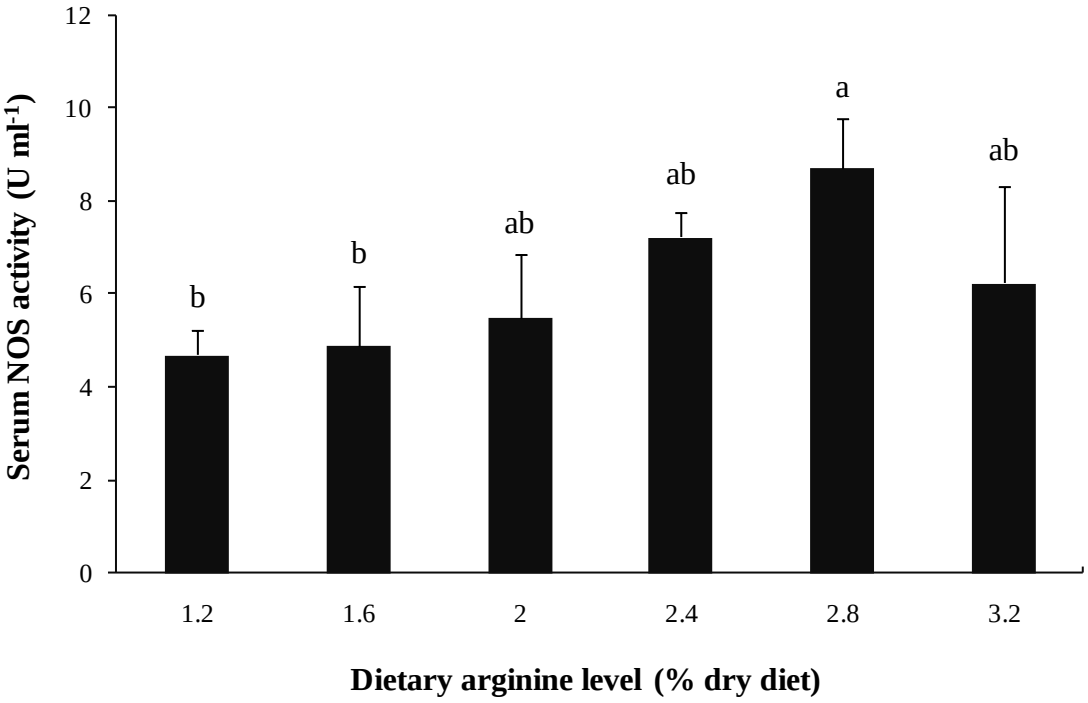

Fig. 3-2. Serum T-NOS activity of red sea bream fed different levels of dietary arginine. Bars with different letters are significantly different ($P < 0.05$).

3.4. Discussion

The results of this study revealed the essentiality of arginine for maximal growth of red sea bream. The lowest growth performance was obtained in fish fed 1.2% arginine and its dietary increment up to 2.4% resulted in significant improvement in growth performance. The estimated value of 2.54% dietary arginine is similar to the reported arginine requirements for Chinook salmon (*Oncorhynchus tshawytscha*), 2.4% (Klein and Halver, 1970); Coho salmon (*Oncorhynchus kisutch*), 2.2-2.5% (Luzzana et al., 1998); Japanese flounder (*Paralichthys olivaceus*), 2-2.5% (Alam et al., 2002a, b) and blunt snout bream (*Megalobrama amblycephala*), 2.46% (Ren et al., 2013). However, this requirement level is higher than those of Indian major carp (*Catla catla*), 1.67% (Zehra and Khan, 2013); Indian catfish (*Heteropneustes fossilis*), 1.63% (Ahmed, 2013) and Nile tilapia (*Oreochromis niloticus L.*), 1.82% (Yue et al., 2013), but lower than that of black sea bream (*Acanthopagrus schlegelii*), 2.79-3.09% (Zhou et al., 2010). The large variations observed in arginine requirement within and among fish species have been reported to be due to the differences in fish size and age, dietary protein sources and levels, the reference amino acid pattern, availability of amino acid and amino acid sources, response criteria, the used mathematical model, and environmental and culture condition. Also, considering the ability of teleostean fish to synthesize arginine from glutamate, the dietary glutamate content may affect the dietary arginine requirement estimation. Furthermore, Ahmed and Khan (2004) pointed out that the metabolic demands of the different pathways can interfere with the arginine requirement for protein synthesis, resulting in differences in arginine requirement level.

Baker (1984) stated that increased growth performance in animals by dietary supplementation of an essential nutrient can be due to increased feed intake and/or metabolic efficiency. In the present study, we did not find any significant difference in the feed intake. However, previous studies reported the significant enhancement in feed intake by increment

of dietary arginine in grouper (*Epinephelus coioides*) (Luo et al., 2007), largemouth bass (*Micropterus salmoides*) (Zhou et al., 2012) and Indian catfish (Ahmed, 2013). Enhancement of growth performance in this study might be due to the improved feed utilization because we found slight improvements of FCR and PER at higher arginine levels. Also, significantly higher PPV was obtained by increment of dietary arginine level and the highest PPV was observed in fish fed 2.4% arginine. Similarly, Zhou et al. (2012) did not find significant effect of dietary arginine level on FCR and PER in yellow grouper, but PPV was significantly elevated by arginine level.

Whole-body and muscle compositions were affected by dietary arginine levels; significantly higher whole-body protein content was achieved at dietary arginine levels of 2.0-2.4% and lipid content decreased at the same levels. A similar tendency to that of whole-body was observed for muscle composition but protein content was not significantly different among treatments. These results are in agreement with previous studies on Indian major carp (Ahmed and Khan, 2004), black sea bream (Zhou et al., 2010) and *H. fossilis* (Khan and Abidi, 2011). Arginine is one of the most versatile amino acids and is a crucial precursor for protein synthesis (Wu and Morris, 1998). In this study whole-body protein content showed a similar trend with PPV and further confirmed that inclusion of an appropriate level of arginine can enhance protein deposition. It has been reported that arginine and its products play important roles in the metabolism of energy substrates (Jobgen et al., 2006; Shantz and Levin, 2007; Montanez et al., 2008). Jobgen et al. (2009) remarked that arginine supplementation enhances lipolysis and inhibits lipogenesis in rats by regulating the expression and function of the key enzymes which are involved in lipid metabolism. The present study further demonstrated that arginine participates in lipid metabolism resulting in decreased lipid deposition.

Hematological parameters are increasingly being taken into account in fish nutrition

studies due to their importance in monitoring the health status of fish (Hrubec et al., 2000; Congleton and Wagner, 2006). Analysis of hematological parameters provides valuable information on metabolic disorders, deficiencies and chronic stress status (Bahmani et al., 2001). Previous studies showed the changes in blood components in response to dietary supplementation of EAAs (Huai et al., 2009; Zhou et al., 2010; Khan and Abidi, 2011). Also, our previous study exhibited the significant effect of dietary valine on red sea bream hematology (Rahimnejad and Lee, 2013). The increased plasma protein in the present study is in agreement with a black sea bream study that showed significant enhancement of serum protein levels by dietary arginine increment (Zhou et al., 2010). AST and ALT are generally used as indicators of cellular damage both in mammals and fishes (Welker and Congleton, 2003; Olsen et al., 2005). Their concentration in plasma increases by several stressors including pollution and ammonia toxicity (Das et al., 2004). In this study, significantly lower ALT levels were recorded at arginine levels of 2.0-2.4% and numerically decreased plasma AST was found. Increment of dietary arginine level led to significant decrease of plasma glucose concentration and supported the previous findings in black sea bream (Zhou et al., 2010). Fu et al. (2005) reported that dietary supplementation of L-arginine enhances the expression of key genes responsible for glucose oxidation in rats. It has been suggested that nitric oxide (NO), a signaling molecule produced from L-arginine by various isoforms of NO synthase (NOS), is involved in regulation of hepatic gluconeogenesis. It was shown that physiological levels of NO stimulate glucose uptake and oxidation in insulin-sensitive tissues and inhibit the synthesis of glucose in target tissues (Jobgen, 2007). In this study increment of dietary arginine level enhanced T-NOS activity and this can be at least partially responsible for reduction of plasma glucose concentration at increased arginine levels.

Arginine has been identified as a potential modulator of both the innate and adaptive immune systems in vertebrates. As mentioned earlier, arginine is used as precursor for the

synthesis of NO and polyamines (Satriano et al., 1999), of which NO plays an important role in the cellular defense mechanisms (Buentello and Gatlin, 1999; Tafalla and Novoa, 2000). Also, it has been shown that incubation of seabream leucocytes with polyamines augments expression of immune-relevant genes (Satriano et al., 1999; Reyes-Becerril et al., 2011). Further, Costas et al. (2011) reported that supplementation of arginine in diets for Senegalese sole increases the expression of immune related genes including HIF-1, HAMP-1, MIP1-alpha and gLYS which play important roles in fish innate immunity. In the present study, innate immunity was positively affected by the increased dietary arginine levels. Serum T-NOS activity was significantly elevated at arginine level of 2.8% and confirmed the results of previous studies on yellow grouper (Zhou et al., 2012), blunt snout bream (Ren et al., 2013) and cobia (Ren et al., 2014). Significantly higher lysozyme activity was recorded in fish groups offered 2.4-3.2% arginine and agreed with the results of the studies on largemouth bass (Zhou et al., 2012) and Senegalese sole (Costas et al., 2011). However, no significant changes were detected in lysozyme activity of Nile tilapia (Yue et al., 2013) and channel catfish (Pohlenz et al., 2014) when they were provided with different dietary arginine levels. Also, our results exhibited the significant enhancement of MPO activity and plasma Ig level. Similarly, Costas et al. (2011) reported the significant enhancement of peroxidase activity in Senegalese sole (*Solea senegalensis*) fed arginine supplemented diets.

In conclusion, the findings in this study clearly demonstrated that dietary arginine not only promotes the growth performance of juvenile red sea bream but also positively affects the hematological and innate immune parameters. The optimal dietary arginine requirement for the maximal growth of red sea bream was estimated at 2.54% dry diet, corresponding to 5.08% of dietary protein.

CHAPTER FOUR

Dietary isoleucine requirement of juvenile olive flounder (*Paralichthys olivaceus*)

4.1. Introduction

Protein is the most important component of fish feeds providing essential amino acids for tissue repair and growth (Luo *et al*., 2006). Proteins and their building blocks, amino acids (AA), play very important roles in the structure and metabolism of living organisms (Meijer, 2003). Fishes cannot synthesize all the AAs and they will need an exogenous source of AAs to meet the requirements (NRC, 2011). A consistent amount of proteins and AAs needs to be incorporated in fish diets as they are continually used by the fish to build new proteins, peptides, free AAs, enzymes, hormones, neurotransmitters and cofactors. Branched-chain amino acids (BCAA), isoleucine, leucine and valine, exert very important roles in certain biochemical reactions and growth. Isoleucine participates in production of certain biochemical compounds which are involved in energy production, and together with the other two BCAA promotes tissue building. It is the first limiting BCAA in meat and bone meal (Wang *et al*., 1997). Isoleucine deficiency results in biochemical malfunction including growth retardation (Ahmed and Khan, 2006; Khan and Abidi, 2007).

It has long been demonstrated that deficiency of dietary proteins and AAs leads to impairment of immune function and increased susceptibility to infectious disease (Li *et al*., 2007). Results from both oral and parenteral feeding studies showed that protein intake and availability of certain dietary AAs are vital for optimal immune function of the intestine and the proximal resident immune cells (Ruth and Field, 2013). A large body of studies has been conducted to assess the role of AAs in immune function of animals including fish (Roch,

1999; Calder, 2006; Grimble, 2006; Kim *et al*., 2007). Early reports suggested that AAs are used as important energy substrates for immune cells (Wu *et al*., 1991a,b,c; Field *et al*., 1994) and antioxidant defense mechanisms (Xue and Field, 2011). Several AAs have been recognized to play significant roles in modulating various immune responses including the activation of lymphocytes, NK cells, and macrophages; proliferation of lymphocytes; regulation of intracellular redox states; gene expression; and production of cytokines (Yoneda *et al*., 2009). BCAA are involved in biosynthesis of glutamine, as part of the immune system, by providing α-amino group (Newsholme and Calder, 1997). Also, the carbon skeletons of BCAA are required by leucocytes for protein synthesis (Waithe *et al*., 1975). It has been reported that deficiency of BCAA results in immune impairment. Petro and Bhattacharjee (1981) reported that mice fed BCAA-deficient diet exhibit increased susceptibility to *Salmonella typhimurium*, impaired antibody production, reductions in serum concentrations of transferrin and complement C3, and increased numbers of bacteria in liver and spleen. Isoleucine has been reported to influence innate immunity including induction of the antimicrobial peptide beta-defensin from Madin–Darby bovine kidney (MDBK) epithelial cells (Fehlbaum *et al*., 2000). Also, it has been shown that isoleucine is incorporated into human leucocyte cellular proteins and lipids (Burns, 1975) and improves the serum complement component 3 (C3) level in mice (Petro and Bhattacharjee, 1980). However, there is only a single available study on immunomodulating effects of isoleucine in fishes; Zhao *et al*. (2013) showed that dietary isoleucine promotes the immune responses in Jian carp (*Cyprinus carpio* var. Jian).

Olive flounder has been the most important cultured marine fish species in Korea. Its total production reached ~37000 tons in 2013 in Korea (Ministry of Maritime Affairs and Fisheries of Korea, 2013). Regarding the importance of olive flounder for Korean aquaculture

industry, this study was conducted to examine the effects of different dietary isoleucine levels on growth, hematology and non-specific immune response of the species.

4.2. Materials and methods

4.2.1. Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 4-1. Six isonitrogenous (45% crud protein) and isocaloric (4.45 kcal g^{-1} gross energy) diets were formulated using fish meal and a mixture of crystalline AAs to contain graded levels of isoleucine (0.48 – 2.78% dry diet). The mixture of crystalline AAs without isoleucine was prepared according to Dabrowski *et al*. (2003) and used as the main protein source and fish meal was included to increase palatability of the semi-purified diets. The basal diet contained a minimum level of isoleucine from fish meal and supplemented with incremental levels (0.5%) of L-isoleucine. Targeted dietary isoleucine concentrations were 0.48, 0.87, 1.43, 1.94, 2.37 and 2.78%. The experimental diets were kept isonitrogenous by decreasing the level of glycine as the isoleucine level was increased. All dry ingredients were thoroughly mixed and after addition of squid liver oil and double distilled water pelleted through a meat chopper machine (SMC-12, Kuposlice, Busan, Korea) in 3 mm diameter. Then, the diets were freeze-dried for 24 h, crushed into desirable particle sizes, sealed in bags and stored at –20 ^{o}C until used.

Table 4-1. Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	**1**	**2**	**3**	**4**	**5**	**6**
White fish meal	20.0	20.0	20.0	20.0	20.0	20.0
Free AA mix[1]	27.5	27.5	27.5	27.5	27.5	27.5
Isoleucine	0.0	0.5	1.0	1.5	2.0	2.5
Glycine	2.5	2.0	1.5	1.0	0.5	0.0
Dextrin	33.5	33.5	33.5	33.5	33.5	33.5
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[2]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin mix[3]	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Dry matter	93.1	93.0	94.0	93.4	94.0	94.0
Protein	44.9	45.2	45.0	45.4	45.6	45.1
Lipid	8.8	8.6	8.8	8.7	8.9	8.9
Ash	3.6	3.8	3.5	3.7	3.5	3.5
Isoleucine	0.48	0.87	1.43	1.94	2.37	2.78

[1]Free amino acid mixture composition: (g per 446 g dry weight mixture): arginine hydrochloride, 15; lysine hydrochloride, 18; methionine, 10; histidine, 7; valine, 12; leucine, 14; phenylalanine, 18; threonine, 8; tryptophan, 2; glutamic acid, 111; glycine, 231.

[2]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3]Vitamin premix (g kg^{-1}): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

4.2.2. Fish and feeding trial

Juvenile olive flounder were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). All the fish were fed the basal diet for one week to be acclimated to the semi-purified diet and the experimental conditions. At the end of the acclimation period, 45 randomly selected fish (averaging at 8.59 ± 0.13 g) were stocked into each polyvinyl circular tanks of 150 L capacity and supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 9 weeks. Uneaten food was siphoned out 30 min after feeding and weighed to determine the feed intake. Growth of fish was measured with three-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish. The water temperature during the feeding trial ranged from 13 to 17 ℃ and the photoperiod was maintained on a 12:12 light:dark schedule.

4.2.3. Sample collection and analyses

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Five intact fish per tank (15 fish per treatment) were selected and kept at −20 ℃ for whole-body composition analysis. Five fish per tank (15 fish per treatment) were randomly captured, anesthetized with 2-phenoxyethanol (200 mg l^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for determination of hematocrit, hemoglobin and

respiratory burst activity. After the above mentioned measurements with whole blood, plasma were separated by centrifugation at 5000 × g for 10 min and stored at −70 °C for determination of total immunoglobulin (Ig) level and blood biochemical parameters including plasma total protein, glucose and cholesterol concentrations and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Another set of blood samples (5 fish per tank, 15 fish per dietary treatment) were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation for 10 min at 5000 × g and stored at −70 °C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities.

