

NON-STARCH POLYSACCHARIDE IN DIETS FOR HYBRID SURUBIM: EFFECTS ON METABOLISM PLASTICITY

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ABSTRACT

This study aimed to analyze the effect of mango meal, a non-starch polysaccharide, as a carbohydrate source on the metabolism plasticity and performance indicators for hybrid surubim (*Pseudoplatystoma fasciatum* x *P. corruscans*), a tropical carnivorous fish. Juveniles (± 14.5 g initial weight) were fed 3% of total biomass for 60 days in four experimental diets replacing 0, 33, 66 and 100% of the cornmeal. Growth by final weight was evaluated, but statistical differences ($p < 0.05$) were not observed among the treatments. The activity of total alkaline protease was statistically lower in the fish group fed 100% cornmeal substitution. However, trypsin levels also decreased when 100% of the cornmeal was replaced by mango meal, but without statistical significance. Zymograms showed bands of protease and amylase activities for all treatments. Cholesterol (0%- 141.83 \pm 46.10; 100%- 71.36 \pm 14.40 mg.dL⁻¹) and total protein (0%- 3.98 \pm 0.94; 100%- 1.70 \pm 1.03 mg.dL⁻¹) in the plasma decreased as mango meal levels increased in the feed. On the other hand, glucose (0%- 105.08 \pm 31.24; 100%- 128.11 \pm 24.51 mg.dL⁻¹) and free amino acids (0%- 24.51 \pm 2.62; 100%- 38.13 \pm 8.94 nmoles.ml⁻¹) in the plasma increased. Although growth was not affected, the carbohydrate source affected fish metabolism, which showed metabolic adaptation by the species. The result suggests that mango meal can replace up to 33% of the cornmeal in diets for *Pseudoplatystoma* sp.

Key words: adaptation; carnivorous; cornmeal; mango meal; physiology; *Pseudoplatystoma fasciatum* x *Pseudoplatystoma corruscans*

POLISSACARÍDEOS NÃO AMILÁCEOS EM DIETAS PARA HÍBRIDO DE SURUBIM: EFEITOS SOBRE A PLASTICIDADE DO METABOLISMO

RESUMO

O objetivo deste estudo foi avaliar o efeito da farinha de manga, um polissacarídeo não amiláceo, como fonte de carboidrato na plasticidade do metabolismo e indicadores de desempenho no surubim híbrido (*Pseudoplatystoma fasciatum* x *P. corruscans*) um peixe carnívoro tropical. Juvenis (± 14.5 g peso inicial) foram alimentados com 3% da biomassa total durante 60 dias, com 4 dietas experimentais substituindo 0, 33, 66 e 100% do farelo de milho. O crescimento final foi avaliado, mas diferenças significativas ($p < 0.05$) não foram observadas entre os tratamentos. A atividade da protease total alcalina foi significativamente menor no tratamento onde houve 100% de substituição do farelo de milho. Entretanto, níveis de tripsina também diminuíram quando 100% do farelo de milho foi substituído por farinha de manga, mas sem diferença significativa. O zimograma mostrou bandas de atividade de protease e amilase em todos os tratamentos. Colesterol (0%- 141.83 \pm 46.10; 100%- 71.36 \pm 14.40 mg.dL⁻¹) e proteína total (0%- 3.98 \pm 0.94; 100%- 1.70 \pm 1.03 mg.dL⁻¹) plasmáticos, diminuíram à medida que os níveis de farinha de manga aumentaram nas dietas. Por outro lado, glicose (0%- 105.08 \pm 31.24; 100%- 128.11 \pm 24.51 mg.dL⁻¹) e aminoácidos livres (0%- 24.51 \pm 2.62; 100%- 38.13 \pm 8.94 nmoles.ml⁻¹) no plasma, aumentaram. Embora o crescimento não foi aumentado, a fonte de carboidrato afetou o metabolismo do animal, o que mostra adaptação metabólica do híbrido. O resultado sugere que farinha de manga pode substituir até 33% de farelo de milho em dietas para *Pseudoplatystoma* sp.

Palavras-chave: adaptação; carnívoro; farelo de milho; farinha de manga; fisiologia; *Pseudoplatystoma fasciatum* x *Pseudoplatystoma corruscans*

INTRODUCTION

Cornmeal is the most commonly used carbohydrate source in fish feed. Although it is not the most expensive ingredient, cornmeal is rich in starch. There are metabolic and performance signs of carbohydrate overdose in several tropical fish (Dabrowski and Portella, 2006). Carnivorous fish diets rich in non-starch polysaccharide showed

improved protein, fat and starch digestibility (Meriac *et al.*, 2014), lower hepatosomatic index (Gatesoupe *et al.*, 2014) and in white sea bream did not show effect on performance or metabolism (Enes *et al.*, 2013).

The level and type of carbohydrate in fish feed affect several metabolic and enzymatic parameters (Kamalan *et al.*, 2012; Saravanan *et al.*, 2012). Digestive and intermediary metabolism assays has been used as a tool to evaluate metabolism adaptation or regulatory mechanisms in different carnivorous fish.

