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Scientific Article

Crude glycerin in feed for *Colossoma macropomum* fish: evaluation of histopathological effects on the liver and kidney

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ABSTRACT

The present study aimed to verify whether the use of crude glycerin in the diet of juvenile *Colossoma macropomum* could promote histopathological changes in the liver and kidneys. 150 juveniles were used, divided into 15 tanks of 1000 liters (five treatments and three repetitions, n = 10). Five different diets with inclusion of crude glycerin (0%; 7.5%; 10%; 12.5%; 15%) were used in exchange for soybean oil and partial replacement of corn bran. The animals were fed to satiation for 60 days. After this period, liver and kidney samples from each group were submitted to histological processing and histopathological analysis. The semi-quantitative method was used to assess the lesions: calculation of Mean Alteration Values (MVA), which assess the occurrence of the lesion, and the Histopathological Alterations Index (HAI), which allows assessing the severity of the lesions. The results indicated significant non-harmful effects, but adaptive responses to the diet. In conclusion, the inclusion of up to 15% crude glycerin can be used without causing significant liver and kidney changes in *C. macropomum* juveniles.

Keywords: Glycerol; Aquaculture; Tambaqui; Fish feed; Histopathology.

Glicerina bruta em rações para peixes tambaqui: avaliação dos efeitos histopatológicos no fígado e rim

Resumo

O presente estudo teve como objetivo verificar se a utilização de glicerina bruta na dieta de juvenis de *Colossoma macropomum* poderia promover alterações histopatológicas no fígado e nos rins. Foram utilizados 150 juvenis, divididos em 15 tanques de 1000 litros (cinco tratamentos e três repetições, n = 10). Foram utilizadas cinco diferentes rações com inclusões de glicerina bruta (0%; 7,5%; 10%; 12,5%; 15%) em troca do óleo de soja e substituição parcial do farelo de milho. Os animais foram alimentados até a saciedade durante 60 dias. Após esse período, amostras de fígado e rim de cada grupo foram submetidas ao processamento histológico e análises histopatológicas. O método semiquantitativo foi usado para avaliar as lesões: cálculo dos Valores Médios de alterações (MVA), que avaliam a ocorrência da lesão e o Índice de Alterações Histopatológicas (IAH), que permite avaliar a gravidade das lesões. Os resultados indicaram efeitos não prejudiciais significativos, mas respostas adaptativas à dieta. Em conclusão a inclusão de até 15% de glicerina bruta pode ser utilizada sem causar alterações hepáticas e renais significativas em juvenis de *C. macropomum*.

Palavras-chave: Glicerol; Aquicultura; Tambaqui; Ração para peixes; Histopatologia.

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INTRODUCTION

High cost with feeding on aquaculture is encouraging research with alternative feed sources, such as agribusiness by-products. Consequently, crude glycerin has been receiving attention as an alternative product, valuable for partial or total replacement of high-cost ingredients on fish feed (Bombardelli et al., 2021a). The term glycerin is applied to purified commercial products usually containing at least 95% of glycerol (Mauerwerk et al., 2021). Glycerin is obtained as a by-product by a transesterification reaction of biodiesel processing (Nomanbhay et al., 2020). However, to use an alternative feed we must consider its availability, quality, lower prices than the conventional ones and the animal's performance. Therefore, the animal's energy and nutritional needs, along with general health and well-being, must be taken into consideration (Santos, L. et al., 2019).

Glycerin is an energy source. In the liver´s intracellular compartment, oxidation of glycerol can occur to produce energy through glycolysis and the Krebs cycle (Santos, L. et al., 2019; Silva et al., 2014). Studies demonstrated that as an energy source, glycerin can be included on feeding, thus decreasing the cost of feed for ruminants (Costa et al., 2020), swines (Martínez-Miró et al., 2021), birds (Avellaneda et al., 2020) and more recently for fish (Bombardelli et al., 2021b; Matos et al., 2016; Theisen et al., 2020). However, most studies focused on the apparent effects, such as the animal's zootechnical performance. Nevertheless, as a new ingredient, the possibility of a deleterious effect cannot be discarded. Hence, liver and kidney evaluation can be used as an indicator of the animal's nutritional and physiological conditions, considering that these organs are responsible for the metabolism and excretion of substances from the digestive system (Rašković et al., 2011). Therefore, liver and kidney alterations can display an instability caused by nutritional factors (Elia et al., 2018) or as indicators of xenobiotic intoxication (Oliveira- Lima et al., 2021).