Analyses of moisture and ash contents of samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Seoul, Korea). Hematocrit was determined by microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of total protein, glucose and cholesterol and activities of ALT and AST were determined by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), then 200 µl of suspension was placed in each well of 96-well plates and 20 µl serum was added subsequently. The reduction in absorbance of the samples was recorded at

570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (*UVM 340, Biochrom, Cambridge, UK*). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

Oxidative radical production by phagocytes during respiratory burst was measured through NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Briefly, blood and NBT (0.2%) (Sigma, USA) were mixed in equal proportion (1:1) and incubated for 30 min at room temperature, then 50 µl was taken out and dispensed into glass tubes. Then, 1 ml dimethylformamide (Sigma) was added and centrifuged at 2000 × *g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as blank.

Serum myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997). Briefly, twenty microliter of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma, USA) in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 µl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in the microplate reader.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein concentration was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules using a 12% solution of polyethylene glycol (Sigma). The difference in protein concentration represents the Ig content.

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

4.2.4. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$. A second-order polynomial regression analysis was used for determination of optimum dietary isoleucine level based on weight gain. Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

4.3. Results

The results showed the improvement of fish growth performance and feed utilization by increment of dietary isoleucine level and significantly ($P < 0.05$) higher values were obtained at dietary isoleucine levels of ≥0.87% (Table 4-2). Feed conversion ratio decreased significantly at isoleucine levels of 0.87-1.94% and significantly higher protein efficiency ratio was found in fish fed 1.43 and 1.94% isoleucine compared to those offered 0.48% isoleucine. The optimum dietary isoleucine level was determined by a second-order polynomial regression analysis on the basis of weight gain and was estimated to be 1.69% diet corresponding to 3.75% dietary protein (Fig. 4-1). Fish survival was not affected by dietary treatments. Also, the results showed no significant effect of dietary isoleucine on fish whole-body composition (Table 4-3).

Higher hematocrit values were obtained by increment of dietary isoleucine level, but the differences were not significant (Table 4-4). Significantly higher plasma total protein and cholesterol concentrations were found in groups of fish fed 1.43-2.37% isoleucine. Also, the results revealed the significant reduction of plasma ALT and AST activities at dietary isoleucine levels of 1.43-1.94% and 0.87-1.94%, respectively.

Non-specific immune response parameters were affected by dietary isoleucine levels (Table 4-5). Significantly higher lysozyme and SOD activities were found in fish fed isoleucine levels of 0.87-2.37% and 1.94-2.37%, respectively, compared to those fed the basal diet. Respiratory burst activity was significantly increased at the levels of 1.43-1.94% isoleucine. The highest Ig level was detected in fish fed 1.94% isoleucine and differed significantly ($P < 0.05$) from that of the fish fed 0.43% isoleucine. However, MPO activity did not significantly differ among experimental groups.

Table 4-2. Growth performance of olive flounder (initial body weight, 8.59 ± 0.13) fed different levels of dietary isoleucine for 9 weeks.

Ile level	WG[1] (%)	SGR[2] (%)	FI[3] (g/fish)	FCR[4]	PER[5]	Survival (%)
0.48	81.54 ± 4.68^{d}	0.95 ± 0.04^{d}	18.71 ± 3.47	2.68 ± 0.61^{a}	0.84 ± 0.22^{b}	80.00 ± 13.5
0.87	119.12 ± 4.07^{ab}	1.25 ± 0.03^{ab}	19.89 ± 1.14	1.92 ± 0.06^{b}	1.14 ± 0.04^{ab}	77.78 ± 7.70
1.43	128.41 ± 4.85^{a}	1.31 ± 0.03^{a}	17.93 ± 0.80	1.64 ± 0.12^{b}	1.33 ± 0.10^{a}	89.63 ± 5.59
1.94	116.80 ± 5.76^{ab}	1.23 ± 0.04^{ab}	18.15 ± 1.23	1.80 ± 0.21^{b}	1.22 ± 0.14^{a}	88.15 ± 16.8
2.37	109.04 ± 4.77^{bc}	1.17 ± 0.04^{bc}	18.47 ± 0.96	1.98 ± 0.18^{ab}	1.10 ± 0.10^{ab}	89.63 ± 8.41
2.78	101.98 ± 5.40^{c}	1.12 ± 0.04^{c}	17.69 ± 1.16	2.05 ± 0.03^{ab}	1.05 ± 0.01^{ab}	91.85 ± 5.13

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1]Weight gain = [(final body weight – initial body weight) / initial body weight × 100]

[2]Specific growth rate = 100 × [(ln final body weight - ln initial body weight) / days]

[3]Feed intake (g/fish) = dry feed consumed (g) / fish

[4]Feed conversion ratio = dry feed fed / wet weight gain

[5]Protein efficiency ratio = wet weight gain / total protein given

Table 4-3. Whole-body composition of olive flounder fed different levels of dietary isoleucine for 9 weeks.

Ile level	Protein	Lipid	Moisture	Ash
0.48	16.78±0.45	1.08±0.16	82.40±0.61	3.48±0.38
0.87	16.83±0.71	1.09±0.03	81.85±0.60	3.25±0.69
1.43	17.55±0.73	0.99±0.05	81.93±0.14	3.56±0.70
1.94	16.97±0.53	1.14±0.19	81.47±0.25	3.33±0.52
2.37	16.80±0.65	1.15±0.12	81.73±0.50	3.65±0.39
2.78	16.87±0.50	1.12±0.04	81.54±0.45	3.51±0.75

Values are mean of triplicate groups and presented as mean ± SD. The lack of superscript letter indicates no significant differences among treatments.

Table 4-4. Hematological parameters of olive flounder (initial body weight, 8.59 ± 0.13) fed different levels of dietary isoleucine for 9 weeks.

Ile level	Ht[1]	Hg[1]	Total protein[3]	ALT[4]	AST[5]	Glucose[6]	Cholesterol[7]
0.48	15.83±1.04	2.36±0.24	1.72±0.15^{c}	57.33±6.66^{a}	105.5±6.40^{a}	19.65±1.94	39.77±8.21^{c}
0.87	16.50±1.80	2.38±0.02	1.87±0.24^{bc}	56.20±4.20^{a}	83.74±5.24^{b}	24.02±4.19	49.12±9.42^{bc}
1.43	17.50±2.18	2.45±0.33	2.58±0.38^{ab}	35.79±2.44^{c}	64.94±7.58^{b}	24.31±3.58	77.55±5.80^{a}
1.94	17.67±1.76	2.59±0.25	2.96±0.28^{a}	43.26±4.95^{bc}	78.00±9.13^{b}	25.19±3.46	67.08±7.57^{ab}
2.37	17.33±0.76	2.30±0.13	2.88±0.21^{a}	55.37±5.37^{ab}	84.88±8.42^{ab}	24.60±2.40	64.85±7.86^{ab}
2.78	17.67±1.04	2.33±0.10	2.53±0.34^{ab}	62.81±2.66^{a}	85.03±7.97^{ab}	23.40±4.89	60.54±8.18^{abc}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1]Haematocrit

[2]Haemoglobin

[3]Total protein (g/dL)

[4] Alanine aminotransferase activity (U/L)

[5] Aspartate aminotransferase (U/L)

[6]Glucose (mg/dL)

[7]Total cholesterol (mg/dL)

Table 4-5. Innate immune response parameters of olive flounder (initial body weight, 8.59 ± 0.13) fed different levels of dietary isoleucine for 9 weeks.

Ile level (% diet)	Lysozyme[1]	NBT[2]	MPO[3]	Ig[4]	SOD[5]
0.48	19.74±2.29^{c}	0.26±0.01^{b}	1.42±0.04	5.89±0.57^{b}	55.22±2.84^{c}
0.87	28.23±2.45^{ab}	0.34±0.03^{ab}	1.49±0.04	8.83±0.73^{ab}	59.06±3.16^{bc}
1.43	28.06±2.13^{ab}	0.41±0.04^{a}	1.51±0.15	10.36±0.21^{ab}	62.54±4.56^{abc}
1.94	34.21±2.48^{a}	0.42±0.08^{a}	1.65±0.09	12.92±3.18^{a}	68.41±1.51^{a}
2.37	33.27±2.59^{a}	0.35±0.03^{ab}	1.45±0.10	10.72±3.56^{ab}	64.58±2.10^{ab}
2.78	24.73±4.57^{bc}	0.37±0.05^{ab}	1.50±0.03	9.75±2.59^{ab}	58.67±3.57^{bc}

Values are mean of triplicate groups and presented as mean ± S.D. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1] Lysozyme (U/ml)

[2]Nitro blue tetrazolium activity (Absorbance)

[3]Myeloperoxidase (Absorbance)

[4]Immunoglobulin (mg/ml)

[5]Superoxide dismutase (% inhibition)

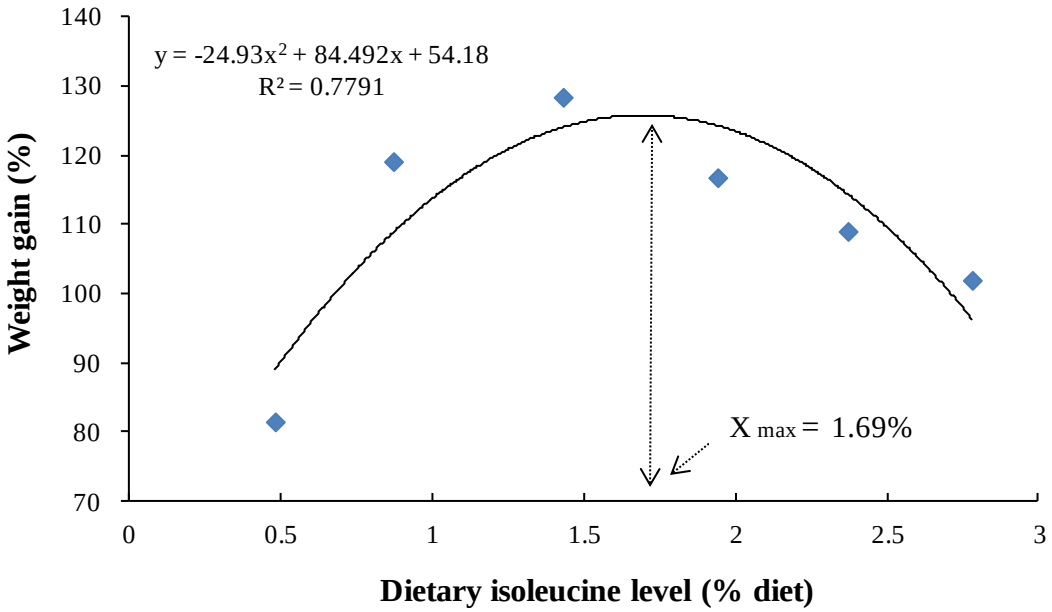

Fig. 4-1. Relationship between weight gain and dietary isoleucine level for olive flounder as described by second-order polynomial regression.

4.4. Discussion

In the present study the fish readily accepted the semi-purified test diets, however, relatively low growth rates were achieved due to low rearing water temperature. The results showed the significant enhancement of fish growth by increment of dietary isoleucine level up to 1.43% and thereafter a decreasing tendency was achieved. The increased fish growth in this study was primarily due to improved feed utilization efficiency as significantly lower FCR and higher PER were obtained by increment of dietary isoleucine level. It has long been demonstrated that a balanced AA profile is required for effective utilization of dietary protein for tissue synthesis (D'Mello, 1994; Yamamoto *et al*., 2000; Berge *et al*., 2002; Green and Hardy, 2002; Goemez-Requeni *et al*., 2003). In this study reduced growth was found in fish groups fed isoleucine deficient diets and those fed higher isoleucine levels than 1.43% indicating imbalances in dietary AA profile. The results showed no significant influence of isoleucine on feed intake. Similarly, previous studies on catla (*Catla catla*) (Zehra and Khan, 2013) and Pacific white shrimp (*Litopenaeus vannamei*) (Liu *et al*., 2014) did not show any significant effect of dietary isoleucine on feed intake. However, Zhao *et al*. (2012) reported the significant increase of feed intake in Jian carp (*Cyprinus carpio* var. Jian) offered incremental dietary isoleucine levels. Quantitative requirement for essential AAs is generally estimated on the basis of fish growth; however regarding the secondary growth of fish in this study the data were not used for determination of optimal isoleucine requirement level and further studies in the future are required. Fish whole-body composition was taken into account in the present study and the results revealed no significant changes regarding to variations in dietary isoleucine level.

Dietary isoleucine requirement of olive flounder, 3.75% of dietary protein, worked out in this experiment is found to be higher than that reported for white sturgeon, 3.0% (Ng and

Hung, 1995); red sea bream, 2.2% (Forster and Ogata, 1998); turbot, 2.60% (Kaushik, 1998); Atlantic salmon, 3.2% (Rollin, 1999) and mrigal carp, 3.12–3.15% (Benakappa and Varghese, 2003, Ahmed and Khan, 2006) but lower than the requirement reported for milkfish, 4.0% (Borlongan and Coloso, 1993) and grass carp, 4.0–4.23% (Di et al., 2009) and comparable to the requirement of rohu, 3.75% (Khan and Abidi 2007) and tilapia 3.45% of dietary protein (NRC 2011). The above discrepancies in amino acid requirements of fish may be affected by fish size and age, adequate levels of other nutrients, flow rate, stock density, and the environmental and culture conditions adopted by different laboratories (Cowey and Luquet, 1983; Kim et al., 1992; Forster and Ogata, 1998; Luzzana, et al. 1998; Abidi and Khan, 2009). Nutrient and energy digestibility, amino acid profile and energy content may also alter the amino acid requirements (Simmons et al., 1999; De Silva et al., 2000).

Hematological parameters are being increasingly taken into account in AA requirement studies because of their sensitivity to dietary manipulations (Hrubec *et al*., 2000; Congleton and Wagner, 2006). Accordingly, in the current study the changes in hematological and blood biochemical parameters were examined and significant improvements could be detected. Zhao *et al*. (2013) reported the significant increase of red and white blood cells count in Jian carp by increment of dietary isoleucine level. In the current study, numerically higher hematocrit values were observed at higher isoleucine levels, however the differences were not significant. Plasma total protein level has been measured frequently as an indicator of physiological condition in fish nutrition studies (Nakagawa *et al*., 2000; Farhangi and Carter, 2001; Watanabe *et al*., 2001; Harikrishnan *et al*., 2003). Total protein in plasma is the most stable component, and few dietary factors have been reported to affect the levels in fish. Plasma total protein is elevated when dietary protein intake increases (Leveille and Sauberlich, 1961) indicating improved physiological condition (Dawson and Bortolotti, 1997). Also, it has been suggested that increased blood protein level is associated with

enhanced innate immune response in fish (Wiegertjes *et al*., 1996). In the current study significant enhancements in plasma total protein level was observed at increased isoleucine levels. There is no available study on the effect of dietary isoleucine on fish plasma total protein level, but in agreement to our study significant enhancements in fish serum/plasma protein levels have been reported following administration of optimum dietary lysine level in black sea bream (*Sparus macrocephalus*) (Zhou *et al*., 2010) and yellow catfish (*Pelteobagrus fulvidraco*) (Cao *et al*., 2012); and optimal dietary valine level in red sea bream (Rahimnejad and Lee, 2013). In general, ALT and AST are mainly distributed in the liver and spleen and play important roles in protein metabolism. Their concentrations in the blood increase when the liver and myocardial cells are damaged or their permeability increased. Both enzymes are used as valuable diagnostic means of stress responses in fish species (De Smet and Blust, 2001; Almeida *et al*., 2002; Choi *et al*., 2007) and their concentration in plasma increases in response to several factors such as pollution and ammonia and nitrite toxications (Das *et al*., 2004). In the current study significant reductions in plasma ALT and AST concentrations were achieved by increment of dietary isoleucine level up to 1.94% indicating an improvement in fish health status. It has been reported that dietary protein affects the plasma cholesterol level (Carroll and Hamilton, 1975; Kritchevsky, 1979; Terpstra *et al*., 1983) and that the main influencing factor is the AA composition of the protein source (Garlich *et al*., 1970; Olsen *et al*., 1970a,b; Coles and McDonald, 1972). In agreement to this notion, Sugiyama *et al*. (1996) reported a significant positive correlation between the plasma total cholesterol level and plasma concentration of valine in rats fed different dietary protein sources. Also, the results of our previous study on red sea bream showed the significant increase of plasma cholesterol concentration by increment of dietary valine level (Rahimnejad and Lee, 2013). Similarly, in the present study plasma cholesterol level elevated significantly when dietary isoleucine level increased.

