Dietary Lysine Requirements of *Colossoma Macropomum* (Cuvier, 1818) Based on Growth Performance, Hepatic and Intestinal Morphohistology and Plasma Biochemistry

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Abstract

This study aimed to determine the dietary lysine requirements of juvenile *Colossoma macropomum* tambaqui based on growth performance. We also evaluated gut and hepatic histomorphometry as well as blood metabolites in accordance with the increased levels of dietary lysine. The juveniles (33.88 ± 2.47 g) were fed until apparent satiation with diets containing 6.60, 9.72, 12.84, 15.96, 19.08 and 22.20 g/kg of lysine. Fish were randomly distributed in groups of 10 fish per tank and assays were performed in triplicate, during 90 days. Tambaqui fed with 15.96 g/kg dietary lysine showed higher final weight (p = 0.001) and optimized feed conversion ratio (p = 0.001). Morphohistological modifications were present in livers of fish fed with low levels of lysine. In the proximal intestine, mucosa layer density was greater at the level of 15.96 g/kg (p = 0.001). In the middle intestine, height (p = 0.001) and perimeter (p = 0.001) of the villi were greater at low levels of lysine (respectively, 9.72 and 12.84 g/kg dietary lysine). Tambaqui fed with 15.96 g/kg of lysine achieved higher plasma protein concentrations (p = 0.01). Using the second-order polynomial regression analysis as support, and based on protein efficiency rate and body weight gain, dietary lysine requirement for juvenile tambaqui was calculated as 15.4–15.6 g/kg of diet (5.7–5.8% of dietary protein).

Introduction

Diets formulated with amino acids that meet the requirements of the fish are necessary in order to support biological development, health and the performance in fish farming (Mai et al. 2006; Zaminhan et al. 2018). Lysine is an essential amino acid that plays an important physiological role in the development of the animal, coming from the metabolic pathway of aspartic acid (Prabhu et al. 2019). Lysine acts strategically in protein synthesis, and influences body growth, weight gain, feed conversion, feed utilization, muscle structure and composition (Madrid et al. 2019).

Dietary lysine may affect the morpho-histological aspects of the liver (Hua et al. 2019). This is because lysine stimulates the synthesis of L-carnitine, which mobilizes long-chain fatty acids into the hepatocytes, where they are catabolized and provide energy for the maintenance and performance of the fish (Yang et al. 2011; Xu et al. 2019).

Similarly, to the liver, intestinal morphology is conditioned by nutrients in the diet (Rotta 2003). The intestine of the fish, structured by proteins such as collagen, is characterized by serosa, muscular, submucosa and mucosa layers, in which there are goblet cells and enterocytes (Yamauchi and Sricholpech 2012). Lysine and other amino acids are absorbed by intestinal villus enterocytes, which are able to increase their area to maximize nutrient capture (Honorato et al. 2013). Through the vascularization of intestinal tissue, lysine is transported to places where it will be metabolized (NRC 2011).

Blood analysis is an effective tool in monitoring the health of fish, and is necessary for animal welfare and production (Higuchi et al. 2011; Pereira et al. 2016; Aride et al. 2016; Aride et al. 2018; Aride et al.

2020). The biochemical components of the plasma reflect functional changes in response to factors such as environmental conditions, physiological stress or nutritional conjunction (Ranzani-Paiva et al. 2013).

The inclusion of crystalline amino acids in fish nutrition should be investigated in order to understand the optimum level of amino acids for species of economic interest (Zhou et al. 2007; Grisdale-Helland et al. 2011; Michelato et al. 2016). The tambaqui, *Colosssoma macropomun* (Cuvier 1818), is a native Amazonian teleost with omnivorous feeding habits, and presents easy handling when in confinement (Araújo-Lima and Gomes 2005). It has good feed conversion, and its flesh has a texture and flavor which is highly appreciated by consumers in northern Brazil (Buzollo et al. 2018).

Although the production of tambaqui throughout the brazilian territory is well established and expanding, the existing nutritional information is insufficient for the elaboration of an intrinsic diet (Rodrigues 2014). Currently, data on species with similar feeding habits to tambaqui is used and, as such, ignores the peculiarities of the digestive tract and physiological aptitudes of the animal (Silva et al. 2018). Thus, this study aimed to determine the dietary lysine requirements for juvenile *Colossoma macropomum* based on growth performance.

Materials And Methods

Ethical approval

The experiment was approved by the ethical review committee on the use of animals at Nilton Lins University (Protocol no.: 003/2017). The study was conducted in accordance with the Brazilian guidelines for animal experiments and was approved by the state government of Amazonas, Brazil. All experiments were conducted according to local and arrive guidelines (Persie du Sert et al. 2020).

Experimental fish

Juvenile tambaqui *Colossoma macropomum* measuring 33.54 ± 1.9 g and 11.72 ± 0.36 cm were obtained from the Balbina fish farming station - Aquaculture Training, Technology and Production Center (Presidente Figueredo, Amazonas, Brazil). In the Aquatic Organisms Production Laboratory, at Nilton Lins University, the fish were randomly distributed into 18 tanks (310^{-L}), with 10 fish per tank, and provided with constant aeration. The fish were fed with commercial diet for omnivorous fish (initial diet: 320.00 g/kg of crude protein, 14 g of lysine) during the acclimation period (ten days).

The water quality was maintained using biological filter. The water used for replacement was stored with continuous aeration in tanks with a capacity of 1000^{-L} and subjected to regular tests to check the chlorine content (Chlorine test, Labcon®, California, USA). Throughout the experiment, dissolved oxygen concentrations (DO), temperature and hydrogen potential (pH) were measured daily with a multiparametric device (Horiba® g-50, Kyoto, Japan). DO, temperature and pH, were maintained, respectively, at 4.95 ± 0.32 mg/L, 26.25 ± 0.06 °C and 5.74 ± 0.07, as recommended for fish performance (Aride et al. 2007). Weekly, nitrite was analyzed using colorimetric kits (Alfakit®, Florianopolis, Brazil),

according to the manufacturer's protocol and maintained between 0.00 and 1.0 mg/L (Schimittou 1993). The juvenile tambaqui were maintained under a constant photoperiod of 12 h light: 12h dark.

Experimental diets and feeding trial

Six experimental diets (270.00 g/kg of crude protein) were formulated with levels of lysine of 6.60, 9.72, 12.84, 15.96, 19.08 and 22.20 g/kg, which represent 0.00, 4.00, 8.00, 12.00, 16.00 and 20.00 g/kg of L-lysine HCl (Ajinomoto®, Chuo, Japan), respectively (Table 1). The diets were extruded (single thread: 2.5 mm; pellets of around 3 mm) at the Aquaculture Laboratory of the National Institute for Amazonian Research (INPA), Manaus, Brazil. For extrusion, all ingredients (Table 1) were finely ground in a knife mill (Tecnal®, Piracicaba, Brazil), homogenized and moistened (20% water) at 50 °C. The product was dried in a forced ventilation oven (55 °C) for 24 hours, subjected to bromatological analysis (AOAC 2005) and then stored. For 90 days, the juvenile tambaqui were fed four times a day (8:00, 11:00, 14:00 and 17:00 hours) until apparent satiety.

Fish sampling

Prior to the feeding trials, nine juvenile tambaqui fed with the initial diet were randomly selected after 24 hours of feeding restriction. Similarly, at the end of the feeding trials, three fish were sampled from each experimental unit. These were anesthetized (benzocaine 100 mg/L) for blood collection by puncture of the caudal vessel using syringes (1 mL) with 15 µL of the anticoagulant heparin sodium 5,000 IU (150 IU/mL). Heparin was diluted in 0.65% saline (1:50) and blood samples were preserved at -4 °C from the time of the collection until centrifugation for obtaining the plasma (Oliveira et al. 2012).