Colossoma macropomum (Costa, 2012), tambaqui, is an endemic Amazonian neotropical fish that belongs to the Characidae family. This species is one of the most desired by fish farms owners for these characteristics: strength, meat with excellent taste and few fish bones (Valenti et al., 2021). Besides, these fish are easy to manage, with a prominent adaptation to intensive culture systems and acceptance of commercial feed. These aspects contributed to *C. macropomum* being one of the main species on aquaculture programs in Brazil (Lima et al., 2020; Santos, E. et al., 2020). Tambaqui fed with the inclusion of crude glycerin (15%) did not decrease the zootechnical performance (Matos et al., 2016). Therefore, in this study we evaluate if the crude glycerin use in the diet of tambaqui juveniles may cause histopathological alterations on liver and kidney.

MATERIAL AND METHODS

Animals and maintenance

Colossoma macropomum juveniles, n = 150 (mean weight = 17.07 ± 6.07g) were obtained from a local fish farm and transferred to the Laboratory. The animals were acclimated for a period of 30 days in tanks with capacity for 5000 liters and constant water flow. Water parameters were monitored daily: temperature (26.2 ± 0.13 °C); dissolved oxygen (7.5 ± 0.16) and pH (6.5 ± 0.5). Animals were maintained in a natural photoperiod and feeding was performed daily with commercial feed *ad libitum.* All procedures were approved by the Animal Ethics Committee (CEUA) by Federal University of Northern Tocantins, Araguaína, TO, Brazil with Protocol no. 23101.002495/2014-82.

Treatments and samples

The experimental design was defined in accordance with our previous study (Matos et al., 2016). Fish were randomly distributed into five groups in three replicates (n = 10/group) in 1000 L capacity tanks. Among them, four groups received feed with inclusion of crude glycerin in substitution of soy oil and the control group received feed with no glycerin (Table 1). Feeding was *ad libitum* twice a day (8 am and 5 pm) for 75 days (15 days of adaptation and 60 days of experimentation). After this period, animals of each treatment were randomly collected, then anesthesia induction was performed with benzocaine (0.1 $g{\cdot}L^{-1}$) and euthanasia was done through medullary section for organ collection.

During the experiment, water parameters were measured and recorded, remaining within the species' comfort range (Table 2).

Histological processing

Liver and kidney samples from the middle portion were washed with saline solution 0.9% and fixed for 2 hours in Bouin solution. Then, we washed the samples in running water and stored them in a container with 70% ethanol. The samples were dehydrated in successive baths of ethanol (80%, 90%, 95% and 100%), clarified with xylol and embedded on paraffin. Subsequently, longitudinal histological slices of 5 µm were made with a manual microtome. The sections were stained with hematoxylin and eosin (HE) (Junqueira, L.C. and Junqueira, L.M., 1983) for histopathological evaluation and with periodic acid–Schiff (PAS) (McManus, 1948) for quantification of liver glycogen. Therefore, we took photomicrographs with a

Table 1. Chemical and percentage composition of experimental feed with different levels of crude glycerin inclusion for juveniles of *Colossoma macropomum.*

	Crude glycerin inclusion levels (%)				
Ingredients	**Control (0)**	**7.5**	**10**	**12.5**	**15**
Corn meal	39	31	29	26	24
Soybean meal (45%)	40	40	40	40	40
Meat and bone meal (40%)	10	10	10	10	10
Rice bran	10	10	10	10	10
Soy oil	0.5	0	0	0	0
Nucleus[1]	0.7	0.7	0.7	0.7	0.7
Requirements					
Crude protein (%)	26	26	26	26	26
Digestible Energy (Kcal kg^{-1})	3127	3126	3127	3128	3129
Calcium (%)	1	1	1	1	1
Phosphorus (%)	0.6	0.6	0.6	0.6	0.6

