Borneo Journal of Marine Science and Aquaculture
Volume: 01 | December 2017, 16 - 24

Dietary leucine requirement for growth and maintenance, and its utilization efficiency for fingerling of Nile tilapia, *Oreochromis niloticus* (Linnaeus)

Seemab Zehra and Mukhtar A. Khan*

Fish Nutrition Research Laboratory, Department of Zoology, Aligarh Muslim University, 202 002 Aligarh, India

*Corresponding author: khanmukhtar@yahoo.com

Abstract

Two separate 12-week feeding trials were conducted to investigate the leucine requirement for maintenance and its utilization efficiency for growth of fingerlings of *Oreochromis niloticus*. In the first experiment, six diets (35% CP; 13.71 kJ g^{-1} DE) containing graded levels of leucine (0.71, 0.95, 1.26, 1.49, 1.72 and 1.95% dry diet) were fed to the fish (3.74±0.11 cm, 0.65±0.05 g) near to satiation to determine its leucine requirement. Highest performance in terms of absolute weight gain (AWG, 5.83 g $fish^{-1}$), protein gain (PG, 1.02 g $fish^{-1}$), leucine gain (LG, 82.19 mg $fish^{-1}$), RNA/DNA ratio (4.74) and best feed conversion ratio (FCR, 1.51) were recorded at 1.49% dietary leucine. Quadratic analysis of AWG, PG, LG against varying levels of leucine indicated the requirement at 1.56, 1.61 and 1.57% dry diet, corresponding to 4.45, 4.60 and 4.48% dietary protein, respectively. In the second experiment, six experimental diets (35.0% CP; 13.8 kJg^{-1} DE) at 5, 25, 45, 65, 85 and 95% of optimum level of leucine determined in experiment I (1.58% dry diet) corresponding to 0.08, 0.39, 0.71, 1.03, 1.34 and 1.50 g of leucine $100g^{-1}$ of dry diet were given to the same sized fish under almost identical laboratory conditions to estimate the leucine maintenance requirement and its utilization efficiency which were calculated by solving the linear equations of protein gain and leucine gain data to leucine intake levels. The maintenance requirement based on protein gain and leucine gain calculated at Y = 0 amounted to 8.23 and 9.62 mg kg^{-1} $BW^{0.7}day^{-1}$. Extrapolating the linear equations of protein gain and leucine gain to the Y intercept showed that zero leucine intake resulted in a net daily loss of 91.9 mg whole body protein and 5.65 mg whole body leucine kg^{-1} $BW^{0.7}$. The maintenance need for leucine represented 6·07% of the total need for leucine. Slope of the leucine gain line showed that the efficiency of leucine utilization above the maintenance level is 58.7%.

Keywords: Leucine, Growth, Maintenance, *Oreochromis niloticus*

Introduction

A major constraint in aquaculture is the unavailability of nutritionally balanced feeds. Fish requires quality protein and nutritionally balanced feeds for maximum growth. Feed plays an important role in successful aquaculture as it amounts to 50- 60% of the total cost of fish production (Daniel, 2017). Dietary protein is the crucial and costliest constituent of fish feeds. It is usually given more attention as it is considered the main nutrient affecting fish growth (NRC, 2011; Zehra and Khan, 2012; Kpogue et al., 2013). Dietary proteins provide essential amino acids necessary for maintenance, growth and reproduction. Also, some amino acids get converted to glucose to supply energy to cells, tissues and body organs. Inadequate availability of essential amino acids may result in poor dietary protein utilization leading to reduced feed efficiency and growth.

Generation of data on essential amino acid requirements of fish and their optimum provision in feeds is crucial as these dietary ingredients play an important role in supporting growth, nutrient deposition and desirable carcass quality. Out of the ten essential amino acids, leucine participates in the synthesis of protein, insulin release, and hampers protein breakdown (Nair et al., 1992, Yan et al., 2010). It provides gluconeogenic precursors through the formation of alanine (Brooks, 1987). It is also involved in the maintenance of nitrogen balance, energy metabolism, blood glucose concentration, growth hormone production and haemoglobin concentration (Norton and Layman, 2006; Gan et al., 2016). Data on the dietary leucine requirements have been generated and published for several fish species including chinook salmon (*Oncorhynchus tshawytscha*), channel catfish (*Ictalurus punctatus*), rainbow trout (*O. mykiss*), Mossambique tilapia (*Oreochromis mossambicus*), white sturgeon (*Acipenser transmontanus)* , rohu (*Labeo rohita*), red sea bream (*Pagrus major*), European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), turbot (*Psetta maxima*), Atlantic salmon (*Salmo salar*), mrigal (*Cirrhinus mrigala*), and yellow croaker (*Pseudosciaena crocea*) (NRC, 2011), *Catla catla* (Zehra and Khan, 2015) and *Portunus trituberculatus* (Huo et al., 2017). Out of several species of tilapia widely farmed in many tropical and subtropical regions of the world, Nile tilapia (*Oreochromis niloticus*) is the predominantly cultured species due to its large size, rapid growth and palatability (Gan et al., 2016). Although data on leucine requirement of fingerling of *O. niloticus* is available (Gan et al., 2016), information on leucine requirement for maintenance has not yet been reported. Thus, the current study was carried out to evaluate the dietary leucine requirement for maintenance and its utilization efficiency for the fingerlings of this fish species.

Requirements of essential amino acids have generally been established through dose response studies. In dose-response experiments, growth of the fish measured against feeding graded levels of amino acid does not reveal its proportions used for maintenance and growth. Hence, a number of authors have suggested that partitioning indispensable amino acid requirements between maintenance and growth would give more insights into the metabolic use of these indispensable amino acids (Shearer, 1995; Fournier et al., 2002; He et al., 2013). For this reason, a factorial approach is being increasingly applied to estimate essential amino acids requirement (NRC, 2011) which is based on information on maintenance requirement and amino acid deposition for body protein and its utilization efficiency. Knowledge of maintenance requirements of utilizing indispensable amino acids for whole-body protein accretion and quantitative relationships between the accretion of protein and amino acids is essential in developing accurate models of amino acid requirements (Edwards et al., 1997). The intake of amino acid resulting in no net protein gain in the animal is assumed to be the maintenance requirement of animal (NRC, 2011). Requirements for maintenance for gain were first estimated for a particular diet by feeding different ration levels. This technique has been used for determining the energy and protein requirements of various fish species including Atlantic salmon (Hauler and Carter, 2001; Helland et al., 2010). For amino acids, instead of using different ration levels, these requirements have mostly been determined by feeding diets containing graded levels of the test amino acid from deficient to excessive (D'Mello, 2003; Moughan and Fuller, 2003; Hua, 2013). Understanding of the utilization efficiency of essential amino acids is necessary for evaluation of their requirements for fish (Kim et al., 2012). The paucity of information on the utilization efficiency of the essential amino acids and their requirements for maintenance is the major drawback in modeling the essential amino acid requirement studies (Kim et al., 2012). Maintenance requirements and utilization efficiencies of amino acids are mostly estimated by linear regression (NRC, 2011) and information on the maintenance needs of leucine and its utilization efficiency in pisciculture is scanty (Rodehutscord et al., 1997; Hua, 2013; NRC, 2011). Therefore, in the present study, data on leucine requirement for maintenance and its utilization efficiency was generated for the fingerlings of this fish species.