This is notable when carbohydrate source or inclusion percentage in the diet is tested (Li *et al.*, 2013; Castro *et al.*, 2015; Meshcheryakova *et al.*, 2016; Kamalan *et al.*, 2017). In order to improve fish welfare and growth, alternative sources of carbohydrates have been tested for use in the fish feed industry (Adamidou *et al.*, 2009; Melo, *et al.*, 2012). It is well known that carnivorous fish do not require carbohydrates in their diet as a nutrient, due to their inadequate regulation of hepatic glucose utilization and production (Moon, 2001; Krogdahl *et al.*, 2004; Enes *et al.*, 2009). However, there is little information about carbohydrates in tropical freshwater carnivorous fish diets (Booth *et al.*, 2013) and their effects on growth performance and metabolism.

Worldwide mango production is over 25 million tons per year (FAO, 2013). The availability of mangos as a food resource might be a promising opportunity for its use as an ingredient in animal feed, since mango is a source of fiber and carbohydrate (Rêgo *et al.*, 2010; Kassahun *et al.*, 2012; Pereira *et al.*, 2013). Care must be taken when using mango as an ingredient in animal feed due to the anti-nutritional factors found in its skin such as polyphenols (Garcia-Magaña *et al.*, 2013).

Hybrid surubim (*Pseudoplatystoma fasciatum* x *Pseudoplatystoma corruscans*) is an important commercial and carnivorous species in South America. Total production in Brazil is over 18,000 tons per year (IBGE, 2016). This study aimed to analyze the effects of a non-starch polysaccharide on performance parameters, digestive enzymes and metabolic responses in a hybrid surubim.

MATERIAL AND METHODS

The UNIVASF (Universidade Federal do Vale do São Francisco- Brazil) local ethics committee approved this study. It was performed in according to the Animal and Human Ethic Commission of UNIVASF (protocol number 0022/260911) directives on animal experiments.

Experimental diets

Four diets containing 0, 10, 20 and 30% mango meal (MM), replaced 0, 33, 66 and 100% of the cornmeal (designated as MM0, MM33, MM66 and MM100), respectively, were formulated. The formulation was made in order to have an isonitrogenous (35%) and isoenergetic (14.88 MJ.kg⁻¹) basis. The dietary formulation, proximate composition and mango meal anti-nutritional factors are presented in Table 1.

Mango meal was made by cutting whole mangos without the seed with a sharp knife and placing them in a forced air recirculation oven at 60°C for 24 h. After removal, the mango was ground into powder in a mill. All ingredients were ground to pass through a 0.5 mm mesh screen and thoroughly mixed before adding hot water (60 °C). Pellets of about 4.0 mm-diameter were made with a meat grinder and placed in a forced air recirculation oven at 60°C for 24 h. After removal from the oven and cooling to room temperature, the feeds were frozen (-18 °C) until the beginning of the trial. Proximate composition of ingredients was determined according to AOAC (1990) for moisture, ash, crude protein and ether extract analyses (Table 2). Polyphenol analyses followed Makkar's (2000) technique.

Fish and Feeding

The fish were obtained from CODEVASF (Companhia de Desenvolvimento do Vale do São Francisco e Parnaíba), a Brazilian federal development company. Juvenile hybrid surubim (*Pseudoplatystoma fasciatum* x *Pseudoplatystoma corruscans*),

Table 1. Mango meal chemical composition and characterization.

Chemical Composition – g.kg ⁻¹		
Crude Protein		4.31
Ether Extract		1.46
Ash		3.92
Fiber		7.08
Antinutritional factors		
	Poliphen g.kg ⁻¹ dry matter	Total tannin g.kg ⁻¹ dry matter
Mango with skin	3.67	2.81

Table 2. Formulation and proximate analysis of experimental diets – (content in dry matter).

Content - g.kg ⁻¹	Experimental Diets			
	MM0	MM33	MM66	MM100
Crude Energy ^c - MJ.kg ⁻¹	19.07	18.99	18.50	18.92
Ingredients				
Fish meal	43.31	43.31	43.31	43.31
Soybean meal	14.01	14.01	14.01	14.01
Corn meal	30.00	20.00	10.00	0.00
Mango meal	0.00	10.00	20.00	30.00
Soybean oil	5.97	5.97	5.97	5.97
Vitamin and Mineral Premix ^a	2.00	2.00	2.00	2.00
Vitamin C	0.01	0.01	0.01	0.01
BHT	0.02	0.02	0.02	0.02
NaCl	0.50	0.50	0.50	0.50
Lysine	2.57	2.57	2.57	2.57
Methionine	1.60	1.60	1.60	1.60
Proximate composition				
Moisture	8.97	7.67	8.19	8.07
Crude Protein	35.54	36.03	35.72	36.07
Crude lipid	12.01	11.74	12.03	12.69
Ash	9.13	10.72	13.25	11.96
NNE ^b	34.35	33.84	30.81	31.21