[1]Levels of micronutrients per kilogram: Folic acid 20.25 mg; Antioxidant 66.15 mg; Cobalt 33.75 mg; Copper 337.50 mg; Iron 337.50 mg; Iodine 50.62 mg; Manganese 1350.00 mg; Methionine 1.20 mg; Calcium pantothenate 315.56 mg; Selenium 10.12 mg; Sodium 55.58 mg; Tyrosine 810.00 mg; Vit. A 216,000 U.I; Vit. B1 45,56 mg; Vit. B2 135.00 mg; Vit. B6 67.50 mg; Vit. C 600 mg; Vit. D3 50.625,00 U.I; Vit. A 506,25 U.I; Vit. C 2,70 mg; Vit. K3 50,62 mg; Vit. B12 675,00 mg; Zinc 3375.00 mg.

Table 2. Physicochemical parameters of water during the experiment with feed containing different levels of crude glycerin inclusion for tambaqui juveniles. Data are expressed as mean and standard deviation.

Parameters	Crude glycerin inclusion levels (%)				
	0	**7.5**	**10**	**12.5**	**15**
pH	06.7±0.4	06,8±0,5	06,5±0,2	06,6±0,3	06,4±0,7
Dissolved oxygen (mg L^{-1})	07.5±0.1	07,1±0,9	07,3±0,6	07,6±0,4	07,2±0,1
Temperature (°C)	26.2±0.1	26,7±0,0	27,1±0,1	25,5±0,2	27,8±0,7
Ammonia (mg L^{-1})	0.7±0.4	0,9±0,8	1,0±0,9	0,9±0,6	1,2±0,8

camera attached to a light microscope (LEICA DM500) with a software (LAZ 2.0).

Histopathological analysis

To identify the histopathological alteration on the liver and kidney, we observed 5 fields of each slide at 40x magnification. To evaluate the tissues, we used two semiquantitative methods: the Mean Values of Alteration (MVA) and the Histopathological Alteration Index (HAI). MVA calculation results from the number of lesions, as described by Schwaiger et al. (1997). Therefore, a grade was given to each animal according to a rank: grade 1 (no pathological alterations), grade 2 (focal lesions) and grade 3 (lesions extended through the organ). To evaluate the degree of liver and kidney alterations we used the Histopathological Alteration Index (HAI) according to Rôxo et al. (2018) (adapted from Poleksic and Mitrovic-Tutundžic, 1994). Each alteration was classified progressively according to the function loss: stage I (for alterations that do not provoke function loss); stage II (severe alterations that do not cause complete loss of function) and stage III (severe alterations that cause complete loss of function). The HAI value was calculated for each animal according to the formula: HAI= (1 x ΣI) + (10 x ΣII) + (100 x ΣIII), where ΣI, II and III correspond respectively to the stages I, II and III. Values between: 0 and 10 stands for tissue normal function, 11 and 20 indicates mild organ lesion,21 and 50 indicates moderate damage, 51 and 99 stands for severe damage; and higher than 100 indicates irreversible tissue damage.

Glycogen quantification

Five fields from each PAS-stained slide were used to quantify liver glycogen. The fields were randomly photographed at 40x magnification and analyzed for each slide. The analyzed fields were distant from great vessels to analyze as many hepatocytes as possible. Glycogen was isolated using an ImageJ® plugin. Total area (µm) occupied by glycogen was quantified and expressed as percentage (Campos et al., 2017).

Statistical analysis

Data was analyzed using the Shapiro-Wilk test to check normality. Then, the data was submitted to analysis of variance (ANOVA). Parametric averages were compared by Tukey's test ($p > 0.05$) and nonparametric data were analyzed with Kruskall Wallis's test. Tests were performed on Graph Pad Prism 5.0 software.

RESULTS

Liver

Table 3 and Fig. 1 show histopathological analysis of the liver. The control group had a characteristic and homogeneous hepatic parenchyma, polygonal hepatocytes with spherical nuclei and central nucleolus arranged in linear cords limited by sinusoids that connected with central veins (Fig. 2a). After the diet with crude glycerin inclusion, we observed some alterations, such as hepatocytes architecture alterations (Fig. 2b),hepatocytes

