UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CENTRO DE AQUICULTURA DA UNESP

DIETARY LYSINE REQUIREMENT IN DIFFERENT GROWTH STAGES OF NILE TILAPIA BY THE DILUTION TECHNIQUE

(Exigência de lisina para tilápia do Nilo em diferentes fases de crescimento pela técnica da diluição)

Rafael de Souza Romaneli

Jaboticabal, São Paulo
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Coorientador: Dr. Thiago Matias Torres do Nascimento

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Jaboticabal, 16 de fevereiro de 2019.

Sumário

Dedicatória

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"Ninguém cruza a nossa vida por acaso e nós não entramos na vida de alguém sem nenhuma razão."
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CERTIFICADO

Certificamos que o projeto intitulado **"Exigência de lisina digestível para tilápia do Nilo, *Oreochromis niloticus,* para diferentes fases de crescimento"**, protocolo nº 012035/17, sob a responsabilidade do Prof. Dr. João Batista Kochenborger Fernandes, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 03 de Agosto de 2017.

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Espécie / Linhagem	Tilápia do Nilo
Nº de animais	945
Peso / Idade	3g / 400g / 800g
Sexo	Masculino
Origem	Piscicultura comercial "Projeto Peixes"

Jaboticabal, 03 de Agosto de 2017.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

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Introdução geral

A produção de peixes de água doce cresce continuamente, dados recentes mostram que em 2016 foram produzidos mundialmente cerca de 51,4 milhões de toneladas. Neste cenário o Brasil representa apenas o 13º lugar entre os produtores, contribuindo com 1,93% da produção mundial. As maiores potências do setor são China e Índia, com 19,93 e 12,57% da produção mundial, respectivamente (FAO, 2018).

No Brasil, a tilápia do Nilo é a espécie mais produzida, representa 58.4% de todo pescado produzido, ou seja, 283,36 mil toneladas em 2017 (IBGE, 2017). O motivo para esta grande representatividade está associado a diversos fatores, como a boa adaptação aos diferentes sistemas de produção (Michelato et al., 2016), rápido crescimento (He et al., 2013), rusticidade (Takashita et al., 2009; He et al., 2013), filé com boas características organolépticas e ausência de espinhas intramusculares em forma de “Y” (Furuya et al., 2005), o que facilita o processamento e a aceitação pelo consumidor. Além disso, a espécie possui potencial de produção tanto em regiões tropicais quanto em subtropicais (Fitzsimmons, 2000).

Diante das qualidades da espécie, o filé é o principal produto da produção de tilápias e a aceitação do mercado pelo filé de tilápia cresce a cada ano (Schulter e Vieira Filho, 2017). Contudo, para que haja um bom desempenho dos animais, a alimentação fornecida deve atender à exigência nutricional da espécie (Ghomi et al., 2012). Neste contexto, a proteína é o nutriente que mais eleva os custos para formulação das dietas (Secco et al., 2002; Wilson et al. 2002), implicando na realização de diversas pesquisas para determinar a exigência nutricional que permita o melhor crescimento do animal e viabilize economicamente a produção (Borin et al., 2002).

Embora a proteína tenha importância na alimentação dos peixes, eles não possuem exigência neste nutriente, mas sim em quantidades balanceadas de aminoácidos que garantem o melhor desempenho zootécnico (Wilson e Poe, 1985; Wilson, 2002; Webster e Lim, 2002).

Os aminoácidos são classificados como essenciais e não essenciais. Os aminoácidos essenciais não são sintetizados pelos peixes e por isso devem estar disponíveis na dieta. Isso significa que eles podem ser disponibilizados através de sua forma sintética, quando suplementados na dieta, ou pela quebra da proteína de origem vegetal ou animal dos ingredientes dietéticos. Por outro lado, os aminoácidos não essenciais são sintetizados pelo organismo do animal (Li et al., 2009). Os aminoácidos são essenciais a diversas funções do organismo, como a síntese de proteína e de outros compostos associados ao metabolismo, incluindo hormônios, neurotransmissores, purinas e enzimas. Também, poderão ser catabolizados para suprir a demanda energética na ausência de gorduras e carboidratos (Ballantyne, 2001).

Os aminoácidos são extremamente importantes para o ótimo crescimento dos peixes, no entanto, o excesso e a deficiência destes componentes na dieta podem provocar prejuízos na produção. A deficiência de lisina, por exemplo, pode promover a diminuição do crescimento e a eficiência alimentar, como também podem provocar a erosão das nadadeiras dorsais e caudais, como constatado em truta arco-íris e carpa comum (Ketola, 1983; Guillaume et al., 1999; NRC, 2011). Os aminoácidos em excesso, podem promover a redução da qualidade da água e impactos negativos ao ambiente aquático, em virtude do aumento da excreção de nitrogênio (Furuya, 2001a; Furuya, 2001b; Henry-Silva et al., 2006).

Deste modo, o conceito de “proteína ideal” é aplicado em diversos trabalhos, cujo objetivo é determinar, através de um aminoácido referência, a exigência de outros aminoácidos de forma balanceada (Green e Hardy, 2002; Rollin et al., 2003; Pezzato et al., 2004; Bomfim et al., 2005; Furuya et al., 2005). A partir desse conceito, a lisina é utilizada como aminoácido de referência em estudos com proteína ideal, porque além da sua importante função na síntese proteica, ela atua em conjunto com outros aminoácidos (Furuya et al., 2004; Furuya et al., 2005; Ball et al., 2007).

Segundo Sandell e Daniel, 1988; Boisen et al., 2000; Hauler e Carter, 2001; Furuya et al., 2004; Rollin et al., 2003; Furuya et al., 2006; Mai et al., 2006 e Gatlin et al., 2007, a lisina é geralmente o aminoácido mais limitante em ingredientes de origem vegetal que substituem a farinha de peixe. Já no corpo e no filé, a lisina é o aminoácido encontrado em maior proporção (Teixeira et al., 2008).

Em quantidades balanceadas com outros aminoácidos, a lisina pode promover algumas vantagens, como a melhora no ganho de peso, conversão alimentar, retenção de nitrogênio e redução da gordura corporal (Bai e Gatlin, 1994; Davies et al., 1997; Berge et al., 1998; Furuya et al., 2006; Marcouli et al., 2006; Nguyen et al., 2014). Além disso a lisina, em ação conjunta com a metionina, promove a síntese da L-carnitina, responsável pelo transporte de ácidos graxos de cadeia longa para dentro da membrana mitocondrial e que gera energia necessária para as funções fisiológicas do animal (Tanphaichitr et al. 1971).

Para determinação do nível adequado de aminoácidos uma opção é a utilização do método de dose-resposta. Nesta metodologia duas técnicas podem ser aplicadas, a suplementação ou a diluição. A técnica da suplementação é utilizada em diversos estudos tendo como objetivo estimar a exigência de aminoácidos, no qual envolve a adição gradual deste componentes em uma dieta basal deficiente no aminoácido limitante (D'Mello, 1982). Contudo, esse procedimento é criticado, uma vez que a suplementação dos níveis do aminoácido testado pode gerar mudanças na relação com outros aminoácidos, podendo provocar respostas não relacionadas ao aminoácido estudado (Fisher e Morris, 1970; Gous, 1980; Gous e Morris, 1985). Outra desvantagem da técnica de suplementação é a dificuldade de obtenção de níveis mais baixos do aminoácido teste (limitante na dieta), sendo necessário aumentar a inclusão de aminoácidos cristalinos nas dietas experimentais (Gous e Morris, 1985).

Já a técnica da diluição, proposta por Fisher e Morris (1970), consiste em promover uma série de diluições de uma dieta com alto teor proteico, limitante no aminoácido teste, com uma dieta isenta de proteína, mantendo os níveis iguais de energia. Com a aplicação do método é possível obter os níveis intermediários do aminoácido teste e manter a relação entre os aminoácidos, colaborando para a avaliação das respostas do aminoácido em estudo, sem a interferência de outros componentes da dieta (Gous e Morris, 1985). Além disso, a técnica evita a inclusão de uma elevada quantidade de aminoácidos sintéticos nas dietas (Abboudi et al., 2007).

Desta forma, os estudos de avaliação de exigência de lisina são de relevância para o avanço da pesquisa em nutrição de peixes, pois o uso da técnica da diluição é uma alternativa para estimar a exigência de lisina e dos demais aminoácidos através da aplicação do conceito de proteína ideal, para tilápias em fases de crescimento ainda não estudadas. A contribuição de informações geradas complementarão os estudos anteriores realizados pela técnica de suplementação, fornecendo subsídios para novas pesquisas que visem por dietas mais precisas e que impactem menos o ambiente aquático.

MANUSCRITO - DIETARY LYSINE REQUIREMENT IN DIFFERENT GROWTH STAGES OF NILE TILAPIA BY THE DILUTION TECHNIQUE

A ser enviado para a revista Aquaculture.

