GROWTH PERFORMANCE, HEMATOLOGY, AND MUSCLE GROWTH IN ISOLEUCINE FED NILE TILAPIA

Dacley Hertes NEU[1], Wilson Rogério BOSCOLO[2], Fernanda Losi Alves de ALMEIDA[3], Micheli ZAMINHAN HASSEMER[3], Jackeline Marcante DALLAGNOL[3], Wilson Massamitu FURUYA[4]

ABSTRACT

The aim of this study was to evaluate the dietary isoleucine requeriments for Nile tilapia juveniles based on growth performance, hematological and biochemical responses, and muscle growth. Three hundred Nile tilapia juveniles (18.09 ± 0.11 g) were distributed in 20 fiberglass tanks and fed with five extruded isoproteic (280 $g.kg^{-1}$ of crude protein) and isoenergetic (3000 $kcal.kg^{-1}$) formulated diets containing 7.00, 8.18, 9.35, 10.53, and 11.70 $g.kg^{-1}$ of isoleucine. No differences were observed ($P > 0.05$) in productive performance, hematological and biochemical parameters, centesimal composition, amino acid body composition, and muscle growth. The isoleucine body retention reduced linearly with increased amounts of isoleucine in the diet. Because protein is the nutrient with the highest cost in fish diets, the results from this study contribute towards the formulation of efficient and cost effective diets for Nile tilapia juveniles.

Key words: essential amino acid; biochemistry; branched-chain amino acid; muscle fibers; *Oreochromis niloticus*

DESEMPENHO PRODUTIVO, HEMATOLOGIA E CRESCIMENTO MUSCULAR DE TILAPIA DO NILO ALIMENTADAS COM ISOLEUCINA

RESUMO

O objetivo desse estudo foi avaliar a exigência dietética de isoleucina para juvenis de tilápia do Nilo com base no desempenho produtivo, respostas hematológicas e bioquímicas e crescimento muscular. Trezentos juvenis de tilápia do Nilo (18,09 ± 0,11g) foram distribuídos em 20 tanques de fibra de vidro e alimentadas com cinco rações extrusadas isoproteicas (280 g kg^{-1} de proteína bruta) e isoenergéticas (3000 kcal kg^{-1}) as dietas foram formuladas continham 7,00; 8,18; 9,35; 10,53 e 11,70 g kg^{-1} de isoleucina. Não foram observadas diferenças ($P>0,05$) no desempenho produtivo, parâmetros bioquímicos e hematológicos, composição centesimal, composição corporal dos aminoácidos e crescimento muscular. A retenção corporal de isoleucina reduziu linearmente com o aumento da quantidade de isoleucina na dieta. A proteína é o nutriente com o maior custo na dieta dos peixes, assim, os resultados deste estudo contribuem para a formulação de dietas mais eficientes e com menores custos para juvenis de tilápia do Nilo.

Palavras chave: aminoácido essencial; aminoácidos de cadeia ramificada; bioquímica; fibras musculares; hematologia; *Oreochromis niloticus*

Artigo Científico: Recebido em 24/08/2016; Aprovado em 17/02/2017

[1]*Universidade Federal da Grande Dourados, Dourados, MS, Brazil. Rodovia Dourados a Itahum, km 12, Aeroporto, CEP: 79804-970. E-mail: dacley_pesca@hotmail.com (author for correspondance).*
[2]*Universidade Estadual do Oeste do Paraná – Unioeste, Toledo, PR, Brazil. Rua da Faculdade 645, Jardim Santa Maria, CEP: 85903-000.*
[3]*Universidade Estadual de Maringá – UEM, Maringá, PR, Brazil. Avenida Colombo, 5790, Jardim Universitário, CEP: 87020-900.*
[4]*Universidade Estadual de Ponta Grossa – UEPG, Ponta Grossa, PR, Brazil. Av. General Carlos Cavalcanti, 4748, Uvaranas, CEP: 84030-900.*

INTRODUCTION

Tilapia is among the various fish species with potential for aquaculture in Brazil and has excelled in intensive farming (FURUYA and FURUYA 2010) because of its productive performance and quality of meat (POPMA and MASSER 1999). Brazil is among the largest tilapia producers worldwide (FAO/ GLOBEFISH, 2013) with the national production estimated in more than 215.000 tons in 2015 (IBGE, 2016).

Protein is the nutrient with the highest cost in fish diets (AHMED and KHAN 2007). Its content, balancing, and availability of amino acids vary in foods (COWEY, 1994). Despite advances in studies on the amino acid requirements for tilapias, the requirements of some amino acids in practical diets for the current tilapia strains in Brazil have not yet been established. The determination of essential amino acid requirements, besides methionine, lysine, and threonine is mainly important in low protein diets and those developed from plant foods. Among the essential amino acids, isoleucine is highlighted because of its important role in protein synthesis and in energy metabolism of skeletal muscle (KHAN and ABIDI, 2007). According to WU (2009), isoleucine acts directly on the synthesis of glutamine, which is the primary energy source for enterocytes and immune cells (CHAMORRO *et al*., 2010; POHLENZ *et al.,* 2012).

In the immune system, isoleucine and other branched amino acids, (leucine and valine) improve the response capacity of lymphocytes against pathogens, however, the mechanism of action involved has not yet been fully clarified (CALDER, 2006). Diets with branched amino acid deficiencies result in decreased response from lymphocytes and stimulating agents, increased susceptibility to infections, and growth reduction (CALDER, 2006).

Several researchers identified the isoleucine requirement in several species of fish (CHANCE *et al*., 1964; BORLONGAN and COLOSO 1993; BENAKAPPA and VARGHESE, 2003). Most studies do not consider metabolic, immune, or physiological abnormalities. Because isoleucine participates in protein synthesis, its dietary inclusion may affect patterns of muscle growth.