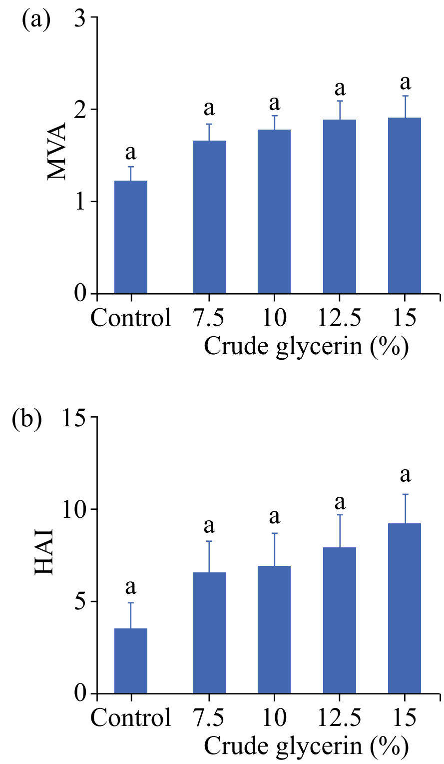

Figure 1. Histopathological evaluation of the liver of *Colossoma macropomum* juveniles after diets with crude glycerin (0%, 7.5%, 10%, 12.5% and 15 %) as a total replacement of soybean oil and partial replacement of corn. (a) Indices from the Mean Value of Alteration (MVA); (b) Histological Alteration Index (HAI) of the liver. The significant difference was considered with $p < 0.05$. Different letters indicate differences among treatments.

Table 3. Evaluation of the occurrence of alterations in the hepatic tissue of *Colossoma macropomum* fed with different levels of crude glycerin inclusion in the diet.

Alteration	Crude glycerin inclusion levels (%)					
	Stage	**0**	**7.5**	**10**	**12.5**	**15**
Hepatocyte atrophy	I	0	0	0+	0	0+
Dilation of sinusoids	I	0	0+	0+	0	0+
Hepatic disorganization	I	0	0+	+	0	0+
Hepatocyte hypertrophy	I	0	0+	+	0+	+
Nuclei hypertrophy	I	0	0+	0+	+	+
Cytoplasmic vacuolization	I	0+	++	+++	++	+++
Congestion	II	0	0+	0+	0	0+
Absence of nucleolus	II	0	0+	0+	0+	0+
Cholestasis	II	0	+	0	0+	0+
Kupffer cells increase	II	0	0	0	0+	+
Necrosis focus	III	0	0	0	0	0

Data expressed as: 0 = non-existent; 0+ = rare occurrence; + = frequent alteration; ++ = very frequent alteration; +++ = occurrence of intense alteration.

hypertrophy and nuclear alterations (Fig. 2f). Table 3 shows the occurrence frequency of these alterations.

The Mean Values of Alteration (MVA) of hepatic alterations were not significant ($p < 0.05$) among the experimental diets (7.5%, 10%, 12.5%, 15% of crude glycerin) and the control (Fig. 1a). These were occasional alterations distributed on the liver, considering that the alterations did not surpass grade 2.

The Histopathological Alteration Index (HAI) did not exhibit significant differences (Fig. 1b). Therefore, hepatic alterations were reversible and did not interfere with the organ function.

Quantification of hepatic glycogen levels exhibited a similarity between control and experimental diet groups, indicating a lack of significant effect ($p < 0.05$) on the energy storage of these fish (Fig. 3).

Kidney

Table 4 and Fig. 4 show histopathological alterations in the kidney of *C. macropomum*. The control group exhibited glomeruli with well-defined Bowman's space, proximal and distal tubules surrounded by interstitial and hematopoietic tissue (Fig. 4a). The most frequent alterations were the ones on the kidney architecture and in the cells (Table 4).

Figure 5 presents kidney´s mean values of alteration and histopathological alteration index. Resembling what was observed on liver analysis, the mean values of alteration (MVA) for the kidney did not present significant differences ($p<0.05$) among

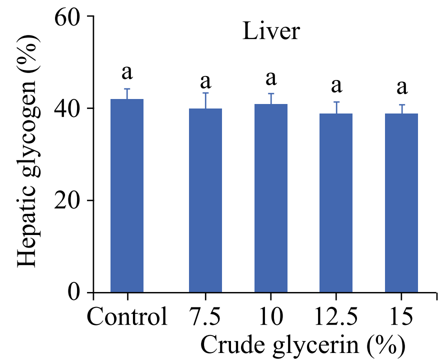

Figure 3. Quantification of liver glycogen of *Colossoma macropomum* juveniles after diets with crude glycerin (0%, 7.5%, 10%, 12.5% and 15 %) as a total replacement of soybean oil and partial replacement of corn. The significant difference was considered with $p < 0.05$. Different letters indicate differences among treatments.

