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Zootechnical performance, degree of steatosis and the genotoxic potential in yellowtail tetra *Astyanax lacustris* fed with different levels of L-carnitine

[*Desempenho zootécnico, grau de esteatose e potencial genotóxico de lambaris* Astyanax lacustris *alimentados com diferentes níveis de L-carnitina*]

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ABSTRACT

L-carnitine perform a major role in transporting long-chain fatty acids into the mitochondria, where they are oxidized. It has been used in animal diets to decrease fat and increase muscle protein. The aim of this study was to evaluate the zootechnical performance, degree of steatosis in the liver, and genotoxic potential in *Astyanax lacustris* fed with different levels of L-carnitine (LC). Yellowtail tetra juveniles (n = 140) were distributed in 20 tanks of 70 L, with seven fish in each, in a water recirculation system with controlled temperature (27±0.1ºC). The treatments with different levels of L-carnitine supplementation were: 0 (control), 250, 500, 750, and 1000 mg of LC per kg of food. The diets were provided twice a day for 60 days. The results showed that the different levels of LC did not affect (P>0.05) weight gain, survival, viscerosomatic index, and the liver hepatocytes showed a normal appearance. However, the use of LC supplementation showed genotoxic potential with a significant difference (P<0.05) for cell alterations when compared to the control at concentrations above 500mg kg-1.

Keywords: cells alterations, fish, food additive, nutrition

RESUMO

A L-carnitina exerce um papel importante no transporte de ácidos graxos de cadeia longa até a mitocôndria para serem oxidados e tem sido incorporada em rações para animais com o objetivo de diminuir a deposição de gordura e aumentar a proteína muscular. O objetivo deste trabalho foi avaliar o desempenho zootécnico, o grau de esteatose no fígado e o potencial genotóxico em Astyanax lacustris alimentados com diferentes níveis de L-carnitina (LC). Juvenis de lambari-do-rabo-amarelo (n=140) foram distribuídos em 20 caixas de 70L, sete peixes em cada, em um sistema de recirculação de água com temperatura controlada (27±0,1ºC). Os tratamentos com os níveis de suplementação foram: 0 (controle), 250, 500, 750 e 1000 mg de LC kg^{-1} de ração. As dietas foram fornecidas duas vezes ao dia, durante 60 dias. Os resultados mostraram que os diferentes níveis de LC não influenciaram (P>0,05) o ganho de peso; a sobrevivência, o índice viscerossomático e os hepatócitos do fígado apresentaram-se com aparência normal. No entanto, a suplementação com LC apresentou potencial genotóxico com diferença significativa (P<0,05) para alterações celulares quando comparada ao controle em concentrações superiores a 500mg kg^{-1}.

Palavras-chave: alterações celulares, peixe, aditivo alimentar, nutrição

INTRODUCTION

Among the various components studied to meet the nutritional demand of fish, L-carnitine (4-N-trimethyl ammonium-3-hydroxybutyric acid) (Scientific…, 2012) plays a major role in transporting long-chain fatty acids into the mitochondria, where they are oxidized. The increased efficiency of energy used from lipids provides an improvement in growth performance in fish, allowing the use of this protein for muscle tissue production (Tonini *et al*., 2011). Thus, it is possible to reduce the time for fish production and increase profits.

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However, studies have shown contradictions about the use of L-carnitine (LC) as an additive to improve weight gain in fish. Some authors have reported positive effects, such as Asgharimoghadam *et al*. (2014) on common carp (*Cyprinus carpio*) at 2500 mg kg^{-1} and Sanchez *et al*. (2021) on Nile tilapia juveniles (*Oreochromis niloticus*). Nevertheless, no significant effects were observed by Yang *et al*. (2009) for hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) at 250 mg kg^{-1}. Therefore, the effect of LC on growth performance must be evaluated for each fish species. No studies were found in the literature evaluating the genotoxicity of LC in fish, which is important to ensure its safety. Consequently, the analysis of genetic damage of exposed organisms, such as micronuclei and nucleus alterations, could be used to evaluate such possible damage.