Muscle growth in fish occurs through mechanisms of hyperplasia and hypertrophy. In hyperplasia, the fusion between activated satellite cells results in the formation of new muscle fibers over existing ones (JOHNSTON, 1999). In hypertrophy, activated satellite cells fuse with existing muscle fibers increasing the number of nuclei, which increases the synthesis of myofibrils and leads to increased muscle fiber area (KOUMANS *et al*., 1994). Although nutrition influences growth, there are no studies on the action of isoleucine on muscle growth in tilapias.

The aim of this study was to evaluate the effects of isoleucine supplementation on growth performance, hematological and blood plasma biochemical parameters, and muscle growth in Nile tilapia juveniles.

METHODS

The experiment was conducted at the Laboratório de Aquicultura do Grupo de Estudos de Manejo na Aquicultura – GEMAq, da Universidade Estadual do Oeste do Paraná - Unioeste – Toledo, PR, Brasil.

This project was approved by the Ethics Committee from the West Paraná State University-Unioeste under the Protocol number 01812 of 2012.

Five rations containing increasing levels of isoleucine (7.00; 8.18; 9.35; 10.53; and 11.70 $g.kg^{-1}$) were formulated (Tables 1 and 2) in accordance with the recommendations proposed by FURUYA (2010) to meet the nutritional requirements of juvenile tilapias. The ingredients were ground in hammer type mills with 0.5 mm diameter sieve. The diet was extruded (Ex-Micro® extruder, ExTeec Company, Ribeirão Preto, Brazil) as 3 mm pellets. The fish were fed four times a day for 70 days (8 and 11 am; 2 and 5 pm) until apparent satiety.

A total of 300 Nile tilapia juveniles were used in the study (18.09 ± 0.11 g). The fish were distributed in 20 500 L fiberglass tanks (15 fish per tank), fitted with a water recirculation system (4 liters of water/minute/tank), central biofilter. Constant aeration was achieved through a central air blower, and constant water temperature was controlled by a thermostat. Water quality parameters such as pH, dissolved oxygen (mg.L-1), and electrical conductivity (µS.cm-1) were measured weekly using a portable digital potentiometer. At the end of the experiment, the fish were submitted to fasting for 24 hours to ensure emptied gastrointestinal tracts. Fish were subsequently numbed in 75 mg.L-1 eugenol (DERIGGI et al. 2006) and individually weighted (g). Three fish from each tank were euthanized in 300 mg.L-1 eugenol and subsequently packed in ice

for removal of visceral fat and liver. After removal of visceral fat and liver, these animals were frozen (-20°C) and forwarded to the Quality Control Laboratory from the Aquaculture Management Studies Group (GEMAq) for further centesimal analysis.

Table 1. Composition ($g.kg^{-1}$) of experimental rations with different levels of isoleucine for Nile tilapia juveniles.

	Dietary isoleucine ($g.kg^{-1}$)				
Ingredient	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Corn flour	257.38	257.38	257.38	257.38	257.38
Wheat meal	250.00	250.00	250.00	250.00	250.00
Soybean meal	124.93	124.93	124.93	124.93	124.93
Rice, broken	100.00	100.00	100.00	100.00	100.00
Fish meal 55	76.09	76.09	76.09	76.09	76.09
Glutamic acid	50.00	47.25	44.50	41.75	39.00
Blood meal	40.00	40.00	40.00	40.00	40.00
Limestone	20.00	20.00	20.00	20.00	20.00
Soy oil	14.80	14.80	14.80	14.80	14.80
Dicalcium phosphate	6.92	6.92	6.92	6.92	6.92
Mineral and vitamin mix[1]	5.00	5.00	5.00	5.00	5.00
L-alanine	40.00	41.50	43.00	44.50	46.00
L-lysine	4.62	4.64	4.66	4.68	4.70
L-isoleucine	0.00	1.20	2.40	3.60	4.80
L-threonine	3.87	3.88	3.89	3.90	3.91
L-tryptophan	0.45	0.46	0.46	0.47	0.47
DL-methionine	1.74	1.75	1.77	1.78	1.80
Salt	3.00	3.00	3.00	3.00	3.00
Propionic acid	1.00	1.00	1.00	1.00	1.00
BHT[2]	0.20	0.20	0.20	0.20	0.20

[1]Composition per kg of product-Premix (DSM-Roche ®): Vit. A, 480,000 IU; Vit. D3, 120,000 IU; Vit. E, 6,000 mg; Vit. K3, 600 mg; Vit. B1, 800 mg; Vit. B2, 800 mg; Vit. B6, 700mg; Vit. B12, 1,600 mg; Folic acid, 240mg;Ca pantothenate, 2,000 mg; Vit. C, 12,000 mg; Biotin, 40mg; Choline, 20,000 mg; Iron, 4,000 mg; Copper, 700mg; Manganese, 2,000 mg; Zinc, 4,800 mg; Iodine, 32 mg; Cobalt, 16
[2]BHT = Butyl Hydroxy Toluene

The growth performance data evaluated include: final average weight (g), weight gain = (final body weight – initial body weight); apparent feed conversion = consume diet/weight gain; protein efficiency ratio = weight gain/consumed protein; protein retention efficiency = (final carcass content x final biomass) – (initial carcass protein content x initial biomass)/consumed protein; survival percentage = (final number of fish/initial number of fish) x 100; hepatosomatic index = (liver weight/ final body weight) x 100; percentage of visceral fat = (visceral fat weight/final body weight) x 100; and body retention of amino acids = (final body amino acids x final body weight) – (initial body amino acids content x initial body weight)/ amino acid consumption.