Subsequently, the fish were euthanized with benzocaine (250 mg/L) and spinal cord dislocation was performed for extraction of the cell tissues (Underwood et al. 2013). After that, they were weighed (g, accuracy: 0.001g; Gehaka®, São Paulo, Brazil) and measured (cm, ichthyometer). The muscle, which was analyzed for proximate composition, was extracted from the dorsolateral region using scissors and cutting blades. In the final period of the feeding trial, the livers and intestines of the fish (n= 54) were weighed (0.001 g) for analysis of the hepatosomatic index and intestinal morphometric analysis. Additionally, visceral fat from the fish was sampled. For morphohistological analyses, the livers and intestines selected in the initial and final period (respectively: n= 9 and n= 54) were fixed in a specific solution.

Growth performance

The growth performance was verified using the following equations:

- Final weight, FW (g) = average final weight of the fish/number of fish;
- Body weight gain, BWG (%) = 100 x [(final weight (g) - initial weight (g))/initial weight (g)]};
- Lysine intake, LI (%) = [dry lysine intake (g)/final fish weight (g)/days fed] x 100;
- Feed intake, FI (%) = [dry feed intake (g)/final fish weight (g)/days fed) x 100;

- Apparent feed conversion, AFC (kg/kg) = dry feed fed (kg)/body weight gain (kg);
- Hepatosomatic index, HSI (%) = [liver weight (g)/fish weight (g)] x 100;
- Visceral fat index, VFI (%) = [visceral fat weight (g)/fish weight (g)] x 100;
- Protein efficiency rate, PER (%) = [body weight gain (g)/protein intake (g)] x 100;
- Feed efficiency rate, FER (%) = [body weight gain (g)/dry feed intake (g)] x 100.

Muscle proximate composition

In the analysis of the proximal composition of the muscles for crude protein and lipids, the extracted tissue was homogenized, dried in an oven at 105 °C and crushed, for verification of nutritional content (AOAC 2005). The crude protein was analyzed using the Microkjehldal method, with steps for digestion and distillation of the matter, followed by titration with acid. Total lipids (ether extract) were verified using the bligh and dyer method, and the total lipids and the ash content were verified by incinerating the organic matter in a muffle furnace at 550 ºC.

Hepatic morphohistology

Liver samples were collected from five fish, per each diet type (n= 30) were packed in histological cassettes and fixed for 24 hours in Davison's solution. These were dehydrated in increasing concentrations of alcohol and embedded in histological resin (Technovit®, 71000, Kulzer Hanau, Germany), according to the manufacturer's protocol. The 3 µm-thick slides were made in triplicate using semiautomatic microtome (Slee®, Cut 5062, Mainz, Germany) at the Functional Morphology Laboratory (FML) of the Federal University of Amazonas. Subsequently, slides from the liver were stained with hematoxylin and eosin (He) in order to analyze the hepatocytes. All slides were analyzed using an optical microscope (Leica®, DM 500, Wetzlar, Germany) with a 32 megapixel camera attached to capture images.

Intestinal morphohistology

Total intestinal weight (TIW), total intestinal length (TIL) and relative intestine length (RIL: intestine length/fish length) were verified (n= 9 intestines per diet), prior to the confection of the slides (Rotta 2003). The intestines were sampled from five fish per diet and segmented in the proximal (PI), middle (MI) and distal (DI) intestine, with cross sections in triplicate and then packed in histological cassettes (n= 310 samples). Subsequently, these were fixed for 24 hours (Davison's solution), dehydrated in alcohol, embedded in histological resin and sectioned at a thickness of 3 µm using a semi-automatic microtome to create the slides. All slides were photographed and analyzed using an optical microscope (Leica®, DM 500, Wetzlar, Germany) with a 32 megapixel camera attached to capture images.

The slides were stained with blue toluidine dye to quantify the fractional volume (based on the delesse principle) or density of the serosa, muscular, submucosa and mucosa layers. In each slide, four villi were selected according to the visibility of the layers (n= 60 villi per diet). Overlapping the digital image,

equidistant points (144) were computed, disregarding empty spaces or content other than the layers and villi analyzed.

To detect the expression of collagen on the intestinal tissue, the sections on the slides were stained with marllory's trichrome to identify the green color of the dye reaction in the presence of the collagen. In parallel, the density of the goblet cells secreting acid mucins was quantified using alcian blue (Ab) dye pH 2.5 on the slides and analyzed using Stepanizer® software (Tschanz et al. 2011). The slides were stained with he to analyze of the height and perimeter of the intestinal villi (n= 60 villi per diet) using Image J® histological evaluation software (Scijava Consortium, Madison, USA). The height was verified by measuring from the base to the apex of each villus, and the perimeter was verified following the outline of the area of villus, as in Ferreira et al. (2014).

Plasma biochemistry

For the plasma biochemical analysis, plasma from the remaining material from the samples obtained after centrifugation (3,000 rpm) for 10 minutes was used. Total proteins (g/dL), glucose (mg/dL), cholesterol (mg/dL) and triglycerides (g/dL) were analyzed using commercial enzymatic-colorimetric assay kits and spectrophotometric readings (Thermo Fisher Scientific®, Waltham, USA). The analyses were performed at the Chemistry Laboratory of the Federal Institute of Espírito Santo, Campus Piúma, ES, according to the manufacturer's specific recommendations (Labtest®, Belo Horizonte, Brazil).

Statistical analysis

The feeding trial was organized using a randomized design, containing a control diet (initial period) and six formulated diets with three replicates. The data were submitted to One-way anova, followed by the Tukey test ($p< 0.01$ and $p< 0.05$) to compare results, which were shown as mean values ± standard deviation. Additionally, the dietary lysine requirement was estimated based on body weight gain and on protein efficiency rate, by second-order polynomial analysis model, given by the equation: $Y = a + bX + cX^2$. The broken-line regression analysis [(Y1 = LU * (X - R); Y = IF (X < R, Y1, L); L = plateau, U = slope and R = breaking point] was used to estimate the requirement of lysine in the diet by feed efficiency rate. The values of X and Y in the equation correspond, respectively, to dietary lysine (g/kg) and the variable analyzed in the current study. All statistical analyses were performed using R® statistical software (r-project, Auckland, New Zealand), version 3.5.3.

Results

Growth performance

The results show that supplementation of dietary lysine significantly affected the performance for final weight (FW) in juvenile tambaqui. The FW of tambaqui fed 15.96 g/kg and 22.20 g/kg of lysine was comparable though higher ($p= 0.010$) than the FW of tambaqui fed with other diets (Table 2). The dietary lysine requirement for tambaqui, based on body weight gain (Figure 1a) was estimated according to the

second-order polynomial analysis at 15.661 g/kg of lysine (5.800% of the diet), calculated using the equation: $y= 20.43378 + 13.00819x – 0.415286x^2$; $r^2= 0.200$.

The lysine intake increased ($p= 0.001$) in parallel to the addition of levels of the dietary lysine, analyzed by second-order polynomial regression, and described by equation: $y= 0.04395736 + 0.01223681x + 6.99e\text{-}06x^2$, $r^2= 0.901$. In contrast, the feed intake of tambaqui was unaffected by dietary lysine levels ($p= 0.118$).

Tambaqui fed 15.96 g/kg of dietary lysine showed lower apparent feed conversion (AFC: 1.45 kg/kg; $p= 0.001$) than those fed with different levels of dietary lysine (Table 2). The levels of dietary lysine 9.72 g/kg and 12.84 g/kg significantly affected ($p= 0.049$) the hepatosomatic index (HSI) of the tambaqui (Table 2). However, the visceral fat index (VFI) was not significantly affected ($p= 0.242$) by the lysine levels in the diet (Table 2).