The yellowtail tetra (*Astyanax lacustris*) is a small native fish species with several requirements for aquaculture, including easy adaptability to captivity, high growth rate, and ease of rearing. This species is mainly used as live bait in sport fishing, for direct human consumption, or even canned (Yasui *et al*., 2020). Additionally, *A. lacustris* has been used as a model organism for several basic and applied studies, such as chromosome manipulation (Nascimento *et al*., 2017, 2020) and ecotoxicological studies (Fernandes *et al*., 2019). Therefore, the aim of this study was to evaluate the growth performance, degree of steatosis, and genotoxic potential in *A. lacustris* fed diets supplemented with LC.

MATERIAL AND METHODS

The experiment was executed in the Aquaculture production laboratory at the Federal University of Grande Dourados (UFGD), Mato Grosso do Sul State, Brazil. The procedures were performed in accordance with the guide for the care and use of laboratory animals of the Committee on Ethics for the Use of Animals at the Federal University of Grande Dourados (UFGD 08/2016).

Yellowtail tetra juveniles (n = 140) were distributed into 20 tanks of 70 L each, with seven fish per tank. The tanks were part of a recirculating system that had controlled water temperature (27 ± 0.1 ºC), biological filter, and constant aeration. The fish were divided into five treatment groups, with four replicates for each treatment. The treatments included different levels of LC in the diet: 0 (control), 250, 500, 750, and 1000 mg of LC per kg of food. To prepare the diets, LC (mg) was first diluted in 100 mL of 70% alcohol and then incorporated into commercial pellets (0.08 mm; 4200 kcal/kg and 45% crude protein) using a sprinkler. The fish were fed twice a day to apparent satiation (ad libitum) for 60 days. Daily, feces and uneaten food were removed, and 20% of the water was renewed. The experiment was conducted under natural conditions with a 12h light:12h dark photoperiod maintained throughout the experiment.

Dissolved oxygen was measured daily using a YSI 55 (YSI Incorporated, Yellow Springs, OH). The mean value obtained was 6.60±0.77 mg L^{-1}. The pH was measured weekly, with a mean value of 7.80±0.5.

For the zootechnical performance analysis, juveniles at 60 days of age were weighted at the beginning of the experiment, showing a mean weight of 2.80 ± 0.05 g. After 60 days, zootechnical indices such as mean final weight (g), weight gain (g), and survival (%) were evaluated in the adults. In addition, one male and one female were randomly selected from each tank, euthanized in a solution of eugenol (1 g L^{-1}), and subsequently dissected to remove the viscera and calculate the viscerosomatic index (VSI), gonadosomatic index (GSI), and hepatosomatic index (HSI) using the following formulas:

$$\text{VSI} = \frac{\text{Viscera weigth}}{\text{Fish weigth}} \text{ x } 100$$

$$\text{GSI} = \frac{\text{Gonad weigth}}{\text{Fish weigth}} \text{ x } 100$$

$$\text{HSI} = \frac{\text{Liver weigth}}{\text{Fish weigth}} \text{ x } 100$$

For the histological analysis, sections of the liver were collected from the euthanized fish. Samples were immediately fixed in Bouin's fixative (300 mL of saturated solution of picric acid + 100 mL formalin + 20 mL glacial acetic acid) for 24h. Afterwards, the fixed tissue was washed in water and stored in 70% ethanol for histological processing. Samples were dehydrated through

increasing ethanol series, cleared in xylene, and embedded in paraffin. The sections (3 µm) were stained with hematoxylin and eosin.

The slides were examined under a microscope (Axioplan 2, Zeiss, USA) equipped with a CCD camera (MC 80 DX, Zeiss, USA) at a magnification of 1000x. The degree of steatosis was assessed using the scoring system adopted by Tessaro *et al*. (2014), where 0 represents the absence of vacuoles, 1 indicates a reduced level, 2 represents an intermediate level, and 3 indicates a severe level. The scoring was performed by two observers to ensure accuracy.