It has been demonstrated that deficiency or excess of dietary protein (Glick *et al*., 1981, 1983; Payne *et al*., 1990) or AAs (Bhargava *et al*., 1970, 1971; Tsiagbe *et al*., 1987a,b) alters immune responses. BCAA have been shown to play very important roles in the immune organ development (Kidd, 2004). Konashi *et al*. (2000) reported that weights of lymphoid organs are modified by either the type of essential AAs or the degree of deficiency; and that feeding the BCAA-deficient diet causes the most severe decrease in both thymus and bursa weights in chickens. Lysozyme as a bactericidal enzyme is well known as an important humoral indicator of innate immunity in fish. It is released by leucocytes and provides protection against both gram-positive and gram-negative bacteria by lysing the 1, 4-beta-linkages in the peptidoglycan layer found in bacteria cell walls (Ellis, 1999). Several factors affect the lysozyme activity in fish including nutritional status (Saurabh and Sahoo, 2008). In the present study, significantly higher lysozyme activities were found by increment of dietary isoleucine level up to 2.37% and thereafter a reduced activity was obtained. Similarly, Zhao *et al*. (2013) reported the significant enhancement in lysozyme activity of Jian carp fed increased dietary isoleucine levels. The respiratory burst is generated by macrophages/monocytes and granulocytes to attack invasive pathogens during phagocytosis and is widely used to evaluate the defense capabilities against pathogens (Dalmo *et al*., 1997). In the current study the respiratory burst activity was measured through NBT assay and significantly higher activities were achieved at isoleucine levels of 1.43-l.94% compared to those fed the basal level. There is no earlier report on the effect of isoleucine on fish respiratory burst activity; however our previous study on red sea bream (Rahimnejad and Lee, 2013) showed the enhanced respiratory burst activity at increased valine levels indicating the role of BCAA. Total immunoglobulins play important roles in innate and acquired immune response (Magnadóttir, 2006) and are regarded as a good indicator for the action of immunonutrients. In this study higher Ig levels were found by increment of dietary isoleucine

and a significant difference was observed between the groups fed 0.48 and 1.94% isoleucine. In agreement to our results, Zhao *et al*. (2013) showed the significant elevation of IgM level in Jian carp fed increased dietary isoleucine level. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen including oxygen ions and peroxides. The over production of ROS may damage cell membranes (Liu *et al*., 2007). Radical scavenging enzymes such as SOD can provide protection against ROS damage. Earlier, Zhao *et al*. (2013) reported the enhancement of SOD activity in Jian carp when dietary isoleucine increased from 0.42 to 0.95%, which is in agreement with the results of the present study.

In conclusion, under the rearing conditions in this study it was shown that supplementation of a proper level of isoleucine is necessary for maximal growth of olive flounder. The most significant finding of this study was that dietary isoleucine influences humoral innate immune response in olive flounder, and that the optimal level for efficient immune function seems to be approximately 2% of diet.

CHAPTER FIVE

Dietary methionine requirement of juvenile olive flounder (*Paralichthys olivaceus*)

5.1. Introduction

Protein quantity and dietary amino acid composition are two major factors that influence growth of fish. Fish do not seem to have a true protein requirement but a well balanced mixture of indispensable amino acids in the diet is essential for their growth and maintenance. Since protein is the most expensive component of feed items, inclusion of its optimum amount will enable us to develop cost-effective dietary formulations. Amino acid balance is one of the most important factors affecting protein quality. Determining the essential amino acid requirements of cultured fish is of extreme importance due to the significant effects of these nutrients on muscle deposition, feed costs and nitrogen pollution (Small and Soares, 1999).

Methionine is the sulphur-containing amino acid required by terrestrial vertebrates as well as various fish species for normal growth and metabolic functions. Methionine takes part in protein synthesis and other important physiological functions (Lovell, 1989). The essential amino acid methionine is important because it is used for protein synthesis, converted to cystine for incorporation into protein, and in the form of S-adenosyl-methionine, it is the principal donor of methyl groups in the body. As S-adenosyl methionine, methionine is the precursor of the polyamines spermine and spermidine which have diverse physiological role related to cell proliferation and growth (Murray et al., 1996). Methionine is also a precursor of choline and Kasper et al. (2000) reported that when methionine is not in excess in the diet

of Nile tilapia, Oreochromis niloticus, choline is required for growth. Methionine is usually the first limiting amino acid in many fish diets, especially those containing higher levels of plant protein sources such as soybean meal, peanut meal and copra meal (Tacon and Jackson, 1985; Dabrowski et al., 1989). The study of sulphur amino acid nutrition in fish has become increasingly important as dietary formulations incorporate lower levels of fish meal and higher levels of plant feedstuffs (Twibell et al., 2000). Studies have demonstrated that supplementation of methionine to plant protein diets is able to improve growth response of many fish species (Mukhopadhyay and Ray, 2001; Takagi et al., 2001; Opstvedt et al., 2003). In fish both excess and restricted dietary methionine content have been reported to affect growth performance, feed intake and carcass quality (Jackson and Capper, 1982; Rumsey et al., 1983; Mambrini et al., 2001; Sveier et al., 2001). Also, cataracts were observed in several salmonid species fed with methionine-deficient diets (Walton et al. 1982; Rumsey et al. 1983; Keembiyehetty and Gatlin, 1993). Thus, to maximize the utilization of cheap plant and animal protein sources, especially those that have low methionine levels, the determination of methionine requirement in semipurified diets and its application to practical diets are important for the development of cost-effective feed.

Aquaculture of the olive flounder is one of the rapidly increased industries in Korea due to the high market value of this species. Knowledge of the dietary essential amino acid (EAA) requirement of aquaculture species like the olive flounder is essential for developing commercial feeds using low levels of marine proteins. Therefore, the present study was conducted to determine dietary methionine requirement of juvenile olive flounder.

5.2. Materials and methods

5.2.1. Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 5-1. Six isonitrogenous (45% crud protein) and isocaloric (4.36 kcal g^{-1} gross energy) experimental diets were formulated to contain different levels of methionine from 0.3 to 1.8%. A mixture of synthetic free amino acids without methionine was prepared according to Dabrowski et al. (2003) and used as the main protein source. The basal diet was formulated to contain 0.3% methionine from fish meal and supplemented with DL-methionine at 0.3% increments. The energy value of each diet was estimated on the basis of physiological fuel value, i.e., 3.99 kcal g^{-1} proteins or carbohydrates and 9.01 kcal g^{-1} lipids (Lee and Putnam, 1973). The experimental diets were kept isonitrogenous and isocaloric using glycine at the expense of methionine. All ingredients were well mixed, pelletized and freeze-dried. The pellets were crushed into desirable particle sizes and stored at -20 °C until use.

Table 5-1. Formulation and proximate composition of the six experimental diets (% dry matter).

	1	**2**	**3**	**4**	**5**	**6**
White fish meal[1]	17.0	17.0	17.0	17.0	17.0	17.0
FAA mix[2]	33.0	33.0	33.0	33.0	33.0	33.0
Methionine[3]	0.0	0.3	0.6	0.9	1.2	1.5
Glycine[4]	1.5	1.2	0.9	0.6	0.3	0.0
Dextrin	33.3	33.3	33.3	33.3	33.3	33.3
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[5]	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin mix[6]	2.5	2.5	2.5	2.5	2.5	2.5
Squid liver oil[7]	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Moisture (%)	11.3	12.4	11.7	12.9	12.2	11.9
Protein (%, DM)	44.7	43.8	44.9	44.2	43.8	44.3
Lipid (%, DM)	8.9	8.5	8.8	8.9	8.7	8.8
Ash (%, DM)	3.1	3.4	3.8	3.8	3.6	3.6

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

[2]Free amino acid mixture composition: (g/1384.11 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 37.8; valine, 37.8 (Fluka, Buchs, Japan); lysine, 45.36; D,L-methionine, 31.5 (WooSung, Daejun, Korea); histidine, 22.05; isoleucine, 28.35; phenylalanine; 56.7; threonine, 25.2; tryptophan, 6.3; proline, 365.4; serine, 365.4; alanine, 362.25 (Sigma Chemicals, St. Louis, MO).

[3]Methionine: Sigma Chemicals, L-Methionine

[4]Glycine: Sigma Chemicals, L-Glycine

[5]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[6]Vitamin premix (g kg^{-1}): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[7]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

5.2.2. Fish and feeding trial

Juvenile olive flounder were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). All the fish were fed the basal diet for one week to be acclimated to the semi-purified diet and the experimental conditions. At the end of the acclimation period, 35 randomly selected fish (averaging at 17.28 ± 0.1 g) were stocked into each polyvinyl circular tanks of 150 L capacity and supplied with filtered seawater at a flow rate of 1.5 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed with one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for eight weeks. Growth of fish was measured with two-week intervals. Feeding was stopped 24 h prior to weighing to minimize handling stress on fish. The water temperature during the feeding trial ranged from 14 to 20 °C and the photoperiod was maintained on a 12:12 light:dark schedule.

5.2.3. Sample collection and analyses

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Five intact fish per tank (15 fish per treatment) were selected and kept at −20 °C for whole-body amino acid composition analysis. The samples were freeze-dried and finely ground using a grinder and the amino acid compositions were analyzed using an automatic amino acid analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

Three fish per tank were randomly captured, anesthetized with 2-phenoxyethanol (200 mg l^{-1}), and blood samples were collected from the caudal vein with heparinized

syringes for determination of hematocrit, hemoglobin and respiratory burst activity. Hematocrit was determined by microhematocrit technique (Brown, 1980) and hemoglobin was measured by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

Oxidative radical production by phagocytes during respiratory burst was measured through NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Briefly, blood and NBT (0.2%) (Sigma, St. Louis, MO, USA) were mixed in equal proportion (1:1) and incubated for 30 min at room temperature. Then 50 µl was taken out and dispensed into glass tubes. One ml of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as the blank.

Analyses of moisture and ash contents of diets were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

5.2.4. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical

significance was determined at $P < 0.05$. A broken-line regression model was used for determination of optimum dietary methionine level based on weight gain (WG). Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

5.3. Results

Fish growth performance was significantly influenced by dietary methionine levels (Table 5-2). The results showed the significant increase of weight gain in the groups fed diets containing 1.5 and 1.8% methionine compared to those fed 0.3% dietary methionine. The protein efficiency ratio increased by increment of methionine level up to 1.5% and lower feed conversion ratio were obtained at higher methionine levels but no significant changes were observed among the experimental groups. The fish survival rate varied from 61 to 85% and no significant differences could be detected. The optimum dietary methionine requirement was evaluated at 1.63% (Fig. 5-1).

The results of hematological analysis revealed a slight improvement in hemoglobin concentration at ≥1.2% methionine levels, although the changes were not significant (Table 5-3). Also, numerically higher respiratory burst activity was found in groups fed higher methionine levels compared to the group fed 0.3% methionine.

Fish whole-body amino acid composition is provided in Table 5-4. The results showed increased accumulation of most essential amino acids at higher methionine levels compared to those fed the basal level but the differences were not significantly different.

Table 5-2. Growth performance of juvenile olive flounder fed the experimental diets with graded levels of methionine for eight weeks.

Met level	FBW[1] (g)	WG[2] (%)	FCR[5]	PER[6]	Survival (%)
0.3	20.54±1.14^{a}	18.70±6.60^{a}	2.22±0.79	0.98±0.29	65.00±14.14
0.6	21.13±1.41^{ab}	22.66±8.41^{ab}	2.16±0.57	0.98±0.26	61.67±5.77
0.9	22.04±1.67^{abc}	27.73±10.59^{ab}	1.83±0.81	1.24±0.48	65.60±8.16
1.2	23.16±1.72^{abc}	33.56±9.95^{ab}	1.61±0.35	1.29±0.25	82.50±10.60
1.5	25.31±1.43^{c}	46.40±9.16^{b}	1.47±0.66	1.54±0.60	85.00±21.22
1.8	25.13±1.40^{bc}	45.62±9.23^{b}	1.56±0.33	1.32±0.27	77.50±3.53

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05).

[1]Final body weight

[2]Weight gain= [(final body weight – initial body weight)/initial body weight × 100]

[3]Feed conversion ratio= dry feed fed/wet weight gain

[4]Protein efficiency ratio= wet weight gain/total protein given

Table 5-3. Hematological parameters of juvenile olive flounder fed the experimental diets with graded levels of methionine for eight weeks.

	Met levels					
	0.3	**0.6**	**0.9**	**1.2**	**1.5**	**1.8**
Hematocrit (%)	23.17±3.54	21.44±4.07	24.33±3.79	23.42±0.12	25.33±1.86	24.61±1.13
Hemoglobin (g/dL)	3.79±0.66	3.68±0.39	3.78±0.54	4.24±0.04	4.19±0.37	3.82±0.19
NBT (absorbance)	0.38±0.02	0.44±0.11	0.42±0.08	0.46±0.09	0.42±0.05	0.46±0.03

Values are mean of triplicate groups and presented as mean ± SD. The lack of superscript letter indicates no significant differences among treatments.

Table 5-4. Whole-body amino acid composition of juvenile olive flounder fed the experimental diets with graded levels of methionine for eight weeks.

	Met levels					
	0.3	**0.6**	**0.9**	**1.2**	**1.5**	**1.8**
EAA						
Arg	6.55±0.60	7.01±0.53	7.04±0.81	6.71±0.53	6.79±0.06	6.63±0.17
His	1.84±0.10	1.91±0.08	2.25±0.52	1.99±0.16	1.97±0.01	1.87±0.06
Ile	3.65±0.13	3.87±0.26	3.77±0.02	3.86±0.24	3.84±0.04	3.75±0.04
Leu	6.67±0.27	7.08±0.54	7.42±1.00	7.03±0.45	6.96±0.09	6.83±0.04
Lys	8.03±0.10	8.90±1.28	8.18±0.23	8.44±0.46	8.68±0.24	8.24±0.22
Met	0.49±0.03	0.68±0.07	0.51±0.01	0.58±0.01	0.63±0.02	0.67±0.15
Phe	3.67±0.17	3.86±0.26	4.33±1.01	3.82±0.31	3.82±0.03	3.74±0.05
Thr	3.86±0.18	4.03±0.08	4.08±0.07	4.03±0.31	4.10±0.03	4.04±0.11

Val	4.46±0.23	4.80±0.43	4.56±0.04	4.73±0.34	4.64±0.04	4.61±0.09
NEAA						
Ala	7.81±0.84	7.41±0.73	7.11±0.16	6.94±0.45	7.02±0.06	7.04±0.24
Asp	8.85±0.76	9.17±0.15	9.28±0.15	9.41±0.30	9.45±0.12	9.45±0.08
Glu	13.78±0.53	14.62±0.66	14.18±0.15	14.01±0.65	14.25±0.03	14.32±0.20
Gly	9.59±0.10	9.36±0.68	8.65±0.69	8.59±0.93	8.95±0.30	8.72±0.46
Pro	9.34±0.81	10.46±0.35	10.43±0.96	8.31±1.40	8.98±0.25	10.12±1.12
Ser	4.32±0.11	4.74±0.47	4.60±0.25	4.32±0.41	4.41±0.02	4.59±0.11
Tyr	2.81±0.09	2.99±0.19	2.88±0.08	2.96±0.15	2.96±0.04	2.88±0.02

Values are mean of triplicate groups and presented as mean ± SD. The lack of superscript letter indicates no significant differences among treatments.

[1]Essential amino acids

[2]Non-essential amino acids

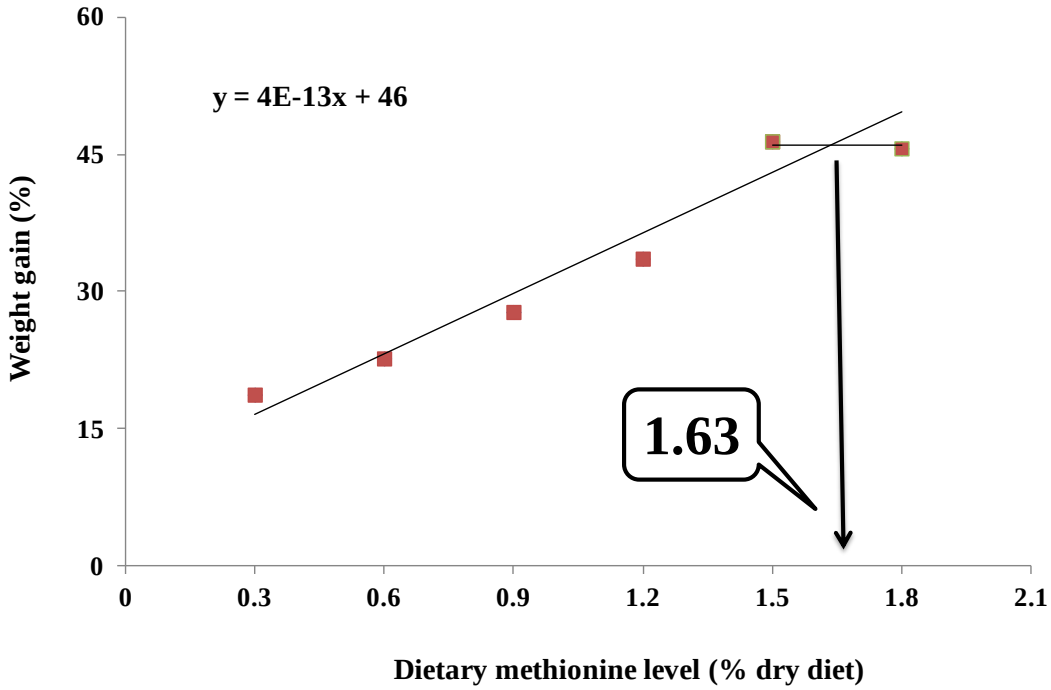

Fig. 5-1. Broken-line regression analysis of weight gain to dietary methionine levels. Each point represents the average of three groups of fish.