^aVitamin and Mineral Premix: Vitamin A = 1.200.000 UI; vitamin D3 = 200.000 UI; vitamin E = 12.000 mg; vitamin K3 = 2400 mg; vitamin B1 = 4800 mg; vitamin B2 = 4800 mg; vitamin B6 = 4000 mg; vitamin B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12.000 mg; vitamin C = 48.000 mg; biotin = 48 mg; choline = 65.000 mg; nicotinic acid = 24.000 mg; Fe = 10.000 g; Cu = 600 mg; Mn = 4000 mg; Zn = 6000 mg; I = 20 mg; Co = 2 mg; Se = 20 mg. ^bNonnitrogen extracts% = 100-(crude protein% + crude lipid% + moisture% + ash%) ^cEnergy (kJ.g⁻¹ diet) = (% crude protein x 23,6) + (% crude lipid x 39,5) + (% carbohydrates x 17,3)

upon arrival in the laboratory, were acclimatized for 20 days and fed a commercial feed (45% crude protein) before the trial. To initiate the trial, all fish were individually weighed (average 14.53 ± 0.40g). After this procedure, they were sorted into 800L PVC tanks, with 10 fish per tank. Three replicate groups of fish were used for each diet. Tanks were built using a totally open water system. Water parameters were monitored weekly (average temperature was 27.5°C; pH 7.3 and dissolved oxygen 5.7 mg.L⁻¹). Fish were fed twice a day, at 3% of total biomass, (0830 h and 1600 h) for 60 days. Each tank was cleaned daily by scrubbing and siphoning of accumulated wastes, algae or any dirt that possibly came from the water.

Sample collection

At the end of the trial fish were starved for 24h. All fish in each tank were individually weighed for performance parameters: final body weight (FBW), weight gain (WG), survival rate (SR) and feed conversion ratio (FCR). Feed consumption (FC) was also measured. Fish were anesthetized in clove oil (8mL.100L⁻¹ of water) and blood samples (1.5 mL) from 4 randomly

selected fish from each tank were taken from the causal vein using heparinized syringe (80 IU). Tested individuals were subsequently euthanized with eugenol.

Glucose was immediately measured with an automatic monitor. In sequence, these blood samples were centrifuged at 500 x g for 10 minutes at 20°C and the plasma stored in a freezer (-20°C) until the beginning of the total cholesterol, total protein, triglycerides and free amino acid analyses.

After blood was drawn, liver and total intestine samples were collected from the same fish and immediately stored in a freezer (-20°C) until the beginning of the following analyses: glycogen and alanine amino transferase analyses (liver), digestive enzymes activity and zymogram.

Performance

Performance was measured by the following equations:

$$WG (g) = [\text{final body weight (g)} - \text{initial body weight (g)}]$$

$$SR (\%) = 100 \times (\text{final fish number} / \text{initial fish number})$$

FCR = [feed consumed (g)/ weight gain (g)]

FC = feed consumed (g)/time

Enzyme Activity

After collection, the intestines were immediately stored at -20°C . In the laboratory, they were homogenized in 0.01M Tris-HCl pH 8.0 buffer using a tissue homogenizer. The resultant preparation was centrifuged at $10,000\times g$ for 10 min at 4°C . The supernatants (crude enzyme extract) were used for analysis assays (Santos *et al.*, 2013).

Total alkaline protease activity

The total enzyme activity of proteases present in the crude extracts was performed using 10 g.L^{-1} azocasein as a substrate, prepared in 10 mM Tris-HCl, pH 8.0. Aliquots containing $30\text{ }\mu\text{L}$ of the crude extract were incubated with $50\text{ }\mu\text{L}$ of substrate solution for 1 hour at 25°C . Then, $240\text{ }\mu\text{L}$ of 100 g.L^{-1} trichloroacetic acid was added to stop the reaction. After 15 minutes the mixture was centrifuged at $8,000\text{ }\times g$ for 5 minutes. The supernatant was collected and $70\text{ }\mu\text{L}$ of it was mixed in $130\text{ }\mu\text{L}$ of 1M sodium hydroxide solution (revealing solution) in microplates.

The absorbance was measured on a microplate reader (x-Mark Bio-Rad) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract with a solution of 10 mM Tris-HCl, pH 8.0 with 0.15 M NaCl added. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

Trypsin Activity (E.C. 3.4.21.4)

The activity of trypsin was determined using 8.0 mM BApNA (N α -benzoyl-DL-arginine-p-nitroanilide) in DMSO (Dimethyl sulfoxide). Intestine crude enzyme extract ($30\text{ }\mu\text{L}$) was incubated with 0.1M Tris-HCl buffer pH 8.0 ($10\text{ }\mu\text{L}$) and respective substrates ($30\text{ }\mu\text{L}$) in a microtiter plate reader (Bio-Rad 680, Japan). The absorbance was measured at 405 nm versus a similarly prepared blank in which 0.1M Tris-HCl pH 8.0 replaced the crude extract sample. Enzyme activity was determined in triplicate. Trypsin units of activity were expressed as a change in absorbance per minute per milligram of protein (Bezerra *et al.*, 2005).

α -Amylase Activity (E.C. 3.2.1.1)

α -Amylase activity from the intestine of *Pseudoplatystoma* sp. was based on the method of Bernfeld (1955) using 20 g.L^{-1} starch solution as a substrate. The enzyme preparations were made in 0.2 M citrate-phosphate buffer, pH at 7.0 at 37°C for 30 minutes. Then the assay was carried out by addition of 3,5-dinitrosalicylic acid (DNSA) at 100°C for 10 minutes.