After the exposure period of 60 days, the genotoxicity assay was performed. Seven fish from each treatment were anesthetized in an ice bath, and a cut was made in their tail fin using surgical scissors to produce a blood smear for the quantification of micronuclei and nuclear morphological alterations. Three blood smears were performed for each fish and left to dry at room temperature (±25 °C). After drying, the material was fixed in 99.8% ethanol for 10 minutes and hydrolyzed in 1N HCl at 60 °C in a water bath for another 10 minutes. The slides were then stained with Schiff's and Fast Green reagents following Schmid's protocol (1975).

The micronuclei and nuclear morphological alterations, including budding, nuclear invagination, chromosomal bridge, pyknosis, binucleated cells, lobed nucleus, karyolitic, and vacuolated nucleus, were counted using a light microscope (Nikon) at a magnification of 1000x (Nikon, Japan). Three thousand cells were counted for each fish to calculate the number of altered cells and the genotoxicity index (GI). Normal and altered erythrocytes are shown in Figure 1.

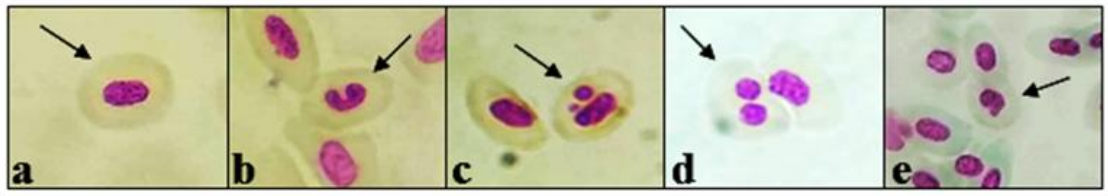

Figure 1. Micrography showing erythrocytes of *Astyanax lacustris*: a) normal cell, b) nuclear invagination, c) micronuclei, d) binucleated cell, e) nuclear budding.

The data on zootechnical performance and histology were analyzed using ANOVA/One-way, followed by the Tukey test (5%), using Statistica 7.0 software. For the genotoxicity assay, a statistical comparison was made between the concentrations and the control using ANOVA, with Tukey's post hoc test ($p \leq 0.05$), using BioEstat 5.0 software.

RESULTS

No differences ($p > 0.05$) for zootechnical performance were observed for the levels of LC (Table 1).

The histological analysis showed no alterations in liver hepatocytes of females and males (Fig. 2) describes the levels of steatosis in liver samples and most of them show normal appearance, with degree of steatosis of just 0 – 1.

Table 1. Zootechnical performance of *Astyanax lacustris* fed different levels of L-carnitine in the diet

Treatment (mg)	FAW(g)	WG(g)	SUR(%)	HSI(%)	GSI(%)	VSI(%)
C	5.51±0.55	2.75±0.48	96.42±3.57	1.17±0.16	6.69±1.96	11.12±2.02
250	5.92±0.38	3.10±0.38	64.28±17.00	0.58±0.12	8.41±2.68	11.9±2.81
500	5.87±0.48	3.04±0.45	78.57±9.22	0.97±0.15	9.58±3.57	14.18±3.81
750	5.48±0.12	2.64±0.12	100.00±0.00	0.78±0.11	6.40±1.86	11.10±1.62
1000	6.05±0.49	3.22±0.51	89.28±10.71	0.80±0.14	7.74±2.51	11.96±2.81
*P value**	0.8406	0.8416	0.1318	0.0741	0.3911	0.5147

FAW: final average weight, WG: weight gain, SUR: survival, HSI: hepatosomatic index, GSI: gonadosomatic index, VSI: viscerosomatic index, C: control group *Different letters indicate significant differences ($P > 0.05$) by Tukey's test (n=4*).