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Resumo

Três experimentos foram conduzidos para determinar a exigência de lisina digestível, mantença e eficiência de utilização da lisina para estabelecer o perfil de aminoácidos essenciais pelo conceito de proteína ideal para diferentes fases de crescimento da tilápia do Nilo. Os peixes foram distribuídos em um delineamento inteiramente ao acaso com oito tratamentos e quatro repetições com 15 peixes por caixa na fase inicial (2,35 ± 0,05 g) e sete tratamentos e três repetições com 11 peixes e 9 peixes por caixa nas fases de crescimento (433,18 ± 12,49 g) e terminação (733,34 ± 11,24 g). Os tratamentos foram organizados em sete dietas isoenergéticas com níveis crescentes de lisina digestível e uma dieta adicional contraprova (3,59; 5,94; 8,85; 12,03; 15,03; 18,74 e 23,05 g kg^{-1}) na fase inicial e seis níveis crescentes de lisina digestível e uma dieta adicional contraprova (3,68; 7,14; 10,40; 13,58; 17,10 e 20,02 g kg^{-1}) para as fases de crescimento e terminação, formulados pela técnica da diluição. O variáveis ganho de peso, deposição de proteína, taxa de eficiência alimentar e rendimento de filé (apenas para as fases de crescimento e terminação) obtiveram maiores respostas mediante ao nível crescente de lisina na dieta. A taxa de eficiência proteica apresentou melhores resultados na dieta contendo 5.94 g kg^{-1} de lisina, na fase inicial e para as fases posteriores, entre os níveis de 10.40 e 13.58 g kg^{-1}. Os níveis ótimos de lisina digestível foram estimados pela variável ganho de peso, resultando nas exigências de 19,4, 14,6 e 13.6 g kg^{-1} (matéria natural) para fases inicial, crescimento e terminação, respectivamente. A exigência de mantença e a eficiência de utilização da lisina foram 19.87 mg $kg^{-0.8}.dia^{-1}$ e 67.68% na fase inicial, 40.26 mg $kg^{-0.8}.dia^{-1}$ e 67.54% na fase de crescimento; 50.25 mg $kg^{-0.8}.dia^{-1}$ e 64.32% na fase de terminação. O perfil ideal de aminoácidos foi determinado para as diferentes fases de crescimento da tilápia do Nilo, sendo importante para a fabricação de dietas com conteúdo aminoacídico balanceado, para juvenis e principalmente para tilápias adultas, no qual pouco se conhece a respeito da exigência de aminoácidos.

Palavras chave: Aminoácidos, dose resposta, exigência de mantença, eficiência de utilização da lisina.

Abstract

Three trials were conducted to determine the requirement of digestible lysine for growth and maintenance and efficiency of lysine utilization and establish the amino acid profile by "ideal protein concept" in different growth stages of Nile tilapia. A completely randomized design was used which consisted of 8 treatments and 4 replicates each and a total of 15 fish with an initial average body weight of 2.35 ± 0.05g were stocked per tank in the started growth phase. The grower and the finisher phase consisted of 7 treatments and 3 replicates each, with 11 (initial average body weight, 433.18 ± 12.49g) and 9 (initial average body weight, 733.34 ± 11.24g) fish stocked per tank, respectively. Isoenergetic diets formulated according to the dilution technique with the supplementation of seven increasing levels of digestible lysine and an additional diet (counter-proof) (3.59, 5.94, 8.85, 12.03, 15.03, 18.74 and 23.05 g kg^{-1}) in the started phase and six increasing levels of digestible lysine and an additional diet (counter-proof) (3.68, 7.14, 10.40, 13.58, 17.10 and 20.02 g kg^{-1}) in the grower and finisher phase, respectively were fed to fish. The body weight gain, protein gain, feed efficiency ratio and fillet yield (only in the grower and finisher phase) were improved with increasing dietary lysine levels in all the trials. Protein efficiency ratio was improved with the supplementation of diets containing 5.94 g kg^{-1} of lysine in the juvenile and from 10.40 to 13.58 g kg^{-1} lysine in the adult tilapias. Based on the weight gain data, the optimum lysine levels were determined to be 19.4, 14.6 and 13.6 g kg^{-1} (as fed basis) in the started, grower and finisher phase, respectively. Maintenance requirement and the efficiency of lysine utilization were 19.87 mg $kg^{-0.8}$ and 67.68% in the started phase, 40.26 mg $kg^{-0.8}$ and 67.54% in the grower phase, and 50.25 mg $kg^{-0.8}.day^{-1}$ and 64.32% in the finisher phase, respectively. The ideal amino acid profile was evaluated in different life stages of Nile tilapia being relevant to improve the quality of practical diets in terms of amino acid balance.

Keywords: Amino acids; dose-response; maintenance requirement; lysine utilization efficiency.

Introduction

Protein composed of balanced quantities of amino acids is an essential nutrient in aquafeeds due to its important role in growth and physiological functions of animals (Wilson and Poe, 1985; Wilson, 2002; Webster and Lim, 2002). Providing the exact amount of dietary amino acids required by fish for maintenance purposes and body protein accretion is essential to achieve maximum growth and nitrogen utilization efficiency. Amongst essential amino acids, lysine is often referred to as the first limiting in feed ingredients with plant feedstuffs sources (Mai et al., 2006; Gatlin et al., 2007). Lysine is commonly used as a reference amino acid in the concept of ideal protein, because it has an important role in protein deposition and metabolic interaction with other amino acids (Ball et al., 2007). The proper supplementation of dietary lysine improves growth performance and health status (Li et al., 2009), nitrogen retention (Cao et al., 2012) and reduces body fat content in fish (Nguyen et al., 2014). As well as, it is responsible for the synthesis of L-carnitine and in combination with methionine, vitamin C and other compounds having an important role to transport the long-chain fatty acids into the mitochondria (Harpaz, 2005).

These advantages are just met, when maintenance demands were satisfied (He et al., 2013). In this context, several studies have been conducted to determined maintenance requirement and utilization efficiency of amino acids of many fish species (Abboudi et al., 2006, 2007; Bodin et al., 2009; He et al., 2013; Kroeckel et al., 2013). These parameters in combination with other inputs obtained by different methodologies are important to elaborate factorial models, that allows the estimation of the essential amino acids requirements at different size of fish (NRC, 2011).

To determine the optimum amino acid level, the most common method used in fish is the dose-response (Madrid et al. 2019). In this procedure two methods can be applied, the supplementation and dilution techniques. The graded supplementation technique is widely used to determined amino acids requirement, which involves the gradual addition of test amino acids in a basal diet deficient in the limiting amino acid (D’Mello, 1982). Even though, this procedure is criticized, since the gradual increase of the levels of test amino acid can causes changes in the amino acid ratio that may result in responses related to another amino acids (Fisher and Morris, 1970; Gous and Morris, 1985). Moreover, at high levels of supplementation the test amino acid could not be the first-limiting in the diet. Another limitation is that to formulate a basal diet with the test amino acid at such a low level, being necessary adding more crystalline amino acids to maintain the other amino acids in adequate proportions (Gous and Morris, 1985).

The dilution technique proposed by Fisher and Morris (1970) is an alternative to supplementation technique, where successive dilutions are made by the mixing of a high protein diet with a nitrogen-free diet in proper proportions maintaining the same energy level. The method allows obtaining the intermediary levels of the test amino acid and maintains the relationship among the amino acids (Gous and Morris, 1985). In addition, the dilution technique allows using a wide range of levels and formulating diets at lowest level without high inclusion of crystalline amino acids, thus enabling determine maintenance requirement.

Among commercially cultured fish species in the world, the Nile tilapia *Oreochromis niloticus* production has been increased because of its fast growth, good disease resistance capability, tolerance to different environmental conditions and high meat quality (Michelato et al., 2016; Xiao et al., 2017). The previous studies regarding the determination of lysine requirements have been based on the graded supplementation method, which in small tilapias are ranging from 13.1 to 21.7g kg^{-1} of lysine in the diet (Santiago and Lovell, 1988; Furuya et al., 2006; Liebert, 2009; Takishita et al., 2009; Bomfim et al., 2010; Furuya et al., 2012; Furuya et al., 2013; Rampe et al., 2014; Diogenes et al., 2016). Little is known about this topic and to our knowledge a single dose-response study is published about adult Nile tilapia reporting the lysine requirement of 14.6 g kg^{-1} (Michelato et al., 2016). Therefore, the current study aimed to assess the optimum digestible lysine requirement for growth and maintenance and efficiency of lysine utilization and the establishment of ideal AA profile for different growth phases of Nile tilapia using dilution technique.

Materials and methods

Ethics on Animal Care and Use

Three feeding assays were conducted at the Aquaculture Center, Sao Paulo State University (UNESP), Jaboticabal, SP, Brazil. The experiments in the started and grower phase, the assays lasted for 44 days, whilst in the finisher phase lasted for 41 days. All procedures described below were according to the ethical guidelines described by the Brazilian College of Animal Experimentation (COBEA) and approved by the Animal Care and Use Committee of the Faculty of Agricultural and Veterinary Sciences, Sao Paulo State University (UNESP) via Protocol No. 012035/17.

Fish and experimental design

Sexually reverted GIFT Nile tilapias used in the present trials were obtained from a local commercial fish farm. Three feeding assays were conducted in order to determine digestible lysine requirements in different growth stages such as starter, grower, and finisher phase. A total of 480 Nile tilapia were used in the starter phase (2.35 ± 0.05 g), 231 fish in grower phase (433.18 ± 12.49g) and 189 fish in finisher phase (733.34 ± 11.24 g). Uniform size fish were randomly assigned to eight treatments with four replicates each (15 fish per replicate) in the starter phase while seven treatments with three replicates each (11 and 9 fish per replicate) in the grower and finisher phase, respectively.