Lysine retention efficiency (LRE) gradually reduced ($p= 0.001$) with increasing dietary lysine. The feed efficiency rate (FER) of tambaqui did not differ ($p= 0.056$) (Table 2) and was estimated using broken-line regression analysis for 15.96 g/kg dietary lysine or 5.91% dietary protein ($y= 22.4063 + 4.850018x\text{-}0.151946x^2$; $r^2= 0.338$). The dietary lysine requirement for tambaqui was estimated using the second-order polynomial analysis for 15,441 g/kg of lysine (5.718% of the diet) based on per (Figure 1b) using the following equation: $y= 0.5418156 - 0.2355635x + 0.00767467x^2$, $r^2= 0.411$.

Proximate composition of muscle tissue

Data for the proximate composition of muscle tissue of juvenile tambaqui (Table 3) showed that an increase dietary lysine did not increase crude protein percentage ($p= 0.051$) and lipids ($p= 0.061$). Fish fed with 22.20 g/kg of dietary lysine demonstrated a higher percentage of ash in the muscle compared to those fed with 6.60, 12.84 and 19.08 g/kg of lysine ($p= 0.010$), but were comparable to fish fed with 9.72 and 15.96 g/kg of lysine in the diet. In parallel, fish fed with 22.20 g/kg of dietary lysine showed a higher percentage of moisture ($p= 0.001$) in the muscle compared to those fed with other diets (Table 3).

Hepatic morphohistology

In the macroscopic analysis of liver tissue in the tambaqui, the livers showed reddish-brown and slightly paler brown tones. Microscopically, the tambaqui parenchyma showed hepatocytes with a polyhedral, irregular round and hexagonal shape, with a centralized nucleus (Figure 2a).

The lipids were visible in the hepatocytes of all fish fed the elaborated experimental diets (Figure 2a). Fish fed diets that were elaborated with higher levels of lysine (15.96, 19.08 and 22.20 g/kg) presented livers with lipid molecules in small quantities. These molecules were generally present close to arteries, arterioles, veins and sinusoids, in isolated or grouped forms (Figure 2a and 2b).

Fish fed diets with 9.72 g/kg of lysine had livers with a higher amount of lipids, showing steatosis hepatodystrophy in low intensity. Additionally, these livers showed edematous degeneration, which is

configured by cellular edema with architectural cortical breakdown, clearing of the cytoplasm and centralized permanence of the nucleus (Figure 2c).

Intestinal morphohistology

The averages of the total intestine weight (TIW), of the total intestine lengths (TIL) and of the relative intestinal lengths (RLI) of the tambaqui did not showed a significant diference (respectively *p= 0.221, p= 0.163, p= 0.590*) in response of increasing dietary lysine (Table 4).

The serosa layers of the proximal (*p*= 0.273), middle (*p*= 0.254) and distal intestine (*p*= 0.172) of tambaqui (Figure 3) from the final period and the initial period of the experiment did not differ significantly. However, one exception was the tambaqui juveniles fed 12.84 g/kg of dietary lysine, which presented a proximal muscle layer of the intestine equivalent to that of the fish from the initial period, and the proximal muscle layer of the tambaqui from the initial period was greater (*p*= 0.001) than those of the final period (Table 5). In the middle portion of the intestine (*p*= 0.350) and the distal intestine (*p*= 0.290), the percentage of muscle layer was not influenced by the different lysine levels in the diet (Table 5).

The submucosa layer (Figure 3) of the proximal intestine (PI) showed significant differences (*p*= 0.001) between the tambaqui fed with the diet of the final period and those fed with the diet of the initial period (Table 5). In the middle intestine, the submucosa layer was smaller (*p*= 0.010) in fish fed 9.72 g/kg of lysine in the diet when compared to those fed diets with 15.96 and 22.20 g/kg of lysine. The mucosa membrane of the pi of fish fed with 15.96 g/kg dietary lysine was statistically different (*p*= 0.001) from those that received 19.08 kg/g of lysine and these showed the greatest density of this layer in the final period (Table 5).

Morphohistological images depicting the presence of collagen protein in the cell layers (serosa, muscular, submucosa and mucosa) that form the intestinal villi where the goblet cells are located are shown in the Figure 3 (a - upper image). Goblet cells (gc; acid glycoproteins) of the PI from fish fed with 22.20 g/kg of dietary lysine showed greater concentrations when compared to the gcs of other fish. In contrast, the concentration of gcs in the MI and DI of juvenile tambaqui did not differ significantly (*p*= 0.561, *p*= 0.213, respectively) with added lysine in the diets (Table 6). The height of the villi of the proximal intestine (PI) was not affected (*p*= 0.100) by increasing dietary lysine (Table 6). In the middle intestine (MI), the lowest and greatest height of the intestinal villi (*p*= 0.010) of the tambaqui were for those that received 19.08 and 9.72 g/kg of dietary lysine, respectively (Table 6).

Plasma biochemistry

The plasma protein levels of tambaqui fed with the initial diet was higher than the plasma protein levels of those fed in the final period with 15.96 g/kg of dietary lysine (*p*= 0.010), as described in Table 7. The blood glucose of the tambaqui was not affected by the dietary lysine (*p*= 0.120).

The blood of tambaqui fed with the initial diet showed lower cholesterol than that of tambaqui sampled in the final period that were fed with 9.72, 15.96 and 22.20 g/kg of dietary lysine (*p*= 0.001). In contrast,

tambaqui sampled in the final period and fed 6.60, 12.84 and 19.08 g/kg of dietary lysine showed lower plasma cholesterol (Table 7). Triglycerides showed no difference among treatments with dietary lysine (p= 0.072).

Discussion

Growth performance

In the present study, the 15.98 g/kg level showed the best results in weight gain in tambaqui juveniles. Abimorad et al. (2010) demonstrated that 16.00 g/kg of dietary lysine provided the best FW of juvenile *Piaractus mesopotamicus* (Holmberg 1987) (pacu), which is similar to tambaqui in biology and behavior. The ideal level of dietary lysine shown for pacu is similar to the ideal level shown in this study with tambaqui.

Silva et al. (2018) indicate a need for 20.00 g/kg total lysine (17.30 g/kg digestible lysine) in order to improve weight gain and protein gain in tambaqui. The dietary lysine estimated by these authors using quadratic plateau and linear response models extrapolates the dietary lysine estimate based on BWG in the present study (15.441 g/kg of lysine) for tambaqui of weight 33.54 ± 1.90 g. However, differences in the lysine requirement for fish with different weights was described by Hua et al. (2019), who performed tests with IW of 9.8 ± 0.0 g (small fish), 58.1 ± 0.4 g (medium fish) and 247.6 ± 1.5 g (large fish). The BWG that occurred in the present study showed a low coefficient of determination, and can be explained by the influence of dietary lysine in 20% of the trend curve. This indicates that the other nutrients in the balanced diet, external factors that exceed lysine or intrinsic metabolic factors of tambaqui, may have contributed to BWG.

The positive increase in lysine intake (LI) in relation to increased dietary lysine confirms the findings in the study by Silva et al. (2018). These authors associated the behavior of the LI with that of the feed intake (FI), which did not variy (inappetence or hyperphagia), and attributed the non-variation to the isoenergetic condition of the diets. Energy imbalance of the diet would increase FI, while insufficient levels of lysine could reduce FI, cause inappetence and decrease performance (Mai et al. 2006; Santos et al. 2010).