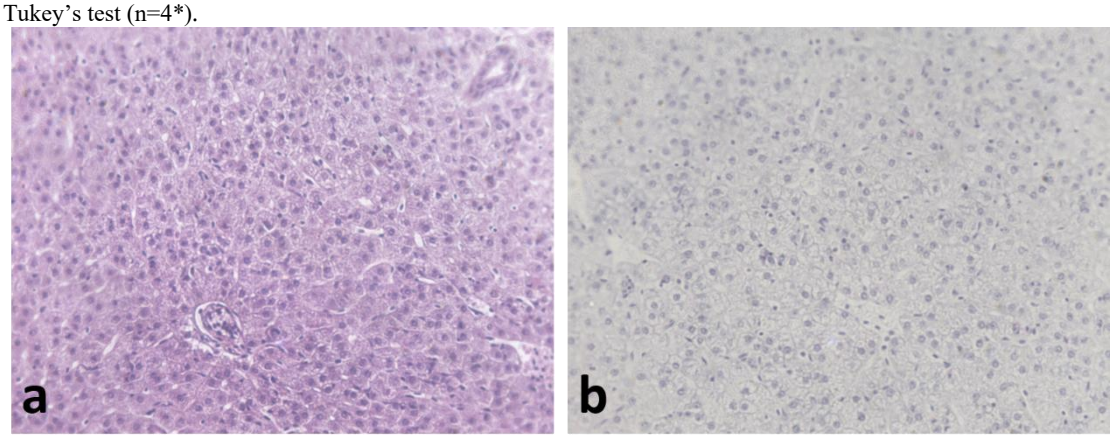

Figure 2. Levels of steatosis in liver of *Astyanax lacustris*: vacuolization score 0 (a) and cellular size alteration; score 1 (b) found in all treatments.

No micronuclei or other alterations such as karyolysis, lobed nucleus, vacuolated nucleus, or pyknosis were observed. Chromosome bridges were detected, but the frequency was similar to that of the control group. Significant differences were observed for nuclear budding (NB) at 1000 mg kg^{-1} compared to the control group ($P<0.01$). Differences were also found for binucleated cells (BC) at 500, 750, and 1000mg kg^{-1} compared to the control group. The data on nuclear invagination showed differences in all concentrations evaluated compared to the control group ($P<0.0001$), and its frequency was dependent on the concentration. Significant differences were also observed for the genotoxicity index, which presented increased genotoxicity at higher concentrations (750 and 1000mg kg^{-1}) (see Table 3).

Table 3. Mean and standard deviation of erythrocytes nuclear alteration in *Astyanax lacustris* after 60 days fed with different levels of L-carnitine in the diet

Treatment (mg)	NB	NI	BC	GI
C	0.00 ± 0.00^{a}	0.50 ± 0.17^{a}	0.00 ± 0.04^{a}	0.57 ± 0.14^{a}
250	0.08 ± 0.05^{a}	1.13 ± 0.17^{b}	0.01 ± 0.02^{ab}	1.24 ± 0.08^{b}
500	0.06 ± 0.00^{a}	1.21 ± 0.14^{b}	0.08 ± 0.05^{b}	1.22 ± 0.14^{b}
750	0.17 ± 0.04^{ab}	1.36 ± 0.20^{b}	0.11 ± 0.07^{b}	1.88 ± 0.38^{c}
1000	0.29 ± 0.13^{b}	2.13 ± 0.83^{c}	0.13 ± 0.05^{b}	2.38 ± 0.94^{c}

NB: nuclear budding, NI: nuclear invagination, BC: binucleated cells, GI: genotoxicity index, C: control group. Distinct superscript letters in the same column indicate significantly difference by Tukey's test ($p \leq 0.05$) (n=5).