The absorbance was measured at 570nm using a microplate reader (Bio-Rad 680). Both substrate-free and an enzyme-free controls were run. The amount of maltose released from this assay was determined from the standard curve using commercial maltose. One milliunit of specific activity was defined as the amount of enzyme needed to release $1\text{ }\mu\text{g}$ maltose per minute per milligram of soluble protein in the enzyme solution at 37°C ($\text{mU}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein).

Zymograms

Protease zymogram

Protease zymography was performed to characterize the protease present in the crude enzyme, in a procedure described by Garcia-Carreño *et al.* (1993). The zymogram was initiated by electrophoresis (SDS-PAGE) under immersion in an ice bath. Crude enzyme extracts were mixed with sample buffer containing 0.5M Tris-HCl at a pH of 6.8, 200 mL.L^{-1} glycerol, 10% SDS, and 5 mL.L^{-1} bromophenol blue. A quantity of $20\text{ }\mu\text{L}$ of the sample buffer mixture was loaded into SDS-PAGE gel with a thickness of 1.0 mm. The gel consisted of 40 g.L^{-1} stacking gel and 125 g.L^{-1} separating gel. Electrophoresis was conducted at a constant current of 12 mA with an electrophoresis buffer comprising Tris-glycine-sodium dodecyl sulfate. After electrophoresis, the gel was immersed in 100 mL of 25 mL.L^{-1} Triton X-100, diluted in 0.1 M Tris-HCl, pH 8.0, for a period of thirty minutes at 4°C to remove the SDS. Then Triton X-100 was removed by washing the gels with 0.1 M Tris-HCl, pH 8.0. The gel was incubated in 100 mL of 30 g.L^{-1} casein diluted in 0.1 M Tris-HCl, pH 8.0, for 30 minutes at 4°C to determine the proteolytic activity. Soon after the gel was kept in the same casein solution at 25°C for 90 minutes to permit the digestion of casein by active fractions. Finally, the gel was stained with a solution composed of 1 g.L^{-1} Coomassie Brilliant Blue, methanol 250 mL.L^{-1} and 100 mL.L^{-1} acetic acid and after 24 hours was bleached in a solution with the same composition but devoid of the dye.

Amylase zymogram

Amylase zymography was carried out according to the modified methodology described by Fernández *et al.* (2001). Enzyme preparations were applied to a 12.5% separating gel. After electrophoresis performed at 4°C and a constant current of 12 mA, the gel was immersed in 2.5 mL.L^{-1} (100 mL) Triton X-100 in 0.1M Tris-HCl (pH 8.0, for 30 min at 4°C) to remove the SDS. Triton X-100 was removed by washing the gel three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Next, the SDS-free, Triton X-100-free gel was incubated with a 20 g.L^{-1} starch solution containing 10 mM phosphate buffer, pH 8.0, and 1 mM CaCl_2 for 60 min at 37°C to allow for the digestion of starch by the active fractions. Finally, the gel was washed with distilled water, stained with a 10 g.L^{-1} iodine/KI solution for 5 minutes and a 130 mL.L^{-1} acetic acid solution was added to stop the reaction.

Plasma and liver metabolites

Metabolite concentration was carried out in plasma and liver tissue extracts. Total plasmatic cholesterol was determined by the enzymatic colorimetric method, using a Labtest®, (Brazil) commercial kit with the reading done at 500 nm wavelength. Ten (10) microliters of plasma sample were incubated for 10 minutes at 37°C and the measurement unit was expressed in mg.dL⁻¹. Total protein was determined by the Biuret method (Hiller, 1948). Twenty microliters of plasma were incubated for 10 minutes at 37°C. The reading was performed at 545 nm and the measurement unit was expressed in g.dL⁻¹. Plasma triglycerides were analyzed by the enzymatic colorimetric method using a commercial kit (Labtest®, Brazil). Ten microliters of plasma samples containing reagent in 1ml of buffer pH 7.0 were incubated for 10 minutes at 37°C. The samples were read at 500nm and the units of measurement were in mg.dL⁻¹.

Glucose in the fish blood was measured at the end of the trial, using an automatic glucose reader (Accucheck®, Brazil). Free amino acids were determined in neutral extracts at 570 nm (Copley, 1941). Glycogen was assayed in alcoholic precipitates from alkaline tissues homogenates (Bidinotto et al., 1997).

Alanine aminotransferase (ALT) activities were determined using a commercial kit (Labtest®, Brazil). The reaction principle of ALT is based on the conversion of alanine to pyruvate by transamination, followed by reduction to lactate by the LDH auxiliary enzyme which is monitored optically by extinction of NADH. The reaction cocktail for ALT contained the following:

100 mM tris-HCl buffer, pH 7.5, 13.7 mM 2-oxoglutarate, 0.18 mM NADH, 0.5 mM L-alanine, 21 mKat LDH.L⁻¹, and a sufficient volume of enzyme. The enzyme activity was expressed in U.L⁻¹.

Statistical Analyses

The statistical model design was completely randomized and the data were analyzed by analysis of variance (ANOVA). The results were expressed as means and standard deviations (SDs), and were subjected to regression analysis, considering the growth performance and metabolic intermediate parameters as the dependent variables. A p-value of <0.05 was considered significant for all statistical methods used. The statistical analyses were performed using the generalized linear model (GLM) procedure (SAS Institute Inc, Cary, NC). Linear, polynomial and exponential models were tested for all regressions, and the best model was selected based on R². For enzymatic activities, significant differences between means were determined by the Tukey's multiple range test.