Fish were acclimated to the laboratory conditions for one week while feeding them with a commercial feed twice a day. In the starter phase, fish were maintained in a recirculation water system, distributed among 32 tanks (180-L) provided in a swimming pool heater and independent aeration by blower. In the grower and finisher phase, fish were distributed among 21 tanks (1500-L) with independent aeration in a flow-through system. During the trial, fish were hand fed three times per day until apparent satiety.

During feeding trials, the water quality parameters including pH, dissolved oxygen and total ammonia were measured weekly while temperature was noted daily. Temperature was noted during the three trials was to be 27.42 ± 0.69 ºC; 29.91 ± 0.34

ºC; 29.12 ± 0.41ºC, respectively, pH was 8.19 ± 0.22; 7.83 ± 0.11; 7.70 ± 0.31, respectively and dissolved oxygen was 6.11 ± 0.55 $mg.L^{-1}$; 4.97 ± 0.42 $mg.L^{-1}$; 5.08 ± 0.83 $mg.L^{-1}$ respectively. The un-ionized ammonia measured in the first stage was 0.0019 ± 0.0001 $mg.L^{-1}$ while in the grower and finisher phase the value of 0.0027 ± 0.0002 $mg.L^{-1}$ was observed. The experimental tanks were regularly cleaned on weekly basis to maintain good water quality.

Experimental diets

Digestible lysine levels were obtained using the dilution technique (Fisher and Morris, 1970), where a concentrated (summit) diet was graded diluted with protein-free diet to achieve the required concentrations of the amino acid under study (Table 2 and 3). Two summit diets were formulated in the current study: one for the starter phase and another one for the grower and finisher phase as shown in Table 1. Regardless of the phase, the summit diet was formulated to exceed 1.5 times the level of lysine recommended by Furuya et al. (2010), whereas the other essential amino acids were provided in a concentration which exceeded in 1.8 times the recommendation of the referred authors, thus producing a relative deficiency of lysine with other amino acids of 30%. Similarly, two protein-free diets, one for the starter phase and other for both the grower and finisher phase, were formulated to be isoenergetic with their respective summit diets (Table 1). The chemical composition of concentrated diet, protein-free diet and experimental diets of all trials are shown in Table 2 and 3, respectively.

In the started phase diets were formulated to contain the digestible lysine levels of 3.59, 5.94, 8.85, 12.03, 15.03, 18.74 and 23.05 g kg^{-1} whilst in the grower and finisher phase they were supplemented at the levels of 3.68, 7.14, 10.40, 13.58, 17.10 and 20.02 g kg^{-1} lysine, respectively as shown in Table 2 and 3. Additionally, a counter-proof treatment was added to each assay to make sure if the counter-proof diet will shows a better response than diet 1 while less response than diet 2. If this happens, lysine will be considering the first limiting amino acid in the diets. This extra treatment was produced using the same dilution of the diet with the lowest lysine content and after that it was supplemented with L-lysine in order to provide the same lysine content as added in the second diet (Table 2 and 3). Prior to feed formulation, all ingredients were analyzed according to standard methods of AOAC (2005). Soybean meal, fish meal, broken rice, corn gluten and maize were analyzed for amino acid content using near-infrared spectroscopy (NIRs) by Evonik Industries, Guarulhos - SP. Feed was formulated based on the digestibility coefficients of Brazilian Tables of Tilapia Nutrition (Furuya et al., 2010). After mixing feed ingredients, diets were ground in a mill, sequentially extruded in an experimental extruder (Exteec, Ribeirao Preto, Sao Paulo, Brazil) and dried in an oven-drying at 55 °C for 24h.

Table 1 Formulation of the basal diets in the started, grower and finisher phase (g kg^{-1} diet).

	Started phase		Grower and Finisher phase	
Ingredients	CD[1]	PFD[2]	CD[1]	PFD[2]
Soybean meal	372.60	-	323.80	-
Fish meal*	284.10	-	249.80	-
Broken rice	30.00	-	130.60	-
Corn gluten	15.60	-	20.00	-
Maize (corn)	222.70	-	196.20	-
Corn starch	-	578.26	-	582.56
Cellulose	-	48.80	-	47.50
Soybean oil	-	156.80	-	153.80
Sugar	-	100.00	-	100.00
L-lysine	5.30	-	5.10	-
DL-methionine	3.40	-	3.00	-
L-threonine	9.40	-	8.70	-
L-tryptophan	1.90	-	1.70	-
L-isoleucine	3.60	-	3.40	-
L-phenylalanine	4.90	-	3.50	-
L-histidine	2.00	-	1.80	-
Vitamin and mineral premix[3]	5.00	5.00	5.00	5.00
Limestone	12.90	48.60	17.20	48.60
Dicalcium phosphate	18.20	54.10	21.80	54.10
Antioxidant (BHT)	0.50	0.50	0.50	0.50
Antifungal	3.00	3.00	3.00	3.00
Salt	5.00	5.00	5.00	5.00

[1]Concentrated diet;
[2]Protein-free diet;
[3] Vitamin and mineral premix (Poli-Nutri) Vitamin A: 860.000 UI/kg, Vitamin D3: 240 000 UI/Kg, Vitamin E: 10.500 UI/Kg, Vitamin K3: 1400mg/kg, Vitamin B1: 2100 mg/Kg, Vitamin B2: 2.150 mg/Kg, Vitamin B6: 2100 mg/Kg, Vitamin B12: 2200mg/Kg, Vitamin C: 25000mg/Kg, Niacin : 10000mg/Kg, calcium pantothenate: 5600mg/Kg, folic acid: 580mg/Kg, biotin: 17mg/Kg, choline chloride: 60000 mg/Kg, inositol: 3750mg/Kg, copper: 1800mg/Kg, manganese: 5000mg/Kg, zinc: 8.000mg/Kg, iodine: 90mg/Kg, selenium: 36mg/Kg, cobalt : 55mg/Kg.

Table 2 Chemical composition (g kg^{-1}) of the experimental diets in the started phase (trial 1).

Treatments (g kg^{-1})		CD[1]	PFD[2]	3.59	5.94	8.85	12.03	15.03	18.74	23.05	6.14*
Dilutions (%)[4]	CD[1]	100	0	17	27	40	53	67	83	100	17
	PFD[2]	0	100	83	73	60	47	33	17	0	83
Chemical composition (g.kg^{-1} as -fed basis)											
Dry matter		912.02	918.27	942.55	946.34	949.98	952.97	951.13	951.36	948.59	951.27
Digestible protein		301.22	3.67	60.51	94.92	139.67	186.53	232.76	290.90	348.30	63.11
Digestible energy (Mj. kg^{-1})		12.4	12.43	12.93	13.04	13.17	13.27	13.27	13.28	13.22	13.22
Digestible lipid		37.56	119.19	112.07	103.40	90.98	85.33	69.50	56.71	46.15	113.10
Ash		95.99	90.90	98.47	99.71	101.48	102.67	103.28	103.48	103.45	99.39
Essential digestible amino acids (g.kg^{-1} as -fed basis)											
Methionine		7.89	<1.00	1.32	2.12	3.18	4.76	6.16	7.68	9.54	1.50
Lysine		18.90	<1.00	3.59	5.94	8.85	12.03	15.03	18.74	23.05	6.14
Threonine		17.09	<1.00	3.02	5.11	7.47	10.57	13.56	17.02	21.46	3.51
Arginine		19.12	<1.00	3.44	5.68	8.39	11.85	14.72	18.53	22.23	3.68
Isoleucine		13.14	<1.00	2.48	4.12	6.22	8.86	11.15	14.05	16.61	2.68
Leucine		20.94	<1.00	3.97	6.25	9.23	12.60	15.47	19.38	23.09	4.08
Valine		12.28	<1.00	2.38	3.81	5.62	7.75	9.61	11.87	14.30	2.50
Histidine		8.03	<1.00	1.47	2.31	3.35	4.68	5.74	7.28	8.74	1.57
Phenylalanine		18.60	<1.00	3.30	5.24	7.56	10.33	12.85	16.02	19.33	3.26
Tryptophan		4.92	<1.00	0.85	1.32	2.09	2.49	3.27	4.34	5.26	0.83
Non-essential amino acids (g.kg^{-1} as -fed basis)											
Glycine		21.39	<1.00	4.05	6.66	9.96	13.88	17.39	21.67	26.29	4.37
Serine		14.52	<1.00	2.78	4.47	6.55	8.65	10.71	13.47	16.09	2.81
Proline		19.58	<0.10	3.47	5.51	8.08	11.94	14.65	18.61	21.87	3.73
Alanine		17.98	<1.00	3.49	5.60	8.30	11.37	14.18	17.68	21.30	3.65
Aspartic acid		30.32	<1.00	5.81	9.33	13.70	18.38	22.81	28.60	34.24	5.92
Glutamic acid		49.55	<1.00	9.45	15.09	22.30	3.55	4.40	5.33	6.51	9.65
Cysteine		3.99	<1.00	0.73	1.14	1.71	2.21	2.76	3.43	4.12	0.74

[1]Concentrated diet;
[2]Protein-free diet
[3]Digestible nutrients according with Brazilian Tables of Tilapia Nutrition (Furuya et al. 2010);
[4]Proportions of concentrated and protein-free diets; * Counter-proof diet (Diet 6.14 g kg^{-1} = diet 3.59 g kg^{-1} + 0.018g L-lysine/kg of feed).