DISCUSSION

Several studies have been conducted to address the effect of LC in fish diets, and some authors have reported a positive impact on weight gain. Sanchez *et al*. (2021) indicated that Nile tilapia juveniles (*O. niloticus*) showed better growth with 500 and 1000 mg kg^{-1} of LC in their diet, and the lipid content in their fillets was reduced. Wang *et al*. (2019) reported increased growth and an antioxidant effect on the defense system when using 400-750 mg kg^{-1} of L-carnitine in the diet of amur minnow (*Phoxinus lagowskii Dybowiskii*). On the other hand, other studies have shown no significant effects of inclusion, such as in channel catfish (*Ictalurus punctatus*) (Burtle and Liu, 1994), rainbow trout (*Oncorhynchus mykiss*) (Chatzifotis *et al*., 1997), and tilapia hybrids (*Oreochromis spp*.) (Yang *et al*., 2009). Despite these contradictory results, it

has been reported that LC dietary supplementation reduces lipid deposition by promoting higher mitochondrial fatty acid β-oxidation, which probably promotes protein synthesis (Li *et al*., 2017).

Changes in liver histology can be easily detected when the food is not appropriate, and such analysis could indicate the nutritional status of the fish (Rašković *et al*., 2011). For example, Tessaro *et al*. (2014) observed an intense level of vacuolization in the hepatocytes of female silver catfish *Rhamdia quelen* by increasing the level of digestive protein in their diet. Chen *et al*. (2010) also found that the inclusion of LC in the diet increased the utilization of lipids from the diet, resulting in decreased lipid content in the liver at higher levels of LC.

The absence of micronuclei indicates that LC did not present mutagenic potential at the tested concentrations. However, several nuclear alterations were observed in erythrocytes. LC has been widely used as a supplement for fish and other animals such as cats, dogs, and humans. Although such studies generally consider LC to be harmless, Araldi *et al*. (2013) showed some genotoxicity of LC administered to Wistar rats, corroborating the genotoxic effect observed in this study. No studies evaluating the genotoxicity of LC in fish were found in the literature, highlighting the importance of this study.

On the other hand, several studies have suggested a protective effect of LC on DNA damage in several test organisms, but at lower concentrations than those tested in the current experiment (200 mg kg^{-1}) (Alzahrani, 2011; Shadboorestan *et al*., 2015; Zakzok *et al*., 2018). Therefore, we hypothesized that higher concentrations of LC could lead to genotoxic effects in exposed organisms. LC at 150 and 500 mg kg^{-1} could induce increased growth, probably due to increased lipid oxidation (Mohseni and Ozorio, 2014).

Moro *et al*. (2020) observed that the inclusion of LC up to 1600mg kg^{-1} in the diet for juvenile Pacu (*Piaractus mesopotamicus*) did not affect weight gain and survival. However, the inclusion of LC at 2000mg kg^{-1} resulted in increased values of the viscerosomatic fat index, carcass lipid content, and thiobarbituric acid in the liver. Thiobarbituric acid, when present at high levels in the liver, indicates a reduction in the antioxidant status of fish tissues and may cause damage to the cytoplasm (Kaur and Jindal, 2017).

Contradictory results are probably related to variations in the chemical composition of LC, different species, stages of development and even by the presence of genotoxicity. According to Lee and Steinert (2003), DNA damage in aquatic organisms can negatively affect the growth rate. Therefore, before using LC for promoting growth, weight gain or reduced the lipid content in the filet in fish, the genotoxic effect must be considered, as the overuse of LC may be associated with the appearance of genetic changes in the cells. DNA is the fundamental unit of inheritance and reproduction, so disturbances in its structure and function can lead to changes in population dynamics. Genetic damage has been linked to the aging process (Kirkwood, 1989), gender differences of populations (Casarett, 1968) and by decreasing the genetic diversity at population level (Guttman, 1994). Therefore, studies that evaluate the genotoxic effect of LC in fish at the molecular and structural level are necessary.

In conclusion, LC supplementation for *A. lacustris* did not affect the zootechnical performance and did not lead to significant liver steatosis. However, the use of LC supplementation showed genotoxic potential with a significant difference ($p<0.05$) for cell alterations when compared to the control at concentrations above 500mg kg^{-1}

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