5.4. Discussion

In this study fish accepted all the semipurified test diets, however in general low growth rates were obtained because the experiment was conducted in low temperature season. On the other hand, in the current study relatively high portion of dietary protein was provided in the form of crystalline amino acids (CAAs). The utilization of CAAs in practical diets of fish depends on the species ability to absorb and use such amino acids for protein synthesis as well as for other physiological (Nguyen and Davis, 2009). Fish do not appear to utilize dietary CAAs as effectively as intact protein, which has frequently been reported by other workers (Robinson et al., 1981; Walton et al., 1982; Fauconneau, 1988; Espe and Lied, 1994; Ahmed et al., 2003).

Most authors reported that the growth of fish or shrimp showed different tendency with the increase in dietary methionine: (1) increased with increasing dietary methionine and remained constant when dietary methionine is higher than requirement (Harding et al. 1977; Coloso et al. 1999; Alam et al. 2001); (2) increased with increasing dietary methionine and decreased when dietary methionine is higher than requirement (Millamena et al. 1996; Akiyama and Arai, 1993; Sveier et al., 2001). In the present study, significant improvement in fish growth performance was observed by increment of dietary methionine level up to 1.5% and plateaued by further increment. The results in the present study showed that weight gain of the juveniles fed methionine-unsupplemented diet was significantly lower than that of the juveniles fed methionine-supplemented diets. This indicates that olive flounder juveniles are able to utilize supplemental methionine from CAA and also indicates that methionine was essential for growth of olive flounder. Dietary methionine deficiency resulted in the

formation of cataracts in some fish species (Walton et al., 1982; Rumsey et al., 1983; Poston, 1986; Keembiyehetty and Gatlin, 1993; Ruchimat et al., 1997; Takagi et al., 2001), but was not observed in the present study. Estkvez et al. (1997) also reported that no cataracts were formed in the eyes of Japanese flounder fed methionine-deficient diets. The poor growth of the methionine-unsupplemented groups may be due to loss of appetite, which resulted in low feed intake, hence depressed growth.

Dose–response experiments with increasing supply of amino acid are accepted in principle as a method for determining dietary amino acid requirements (Cowey, 1995). The present study indicated that the optimum dietary methionine requirement of juvenile olive flounder was estimated to be 1.63% of diet (3.62% of dietary protein) based on growth performance. This value is similar to those previously reported for other fish species, such as Chinook salmon (1.6% of diet, 4.0% of dietary protein) (Halver et al., 1959), gilthead sea bream (1.4% of diet, 4.0% of dietary protein) (Luquet and Sabaut, 1974). However, it is higher than those estimated for channel catfish (0.6% of diet) (Harding et al., 1977), rainbow trout (0.52%) (Kim et al., 1992), red drum (1.06%) (Moon and Gatlin, 1991), sea bass (1.0%) (Thebault et al., 1985), hybrid striped bass (1.0%) (Keembiyehetty and Gatlin, 1993), yellow perch (1.0%) (Twibell et al., 2000), Nile tilapia (0.75%) (Santiago and Lovell, 1988) and yellowtail (1.11%) (Ruchimat et al., 1997). There are many factors that may affect measured amino acid requirements, including species, the fish size, diet type (purified, semi-purified, practical), dietary protein and energy level, statistical method, palatability, feeding regime and environmental conditions (Tacon and Cowey, 1985; Rodehutscord et al., 1997). Because a portion of dietary methionine can be converted to cystine when

necessary in the body, the presence of dietary cystine reduces the amount of methionine required for maximum growth. Thus, the requirement for total sulfur amino acids can be met by either methionine alone or the proper mixture of methionine and cystine (Ahmed et al., 2003).

In the current study hemoglobin and hematocrit content were not affected by dietary methionine level. However, Zhou et al. (2006) reported the significant effect of methionine on hemoglobin, hematocrit and leucocyte counts of Juvenile Cobia (*Rachycentron canadum*) where lower values were obtained for the group fed methionine deficient diet. Also, Ruchimat et al. (1997) pointed out that dietary methionine level significantly influenced hemoglobin and haematocrit content in yellowtail (*Seriola quinqueradiata*).

A relationship between tissue EAAs levels and dietary requirements has been suggested (Cowey, 1994). The results of a study on juvenile Chinese sucker (*Myxocyprinus asiaticus*) (Chu et al., 2014) revealed the significant increase of all essential amino acids accumulation in fish muscle at increased dietary methionine levels. Also, Niu et al. (2013) showed that dietary methionine level influences whole-body amino acid composition of pompano (*Trachinotus ovatus*.); concentration of all the essential amino acids, except for arginine, increased by dietary methionine increment. They suggested that methionine deficiency in diet could inhibit methionine participating in protein synthesis and reduce its level in amino acid pool in muscle. In the present study, concentration of most essential amino acids was increased in whole-body although this increment was numerical.

In conclusion, the results in this study showed that inclusion of an appropriate level of

methionine is required for optimal growth of olive flounder. The optimum requirement level of methionine was estimated to be 1.63% of diet (3.62% of dietary protein) for juvenile olive flounder.

CHAPTER SIX

Comparison of free and dipeptide lysine utilization in diets for juvenile olive flounder (*Paralichthys olivaceus*)

6.1. Introduction

The quality of dietary protein is determined by its amino acid (AA) composition and availability (Wilson and Cowey, 1985; Wilson and Poe, 1985). Quantitative estimation of essential amino acid requirements of cultured fish species is detrimental for achievement of optimum growth and feed utilization, cost-effective diet formulation, and desirable carcass quality (NRC, 2011). It has long been demonstrated that a balanced AA profile is required for effective utilization of dietary protein for tissue synthesis (D'Mello, 1994; Yamamoto et al., 2000; Berge et al., 2002; Green and Hardy, 2002; Gómez-Requeni et al., 2003). Amino acids can be supplied either in the forms of protein-bound, free amino acids or peptides in formulated diets. It has been shown that absorption of peptides and free AA (FAA) are the major transport routes for protein utilization in mammals (Abidi, 1997; Ganapathy et al., 1994). The current understanding of protein utilization in vertebrates centers on absorption of peptides in the intestine as a major route of transport. In fishes it was shown that small peptides and FAA are absorbed faster in comparison to protein (Rust et al., 1993; Rønnestad et al., 2003). There is evidence that a single peptide can be more efficiently absorbed than the mixture of identical amino acids both in fish (Reshkin and Ahearn, 1991; Boge et al., 2002) and mammals (Matthews, 1991). The results of previous studies on common carp

(*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) showed the inferior utilization of amino acids from FAA-based diets compared to those from protein-based diets (Murai et al., 1982; 1983; Kaushik and Dabrowski, 1983; Dabrowski et al., 2003). Increased deamination or higher total dietary nitrogen excretion through gill and kidney have been suggested to be the reason for the secondary utilization of FAA-based diets compared to the protein-based diets (Dabrowski et al., 2003). It is generally thought that the inefficiency of free AA in fish is due to its faster uptake and subsequent catabolism compared to those from intact protein (Murai et al., 1987; Cowey and Walton, 1988; Dabrowski et al., 2003, Rønnestad et al., 2000; Dabrowski et al., 2007). Another explanation was proposed by higher leaching loss of free AA than bound AA in aquatic environments prior to ingestion (Zarate and Lovell, 1997).

Dabrowski et al. (2003) showed for the first time that a synthetic dipeptide-based diet can support growth of rainbow trout in the early stages while a free AA-based diet did not. Peptides have different transport systems from FAA. It has been reported that tetra- and larger peptides do not cover the nitrogen requirements in the absence of pancreatic enzymes or lack of brush border peptidase activity (Grimble, 1994; Daniel, 2004), whereas di- or tripeptides have specific peptide transporters (Doring et al., 1998). These transporters were demonstrated to be expressed in teleost larvae prior to exogenous feeding (Verri et al., 2003).

Lysine has been identified as an essential amino acid for all the studied fish species (NRC, 2011). It is of great importance as it is found in the highest concentration in the carcass of many fish species (Wilson and Cowey, 1985; Wilson and Poe, 1985; NRC, 1993). Lysine deficiency has been reported to result in decreased growth and low feed

efficiency (Ahmed and Khan, 2004; Wang et al., 2005; Zhou et al., 2008). It is the most limiting amino acid in protein sources, particularly plant proteins, used in aquafeeds (Deng et al., 2010).

Olive flounder, *Paralichthys olivaceus*, is one of the most important marine cultured species in Korea, Japan and China. Its total production reached ~37000 tons in 2013 in Korea (Ministry of Maritime Affairs and Fisheries of Korea, 2013). Dietary lysine requirement of the species was determined by Forster and Ogata (1998) using free L-lysine as amino acid source. Kim and Lee (2013) showed that juvenile olive flounder can utilize dipeptide leucine more efficiently than its free form for protein synthesis. Accordingly, this study was conducted to compare the utilization efficiency of free and dipeptide forms of lysine in diets for juvenile olive flounder.

6.2. Materials and methods

6.2.1. Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 6-1. Five isonitrogenous (47% crud protein) and isocaloric (4.38 kcal g^{-1} gross energy) experimental diets were formulated to contain different levels and forms of lysine amino acid. A mixture of synthetic free amino acids without lysine was prepared according to Dabrowski et al. (2003) and used as the main protein source. A control diet was formulated to contain basal level of lysine (0.5%) from fish meal and supplemented with 0.5 or 1.0% of lysine either in free or dipeptide forms. Lysine-Glycine (LG) (Bachem, Torrance, CA, USA) was used as the dipeptide form and crystalline L-lysine (L-Lys) (Sigma Chemicals, St. Louis, MO, USA) was used as the free form (FL). The experimental diets were kept isonitrogenous and isocaloric using glycine at the expense of lysine. All ingredients were well mixed, pelletized and freeze-dried. The pellets were crushed into desirable particle sizes and stored at -20 °C until use.

Table 6-1. Composition and proximate analysis of the experimental diets (% dry matter).

	Control	L-Lys		Lys-Gly	
Ingredient	**0.5**	**1.0**	**1.5**	**1.0**	**1.5**
White fish meal	10	10	10	10	10
Free AA mix[1]	38.5	38.5	38.5	38.5	38.5
Lys-Gly	0.0	0.0	0.0	0.5	1.0
L-Lys	0.0	0.5	1.0	0.0	0.0
L-Glycine	1.0	0.5	0.0	0.5	0.0
Dextrin	32	32	32	32	32
Choline chloride	1.0	1.0	1.0	1.0	1.0
Mineral mix[2]	2.0	2.0	2.0	2.0	2.0
Vitamin mix[3]	2.0	2.0	2.0	2.0	2.0
Taurine	1.0	1.0	1.0	1.0	1.0
Squid liver oil	12.5	12.5	12.5	12.5	12.5
Proximate composition					
Dry matter	93.5	93.0	94.1	93.8	93.5
Crude protein	47.0	46.5	47.0	46.8	46.7
Lipid	8.7	8.8	8.8	8.6	8.5
Ash	3.8	3.7	3.7	3.6	3.8

[1]Free amino acid mixture composition (g per 446 g dry weight mixture): arginine hydrochloride, 15; valine, 12; methionine, 10; histidine, 7; isoleucine, 9; leucine, 14; phenylalanine, 18; threonine, 8; tryptophan, 2; glutamic acid, 111; glycine, 240.

[2]Mineral premix (g kg^{-1} mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3]Vitamin premix (g kg^{-1} mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4;

Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

6.2.2. Fish and feeding trial

Juvenile olive flounder were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). All the fish were fed the basal diet for one week to be acclimated to the semi-purified diet and the experimental conditions. At the end of the acclimation period, 20 randomly selected fish (averaging at 5.41 ± 0.16 g) were stocked into each polyvinyl circular tanks of 20 L capacity and supplied with filtered seawater at a flow rate of 1.5 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed with one of the test diets at a ratio of 3% of body mass (twice a day, 09:00 and 17:00 h) for six weeks. Growth of fish was measured with three-week intervals. Feeding was stopped 24 h prior to weighing to minimize handling stress on fish. The water temperature during the feeding trial ranged from 14 to 20 °C and the photoperiod was maintained on a 12:12 light:dark schedule.

6.2.3. Sample collection and analyses

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Five intact fish per tank (15 fish per treatment) were selected and kept at −20 °C for whole-body amino acid composition analysis. The samples were freeze-dried and finely ground using a grinder and the amino acid compositions were analyzed using an automatic amino acid analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

Another set of five fish per tank were sampled for determination of organosomatic indices including hepatosomatic index (HSI) and viscerosomatic index

(VSI), and relative intestine length (RIL).

Analyses of moisture and ash contents of diets were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

6.2.4. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way ANOVA in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was compared with Tukey's HSD multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. In two-way ANOVA, to isolate the effects of molecular form from lysine level only the diets with lysine supplementation were considered. Data were presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

6.3. Results

The results of fish growth performance fed the experimental diets with different lysine molecular forms and levels are provided in Table 6-2. The results showed the significant increase of fish growth by increment of dietary lysine level ($P < 0.05$, one-way ANOVA) and the highest growth performance was found in fish fed 1.5% LG. Feed utilization was significantly affected by dietary treatments (one-way ANOVA); the highest protein efficiency ratio (PER) was obtained in fish fed 1.5% LG. Also, the results showed the significant effects of both lysine level and molecular form on weight gain and PER ($P < 0.05$, two-way ANOVA); however no interaction could be found between dietary lysine level and form ($P > 0.05$, two-way ANOVA). Fish survival rate varied from 86 to 93% and was not significantly ($P > 0.05$) different among all the treatments (Table 6-2).

Organosomatic indices of fish fed the experimental diets are presented in Table 6-3. Significantly higher HSI and VSI were obtained in fish fed LG contacting diets and free form did so at 1.5% ($P < 0.05$, one-way ANOVA). Also, the two indices were significantly affected by both lysine form and level ($P < 0.05$, two-way ANOVA). However, RIL did not significantly differ among treatments (Table 6-3).

Fish whole-body amino acid composition was significantly affected by dietary treatments (Table 6-4). Significantly higher arginine levels were found in fish fed LG containing diets (one-way ANOVA) and a significant interaction was observed for lysine level and form ($P = 0.009$, two-way ANOVA). The results of two-way ANOVA analysis showed the significant effect of lysine level on whole-body valine and aspartic acid contents. Also, alanine content was significantly increased in fish offered 1.5% LG

(one-way ANOVA) and significantly influenced by both lysine level and form.

Table 6-2. Growth performance of olive flounder (initial body weight, 5.41±0.16 g) fed the experimental diets containing different lysine levels and molecular forms for 6 weeks.

	Dietary treatment					SEM[1]	P-values	P-values	P-values
	Control	L-Lys		Lys-Gly			Lys form	Lys level	Lys form×level
	0.5	1.0	1.5	1.0	1.5				
FBW[2]	6.57^{c}	7.70^{b}	8.73^{a}	8.15^{ab}	8.87^{a}	0.45	0.191	0.003	0.463
WG[3]	26.44^{c}	43.30^{b}	57.06^{ab}	49.72^{b}	64.92^{a}	6.62	0.034	0.001	0.787
PER[4]	0.55^{c}	0.84^{b}	1.07^{ab}	0.94^{ab}	1.21^{a}	0.12	0.023	0.000	0.677
Survival (%)	86.6	86.7	91.7	93.3	93.3	15.2	0.772	0.911	0.609

Each value is the mean of triplicate groups. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Pooled standard error of the mean

[2]Final body weight

[3]Weight gain = final mean body weight - initial mean body weight

[4]Protein efficiency ratio = Wet weight gain/total protein fed

Table 6-3. Organosomatic indices of olive flounder (initial body weight, 5.41±0.16 g) fed the experimental diets containing different lysine levels and molecular forms for 6 weeks.

	Dietary treatment					SEM[1]	P-values	P-values	P-values
	Control	L-Lys		Lys-Gly			Lys form	Lys level	Lys form×level
	0.5	1.0	1.5	1.0	1.5				
HSI[2]	2.05^{c}	2.18^{c}	3.21^{ab}	3.0^{b}	3.95^{a}	0.51	0.003	0.000	0.605
VSI[3]	3.49^{b}	3.95^{b}	5.63^{a}	5.61^{a}	5.83^{a}	0.84	0.026	0.021	0.059
RIL[4]	61.2	63.6	65.9	61.5	64.1	5.24	0.360	0.245	0.978

Each value is the mean of triplicate groups. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Pooled standard error of the mean

[2]Hepatosomatic index = 100 × (liver weight/body weight)

[3]Viscera somatic index = 100 × (viscera weight/body weight)

[4]Relative intestine length = 100 × (intestine length/total body length)

Table 6-4. Whole-body amino acid composition of olive flounder fed the experimental diets containing different lysine levels and molecular forms for 6 weeks.