Three fish from each experimental unit were randomly selected for blood sampling after being anesthetized in eugenol ($60\ mg.L^{-1}$). An aliquot of 2.0 ml of blood was collected through caudal puncture using a heparinized syringe; 0.5 ml was used for hematological analyses and 1.5 ml for biochemical analyses. Whole blood samples were used for total count of red blood cells using a Neubauer chamber, the identification of the hemoglobin rate through the methodology described by Collier (1944), percentage

of hematocrit following the methodology of Goldenfarb et al. (1971), and hematimetric indexes such as MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) according to Wintrobe (1934): MCV (fL) = (hematocrit x 10/erythrocytes); (MCH (pg) = (hemoglobin x 10/ erythrocytes); and MCHC ($g.dL^{-1}$) = (hemoglobin x 100/hematocrit).

Table 2. Chemical composition ($g.kg^{-1}$) of the experimental diets containing graded of isoleucine for Nile tilapia juveniles (based in natural matter).

	Dietary isoleucine ($g.kg^{-1}$)				
Ingredients	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Digestible energy ($kcal.kg^{-1}$)	3000.00	3003.70	3007.40	3011.10	3014.80
Crude Protein	280.00	280.00	280.00	280.00	280.00
Calcium	15.00	15.00	15.00	15.00	15.00
Crude fiber	35.96	35.96	35.96	35.96	35.96
Total phosphorus	8.11	8.11	8.11	8.11	8.11
Available phosphorus	5.47	5.47	5.47	5.47	5.47
Crude lipid	40.42	40.42	40.42	40.42	40.42
Essential amino acids[1]					
Arginine	12.50	12.40	11.30	12.60	12.40
Phenylalanine	10.40	10.70	10.50	10.40	10.30
Histidine	6.02	5.72	5.69	5.79	5.98
Isoleucine	7.00	8.18	9.35	10.53	11.70
Leucine	16.90	17.00	17.20	17.00	16.70
Lysine	14.57	14.70	14.92	14.69	15.11
Methionine	5.07	5.11	5.23	5.13	5.34
Threonine	11.86	11.64	12.08	11.82	11.86
Tryptophan	3.70	3.65	3.56	3.67	3.71
Valine	10.24	10.39	10.65	10.65	10.48
Non-essential amino acids[1]					
Aspartic acid	18.54	18.55	18.84	18.64	18.56
Glutamic acid	82.34	82.45	80.51	76.86	72.41
Alanine	50.12	51.32	57.15	55.49	56.89
Cysteine	2.81	2.83	2.82	2.84	2.80
Glycine	10.59	10.48	11.02	10.54	10.69
Serine	9.88	9.89	9.94	9.98	9.72
Tyrosine	6.98	7.46	7.19	7.25	7.20

[1]Analysis conducted by *Ajinomoto Animal Nutrition*

Blood smears were prepared on glass slides, which were air-dried and stained by the method ROSENFELD (1947). Total leukocytes and thrombocytes were counted through the indirect method (RANZANI-PAIVA *et al.*, 2013): total leukocytes (µl) = (white blood counted on the smear x erythrocytes counted in Neubar chamber)/ 2000; and total thrombocytes (µl) = thrombocytes counted on the smear x erythrocytes counted in Neubar chamber)/2000.

The differential leukocyte count consisted of determining the existing proportion among lymphocytes, neutrophils, and monocytes. One hundred leukocytes were counted in a light microscope with a 100X immersion objective. Counts were expressed in percentages. The blood plasma biochemical analysis included total proteins ($g.dL^{-1}$), triglycerides ($mg.dL^{-1}$), total cholesterol ($mg.dL^{-1}$), high density lipoprotein (HDL, ($mg.dL^{-1}$)), glucose ($mg.dL^{-1}$), very low density lipoprotein (VLDL, ($mg.dL^{-1}$), low density lipoprotein (LDL, ($mg.dL^{-1}$)), and urea ($mg.dL^{-1}$). Samples for differential leukocyte count were centrifuged at 2,500 rpm for five minutes. Specific commercial kits from *Gold Diagnostic Analyses*® (Analisa Diagnóstica LTDA Company, Belo Horizonte, Brazil) were used in the analysis;

results were read in a spectrophotometer according to manufacturer's instructions.

Two fish from each experimental unit were used in the evaluation of muscle growth. These fish were anesthetized and a sample of white dorsal muscle above the lateral line was obtained using a razor. These samples were fixed in 10% buffered formalin for 24 hours and processed for inclusion in paraffin. The cross-sections (6 µm), obtained using a microtome, were stained with hematoxylin and eosin. The morphometry analysis used a system of image analyses that determined the smallest diameter of 200 muscle fibers per animal. Fiber diameters were distributed into classes (< 20 µm, 20-50 µm, and >50 µm) (ALMEIDA *et al*., 2008). These analyses were conducted at the Histology Laboratory, Department of Morphological Sciences (DCM) at the Maringá State University (UEM).

The centesimal composition analysis followed the methods established by AOAC (1995) for moisture analysis (pre-drying at 55ºC for 72 hours followed by drying at 105ºC for eight hours), proteins (Kjeldhal method), ether extract (Soxhlet Extractor with ether as a solvent), and mineral matter (calcination of samples at 550ºC for 6 hours). The fish were sent to the laboratory with head and scales but without the visceral content. The pre-dried material was forwarded to the Ajinomoto Biolatina Laboratory for amino acid analyses by HPLC (Hitashi L-8800). The tryptophan content was determined after acid hydrolysis.

Experimental design was completely randomized with five treatments and four replications; one experimental unit consisted of one aquarium containing 15 fish. Data were subjected to regression analysis at 5% probability through the *Statistic 7.1* computational program (STATSOFT, 2005).

RESULTS

Average temperature was 25.13 ± 1.23ºC; average dissolved oxygen was 4.44 ± 0.55 mg.L^{-1}; average pH was 7.52 ± 0.14; average electrical conductivity was 135.28 ± 28.80 µS.cm^{-1}.

The consumption of diets with different levels of isoleucine did not influence (P > 0.05) the growth performance of the fish in any of the evaluated parameters (Table 3).