Methodology

Preparation of test diets

In experiment I, six amino acid test diets (L1, L2, L3, L4, L5 and L6) using casein (fat-free), gelatin and L-crystalline amino acids with varying levels of leucine (0.75, 1.0, 1.25, 1.5, 1.75 and 2.0% dry diet) were prepared to determine the leucine requirement of fingerlings of *O. niloticus*. The diets contained 35% crude protein and 13.71 kJ g^{-1} digestible energy. Casein and gelatin were used as intact protein sources and provided 0.75% leucine in the basal diet (L1, Table 1).

Table 1. Composition of the basal diet used in experiment I

Ingredients	g/100g dry diet
Casein (fat-free; crude protein - 76%)	7
Gelatin (crude protein - 96%)	2.33
Dextrin	31.63
Amino acid mixture*	28.56
Corn oil	5
Cod liver oil	2
Mineral mix**	4
Vitamin mix***	3
α- Cellulose	5.48
Chromic oxide	1
Carboxymethyl cellulose	10
Total	100
Analyzed crude protein	35.15
Analyzed crude lipid	6.98
Digestible energy≠ (kJ g-1, dry diet)	13.71
Digestibility of the basal diet (%)	97.3

* Amino acid mixture (% dry diet): arginine 1.798; histidine 0.54; isoleucine 2.38; leucine 0; lysine 1.854; methionine 1.19; cystine 0.812; phenylalanine 1.813; tyrosine 1.213; threonine 1.224; tryptophan 0.469; valine 2.042; alanine 1.567; aspartic acid 0.634; glutamic acid 0.773; proline 1.894; serine 0.473; glycine 7.881 (Loba Chemie, India). Halver (2002).

** Mineral mixture (g/100g of mineral mixture): calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 2.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride. $6H_2O$0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate. H_2O 0.080; cobalt chloride. $6H_2O$0.100; zinc sulphate. $7H_2O$ 0.40.

*** Vitamin mixture (g/100g dry diet): choline chloride 0.500; inositol 0.200; ascorbic acid 0.100;Ú niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; thiamin hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose.

≠ The physiological fuel values of 18.82, 14.64 and 35.55 kJ g^{-1} for protein, carbohydrate and fat were used to calculate digestible energy (Jauncey 1982).

The amino acid composition of the test diets was identical to that of 35% whole chicken egg protein excluding the test amino acid leucine. Incremental levels of crystalline leucine were added to basal diet (L1) at 0.25 (L2), 0.5 (L3), 0.75 (L4), 1.0 (L5) and 1.25% (L6) to make the intended concentrations of leucine in the amino acid test diets. The L-leucine contents of experimental diets were analyzed and found to be 0.71, 0.95, 1.26, 1.49, 1.72 and 1.95% of the dry diet. The levels of leucine in the amino acid test diets were taken on the basis of information available on other freshwater fishes (NRC, 2011, Zehra and Khan, 2015, Gan et al., 2016). Level of protein in the test diets was set based on the reported requirement of the fingerlings of this fish species (35%, Abdelghany, 2000). The protein content of experimental diets was kept constant by adjusting the levels of glycine. Dextrin was used to maintain the constant energy content of all the experimental diets. The amino acid composition of the basal diet was analyzed and is depicted in Table 2. Test diets were prepared as per the method of Zehra and Khan (2015).

In Experiment II, leucine maintenance requirement and its utilization efficiency for growth of fingerlings of *O. niloticus* were estimated. Six experimental diets (35% crude protein; 13.6 kJ/g digestible energy) at 5, 25, 45, 65, 85 and 95% of leucine requirement (1.58% dry diet) determined in experiment I were prepared by adding crystalline L-leucine at 0.08 (D1), 0.39 (D2), 0.71 (D3), 1.03 (D4), 1.34 (D5) and 1.50% of the dry diet (D6). A protein-free diet (PF), devoid of all amino acids was also fed to fish to measure the protein

Table 2. Analyzed amino acid profile of the basal diet used in experiment I

	Essential Amino Acids									
	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
Composition*	2.23	0.73	2.78	0.71	2.53	1.45	2.21	1.51	0.52	2.57
	Non-Essential Amino Acids									
	Cystine	Tyrosine	Alanine	Aspartic acid		Glutamic acid	Glycine	Proline	Serine	
Composition*	0.83	1.58	2.05	1.19		2.48	8.58	2.89	0.59	

*Analyzed amino acid composition of the basal diet (% dry diet)

and leucine losses in fingerlings of Nile tilapia. This protein-free diet contained optimum levels of vitamins, minerals and energy to prevent any energy limitation. The composition of the basal (D1) and protein-free (PF) diets used in experiment II is given in Table 3.

Table 3. Composition of protein free (PF) and basal diets (D1) used in experiment II

Ingredients	PF (g/100g dry diet)	D1 (g/100g dry diet)
Dextrin	75	31.56
Amino acid mixture*	0	37.19
Corn oil	5	5
Cod liver oil	2	2
Mineral mix**	4	4
Vitamin mix***	3	3
α- Cellulose	0	6.25
Chromic oxide	1	1
Carboxymethyl cellulose	10	10
Total	100	100
Digestible energy≠ (kJ/g, dry diet)	13.46	13.86
Digestibility of the basal diet (%)	-	96.2
Analyzed crude protein	-	32.96
Analyzed crude lipid	7.01	6.95

* Amino acid mixture (% dry diet): arginine 2.24; histidine 0.740; isoleucine 2.801; leucine 0.06; lysine 2.521; methionine 1.44; cystine 0.84; phenylalanine 2.205; tyrosine 1.575; threonine 1.513; tryptophan 0.525; valine 2.555; alanine 2.015; aspartic acid 1.206; glutamic acid 2.476; proline 2.94; serine 0.601; glycine 8.94; (Loba Chemie, India).Halver (2002).

** Mineral mixture (g/100g of mineral mixture): calcium biphosphate 13.57; calcium lactate 32.69; ferric citrate 02.97; magnesium sulphate 13.20; potassium phosphate (dibasic) 23.98; sodium biphosphate 08.72; sodium chloride 04.35; aluminium chloride $6H_2O$0.0154; potassium iodide 0.015; cuprous chloride 0.010; manganous sulphate H_2O 0.080; cobalt chloride $6H_2O$ 0.100; zinc sulphate $7H_2O$ 0.40

*** Vitamin mixture (g/100g dry diet) choline chloride 0.500; inositol 0.200; ascorbic acid 0.100; niacin 0.075; calcium pantothenate 0.05; riboflavin 0.02; menadione 0.004; pyridoxine hydrochloride 0.005; folic acid 0.0015; biotin 0.0005; alpha-tocopherol 0.04; vitamin B_{12} 0.00001; 2 g α-cellulose.