Apparent feed conversion (AFC) is an essential index for fish farming and varies between 1.0 and 2.4 depending on differences such as age, production technology, source of nutrients and composition of the fish's diet (Torrinsen et al. 2011; Fry et al. 2018). The AFC value shown in this study (1.45 kg/kg) is similar to that of 1.44 kg/kg and 1.49 kg/kg as shown by Furuya et al. (2006) and by Michelato et al. (2016), respectively for juvenile *Oreochromis niloticus* (Linnaeus 1758) (Nile tilapia). However, the AFC is lower than that shown for pacu (1.8 kg/kg), for *Rachycentron canadum* (Linnaeus 1766) (1.10 kg/kg) (Beijupirá), and for the initial stage of tambaqui (1.66 kg/kg) studied, in this order, by Abimorad et al. (2010), Ahou et al. (2007) and Silva et al. (2018).

Although there is a significant difference between the hepatosomatic index (HSI) of fish fed with 9.72 and 12.84 g/kg of dietary lysine (higher and lower HSI, respectively), there were no differences among their

levels of growth performance. Hansen et al. (2011) demonstrated that for *Gadus morhua* (Linnaeus 1758) (Atlantic cod) the highest levels of lysine generated the lowest levels of HSI. This corroborates with the proportionally inverse results detected by Zhou et al. (2007) for Beijupirá (or cobia) and by Bicudo et al. (2009) for pacu.

The deficiency in lysine influences the reduction of carnitine biosynthesis, decreases the transport and β-oxidation of fatty acids, and increases the storage of lipids in the liver (Sika and Layman 1995). However, studies by Furuya et al. (2013) with tilapia showed no interference of lysine in HSI. Similarly, Grisdale-Helland et al. (2011), who researched the lysine requirement for Atlantic cod, did not detect any interference in HSI either. The concentration of proteins and amino acids in the diet affects the synthesis and hydrolysis of hepatic glycogen, and is able to retain the glucose in hepatocytes. This retention contributes to the increase in the weight of the liver, consequently increasing HSI (Soares et al. 2011). For the visceral fat index (VFI), the data from the present study reaffirm the data by Furuya et al. (2004) and by Takishita et al. (2009) in studies with Nile tilapia. Additionally, these data corroborate information for VFI in tambaqui in the study by Silva et al. (2018).

The lysine retention efficiency (LRE) values shown in the present study decreased proportionally with the increase in the levels of dietary lysine. The behavior of the efficient deposition of amino acids and proteins is associated with catabolism and with the nutritional needs during the growth of the fish's body (Azevedo et al. 2004; Bermudes et al. 2010; Hua et al. 2019).

The protein efficiency rate (PER) decreased proportionally to the increase in dietary lysine after reaching the level estimated at 15.441 g/kg of lysine, indicating the point of requirement for optimal use of protein deposition in the body of juvenile tambaqui. This point is close to the optimal body weight gain (BWG: 15.661 g/kg dietary lysine) shown in the current study. In addition, the behavior of the per effect on the fish's body before and after reaching the requirement for dietary lysine is similar to that of BWG. For the feed efficiency rate (FER) of juvenile tambaqui, the increased levels of dietary lysine occurred up to the estimated level of 15.96 g/kg of lysine, followed by plateau formation. This level coincides with the tested lysine level which showed optimum expression of the AFC. This is because the maximum fer estimate is reached when the fish expresses its potential for protein deposition (Bureau et al. 2000; Bomfim et al. 2010).

Proximate composition of muscle tissue

The proximal composition of the fish muscle varies between 15 and 24% for proteins, and between 1 and 2% for Ash. These values may be different between species and within the same species, since it is influenced by age, growing conditions, body portion and feed intake (Arbeláez-Rojas et al. 2002). No statistical variation was observed in the muscle composition of tambaqui fed with different lysine levels in the current study in relation to the percentage of proteins and lipids, however, it was expressed in the ash and moisture levels.

Ash complexed with amino acids tends to increase the absorption of the nutrient in the intestine, providing transport through mucosa membranes. In parallel to this, the formation of insoluble compounds with possible anti-nutritional factors in the diet are avoided (Barros et al. 2004).

In the muscles of fish that received diets containing 22.20 g/kg of lysine, the percentage of moisture exceeded the others and presented a significant difference. The data show similarities to those shown by Oetterer et al. (2004) for red tilapia (79.20%) and for nile tilapia (78.43%). In contrast, they are lower than the values shown by Arbeláez-Rojas et al. (2002) at time 0 (zero) of the study of juvenile tambaqui (86.2 ± 10.9 g and 15.1 ± 0.5 cm) in intensive and super-intensive agriculture.

Hepatic morphohistology

The formats of the hepatocytes that were verified corroborate the structure characterized for tambaqui by Costa et al. (2012) and for pacu by Fujimoto et al. (2008). Microscopically, the tambaqui parenchyma showed hepatocytes with a polyhedral, irregular round and hexagonal shape, with a centralized nucleus (Figure 2a). The reddish brown color presented by the liver tissues occurs due to the abundant vascularization of the organ and indicates normal tissue. However, the brown color in a slightly paler tone suggests hydropic degeneration. It is caused by ionic and homeostatic imbalance, and can generate pallor, turgidity and weight increase in the tissue, as evidenced in the analysis of the HSI of the tambaqui treated with 9.72 g/kg, though it is reversible.

Rocha et al. (2010) analyzed the liver of the bream *Brachyplatystoma rousseauxii* (Castelnau 1855) and detected the hepatic condition of steatosis, characterized by small drops of lipids, isolated or non-isolated, located close to blood vessels, and the same pattern was seen in the present study. Insufficient levels of proteins and amino acids in the diet can promote hepatic steatosis, since proteins act in the transport and uptake of lipids in the liver, and amino acids, such as lysine, synthesize metabolites that oxidize them (Furuya et al. 2013). Juvenile tambaqui fed with lower levels of dietary lysine (9.72 and 12.84 g/kg of lysine) had lipids stored in greater concentration in hepatocytes, had greater liver damage, according to the analysis of performance. The low lipid concentration in the hepatocytes of fish fed with 15.96, 19.08 and 22.20 g/kg of dietary lysine did not affect the growth performance of the juvenile tambaqui.

Intestinal morphohistology

Data from the intestinal morphometric analysis of the tambaqui in this study showed that they were not affected by the lysine levels in the diet. However, the relative intestinal length was 1.20 to 1.37 cm/cm, which is in accordance with 0.6 to 8.0 cm/cm as recommended for omnivorous fish (Rotta 2003; Ferreira et al. 2014).

The absorption of amino acids, monosaccharides and fatty acids is performed by the proximal intestine (PI), while the absorption of macromolecules by pinocytosis occurs in the distal intestine (DI). This characteristic corroborates what was observed in this study. Mucosa, submucosa and muscle layers of

the villi of the pi varied among fish fed with initial diets and fed with the diets of the final period. Tambaqui fed with 15.96 kg/kg of lysine showed statistically greater cell density among the fish fed with the experimental diets, indicating a greater ability to absorb dietary lysine. The data from this study validate the information presented in the performance for feed conversion, which expressed the best response with 15.96 g/kg of lysine. The middle intestine (MI) showed alterations in the submucosa, but it was not possible to associate this variation with the diet.

In the intestinal segments of the tambaqui, the collagen shown in the cell layers corroborates the description by Honorato et al. (2013) for the intestine of nile tilapia. Collagen is a synthesized protein and essentially composed of the amino acids glycine, proline and lysine. Thus, diets with high levels of lysine tend to increase the synthesis of this protein, which in insufficient quantities can limit animal growth. The mucin protein secreted by goblet cells was more active in the pi of fish fed 22.20 g/kg of lysine in the diet. Acid mucins are influenced by the type of diet and form barriers against bacteria and agents that limit absorption (Rocha et al. 2016).