Table 3. Growth performance of Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

Parameter	Isoleucine					CV	Effect
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5		
Initial weight (g)	18.09	18.12	18.09	18.11	18.06	0.60	NS
Weight gain (g)	83.05	86.68	80.85	74.31	77.42	9.84	NS
Feed conversion (g/g)	1.05	1.13	1.05	1.17	1.06	10.77	NS
Hepatossomatic index (%)	2.00	2.23	2.27	1.84	1.59	21.16	NS
Visceral fat (%)	2.85	2.83	3.04	2.72	2.93	15.05	NS
Survival (%)	95.00	78.33	95.00	88.33	90.00	12.90	NS
Protein efficiency ratio (%)	2.22	2.00	2.22	1.98	2.14	11.46	NS
Protein retention efficiency (%)	46.93	49.90	54.37	47.93	55.55	12.49	NS

CV = coefficient of variation (%); NS = not significant (P > 0.05).

The consumption of diets with increasing levels of isoleucine did not influence (P > 0.05) the body composition of fish with respect to moisture, protein, lipids, and mineral matter (Table 4).

Table 4. Body composition of Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

Parameter	Isoleucine					CV	Effect
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5		
Moisture	71.82	70.67	71.60	70.60	72.65	2.96	NS
Crude protein	14.77	15.59	16.50	15.72	16.59	15.24	NS
Lipids	8.80	8.75	8.52	8.97	8.56	11.49	NS
Mineral matter	3.45	3.36	3.54	3.63	4.18	9.91	NS

CV = coefficient of variation (%); NS = not significant (P > 0.05).

No significant differences were observed (P > 0.05) for specific biochemical and hematological parameters in Nile tilapia juveniles fed with diets containing different levels of isoleucine (Table 5).

Table 5. Blood plasma biochemical and hematological parameters in Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

Biochemical parameter	Isoleucine					CV	Effect
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5		
Glucose (mg.dL^{-1})	73.54	75.47	71.89	84.34	78.13	16.57	NS
Total cholesterol (mg.dL^{-1})	156.86	146.13	159.06	171.49	137.86	12.67	NS
Triglycerides (mg.dL^{-1})	213.00	215.83	209.74	231.08	155.61	25.46	NS
HDL (mg.dL^{-1})	63.77	64.33	63.51	60.35	68.67	22.15	NS
LDL (mg.dL^{-1})	51.09	43.29	55.45	52.43	37.51	26.57	NS
VLDL (mg.dL^{-1})	42.60	43.17	41.95	46.22	31.12	25.46	NS
Total protein (g.dL^{-1})	4.62	4.59	4.59	4.66	4.66	6.88	NS
Urea (mg.dL^{-1})	10.05	9.40	10.28	10.39	10.76	7.45	NS
Hematologic							
Erythrocytes (10^6. µl^{-1})	1.91	1.94	1.95	2.00	1.99	6.82	NS
Hematocrit (%)	35.87	36.16	34.50	37.58	36.88	5.78	NS
Hemoglobin (g.dL^{-1})	7.02	7.12	7.14	6.57	7.05	16.89	NS
MCV (fL)	188.05	187.10	178.42	189.11	186.03	7.48	NS
HCM (µg)	36.70	36.95	36.81	33.58	35.62	20.22	NS
MCHC (g.dL^{-1})	19.53	19.74	20.68	17.54	19.04	16.88	NS

HDL = high-density lipoprotein; LDL = low density lipoproteins; VLDL = very low density lipoproteins; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; CV = coefficient of variation (%); NS = not significant (P > 0.05).

The consumption of diets with increasing levels of isoleucine did not significantly change (P > 0.05) the total counts of leukocytes and thrombocytes. Leukocyte counts ranged from 20,461 to 29,945 µl^{-1} and thrombocytes ranged from 27,475 to 37,548 µl^{-1}. Lymphocytes accounted for the largest portion of the leukocyte count with average values of 90%, followed by neutrophils (9%), and monocytes (from 1 to 4%) (Table 6). The carcass composition and amino acid retention in Nile tilapia juveniles are presented in Table 7. The body composition of the 10 essential amino acids evaluated was not influenced by the levels of isoleucine in the diet (P > 0.05). The results from body retention of essential amino acids showed, isoleucine was the only amino acid that showed significant differences correlated with feeding levels in the diet (P < 0.05). The isoleucine body retention reduced with increased levels of this amino acid in the diet.

Table 6. Average values of leucocytes, thrombocytes and differential leucocytes percentage (lymphocytes, neutrophils, and monocytes) in Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

Parameter	Isoleucine					CV	Effect
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5		
Total leukocytes (µL^{-1})	25641	29191	20461	29945	22753	27.37	NS
Total thrombocytes (µL^{-1})	30778	34625	37548	32796	27475	36.72	NS
Lymphocytes (%)	89.83	90.00	90.12	90.12	89.83	0.95	NS
Neutrophils (%)	9.17	8.87	9.62	9.50	9.50	9.72	NS
Monocytes (%)	4.25	1.00	1.00	1.00	2.00	95.53	NS

CV = coefficient of variation (%); NS = not significant (P > 0.05).

