

# Comparison of Free and Dipeptide Lysine Utilization in Diets for Juvenile Olive Flounder *Paralichthys olivaceus*

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## Abstract

We compared the utilization efficiency of free lysine (FL) and dipeptide lysine–glycine (LG) in terms of growth performance and whole-body amino acid composition in olive flounder *Paralichthys olivaceus*. A basal experimental diet was formulated to contain 0.5% (basal) lysine from fish meal, and four other diets were prepared by supplementing 0.5% or 1.0% of either FL or LG. The experiment was performed in triplicate, and 20 randomly selected fish averaging  $5.41 \pm 0.16$  g were fed one of the test diets at the rate of 3% BW/day twice daily for 6 weeks. At the end of the feeding trial, fish fed the basal diet showed significantly less weight gain than did the other groups. The results of a two-way ANOVA showed that both lysine level ( $P = 0.001$ ) and type ( $P = 0.034$ ) influenced growth rate; however, we found no significant interaction between lysine level and form ( $P > 0.05$ ). Our results revealed a significant improvement in protein efficiency ratio (PER) with each increment of dietary lysine, and the groups fed LG-supplemented diets showed higher PER than did those offered FL. Hepatosomatic and viscerosomatic indices were significantly influenced by lysine level and form, and higher values were recorded in fish fed diets containing LG. Significantly higher whole-body arginine levels were found in LG-fed groups, and a significant interaction was observed between lysine level and form ( $P = 0.009$ ). Whole-body valine and aspartic acid contents were affected by lysine level, and alanine concentration was influenced by both lysine level and form. Our findings indicate that juvenile olive flounder can utilize LG more efficiently than FL for protein synthesis.

**Key words:** Olive flounder, Lysine, Dipeptide, Free amino acid, Growth performance

## 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 the essential AA requirements of cultured fish species is very important to achieve optimum growth and feed utilization, cost-effective diet formulation, and desirable carcass quality (NRC, 2011). It is well established that a balanced AA profile is required for the effective use of dietary protein in tissue synthesis (D'Mello, 1994; Yamamoto et al., 2000; Berge et al., 2002; Green and Hardy, 2002; Gómez-Requeni et al., 2003). AA can be supplied as protein-bound AA, free AA, or peptides in formulated diets. Absorption of peptides and free AA (FAA)

are the major transport routes for protein use in mammals (Ganapathy et al., 1994; Adibi, 1997). The current understanding of protein use in vertebrates centers on the absorption of peptides in the intestine as a major route of transport. In fishes, small peptides and FAA are absorbed faster than protein (Rust et al., 1993; Rønnestad et al., 2003), and there is evidence that single peptides can be more efficiently absorbed than a mixture of identical AA in both fish (Reshkin and Ahearn, 1991; Boge et al., 2002) and mammals (Matthews, 1991). Previous studies on common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* showed inferior utilization of AA from FAA-based diets vs. that from protein-based diets (Murai,

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1982; Kaushik and Dabrowski, 1983; Dabrowski et al., 2003), possibly because of deamination or higher total dietary nitrogen excretion through the gills and kidneys (Dabrowski et al., 2003). It is generally thought that the inefficiency of free AA use in fish is due to faster uptake and subsequent catabolism compared with intact protein (Cowey and Walton, 1988; Rønnestad et al., 2000; Dabrowski et al., 2003; Dabrowski et al., 2007). Another proposed explanation is higher leaching loss of free AA compared with bound AA in aquatic environments prior to ingestion (Zarate and Lovell, 1997).

Dabrowski et al. (2003) showed that a synthetic dipeptide-based diet could support the early stages of rainbow trout growth, whereas a free AA-based diet could not. Peptides have different transport systems than FAA has. Tetra- and larger peptides do not cover nitrogen requirements in the absence of pancreatic enzymes or brush border peptidase activity (Grimble, 1994; Daniel, 2004), whereas di- or tripeptides have specific peptide transporters (Doring et al., 1998). These transporters are expressed in teleost larvae prior to exogenous feeding (Verri et al., 2003).

Lysine has been identified as an essential AA for all studied fish species (NRC, 2011) and has been found in high concentrations in the carcasses of many fishes (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 the protein sources, particularly plant proteins, used in aquafeeds (Deng et al., 2010).