RESULTS

In this study the influence of a non-starch polysaccharide, carbohydrate source on a tropical carnivorous siluriformes fish could be observed. None of the performance parameters were affected by dietary treatments (p<0.005) (Table 3). No death was noted during the trial.

Table 3. Growth performance and metabolic intermediates of hybrid surubim *Pseudoplatystoma* sp. fed diets containing various levels of mango meal in replacement for corn meal.

Parameters	MM0	MM33	MM66	MM100	p-value	Regression		Equation	R ²
						Linear	Quadratic		
Performance									
FBW -g.fish	32.50±1.37	31.35±1.79	30.09±2.81	30.09±2.18	0.4771	-	-	-	-
WG -g	17.62±1.11	17.08±1.60	15.84±2.81	15.41±1.92	0.5116	-	-	-	-
SR-%	100	100	100	100	-	-	-	-	-
FCR	0.76±0.04	0.75±0.06	0.82±0.17	0.86±0.09	0.5183	-	-	-	-
FC-g.day ⁻¹	4.4±0.12	4.2±0.06	4.2±0.15	4.4±0.12	0.1498	-	-	-	-
Values are means ± SD. FBW = Final body weight, WG = weight gain, SR = survival rate, FCR = feed conversion rate, FC = feed consumption									

Among the intestinal enzyme activities analyzed, it was observed that amylase was the lowest one, independent of group treatment. Total protease (MM0 0.139±0.03A; MM33 0.154±0.05A; MM66 0.154±0.06A; MM100 0.035±0.00B U.mg⁻¹), trypsin (MM0 0.812±0.49; MM33 0.559±0.57; MM66 0.781±0.32; MM100 0.325±0.22 U.mg⁻¹) and amylase activity (MM0 0.008±0.00; MM33 0.009±0.00; MM66 0.011±0.00; MM100 0.007±0.00 um.min⁻¹.mg⁻¹) were lowest (p<0.05) in the group where 100% of the cornmeal was replaced by mango meal (Figure 1). A statistical difference was only observed in the total protease activity analysis.

A caseinolytic zymogram was prepared to compare the proteolytic activities of the treatments (Figure 2A). Similar patterns were observed for MM0 and MM33. Five caseinolytic bands were observed for MM0 and MM33; six for MM66, five of them with higher intensity (A) and two for MM100. The amylase zymogram (Figure 2B) revealed that the surubim *Pseudoplatystoma* sp. has two types of amylase. The intensity of one of the amylases was always higher than the other. This can also be observed in the fourth line, which is the MM100 treatment; the activity was a little lower than the others.

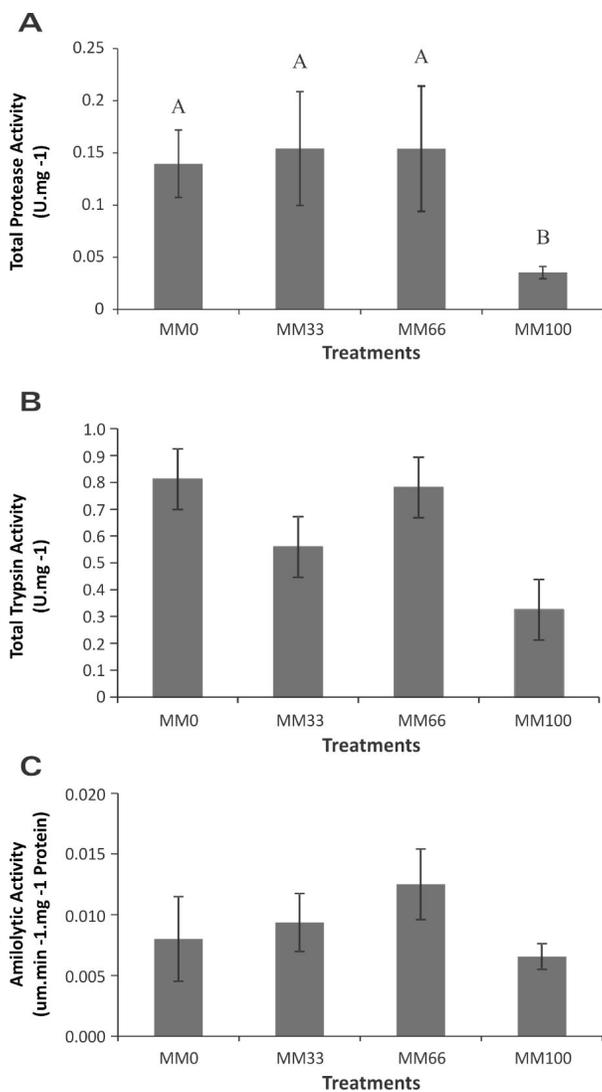


Figure 1. Intestine digestive activity (means±SD) of surubim juvenile fed diets containing mango meal replacing corn meal. **A.** Total alkaline protease activity. **B.** Trypsin activity. **C.** Amylase activity. Different letters in the same line indicate statistical difference ($P<0.05$) among treatments (Tukey).