≠ The physiological fuel values of 18.82, 14.64 and 35.55 kJ g^{-1} for protein, carbohydrate and fat were used to calculate digestible energy (Jauncey 1982).

Amino acid analysis of experimental diets revealed the L-leucine content to be 0.08, 0.37, 0.70, 1.05, 1.32 and 1.47% of the dry diet. The analyzed amino acid composition of the experimental diet (D1) is given in Table 4. The crystalline L-amino acids were coated with carboxymethyl cellulose to provide sufficient water stability which was checked and found to be 95%.

Fish maintenance and husbandry

Fingerlings of *O. niloticus* were obtained from fish farm, Naihaty, North 24 Parganas, West Bengal and were acclimatized in indoor cylindrical aqua-blue coloured (Plastic Crafts Corp, Mumbai, India) fish tanks (1.22m in diameter, 0.91m in height; water volume 600 L) for two weeks. During this period, the fish were fed basal diet (L1). The fingerlings (3.75±0.15 cm, 0.66±0.04 g) were taken from the above acclimated fish lot and shifted to 70 L circular polyvinyl tanks (water volume 55 L). A continuous water flow-through system was maintained at 1-1.5 L min^{-1}. Fish were stocked in triplicate groups at the rate of 25 fish per tank for each dietary treatment level. The test diets in the form of dry crumbles were fed to fish thrice daily at 08:00, 12:30 and 17:30h to satiety. The initial and final weights of fish were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with tricaine methane sulfonate (MS-222). Feed was not offered to fish on the day they were weighed. The length of the experiment was 12 weeks. Water quality parameters were estimated on a daily basis according to APHA (1992). The water temperature was recorded in the range of 27.1-28.1ºC. Dissolved oxygen varied from 6.2-7.2 mg L^{-1}. Free carbon dioxide, pH, total ammonia nitrogen, nitrites and total alkalinity were in the range of 5.7-9.3 mg L^{-1}, 7.3-7.5, 0.29-0.32 mg L^{-1}, 0.04-0.08 mg $L^{-1}$and 72.9-81.3mgL^{-1}, respectively.

Biochemical analyses

Proximate composition of experimental diets, and that of initial and final carcass was analyzed using standard methods of AOAC (1995) as detailed earlier (Zehra and Khan, 2013). Amino acid analyses of casein, gelatin, experimental diets, and initial and final carcass were done by hydrolyzing 0.3 mg sample in 1 ml of 6 N HCl for about 22 h. The sample thus obtained was diluted in 0.02 N HCl and injected in an automatic amino acid analyzer (Hitachi L-8800, Tokyo, Japan). Recovery hydrolysis was performed in 4 N methanesulfonic acid for the analysis of tryptophan and in performic acid for the recovery of sulphur amino acids. Chromium oxide was included in the test diets as an inert marker for digestibility determinations. The test diets were taken without difficulty and fish quickly consumed the feed offered. Faeces were collected daily 2 hr after each feeding through pipetting using Pasteur pipette. Faecal collection continued until it was judged that sufficient sample had been collected for chemical analysis. Collected faecal matter was rinsed gently in distilled water for a few seconds to separate other materials such as dirt particles (Zhang et al., 2010). Care was taken to prevent breaking up of faecal strands while pipetting and rinsing in order to avoid the possible loss of nutrients from the faeces. Feacal samples from each tank were immediately frozen and stored at -20°C until analyzed. Diets and feacal samples were analyzed in triplicate for chromic oxide following the method of Bolin et al. (1952) after perchloric acid digestion. Digestibility of

Table 4. Analyzed amino acid profile of the basal diet used in experiment II*

	Essential Amino Acids									
	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
Composition*	2.21	0.74	2.76	0.06	2.54	1.42	2.19	1.53	0.54	2.54
	Non-Essential Amino Acids									
	Cystine	Tyrosine	Alanine	Aspartic acid		Glutamic acid	Glycine	Proline	Serine	
Composition*	0.81	1.56	2.08	1.21		2.45	8.96	2.87	0.57	

* Analyzed by Hitachi L-8800 Automatic Amino Acid Analyzer

protein-free diet has not been determined because of inability of collecting faeces. At the start of the experiments, 60 fish were randomly sampled, sacrificed and pooled. Six subsamples of a pooled sample were analyzed for initial carcass composition. At the end of the experiments, 20 fish specimens from each replicate of dietary treatments were randomly sacrificed with an overdose of the MS-222 and pooled individually. Three subsamples of the pooled samples were analyzed for final carcass composition. Towards the termination of the experiment, five fish from each replicate of the treatment group were randomly sacrificed and muscle was removed and pooled. Three subsamples of the pooled muscle samples for each replicate of the treatment group were used for the determination of RNA and DNA by using the methods of Schneider (1957) and Ashwell (1957) as adopted by Mustafa and Mittal (1982).

Assessment of performance of fish

Growth performance of fish fed test diets was measured by calculating following parameters:

Metabolic body weight (MBW)	=	[(Initial mean body weight/1000)$^{0.7}$ + (Final mean body weight/ 1000)$^{0.7}$]/2
Feed conversion ratio (FCR)	=	Dry feed intake/Wet weight gain
Feed efficiency (FE)	=	Wet weight gain/Dry feed intake
Leucine intake (mg kg^{-1} BW$^{-0.7}$day^{-1})	=	Total dry feed fed (g fish^{-1}) x dietary leucine content/Metabolic body weight (kg BW$^{0.7}$)/Total no. of experimental days
Protein gain (PG, g fish^{-1})	=	Final body protein x final body weight-initial body protein x initial body weight
Leucine gain (LG, mg fish^{-1})	=	Final body leucine content x final body weight-initial body leucine content x initial body weight x 1000
Apparent total digestibility of the basal diet (%)	=	100-[100×(% marker in feed/% marker in feces)]
Apparent leucine digestibility (%)	=	100-[100×(% marker in feed/% marker in feces) x (% leucine in feces/%leucine in feed)]

The growth data were subjected to one-way analysis of variance (Sokal and Rohlf, 1981). Differences among treatment means were determined by Tukey's honestly significant difference (HSD) test at a $P<0.05$ level of significance. Quadratic regression model was used to estimate the dietary leucine requirement of the fingerlings (Shearer, 2000). The quadratic equation employed was $Y=aX^2+bX+c$. Maintenance requirement for leucine was calculated by the linear regressing model of protein and leucine gain versus leucine intake levels where y-axis (PG and LG) is zero (NRC, 2011). The linear equation employed was $Y=aX+b$. Data were scaled using the metabolic body weight ($MBW^{0.7}$) exponents of 0.7 for protein gain, leucine gain and leucine intake data (Helland et al., 2011). Statistical software Origin (version 6.1; Origin Software, San Clemente, CA) was used for analyses.