Table 7. Essential amino acid body composition (g per 16 g of N) and body retention (%) in Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

	Isoleucine						
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**CV**	**Effect**
	Carcass essential amino acids composition						
Arginine	6.48	6.20	5.94	6.55	5.77	5.69	NS
Phenylalanine	3.78	3.71	3.54	3.91	3.42	5.85	NS
Histidine	2.17	2.16	2.00	2.18	1.89	8.94	NS
Isoleucine	3.66	3.64	3.34	3.61	3.19	9.16	NS
Leucine	6.67	6.54	6.22	6.81	5.96	6.10	NS
Lysine	6.47	6.93	6.55	6.91	6.03	9.03	NS
Methionine	2.30	2.30	2.24	2.33	2.02	8.34	NS
Threonine	4.06	4.07	3.77	4.20	3.69	5.80	NS
Tryptophan	0.84	0.84	0.74	0.82	0.72	11.60	NS
Valine	4.25	4.22	3.93	4.39	3.84	6.01	NS
	Carcass essential amino acid retention						
Arginine	65.50	64.04	73.15	60.85	64.74	13.46	NS
Phenylalanine	43.49	41.04	43.51	41.00	42.73	11.52	NS
Histidine	42.66	28.33	46.74	43.46	42.55	23.00	NS
Isoleucine	61.40	50.89	45.90	38.25	36.82	21.78	Linear
Leucine	46.83	45.72	47.17	43.78	46.48	10.73	NS
Lysine	54.56	58.23	61.27	57.22	56.52	12.20	NS
Methionine	54.05	53.60	57.89	53.12	51.30	12.33	NS
Threonine	42.75	42.44	42.59	39.90	41.52	10.76	NS
Tryptophan	25.75	26.67	26.65	25.07	25.28	12.83	NS
Valine	48.62	47.49	47.27	44.25	47.13	11.29	NS
			Equations			Value of P	
Isoleucine Retention			$Y = -5.26 x + 95.84$; $R^2 = 0.77$			0.000001	

CV = coefficient of variation (%); NS = not significant (P > 0.05).

The isoleucine diet did not influence (P > 0.05) the frequencies of muscle fibers in any of the three established classes of fiber diameters (< 20, between 20 and 50 µm, and > 50 µm) (Table 8).

Table 8. Frequency distribution of muscle fibers in three classes of fiber diameters (< 20 µm, between 20 and 50 µm, and > 50 µm) in Nile tilapia juveniles fed with diets containing increasing levels of isoleucine.

		Isoleucine						
Diameter class	**«**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**CV**	**Effect**
< 20µm	36.46	10.77	16.50	13.31	11.77	17.66	43.90	NS
20-50µm	62.78	75.43	71.60	71.58	69.03	74.28	9.36	NS
> 50µm	1.00	13.80	11.89	15.10	19.20	8.05	56.96	NS

CV = coefficient of variation (%); NS = not significant (P > 0.05).

DISCUSSION

The water quality parameters monitored during the experimental period remained within ranges that are suitable for the species. Tilapias show maximum growth when raised at temperatures between 24 and 30ºC (EL-SAYED, 2006; COA *et al.*, 2017), dissolved oxygen levels above 4 $mg.L^{-1}$, pH close to neutral (POPMA and LOVSHIN, 1995), and electrical conductivity between 20 and 150 $\mu S.\ cm^{-1}$ (ZIMMERMANN *et al.*, 2001). In the present study, we did not observe any effect of different levels

of dietary isoleucine on the growth performance of fishes. The diet with 7.00 $g.kg^{-1}$ isoleucine was sufficient for the requirements of Nile tilapia juveniles for this amino acid. This observed requirement was less than those reported by SANTIAGO and LOVELL (1988) for Nile tilapia larvae (8.70 $g.kg^{-1}$ of isoleucine in the diet), AHMED and KHAN (2006) for *Cirrhinus mrigala* fingerlings (12.6 $g.kg^{-1}$), and KHAN and ABIDI (2007) for *Labeo rohita* fingerlings (between 15.2 and 15.9 $g.kg^{-1}$ of isoleucine in the diet).

Isoleucine requirements determined in this study may be less than those observed in other studies because of the practical diet used, a diet which is more palatable than purified diets to some species of fish (GRIFFIN *et al.,* 1992).

Although TISCHLER *et al*. (1982) reported that leucine is responsible for protein synthesis, WU (2009) pointed out that supplementation with the three branched chain amino acids (leucine, isoleucine, and valine) is needed to potentiate the role of leucine in muscle growth: a possible imbalance can occur when the diet is only supplemented with leucine. An imbalance among the branched amino acids was not observed in this study because no effects of isoleucine supplementation on protein and amino acids body retention were observed.

The high protein retention values (above 45%) observed in the fish in all treatments suggests an efficient utilization of the dietary amino acids and indicates that their levels and balancing were adequate to allow the use of protein.

The body composition results for moisture, proteins, lipids, and mineral matter in Nile tilapia juveniles (Table 4) were not influenced by the levels of isoleucine in the diet ($P > 0.05$), possibly because these diets were formulated to contain the same levels of protein and energy. Although isoleucine functions as an energy fuel particularly in the skeletal muscle (KOHLMEIER, 2003), our results suggest that the formulated diets were adequate to meet the requirements of this amino acid because the fish fed with different levels of isoleucine did not present differences in visceral fat, body lipids, and blood plasma biochemistry composition. Glucose values observed ranged from 71.89 to 84.34 $mg.dL^{-1}$ (Table 5) and are within the recommended reference values (from 39 to 96 mg. dL^{-1}) for tilapias raised in intensive systems (HRUBEC *et al.,* 2000). Glucose is a common parameter used to evaluate stress in fish (IWAMA, 1998). The continuous weight gain observed in the fish during the experimental period in this study indicates a lack of stress resulting from the provision of a suitable environment, consistent feeding, comfortable temperatures, and continuous aeration with oxygen levels averaging 4.44 $mg.L^{-1}$.

Cholesterol is a complex substance with many functions in the body, however, when metabolism problems occur, cholesterol blood concentration can rise (LUDKE and LOPES 1999). The observed cholesterol values (137.1 to 171.53 $mg.dL^{-1}$) are lower than the 74.42 to 221.19 $mg.dL^{-1}$ range reported by NEU *et al*. (2013) in a study using increasing levels of glycerol in diets for Nile tilapias. However, according to HRUBEC *et al*. (2000), the values in the present study are within the established reference values for the same species.