Table 3 Chemical composition (g kg^{-1}) of the experimental diets in the grower (trial 2) and finisher (trial 3) phase.

		CD[1]	PFD[2]	3.68	7.14	10.40	13.58	17.10	20.02	6.85*
Dilutions (%)[4]	CD[1]	100	0	17	33	50	67	83	100	17
	PFD[2]	0	100	83	67	50	33	17	0	83
Chemical composition (g.kg^{-1} as -fed basis)										
Dry matter		908.11	923.48	944.85	945.16	941.32	942.28	938.86	934.62	940.07
Digestible protein		281.61	5.45	54.76	102.75	153.50	204.46	252.81	300.71	56.45
Digestible Energy (Mj. kg^{-1})		12.16	12.36	12.91	12.96	12.95	13.00	12.95	12.90	12.89
Digestible lipid		38.15	118.22	111.61	94.27	82.91	66.59	52.30	35.87	109.88
Ash		97.98	89.96	95.55	97.60	98.68	99.00	101.34	103.57	94.44
Essential digestible amino acids (g.kg^{-1} as -fed basis)										
Methionine		6.65	<1.00	1.45	2.72	4.11	5.38	6.65	7.90	1.32
Lysine		17.88	1.2	3.68	7.14	10.40	13.58	17.10	20.02	6.85
Threonine		14.88	<1.00	3.31	6.56	9.53	12.23	15.08	17.66	3.10
Arginine		17.47	<1.00	3.33	6.61	9.97	13.15	16.75	19.67	3.21
Isoleucine		13.08	<1.00	2.58	5.14	7.40	9.82	12.20	14.29	2.41
Leucine		19.23	2.1	3.72	7.07	10.62	14.04	17.39	20.78	3.56
Valine		11.57	1.2	2.26	4.30	6.44	8.65	10.69	12.68	2.15
Histidine		7.24	<1.00	1.41	2.71	3.97	5.15	6.40	7.45	1.34
Phenylalanine		14.73	1.9	2.92	5.65	8.38	11.04	13.68	16.37	2.75
Tryptophan		4.05	<1.00	0.77	1.49	2.23	2.90	3.59	4.26	0.72
Non-essential amino acids (g.kg^{-1} as -fed basis)										
Glycine		19.35	1	3.95	7.64	11.27	14.72	18.30	21.54	3.71
Serine		13.58	<1.00	2.53	4.99	7.33	9.55	11.89	14.20	2.41
Proline		16.37	1.2	3.35	6.47	9.78	12.91	16.23	19.73	3.36
Alanine		16.78	1.4	3.36	6.43	9.53	12.50	15.49	18.33	3.18
Aspartic acid		28.08	1.6	5.39	10.42	15.42	20.21	25.27	29.91	5.13
Glutamic acid		45.87	3.5	8.80	16.87	25.21	33.40	41.73	49.42	8.38
Cysteine		3.67	<1.00	0.71	1.33	2.01	2.64	3.33	3.96	0.70

[1]Concentrated diet;
[2]Protein-free diet;
[3]Digestible nutrients according with Brazilian Tables of Tilapia Nutrition (Furuya et al. 2010);
[4]Proportions of concentrated and protein-free diets; * Counter-proof diet (D7(6.85 g kg^{-1}) = D1(3.68 g kg^{-1}) + 0.058g L-lysine/kg of feed).

Samples collection and chemical analyses

At the beginning of each feeding assay, 15 fish were randomly sampled for whole-body composition and amino acid analyses. Fish were fasted for 24h and euthanized using benzocaine (4-Aminobenzoic acid ethyl ester; Sigma - Aldrich, Brazil) overdose 175 mg L^{-1} in the started phase and 300 mg L^{-1} in the grower and finisher phase. Five fish per tank were randomly collected at the end of each assay except the started phase where eleven fish per treatment were sampled due the small size of fish and stored at -20 ºC for further whole-body composition analysis. For all the phases assessed, four fish were sampled to determine the hepatosomatic index (HIS). The fish being used for the analysis of HIS were first subjected to calculate the fillet yield in the grower and finisher phase.

Chemical analyses of diets and fish were determined according to the standard methods of AOAC (2005). Total lipid in fish body was determined by extraction with petroleum ether in a fat extractor (Ankon technology®) and in diets by acid hydrolysis technique. Crude protein was determined by the method of Dumas in a Leco 528 LC apparatus (Etheridge et al., 1998). Dry matter was determined by oven-drying the samples at 105 °C for 12h. Ash was determined by combustion in a muffle furnace at 550ºC. The amino acid content was determined using High Performance Liquid Chromatograph (HPLC) in the diets (Evonik Industries, Essen, Germany) and in the fish body (CBO - Laboratory, University of Campinas (UNICAMP), SP, Brazil) after acid hydrolysis. Tryptophan was determined after alkaline hydroxylation of the sample with lithium hydroxide.

Calculations

Body weight gain (BWG), feed intake (FI), feed efficiency ratio (FCR), protein efficiency ratio (PER) and survival rate (SR) was calculated in all of the three phases while the fillet yield (FY) was just calculated in the grower and finisher phase, according to the following formulas:

Body weight gain (g) = final body weight – initial body weight

Feed intake (g) = dry feed consumed x numbers of $fish^{-1}$

Feed efficiency ratio ($g.g^{-1}$) = body weight gain x feed intake^{-1}

Protein efficiency ratio ($g.g^{-1}$) = body weight gain x digestible protein intake^{-1}

Fillet yield (%) = 100 x fillet weight x body weight^{-1}

Survival rate (%) = 100 x number of final fish x number of initial fish^{-1}

Protein gain (g) = (final protein content in the body x final live weight) – (initial protein content in the body x initial live weight)

Lysine gain (g) = (final lysine content in the body x final body protein weight - initial lysine content in the body x initial body protein weight)

After determining the optimum level of digestible lysine, the percentage of amino acids based on whole-body protein was established. The amino acid profile, called the “ideal EAA ratio”, was determined based on the ratio of body amino acids of Nile tilapia according to the equation suggested by Arai (1981) : $(EAA/\sum EAA)*1000$ = (essential amino acid/ total sum of essential amino acids + cystine + tyrosine) x 1000. The other amino acids requirements were stablished according to Kaushik’s equation, (Kaushik, 1998): (digestible lysine requirement / the lysine value obtained by Arai’s formula) x the value of the amino acid (except lysine) estimated by Arai’s formula (1981).

Maintenance and efficiency of lysine utilization

The lysine maintenance requirement was estimated by monomolecular model (Kuhi et al. 2001), considering the lysine gain mg $kg^{-0.8}$ day^{-1} (dependent variable) and lysine intake mg $kg^{-0.8}.day^{-1}$ (independent variable), as in the following equation:

$$L = L_{max}*(1-e^{(-k*(x-m))})$$

Where, L is the lysine deposition, L_{max} is the maximum lysine deposited in the body (mg $kg^{-0.8}$ day^{-1}), k is a fractional rate parameter, x is the lysine intake (mg $kg^{-0.8}$ day^{-1}) and m is the lysine intake at maintenance.

The efficiency of lysine utilization was obtained by the relationship between the parameters L and R (k_{lys}= (L / R) x100) generated by broken-line model (Y = L+ U (R – X_{adj}), where L is the breakpoint on the y-axis, R is the breakpoint on the x-axis, U is the slope and X_{adj} is the digestible lysine intake adjusted for the maintenance. The X_{adj} was calculated as follow:

$$X_{adj} = (m - LI) \times BW^{0.8}$$

Where, *m* is the maintenance requirement for lysine (mg $kg^{-0.8}$ day^{-1}), *LI* is the lysine intake (mg $kg^{-0.8}$ day^{-1}) and $BW^{0.8}$ is the metabolic weight. The metabolic weight exponent used in this work was 0.8, which was according to a previous study for Nile tilapia (Trung et al., 2011).

Statistical analysis

Data were analyzed by ANOVA using the GLM procedure. Overall treatment effects were accepted as statistically significant when $P<0.05$. Differences between performance traits of fish fed by the lowest digestible lysine level with those fed counter-proof diets were compared using Dunnett's test. Collected data of growth response were fitted by broken-line model using NLMIXED procedure and body weight gain chose to determine the optimum level of digestible lysine. All procedures were conducted using SAS 9.4 (SAS Inst. Inc., Cary, NC).

Results

Growth responses

In all phases assessed, fish fed with counter-proof treatment exhibited improvements in growth performance compared with those fed with diets containing the lowest lysine level according to Dunnett's test ($P < 0.05$). This confirms that indeed lysine was the first limiting amino acid in experimental diets (Tables 4 and 5). The mortality rates were recorded throughout the grower and finisher phase and were observed unaffected by the dietary digestible lysine ($P > 0.05$) as shown in Table 5. No mortality was observed in the started phase (Table 4). Growth performance parameters of Nile tilapia in the started phase are showed in (Table 4) and those of grower and finisher phase (Table 5).

According to broken-line model, in the first trial (started phase) the better body weight gain (52.90 g) and protein gain (8.02 g) were estimated to be 19.37 and 20.09 g kg^{-1} of lysine, respectively, (Fig. 1 and Table 6). Feed intake (FI) (50.93 g) showed an improvement at 17.39 g kg^{-1} and feed efficiency (FE) (1.01 $g.g^{-1}$) at 15.42 g kg^{-1} of lysine (Table 6). Protein efficiency ratio (PER) was not possible to be fitted by broken-line model in all essays; however the high values were showed at 5.90 and 8.80 g kg^{-1} of lysine (Table 4).