Results

Experiment I: Leucine requirement for growth of fingerlings of O. niloticus

AWG, FCR, PG, LG and RNA/DNA ratio of fingerlings of *O. niloticus* were significantly affected as the amounts of dietary leucine increased up to 1.49% as shown in Table 5. The leucine requirement of the fingerlings attained by quadratic regression analysis of AWG exhibited the requirement at 1.56% of dry diet (Figure 1). On subjecting PG to quadratic analysis, leucine requirement was reflected at 1.61% dry diet (Figure 2). The quadratic response of LG data versus dietary leucine levels has shown the requirement at 1.57% dry diet (Figure 3). Survival was not affected and was 100% in all the treatments.

Review of data on carcass composition of fingerlings of *O. niloticus* fed different quantities of leucine in the present study clearly indicated marked changes in protein, fat and moisture contents except for carcass ash which remained almost stable in all the treatments (Table 6). A positive association of carcass protein to that of dietary leucine levels was achieved up to 1.49% (L4). Carcass fat showed positive association with the increasing levels of dietary leucine from 0.71 (L1) to 1.95% (L6). However, a reverse pattern of moisture content to that of carcass fat was recorded.

Table 5. Growth performance of fingerlings of *O. niloticus* fed diets with different quantities of leucine in experiment I*

	Analyzed dietary leucine levels (% dry diet)					
	0.71 (L1)	***0.95 (L2)***	***1.26 (L3)***	***1.49 (L4)***	***1.72 (L5)***	***1.95 (L6)***
Average initial weight (g)	0.66±0.01 [a]	0.65±0.03[a]	0.66±0.03 [a]	0.65±0.02 [a]	0.66±0.04 [a]	0.66±0.11 [a]
Average final weight (g)	2.87±0.05[e]	4.11±0.02[d]	5.22±0.06[c]	6.48±0.02[a]	5.55±0.07[b]	5.34±0.02[bc]
AWG (g fish^{-1})	2.21±0.02[e]	3.45±0.02[d]	4.56±0.04[c]	5.83±0.05[a]	4.89±0.04[b]	4.67±0.03[bc]
FI (g fish^{-1})	7.69±0.06[b]	8.65±0.11[a]	8.98±0.06[a]	8.80±0.09[a]	8.16±0.05[a]	8.03±0.12[a]
FCR	3.48±0.05[a]	2.51±0.02[b]	1.97±0.01[c]	1.51±0.02[e]	1.67±0.05[d]	1.72±0.02[d]
PG (g fish^{-1})	0.27±0.02[e]	0.50±0.01[d]	0.74±0.03[c]	1.02±0.04[a]	0.86±0.02[b]	0.83±0.05[b]
LG (mg fish^{-1})	42.85±0.94[f]	61.17±1.17[e]	76.28±1.64[d]	82.19±1.71[a]	79.42±1.52[b]	75.11±1.69[c]
RNA/DNA ratio	2.39±0.03[f]	3.56±0.07[e]	3.92±0.06[d]	4.74±0.08[a]	4.51±0.05[b]	4.37±0.02[c]

*Mean values of 3 replicates ± SEM. Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

Table 6. Carcass composition (%wet basis) of fingerlings *O. niloticus* fed diets with different quantities of leucine in experiment I*

	Analyzed dietary leucine levels (% dry diet)						
	Initial	***0.71 (L1)***	***0.95 (L2)***	***1.26 (L3)***	***1.49 (L4)***	***1.72 (L5)***	***1.95 (L6)***
Moisture	77.21±0.48	78.13±0.49[a]	77.11±0.72[b]	76.58±0.61[c]	75.26± 0.52[d]	74.11± 0.39[e]	73.15± 0.47[f]
Protein	12.48±0.13	12.24±0.04[d]	14.26±0.09[c]	15.71±0.07[b]	16.92±0.12[a]	16.89±0.08[a]	17.11±0.15[a]
Fat	3.48± 0.11	3.17± 0.04[f]	3.48± 0.07[e]	3.81± 0.05[d]	4.47± 0.07[c]	4.82± 0.02[b]	5.38± 0.06[a]
Ash	2.51± 0.04	2.46±0.04[a]	2.49± 0.03[a]	2.41±0.05[a]	2.43± 0.03[a]	2.47± 0.02[a]	2.46± 0.03[a]

*Mean values of 3 replicates ± SEM. Mean values sharing the same superscripts in the same row are insignificantly different (P>0.05).

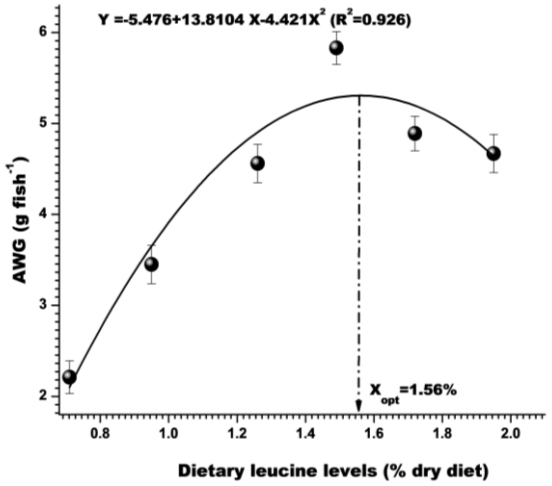

Figure 1. Quadratic regression analysis of absolute weight gain to different levels of dietary leucine

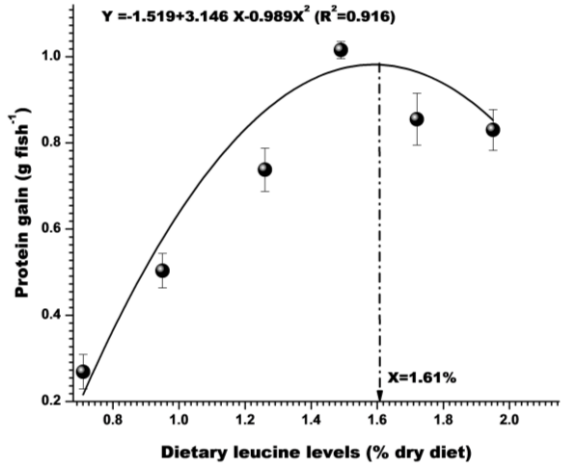

Figure 2. Quadratic regression analysis of protein gain to different levels of dietary leucine

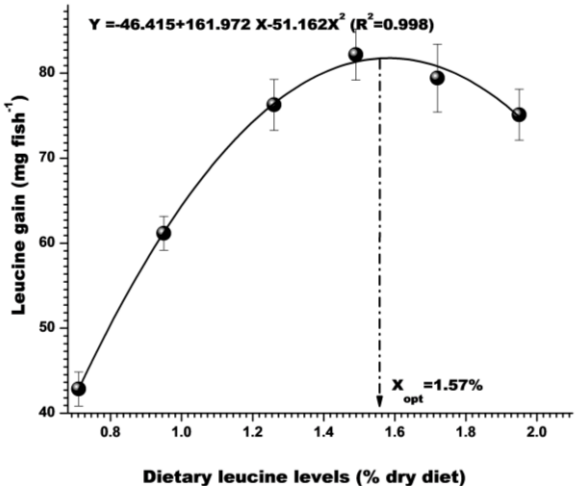

Figure 3. Quadratic regression analysis of leucine gain to different levels of dietary leucine