The height and perimeter of the villi of the PI did not vary according to diet, in contrast, the villi of the DI showed great variability, which made it impossible to associate these aspects with the levels of dietary lysine. In the MI, the greater villus height shown in fish fed with 9.72 g/kg dietary lysine was equivalent to that of fish fed with 6.60 g/kg dietary lysine. This suggests a strategy for increasing nutrient uptake in diets with insufficient levels of lysine. In the MI, the greater villus height shown in fish fed with 9.72 g/kg dietary lysine was equivalent to that of fish fed with 6.60 g/kg dietary lysine.

This suggests a strategy for increase the uptake of nutrients in diets with insufficient levels of lysine. Diets with 6.60 g/kg of lysine were prepared without the inclusion of l-lysine. diets with 9.72 g/kg of lysine have 4.00 g/kg of L-lysine in total lysine. Competition at the absorption sites between intact lysine and L-lysine, or the action of adverse variables on fish metabolism, may have caused a greater imbalance at the level of 9.72 g/kg, which generates greater urgency in the capture of nutrients. According to Rotta et al. (2003), crystalline amino acids are absorbed more slowly. However, Nguyen and Davis (2016) found no difference in the performance of channel catfish, or american catfish *Ictalurus punctatus* (Rafinesque 1818) and nile tilapia *Oreochromis niloticus* when fed with L-lysine and with intact lysine.

Plasma biochemistry

The protein content in the blood tissue of tropical fish in fish farming is between 2.3 and 8.2 g/dL. Thus it possible to assume the values shown in this study to be normal (Tavares-Dias and Moraes, 2003; Tavares-Dias and Mataqueiro 2004). The experimental diets were rich in amino acids, which are structural components of proteins, thus we expected an increase in plasma protein concomitant with the increase in dietary lysine levels. However, blood data showed a higher protein concentration in fish fed with 15.96 g/kg of dietary lysine, with an increase of 56.8% in relation to tambaqui not fed with experimental diets (p= 0.010).

Adesola et al. (2017) investigated the lysine requirement for juvenile african dusky kob *Argyrosomus japonicus* (Griffiths and Heemstra 1995), and observed no differences for total proteins, triglycerides, glucose and cholesterol. Similarly, the same occurred in the current study in regards to triglycerides and glucose, which showed no difference, however, a difference was observed among plasma cholesterol concentrations in the different groups.

The juvenile tambaqui showed normal or high cholesterol levels. Although it is possible to link the variations in cholesterol concentration to the diets, they may have been secondary responses to stress conditions and adaptations to the environment (Ferreira et al. 2011). Luo et al. (2006) detected cholesterol between 2.11 ± 0.19 and 2.97 ± 0.28 mmol/L for the southeast asian grouper *Epinephelus coioides* (Hamilton 1822) with IW of 15.84 ± 0.23 g, fed with different levels of lysine. The results showed significant differences, but with variations that do not characterize them as an effect of the diet on the total cholesterol levels. This description corroborates what was verified in this study, where, possibly, the alterations among the means of total cholesterol were influenced by factors that were not limited to diet.

In conclusion, juvenile tambaqui (*Colossoma macropomum*) fed dietary lysine (g/kg) showed better growth performance (15.4g lysine / kg of diet). The dietary lysine levels influenced liver morphohistology, density of the intestinal cell layers, villus height and perimeter, and the secretion of acid mucins by goblet cells. Similarly, biochemical responses were affected by diet. In the current study, dietary lysine requirement for juvenile tambaqui was estimated at 15.4 – 15.6 g/kg of diet (5.7%–5.8% of dietary protein).

Declarations

Ethical approval

The experiment was approved by the ethical review committee on the use of animals at Nilton Lins University (Protocol no.: 003/2017). The study was conducted in accordance with the Brazilian guidelines for animal experiments and was approved by the state government of Amazonas, Brazil. All experiments were conducted according to local and arrive guidelines (Persie du Sert et al. 2020).

Ethics approval and consent to participate

The experiment was developed in accordance with the rules of ethical principles for animal experimentation approved by the National Council for the Control of Animal Experimentation (CONCEA), subject to approval by the Ethics Commission on the Use of Animals (ECUA) of the Federal University of Amazonas under approval No. 005/2016 and ECUA of the Nilton Lins University under approval No. 003/2017. All experiments were conducted according to local and ARRIVE guidelines.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

Authors contributions

ARSL and MSN conceived the study. WLPD, MRFMB and ATO designed the study; ARSL and MSN undertook laboratorial analyses. ARSL, JPL, PHRA, WMF and ATO drafted the paper with contributions from all other authors. All authors read and approved the final manuscript.

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Tables

Table 1
Formulation and chemical composition of the experimental diets (g/kg of diet) with increasing levels of lysine for juvenile tambaqui *Colossoma macropomum*.

Ingredients[1] (g/kg)	**Dietary lysine (g/kg)**					
	6.60	**9.72**	**12.84**	**15.96**	**19.08**	**22.20**
Corn	356.20	360.20	364.20	368.20	372.20	376.20
Corn gluten 60	230.00	230.00	230.00	230.00	230.00	230.00
Rice flour	120.00	120.00	120.00	120.00	120.00	120.00
Wheat bran	120.00	120.00	120.00	120.00	120.00	120.00
Fish meal	60.00	60.00	60.00	60.00	60.00	60.00
Dicalcium phosphate	20.00	20.00	20.00	20.00	20.00	20.00
Soy oil	10.00	10.00	10.00	10.00	10.00	10.00
Ashe and vitamin supplement [3]	7.00	7.00	7.00	7.00	7.00	7.00
Calcitic limestone	5.00	5.00	5.00	5.00	5.00	5.00
Sodium bicarbonate	2.00	2.00	2.00	2.00	2.00	2.00
Antifungal	2.00	2.00	2.00	2.00	2.00	2.00
BHT Antioxidant [4]	0.20	0.20	0.20	0.20	0.20	0.20
Mix of crystalline amino acids [5]	17.60	17.60	17.60	17.60	17.60	17.60
L-glutamic acid	50.00	42.00	34.00	26.00	18.00	10.00
L-lysine	0.00	4.00	8.00	12.00	16.00	20.00
Nutrients [1]						
Dry matter [2] (g/kg)	944.90	952,40	957.20	943.10	937.20	944.80

[1]Composition calculated according to Furuya et al. (2006) and Rostagno et al. (2011)

[2]Composition analyzed (AOAC, 2005)

[3]Ash and vitamin supplement (composition per kg of product): Selenium: 75.00 mg; copper: 2,000.00 mg; choline chloride: 125.00 g; manganese: 3750.00 mg; zinc: 20.00 g; iron: 15.00; iodine: 125.00 mg; niacin: 7,800.00 mg; folic acid: 750.00 mg; pantothenic acid: 3,750.00 mg; biotin: 125.00 mg; vitamin C 53.00 g; iodine: 125.00 g; vitamin A: 2,000.000.00 IU; vitamin D3: 500,000.00 IU; vitamin E: 15,000.00 IU; vitamin K3: 1,000.00 mg; vitamin B1: 2,500.00 mg; vitamin B2: 2,500.00 mg; vitamin B6: 2,000.00 mg; vitamin B12: 5,000.00 mg.

[4]BHT: Butyl-hydroxy-toluene

[5]Mix of crystalline amino acids (g/kg): Dl-methionine: 2.50 g; L-arginine: 2.50 g; L-histidine: 2.50 g; L-isoleucine: 2.50 g; L-phenylalanine: 2.50 g; L-threonine: 2.50 g; L-triptophan: 1.60 g; L-valine: 1.00 g.