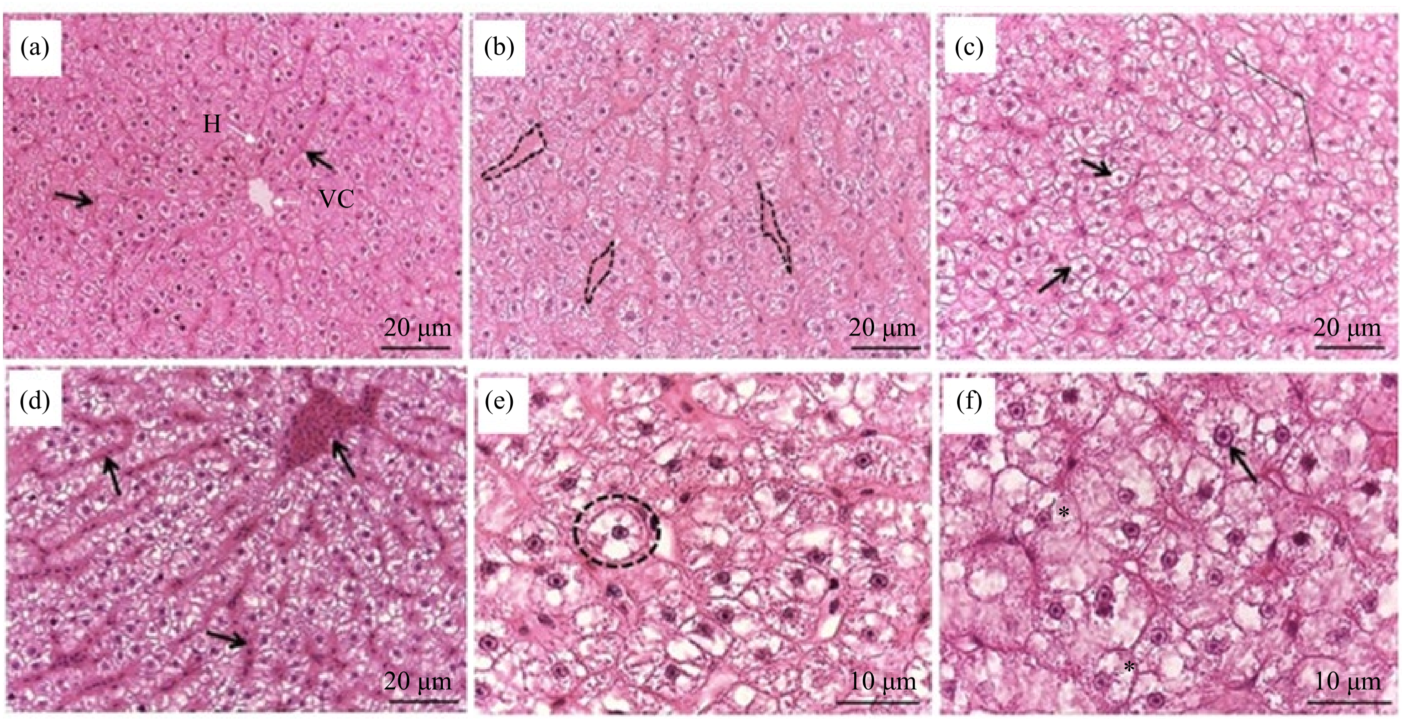

Figure 2. Representative photomicrographs of the histopathology of *Colossoma macropomum* liver, after diets with crude glycerin (0%, 7.5%, 10%, 12.5% and 15%) as a total replacement of soybean oil and partial replacement of corn. (a) Control: no alterations, hepatocyte (H); central lobular vein (VC); sinusoid capillaries (arrow); (b) 7.5%: Dilation of sinusoids (dashed area); (c) 10%: cytoplasmic vacuolization (arrow) and cordonal disorganization; (d) 12.5%: congestion (arrow); (e) 15%: cellular hypertrophy (dashed area); (f) 15%: nuclear hypertrophy (arrow) and vacuolization (asterisk). HE is stained.