Experiment II: Leucine requirement for maintenance and its efficiency of utilization

Absolute weight gain (g fish^{-1}), feed efficiency, protein gain (g kg^{-1} BW$^{0.7}$day^{-1}) and leucine gain (mg kg^{-1} BW$^{0.7}$day^{-1}) increased linearly with the increasing levels of dietary leucine as described in Table 7. Leucine intake increased linearly with the increasing concentrations of dietary leucine but feed intake (g fish^{-1}) increased only up to 65% (D4) of the ideal leucine level (1.58%), after which it tended to plateau (Table 7). Fish fed protein-free diet showed reduction (negative value) in weight gain. Protein gain and leucine gain data were subjected to linear regression analysis against digestible leucine intake levels (mg kg^{-1}BW$^{0.7}$day-^{1}) as an independent variable to work out the leucine maintenance requirement of *O. niloticus*. The linear equations based on metabolic body weight (kg BW$^{0.7}$) for PG (Figure 4) and LG (Figure 5) against digestible leucine intake levels were Y=-0.0919+0.01116X (R^2=0.965) and Y=-5.65+0.587X (R^2=0.971), respectively.

Table 7. Growth performance of fingerlings of *O. niloticus* fed diets at 5, 25, 45, 65, 85 and 95% of optimum level of leucine to estimate leucine maintenance requirement and its utilization efficiency*

	Analyzed dietary leucine levels (% dry diet)						
		0.08 (D1)	0.37 (D2)	0.70 (D3)	1.05 (D4)	1.32 (D5)	1.47 (D6)
	Digestible leucine intake levels (mg kg BW$^{-0.7}$ day^{-1})						
	PF	5.34 (D1)	28.84 (D2)	48.62 (D3)	71.07 (D4)	72.59 (D5)	76.67 (D6)
Average Initial weight (g)	0.66±0.02 a	0.66±0.02a	0.66±0.03 a	0.66±0.02 a	0.66±0.08 a	0.66±0.11 a	0.65±0.05 a
Average Final weight (g)	0.31±0.02 g	0.85±0.05f	2.29±0.02 e	3.97±0.06 d	5.24±0.02 c	6.81±0.07b	7.55±0.04 a
MBW (kg BW$^{0.7}$) **	0.005	0.007	0.01	0.013	0.015	0.018	0.019
AWG (g fish^{-1}) ¶	-0.35±0.01 g	0.19±0.02f	1.63±0.03 e	3.31±0.05 d	4.58±0.07 c	6.15±0.07 b	6.91±0.06 a
FE	-0.63±0.07 g	0.044±0.0 f	0.23±0.02 e	0.32±0.01 d	0.50±0.02 c	0.66±0.05b	0.75±0.02a
FI (g fish^{-1})	0.51±0.09 e	4.27±1.13 d	7.08±1.13 c	8.21±1.13 b	9.24±1.13 a	9.24±1.13 a	9.25±1.13a
LI (mg kg^{-1}MBW$^{-0.7}$day^{-1})	0	5.81±1.13 f	31.18±1.18 e	52.63±1 d	77.01±1 c	80.66±1 b	85.19±0.9a
PG (g kg MBW$^{-0.7}$day^{-1}) ***	-0.099±0.01g	-0.03±0.01 f	0.17±0.02 e	0.43±0.02 d	0.60±0.01 c	0.75±0.02 b	0.86±0.01a
LG (mg kg MBW$^{-0.7}$day^{-1})≠	-6.91±0.09 g	-1.01±0.09 f	10.14±0.08 e	18.22±0.08 d	32.87±0.0c	39.62±0.1b	42.70±0.1a
Digestibility of leucine (%)	-	92.1±1.25	92.5±1.21	92.4±0.98	92.3±1.35	90.1±1.24	90.3±0.99

* Mean values of 3 replicates ± SEM. Mean values sharing the same superscripts in the same row are not significantly different (P>0.05).
** Metabolic body weight (kg BW$^{0.7}$)= [(Initial mean body weight/1000)$^{0.7}$+(Final mean body weight/1000)$^{0.7}$]/2.
***Protein gain (g kg BW$^{-0.7}$day^{-1})= Final body protein x final body weight–initial body protein x initial body weight/Metabolic body weight (kg BW$^{0.7}$)/Total no. of experimental days.
≠ Leucine gain (mg kgBW$^{-0.7}$day^{-1})= Final body leucine content x final body weight–initial body leucine content x initial body weight/Metabolic body weight (kg BW$^{0.7}$)/Total no. of experimental days x 1000.

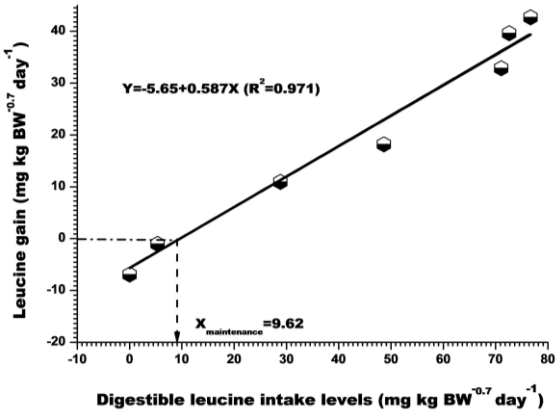

Figure 4. Linear relationship of leucine gain to digestible leucine intake levels.

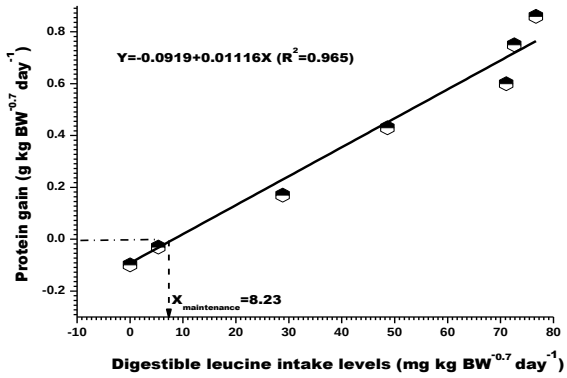

Figure 5. Linear relationship of protein gain to digestible leucine intake levels.