Ingredients[1] (g/kg)	Dietary lysine (g/kg)					
	6.60	**9.72**	**12.84**	**15.96**	**19.08**	**22.20**
Gross energy (MJ/kg)	15.07	15.12	15.18	15.23	15.28	15.34
Crude protein [2] (g/kg)	266.20	271.60	264.00	269.70	268.30	276.70
Crude lipids [1] (g/kg)	40.00	40.00	40.00	40.00	40.00	40.00
Ash [2] (g/kg)	50.80	49.90	50.40	51.30	51.40	49.10
Calcium [1] (g/kg)	11.00	11.00	11.00	11.00	11.00	11.00
Available phosphorus [1] (g/kg)	7.00	7.00	7.00	7.00	7.00	7.00

[1]Composition calculated according to Furuya et al. (2006) and Rostagno et al. (2011)

[2]Composition analyzed (AOAC, 2005)

[3]Ash and vitamin supplement (composition per kg of product): Selenium: 75.00 mg; copper: 2,000.00 mg; choline chloride: 125.00 g; manganese: 3750.00 mg; zinc: 20.00 g; iron: 15.00; iodine: 125.00 mg; niacin: 7,800.00 mg; folic acid: 750.00 mg; pantothenic acid: 3,750.00 mg; biotin: 125.00 mg; vitamin C 53.00 g; iodine: 125.00 g; vitamin A: 2,000.000.00 IU; vitamin D3: 500,000.00 IU; vitamin E: 15,000.00 IU; vitamin K3: 1,000.00 mg; vitamin B1: 2,500.00 mg; vitamin B2: 2,500.00 mg; vitamin B6: 2,000.00 mg; vitamin B12: 5,000.00 mg.

[4]BHT: Butyl-hydroxy-toluene

[5]Mix of crystalline amino acids (g/kg): Dl-methionine: 2.50 g; L-arginine: 2.50 g; L-histidine: 2.50 g; L-isoleucine: 2.50 g; L-phenylalanine: 2.50 g; L-threonine: 2.50 g; L-triptophan: 1.60 g; L-valine: 1.00 g.

Table 2
Average performance values and nutritional rates of juvenile tambaqui *Colossoma macropomum* fed diets containing increasing levels of lysine.

	Dietary lysine (g/kg)						
Growth performance	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
Initial weight (g)	31.00 ± 2.57	32.2 ± 2.33	32.96 ± 2.29	33.90 ± 2.40	34.43 ± 0.89	38.80 ± 2.89	0.073
Final weight (g)	58.09 ± 3.14^{a}	68.02 ± 6.42^{a}	67.84 ± 2.98^{a}	80.69 ± 4.68^{b}	70.79 ± 5.93^{a}	79.86 ± 7.43^{b}	0.010*
Body weight gain (%)	87.39 ± 11.13	111.24 ± 10.48	105.79 ± 7.45	138.01 ± 31.96	105.59 ± 22.10	105.84 ± 27.22	0.282
Lysine intake (g/fish)	0.12 ± 0.00^{a}	0.16 ± 0.01^{ab}	0.21 ± 0.00^{bc}	0.22 ± 0.01^{c}	0.30 ± 0.02^{d}	0.31 ± 0.02^{d}	0.001*
Feed intake (g/fish)	1.09 ± 0.00	1.02 ± 0.10	0.99 ± 0.04	0.93 ± 0.03	0.98 ± 0.04	0.98 ± 0.00	0.118
AFC (kg/kg)	2.12 ± 0.12^{a}	1.76 ± 0.14^{a}	1.74 ± 0.10^{a}	1.45 ± 0.11^{b}	1.73 ± 0.81^{a}	1.74 ± 0.20^{a}	0.001*
HSI (%)	1.82 ± 0.15^{ab}	2.49 ± 0.57^{a}	1.16 ± 0.34^{b}	1.59 ± 0.14^{ab}	1.94 ± 0.35^{ab}	2.17 ± 0.45^{ab}	0.049*
VFI (%)	2.88 ± 0.32	3.14 ± 0.12	2.58 ± 0.38	2.31 ± 0.38	3.08 ± 0.52	3.03 ± 0.35	0.242
Nutritional rates							
LRE (%)	7.15 ± 0.43^{d}	5.88 ± 0.46^{d}	4.49 ± 0.23^{c}	4.31 ± 0.35^{cb}	3.02 ± 0.43^{ab}	2.60 ± 0.32^{a}	0.001*
PER (%)	1.77 ± 0.12	2.12 ± 0.28	2.18 ± 0.12	2.52 ± 0.16	2.15 ± 0.17	2.01 ± 0.25	0.100
FER (%)	47.15 ± 2.85	57.03 ± 3.48	57.11 ± 4.48	68.75 ± 5.59	57.03 ± 6.92	57.59 ± 8.22	0.056

AFC: Apparent feed conversion; HSI: Hepatosomatic index; VFI: visceral fat index; PER: Protein efficiency rate; FER: Feed efficiency rate; LRE: Lysine retention efficiency. Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Table 3
Muscle proximate composition of juvenile tambaqui (*Colossoma macropomum*) fed diets containing increasing levels of lysine.

	Dietary lysine (g/kg)						
Variables (%)	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
Chemical composition							
Crude protein	18.26 ± 0.65	16.94 ± 0.43	14.28 ± 0.68	16.34 ± 1.94	18.13 ± 0.36	16.80 ± 0.05	0.051
Lipids	2.53 ± 0.16	1.62 ± 0.21	2.25 ± 0.01	2.51 ± 0.73	2.08 ± 0.45	1.75 ± 0.06	0.061
Ashes	1.27 ± 0.03^{a}	1.30 ± 0.03^{ab}	1.22 ± 0.01^{a}	1.31 ± 0.04^{ab}	1.16 ± 0.01^{a}	1.28 ± 0.01^{b}	0.010*
Moisture	76.16 ± 0.01^{a}	77.26 ± 0.01^{a}	77.75 ± 0.01^{a}	77.95 ± 0.11^{a}	77.49 ± 0.12^{a}	79.12 ± 0.04^{b}	0.001*

Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Table 4
Morphometry of the intestines of juvenile tambaqui (*Colossoma macropomum*) fed diets containing different levels of lysine.

	Dietary lysine (g/kg)						
Intestine	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
TIW (g)	0.41 ± 0.11	0.56 ± 0.10	0.35 ± 0.09	0.40 ± 0.01	0.50 ± 0.15	0.57 ± 0.07	0.221
TIL (cm)	17.67 ± 2.44	20.66 ± 0.50	18.71 ± 1.34	20.54 ± 2.11	22.81 ± 2.96	22.04 ± 1.68	0.163
RLI (cm/cm)	1.25 ± 0.11	1.33 ± 0.05	1.20 ± 0.09	1.27 ± 0.11	1.37 ± 0.13	1.33 ± 0.04	0.590

TIW: Total intestine weight; TIL: Total intestine length; RLI: Relative length of the intestine. Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Table 5

Density (fractional volume) of the serosa, muscular, submucosa and mucosa cell layers of the proximal, middle and distal portions of the intestinal villi of the juvenile tambaqui (*Colossoma macropomum*) fed diets with lysine (g/kg).