	Dietary treatment					SEM[1]	P-values	P-values	P-values
	Control	L-Lys		Lys-Gly			Lys form	Lys level	Lys form×level
	0.5	1.0	1.5	1.0	1.5				
EAA									
Arg	6.59[b]	6.67[ab]	6.72[ab]	6.77[a]	6.77[a]	0.08	0.619	0.085	0.009
His	1.78	1.77	1.72	1.79	1.78	0.32	0.794	0.831	0.887
Ile	4.25	4.21	4.25	4.29	4.28	0.08	0.222	0.687	0.485
Leu	7.50	7.34	7.43	7.44	7.40	0.11	0.541	0.694	0.275
Lys	8.53	8.20	8.41	8.33	8.48	0.21	0.331	0.091	0.768
Met	0.72	0.73	0.74	0.75	1.09	0.22	0.139	0.145	0.171
Phe	4.15	4.10	4.10	4.15	4.12	0.05	0.264	0.641	0.654
Thr	4.20	4.27	4.24	4.27	4.25	0.08	0.751	0.545	0.909
Val	4.98	4.91	5.02	4.89	5.06	0.11	0.819	0.021	0.536
NEAA									
Ala	7.02[b]	7.15[ab]	7.14[ab]	7.19[ab]	7.31[a]	0.14	0.046	0.001	0.002
Asp	9.33[b]	9.79[ab]	10.15[a]	9.42[ab]	9.98[a] [b]	0.35	0.749	0.001	0.353
Glu	14.71	14.89	14.83	14.81	14.71	0.10	0.104	0.170	0.745

Gly	7.96	7.93	7.85	7.91	7.48	0.41	0.317	0.197	0.363
Pro	4.82	7.21	5.85	6.59	6.57	1.62	0.871	0.054	0.059
Ser	4.73	4.72	4.81	4.71	4.56	0.16	0.077	0.698	0.091
Tyr	3.24	3.21	3.20	3.26	3.25	0.06	0.102	0.706	0.874

Each value is the mean of triplicate groups. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Pooled standard error of the mean

6.4. Discussion

In the present study, the fish accepted all the experimental semi-purified diets, however, because the feeding trial was undertaken in low temperature season relatively low growth rates were achieved. The results showed the significant enhancement of fish growth by increment in dietary lysine level indicating the essentiality of lysine for maximal growth of juvenile olive flounder. Forster and Ogata (1998) reported that the lysine requirement of olive flounder is 1.5-2.1% of diet. Accordingly, in this study a range of 0.5 to 1.5% lysine were considered to compare efficiency of different forms of lysine in either free or dipeptide.

Dabrowski et al. (2003) reported for the first time that a dipeptide based diet can support the growth performance of rainbow trout at early life stages while a FAA based diet could not. In the current study, the fish fed LG supplemented diets showed higher growth than those offered FL and the highest weight gain was obtained in group fed 1.5% LG. Similarly, Kim and Lee (2013) showed that dipeptide form of leucine performs better compared to the free form and higher weigh gain were achieved in all the supplementation levels of leucine than free form. In contrary, Kim et al. (2012) reported a similar efficiency for dipeptide and free forms of phenylalanine when they were supplemented in diets for red sea bream (*Pagrus major*). Also, Tesser et al. (2005) found that juvenile South American pacu (*Piaractus mesopotamicus*) can utilize dipeptide arginine with similar efficiency for growth. Further, the results of a study on koi carp (*Cyprinus carpio*) revealed no significant difference in fish growth performance when they were provided free or dipeptide form of amino acids (Kwasek et al., 2010). Absorption of free amino acids is faster than protein-bound amino acids and

may result in amino acid imbalances and subsequently low protein utilization (Rønnestad et al., 2000). Moreover, an excess level of di- or tri-peptides can be similarly damaged due to either the saturation of their transport mechanisms (Verri et al., 2003) or their instant hydrolysis to free amino acids (Carvalho et al., 2004). However, better absorption of single peptide compared to a mixture of equal free amino acid has been reported in rainbow trout (*Salmo gairdneri R.*) (Boge et al., 1981). It has been earlier reported that substitution of protein-bound amino acids by di- and tripeptides up to 20% enhances the growth performances of European sea bass larvae, while higher substitution levels decreased growth (ZamboninoInfante et al., 1997). Composition of the basal diet is one of the most important factors influencing the results of dipeptide amino acids supplementation. In the present study utilization efficiency of the two lysine forms were examined by their supplementation to free AA-based diets, while in the previous studies by Tesser et al. (2005) and Kwasek et al. (2012) the comparisons were made by their supplementation to whole-protein based diets. The differences in the availability of AA from free AA and whole-protein based diets can be at least partially responsible for the observed variations. Also, it has been suggested that the use of different dipeptides can profoundly affect the absorption characteristics by switching the amino acid sequence (Daniel, 2004). Cytosolic and brush border aminopeptidases are involved in the utilization of dietary peptides (Cahu and ZamboninoInfante, 1995; Kurokawa and Suzuki, 1998). However, further studies in the future are required to clarify the exact mechanism through which dipeptides mediate the specific peptide transporters in intestinal brush border epithelium.

The results of two-way ANOVA exhibited that both lysine level and form

affected weight gain of olive flounder in this study. Kim and Lee (2013) showed that molecular form of leucine is the main influencing factor of olive flounder growth performance. This variation can be due to the difference in fish size, feeding period, different requirement levels of the species for leucine and lysine, and changes in experimental conditions.

The study of HSI and VSI provides important information on the metabolism of the fishes, related to digestion and absorption and synthesis and secretion of digestive enzymes (McLaughlin, 1983). HSI is used as an indicator of general nutritional status (Busacker et al., 1990). In the current study significantly higher HSI and VSI values were obtained by increment of dietary lysine level and this was more evident when dipeptide form was used indicating higher energy budget in LG fed fish.

The whole-body amino acid profile of the fish has been commonly used as a good indicator of fish amino acid requirement (Wilson and Cowey, 1985; Bicudo and Cyrino, 2009). Several studies have attested that whole-body essential amino acid profile provides a good estimation of the optimum essential amino acid profile of fish diets (Akiyama et al., 1997; Green and Hardy, 2002). Aragão et al. (2004) showed that juvenile Senegale sole (*Solea senegalensis*) exhibit improved general amino acid deposition in the body when they were offered a diet supplemented with dipeptide essential amino acids. The results of the current study showed the significant effect of dietary treatments on fish whole-body composition. Higher accumulation of arginine in fish whole-body was found when they were provided LG, and a significant interaction was detected for lysine level and form. A similar tendency was observed for alanine concentration, where higher values were obtained for LG fed groups. In agreement to

our results, Kwasek et al. (2010) found significantly lower concentrations of threonine, arginine, valine, methionine, isoleucine, leucine, phenylalanine and lysine in the whole-body of koi carp fed free amino acid based diet compared to those fed dipeptide based diet. However, the results of previous studies on common carp (*Cyprinus carpio L.*) (Zhang et al., 2006), red sea bream (Kim et al., 2012) and olive flounder (Kim and Lee, 2013) did not show any significant changes in whole-body/muscle amino acid composition when they were provided with free or dipeptide forms of amino acids. Dabrowski et al. (2005) stated that the differences in fish muscle free amino acid composition offered different dietary amino acid sources are due to differences in amino acid absorption rates from free, dipeptide or protein dietary sources that lead to uneven accumulation rates and post-prandial peak times for muscular free amino acid and consequently result in different metabolic handling of the amino acids and availability for protein synthesis. The findings in this study show that the availability of amino acids could be better in the fish when they are fed with dipeptide forms than free forms.

In conclusion, the findings in this study showed that juvenile olive flounder can utilize lysine more efficiently for growth if it is provided in dipeptide form than free form. Also, it was shown that the use of dipeptide form can improve retention of amino acids. This study provides more evidence for the notion that the previously published data on amino acid requirements using crystalline/free amino acids were likely to be over-estimated and need to be reevaluated using dipeptides as amino acid source.

SUMMARY

In the first experiment (chapter 2), a 12-week feeding trial was conducted to determine dietary valine requirement of red sea bream. Six diets were formulated with graded levels of valine (0.27, 0.79, 1.22, 1.69, 2.04 and 2.38% diet) and fed to triplicate groups of fish (32.04 ± 0.2 g) to apparent satiation. Significantly higher growth and feed utilization were obtained at valine levels of ≥0.79%. Non-specific immune responses of fish were significantly improved by increment of dietary valine up to 2.04%. Significantly lower postprandial ammonia excretion levels were obtained at higher valine levels. The optimum valine requirement level was estimated at 0.9% of diet. The second experiment (chapter 3), was undertaken to evaluate dietary arginine requirement of red sea bream (13.3 ± 0.2 g). Six diets were prepared to contain 1.2, 1.6, 2.0, 2.4, 2.8 and 3.2% arginine, and fed to triplicate groups of fish (13.3 ± 0.2 g) to apparent satiation for 9 weeks. Fish fed ≥ 2.0% arginine showed significantly higher growth than those fed 1.2% arginine. Significant improvement in protein productive value was found at 2.4% arginine. Fish hematology was influenced by arginine levels. Significant improvement in fish innate immunity was found at increased arginine levels. The optimum dietary arginine level was estimated to be 2.54% of diet. The third experiment (chapter 4) was carried out to quantify isoleucine requirement of olive flounder. Six experimental diets were formulated to contain graded levels of isoleucine (0.48, 0.87, 1.43, 1.94, 2.37 and 2.78% diet) and fed to triplicate groups of fish to apparent satiation for 9 weeks. The highest growth was obtained at 1.43% isoleucine. The results showed the significant increase of plasma total protein and cholesterol concentrations at higher

isoleucine levels. Significant reductions in plasma alanine aminotransferase and aspartate aminotransferase activities were detected by increment of isoleucine level. Fish innate immunity was significantly enhanced at increased isoleucine levels. Dietary isoleucine requirement was estimated to be 1.69% of diet. The forth experiment (chapter 5) was conducted to quantify dietary methionine requirement of olive flounder. Six diets were formulated to contain graded levels of methionine from 0.3 to 1.8% and fed to triplicate groups of fish (17.28±0.1 g) to apparent satiation for 8 weeks. Fish fed ≥1.5% methionine exhibited significantly higher growth than those fed 0.3% methionine. Increment of dietary methionine level resulted in a numerical increase of most whole-body essential amino acids. The optimal dietary methionine level was suggested to be 1.63% diet. Finally, in the last experiment (chapter 6), utilization efficiency of free lysine (FL) and dipeptide lysine-glycine (LG) was compared in diets for olive flounder. A basal experimental diet contained 0.5% lysine from fish meal and four other diets were prepared by supplementing 0.5 or 1.0% of either FL or LG. The fish (5.41±0.16 g) were fed one of the diets at the rate of 3% BW/day for six weeks. The lowest weight gain was found in fish fed the basal diet and differed significantly from that of others. The results of two-way ANOVA showed that both lysine level ($P = 0.001$) and type ($P = 0.034$) influence fish weight gain. Also, the results revealed the significant improvement of protein efficiency ratio (PER) by increment of dietary lysine level, and the groups fed LG supplemented diets showed higher PER values. Significantly higher whole-body arginine level was found in LG fed groups. Whole-body valine and aspartic acid contents were affected by lysine level. Our findings showed that juvenile olive flounder can utilize LG more efficiently than FL for protein synthesis.

REFERENCES

Adibi SA. 1997. The oligopeptide transporter (Pept-1) in human intestine: biology and function. Gastroenterology 113, 332–340.

Abidi, S.F., Khan, M.A., 2004a. Dietary valine requirement of Indian major carp, *Labeo rohita* (Hamilton). Journal of Applied Ichthyology 20, 118–122.

Abidi, S.F., Khan, M.A., 2004b. Dietary histidine requirement of Indian major carp, *Labeo rohita* (Hamilton). The Israeli Journal of Aquaculture-Bamidgeh 56, 200–208.

Abidi SF, Khan MA (2009) Dietary arginine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton) based on growth, nutrient retention efficiencies, RNA/DNA ratio and body composition. J Appl Ichthyol 25:707–714.

Adhikari, S., Sarkar, B., Chatterjee, A., Mahapatra, C.T., Ayyappan, S., 2004. Effects of cypermethrin and carbofuran on certain hematological parameters and prediction of their recovery in a freshwater teleost, *Labeo rohita* (Hamilton). Exotoxicology and Environmental Safety 58, 220–226.

Ahmed, I., Khan, M.A., Jafri, A.K., 2003. Dietary methionine requirement of fingerling India major carp, Cirrhinus mrigala (Hamilton). Aquac. Int. 11, 449–462.

Ahmed, I., Khan, M.A., 2004. Dietary arginine requirement of fingering India major carp, *Cirrhinus mrigala* (Hamilton). Aquaculture 235, 499–511.

Ahmed I and Khan MA. 2004. Dietary lysine requirement of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). Aquaculture 235(1–4), 499–511.

Ahmed, I., Khan, M.A., Jafri, A.K., 2004. Dietary threonine requirement of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). Aquaculture Research 35, 162–

170.

Ahmed, I., Khan, M.A., 2006. Dietary branched-chain amino acid valine, isoleucine and leucine requirements of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). British Journal of Nutrition 96, 450–460.

Ahmed, I., 2013. Dietary arginine requirement of fingerling Indian catfish (*Heteropneustes fossilis*, Bloch). Aquacult. Int. 21, 255–271.

Akiyama, T. and S. Arai. 1993. Amino acid requirements of chum salmon fry and supplementation of amino acids to diet. Pages 35-48 in M. R. Collie and J. P. McVey, editors. Proceedings of 20th US.-Japan Symposium on Aquaculture Nutrition. UJNR Department of Commerce, Newport, Oregon, USA.

Akiyama T, Oohara I and Yamamoto T. 1997. Comparison of essential amino acid requirement with A/E ratio among fish species (Review Paper). Fisheries Sci 63, 963-970.

Alam, M.s., Teshima, S., Ishikawa, M., Koshio, S., Yaniharto, D., 2001. Methionine requirement of juvenile Japanese flounder Paralichthys olivaceus by the oxidation of radioactive methionine. Aquac. Nutr. 7, 201–209.

Alam, Md.S., Teshima, S., Koshio, S., Ishikawa, M., 2002a. Arginine requirement of juvenile Japanese flounder *Paralichthys olivaceus* estimated by growth and biochemical parameters. Aquaculture 205, 127–140.

Alam, Md.S., Teshima, S., Yaniharto, D., Koshio, S., Ishikawa, M., 2002b. Influence of different amino acid patterns on growth and body composition of juvenile Japanese flounder, *Paralichthys olivaceus*. Aquaculture 210, 359–369.

Alam, Md.S., Teshima, S.I., Yaniharto, D., Sumule, O., Ishikawa, M., Koshio, S., 2005. Assessment of reference dietary amino acid pattern for juvenile red sea bream, *Pagrus major*. Aquacult. Int. 13, 369–379.

Almeida, J.A., Diniz, Y.S., Marques, S.F.G., Faine, L.A., Ribas, B.O., Burneiko, R.C., Novelli, E.L.B., 2002. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. Environment International 27, 673–679.

Anderson, D.P., Siwicki, A.K., 1995. Basic haematology and serology for fish health programs. In: Shariff, M., Arthur, J.R., Subasinghe, R.P. (Eds.), Diseases in Asian Aquaculture II. Philippines fish health section, Asian Fisheries Society, Manila, p. 185.

AOAC (Association of Official Analytical Chemists), 1995. Official Methods of Analysis of Official Analytical Chemists International 16th ed. Association of Official Analytical Chemists. Arlington, VA, USA.

Aragão C, Conceicao LEC, Martins D, Rønnestad I, Gomes E and Dinis T. 2004. A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (*Solea senegalensis*). Aquaculture 233, 293–304.Aschkenasy, A., 1979. Prevention of the immunodepressive effects of excess dietary leucine by isoleucine and valine in the rat. Journal of Nutrition 109, 1214–1222.

Bahmani, M., Kazemi, R., Donskaya, P., 2001. A comparative study of some hematological features in young reared sturgeons *Acipenser persicus* and *Huso huso*. Fish. Physiol. Biochem. 24, 135–140.

Bain, P.J., 2003. Liver. In: Latimer, K.S., Mahafey, E.A., Prasse, K.W. (Eds.), Duncan &

Prasse's Veterinary Laboratory Medicine: Clinical Pathology, Iowa State Press., Ames, IA, USA. pp. 193–214.

Baker, D.H., 1984. Equalized versus ad libitum feeding. Nutr. Rev. 42, 269–273.

Benakappa S, Varghese TJ (2003) Isoleucine, leucine and valine requirement of juvenile Indian major carp, Cirrhinus cirrhosus (Bloch, 1975). Acta Ichthyol Piscat 33:161–172.

Berge, G.E., Lied, E., Sveier, H., 1997. Nutrition of Atlantic salmon (*Salmo salar*): the requirement and metabolism of arginine. Comp. Biochem. Physiol. 117A, 501–509.

Berge, G.E., Sveier, H., Lied, E., 2002. Effects of feeding Atlantic salmon (*Salmo salar* L.) imbalanced levels of lysine and arginine. Aquaculture Nutrition 8, 239–248.

Bhargava, K.K., Hanson, R.P. and Sunde, M.L. 1970. Effects of methionine and valine on antibody production in chickens infected with Newcastle disease virus. Journal of Nutrition, 100: 241–248.