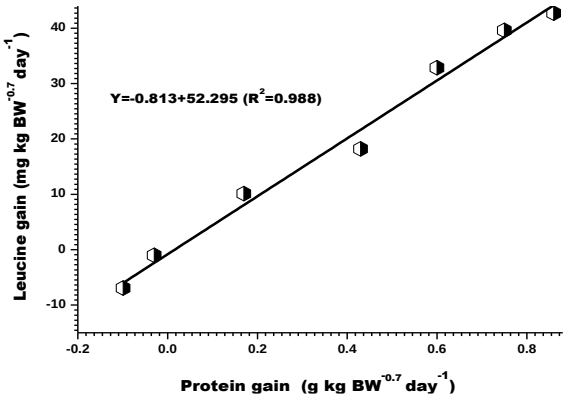

Figure 6. Linear relationship of protein gain to leucine gain.

The maintenance requirement for PG and LG were found to be 8.23 and 9.62 mg kg^{-1} BW$^{0.7}$day^{-1}. Slope of the line representing leucine gain showed that the efficiency of leucine utilization above maintenance was 58.7%. Extrapolating the linear equations of protein gain and leucine gain to the Y intercept showed that zero leucine intakes resulted in a net daily loss of 91.9 mg whole body protein and 5.65 mg whole body leucine per kg MBW. These values are not significantly different compared with the losses (6.91 mg whole-body leucine and 99 mg whole body protein per kg MBW) observed in fingerlings of Nile tilapia fed the PF diet. The inverse value of the slope from the best-fit linear regression equations of protein gain data versus leucine intake levels indicated that 89.61 mg leucine intake is required for 1 g protein deposition in the fingerlings. The equation obtained by linear regression analysis of protein gain (X axis) versus leucine gain (Y axis) was Y=-0.813+52.295 X (R^2=0.988) as illustrated in Figure 6. Thus,

each 1 g increase in protein gain resulted in an increase of 52.29 mg of leucine, indicating that leucine concentration in the whole body protein gain was constant (5.23%) at all levels of leucine intake. The linear equation of protein gain to leucine gain showed that zero protein gain resulted in negative value of leucine gain (-0.813), suggesting a higher maintenance leucine requirement for zero leucine gain than for zero protein gain.

Discussion

The average leucine requirement of fingerling of *O. niloticus* determined at 1.58% dry diet, equivalent to 4.51% dietary protein is higher than the requirement reported for other fish species including *Cyprinus carpio,* 3.3% (Nose, 1979), *O. kisutch,* 3.4% (Arai and Ogata, 1993), *A. transmontanus,* 4.3% (Ng and Hung, 1995),red seabream, 4.2%, *C. major,* 3.9% (Forster and Ogata, 1998), *O. mykiss,* 4.4% (Kaushik, 1998), *C. mrigala,* 3.9% (Ahmed and Khan, 2006), *I. punctatus,* 4.5%; *C. carpio,* 4.4% (NRC, 2011), *O. niloticus,* 4.31% (Gan et al., 2016); lower than the requirement of *S. salar,* 5.2% (Rollin, 1999); *L. crocea,* 6.8% (Yan et al., 2010) and almost similar to the requirement reported for *L. rohita,* 4.7% (NRC, 2011). The variations in the amino acid requirements may also be due to differences in fish age, size, flow rate, stocking density, environmental conditions, feeding levels and general culture conditions (NRC, 2011; Zehra and Khan, 2015). Dietary metabolizable energy, availability of dietary amino acids, and antagonism and imbalance among amino acids may also be responsible for the wide variations in amino acid requirements (Ishibashi and Ohta, 1999). Variation in the leucine requirement in the above studies may be due to the differences in size and age of the fish.

In this study, decline in growth parameters of fish beyond 149% dietary leucine may be due to amino acid toxicity or dietary amino acid imbalance as evident in earlier studies (Benakappa and Varghese, 2003; Ahmed and Khan, 2006; Yan et al., 2010, Gan et al., 2016). The RNA/DNA ratio has been considered as a sensitive indicator of nutritional condition in several fish species (Mustafa, 1977; Mustafa and Jafri, 1977; Mustafa and Mittal, 1982; Mustafa and Zofair, 1985; Bulow, 1987; Mustafa et al., 1991; Buckley et al., 1999; Zehra and Khan, 2015, 2017). In this study, maximum value of RNA/DNA ratio obtained in fish fed 1.49% dietary leucine indicates that this level might be optimum to maximize protein synthesis in fingerlings of *O. niloticus.* Similarly, carcass protein and protein gain improved up to 1.49% of leucine of the dry diet indicating better dietary amino acid balance leading to maximum protein gain at the above level of dietary leucine.

Another important aspect of refining the amino acid requirement is determining the maintenance requirement and utilization efficiency of amino acids. In the experiment II of this study, the estimate of maintenance requirement for leucine was found to be 8.92 mg kg^{-1} $BW^{0.7}$/day. Rodehutscord et al. (1997) have reported considerably high maintenance requirements (82.6 mg leucine kg^{-1} day^{-1}) for leucine in rainbow trout. However, the maintenance requirement for leucine by Hua (2013) was estimated to be 9.1 mg kg^{-1} $BW^{0.75}day^{-1}$ which is comparable to the maintenance requirement obtained in the present study. Helland et al. (2010) have reported that Atlantic salmon required 20.9 mg kg^{-1} $BW^{0.7}day^{-1}$ leucine for maintenance. These estimates indicate that maintenance requirements cannot be assumed equal as it may be species specific.

In this study, the whole-body leucine loss in fish fed protein-free diet was 6.91 mg kg^{-1} BW day^{-1}. This value is lower than the reported value of leucine loss (14.9 mg kg^{-1} BW day^{-1}) in Atlantic salmon (Helland et al., 2010). The exact value for the efficiency term is crucial to the application of the factorial method for the estimation of amino acid requirements (Fuller, 1994). There are contradictory findings about the efficiency of amino acid utilization, i.e., whether it decreases or remains constant as amino acid intake above maintenance increases (Fisher et al., 1973; Heger and Frydrych, 1985; Baker, 1991; Fuller and Garthwaite, 1993; Gahl et al., 1996; Rodehutscord and Pack, 1999; Baker, 2003). In the present study, the efficiency of leucine above maintenance was 58.7% and found to be constant at all levels of leucine intake between 5% and 95% of its requirement (1.58%). Thus, leucine utilization showed no evidence of declining efficiency probably due to the fact that leucine intake even at the highest level (95% of optimum) was lower than the overall needs of Nile tilapia fingerlings. Similar findings of constant efficiency have also been reported by other studies such as on valine, 73% (Baker et al., 1996); threonine, 82% (Edwards et al., 1997); threonine, 76% (Rollin et al., 2006); threonine, 77% (Abboudi et al., 2007) and arginine, 49%; histidine, 65%; isoleucine, 62%; leucine, 58%; lysine, 62%; methionine, 53%; phenylalanine, 59%; threonine, 60%; tryptophan, 45%; valine, 58% (Helland et al., 2010). These estimates indicate that indispensable amino acids have different efficiencies of utilization probably because of different turnover rates for the amino acids in body tissue pools (Edwards et al., 1997).