	Dietary lysine (g/kg)							
Intestinal layers (%)	Initial diet	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
Serosa								
Proximal	10.75 ± 4.46	13.59 ± 4.24	10.62 ± 3.32	11.94 ± 4.33	13.86 ± 3.50	14.78 ± 5.25	9.77 ± 6.00	0.273
Middle	6.38 ± 4.68	8.87 ± 5.67	7.15 ± 2.16	9.09 ± 4.49	8.09 ± 6.59	5.83 ± 3.41	6.07 ± 3.43	0.254
Distal	13.66 ± 4.38	11.75 ± 3.27	8.83 ± 4.61	9.09 ± 4.66	12.54 ± 5.91	10.59 ± 4.17	9.99 ± 3.50	0.172
Muscular								
Proximal	12.86 ± 2.74^{b}	21.20 ± 4.76^{a}	14.52 ± 6.86^{a}	15.91 ± 6.35^{b}	16.89 ± 5.52^{a}	15.34 ± 1.36^{a}	12.51 ± 4.81^{a}	0.001*
Middle	8.81 ± 4.61	11.09 ± 4.59	12.28 ± 4.51	11.56 ± 4.55	10.94 ± 5.90	10.01 ± 5.90	12.78 ± 5.97	0.350
Distal	16.57 ± 9.10	18.77 ± 9.11	15.41 ± 7.13	11.56 ± 7.11	15.51 ± 7.21	15.19 ± 7.02	13.79 ± 7.59	0.290
Submucosa								
Proximal	16.24 ± 4.97^{b}	28.80 ± 5.27^{a}	12.21 ± 5.11^{a}	17.05 ± 5.39^{a}	17.13 ± 5.20^{a}	19.31 ± 4.96^{a}	13.50 ± 4.29^{a}	0.001*
Middle	7.91 ± 4.65^{ab}	10.63 ± 5.03^{ab}	8.95 ± 4.69^{b}	13.22 ± 6.18^{ab}	9.61 ± 6.13^{a}	8.70 ± 6.00^{ab}	11.27 ± 6.02^{a}	0.010*
Distal	13.48 ± 3.89	17.51 ± 2.40	14.55 ± 2.39	13.22 ± 3.68	15.22 ± 3.83	13.67 ± 9.28	14.92 ± 4.21	0.141
Mucosa								
Proximal	59.96 ± 3.29^{a}	36.41 ± 6.13^{bc}	62.62 ± 1.33^{bc}	55.10 ± 1.10^{bc}	52.03 ± 8.32^{b}	50.57 ± 3.51^{c}	64.21 ± 1.26^{bc}	0.001*
Middle	76.90 ± 5.61	69.41 ± 6.56	71.62 ± 7.24	66.13 ± 8.63	71.35 ± 10.11	75.46 ± 9.96	69.88 ± 10.31	0.162
Distal	56.29 ± 3.89	51.96 ± 3.44	61.22 ± 4.49	66.13 ± 4.38	56.73 ± 3.25	60.55 ± 2.40	61.30 ± 03.67	0.230

Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Table 6
Goblet cells (GC), height and perimeter of intestinal villi in the proximal, middle and distal portions of juvenile tambaqui (*Colossoma macropomum*) fed diets with lysine (g/kg).

	Dietary lysine (g/kg)						
	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
AI							
height (µm)	143.91 ± 72.92	141.58 ± 65.04	148.28 ± 61.48	136.95 ± 89.50	121.28 ± 30.39	126.12 ± 17.87	0.100
Perimeter (µm)	451.60 ± 48.99	453.28 ± 18.36	451.25 ± 89.05	417.75 ± 20.46	368.62 ± 18.00	385.27 ± 51.59	0.050
GC (mm²)	6.50 ± 0.08^{a}	7.71 ± 0.03^{a}	8.92 ± 0.02^{a}	6.99 ± 0.02^{a}	4.33 ± 0.01^{a}	31.82 ± 0.01^{b}	0.001*
MI							
height (µm)	150.68 ± 12.22^{ab}	537.32 ± 28.88b	136.09 ± 65.26^{a}	111.72 ± 56.21^{a}	88.57 ± 44.62^{a}	98.93 ± 48.61^{a}	0.010*
Perimeter (µm)	400.27 ± 20.02^{ab}	357.46 ± 17.72^{ab}	450.40 ± 21.62^{a}	421.86 ± 21.96^{ab}	339.97 ± 16.91^{ab}	335.21 ± 16.37^{b}	0.040*
GC (mm²)	2.17 ± 1.46	20.25 ± 1.61	22.18 ± 2.42	25.32 ± 2.43	15.91 ± 2.53	14.95 ± 9.48	0.561
PI							
height (µm)	161.94 ± 7.94^{a}	73.78 ± 35.63^{ab}	71.02 ± 36.99^{b}	159.98 ± 84.40^{ac}	95.36 ± 46.47^{abc}	103.56 ± 51.705^{abc}	0.020*
Perimeter (µm)	424.09 ± 20.93^{c}	287.08 ± 11.12^{ab}	291.29 ± 13.721^{ab}	193.80 ± 73.28^{a}	321.52 ± 12.81^{bc}	326.59 ± 46.62^{bc}	0.001*
GC (mm²)	2.65 ± 0.05	1.69 ± 0.01	13.98 ± 0.02	10.37 ± 0.02	12.06 ± 0.04	20.98 ± 0.04	0.213

AI: Proximal intestine; MI: Middle Intestine; PI: Distal Intestine. GC: Goblet cells. Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Table 7
Blood biochemistry of the juvenile tambaqui (*Colossoma macropomum*) fed diets containing lysine.

		Dietary lysine (g/kg)						
Biochemical component	Initial diet	6.60	9.72	12.84	15.96	19.08	22.20	*p* value
Proteins (g/dL)	3.17 ± 3.53^{a}	5.50 ± 2.22^{ab}	4.27 ± 3.76^{ab}	6.03 ± 1.16^{ab}	7.35 ± 2.09^{b}	6.61 ± 1.09^{ab}	4.39 ± 1.79^{ab}	0.010*
Glucose (mg/dL)	73.88 ± 5.57	156.46 ± 2.60	133.43 ± 3.27	114.54 ± 0.20	102.55 ± 2.92	108.57 ± 1.18	147.98 ± 3.77	0.120
Cholesterol (mg/dL)	338.26 ± 5.42^{c}	199.65 ± 8.64^{ab}	321.83 ± 1.48^{bc}	147.06 ± 2.88^{a}	297.72 ± 1.30^{bc}	207.52 ± 1.04^{ab}	241.96 ± 1.15^{ac}	0.001*
Triglycerides (g/dL)	252.86 ± 1.09	308.58 ± 1.48	313.93 ± 6.60	130.17 ± 0.50	350.55 ± 1.44	241.05 ± 9.74	261.26 ± 1.20	0.072

Dissimilar superscripted letters in rows indicate significant differences by Tukey's test ($p < 0.05$).