Bhargava, K.K., Hanson, R.P. and Sunde, M.L. 1971. Effects of threonine on growth and antibody production in chickens infected with Newcastle disease virus. Poultry Science, 50: 710–713.

Bicudo AJA and Cyrino JEP. 2009. Estimating amino acid requirement of Brazilian freshwater fish from muscle amino acid profile. J. World Aquacult. Soc 40, 818-823.

Boisen, S., Hvelplund, T., Weisbjerg, M.R., 2000. Ideal amino acid profiles as a basis for feed protein evaluation. Livestock Production Science 64, 239–251.

Boge G, Rigal A and Peres G. 1981. Rates of in vivo intestinal absorption of glycine and glycyglycine by rainbow trout (*Salmo gairdneri R.*). Comp. Biochem. Physiol 69,

455-459.

Boge G, Roche H and Balocco C. 2002. Amino acid transport by intestinal brush border vesicles of a marine fish, Boops salpa. Comp. Biochem. Physiol B 131, 19-26.

Borlongan IG, Coloso RM (1993) Requirements of juvenile milkfish (Chanos chanos Forsskal) for essential amino acids. J Nutr 123:125–132.

Brown, B.A., 1980. Routine hematology procedures. In: Brown, B.A. (Ed.), Hematology, Principles and Procedures, Lea and Febiger, Philadelphia, PA, USA, pp.71–112.

Buentello, J.A., Gatlin III, D.M., 1999. Nitric oxide production in activated macrophages from channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media. Aquaculture 179, 513–521.

Buentello, J.A., Gatlin, D.M., 2001. Effects of elevated dietary arginine on resistance of channel catfish to exposure to *Edwardsiella ictaluri*. J. Aquat. Anim. Health. 13, 194–201.

Buentello, J.A., Reyes-Becerril, M., Romero-Geraldo, M.J., Ascencio-Valle, F.J., 2007. Effects of dietary arginine on hematological parameters and innate immune function of channel catfish. J. Aquat. Anim. Heal. 19, 195–203.

Busacker, G.P., Adelman, I.R., Goolish, E.M., 1990. Growth. In: Schreck, C.B., Moyle, P.B. (Eds.), Methods for Fish Biology. American Fisheries Society, Bethesda (MD), pp. 363– 387.

Cahu N and ZamboninoInfante J. 1995. Effect of the molecular-form of dietary nitrogen supply in sea bass larvae-response of pancreatic-enzymes and intestinal peptidases. Fish. Physiol. Biochem 14, 209-214.

Cai, T., Wermerskirchen, J., Adelman, I.R., 1996. Ammonia excretion rate indicates dietary protein adequacy for fish. The Progressive Fish-Culturist 58, 124–127.

Calder, P.C., 2006. Branched-chain amino acid and immunity. Journal of Nutrition 136, 288S–293S.

Cao, J.M., Chen, Y., Zhu, X., Huang, Y.H., Zhao, H.X., Li, G.L., Lan, H.B., Chen, B., Pan, Q., 2012. A study on dietary L-lysine requirement of juvenile yellow catfish *Pelteobagrus fulvidraco*. Aquaculture Nutrition 18, 35–45.

Carroll, K.K., Hamilton, R.M.G., 1975. Effects of dietary protein and carbohydrate on plasma cholesterol levels in relation to atherosclerosis . Journal of Food Science 40, 18–23.

Carvalho AP, Sa R, Oliva-Teles A and Bergot P. 2004. Solubility and peptide profile affect the utilization of dietary protein by common carp (*Cyprinus carpio*) during early larval stages. Aquaculture 234, 319-333.

Cheng, Z.Y., Buentello, A., Gatlin, D.M., 2011. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. Aquaculture 319, 247–252.

Cho, C.Y., Kaushik, S.J., 1985. Effects of protein intake on metabolizable and net energy values of fish diets. In: Cowey, C.B., Mackie, A.M., Bell, J.B. (Eds.), Nutrition and Feeding in Fish, Academic Press., New York, pp. 95–118.

Choi, C.Y., Min, B.H., Jo, P.G., Chang, Y.J., 2007. Molecular cloning of PEPCK and stress response of black porgy (*Acanthopagrus schlegeli*) to increased temperature in freshwater and seawater. General and Comparative Endocrinology 152, 47–53.

Chu, Z.J., Gong, Y., Lin, Y.C., Yuan, Y.C., Cai, W.J., Gong, S.Y., Luo, Z., 2014. Optimal

dietary methionine requirement of juvenile Chinese sucker, *Myxocyprinus asiaticus*. Aquaculture Nutrition, 20: 253-264.

Cole, D.J.A., Van Lunen, T.A., 1994. Ideal amino acid patterns. In: D'Mello, J.P.F. (Ed.), Amino Acids in Farm Animal Nutrition, Wallingford: CAB International, pp. 99–112.

Coles, B.L. and McDonald, I. 1972. The influence of dietary protein on dietary carbohydrate: lipid interrelationships. Nutrition and Metabolism, 14: 238–244.

Coloso, R.M., Murillo-Gurrea, D.P., Borlongan, I.G., Catacutan, M.R., 1999. Sulphur amino acid requirement of juvenile Asian Sea bass *Lates calcarifer.* Journal of Applied Ichthyology 15, 54–58.

Congleton, J.L., Wagner, T., 2006. Blood-chemistry indicators of nutritional status and food intake in juvenile salmonids. J. Fish Biol. 69, 473–490.

Costas, B., Conceição, L.E.C., Dias, J., Novoa, B., Figueras, A., Afonso, A., 2011. Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). Fish & Shellfish Immunology 31, 838–847.

Cowey, C.B., 1979. Protein and amino acid requirements of finfish. In: Halver, J.E., Tiews, K. (Eds.), Finfish nutrition and Fishfeed Technology, Hamburg, Germany, pp. 3–16.

Cowey, C.B., 1994. Review—Amino acid requirements of fish: a critical appraisal of present values. Aquaculture 124, 1–11.

Cowey, C.B. (1995) Protein and amino acid requirements: a critique of methods. J. Appl. Ichthyol., 11, 199-204.

Cowey CB, Luquet P (1983) Physiological basis of protein requirements of fishes. Critical analysis of allowances. In: Prion R, Arnal M, Bonin D (eds) Proceedings of the fourth international symposium on protein metabolism and nutrition. Clermont-Ferrand, France, 5–9 September. INRA Publications, Les Colloques de 1' INRA, France, pp 365–384.

Cowey CB and Walton MJ. 1988. Studies on the uptake of (^{14}C) amino acids derived from both dietary (^{14}C) protein and dietary (^{14}C) amino acids by rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol 33, 293-305.

Dabrowski, K., 1986. A new type of metabolism chamber for the determination of active and postprandial metabolism of fish, and consideration of results for coregonids and salmon juveniles. Journal of Fish Biology 28, 105–117.

Dabrowski, K., Poczyczynski, P., Köck, G., Berge, B., 1989. Effect of partially or totally replacing fish meal protein by soybean meal protein on growth, food utilization and proteolytic enzyme activities in rainbow trout (Salmo gairdneri). New in vivo test for exocrine pancreatic secretion. Aquaculture 77, 29–49.

Dabrowski, K., Lee, K.J., Rinchard, J., 2003. The smallest vertebrate, teleost fish, can utilize synthetic dipeptide-based diets. Journal of Nutrition 133, 4225–4229.

Dabrowski K, Terjesen BF, Zhang Y, Phang JM and Lee KJ. 2005. A concept of dietary dipeptides: a step to resolve the problem of amino acid availability in the early life of vertebrates. The Journal of Experimental Biology 208, 2885-2894.

Dabrowski K, Arslan M, Terjesen BF and Zhang Y. 2007. The effects of dietary indispensable amino acid imbalances on feed intake: Is there a sensing of deficiency and neural signaling present in fish? Aquaculture 268, 036-142.

Dalmo, R.A., Ingebrigtsen, K. and Bogwald, J. 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). Journal of Fish Diseases, 20: 241–273.

Daniel H. 2004. Molecular and integrative physiology of intestinal peptide transport. Annu. Rev. Physiol 66, 361–384.

Das, P.C., Ayappan, A.J., Jena, J.K., Das, B.K., 2004. Acute toxicité of ammonia and sub lethal effects on marigal (*Cirrhinus mrigala*) Hamilton, Aquate. Res 35,134–143.

Dawson, R.D. and Bortolotti, G.R. 1997. Total plasma protein level as an indicator of condition in wild American kestrels (*Falco sparverius*). Canadian Journal of Zoology, 75: 680–686.

Deng DF, DominyW, Ju ZY, Koshio S, Murashige R and Wilson RP. 2010. Dietary lysine requirement of juvenile Pacific threadfin (*Polydactylus sexfilis*). Aquaculture 308(1), 44–48.

De Silva SS, Gunasekera RM, Gooley G (2000) Digestibility and amino acid availability of three protein rich ingredient incorporated diets by Murray cod *Maccullochella peelii* peelii (Mitchell) and Australian shortfin eel *Anguilla australis* Richardson. Aquac Res 31:195–205.

De Smet, H., Blust, R., 2001. Stress responses and changes in protein metabolism in Carp *Cyprinus carpio* during cadmium exposure. Ecotoxicology and Environmental Safety 38, 255–262.

Di SX, Li L, Hua W, Wen G, Shui WQ, Hui X (2009) Study on isoleucine requirement for juvenile grass carp, *Ctenopharyngodon idellus*. J Fish China 33:813–822.

Dong, M., Feng, L., Kuang, S.Y., Liu, Y., Jiang, J., Hu, K., Jiang, W.D., Li, S.H., Tang, L., Zhou, X.Q., 2012. Growth, body composition, intestinal enzyme activities and microflora of juvenile Jian carp (*Cyprinus carpio* var. Jian) fed graded levels of dietary valine. Aquaculture Nutrition 19, 1–14.

D'Mello, J.P.F. 1994. Amino acid imbalances, antagonisms and toxicities. In: D'Mello, J.P.F. (Ed.), Amino Acids in Farm Animal Nutrition, Wallingford: CAB International., pp. 63–97.

Doring F, Will J, Amasheh S, Clauss W, Ahlbrecht H and Daniel H. 1998. Minimal molecular determinants of substrates for recognition by the intestinal peptide transporter. J. Biol. Chem 273, 23211–23218.

Ellis, A.E., Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, W.B., Van muiswinker, W.B., 1990. Serum antiprotease in fish. Techniques in Fish Immunology. SOS Publication, Fair Haven, U.S.A., pp. 95–99.

Ellis, A. E. 1999. Immunity to bacteria in fish. Fish & Shellfish Immunology, 9: 291–308.

Espe, M., Lied, E., 1994. Do Atlantic salmon (Salmo salar) utilize mixtures of free amino acids to the same extent as intact protein sources for muscle protein synthesis? Comp. Biochem. Physiol. 107A, 249– 254.

EstCvez, A., M. Sameshima, M. Ishikawa, and A. Kanazawa. 1997. Effect of diets containing low levels of methionine and oxidized oil on body composition, retina structure and pigmentation success of Japanese flounder. Aquaculture Nutrition 3:201-216.

Evoy, D., Lieberman, M.D., Fahey III, T.J., Daly, J.M., 1998. Immunonutrition: the role

of arginine. Nutrition 14, 611–617.

Farhangi, M. and Carter, C.G. 2001. Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angusti folius*). Aquaculture Research, 32 (1): 329–340.

Fauconneau, B., 1988. Partial substitution of protein by a single amino acid or an organic acid in rainbow trout diets. Aquaculture 79, 29–36.

Fehlbaum, P., Rao, M., Zasloff, M. and Anderson, G.M. 2000. An essential amino acid induces epithelial ß-defensin expression. Proceedings of the National Academy of Sciences, 97: 12723–12728.

Field, C.J., Wu, G. and Marliss, E.B. 1994. Enhanced metabolism of glucose and glutamine in mesenteric lymph node lymphocytes from spontaneouslydiabetic BB rats. Canadian Journal of Physiology and Pharmacology, 72: 827–832.

Forster I., Ogata H.Y., 1998. Lysine requirement of juvenile Japanese flounder *Paralichthys olivaceous* and juvenile red sea beam *Pagrus major*. Aquaculture 161, 131–142.

Fournier, V., Gouillou-Coustans, M.F., Metailler, R., Vachot, C., Moriceau, J., LeDelliou, H., Huelvan, C., Desbrugeres, E., Kaushik, S.J., 2003. Excess arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss* and turbot *Psetta maxima*. Aquaculture 217, 559–576.

Fu, W.J., Haynes, T.E., Kohli, R., Hu, J., Shi, W., Spencer, T.E., Carroll, R.J., Meininger, C.J., Wu, G., 2005. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J. Nutr. 135, 714–721.

Galli, F., 2007. Amino acid and protein modification by oxygen and nitrogen species. Amino Acids 32, 497–499.

Garlich, J.D., Bazzano, G. and Olsen, R.E. 1970. Changes in plasma free amino acid concentrations in human subjects on hypercholesterolemic diets. The American Journal of Clinical Nutrition, 23: 1626–1683.

Ganapathy V, Brandsch M and Leibach FH. 1994. Intestinal transport of amino acids and peptides. In: Physiology of gastrointestinal tract (Ed. L. R. Johnson and J. Christensen). Raven Press, New York, USA. Pp. 1773-1794.

Glick, B., Day, E.J. and Thompson, D. 1981. Calorie–protein deficiencies and the immune response of the chicken I. Humoral immunity. Poultry Science, 60: 2494–2500.

Glick, B., Taylor, R.L.Jr., Martin, D.E., Watabe, H., Day, E.J. and Thompson, D. 1983. Calorie–protein deficiencies and the immune response of the chicken Π. Cell-mediated immunity. Poultry Science, 62: 1889–1893.

Gómez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., M edale, F., Corraze, G., Panserat, S., Martin, S.A.M., Houlihan, D.F., Kaushik, S.J., Perez-Sanchez, J., 2003. Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotropic axis responsiveness of gilthead sea bream (*Sparus aurata*). Aquaculture 220, 749–767.

Green, J.A., Hardy, R.W., 2002. The optimum dietary essential amino acid pattern for rainbow trout (*Oncorhynchus mykiss*), to maximize nitrogen retention and minimize nitrogen excretion. Fish Physiology and Biochemistry 27, 97–108.

Grillo, A.M.A., Colombatto, S., 2007. S-Adenosylmethionine and radical based

catalysis. Amino Acids 32, 197–202.

Grimble G. 1994. The significance of peptides in clinical nutrition. Annu. Rev. Nutr 14, 419-447.

Grimble, R.F. 2006. The effects of sulfur amino acid intake on immune function in humans. Journal of Nutrition, 136: 1660S–1665S.

Halver, J.E., Delong, D.C. & Mertz. E.T. (1959) Methionine and cystine requirements of chinook salmon. Fed. Proc., 18, 2076.

Harding, D. E., O. W. Allen, and R. P. Wilson. 1977. Sulfur amino acid requirement of channel catfish: L-methionine and L-cystine. J. Nutr. 107:2031.

Hardy, R.W. and Tacon, A.G.J. 2002. Fish meal: historical uses, production trends, and future outlook for sustainable supplies. In: Stickney, R.R and MacVey, J.M. (Eds.), Responsible Marine Aquaculture, CABI Publishing, Wallingford, UK, 311–325 pp.

Harikrishnan, R., Nisha Rani, M. and Balasundaram, C. 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture, 221: 41–50.

Higgs, D.A., Dosanjh, B.S., Prendergast, A.F., Beams, R.M., Hardy, R.W. Riley, W., Deacon, G., 1995. Use of rapeseed/canola protein products in finfish diets. In: D.J. Sessa (Ed.), Nutrition and utilization of technology in aquaculture. AOCS Press, Champaign USA, pp. 130–156.

Hird, F.J., 1986. The importance of arginine in evolution. Comp. Biochem. Physiol. 85B: 285–288.

Holecek, M., Sprongl, L., Tilser, I., 2001. Metabolism of branched-chain amino acids in starved rats: The role of hepatic tissue. Physiological Research 50, 25–33.

Hrubec, T.C., Cardinale, J.L., Smith, S.A., 2000. Hematology and plasma chemistry

reference intervals for cultured Tilapia (*Oreochromis hybrid*). Vet. Clin. Pathol. 29, 7–12.

Huai, M.Y., Tian, L.X., Liu, Y.J., Xu, A.L., Liang, G.Y., Yang, H.J., 2009. Quantitative dietary threonine requirement of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Boone) reared in low-salinity water. Aquac. Res. 40, 904–914.

Huang, S.S.Y., Oo, A.N., Higgs, D.A., Brauner, C.J., Satoh, S., 2007. Effect of dietary canola oil level on the growth performance and fatty acid composition of juvenile red sea bream, *Pagrus major*. Aquaculture 271, 420–431.

Hughes, S.G., Rumsey, G.L., Nesheim, M.C., 1983. Dietary requirements for essential branched-chain amino acids by lake trout. Transactions of the American Fisheries Society 112, 812–817.

Hultmark, D., 1980. Insert immunity: purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. European Journal of Biochemistry 106, 7 –16.

Jackson, A.J., Capper, B.S., 1982. Investigations into the requirements of the tilapia Sarotherodon mossambicus for dietary methionine, lysine and arginine in semi-synthetic diets. Aquaculture 29, 289–297.