The inverse value of the slope from the best-fit linear regression equations of protein gain data versus leucine intake levels indicated that 89.61 mg leucine intake is required for each 1 g protein deposition in the fingerling of Nile tilapia. Each 1 g increase in protein gain resulted in an increase of 52.29 mg of leucine as depicted by the linear regression of leucine gain to protein gain data. Thus, leucine utilization efficiency was found to be 58.4% (52.29/89.61 x 100). This value is in agreement with the 58.7% leucine utilization efficiency estimated from linear regression of leucine gain data versus digestible leucine intake levels (Figure 6).

Graded dosing below the requirement of an indispensable amino acid in growing animals does not result in a straight-line response in feed intake (Han and Baker, 1991; 1993; Hahn et al., 1995; Baker et al., 1996; Rollin et al., 2006). In the second experiment of this study, feed intake showed a linear increase up to 65% of the ideal level of dietary leucine requirement for maximal growth, after which

it remained almost constant (Table 7). This may probably be due to the fact that metabolizable energy needs to support maintenance and protein gain at this level. Beyond this level, gain in body weight and body protein result only from increasing concentrations of the dietary leucine. Below 65% of the ideal leucine level, increased leucine intake resulted from both increased feed intake and increased leucine concentrations in the diet. In spite of everything, protein and leucine gain increased linearly both below and above the 65% of ideal leucine level. Hence, the fish deposited both protein and leucine in response to how much leucine was consumed, irrespective of what caused the leucine consumption to increase.

In conclusion, the efficiency of leucine utilization and maintenance requirement for leucine in experiment II was estimated to be 58.4% and 8.92 mg kg^{-1} $BW^{0.7}$ day^{-1}, respectively and total requirement for dietary leucine determined in experiment I was found to be 1·58% (146.95 mg kg^{-1} $BW^{0.7}$ day^{-1}) of diet representing 6·07% maintenance need of the total dietary leucine requirement for Nile tilapia fingerlings. The information generated during this study would be helpful in formulating leucine balanced diet for the intensive culture of this fish species.

Acknowledgments

The authors are thankful to the Chairman, Department of Zoology, A. M. U., Aligarh, India for providing necessary laboratory facilities. We also gratefully acknowledge the financial assistance of University Grant Commissions awarded to one of us (Seemab Zehra) and generous funding received under DST-FIST Programme, New Delhi, in the priority area of Fish Nutrition.

References

Abboudi, T., Ooghe, W., Larondelle, Y. et al. (2007). Determination of the threonine requirement for maintenance in Atlantic salmon (*Salmo salar* L.) fry with the diet dilution procedure. **Aquaculture Nutrition** 13, 281-290.

Abdelghany, A.E. (2000). Tilapia aquaculture in the 21st century. In, **Optimum dietary protein requirements for *Oreochromis niloticus* L. fry using formulated semi-purified diets.** K. Fitzsimmons & J.C. Filho (eds.). Proc. from the 5th International Symposium on Tilapia in Aquaculture. Rio de Janeiro, Brasil, 3-7 September 2000. pp. 101-108.

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, 50-460.

AOAC. (1995). **Association of Official Analytical Chemists**, Official Methods of Analysis.16th ed. (Patricia Cuniff, editors). Arlington, V.A

APHA. (1992). Standard methods for the examination of water and wastewater, **American Public Health Association (1992)** 18th ed. 1268, pp. Washington DC, APHA

Arai, S. & Ogata, H. (1993). Quantitative amino acid requirements of fingerling *Coho salmon*. In, **Proceedings of the Twentieth U.S. Japan Symposium on Aquaculture Nutrition,** UJNR (ed. By Collie, M.R. and McVey, J.P.), pp. 19-28. Department of Commerce, Newport, OR

Ashwell, G. (1957). Colorimetric analysis of sugars: cysterine reaction of DNA. In, **Methods of enzymology.** Calowick S.P. & Kaplon N.O. (eds.). Academic press, Vol. 3, New-York, New York

Baker, D.H. (2003). Ideal amino acid patterns for broiler chicks. Pages 223-235 in JPF D'Mello, editor. **Amino Acid in Animal Nutrition**, 2nd ed., Wallingford, UK, CAB International

Baker, D.H., Fernandez, S.R., Parsons, C.M. et al. (1996). Maintenance requirement for valine and efficiency of its use above maintenance for accretion of whole-body valine and protein in young chicks**. Journal of Nutrition** 126, 1844-1851.

Benakappa, S. & Varghese, T.J. (2003). Isoleucine, leucine and valine requirement of juvenile Indian major carp, *Cirrhinus cirrhosus* (Bloch, 1975). **Acta Ichthyologica et Piscatoria** 33, 161-172.

Bolin, D.W., King, R.P. & Klosterman, E.W. (1952). A simplified method for the determination of chromic oxide Cr_2O_3 when used as an index substance. **Science** 116, 634-635.

Brooks, G.A. (1987). Amino acid and protein metabolism during exercise and recovery. **Medicine and Science in Sports and Exercise** 19, S150-S156.

Buckley, L.J., Caldarone, E. & Ong, T.L. (1999). RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. **Hydrobiologia** 401, 265-277.

Bulow, F.J. (1987). The age and growth of fish. In, **RNA-DNA ratio as indicators of growth in fish, a review**, R.C. Summerfelt and G.E. Hall (eds.), pp. 45-64. Iowa State University Press, Ames, Iowa

D'Mello, JPF. (2003). Amino acids in animal nutrition, 2nd edn. In**, Responses of growing poultry to amino acids**. J.P.F. D'Mello editor., Pages 237-263 CAB International, Wallingford, Oxon, UK

Daniel, N. (2017). Status of aquaculture with respect to nutrition and feed. **International Journal of Fisheries and Aquatic Studies** 5, 333-345.

Edwards, H.M. III, Baker, D.H., Fernandez, S.R. et al. (1997). Maintenance threonine requirement and efficiency of its use for accretion of whole-body threonine and protein in young chicks. **British Journal of Nutrition** 78, 111-119.

Fisher, C., Morris T.R., Jennings, R.C. (1973). A model for the description and prediction of the response of laying hens to amino acid intake. **British Poultry Science** 14, 469-484.

Forster, I. & Ogata, H.Y. (1998). Lysine requirement of juvenile Japanese flounder *Paralichthys olivaceus* and juvenile red sea bream *Pagrus major*. **Aquaculture** 161, 131-142.

Fournier, V., Gouillou-Coustans, M.F., Metailer, R. et al. (2002). Protein and arginine requirements for maintenance and nitrogen gain in four teleosts. **British Journal of Nutrition**, 87, 459–469.

Fuller, M.F. & Garthwaite, P. (1993). The form of response of body protein accretion to dietary amino acid supply. **Journal of Nutrition** 123, 957-963.

Fuller, M.F. (1994). Amino Acids in Farm Animal Nutrition. In, **Amino acid requirements for maintenance, body protein accretion and reproduction.** (JPF D'Mello, ed). Pages 155-184. Wallingford, Oxon, CAB International

Gahl, M., Finke, M.D. & Crenshaw, T.D. (1996). Efficiency of lysine or threonine retention in growing rats fed diets limiting in either lysine or threonine. **Journal of Nutrition** 126, 3090-3099.