In the grower phase, the higher body weight gain (193.43 g) was estimated at 14.59 g kg^{-1} and protein gain (34.64 g) at 17.86 g kg^{-1} of lysine, (Fig. 2 and Table 6). The optimum lysine level for the FI (270.50 g) and FE (0.69 $g.g^{-1}$) were obtained at 12.62 and 12.12 g kg^{-1} (Table 6). PER was improved at 10.40 g kg^{-1} of lysine (Table 5).

In the last trial (finisher phase), the optimum level of digestible lysine for BWG (257.75 g) and PG (44.56 g) was estimated to be 13.6 and 14.36 g kg^{-1}, (Fig. 3 and Table 6). The better values of FI (451.09 g) and FE (0.58 $g.g^{-1}$) were obtained at 12.77 and 11.94 g kg^{-1} of lysine (Table 6). A similar values of PER was found at 10.40 and 13.60 g kg^{-1} of lysine and the fillet yield was higher when fish were fed with 20.0 g kg^{-1} of lysine in the grower and finisher phase (Table 5). Hepatosomatic index (HSI) was affected by diets in the started and finisher phase, although in the grower phase HSI was not affected by dietary lysine levels (Table 4 and 5).

Table 4 Growth performance of Nile tilapia in the started phase fed graded levels of dietary digestible lysine.

Treatments (g kg^{-1} as-fed basis)	3.60	5.90	8.80	12.00	15.00	18.70	23.00	6.10[a]	*P* value
Initial Body Weight (g)	2.36 ± 0.05	2.35 ± 0.09	2.33 ± 0.06	2.33 ± 0.05	2.36 ± 0.06	2.34 ± 0.04	2.36 ± 0.04	2.36±0.05	0.9782
Body Weight Gain(g)	0.77 ± 0.07	2.54 ± 0.15	9.63 ± 0.84	25.23 ± 2.07	41.44 ± 2.81	50.05 ± 1.88	52.90 ± 2.16	1.00 ± 0.09*	<0.0001
Protein gain (g)	0.07 ± 0.01	0.26 ± 0.01	1.19 ± 0.08	3.42 ± 0.28	5.77 ± 0.34	7.45 ± 0.31	8.02 ± 0.29	0.08 ± 0.01	<0.0001
Feed Intake (g.fish^{-1})	3.5 ± 0.14	5.13 ± 0.25	13.82 ± 1.00	31.01 ± 2.43	44.35 ± 3.29	50.10 ± 0.98	51.76 ± 1.71	3.52±0.20	<0.0001
Feed Efficiency Ratio (g.g^{-1})	0.22 ± 0.02	0.50 ± 0.01	0.70 ± 0.02	0.81 ± 0.01	0.93 ± 0.01	1.00 ± 0.02	1.02 ± 0.02	0.28 ± 0.02*	<0.0001
Protein Efficiency Ratio (g.g^{-1})	3.62 ± 0.29	5.22 ± 0.09	4.98 ± 0.15	4.36 ± 0.07	4.02 ± 0.06	3.43 ± 0.06	2.93 ± 0.06	4.49 ± 0.31*	<0.0001
HSI (%)[1]	1.18 ± 0.71	2.07 ± 0.51	2.53 ± 0.78	2.21 ± 0.57	2.45 ± 0.12	1.79 ± 0.09	2.54 ± 0.31	1.25 ± 0.57	0.0027
Survival (%)	100	100	100	100	100	100	100	100	1

[a] Counter-proof diet
[1] Hepatosomatic index
* Counter-proof diet was statistically different than diet with 3.60 g kg^{-1} to BWG, FE and PER based on Dunnett's test (P <0.05)
Values were expressed as mean (± SD)

Table 5 Growth performance of Nile tilapia in the grower and finisher phases fed graded levels of dietary digestible lysine.

Treatments (g kg^{-1} as-fed basis)	3.70	7.10	10.40	13.60	17.10	20.00	6.90[a]	*P* value
Grower phase								
Initial Body Weight (g)	432.79 ± 23.28	433.70 ± 16.26	433.85 ± 21.91	432.61 ± 7.52	430.71 ± 2.25	433.38 ± 11.61	435.39 ± 7.17	0.9999
Body Weight Gain (g)	-8.06 ± 0.34	33.76 ± 5.80	130.60 ± 28.27	169.00± 21.73	186.80 ± 2.98	200.06 ± 17.95	-6.79 ± 0.20 *	<0.0001
Protein gain (g)	-5.64 ± 0.38	-0.50 ± 1.11	14.88 ± 2.44	23.33 ± 4.43	31.37 ± 0.16	34.64 ± 3.41	-3.93 ± 0.44 *	<0.0001
Feed Intake (g. fish^{-1})	117.03 ± 5.49	131.79 ± 9.96	245.18 ± 33.62	274.97 ± 39.39	265.19 ± 11.92	271.34 ± 9.89	91.50 ± 3.73	<0.0001
Feed Efficiency Ratio (g.g^{-1})	-0.07 ± 0.00	0.26 ± 0.03	0.53 ± 0.05	0.62 ± 0.05	0.71 ± 0.03	0.74 ± 0.04	-0.07 ± 0.00	<0.0001
Protein Efficiency Ratio (g.g^{-1})	-1.26 ± 0.09	2.48 ± 0.25	3.44± 0.31	3.02 ± 0.25	2.79 ± 0.11	2.45 ± 0.13	-1.32 ± 0.08	<0.0001
Fillet Yield (%)	28.69 ± 1.04	29.04 ± 0.59	28.80 ± 0.67	31.16 ± 1.28	31.73 ± 1.57	32.49 ± 1.39	28.87 ± 0.67	0.0018
HSI (%)[1]	1.46 ± 0.31	1.39 ± 0.18	1.38 ± 0.16	1.41 ± 0.18	1.15 ± 0.04	1.00 ± 0.15	1.34 ± 0.06	0.0515
Survival (%)	96.97 ± 5.25	90.91 ± 9.09	96.97 ± 5.25	100 ± 0.00	96.97 ± 5.25	96.97 ± 5.25	87.88 ± 5.25	0.2306
Finisher phase								
Initial Body Weight (g)	739.70 ± 9.68	723.19 ± 14.31	747.89 ± 17.14	730.26 ± 3.48	731.94 ± 5.79	731.54 ± 0.72	728.89 ± 6.31	0.1407
Body Weight Gain (g)	-30.95 ± 2.25	44.29 ± 18.17	162.83 ± 13.14	263.07 ± 22.16	232.06 ± 16.69	283.35 ± 21.41	-22.85 ± 2.31 *	<0.0001
Protein gain (g)	-13.80 ± 0.94	1.16 ± 3.18	19.58 ± 1.67	42.69 ± 5.57	40.13 ± 5.20	48.99 ± 2.75	-7.55 ± 0.75 *	<0.0001
Feed Intake (g. fish^{-1})	172.55 ± 9.33	244..76 ± 39.13	387.70 ± 22.77	467.28 ± 23.73	417.58 ± 16.62	468.41 ± 32.87	125.97 ± 6.87	<0.0001
Feed Efficiency Ratio (g.g^{-1})	-0.18 ± 0.02	0.18 ± 0.05	0.42 ± 0.03	0.56 ± 0.02	0.56 ± 0.02	0.60 ± 0.01	-0.18 ± 0.02	<0.0001
Protein Efficiency Ratio (g.g^{-1})	-3.28 ± 0.33	1.71 ± 0.49	2.74 ± 0.18	2.76 ± 0.27	2.20 ± 0.07	2.01 ± 0.04	-3.22 ± 0.39	<0.0001
Fillet Yield (%)	28.85 ± 0.83	29.80 ± 1.12	31.72 ± 0.89	31.41 ± 1.27	33.23 ± 0.57	34.56 ± 0.46	29.27 ± 1.06	<0.0001
HSI (%)[1]	3.12 ± 0.47	2.82 ± 0.30	2.71 ± 0.20	2.45 ± 0.24	2.01 ± 0.43	1.52 ± 0.48	1.84 ± 0.21	0.0006
Survival (%)	96.97 ± 5.25	100 ± 0.00	93.94 ± 10.50	90.91 ± 15.75	96.97 ± 5.25	100 ± 0.00	93.94 ± 5.25	0.7702

[a] Counter-proof diet
[1] Hepatosomatic index
* Counter-proof diet was statistically higher than diet with 3.70 g kg^{-1} in both phases for BWG and PG based on Dunnett's test. (P <0.05).
Values were expressed as mean (± SD).

Table 6 Equations fitted by broken-line model for feed intake, feed efficiency ratio and protein gain of Nile tilapia feed with increased level of digestible lysine.