Table 4. Evaluation of alterations occurrence in the kidney tissue of *Colossoma macropomum* fed with different levels of crude glycerin inclusions on the diet.

Alteration	Crude glycerin inclusion levels (%)					
	Stage	0	7.5	10	12.5	15
Tubular disorganization	I	0	0	0+	0+	+
Nuclear hypertrophy	I	0+	0+	0+	0+	0+
Glomerular dilation	I	0	0+	+	+	+
Melanomacrophages centers	I	0	0+	0+	+	0+
Tubular lumen dilation	II	0	0	0+	0+	0+
Focal necrosis	III	0	0	0	0	0

Data expressed as: 0 = non-existent; 0+ = rare occurrence; + = frequent alteration; ++ = very frequent alteration; +++ = occurrence of intense alteration.

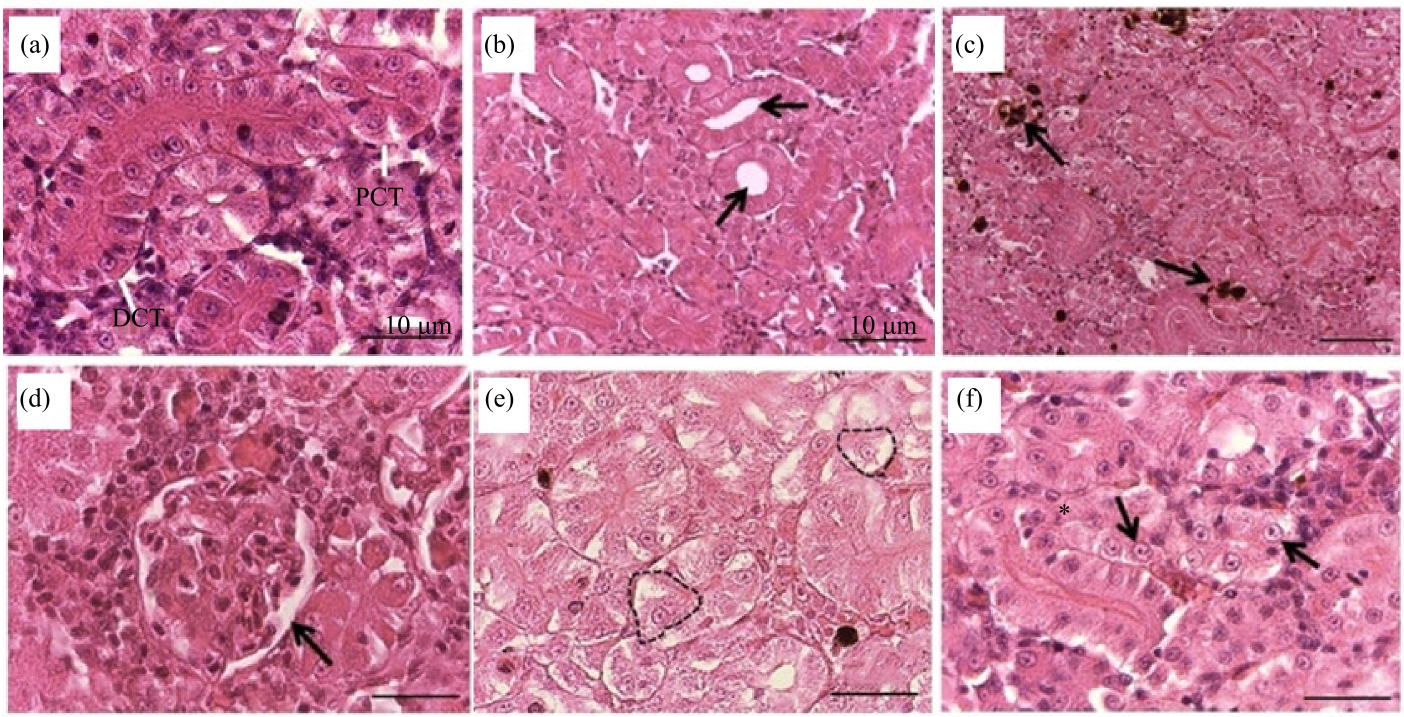

Figure 4. Representative photomicrographs of the kidney's histopathology of *Colossoma macropomum*, after diets with crude glycerin (0%, 7.5%, 10%, 12.5% and 15%) as total replacement of soybean oil and partial replacement of corn. (a) Control: no changes, proximal convoluted tubule (PCT); distal convoluted tubule (DCT); (b) 10%: Dilation of the tubular lumen (arrow); (c) 12.5%: increase in the frequency of melanomacrophage centers (arrow); (d) 12.5%: glomerulus with increased Bowman's capsule space (arrow); (e) 15%: cellular hypertrophy (dashed area); (f) 15%: nuclear hypertrophy (arrow). HE is stained.

the groups of experimental diet with crude glycerin and control (Fig. 5a). Alterations were scarcely distributed on the kidney parenchyma and did not surpass grade 2. The Histopathological Alteration Index (HAI) did not exhibit significant differences (Fig. 5b). Hence, kidney alterations were not severe and did not interfere with organ function.

DISCUSSION

Most studies that use feeds containing alternative ingredients focus only on the evaluation of zootechnical data, thus lacking an assessment of the general health and even the well-being of the animals. For this reason, the present study investigated the histopathology of vital organs for fishes that were fed with feed

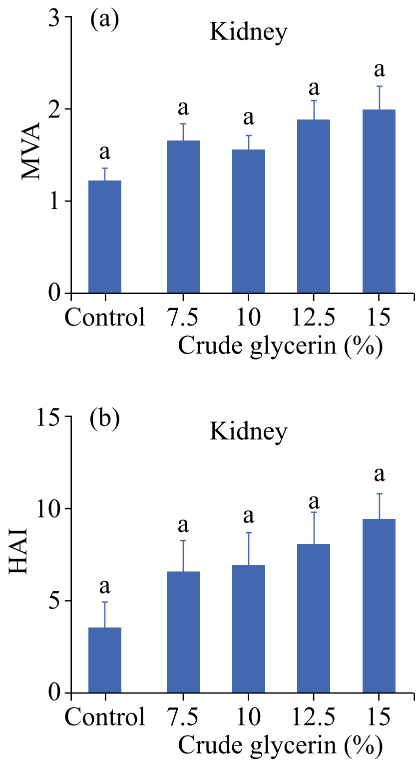