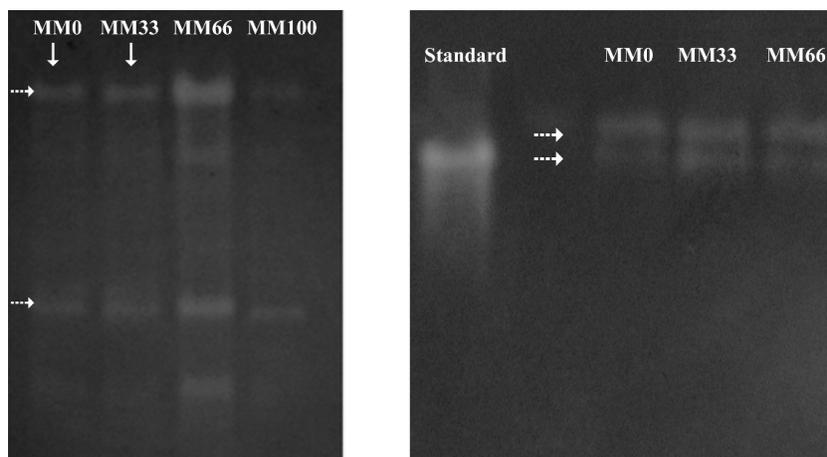


Figure 2. **A.** Zymogram of digestive protease (3% casein as substrate) of intestine enzyme extract from surubim *Pseudoplatystoma corruscans* fed on diets containing different inclusion levels of mango meal. Lanes correspond to dietary mango meal inclusion (%): MM0%; MM33%; MM66%; MM100%. **B.** Zymogram of digestive amylase (2% starch as substrate) of intestine enzyme extract from surubim *Pseudoplatystoma corruscans* fed on diets containing different inclusion levels of mango meal. Lanes correspond to dietary mango meal inclusion (%): MM0%; MM33%; MM66%; MM100%.

Metabolic parameters were influenced by the treatments ($p < 0.005$) and showed linear regression (Table 4), except for triglycerides. In the treatment with 100% mango meal inclusion, total cholesterol was lower. As mango level increased in the diets, glucose and free amino acids in the plasma also increased. For both parameters analyzed, the lowest levels were observed at treatments with 0 and 33% inclusion of mango meal. Liver glycogen level was higher in the dietary treatments with 0% inclusion of mango meal. It showed a quadratic effect among treatments, with the lowest level observed in the treatment with 66% mango meal. Alanine aminotransferase also showed a quadratic effect in the four dietary treatments evaluated. The lowest level was observed at treatment with 33% inclusion of mango meal.

Table 4. Growth performance and metabolic intermediates of hybrid surubim *Pseudoplystoma* sp. fed diets containing various levels of mango meal in replacement for corn meal.

Parameters	MM0	MM33	MM66	MM100	p-value	Regression		Equation	R ²
						Linear	Quadratic		
Metabolism Blood Plasma									
TC-mg.dL ⁻¹	141.83±46.10	104.87±37.77	81.32±26.49	71.36±14.40	0.0053	0.0008	0.4040	126.72±7.14-0.59±0.11X	0.9687
TP-mg.dL ⁻¹	3.98±0.94	4.13±0.68	3.28±0.46	1.70±1.03	<.0001	<.00001	0.0012	4.39±0.13-0.02±0.01X	0.8085
TG- mg.dL ⁻¹	163.87±10.98	139.95±17.58	121.77±22.62	110.69±25.23	0.3733	-	-	-	-
G- mg.dL ⁻¹	105.08±31.24	100.11±14.84	141.11±36.09	128.11±24.51	0.0043	0.0038	0.5294	102.09±5.11+0.33±0.08X	0.5401
FAA-nmols.ml ⁻¹	24.51±2.62	23.51±4.03	41.10±8.44	38.13±8.94	0.0009	0.0005	0.5154	23.41±1.59+0.14±0.02X	0.6384
Liver									
GC-umols glucose.g ⁻¹	39.91±8.28	32.49±4.10	15.70±4.45	20.30±4.24	<.0001	<.00001	0.0074	41.45±1.64-0.49±0.07X+0.003±0.001X ²	0.8719
ALT-U.g protein ⁻¹	5.01±1.80	3.92±1.13	4.58±1.49	4.58±1.49	0.0001	0.0018	<.0001	5.59±0.32-0.13±0.01X+0.001±0.0001X ²	0.8969

Values are means ± SD. TC = total cholesterol, TP = total protein, TG = triglycerides, G = glucose, FAA = free amino acids, GC = liver glycogen, ALT = liver alanine aminotransferase

physiological importance for growth and metabolic maintenance (Adamidou *et al.*, 2009; Polakof *et al.*, 2012). Moreover, it is important for farm profitability if performance parameters are improved (Besson *et al.*, 2014).

In the present study, there was no statistical difference among the performance parameters evaluated, during the 60-day trial. When cornmeal was replaced by mango meal with skin, juvenile tilapia performance was negatively influenced by this source of carbohydrate used in the feed when fed at 66 and 100% inclusion (Souza *et al.*, 2013). However, such results are not the same for all fish species and the natural feeding behavior must be considered. Fruit skin has a high amount of tannins, which is an anti-nutritional factor, but some fish species usually can tolerate certain amount of tannins (Francis *et al.*, 2001; Siddhuraju and Becker 2001). These substances are enzyme inhibitors that bind to proteases at their substrate loci and negatively affect fish growth; some species can compensate for this by increasing trypsin activity in the intestine (Francis *et al.*, 2001; Zheng *et al.*, 2012).