Gan, L., Zhou, L. L., Li & Yue, Y. R. (2016). Dietary leucine requirement of Juvenile Nile tilapia, *Oreochromis niloticus*. **Aquaculture Nutrition** 22, 1040–1046.

Hahn, J.D., Biehl, R.R. & Baker, D.H. (1995). Ideal digestible lysine level for early and late finishing swine. **Journal of Animal Science** 73,773-784.

Halver, J.E. (2002). Fish Nutrition. In, **The vitamins**. (J. E. Halver and R. W. Hardy eds) Pages 61-141. Academic Press, San Diego, CA

Han, Y. & Baker, D.H. (1991). Lysine requirement of fast and slow growing broiler chicks. **Poultry Science** 70, 2018-2114.

Han, Y. & Baker, D.H. (1993). Effects of sex, heat stress, body weight and genetic strain on the lysine requirement of broiler chicks. **Poultry Science** 72, 701-708.

Hauler, R.C. & Carter, C.G. (2001). Lysine deposition responds linearly to marginal lysine intake in Atlantic salmon (*Salmo salar* L.) parr. **Aquaculture Research** 32, 147-156.

He, J.Y., Tian, L.X., Lemme, A. et al. (2013). Methionine and lysine requirements for maintenance and efficiency of utilization for growth of two sizes of tilapia (*Oreochromis niloticus*). **Aquaculture Nutrition** 19, 629–640.

Heger, J. & Frydrych, Z. (1985) Efficiency of utilization of essential amino acids in growing rats at different levels on intake. **British Journal of Nutrition** 54, 499-508.

Helland, B.G., Gatlin III, D.M., Corrent, E. et al. (2011). The minimum dietary lysine requirement, maintenance requirement and efficiency of lysine utilization for growth of Atlantic salmon smolts. **Aquaculture Research** 42, 1509-1529.

Helland, S.J., Hatlen, B. & Grisdale-Helland, B. (2010). Energy, protein and amino acid requirements for maintenance and efficiency of utilization for growth of Atlantic salmon post-smolts determined using increasing ration levels. **Aquaculture** 305, 150-158.

Hua, K. (2013). Estimating maintenance amino acids requirements of fish through a nonlinear mixed modeling approach. **Aquaculture Research** 44, 542–553.

Ishibashi, T. & Ohta, Y. (1999). Recent advances in amino acid nutrition for efficient poultry production, Review. **Asian Australasian Journal of Animal Science** 12, 1298-1309.

Jauncey, K. (1982). The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). **Aquaculture** 27, 43-54.

Kaushik, S.J. (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. **Aquaculture of Live Research** 11, 355-358.

Kim, S.S., Rahimnejad, S., Song, J.W. et al. (2012). Comparison of Growth Performance and Whole-body Amino Acid Composition in Red Seabream (*Pagrus major*) Fed Free or Dipeptide Form of Phenylalanine. **Asian Australasian Journal of Animal Science** 25, 1138-1144.

Kpogue, D., Gangbazo, H. & Fiogbe, E. (2013). A Preliminary Study on the Dietary Protein Requirement of *Parachanna obscura* (Günther, 1861) Larvae. **Turkish Journal of Fish and Aquatic Science** 13, 111-117.

Moughan, P.J. & Fuller, M.F. (2003). Amino Acids in Animal Nutrition. In, **Modeling amino acid metabolism and the estimation of amino acid requirements. 2nd edn** (ed. J.P.F. D'Mello), CABI International, Wallingford, UK, pp. 187-202.

Mustafa, S. & Mittal, A. (1982). Protein, RNA and DNA levels in liver and brain of starved catfish *Clarias batrachus*. **Japanese Journal of Ichthyology** 28, 396-400.

Mustafa, S., Lagardere, J.P. & Pastoureaud, A. (1991). Condition indices and RNA,DNA ratio in overwintering European sea bass, *Dicentrarchus labrax*, in salt marshes along the Atlantic coast of France. **Aquaculture** 96, 367-374.

Nair, K.S., Schwartz, R.G. & Welle, S. (1992). Leucine as a regulator of whole body and skeletal muscle protein metabolism in humans. **American Journal of Physiology** 263, E928-E934.

National Research Council (NRC) (2011) **Nutrient Requirements of Fish and shrimps.** National Academy Press, Washington, DC

Ng, W.K. & Hung, S.S.O. (1995). Estimating the ideal dietary essential amino acid pattern for growth of white sturgeon, *Acipenser transmontanus* (Richardson). **Aquaculture Nutrition** 1, 85-94.

Ng, W.K., Hung, S.S.O. & Herold, M.A. (1996). Poor utilization of dietary free amino acids by white sturgeon. **Fish Physiology and Biochemistry** 15, 131-142.

Norton, L.E. & Layman, D.K. (2006). Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. **Journal of Nutrition** 136, 533–537.

Nose, T. (1979). Finfish Nutrition and Fish feed Technology. In, **Summary report on the requirements of essential amino acids for carp.** (Halver J.E. and Tiews K., editors) Pages 145-156 Heenemann, Berlin, Germany

Rodehutscord, M. & Pack, M. (1999). Estimates of essential amino acid requirements from dose-response studies with rainbow trout and broiler chicken, effect of mathematical model. **Archives of Animal Nutrition** 52, 223-244.

Rodehutscord, M., Becker, A., Pack, M. et al. (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. **Journal of Nutrition**126, 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., Wauters, J.B., Bodin, N. et al. (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.

Schneider, W.C. (1957). Determination of nucleic acids in tissue by pantose analysis. In, **Methods of enzymology.** Calowick S.P. & Kaplon N.O. (eds.). Academic press, New York, p 680

Shearer, K.D. (1995). The use of factorial modeling to determine the dietary requirements for essential elements in fishes. **Aquaculture** 133, 57–72.

Shearer, K.D. (2000). Experimental design, statistical analysis and modeling of dietary nutrient requirement studies for fish, a critical review. **Aquaculture Nutrition** 6, 91-102.

Sokal, R.R., Rohlf, F.J. (1981).**Biometry**. W.H. Freeman and Company, New York, 859 p.

Yan, L., Qinghui, A., Kangsen, M. et al. (2010). Dietary leucine requirement for juvenile large yellow croaker *Pseudosciaena crocea* (Richardson, 1846). **Journal of Ocean University of China** 9, 371-375.

Zehra, S. & Khan, M.A. (2015), Dietary Leucine requirement of fingerling *Catla catla* (Hamilton) based on growth, feed conversion ratio, RNA/DNA ratio, Leucine gain, blood indices and carcass composition. **Aquaculture International** 23, 577-595.

Zhang, J.Z., Zhou, F., Wang, L. et al. (2010). Dietary protein requirement of juvenile black sea bream, *Sparus macrocephalus*. **Journal of the World Aquaculture Society** 41, 151-164.