Olive flounder *Paralichthys olivaceus* is an important marine cultured species in Korea, Japan, and China. Its total production reached ~37,000 tons in 2013 in Korea (Ministry of Maritime Affairs and Fisheries of Korea, 2013). Dietary lysine requirements for the species were determined by Forster and Ogata (1998) using free L-lysine as the AA source. Kim and Lee (2013) showed that juvenile olive flounder can use dipeptide leucine more efficiently than its free form for protein synthesis. Accordingly, we compared the utilization efficiency of free and dipeptide forms of lysine in juvenile olive flounder diets.

## Materials and Methods

### Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 1. Five isonitrogenous (47% crude protein) and isocaloric (4.38 kcal g<sup>-1</sup> gross energy) experimental diets were formulated to contain different levels and forms of lysine. A mixture of synthetic free AA 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 levels of lysine (0.5%) from fish meal and

supplemented with 0.5% or 1.0% lysine in either the free or the dipeptide form. 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) as the free form (FL). The experimental diets were kept isonitrogenous 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.

### Fish and experimental conditions

Juvenile olive flounder were transported from a private hatchery to the Institute of Marine Sciences at Jeju National University (Jeju, South Korea). All fish were fed the basal diet for 1 week to allow acclimation to both the semi-purified diet and the experimental conditions. At the end of the acclimation period, 20 randomly selected fish (averaging 5.41 ± 0.16 g) were stocked into polyvinyl circular tanks (20-L capacity), supplied with filtered seawater at a flow rate of 1.5 L min<sup>-1</sup>, and aerated to maintain sufficient dissolved oxygen. Triplicate groups of fish were hand-fed with one of the test diets at a ratio of 3% of their body mass (twice a day, 09:00 and 17:00 h) for 6 weeks. The growth of fish was measured at 2-week intervals. Feeding was stopped 24 h prior to weighing to minimize handling stress. 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.

### Sample collection and analyses

At the end of the feeding trial, all fish in each tank were bulk-weighed and counted for 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 their 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), viscerosomatic index (VSI), and relative intestine length (RIL).

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

### Statistical analysis

All experimental diets were assigned by a completely ran-

domized design. Data were analyzed by one-way ANOVA using SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA). When an ANOVA identified differences among groups, the means were compared using 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. To isolate the effects of molecular form from lysine level in the two-way ANOVAs, only diets with lysine supplementation were considered. Data are presented as means  $\pm$  SD. Percentage data were arcsine transformed before statistical analysis.

## Results

Results showed significant improvement in fish growth with each increment of dietary lysine ( $P < 0.05$ , one-way ANOVA), and the highest growth performance occurred in fish fed 1.5% LG (Table 2). Feed utilization was significantly affected by dietary treatment, and the highest protein efficiency ratio (PER) was obtained in fish fed 1.5% LG (one-way ANOVA). We also found significant effects of both lysine level and form on weight gain and PER ( $P < 0.05$ , two-way ANOVA), but no

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

Ingredient	Control 0.5	L-Lys		Lys-Gly	
		1.0	1.5	1.0	1.5
White fish meal	10	10	10	10	10
Free AA mix <sup>1</sup>	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 <sup>2</sup>	2.0	2.0	2.0	2.0	2.0
Vitamin mix <sup>3</sup>	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
<i>Proximate composition</i>					
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	9.30	9.46	9.35	9.17	9.09
Ash	3.8	3.7	3.7	3.6	3.8

<sup>1</sup>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.

<sup>2</sup>Mineral premix (g kg<sup>-1</sup> mixture): MgSO<sub>4</sub>·7H<sub>2</sub>O, 80.0; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 20.0; Ca-lactate, 356.5; CuCl<sub>2</sub>, 0.2; AlCl<sub>3</sub>·6H<sub>2</sub>O, 0.15; Na<sub>2</sub>SeO<sub>3</sub>, 0.01; MnSO<sub>4</sub>·H<sub>2</sub>O, 2.0; CoCl<sub>2</sub>·6H<sub>2</sub>O, 1.0.