Jobgen, W.S., Fried, S.K., Fu, W.J., Meininger, C.J., Wu, G., 2006. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. Journal of Nutritional Biochemistry 17, 571–588.

Jobgen, W.S., 2007. Dietary l-arginine supplementation reduces fat mass in diet-induced obese rats. PhD dissertation, Univ. of Texas A&M.

Jobgen, W., Fu, W.J., Gao, H., Li, P., Meininger, C.J., Smith, S.B., Spencer, T.E., Wu, G.,

2009. High fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. Amino Acids 37, 187–198.

Kasper C.S., White M.R. & Brawn P.B. (2000) Choline is required by tilapia when methionine is not in excess. Journal of Nutrition 130, 238–244.

Kaushik SJ (1998) Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat Living Resour 11:355–358.

Kaushik SJ and Dabrowski K. 1983. Postprandial metabolic changes in larval and juvenile carp (*Cyprinus carpio*). Reprod. Nutr. Develop 23, 223-234.

Kaushik, S.J., Dabrowski, K., 1984. Postprandial metabolic changes in larval and juvenile carp (*Cyprinus carpio*). Reproductive Nutrition Development 23, 223–234.

Keembiyehetty C.N. & Gatlin DM III (1993) Total sulphur amino acid requirement of juvenile hybrid striped bass (Morone chrysops x M. Saxatilis). Aquaculture 110, 331–339.

Khan, M.A. and Abidi, S.F. 2007. Dietary isoleucine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton). Aquaculture Nutrition, 13: 424–430.

Khan, M.A., Abidi, S.F., 2011. Dietary arginine requirement of *Heteropneustes fossilis* fry (Bloch) based on growth, nutrient retention and haematological parameters. Aquacult. Nutr. 17, 418–428.

Kidd, M.T. 2004. Nutritional modulation of immune function in broilers. Poultry Science, 83: 650–657.

Kim KI, Kayes TB, Amundson CH (1992) Requirements for lysine and arginine by

rainbow trout (*Oncorhynchus mykiss*). Aquaculture 106:333–344.

Kim SS, Rahimnejad S, Song JW and Lee KJ. 2012. Comparison of growth performance and whole-body amino acid composition in red seabream (*Pagrus major*) fed free or dipeptide form of phenylalanine. Asian-Aust. J. Anim. Sci 25(8), 1138 – 1144.

Kim SS and Lee KJ. 2013. Comparison of leucine requirement in olive flounder (*Paralichthys olivaceus*) by free or synthetic dipeptide forms of leucine. Animal Feed Science and Technology 183, 195– 201.

Kim, S.W., Mateo, R.D., Yin, Y.L. and Wu, G. 2007. Functional amino acids and fatty acids for enhancing production performance of sows and piglets. Asian-Australasian Journal of Animal Science, 20: 295–306.

Klein, R.G., Halver, J.E., 1970. Nutrition of salmonoid fishes: arginine and histidine requirements of Chinook and coho salmon. J. Nutr. 100, 1105–1110.

Konashi, S., Takahashi, K. and Akiba, Y. 2000. Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. British Journal of Nutrition, 83: 449–456.

Kritchevsky, D., 1979. Vegetable protein and atherosclerosis. Journal of the American Oil Chemists' Society 56, 135–140.

Kurokawa T and Suzuki T. 1998. Development of intestinal brush border aminopeptidase in the larval Japanese flounder *Paralichthys olivaceus*. Aquaculture 162, 113-124.

Kwasek K, Zhang Y and Dabrowski K. 2010. Utilization of dipeptide/protein based diets in larval and juvenile Koi carp-post-prandial free amino acid levels. J. Anim.

Physiol. Anim. Nutr 94, 35-43.

Kwasek K, Terova G, Wojno M, Dabrowski K and Wick M. 2012. The effect of dietary dipeptide lysine–glycine on growth, muscle proteins, and intestine PepT1 gene expression in juvenile yellow perch. Rev. Fish. Biol. Fisheries 22, 797–812.

Lee, D.J., Putnam, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. Journal of Nutrition 103, 916-922.

Leveille, G.A. and Sauberlich, H.E. 1961. Influence of dietary protein level on serum protein components and cholesterol in the growing chick. Journal of Nutrition, 74: 500–504.

Li, P., Yin, Y.L., Li, D., Kim, S.W., Wu, G., 2007. Amino acids and immune function. Br. J. Nutr. 98, 237–252.

Lim, S.J., Lee, K.J., 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus.* Aqauculture 290, 283–289.

Liu, Y., Wang, W.N., Wang, A.L., Wang, J.M. and Sun, R.Y. 2007. Effects of dietary vitamin E supplementation on antioxidant enzyme activities in *Litopenaeus vannamei* (Boone, 1931) exposed to acute salinity changes. Aquaculture, 265: 351–358.

Liu, F.J., Liu, Y.J., Tian, L.X., Li, X.F., Zhang, Z.H., Yang, H.J. and Du, Z.Y. 2014. Quantitative dietary isoleucine requirement of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Boone) reared in low-salinity water. Aquaculture International. DOI 10.1007/s10499-014-9761-y.

Lovell, R. T. 1989. Nutrition and feeding of fish. Van Nostrand Reinhold, New

York, New York, USA.

Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., Tan, X.Y., Yang, H.J., Liang, G.Y. and Liu, D.H. 2006. Quantitative L L-lysine requirement of juvenile grouper *Epinephelus coioides*. Aquaculture Nutrition, 12: 165–172.

Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., Tan, X.Y., Yang, H.J., 2007. Effects of dietary arginine levels on growth performance and body composition of juvenile grouper *Epinephelus coioides*. J. Appl. Ichthyol. 23, 252–257.

Luquet, P. & Sabaut, J.J. (1974) Nutrition azotee et croissance chezla daurade et la truite. In: Actes Des Colloques, pp. 243-253. Colloques sur l'Aquaculture, 1. Brest.

Luzzana, U., Hardy, R.W., Halver, J.E., 1998. Dietary arginine requirement of fingerling coho salmon (*Oncorhynchus kisutch*). Aquaculture 163, 137–150.

Magnadóttir, B., Jonsdottir, H., Helgason, S., Bjornsson, B., Jørgensen, T., Pilströ, L., 1999. Humoral immune parameters in Atlantic cod *Gadus morhua* L I: the effects of environmental temperature. Comparative Biochemistry and physiology 122, 173–180.

Maheswaran, R., Devapaul, A., Muralidharan, S., Velmurugan, B., Ignacimuthu, S., 2008. Haematological studies of freshwater fish, *Clarias batrachus* (L.) exposed to mercuric chloride. International Journal of Integrative Biology 2, 49–54.

Mai, K., Mercer, J.P., Donton, J., 1994. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus* hannai Ino. II. Amino acid composition of abalone and six species of macroalgae with an assessment of their nutritional value. Aquaculture 128, 115–130.

Mambrini M., Roem A.J., Crave `di J.P., Lalle`s J.P. & Kaushik S.J. (2001) Effects of

replacing fish meal with soy protein concentrate and of dl-methionine supplementation in high-energy extruded diets on the growth and nutrient utilization of rainbow trout, Oncorhynchus mykiss. Journal of Animal Sciences 77, 2990–2999.

Matthews D. 1991. Protein absorption. New York: Wiley-Liss.

McLaughlin PA. 1983. Internal anatomy. In: Bliss DE & Mantel TH (Eds.). The Biology of Crustacea. Academic Press, New York, 5, 1-52, 479.

Meijer, A.J., 2003. Amino Acids as Regulators and Components of Non-proteinogenic Pathways. Journal of Nutrition 133, 2057S–2062S.

Millamena, O.M., Bautista-Temel, M.N. & Kanazawa, A. (1996) Methionine requirement of juvenile tiger shrimp Penaeus monodon Fabricius. Aquaculture,143 , 403–410.

Ministry of Maritime Affairs and Fisheries, 2013. Aquaculture Statistic from Ministry of Maritime Affairs and Fisheries of Korea.

Montanez, R., Rodriguez-Caso, C., Sanchez-Jimenez, F., Medina, M.A., 2008. In silico analysis of arginine catabolism as a source of nitric oxide or polyamines in endothelial cells. Amino Acids 34, 223–229.

Moon, H.Y. & Gatlin, D.M. III (1991) Total sulfur amino acid requirement of juvenile red drum, Sciaenops ocellatus. Aquaculture, 95, 97-106.

Mukhopadhyay, N., Ray, A.K., 2001. Effects of amino acid supplementation on the nutritive quality of fermented linseed meal protein in the diets of rohu, Labeo rohita, fingerlings. J. Appl. Ichthyol. 17, 220–226.

Murai T. 1982. Effect of coating amino acids with casein supplmented to gelatin diet on

plasma free amino acids of carp. Bull. Jpn. Soc. Sci. Fish 48, 703-710.

Murai T, Hirasawa Y, Akiyama T and Nose T. 1983. Effects of dietary pH and electrolyte concentration on the utilization of crystaline amino acids in fingerling carp. Bull. Jpn. Soc. Sci. Fish 49, 1377-1380.

Murray, R.K., Granner, D.K., Mayes, P.A. & Rodwell, V.W. (1996) Harper's Biochemistry, 24th edn. Appleton & Lange, Stamford, CT, 868 spp.

Murai T, Ogata H, Hirasawa Y, Akiyama T and Nose T. 1987. Portal absorption and hepatic uptake of amino acids in rainbow trout force-fed complete diets containing casein or crystalline amino acids. Bull. Jpn. Soc. Sci. Fish 53, 1847-1859.

Nakagawa, H., Mustafa, M.G., Takii, K., Umino, T. and Kumai, H. 2000. Effect of dietary catechin and Spirulina on vitamin C metabolism in red sea bream. Fisheries Science, 66: 321–326.

Newsholme, E.A., Calder, P.C., 1997. The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition 13, 728–730.

Ng WK, Hung SSO (1995) Estimating the ideal dietary essential amino acid pattern for growth of white sturgeon, *Acipenser transmontanus* (Richardson). Aquac Nutr 1:85–94.

Nguyen, T.N., Davis, A., 2009. Evaluation of alternative protein sources to replace fish meal in practical diets for juvenile tilapia, *Oreochromis* spp 2009. J. World Aquac. Soc. 40 (1), 113–121.

Niu, J., Du, Q., Lin, H.Z., Cheng, Y.Q., Huang, Z., Wang, Y., Wang, J., Chen, Y.F., 2013. Quantitative dietary methionine requirement of juvenile golden pompano

Trachinotus ovatus at a constant dietary cystine level. Aquaculture Nutrition 19: 677-686.

Nose, T., 1979. Summary report on the requirements of essential amino acids for carp. In: Halver, J.E., Tiews, K. (Eds.), Finfish Nutrition and Fishfeed Technology, Heinemann., Berlin, pp. 145–156.

NRC National Research Council , 1993. Nutrient requirements of fish. National Academy Press, Washington, DC, USA, 114 p.

NRC., 2011. Nutrient Requirements of Fish. Natl Academy Press, Washington, DC, USA.

Olsen, R.E. Bazzano, C. and D'Elia, J.A. 1970a. The effects of large amounts of glutamic acid upon serum lipids and sterol metabolism in man. Transactions of the Association of American Physicians, 83: 196–210.

Olsen, R.E., Nichaman, M.Z., Nittka, J. and Eagles, A. 1970b. Effect of amino acid diets upon serum lipids in man. The American Journal of Clinical Nutrition, 23: 1614–1625.

Olsen, R.E., Sundell, K., Mayhew, T.M., Myklebust, R., Ringø, E., 2005. Acute stress alters intestinal function of rain bow trout, *Oncorhynchus mykiss* (W). Aquaculture 250, 480-495.

Opstvedt, J., Aksnes, A., Hope, B., Pike, I.H., 2003. Efficiency of feed utilization in Atlantic salmon (Salmo salar) fed diets with increasing substitution of fish meal with vegetable proteins. Aquaculture 221, 365–379.

Payne, C.J., Scott, T.R., Dick, J.W. and Glick, B. 1990. Immunity to *Pasteurella multocida* in protein-deficient chickens. Poultry Science, 69: 2134–2142.

Petro, T.M., Bhattacharjee, J.K., 1981. Effect of dietary essential amino acid limitations upon susceptibility to *Salmonella typhimurium* and the effect upon humoral and cellular immune response in mice. Infection and Immunity 32, 251–259.

Pohlenz, C., Buentello, A., Helland, S.J., Gatlin III, D.M., 2014. Effects of dietary arginine supplementation on growth, protein optimization and innate immune response of channel catfish *Ictalurus punctatus* (Rafinesque 1818). Aquac. Res. doi:10.1111/j.1365-2109.2012.03252.x.

Poston H.A. (1986) Response of rainbow trout to source and level of supplemental dietary methionine. Comparative Biochemistry & Physiology 83A, 739–744.

Quade, M.J., Roth, J.A., 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology 58, 239–248.

Rahimnejad, S., Lee, K.J., 2013. Dietary valine requirement of juvenile red sea bream *Pagrus major*. Aquaculture 416-417, 212–218.

Ren, M., Lia, Y., Xie, J., Liu, B., Zhou, Q., Ge, X., Cui, H., Pan, L., Chen, R., 2013. Dietary arginine requirement of juvenile blunt snout bream, *Megalobrama amblycephala*. Aquaculture, 414-415, 229–234.

Ren, M., Ai, Q., Mai, K., 2014. Dietary arginine requirement of juvenile cobia (*Rachycentron canadum*). Aquac. Res. 45, 225–233.

Reshkin S and Ahearn G. 1991. Intestinal glycyl-L-phenylalanine and L-phenylalanine trasnport in a euryhaline teleost. Am. J. Physiol 260, R563-R569.

Reyes-Becerril, M., Ascencio-Valle, F., Tovar-Ramírez, D., Meseguer, J., Esteban, M.A., 2011. Effects of polyamines on cellular innate immune response and the

expression of immune-relevant genes in gilthead seabream leucocytes. Fish & Shellfish Immunology 30, 248–254.

Robinson, E.H., Wilson, R.P., Poe, W.E., 1981. Arginine requirement and apparent absence of a lysine–arginine antagonism in fingerling channel catfish. J. Nutr. 111, 46– 52.

Roch, P. 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. Aquaculture, 172: 125–145.

Rodehutscord, M., Becker, A., Pack, M., Pfeffer, E., 1997. Response of rainbow trout (*Oncorhynchus mykiss*) to supplements of individual essential amino acids in a semipurified diet, including an estimate of the maintenance requirement for essential amino acids. Joural of Nutrition 127, 1166–1175.

Rollin X (1999) Critical study of indispensable amino acids requirements of Atlantic salmon (*Salmo salar* L.) fry. PhD thesis, Universite catholique de Louvain, Louvain, Belgium.

Rollin, X., Mambrini, M., Abboudi, T., Larondelle, Y., Kaushik, S.J., 2003. The optimum dietary indispensable amino acid pattern for growing Atlantic salmon (*Salmo salar* L.) fry. British Journal of Nutrition 90, 865–876.

Rollin, X., Wauters, J.B., Bodin, N., Larondelle, Y., Ooghe, W., Wathelet, B., Abboudi, T., 2006. Maintenance threonine requirement and efficiency of its use for accretion of whole-body threonine and protein in Atlantic salmon (*Salmo salar* L.) fry. British Journal of Nutrition 95, 234–245.

Rønnestad I, Conceicao LEC, Aragai C and Dinis MT. 2000. Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal

sole (*Solea senegalensis*). J. Nutr 130, 2809-2812.

Rønnestad I, Tonheim SK, Fyhn HJ, Rojas-Garcia CR, Kamisaka Y and Koven W. 2003. The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings. Aquaculture 227, 147-164.

Roth, E., 2007. Immune and cell modulation by amino acids. Clin. Nutr. 26, 535–544.

Ruchimat T., Masumoto, T., Hosokawa H. & Shimeno S. (1997) Quantitative methionine requirement of yellowtail (*Seriola quinquiradiata*). Aquaculture 150, 113–122.

Rumsey, G.L., Page, J.W., Scott, M.L., 1983. Methionine and cystine requirements of rainbow trout. Prog. Fish-Cult. 45, 139– 143.

Rust MB, Hardy RW and Stickney RR. 1993. A new method for force feeding larval fish. Aquaculture 116, 341-352.

Ruth, M.R. and Field, C.J. 2013. The immune modifying effects of amino acids on gut-associated lymphoid tissue. Journal of Animal Science and Biotechnology, 4: 27.Santiago, C.B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. Journal of Nutrition 118, 1540–1546.

Santiago, C.B. & Lovell, R.T. (1988) Amino acids requirements for growth of Nile tilapia. J. Nutr., 118, 1540-1546.

Satriano, J., Ishizuka, S., Archer, D.C., Blantz, R.C., Kelly, C.J., 1999. Regulation of intracellular polyamine biosynthesis and transport by NO and cytokines TNF-alpha and IFN-gamma. Am. J. Phys. 276, C892–C899.

Saurabh, S. and Sahoo, P.K. 2008. Lysozyme: An important defence molecule of fish innate immune system. Aquaculture Research, 39: 223–239.