Although this study showed that the carbohydrate source evaluated did not influence performance, *Pseudoplystoma corruscans* growth is affected by the energy source of feed ingested (Arslan *et al.*, 2009). A diet for a carnivorous, tropical fish

DISCUSSION

In the present study, the change in the qualitative pattern of carbohydrate source modified the metabolism of a carnivorous tropical fish. There was a physiological adaptation to the diet, which showed metabolic plasticity, although growth was not affected.

Carbohydrate inclusion may improve the protein efficiency of fish feed (Kamalan *et al.*, 2012). It is known that the amount of carbohydrate influences the performance, digestive enzymes, digestibility and metabolism of carnivorous fish (Ren *et al.*, 2011). Alternative carbohydrate sources also impact the performance and metabolism of carnivorous fish species (Adamidou *et al.*, 2009; Enes *et al.*, 2013). Searching for an alternative carbohydrate source for commercial fish feed has

might attend the energy requirement for the species using either carbohydrates or lipids, but the correct fatty acid and amino acid profile is essential for optimal performance and survival. Since no deformities were observed in the fish from this study, the fatty acid profile from the fish oil of the fishmeal used and added soybean oil probably provided the requirements for this species.

High protease activity, especially trypsin, is an indicator of growth in fish (Torrissen-Rungruangsak *et al.*, 2006). In this study, protease activity was lower in the group that had 100% of the cornmeal replaced by mango meal while growth parameters were not different. There was no difference among digestive enzyme levels in the treatments from 0 through 66% of cornmeal replaced by mango meal. The differences observed are probably due to the random selection of fish sampled for the assays. Other hypothesis is that the hybrid tested did not show the metabolic plasticity to compensate protease inhibition by mango skin anti-nutritional factors, increasing trypsin activity in the intestine (Francis *et al.*, 2001; Zheng *et al.*, 2012).

In carnivorous fish species, protease activity will always be higher when compared to other digestive enzyme activity no matter what the macronutrient sources are, due to their natural feeding behavior (Lundstedt *et al.*, 2004). Digestion potential

ratio analysis shows that a carnivorous fish exhibits higher amylase activity than an herbivore (1:1.28) and an herbivore shows higher activity of acid protease than a carnivore (1:1.25) (Chacrabarti *et al.*, 1995). It seems that fish in general have an evolutionary adaptation to digest any kind of food, which is clearly, an advantage over any kind of food deprivation.

Juvenile tilapia fed diets with 50% rapeseed meal inclusion, which is rich in anti-nutritional factors such as tannins, also had decreased their protease activity when compared to other plant protein sources without high amounts of anti-nutritional factors (Lin *et al.*, 2010). Total alkaline protease activity is the sum of specific proteases such as trypsin, chymotrypsin and others, which cleave the peptides of protein in the feed into amino acids that are then bioavailable for fish growth or fish metabolism. Trypsin activity is understood to be a key factor in supplying amino acids and peptides for fish growth (Sunde *et al.*, 2001). Also, trypsin activity and growth rate are linked through the effect of trypsin effect on the capacity of fish to convert feed into body components (Lemieux *et al.*, 1999). As can be seen from the results found in the present work, the highest trypsin activity was found in the group where 66% of the cornmeal was replaced by mango meal. In this same group, free amino acids in the plasma were also higher than the other groups, which might also indicate that amino acids from feed protein were available for fish growth.

Different amounts of the dietary macronutrients used in carnivorous fish feed influence the activity of digestive enzymes, including amylase and lipase. Indeed, digestive tract pH may also affect it (Pérez-Jiménez *et al.*, 2009). Nevertheless, there are also studies showing that the amount of starch in the feed does not influence protease or amylase activity in carnivorous fish (Couto *et al.*, 2012). In the present study, amylase activity was not statistically different among the tested groups. This may be due to the amount of carbohydrates and the saccharide profile found in the feed (Pérez-Jiménez *et al.*, 2009; Couto *et al.*, 2012). However, in tilapia different plant-based feeds did not affect amylase activity (Lin *et al.*, 2010).

Zymography has proven to be an effective tool in detecting changes in digestive enzyme activity in fish subjected to different diets (Santos *et al.*, 2013). Both protease and amylase zymograms showed the same enzyme activity found in the crude extract assays. The inhibitory effect of the anti-nutritional factors found in the diets with 100% mango meal was also confirmed. In short, there was a clear physiological adaptation of surubim digestive enzymes in response to the different carbohydrate profiles found in the diet. This adaptation promoted alterations in feed nutrient bioavailability that changed the metabolic profile of the fish.

The metabolic responses of the surubim were highly influenced by the level of mango meal. The same was observed for tambaqui (*Colossoma macropomum*) also fed with increasing levels of mango meal (Souza *et al.*, 2018). Fish total cholesterol decreased with the increase of mango meal, as has also been observed in mammals (Salgado *et al.*, 2008). Fruits in general have high amounts of soluble fibers that irreversibly link to cholesterol and to bile acids carrying them to the feces, thus cholesterol is not absorbed in the liver (Zhang *et al.*, 2011).