Triglycerides concentrations between 155.61 and 231.08 $mg.dL^{-1}$ were observed. BORGES *et al.* (2004) report concentrations between 138 and 546 $mg.dL^{-1}$ as normal values for *Rhamdia quelen*. Cholesterol and triglycerides are transported to the plasma through lipoproteins (SCHIAVO *et al.* 2003), which also have a role in lipid metabolism (METCALF *et al*., 1999). The high-density lipoprotein (HDL) was identified in higher concentrations than the other lipoproteins of low and very low density (LDL and VLDL) in the blood, similar to the values reported by METCALF *et al.* (1999). Considering that isoleucine plays a central role as energy fuel and that no differences were observed between treatments, we infer that the lowest concentration tested in the diet meets all the requirements for this species.

The total blood protein contents showed minor alterations that were not a result of the inclusion of dietetic isoleucine. The observed values ranged between 4.59 and 4.66 $g.dL^{-1}$, which are within the range between 2.7 and 5.0 $g.dL^{-1}$ proposed by MANUEL *et al.* (2007), and similar to 4.6 $g.dL^{-1}$ reported by TAVARES-DIAS *et al*. (2008) for *Leporinus macrocephalus.*

Plasma urea ranged from 9.40 to 10.76 $mg.dL^{-1}$, values that are not statistically significant. EL-HAWARRY (2012) reported that the average urea in Nile tilapia and blue tilapia maintained in semi-intensive systems is 6.10 and 6.80 $mg.dL^{-1}$, respectively. NICULA *et al*. (2010) report that salmonids and cyprinids present average values of blood urea between 18.30 and 12.16 $mg.dL^{-1}$, respectively, higher than those observed in our study with tilapias. This may result from the ability of fish to excrete nitrogen to maintain their osmotic activity.

In the current study, the contents and proportions

of amino acids in the diet that was not supplemented with isoleucine were sufficient for the maintenance of all hematological parameters (Table 5). They were within the reference range proposed by HRUBEC *et al*. (2000) and BITTENCOURT *et al*. (2003) for tilapias. Leukocyte counts (Table 6) ranged from 20,461 to 29,945 μL^{-1}, thrombocytes ranged from 27,475 to 37,548 μL^{-1}; the differential leukocyte count showed the largest quantity of lymphocytes, approximately 90%, which are the most numerous cells in the bloodstream in several teleost species (RANZANI-PAIVA and SILVA-SOUZA, 2004), including tilapias.

None of the ten essential amino acid concentrations was altered because of the inclusion of isoleucine in the fish diet (Table 7). The body proportion between branched chain amino acids and essential amino acids was around 35%, similar to that described by HARPER *et al*. (1984) as the amount of branched amino acids in muscle protein. The proportion of isoleucine in the present study corresponded to 9% of essential amino acids, similar to the value described by TROSVIK *et al.* (2013).

In this study, the retention of any amino acid, except isoleucine, was not influenced by the levels of dietetic isoleucine. A low efficiency in arginine retention was detected by ZHOU *et al.* (2012) who observed an inverse relationship between the level of arginine in the diet and its retention. In this study, regardless of the relationship between leucine, isoleucine, and valine (branched amino acids), the increased levels of isoleucine in the diet did not affect the utilization of the remaining branched amino acids.

Muscle fibers with diameters smaller than 20 μm indicate hyperplasia, those with diameters larger than 50 μm indicate hypertrophy (VALENTE *et al*., 1999; ROWLERSON and VEGGETTI, 2001). In the present study, muscle growth occurred both by hyperplasia and hypertrophy during the experimental period in all treatments. This result indicates that hypertrophy and hyperplasia contributed equally to muscle growth and is consistent with growth performance in which no differences are observed between treatments.

Muscle growth can be influenced by several external factors such as nutrition (JOHNSTON *et al*., 2006). Studies on amino acid supplementation in fish show controversial results because they are dependent on species and stage of development. In this study, the different levels of isoleucine did not promote changes in the contribution of hypertrophy and hyperplasia to muscle growth in Nile tilapia juveniles. AGUIAR *et al*. (2005) observed similar results showing that different levels of dietary lysine did not influence the growth of white fibers in Nile tilapia larvae.

Because fish have indeterminate growth and fiber hyperplasia extends over a long period (ROWLERSON and VEGGETTI, 2001), it is difficult to determine the final weight of the adult fish. VALENTE *et al*. (1999) demonstrated a negative correlation between the size of muscle fibers with less than 25 μm in diameter and body size in trout. In this study, fibers with less than 20 μm in diameter occurred in all treatments; however, the correlation analysis was not performed because no differences on growth performance of tilapias fed with different levels of dietary isoleucine were observed.

CONCLUSION

Signs of dietary isoleucine deficiency were not observed in this study as differences in growth performance, hematological or biochemical responses, and muscle growth were not detected. Therefore, the lowest isoleucine level tested (7.00 $g.kg^{-1}$) is enough to meet the isoleucine dietary requirements of Nile tilapia juveniles. Protein is the nutrient with the highest cost in fish diets, the results from this study contribute towards the formulation of efficient and cost effective diets for Nile tilapia juveniles.

REFERENCES

AGUIAR, D.H.; BARROS, M.M.; PADOVANI, C.R.; PEZZATO, L.E.; DAL, PAI-SILVA, M. 2005 Growth characteristics of skeletal muscles tissue in *Oreochromis niloticus* larvae fed on a lysine supplemented diets. *Journal of Fish Biology, 67*(5): 1287-1298.

AHMED, I.; KHAN, M. 2006 Dietary branched-chain amino acid valine, isoleucine and leucine requirements of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). *British Journal of Nutrition, 96*(3): 450-460.