Variable	Equation	Optimum level of digestible lysine (g kg^{-1})	R^2
Started phase			
Body weight gain	(52.90 – 3.60 (19.37 – x)	19.37	0.97
Protein gain	(8.02 - 0.53 (20.09 - x)	20.09	0.97
Feed intake	(50.93 -3.77 (17.39 – x)	17.39	0.97
Feed efficiency ratio	(0.99 – 0.07 (13.80 – x)	13.80	0.96
Grower phase			
Body weight gain	(193.43 – 19.03 (14.59 – x)	14.59	0.95
Protein gain	(34.64 -2.94 (17.86 - x)	17.86	0.97
Feed intake	(270.50 – 19.05 (12.62 – x)	12.62	0.87
Feed efficiency ratio	(0.69 – 0.09 (12.121 – x)	12.12	0.97
Finisher phase			
Body weight gain	(257.75 – 30.25 (13.60 -x)	13.60	0.96
Protein gain	(44.56 -5.68 (14.36 - x)	14.36	0.96
Feed intake	(451.09 – 32.06 (12.77 – x)	12.77	0.93
Feed efficiency ratio	(0.58 – 0.09 (11.94 – x)	11.94	0.98

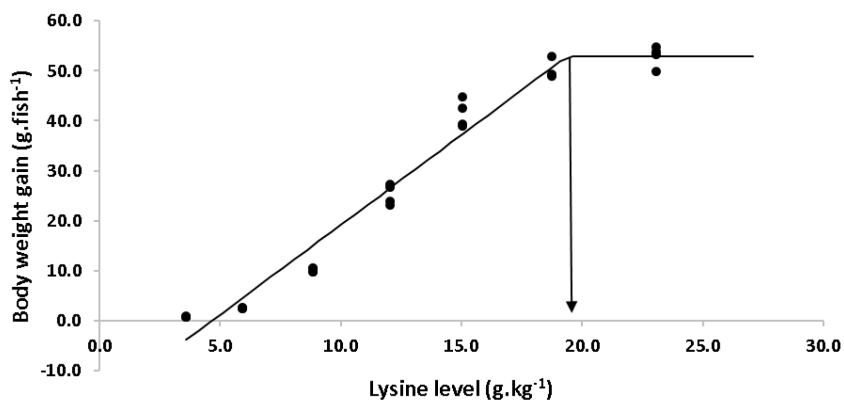

Figure 1 - Body weight gain in started phase (Y = 52.90 + (-3.60 * (19.37 - lysI)); R^2= 0.97), fitted by linear response plateau, fed with different levels of digestible lysine during 44 days. Each point represent one group of fish (N=15). Where lysI = lysine in intake (g kg^{-1}).

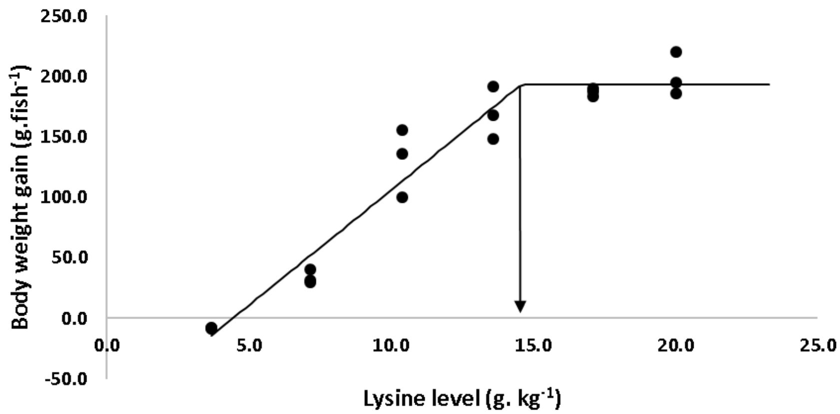

Figure 2 - Body weight gain for grower phase (Y = (193.43 + (-19.03)*(14.59 -lysI)); R^2= 0.95), fitted by linear response plateau, fed with different levels of digestible lysine during 44 days. Each point represent one group of fish (N=11). Where lysI = lysine intake (g kg^{-1}).

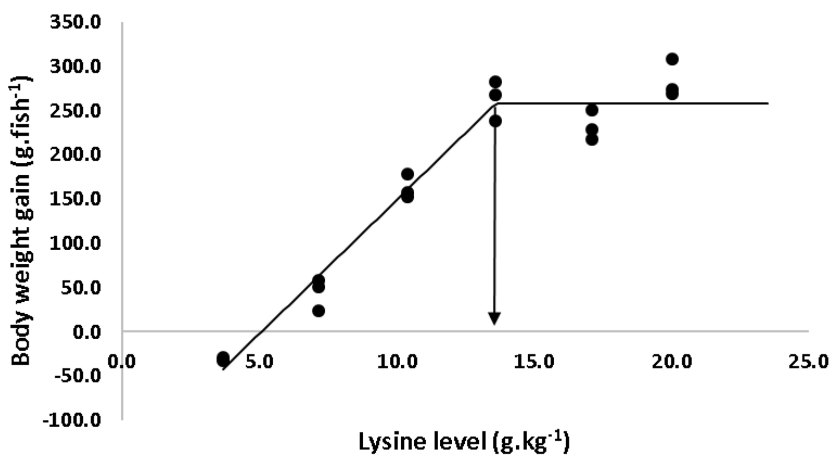

Figure 3 - Body weight gain in finisher phase (Y = 257.75 + (-30.25 * (13.60 - lysI)); R^2= 0.96), fitted by linear response plateau, fed with different levels of digestible lysine during 41 days. Each point represent one group of fish (N=9). Where lysI = lysine intake (g kg^{-1}).

Maintenance and efficiency of lysine utilization

The lysine requirements of maintenance in the started, grower and finisher phase were defined at 19.87, 40.26 and 50.25 mg $kg^{-0.8}$ day^{-1}, respectively, (Fig. 4). The efficiency of lysine utilization was estimated at 67.68% (klys=12.13/17.92) in the started phase (y=12.12 -0.67 (17.92- X_{adj})), 67.54% (klys=56.30/83.37) in the grower phase (y=56.30 -0.68 (83.37- X_{adj})) and 64.32% (klys=83.97/130.54) in the final growing phase (y=83.97 -0.67 (130.54- X_{adj})).

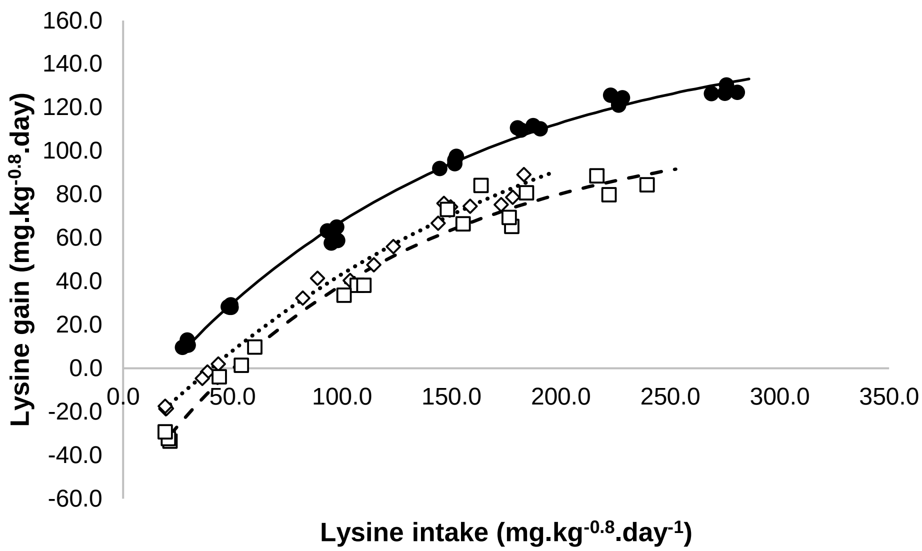

Figure 4 - Lysine requirement for maintenance in the started phase (black balls) [y=157.60*(1-exp(-0.01*(x-19.87)))], grower phase (diamonds) [y=162.6*(1-exp(-0.01*(x-40.26)))] and finisher phase (squares) [y=112*(1-exp(-0.01*(x-50.25)))] fitted by the monomolecular model.

Ideal protein concept

The optimum level of digestible lysine based on body weight gain determined in this dose-response study was used to calculate the ideal essential amino acid profile for Nile tilapia in different phases, as shown in Table 7.

Table 7 The requirement of digestible essential amino acids (EAA) for different growth stages of Nile tilapia calculated by the concept of "ideal protein".

	Started phase			Grower phase			Finisher phase		
EAA	% aacwbp[1]	(ARAI, 1981)[2]	IAAP ($g.kg^{-1}$)[3]	% aacwbp[1]	(ARAI, 1981)[2]	IAAP ($g.kg^{-1}$)[3]	% aacwbp[1]	(ARAI, 1981)[2]	IAAP ($g.kg^{-1}$)[3]
Histidine	2.43	54.25	6.84	2.20	48.07	4.50	2.21	49.38	4.22
Arginine	6.03	134.70	16.97	6.58	143.53	13.44	6.89	153.57	13.12
Threonine	4.11	91.73	11.56	4.17	90.99	8.52	4.10	91.48	7.82
Valine	4.18	93.39	11.77	4.23	92.40	8.65	4.08	90.98	7.77
Methionine	2.24	50.09	6.31	2.36	51.57	4.83	2.07	46.26	3.95
Isoleucine	3.73	83.27	10.49	3.84	83.73	7.84	3.63	81.01	6.92
Leucine	6.48	144.75	18.24	6.56	143.06	13.40	6.29	140.33	11.99
Phenylalanine	3.76	83.85	10.56	3.77	82.30	7.71	3.63	81.04	6.92
Lysine[4]	6.90	153.98	**19.40**	7.15	155.92	**14.60**	7.14	159.17	**13.60**
Tryptophan	1.15	25.63	3.23	1.25	27.23	2.55	1.03	22.91	1.96
Tyrosine	2.53	56.59	7.13	2.45	53.46	5.01	2.38	53.10	4.54
Cystine	1.24	27.78	3.50	1.27	27.73	2.60	1.38	30.76	2.63
ΣAAE+Cis+Tir[5]	44.79			45.83			44.83		

[1]% Amino acid content in whole- body protein (Fish analyzed: n =20)
[2] AAE/ ∑AAE x 1000 (Arai, 1981)
[3] Ideal amino acid profile ($g\ kg^{-1}$) (Kaushik, 1998)
[4] Sum of essential amino acids + cystine + tyrosine
*Amino acid requirement based on weight gain

Whole-body composition

In whole-body composition, moisture, crude protein, total lipid and ash were statistically affected in the started and grower phase by dietary lysine levels as shown in Table 8 and 9. Also in the finisher phase, whole body composition was statistically affected by dietary lysine levels, except moisture.