<sup>3</sup>Vitamin premix (g kg<sup>-1</sup> mixture): L-ascorbic acid, 121.2; DL- $\alpha$  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-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

**Table 2.** Growth performance of olive flounder *Paralichthys olivaceus* (initial body weight, 5.41  $\pm$  0.16 g) fed the experimental diets containing different lysine levels and molecular forms for 6 weeks

	Dietary treatment					SEM <sup>1</sup>	P-values Lys form	P-values Lys level	P-values Lys form×level
	Control 0.5	L-Lys		Lys-Gly					
		1.0	1.5	1.0	1.5				
FBW <sup>2</sup>	6.57 <sup>c</sup>	7.70 <sup>b</sup>	8.73 <sup>a</sup>	8.15 <sup>ab</sup>	8.87 <sup>a</sup>	0.45	0.191	0.003	0.463
WG <sup>3</sup>	26.44 <sup>c</sup>	43.30 <sup>b</sup>	57.06 <sup>ab</sup>	49.72 <sup>b</sup>	64.92 <sup>a</sup>	6.62	0.034	0.001	0.787
PER <sup>4</sup>	0.55 <sup>c</sup>	0.84 <sup>b</sup>	1.07 <sup>ab</sup>	0.94 <sup>ab</sup>	1.21 <sup>a</sup>	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.

<sup>1</sup>Pooled standard error of the mean

<sup>2</sup>Final body weight (g)

<sup>3</sup>Weight gain (%) = final mean body weight - initial mean body weight

<sup>4</sup>Protein efficiency ratio = Wet weight gain/total protein fed

interaction between them ( $P > 0.05$ , two-way ANOVA). The survival rate ranged from 86 to 93% and did not significantly differ among treatments.

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

Whole-body amino acid composition was significantly affected by dietary treatment (Table 4). Significantly higher arginine levels were found in fish fed diets containing LG (one-way ANOVA), and we found a significant interaction between lysine level and form ( $P = 0.009$ , two-way ANOVA). Two-way ANOVA analysis showed a significant effect of lysine level on whole-body valine and aspartic acid contents. Moreover, alanine content was significantly higher in fish offered 1.5% LG (one-way ANOVA), and it was significantly influenced by both lysine level and form.

**Table 3.** Organosomatic indices of olive flounder *Paralichthys olivaceus*, (initial body weight,  $5.41 \pm 0.16$  g) fed the experimental diets containing different lysine levels and molecular forms for 6 weeks

	Dietary treatment					SEM <sup>1</sup>	P-values Lys form	P-values Lys level	P-values Lys form×level
	Control 0.5	L-Lys		Lys-Gly					
		1.0	1.5	1.0	1.5				
HSI <sup>2</sup>	2.05 <sup>c</sup>	2.18 <sup>c</sup>	3.21 <sup>ab</sup>	3.0 <sup>b</sup>	3.95 <sup>a</sup>	0.51	0.003	0.000	0.605
VSI <sup>3</sup>	3.49 <sup>b</sup>	3.95 <sup>b</sup>	5.63 <sup>a</sup>	5.61 <sup>a</sup>	5.83 <sup>a</sup>	0.84	0.026	0.021	0.059
RIL <sup>4</sup>	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.

<sup>1</sup>Pooled standard error of the mean

<sup>2</sup>Hepatosomatic index =  $100 \times (\text{liver weight/body weight})$

<sup>3</sup>Viscera somatic index =  $100 \times (\text{viscera weight/body weight})$

<sup>4</sup>Relative intestine length =  $100 \times (\text{intestine length/total body length})$

**Table 4.** Whole-body amino acid composition (% protein) of olive flounder *Paralichthys olivaceus*, fed the experimental diets containing different lysine levels and molecular forms for 6 weeks

	Dietary treatment					SEM <sup>1</sup>	P-values Lys form	P-values Lys level	P-values Lys form×level
	Control 0.5	L-Lys		Lys-Gly					
		1.0	1.5	1.0	1.5				
<i>EAA</i>									
Arg	6.59 <sup>b</sup>	6.67 <sup>ab</sup>	6.72 <sup>ab</sup>	6.77 <sup>a</sup>	6.77 <sup>a</sup>	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
<i>NEAA</i>									
Ala	7.02 <sup>b</sup>	7.15 <sup>ab</sup>	7.14 <sup>ab</sup>	7.19 <sup>ab</sup>	7.31 <sup>a</sup>	0.14	0.046	0.001	0.002
Asp	9.33 <sup>b</sup>	9.79 <sup>ab</sup>	10.15 <sup>a</sup>	9.42 <sup>ab</sup>	9.98 <sup>ab</sup>	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.

<sup>1</sup>Pooled standard error of the mean.