ALMEIDA, F.L.A.; CARVALHO, R.F.; PINHAL, D.; PADOVANI, C.R.; MARTINS, C.; DAL,

PAI-SILVA M. 2008 Differential expression of myogenic regulatory factor MyoD in pacu skeletal muscle (*Piaractus mesopotamicus* Holmberg 1887: Serrasalminae, Characidae, Teleostei) during juvenile and adult growth phases. *Micron, 39*(8): 1306-1311.

AOAC. 1995 *Oficial Methods of Analysis of the Association of Official Analytical Chemists*. 30p. AOAC, Arlington.

BENAKAPPA. S.; VARGHESE, T.J. 2003 Isoleucine, leucine and valine requirement of juvenile Indian major carp, *Cirrhinus cirrhosus* (Bloch, 1795). *Acta Ichthyologica Piscatoria, 33*(2): 161-172.

BITTENCOURT, N.L.R.; MOLINARI, L.M.; SCOARIS, D.O.; PEDROSO, R.B.; NAKAMURA, C.V.; UEDA-NAKAMURA, T.; ABREU – FILHO, B.A.; DIAS, FILHO, B.P. 2003 Haematological and biochemical values for Nile tilapia *Oreochromis niloticus* cultured in semi-intensive system. *Acta Scientiarum Biological Sciences, 25*(2): 385-389.

BORGES, A.; SCOTTI, L.V.; SIQUEIRA, D.R.; JURINITZ, D.F.; WASSERMANN, G.F. 2004 Hematologic and serum biochemical values for jundiá (*Rhamdia quelen*). *Fish Physiology Biochemistry, 30*(1): 21–25.

BORLONGAN, I.G.; COLOSO, R.M. 1993 Requirements of juvenile milkfish (*Chanos chanos*) for essential amino acids. *Journal Nutrition, 123*(1): 125-132.

CALDER, P.C. 2006 Branched-chain amino acids and immunity. *Journal Nutrition, 136*(1): 288-293.

CHAMORRO, S.; BLAS, C.; GRANT, G.; BADIOLA, I.; MENOYO, D.; CARABAÑO, R. 2010 Effect of dietary supplementation with glutamine and a combination of glutamine-arginine on intestinal health in twenty-five-day-old weaned rabbits. *Journal of Animal Science, 88*(1): 170-180.

CHANCE, R.E.; MERTZ, E.T.; HALVER, J.E. 1964 Isoleucine, leucine, valine and phenylalanine requirements of Chinook salmon and interrelations between isoleucine and leucine for growth. *Journal Nutrition, 83*(1): 177-185.

COLLIER, H.B. 1944 The standardization of blood haemoglobin determinations. *Canadian Medical Association Journal, 50*(6): 550-552.

COWEY, C.B. 1994 Amino acid requirements of fish: a critical appraisal of present values. *Aquaculture, 124*(1-4): 1-11.

DERIGGI, G.F.; INOUE – LAKA.; MORAES, G. 2006 Stress responses to handling in Nile tilapia (*Oreochromis niloticus* Linnaeus): assessment of eugenol as an alternative anesthetic. *Acta Scientiarum Biological Sciences, 28*(3): 269-274.

EL-HAWARRY, W. 2012 Biochemical and non-specific immune parameters of healthy Nile tilapia (*Oreochromis niloticus*), blue tilapia (*Oreochromis aureus*) and their interspecific hybrid (male *O. aureus* x female *O. niloticus*) maintained in semi-intensive culture system. *Online Journal of Animal and Feed Research, 2*(1): 84-88.

EL-SAYED, A.F.M. 2006 *Tilapia culture*. CABI Publishing, Wallingford, 277p.

FAO/GLOBEFISH 2013 Globefish highlights: A quarterly updates on word seafood market. *FAO/Globefish, 3*(1): 61p.

FURUYA, W.M. 2010 *Tabelas brasileiras para a nutrição de tilápias*. GFM, Toledo, 100p.

FURUYA, W. M.; FURUYA, V.R.B. 2010 Nutritional innovation on amino acids supplementation in Nile tilapia diets. *Revista Brasileira de Zootecnia, 39*(1): 88-94.

GOLDENFARB, P.B.; BOWYER, F.P.; HALL, E.; BROSIUS, E. 1971 Reproducibility in the hematology laboratory: the microhematocrit determinations. *American Journal of Clinical Pathology, 56*(1): 35-39.

GRIFFIN, M.E.; BROWN, P.B.; GRANT, A.L. 1992 The dietary lysine requirement of juvenile hybrid striped bass. *Journal Nutrition, 122*(2): 1332-1337.

HARPER, A.E.; MILLER, R.H.; BLOCK, K.P. 1984 Branched-chain amino acid metabolism. *Annual*

Review of Nutrition, 4(1): 409-454.

HRUBEC, T.C.; CARDINALE, J.L.; SMITH, S.A. 2000 Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis hybrid*). *Veterinary Clinical Pathology, 29*(1): 7-12.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA – IBGE 2015 *Produção da pecuária municipal 2015, 43*(1): 1-49.

IWAMA, G.K. 1998 Stress in fish. *Annals of the New York Academy of Sciences, 851*(1): 304-310.

JOHNSTON, I.A. 1999 Muscle development and growth: potential implication for flesh quality in fish. *Aquaculture, 177*(1): 99–115.

JOHNSTON, I.A. 2006 Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology, 209*: 2249-2264.

KHAN, M.A.; ABIDI, S.F 2007 Dietary isoleucine requirement of fingerling Indian major carp, L*abeo rohita* (Hamilton). *Aquaculture Nutrition, 13*(6): 424-430.

KOUMANS, J.T.M.; AKSTER, H.A.; WITKAM, A.; OSSE, J.W.M. 1994 Numbers of muscle nuclei and myosatellite cell nuclei in red and white axial muscle during growth of the carp (*Cyprinus carpio*). *Journal of Fish Biology, 44*(3): 391–408.

KOHLMEIER, M. 2003 *Amino acids and nitrogen compounds. Nutrient Metabolism*. Academic Press, London, 840p.