Table 8 Whole-body proximate composition (g 100 g^{-1}) of Nile tilapia, corresponding to started phase, fed graded levels of dietary lysine.

	Initial	Dietary lysine levels (g kg^{-1})							
		3.59	5.94	8.85	12.03	15.03	18.74	23.05	*P* value
Moisture	78.09	73.75 ± 0.77	72.77 ± 0.48	72.12 ± 0.14	71.14 ± 0.34	71.29 ± 0.29	72.24 ± 0.17	73.45 ± 0.29	<0.0001
Crude Protein	13.52	13.06 ± 0.11	12.16 ± 0.14	12.26 ± 0.27	13.63 ± 0.11	13.96 ± 0.13	14.86 ± 0.15	15.14 ± 0.2	<0.0001
Crude Lipid	4.61	7.51 ± 0.61	9.40 ± 0.37	11.24 ± 0.53	11.47 ± 0.4	10.67 ± 0.31	8.96 ± 0.14	7.30 ± 0.13	<0.0001
Ash	3.23	5.00 ± 0.12	4.43 ± 0.13	3.65 ± 0.19	3.58 ± 0.05	3.58 ± 0.07	3.58 ± 0.08	3.64 ± 0.13	<0.0001

Values were expressed as mean (± SD).

Table 9 Whole-body proximate composition (g 100 g^{-1}) of Nile tilapia, corresponding to grower and finisher phases, fed graded levels of dietary lysine.

		Dietary lysine levels (g kg^{-1})						
	Initial	3.68	7.14	10.40	13.58	17.10	20.02	*P* value
Grower phase								
Moisture	68.26	65.83 ± 1.29	66.02 ± 0.55	66.65 ± 0.59	67.93 ± 1.02	69.05 ± 0.34	69.41 ± 0.56	0.0003
Crude Protein	15.84	14.81 ± 0.04	14.59 ± 0.11	14.81 ± 0.47	15.27 ± 0.50	16.13 ± 0.10	16.73 ± 0.16	<0.0001
Crude Lipid	11.29	13.49 ± 1.00	13.46 ± 0.61	13.69 ± 0.68	12.24 ± 0.90	10.80 ± 0.36	10.04 ± 0.47	<0.0001
Ash	3.73	4.99 ± 0.19	4.77 ± 0.10	4.58 ± 0.18	4.11 ± 0.14	4.11 ± 0.12	4.21 ± 0.17	<0.0001
Finisher phase								
Moisture	68.16	65.18 ± 0.45	65.70 ± 0.31	65.83 ± 0.24	64.96 ± 0.91	66.99 ± 0.24	66.97 ± 1.00	0.1135
Crude Protein	15.27	13.99 ± 0.16	14.54 ± 0.18	14.69 ± 0.23	15.52 ± 0.44	15.75 ± 0.26	15.84 ± 0.32	<0.0001
Crude Lipid	11.85	14.36 ± 0.42	14.11 ± 0.66	14.59 ± 0.31	14.20 ± 0.17	12.19 ± 0.11	11.97 ± 0.32	0.0059
Ash	5.00	5.95 ± 0.57	5.27 ± 0.51	4.44 ± 0.15	4.87 ± 0.57	4.58 ± 0.09	5.50 ± 0.84	0.0234

Values were expressed as mean (± SD).

Discussion

Dietary lysine supplementation has significantly improved the growth performance and feeding aspects of fish in several works (Michelato et al., 2016; Ye et al., 2017; Madrid et al., 2019). In the present study, body weight gain (BWG) and protein gain (PG) was significantly increased among the different life stages of Nile tilapia. However, the lowest level of lysine resulted in lower BWG and PG in the grower and finisher phase that may be related to the lack of enough amounts of dietary lysine for maintenance demands (Table 5). Feed intake increased in response to the dietary lysine among different life stages. On the other hand, feed efficiency ratio (FER) and protein efficiency ratio (PER) showed a decreased behavior throughout the growth stages. The same observations were noticed by Abdel-Tawwab et al. (2010) in Nile tilapia, Árnason et al. (2010) in Atlantic cod (*Gadus morhua L.*) and Ye et al. (2017) in Gibel carp (*Carassius auratus gibelio*). The higher FER and PER observed in juvenile Nile tilapia than adults could be related to the high demand of small fish for nutrients to maintain fast growth rate (Sá et al. 2008) and thus, juvenile fish use nutrients more efficiently than large fish. Fillet yield (FY) was enhanced with the fish size as noticed in grower (32.49%) and finisher phase (34.56%) at high dietary lysine level (Table 4 and 5). In concordance with the present study, Michelato et al. (2016) has found the maximum FY of 30.69% for Nile tilapia with initial weight of about 270 g and final weight 570 g, indicating a possible improved of FY not only by lysine levels, but also in relation to body size.

Many studies have been done in the last two decades about lysine requirement of Nile tilapia. Santiago and Lovell (1988) recommend the optimum lysine level at 14.3g kg^{-1} of total lysine (5.1% of dietary protein) (0.04 to 1.65 g of live weight). Furuya et al. (2006) estimated for the same specie 14.4 g kg^{-1} of digestible lysine (as-fed basis) (5.23% of dietary protein) (5 to 38 g). A high level of digestible lysine was obtained by Takishita et al. (2009) for the maximum weight gain 21.7 g kg^{-1} (as-fed basis) (5.93% of dietary protein) (1 to 11 g). In contrast, Bomfim et al. (2010) and Rampe et al. (2014) recommended a low level at 17.0 and 18.4 g kg^{-1} (as-fed basis) (6.18 and 6.86% of dietary protein), respectively, at similar size of fish and parameter evaluated. In the present study, the requirement estimated based on weight gain in the started phase was 19.4 g kg^{-1} (as-fed basis) (6.36% of dietary protein) which is close to the requirement established by Takishita et al. (2009) and Rampe et al. (2014).

Liebert (2009) using an exponential N-utilization model reported 16.3 g kg^{-1} (DM) (4.8% of dietary protein) of total lysine for 187 mg daily protein deposition for juvenile Nile tilapia (50 g BW, feed intake at 3% of BW). The results obtained by Furuya et al. (2012, 2013), recommended 15.21 g kg^{-1} of digestible lysine (as-fed basis) (5.41 of dietary protein) (1.44 to 5.78 g) based on maximum protein gain and 13.1 g kg^{-1} (as-fed basis) (4.68 % of dietary protein) (87 to 206 g) in balanced diets for arginine to lysine ratio for maximum weight gain. Diogenes et al. (2016) proposing the ideal amino acids profile have found 15.6 g kg^{-1} (5.01% of dietary protein) using the deletion technique (20 to 56 g). This discrepancy in the recommended levels for Nile tilapia, may be related to the technique used, the size of the fish, genetic improvements, the type of food resource, the environment conditions or the model used in each study.

In the grower phase, the optimum level of digestible lysine based on weight gain that we found in our study was 14.6 g kg^{-1} (as-fed basis)(6.70% of dietary protein), being similar to that determined by Michelato et al. (2016) who estimated 14.6 g kg^{-1} (DM)(5.8% of dietary protein) of crude lysine based on maximum fillet yield (IW: 275 g). These requirements seem to be similar, however the nature (fed basis) of lysine tested was different. In the finisher phase the lysine requirement was determined to be 13.6 g kg^{-1} (6.64% of dietary protein). For larger fish a little is known about its dietary lysine requirement, thus it is difficult to compare the present findings of the species under study to other species of large sizes. According to the existing literature the requirements established for Nile tilapia above 100 g are around 13.8 g kg^{-1} of dietary digestible lysine (Furuya, 2010) which is close to that determined in the present study during finisher phase. The lysine requirements established in this study was declined in relation to the body size of Nile tilapia. The same observations were reported in previous works conducted by Abdel-Tawwab et al. (2010) in Nile tilapia, Árnason et al. (2010) in Atlantic cod and Ye et al. (2017) in Gibel carp to determine protein requirement.