Shah, S.L., Altindag, A., 2005. Alterations in the immunological parameters of tench (*Tinca tinca* L. 1758) after acute and chronic exposure to lethal and sub lethal treatments with mercury, cadmium and lead. Turkish Journal of Veterinary and Animal Sciences 29, 1163–1168.

Shantz, L.M., Levin, V.A., 2007. Regulation of ornithine decarboxylase during oncogenic transformation: mechanisms and therapeutic potential. Amino Acids 33, 213–223.

Shimomura, Y., Yamamoto, Y., Bajotto, G., Sato, J., Murakami, T., Shimomura, N., Kobayashi, H., Mawatari, K., 2006. Nutraceutical effects of branched-chain amino acids on skeletal muscle. Journal of Nutrition 136, 529S–532S.

Simmons L, Moccia RD, Bureau DP, Sivak JG, Herbert K (1999) Dietary methionine requirement of juvenile Arctic charr, Salvelinus alpinus (L). Aquac Nutr 5:93–100.

Small, B.C., Soares Jr, J.H., 1999. Quantitative dietary threonine requirement of juvenile striped bass *Morone saxatilis*. Journal of the World Aquaculture Society 30, 319–323.

Siwicki, A.K., Anderson, D.P., 1993. Nonspecific defense mechanisms assay in fish: II. potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. Fish Disease Diagnosis and Prevention Methods 105-112. Olsztyn, Poland.

Small, B.C., Soares Jr., J.H., 1999. Quantitative dietary threonine requirement of juvenile striped bass *Morone saxatilis*. J. World Aquac. Soc. 30, 319–323.

Sugiyama, K., Kanamori, H., Akachi, T., Yamakawa, A., 1996. Amino acid composition of dietary proteins affects plasma cholesterol concentration through alteration of

hepatic phospholipid metabolismin rats fed a cholesterol-free diet. Nutritional Biochemistry 7, 40–48.

Sveier H., Nordas H., Berge G.E. & Lied E. (2001) Dietary inclusion of crystalline d- and l-methionine: effects on growth, feed and protein utilization, and digestibility in small and large Atlantic salmon. Aquaculture Nutrition 7, 169–181.

Tacon, A.G. & Cowey, C.B. (1985) Protein and amino acid requirements. In: Fish Energetics: and New Perspectives (Tytler, P. & Calow, P. eds), pp. 155±183. Croom-Helm, London, UK.

Tacon, A.G., Jackson, A.J., 1985. Utilization of conventional and unconventional protein sources in practical fish feeds. In: Cowey, C.B., Mackie, A.M., Bell, J.G. (Eds.), Nutrition and Feeding in Fish. cademic Press, London, pp. 119–145.

Tacon, A.G.J. and Metian, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture, 285: 146–158.

Tafalla, C., Novoa, B., 2000. Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. Dev. Comp. Immunol. 24, 623–631.

Takagi, S., Shimeno, S., Hosokawa, H., Ukawa, M., 2001. Effect of lysine and methionine supplementation to a soy protein concentrate diet for red sea bream Pagus major. Fish. Sci. 67, 1088–1096.

Terpstra, A.H.M., Hermus, R.J.J., West, C.E., 1983. The role of dietary protein in cholesterol metabolism. World Review of Nutrition and Dietetics 42, 1–55.

Tesser M, Terjesen BF, Zhang Y, Portella MC and Dabrowski K. 2005. Free- and

peptide-based dietary arginine supplementation for the South American fish pacu (*Piaractus mesopotamicus*). Aquacult. Nutr 11, 443-453.

Thebault, H., Alliot, E. & Pastoureaud, A. (1985) Quantitative methionine requirement of juvenile seabass (*Dicentrarchus labrax*). Aquaculture, 50, 75-87.

Tsiagbe, V.K., Good, M.E., Harper, A.E. and Sunde M.L. 1987a. Efficacy of cysteine in replacing methionine in the immune responses of broiler chickens. Poultry Science, 66: 1138–1146.

Tsiagbe, V.K., Cook, M.E., Harper, A.E. and Sunde, M.L. 1987b. Enhanced immune responses in broiler chickens fed methionine-supplemented diets. Poultry Science, 66: 1147–1154.

Tsukishiro, T., Shimizu, Y., Higuchi, K., Watanabe, A., 2000. Effect of branched-chain amino acids on the composition and cytolytic activity of liver-associated lymphocytes in rats. Journal of Gastroenterology and Hepatology 15, 849–859.

Twibell, R.G., Wilson, K.A., Brown, P.B., 2000. Dietary sulfur amino acid requirement of juvenile yellow perch fed maximum cystine replacement value for methionine. Journal of Nutrition 630, 612–616.

Verri T, Kottra G, Romano A, Tiso N, Peric M, Maffia M, Boll M, Argenton F, Daniel H and Storelli C. 2003. Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1-type peptide transporter. FEBS Lett 549, 115-122.

Waithe, W.I., Dauphinais, C., Hathaway, P., Hirschhorn, K., 1975. Protein synthesis in stimulated lymphocytes. II. Amino acid requirements. Cellular Immunology 17, 323–334.

Walton, M.J., Cowey, C.B., Adron, J.W., 1982. Methionine metabolism in rainbow trout

fed diets of differing methionine and cystine content. J. Nutr. 112, 1525– 1535.

Wang S, Liu YJ, Tian LX, Xie MQ, Yang HJ, Wang Y and Liang GY. 2005. Quantitative dietary lysine requirement of juvenile grass carp *Ctenopharyngodon idella*. Aquaculture 249(1–4), 419–429.

Wang, X., Castanon, F. and Parsons, C.M. 1997. Order of amino acid limitation in meat and bone meal. Poultry Science, 76: 54–58.

Watanabe, T., Aoki, H., Watanabe, K., Maita, M., Yamagata, Y. and Satoh, S. 2001. Quality evaluation of different types of non-fish meal diets for yellowtail. Fisheries Science, 67: 461–469.

Welker, T.L., Congleton, J.L., 2003. Relationship between dietary lipid source, oxidative stress, and the physiological response to stress in sub-yearling chinook salmon (*Oncorhynchus tshawytscha*). Fish. Physiol. Biochem. 29, 225–235.

Wiegertjes, G.F., Stet, R.J.M., Parmentier, H.K. and Van Muiswinkel, W.B. 1996. Immunogenetics of disease resistance in fish; a comparable approach. Developmental and Comparative Immunology, 20: 365–381.

Wilson, R.P., Allen, O.W.Jr, Robinson, E.H., Poe, W.E., 1978. Tryptophan and threonine requirements of fingerling channel catfish. Journal of Nutrition 108, 1595–1599.

Wilson RP and Cowey CB. 1985. Amino acid composition of whole body tissue of rainbow trout and Atlantic salmon. Aquaculture 48, 373-376.

Wilson, R.P., Poe, W.E., Robinson, E.H., 1980. Leucine, isoleucine, valine and histidine requirements of fingerling channel catfish. Journal of Nutrition 110, 627–633.

Wilson RP and Poe WE. 1985. Relationship of whole body and egg essential amino acid patterns to amino acid requirement patterns in channel catfish, *Ictalurus punctatus*.

Comp. Biochem. Physiol 80, 385-388.

Wu, G.Y., Field, C.J. and Marliss, E.B. 1991a. Glutamine and glucose metabolism in thymocytes from normal and spontaneously diabetic BB rats. Biochemistry and Cell Biology, 69: 801–808.

Wu, G.Y., Field, C.J. and Marliss, E.B. 1991b. Elevated glutamine metabolism in splenocytes from spontaneously diabetic BB rats. Biochemical Journal, 274: 49–54.

Wu, G.Y., Field, C.J. and Marliss, E.B. 1991c. Glutamine and glucose metabolism in rat splenocytes and mesenteric lymph node lymphocytes. American Journal of Physiology, 260: E141–E147. Wu, G.Y., Morris, S.M., J.r., 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336, 1–17.

Wu, G., 2010. Functional amino acids in growth, reproduction, and health. Advances in Nutrition: An International Review Journal 1, 31–37.

Xue, H. and Field, C.J. 2011. New role of glutamate as an immunoregulator via glutamate receptors and transporters. Frontiers in Bioscience (Scholar Edition), 3: 1007–1020.

Yamamoto, T., Shima, T., Furuita, H., Shiraishi, M., Sanchez-Vasquez, F.J., Tabata, M., 2000. Self-selection of diets with different amino acid profiles by rainbow trout (*Oncorhynchus mykiss*). Aquaculture 187, 375–386.

Yang, S., Liou, C., Liu, F., 2002. Effects of dietary protein level on growth performance, carcass composition and ammonia excretion in juvenile silver perch (*Bidyanus bidyanus*). Aquaculture 213, 363–372.

Yoneda, J., Andou, A. and Takehana, K. 2009. Regulatory Roles of Amino Acids in

Immune Response. Current Rheumatology Reviews, 5(4): 252–258.

Yue, Y., Zou, Z., Zhu, J., Li, D., Xiao, W., Han, J., Yang, H., 2013. Effects of dietary arginine on growth performance, feed utilization, haematological parameters and non-specific immune responses of juvenile Nile tilapia (*Oreochromis niloticus* L.). Aquac. Res. doi:10.1111/are.12333.

ZamboninoInfante JL, Cahu CL and Peres A. 1997. Partial substitution of di- and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* larval development. J. Nutr 127, 608-614.

Zarate DD and Lovell RT. 1997. Free lysine (L-lysine·HCL) is utilized for growth less efficiently than protein-bound lysine (soybean meal) in practical diets by young channel catfish (*Ictalurus punctatus*). Aquaculture159, 87-100.

Zehra, S., Khan, M.A., 2013. Dietary Arginine Requirement of Fingerling Indian Major Carp, *Catla catla* (Hamilton). J. World Aquac. Soc. 44 (3), 363–373.

Zehra, S. and Khan, M.A. 2013. Dietary isoleucine requirement of fingerling catla, *Catla catla* (Hamilton), based on growth, protein productive value, isoleucine retention efficiency and carcass composition. Aquaculture International. DOI 10.1007/s10499-013-9627-8.

Zhang Y, Dabrowski K, Hliwa P and Gomulka P. 2006. Indispensable amino acid concentrations decrease in tissues of stomachless fish, common carp in response to free amino acid- or peptide-based diets. Amino Acids 31, 165-172.

Zhao, J., Liu, Y., Jiang, J., Wu, P., Chen, G., Jiang, W., Li, S., Tang, L., Kuang, S., Feng, L. and Zhou, X. 2012. Effects of dietary isoleucine on growth, the digestion and absorption capacity and gene expression in hepatopancreas and intestine of

juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture, 368–369: 117–128.

Zhao, J., Liu, Y., Jiang, J., Wu, P., Jiang, W., Li, S., Tang, L., Kuang, S., Feng, L. and Zhou, X. 2013. Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish & Shellfish Immunology, 35: 572–580.

Zhou, H., Chen, N., Qiu, X., Zhao, M., Jin, L., 2012. Arginine requirement and effect of arginine intake on immunity in largemouth bass, *Micropterus salmoides*. Aquacult. Nutr. 18, 107–116.

Zhou, F., Shao, J., Xu, R., Ma, J. and Xu, Z. 2010. Quantitative L-lysine requirement of juvenile black sea bream (*Sparus macrocephalus*). Aquaculture Nutrition, 16: 194–204.

Zhou, F., Xiong, W., Xiao, X.J., Shao, J.Q., Bergo, N.O., Hua, Y., Chai, X., 2010. Optimum arginine requirement of juvenile black sea bream, *Sparus macrocephalus*. Aquac. Res. 41, 418–430.

Zhou, Q.C., Wu, Z.H., Tan, B.P., Chi, S.Y. & Yang, Q.H. (2006) Optimal dietary methionine requirement for Juvenile Cobia (Rachycentron canadum). Aquaculture, 258, 551–557.

Zhou, Q.C., Zeng, W.P., Wang, H.L., Xie, F.J., Wang, T., Zheng, C.Q., 2012. Dietary arginine requirement of juvenile yellow grouper *Epinephelus awoara*. Aquaculture 350, 175–182.

Zhou XQ, Zhao CR, Jiang J, Feng L and Liu Y. 2008. Dietary lysine requirement of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquacult Nutr14(5), 381–386.

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LIST OF PUBLICATIONS

1. Cha, J.H., **Rahimnejad, S**., Lee, B.J., Yang, S.Y., Lee, K.J., 2014. Effects of dietary supplementation of *Bacillus subtilis* on growth performance, feed utilization, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* (In preparation).
2. **Rahimnejad, S**., Lee, K.J. Dietary isoleucine influences non-specific immune response in juvenile olive flounder (*Paralichthys olivaceus*). Turkish Journal of Fisheries and Aquatic Sciences (Under review).
3. **Rahimnejad, S**., Lee, K.J. Dietary arginine requirement of juvenile red sea bream *Pagrus major*. Aquaculture (Under review).
4. **Rahimnejad, S**., Lee, K.J. Comparison of free and dipeptide lysine utilization in diets for juvenile olive flounder (*Paralichthys olivaceus*). Fisheries and Aquatic Sciences (Under review).
5. Kim, S,S., Khosravi, S., **Rahimnejad, S**., Lee, K.J., 2014. Growth performance, feed utilization and body composition of parrot fish (*Oplegnathus fasciatus*) fed graded levels of dietary lipid. Journal of Applied Ichthyology (Under review).
6. Khosravi, S., Bui, H.T.D., **Rahimnejad, S**., Herault, M., Fournier, V., Jeong, J.B., Lee, K.J., 2014. Effect of dietary hydrolysate supplementation on growth performance, non-specific immune response and disease resistance of olive flounder (*Paralichthys olivaceus*) challenged with *Edwardsiella tarda*. Aquaculture Nutrition (Accepted, in press).

7. Kim, K.W., Kim, S,S., Khosravi, S., **Rahimnejad, S**., Lee, K.J. 2014. Evaluation of *Sargassum fusiforme* and *Ecklonia cava* as dietary additives for olive flounder (*Paralichthys olivaceus*). Turkish Journal of Fisheries and Aquatic Sciences, 14: 321-330.

8. Shin, C.H., Bui, H.T.D., **Rahimnejad, S**., Yoo, B.W., Lee, B.K., Ahn, H.J., Choi, S.I., Choi, Y.J., Park, Y.H., Kim, J.D., Lee, K.J., 2014. Effects of dietary supplementation of Barodon on growth performance, innate immunity and disease resistance of juvenile olive flounder, *Paralichthys olivaceus*, against *Streptococcus iniae*. Journal of the World Aquaculture Society, 45(3): 258-268.

9. Shin, C.H., Cha, J.H., **Rahimnejad, S**., Jeong, J.B., Yoo, B.W., Lee, B.K., Ahn, H.J., Choi, S.I., Choi, Y.J., Park, Y.H., Kim, J.D., Lee, K.J., 2014. Effects of dietary supplementation of Barodon, an anionic alkali mineral complex, on growth performance, feed utilization, innate immunity, goblet cell and digestibility in olive flounder (*Paralichthys olivaceus*). Asian-Australasian Journal of Animal Science, 27: 383-390.

10. Rahimnejad, S., Lee, K.J., 2013. Dietary valine requirement of juvenile red sea bream *Pagrus major*. Aquaculture, 416–417: 212–218.

11. Lee, K.J., **Rahimnejad, S**., Madison, S.P., Barrows, F.T., Smiley, S., Bechtel, P.J., Hardy, R.W., 2013. Salmon testes meal as a functional feed additive in fish meal and plant protein-based diets for rainbow trout (*Oncorhynchus mykiss Walbaum*) and Nile tilapia (*Oreochromis niloticus* L.) fry. Aquaculture Research. doi:10.1111/are.12313.

12. Cha, J.H., **Rahimnejad, S.,** Yang, S.Y., Kim, K.W., Lee, K.J., 2013. Evaluations of Bacillus spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against Streptococcus *iniae* and as water additives. Aquaculture, 402–403: 50–57.

13. Kim, S,S., **Rahimnejad, S.,** Kim, K.W., Lee, K.J., 2013. Partial Replacement of Fish Meal with *Spirulina pacifica* in Diets for Parrot Fish (*Oplegnathus fasciatus*). Turkish Journal of Fisheries and Aquatic Sciences, 13: 197-204.

14. Kim, S,S., **Rahimnejad, S.,** Kim, K.W., Lee, B.J., Lee, K.J., 2013. Effects of Dietary Supplementation of Spirulina and Quercetin on Growth, Innate Immune Responses, Disease Resistance Against *Edwardsiella tarda*, and Dietary Antioxidant Capacity in the Juvenile Olive Flounder *Paralichthys olivaceus*. Fisheries and Aquatic Sciences 16(1): 7-14.

15. Kim, S,S., **Rahimnejad, S.,** Song, J.W., Lee, K.J., 2012. Comparison of growth performance and whole-body amino acid composition in red seabream (*Pagrus major*) fed free or dipeptide from of phenylalanine. Asian-Australasian Journal of Animal Science 25(8): 1138-1144.

16. Rahimnejad, S., Agh, N., Kalbasi, M.R., Khosravi, S., 2012. Effect of dietary bovine lactoferrin on growth, haematology and non-specific immune response in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Research, 43: 1451–1459.