## Discussion

Fish in our study accepted the semi-purified diets but grew relatively slowly because the trials occurred in low temperature season. Our results show a significant enhancement of fish growth with each increment of dietary lysine, indicating the importance of lysine for maximal growth in juvenile olive flounder. Forster and Ogata (1998) reported that the lysine requirement of olive flounder is 1.5–2.1% of their diet. Accordingly, we used a range of 0.5 to 1.5% lysine to compare the efficiency of its free and dipeptide forms.

Dabrowski et al. (2003) reported that a dipeptide-based diet could support the early-life growth performance of rainbow trout, whereas a FAA-based diet could not. In our study, fish fed LG supplemented diets showed higher growth than those fed FL, and the highest weight gain occurred in those fed 1.5% LG. Similarly, Kim and Lee (2013) found higher weight gain at all supplementation levels of the dipeptide form of leucine than of the free form. In contrast, Kim et al. (2012) reported similar efficiency for the dipeptide and free forms of phenylalanine when they supplemented in diets for red sea bream *Pagrus major*. Furthermore, Tesser et al. (2005) found that juvenile South American pacu *Piaractus mesopotamicus* can utilize dipeptide arginine for growth with similar efficiency. Moreover, the results of a study on koi carp *Cyprinus carpio* revealed no difference in growth performance between free and dipeptide AA (Kwasek et al., 2010). Absorption of free AA is faster than that of protein-bound AA, and may result in AA imbalances and subsequent low protein utilization (Rønnestad et al., 2000). Moreover, an excess of di- or tripeptides can be similarly problematic due to the saturation of their transport mechanisms (Verri et al., 2003) or their instant hydrolysis to free AA (Carvalho et al., 2004). However, better absorption of single peptides compared with an equivalent mixture of free AA has been reported in rainbow trout (Boge et al., 1981). It has also been reported that the substitution of protein-bound AA by di- and tripeptides at rates up to 20% enhanced the growth performance of European sea bass larvae, whereas higher substitution levels decreased growth (ZamboninoInfante et al., 1997).

Composition of the basal diet is an important factor influencing the results of dipeptide AA supplementation. In this study, the utilization efficiencies of the two lysine forms were examined by supplementing them into a free AA-based basal diet, whereas previous studies have supplemented whole-protein based diets (Tesser et al., 2005; Kwasek et al., 2012). The differences in AA availability from free AA and whole-protein-based diets may at least partially explain the observed variations. Moreover, it has been suggested that the use of different dipeptides can profoundly affect absorption characteristics by switching the AA 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 are

required to clarify the mechanism by which dipeptides mediate specific peptide transporters in the intestinal brush border epithelium.

We showed that both lysine level and form affected the weight gain of olive flounder. Kim and Lee (2013) reported that the molecular form of leucine, and not its level, was the main factor in olive flounder growth performance. This variation may be due to differences in fish size, feeding period, dietary requirements, and experimental conditions.

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

The whole-body AA profile has been used as a standard indicator of fish AA requirements (Wilson and Cowey, 1985; Bicudo and Cyrino, 2009). Several studies have shown that whole-body essential AA profiles provide a good estimate of the optimum essential AA profile in fish diets (Akiyama et al., 1997; Green and Hardy, 2002). Aragão et al. (2004) showed that juvenile Senegal sole *Solea senegalensis* exhibited improved general AA deposition in the body when they were offered a diet containing dipeptide AA. Our results showed a significant effect of dietary treatments on whole-body AA composition. We found higher whole-body accumulation of arginine when fish were provided LG, and detected a significant interaction between lysine level and form. A similar tendency was observed for alanine concentration, where higher values were obtained for LG fed groups. In line with 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 a free AA-based diet compared with those fed a 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 AA composition when the fish were provided with free or dipeptide forms of AA. Dabrowski et al. (2005) proposed that the differences in muscle free AA composition in fish offered different dietary AA sources are due to differences in the absorption rates for free and dipeptide AA, leading to uneven accumulation rates of muscle free AA, ultimately resulting in different metabolic rates and AA availability for protein synthesis. Our findings show that AA availability could improve in fish fed with dipeptide forms vs. those fed free forms.

In conclusion, juvenile olive flounder can utilize lysine more efficiently for growth if it is provided in dipeptide form, and the dipeptide form can improve AA retention in fish. Overall, we provide further evidence for the notion that previ-



ously published data on AA requirements using free AA are likely to be overestimated and need to be reevaluated using dipeptides as the AA source.

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