A high percentage of lysine in the dietary protein was obtained in our study due to necessity to exceed the lysine proportion 1.5 times more than that recommended in the literature in the summit diet. Similarly, Bomfim et al. (2010) and Rampe et al. (2014) have reported high values of 6.18 and 6.86% lysine of dietary protein for the same species, respectively. However, in several studies reported that the use of essential amino acids as percentage of dietary protein is not adequate, because even in different protein levels, the lysine requirement was quite similar, thus EAA requirement was not dependent of dietary protein levels (Webb and Gatlin, 2003; Abboudi et al., 2006; Nang Thu et al., 2009). According to NRC (2011), expressing amino acids requirement as percentage of protein is not a reliable measure due to the different proportions of nitrogen compounds presents in the dietary protein. The requirements established in our study by dilution technique were consistent to other studies performed by Nile tilapia. The study conducted by Abboudi et al. (2007) estimating the threonine requirement for maintenance in Atlantic salmon (*Salmo salar L*.) and Liebert and Benkendorff (2007a, b) used the principles of dilution technique to determined lysine, methionine and threonine requirements of Nile tilapia. Based on the results of their studies, dilution technique is a reliable method to determine amino acid requirements.

In the present study, the lysine maintenance requirement was obtained by monomolecular model at 19.87, 40.26 and 50.25 mg $kg^{-0.8}$ day^{-1} in the started, grower and finisher phase, respectively. According to Hau et al. (2013), monomolecular model is considering a reliable method to estimate amino acid maintenance due its good fit in comparison to other models. In concordance with our findings, similar values were found for juvenile Atlantic salmon by Abboudi et al. (2006) at 20 mg $kg^{-0.75}$.day^{-1} and for juvenile Rainbow trout by Bodin et al. (2009) at 21 mg $kg^{-0.75}$ day^{-1} in comparison to our first trial. He et al. (2013) has found a smaller value than the started phase at 16.9 mg $kg^{-0.7}$ day^{-1}. In addition, a high lysine maintenance requirement was established by the same authors for adult Nile tilapia (IW: 165 g) at 68.8 mg $kg^{-0.7}$ day^{-1} than the requirements we determined in the grower and finisher phase. These differences could be related to the metabolic weight exponent used, the methodology and/ or the formulation of diets. The metabolic exponent used in our study ($BW^{0.8}$) was according to Trung et al. (2011), in which the authors have found 0.854 as exponent of weight for protein metabolism and 0.849 for energy metabolism for Nile tilapia at 32ºC. However, to keep the consistency between both parameters, the same authors adopted 0.8 as a metabolic exponent in their study.

In our work, the amino acid maintenance has increased among life stages (Fig. 1) and a similar behavior was reported by He et al. (2013) for the same species. Einen and Roem (1997) reported the increased in the maintenance requirement during life stages due to the high demand in the maintenance energy requirement for slow-growing fish (large fish) in compare to fast-growing fish (small fish). Other researchers has reported the same relationship between increase of maintenance demands to the increase of the fish body size (Rodehutscord et al., 1997; Glencross, 2008; Grisdale-Helland et al., 2011b). The above arguments collaborate to explain the differences in maintenance requirement throughout the growth stages**.**

The efficiency of lysine utilization that we have found in the present study was contrary to maintenance such as in the started and grower phase that it was quite similar 67.68%, 67.54%, respectively, which sweetly dropped to 64.32% in the finisher phase. The same trend was observed by He et al. (2013) who determined the efficiency of lysine utilization for two growth stages of Nile tilapia at 72% for juveniles and 52% for adults and by Hua, et al. (2019) that found values of 68%, 63% and 47% in different sizes of Nile tilapia. In the present study the efficiency of lysine utilization timidly decreased, but does not considerably differed among life stages, being contrary to previous researches described above. In literature some aspects that can affect lysine utilization efficiency are mentioned, as the different protein sources in the diets (Nang Thu et al., 2009), dietary protein level (Rodehutscord et al., 2000) and dietary energy level (Encarnação et al., 2004, 2006). In the present study, the dilution technique was used and in compare to the supplementation technique, in this method, diets are not isoproteic. In the dilution technique, protein levels increase gradually in the same trend that all amino acids among diets to keep the ideal ratio between then. Castillo et al. (2017) working with different sizes of Nile tilapia found also a small variability in their results of efficiency of protein utilization (52, 51, 51 and 50%) using the dilution technique. Thus, those differences in lysine utilization efficiency values for Nile tilapia could be related to the feed formulation applied. According to our results, we cannot suggest that Nile tilapia possibly use lysine similarly during the life stages, so more studies must be done to validate this consideration.

In the present study body protein content in response to different dietary digestible lysine was considerably improved as shown in Table 8 and 9. In agreement to our findings, several authors found the same positive effect on body protein content in response to diet lysine in grass carp (*Ctenopharyngodon idella*), jundiá (*Rhamdia quelen*), cobia (*Rachycentron canadum*), atlantic cod (*Gadus morhua*) and silver perch (*Bidyanus bidyanus*) (Wang et al. 2005; Montes-Girao and Fracalossi, 2006; Zhou et al. 2007; Grisdale-Helland et al. 2011; Yang et al. 2011). Michelato et al. (2016) studying Nile tilapia and Peres and Oliva-Teles (2008) evaluating Turbot (*Scophthalmus maximus*) nutrition reported no significant differences regarding protein composition in response to lysine supplementation.

Body lipid content was effected in all trials to the dietary lysine, having decreasing behavior at high levels of digestible lysine. Bicudo et al. (2009) and Abimorad et al. (2010) in pacu juveniles (*Piaractus mesopotamicus*) observed the same behavior in body lipid content in response to dietary lysine. Deng et al. (2010) in juvenile Pacific threadfin (*Polydactylus sexfilis*) found a high body lipid content in the treatment with the lowest level of lysine, reporting the possible reason to a decrease in carnitine synthesis that could have impaired lipid metabolism due to lysine-deficient diet. Similarly, hepatosomatic index (HSI) was reduced in response to dietary lysine levels in the initial and finisher phase as shown in Table 4 and 5. Walton et al. (1984) found a same response in diets with deficient-lysine in response to dietary L-carnitine. The changes in HIS with increased levels of lysine were also observed in grass carp, cobia, turbot and silver perch (Wang et al. 2005; Zhou et al. 2007; Perez and Oliva -Teles, 2008 and Yang et al., 2011).

According to our findings, ideal amino acid profile was determined for all trials based on digestible lysine requirements obtained (Table 7). The ideal protein concept has been used in many researches to establish the ideal amino acids profile to provide the exact proportions of amino acids, resulting in diets with low-protein content and consequently reducing the nitrogen wastes (Arai, 1981; Montes-Girao and Fracalossi, 2006; Bicudo et al., 2009; Abimorad et al., 2010; Madrid et al., 2019). This procedure was applied in our study to provide more information regarding amino acids with little information for Nile tilapia adults as well as to improve the practical diets for juvenile tilapia.

Conclusion

In conclusion, the optimum lysine level established in the present study for Nile tilapia was in agreement with previous literature for the same species. The digestible lysine requirements determined in different growth stages based on weight gain were 19.4, 14.6 and 13.6 g kg^{-1} (on fed basis) in the started, grower and finisher phase, respectively. The maintenance requirement and efficiency of lysine utilization established was 19.87 mg $kg^{-0.8}$ day^{-1} and 67.68% in the started phase, 40.26 mg $kg^{-0.8}$ day^{-1} and 67.54% in the grower phase; 50.25 mg $kg^{-0.8}.day^{-1}$ and 64.32% in the finisher phase. The ideal AA profile determined in our work may help to improve practical diets for Nile tilapia in different life stages through adequate amino acids balance with low dietary protein levels. Moreover, the present profile also provides information that may be important to determine amino acids requirements for large fish in future researches.

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Lista de figuras

Lista de tabelas

Considerações Finais

Em conclusão aos trabalhos desenvolvidos, foi possível estimar a exigência de lisina digestível para diferentes fases de crescimento com a aplicação da técnica de diluição. Os dados obtidos no presente estudo irão colaborar para a elaboração de modelos fatoriais, cujo produto final será o desenvolvimento de uma planilha eletrônica. No entanto, algumas considerações sobre a aplicação do método de formulação devem ser apontados. Primeiro, a variação dos níveis de gordura nas dietas, no qual estão relacionadas a alta inclusão de óleo de soja na dieta “isenta em proteína”, medida aplicada para manter as dietas isoenergética. A técnica preconiza manter os mesmo níveis dos nutrientes entre as dietas, no entanto com a grande discrepância entre os níveis de gordura, o procedimento realizado foi de encontro aos princípios do método. Deste modo, para estudos futuros será necessário estabelecer medidas que resolvam este problema.

A segunda consideração é sobre a utilização de valores mais baixos para manter a deficiência relativa do aminoácido estudado. No presente estudo, a lisina foi formulada para extrapolar 50% da exigência recomendada em literatura e os demais aminoácidos essências foram extrapolados em 80% da exigência, com isso foi obtido uma deficiência relativa de 30%. A medida foi aplicada para termos certeza que a lisina seria limitante nas dietas experimentais, visto que, há uma grande discrepância entre os dados de exigência de lisina em literatura. Como sugestão para futuros estudos, sugere-se a utilização de valores mais baixos, como 20% acima da exigência para o aminoácido em estudo e 40% para os demais aminoácidos, procedimento aplicado em estudos para aves e suínos. Isto diminuiria a porcentagem de lisina em relação à proteína, no qual apresentou-se elevada em relação a outros estudos. Como também, facilitaria a formulação das dietas por não precisar atingir níveis tão altos de aminoácidos.

Portanto, a técnica da diluição atentou as demandas do presente estudo, sendo aplicável em pesquisas que visem estimar a exigência de aminoácidos. No entanto, mais estudos devem ser realizados com peixes para levantar informações sobre a aplicabilidade da técnica.

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