Figure 5. Histopathological evaluation of the kidney of *Colossoma macropomum* juveniles after diets with crude glycerin (0%, 7.5%, 10%, 12.5% and 15 %) as a total replacement of soybean oil and partial replacement of corn. (a) Indices from the Mean Value of Alteration (MVA); (b) Histological Alteration Index. The significant difference was considered with $p < 0.05$. Different letters indicate differences among treatments.

containing crude glycerin, given that zootechnical data were not significantly affected (data published in Matos et al., 2016).

Water parameters analyzed throughout the experiment were not influenced by the diets tested, reflecting the meticulous care with experimental variables. These parameters were maintained in a healthy pattern for this species (Santos, R. et al., 2021).

The liver is an important organ in the digestion and metabolism of nutrients. It is an organ highly susceptible to changes in the nutritional status of fish, whereas the quality of the diet directly interferes with its morphofunctional state (Rašković et al., 2011). Morphological changes in the liver can be triggered by chemicals, medications and even an unbalanced diet, which may result in adaptations, injuries and even cell death (Rôxo et al., 2018). In the present study, the general morphological pattern of the liver of juveniles *C. macropomum* was like that found in other freshwater teleost, presenting a characteristic architecture, rich in relatively hexagonal hepatocytes with a central nucleus and surrounded by sinusoid capillaries, as reported by Costa et al. (2012). However, the results showed diets induced homeostatic adjustments, which are adaptive strategies to maintain cellular function. The observed changes such as atrophy or hypertrophy of hepatocytes reflect the functional state of the liver and are notably different from hyper or hypofunction. Cellular or nuclear hypertrophy indicates increased cellular activity due to the presence of a chemical compound or increased metabolic activity (Takashima and Hibiya, 1995). Feed containing crude glycerin may have provided extra energy by altering the functional activity of hepatocytes.