Figures

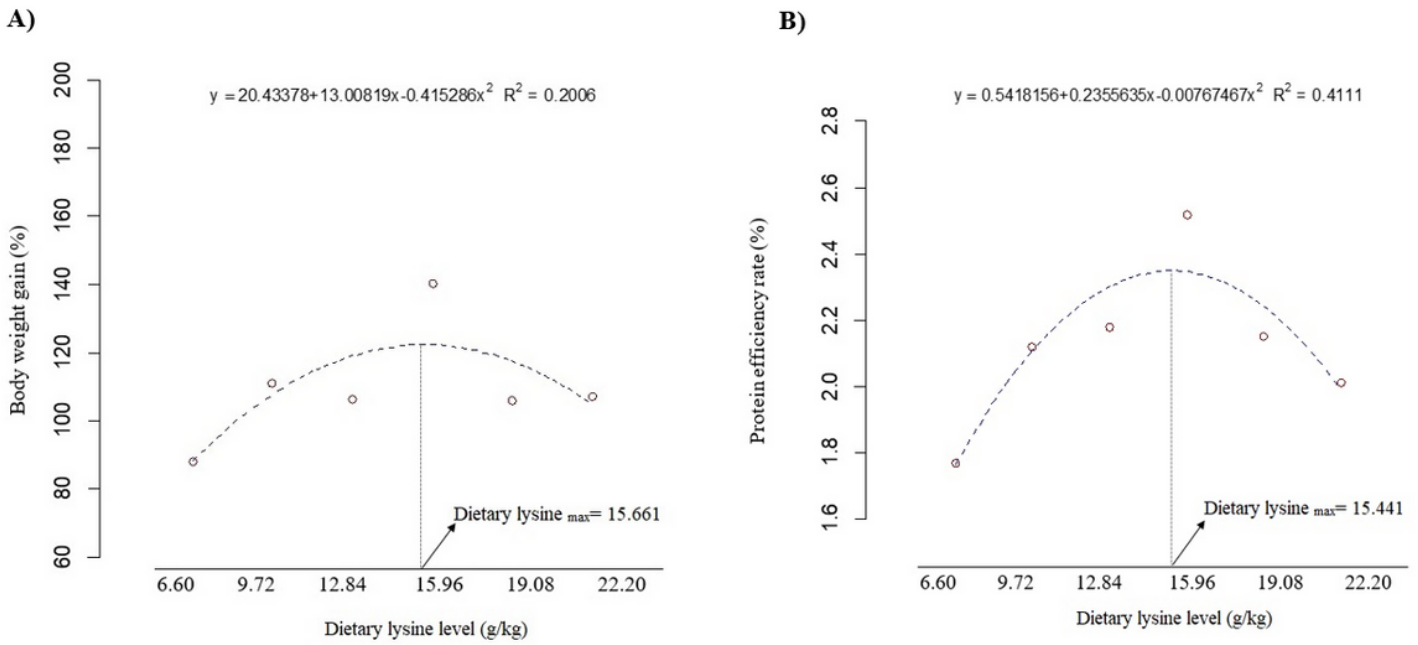

Figure 1

Estimated optimal dietary lysine (g/kg) required by juvenile tambaqui (Colossoma macropomum) as determined by second-order polynomial regression based on body weight gain (15.661 g/kg; 1a) and based on protein efficiency rate (15.441 g/kg; 1b).

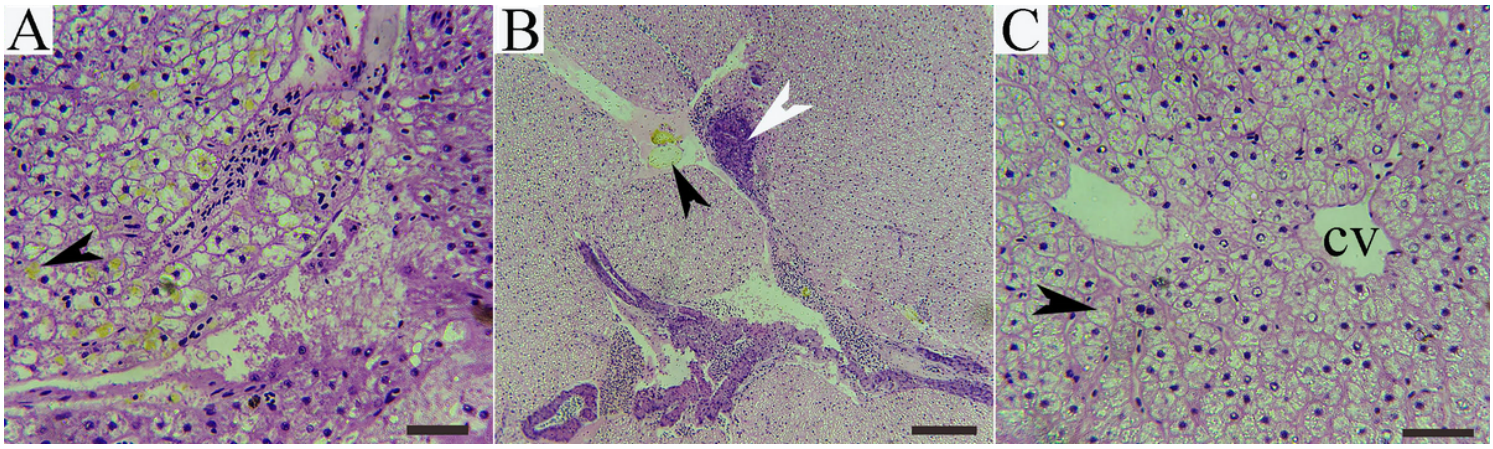

Figure 2

a-c. Photomicrograph of the hepatopancreas of tambaqui (Colossoma macropomum) fed with 9.72 g/kg of dietary lysine: a) multifocal presence of lipid droplets (black arrowhead) in hepatocytes near blood vessels. hepatocytes with polyhedral shape, irregular round and hexagonal, with centralized nucleus. b) lipids in the portal space (black arrowhead) and pancreas (white arrowhead). c) hydropic degeneration characterized by cell edema, cord breakdown and hepatocytes with clear cytoplasm (black arrowhead). key: central vein (cv). bar scales = 500 µm (b) and 50 µm (a and c).

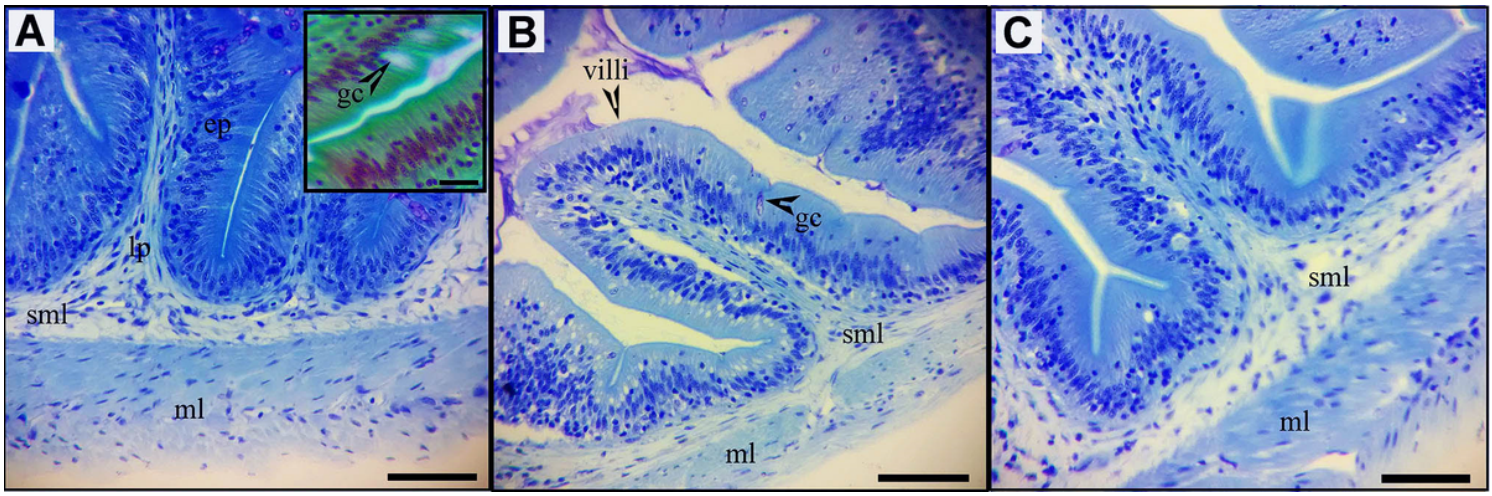

Figure 3

a-c. Representative photomicrograph of the anterior (a), middle (b) and posterior (c) intestine of the tambaqui (Colossoma macropomum) showing epithelium (ep), lamina propria (lp), submucosa layer (cml) and muscularis layer (ml). in the anterior intestine (upper image), the goblet cell (gc) is shown. stained with toluidine blue. scale bars indicate 50 µm, except in upper image in (a) where scale bar = 30 µm.