LUDKE, M.C.M.M.; LOPEZ, J. 1999 Colesterol e composição dos ácidos graxos nas dietas para humanos e na carcaça suína. *Ciência Rural*, 29(1): 181-187.

MANUEL, M.J.; MILLER, D.L.; MERRILL, A.L. 2007 Hematology and plasma biochemical values of healthy hybrid tilapia (*Oreochromis aureus* x *Oreochromis nilotica*) maintained in a recirculation system. *Journal of Zoo and Wildlife Medicine, 38*(3): 420-424.

METCALF, V.J.; BRENNAN, S.O.; CHAMBERS, G.; GEORGE, P.M. 1999 High density lipoprotein (HDL), and not albumin, is the major palmitate binding protein in New Zeland long-finned (*Anguilla dieffenbachia*) and short-finned eel (*Anguilla australis schmidtii*) plasma. *Biochimica et Biophysica Acta, 1429*(2): 467-475.

NEU, D.H.; FURUYA, W.M.; BOSCOLO, W.R.; POTRICH, F.R.; LUI, T.A.; FEIDEN, A. 2013 Glycerol inclusion in the diet of Nile tilapia (*Oreochromis niloticus*) juveniles. *Aquaculture Nutrition, 19*(2): 211-217.

NICULA, M.; BURA, M.; SIMIZ, E.; BANATEAN-DUNEA, I.; PATRUICA, S.; MARCU, A.; LUNCA, M.; SZELEI, Z. 2010 Researches concerning references values assessment of serum biochemical parameters in some fish species from *Acipenseridae*, *Cyprinidae*, *Esocidae*, and *Salmonidae* family. *Journal Animal Science and Biotechnology, 43*(1): 498-505.

POHLENZ, C.; BUENTELLO, A.; MWANGI, W.; GATLIN III, D.M. 2012 Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells. *Fish Shellfish Immunology, 32*(5): 762-768.

POPMA, T.J.; LOVSHIN, L.L. 1995 *Worldwide Prospects for Commercial Production of Tilapia*. International Center for Aquaculture and Aquatic Environments, Auburn, 42p.

POPMA, T.J.; MASSER, M. 1999 *Tilapia: life history and biology*. SRAC Publication, 283, 4p.

RANZANI-PAIVA. M.J.T.; SILVA-SOUZA, A.T. 2004 Hematologia de peixes brasileiros. In: *Sanidade de Organismos Aquáticos.* (eds. RANZANI-PAIVA, M.J.T., TAKEMOTO, R.M., LIZAMA, M.) Varela, São Paulo: p.89-120.

RANZANI-PAIVA, M.J.T., BENITES DE PÁDUA, S., TAVARES-DIAS, M., EGAMI, M.I. 2013 *Métodos para análise hematológica em peixes.* Eduem, Maringá, Paraná, 140p.

ROSENFELD, G. 1947 Corante pancrômico para hematologia e citologia clínica. Nova combinação dos componentes do May-Grünwald e do Giemsa num só corante de emprego rápido. *Mem***órias** *do Instituto Butantan, 20*(1): 329-334.

ROWLERSON, A.; VEGGETTI, A. 2001 Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: *Muscle Development and Growth.* (ed. JOHNSTON, I.A.). Academic Press, London. p.103-139.

SANTIAGO, C.B.; LOVELL, R.T. 1988 Amino acid requirement for growth of Nile tilapia. *Journal Nutrition, 118*(12): 1540-1546.

SCHIAVO, M.; LUNARDELLI, A.; OLIVEIRA, J.R. 2003 Influência da dieta na concentração sérica de triglicerídeos. *Jornal Brasileiro de Patologia e Medicina Laboratorial, 39*(4): 283-288.

STATSOFT, Inc. 2005 *STATISTICA* (data analysis software system), version 7.1.

TAVARES-DIAS, M.; MORAES, F.R.; IMOTO, M.E. 2008 Hematological parameters in two freshwater teleosts, *Leporinus macrocephalus* (Anostomidae) and *Prochilodus lineatus* (Prochilodontidae). *Biosciense Journal, 24*(3): 96-101.

TISCHLER, M.E.; DESAUTELS, M.; GOLDBERG, A.L. 1982 Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? *The Journal of Biological Chemistry, 257*(4): 1613–1621.

TROSVIK, K.A.; WEBSTER, C.E.; THOMPSON, K.R.; METTS, L.E.; GANNAM, A.; TWIBELL, R. 2013 Effects on growth performance and body composition in Nile tilapia, *Oreochromis niloticus*, fry fed organic diets containing yeast extract and soybean meal as a total replacement of fish meal without amino acid supplementation. *Biological Agriculture and Horticulture, 29*(3): 173-185.

VALENTE, L.M.P.; ROCHA, E.; GOMES, E.F.S.; SILVA, M.W.; OLIVEIRA, M.H.; MONTEIRO, R.A.F.; FAUCONNEAU, B. 1999 Growth and dynamics of white and red muscle fibres in fast- and slow-growing strains of rainbow trout. *Journal Fish Biology, 55*(4): 675-691.

WINTROBE, M.M. 1934 Variations in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. *Folia Haematologica, 51*(3): 32-49.

WU, G. 2009 Amino acids: metabolism, functions and nutrition. *Amino acids, 37*(1): 1-17.

ZHOU, H.; CHEN, N.; QIU, X.; ZHAO, M.; J.I.N, L. 2012 Arginine requirement and effect arginine intake on immunity in largemouth bass, *Micropterus salmoides*. *Aquaculture Nutrition, 18*(2): 107-116.

ZIMERMANN, S.; RIBEIRO, R.P.; VARGAS, L.; MOREIRA, H.L.M. 2001 *Fundamentos da moderna aquicultura.* ULBRA, Canoas, 200p.