Total glucose in the plasma was lower in fish groups that had the lowest amounts of mango meal in their diets. In those same groups, liver glycogen was higher than in the other groups. In the groups where mango meal inclusion was higher, higher glucose levels and lower glycogen levels were observed. Glucose maintenance was not regulated by hepatic glycogen. Liver glycogen reduction for glucose maintenance has already been observed in tambaqui, *Colossoma macropomum* (Almeida *et al.*, 2011). The effect of higher mango meal on glycemia might be due to gluconeogenesis from other substrates, such as free amino acids. Unlike the results observed in this study, rainbow trout fed low and high amounts of starch did not have altered levels of glucose and total free amino acids, while triglycerides did change (Kamalan *et al.*, 2012).

Triglyceride levels in sea bass fed a non-starch polysaccharide feed diet was lower than fish fed a starch polysaccharide diet (Gatesoupe *et al.*, 2014). Triglycerides, which indicate that excess carbohydrate from the diet was not used as an energy source and was converted into fatty acids and glycerol, showed a slight change when intra-peritoneal glucose was injected in rainbow trout. Only 24h after the injection the level of this parameter started to increase and was about the same as 2h after the injection (Enes *et al.*, 2012). This proves that glucose influences the level of triglycerides, but nothing has been specified about the influence of the carbohydrate source. However, carbohydrate concentration may affect triglyceride levels (Correa, *et al.*, 2007). Levels of glucose in fish have been related to their time tolerance rather than to temperature, fish species and its feeding habit related to the diet (Enes *et al.*, 2012).

Kumar *et al.* (2009) found that carp fed diets with non-gelatinized corn had lower glucose levels than the groups fed gelatinized corn. This is similar to the result found in this study, since the treatments with lower mango meal had higher amounts of starch. Mango meal is composed of short chain, non-starch polysaccharides (saccharide and fructose) which improve its digestibility and energy availability to the animal (Couto *et al.*, 2012). When liver glycogen concentration is low, it might mean that glucose available in the body is being used for metabolic energy (Enes *et al.*, 2009). It has already been proven for carnivorous fish that the amount of carbohydrates in the diet influences liver glycogen and plasma glucose, while the source of carbohydrates does not (Ren *et al.*, 2011; Kamalan *et al.*, 2012). Recent studies have shown that there is no difference between ingested carbohydrates and gene expression of enzymes from the gluconeogenesis pathway (Enes *et al.*, 2009; Li *et al.*, 2013). The fact that the values of ALT have not changed among the different treatments tested means that no damage was caused in the liver (Fuentealba *et al.*, 2011) or amino acid availability (Men *et al.*, 2014). Also, the carbohydrate source and the amount evaluated did not influence transamination by ALT. The activities of transaminases and deaminases are useful in evaluating the feeding status in some fish (Melo *et al.*, 2006; Moyano *et al.*, 1991; Meshcheryakova *et al.*, 2016). Vegetable or animal diet sources can affect ALT values, as it was observed for Japanese seabass (Men *et al.*, 2014). Therefore, it is important to evaluate this enzyme activity to design a metabolism regulation due to different ingredients sources.

Gelatinized and non-gelatinized starch inclusion in diet for *Labeo rohita* trials, resulted in activity of ALT and AST modulated by the reduction of hepatic enzyme activities in diets with higher concentrations of non-gelatinized starch (Kumar et al., 2010). Thus, dietary modification of carbohydrates can improve the utilization of this energy source by fish (Yengkokpam et al., 2006). The amount and type of energy or protein macronutrients in fish diets, may alter the activity of the transaminase enzymes, leading the fish to a metabolic adaptation. *Micropterus salmoides* fed diets with different carbohydrates sources, showed an increase in ALT activity with cassava starch and wheat starch, and lower transamination activities with pea starch and corn with high amylose maize (Song et al., 2018). Also, increased ALT and hepatic AST activity was observed by protein increase in *Carassius auratus* feed (Ye et al., 2016). Therefore, in this study the inclusion of mango flour in the proportion of 33% reduced the transamination process. In fish that received other diets, there was an increase in ALT activity, which can not be considered as a high transamination process, since there are still no refractive values to classify the degree of transamination.

Total protein in the plasma was lower than in the groups with 0 and 33% inclusion levels of mango meal in the feed. This fact might also indicate a change in protein and free amino acid metabolism and their synthesis in the liver (Yousef et al., 2006). Total plasma protein is related to globulin levels, so the lower levels found in the MM66 and MM100 groups might indicate a reduction in fish immunity.

CONCLUSION

Although growth performance was not affected during the 60 days trial, the decrease in plasma protein and glycogen, as well as the increase in glucose in the treatments MM66 and MM100, showed negative metabolic responses to these diets. Therefore, feed nutrients caused metabolic changes, even in 60-day feeding trials. In addition, different carbohydrate sources influenced the digestive enzyme profiles.

Since no difference in growth performance was observed, changes in metabolism maintain energy levels during fish growth. These changes are a sign that performance can be compromised during long-term feeding periods with feeds that contain more than 33% inclusion of mango meal. It was proven through the analyses performed that growth performance alone cannot answer for the inclusion of an alternative ingredient in fish feed. The hybrid studied showed high metabolism plasticity when fed different carbohydrate sources.

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