The liver can also, in response to an energy surplus, increase hepatic glycogen deposition or, due to energy mobilization promoted by stress, reduce its activity (Takashima and Hibiya, 1995). Thus, the quantification of glycogen levels is a good biomarker of the body's energy state (Ansaldo et al., 2006). The analysis of this reserve showed that crude glycerin in the diet, even at the highest level of inclusion (15%) does not significantly alter the deposition of glycogen on the liver of tambaqui. The vacuolization observed in hepatocytes may be lipid granules; however, it needs further investigation. Similarly, Bombardelli et al. (2021a) observed a significant degree of hepatocytes vacuolization on females of tilapia fed with feed with a considerable amount of crude glycerin (80, 120, 160 $g \cdot kg^{-1}$).

The alterations observed in the liver parenchyma were low frequency and occasional, although it did not present statistical difference, it may be indicative of toxic residue presence in the ingredient used, as it tended to increase according to the level of inclusion. In this context, it should be noted that the composition of crude glycerin may have some impurities, among which methanol and some metals, even in small amounts, can interfere with the metabolism of animals (Moesch et al., 2016). Thus, the need to increase knowledge about this product in feed emerges. There are few investigations about the histopathology of animals fed with crude glycerin.

Few studies have investigated the histopathology of animals fed crude glycerin. Among the few works performed, it is possible to observe that the results corroborate those found in the present study. Moesch et al. (2016), when studying the replacement of corn bran by crude glycerol in diets for fingerlings of tilapia at concentrations of 0, 20, 40, 60, 80 and 100%, concluded that there were no differences around hepatocytes. Similarly, the use of crude glycerol (60, 120, 180 and 240 $g \cdot kg^{-1}$) as an energy source to replace corn in diets for tilapia in the fattening phase did not affect liver morphology (Santos, L. et al., 2019). On juveniles of the catfish hybrid "Pintado-da-Amazônia" (female *Pseudoplatystoma punctifer* x male *Leiarius marmoratus*) diets with crude glycerin did not alter liver morphology (Rôxo et al., 2018). For other monogastric, such as growing pigs, feeding with the addition of up to 10% glycerin did not induce liver damage (Lammers et al., 2008).

Morphological assessments in the kidney are generally investigated on fish when there are changes in the diet, as it is an organ that performs the filtration and excretion of substances from digestion (Elia et al., 2018). The histopathological evaluation of the kidney, after diets containing crude glycerin, showed changes that are not harmful to the organ and that are still reversible. Rôxo et al. (2018) carried out an evaluation of the kidney tissue after the inclusion of crude glycerin in the diet of the Amazon catfish hybrid and found that such diet does not cause histopathological changes in the kidney and can be included in the diet (up to 125 $g{\cdot}kg^{-1}$). Likewise, on ruminants, Leão et al. (2012) did not observe histopathological alterations in kidney samples from cattle fed with crude glycerin.

Our results present relevant information, which is in conformity with other studies and demonstrates that crude glycerin has a considerable potential as an alternative ingredient. Considering that glycerin is a by-product of biofuel production, and there is a recent increase in this industry, using glycerin on fish feed can reduce costs and improve profit of both businesses and, therefore, reduce its presence on the environment (Balen et al., 2014).

CONCLUSION

Fish feed containing crude glycerin (up to 15%) as an exchange for soy oil did not cause harmful alterations on the liver and kidney of juveniles of *C. macropomum*, considering there are no significant histopathological effects on these organs.

ETHICAL APPROVAL

Approved by the Ethics Committee for the Use of Animals at UFNT/Araguaína (protocol 23101.002495/2014-82).

CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

All datasets were generated or analyzed in the current study.

AUTHOR CONTRIBUTIONS

Conceptualization: Moron SE; **Data curation:** Santos ELR; **Formal analysis:** Santos ELR; **Acquisition of funding:** Not applicable; **Research:** Santos ELR; **Methodology:** Santos ELR; **Project administration:** Not applicable; **Resources:** Moron SE; **Software:** Santos ELR; **Supervision:** Moron SE; **Validation:** Moron SE; **Viewing:** Moron SE; **Writing - Preparation of original draft:** Santos ELR; Moron SE; **Writing - Proofreading and editing:** Santos ELR; Moron SE.

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