Int Aquat Res
https://doi.org/10.1007/s40071-019-0220-2

ORIGINAL RESEARCH

Effects of aflatoxin B_1 on performance and health of tambaqui fingerlings (*Colossoma macropomum*)

Etelvina M. C. G. Nunes · Maria M. G. Pereira · Amilton P. R. Costa · María N. B. A. Araripe · Rodrigo M. Calvet · Carina M. Pereyra · Maria L. X. Azevedo · Raizza E. E. Pinheiro · Verbena C. Alves · Airton M. Conde Júnior · Lidiane S. N. Ramos · Joao B. Lopes · Camila G. Marinho · María C. S. Muratori

Received: 20 November 2018 / Accepted: 2 March 2019

Abstract The detection of mycotoxins in feeds and their ingredients in aquaculture gained prominence due to losses caused in production and animal health, mainly the occurrence of aflatoxin (AFB_1). The aim of this study was to evaluate the effects of AFB_1 on the performance of tambaqui fingerlings (*Colossoma macropomum*). Four hundred tambaqui were used. Four different treatments were evaluated: treatment T1, considered as the control treatment (CT) with 3.84 μg kg^{-1}; treatment T2, treatment T3 and treatment T4 with 500, 1000 and 2000 μg kg^{-1} of AFB_1, respectively. The AFB_1 of the samples (muscle, liver and kidney) was detected by high-pressure liquid chromatography. Four fingerlings from each treatment for histological analysis were examined. Moreover, the performance parameters (weight gain, feed conversion and feed intake) were studied. The levels of toxins used in T2, T3 and T4 represent a reduction in the growth of 14%, 35% and 45%, respectively. The T3 and T4 showed the lowest weight gain (78%) and the worst feed conversion. Aflatoxin B_1 in muscle (3.28 μg kg^{-1}) and kidneys (8.8 μg kg^{-1}) in the T3, as well as liver (4.4 μg kg^{-1}) and kidney (4.08 μg kg^{-1}) in T4, was detected. Histopathological changes in liver and kidney tissues of fingerlings were more pronounced in T3 and T4. Fingerlings that consume feed contaminated with AFB_1 in concentrations higher than 500 μg kg^{-1} present decreases in growth, reduction in weight gain and feed intake with increased

E. M. C. G. Nunes · M. M. G. Pereira · A. P. R. Costa · M. L. X. Azevedo · R. E. E. Pinheiro · V. C. Alves · M. C. S. Muratori
Departamento de Morfofisiologia Veterinária, Centro de Ciências Agrárias, Universidade Federal do Piauí, Teresina, Piauí 64049-550, Brazil

M. N. B. A. Araripe · J. B. Lopes · C. G. Marinho
Departamento de Zootecnia, Centro de Ciências Agrárias, Universidade Federal do Piauí, Teresina, Piauí 64049-550, Brazil

R. M. Calvet
Instituto Federal de Educação, Ciência e Tecnologia do Maranhão (IFMA), São Luís, Brazil

C. M. Pereyra
Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 km, 601, 5800 Río Cuarto, Córdoba, Argentina

C. M. Pereyra (✉)
Member of Consejo Nacional de Investigaciones Científicas y Tecnológicas (CIC-CONICET), Buenos Aires, Argentina
e-mail: cpereyra@exa.unrc.edu.ar; carinapereyra06@gmail.com

A. M. Conde Júnior
Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal do Piauí, Teresina, Piauí, Brazil

L. S. N. Ramos
Instituto Federal de Educação, Ciência e Tecnologia do Piauí (IFPI), Teresina, Brazil

Published online: 09 March 2019

feed conversion. The consumption of feed contaminated with 1000 and 2000 μg kg^{-1} of AFB_1 caused severe deterioration in the hepatic and renal tissues.

Keywords Aquaculture · Aflatoxin B_1 · Weight gain · Mortality · Histopathology

Introduction

Aquaculture continues to grow faster than other major food production sectors (FAO 2018). Brazil obtained the third position in production of aquaculture in America with average growth of 27.5% per year from 2005, and the contribution of Piauí state was 11174 kilograms in 2010, representing a growth of 12.3% (FAO 2012; Ministério da Pesca e Aquicultura 2012). The tambaqui fish (*Colossoma macropomum*) is an Amazonian species cultivated in the Piauí state with a total production of 40 million of fingerlings in fish farming for producers (Ulrich Saint-Paul 2017).

This increase in aquaculture production must be supported by a corresponding increase in the production of formulated diets for the cultured aquatic animals. For most aquaculture systems, the cost of feed constitutes 30–60% of the operational costs of the farm (Ng 2003). The primary objective in fish nutrition is to provide a nutritionally balanced mixture of ingredients as finished feed to support the maintenance, growth, reproductive performance, flesh quality and health of the animals at an acceptable cost (NRC 1993). The improvement in the formulation and preparation of diets for fish requires the detection of factors that negatively affect the quality of feed, to avoid production losses (Conroy 2000). For fish farm, the quality of food in the different production phases and animal health are essential factors to ensure good animal performance and avoid economic losses. Balanced rations should be carefully stored so that they can guarantee safe food that favors the growth of fish.

Mycotoxins are fungal secondary metabolites associated with severe toxic effects to vertebrates and produced by important fungi including *Aspergillus*, *Penicillium, Fusarium* and *Alternaria* species (Kabak et al. 2006). Many feed ingredients used in aquaculture, such as cottonseed, peanuts, corn, soybean, maize, rice, dried fish, shrimp and fish meals, have been found to be frequently contaminated with mycotoxins, including aflatoxins (Spring and Fegan 2005). Aflatoxin B_1 (AFB_1) was the first of the mycotoxins to be investigated in aquaculture. As in other animal species, aflatoxin exerts carcinogenic effects in fish (Spring and Fegan 2005). Different researchers demonstrated the presence of AFB_1 in shrimp and fish feed (Bautista et al. 1994; Abdelhamid et al. 1998, Hashimoto et al. 2003; Buck 2005; Calvet et al. 2009; Nunes et al. 2015).

The number of studies addressing the effects of aflatoxins in aquatic species is very limited. The difficulty in accurately diagnosing aflatoxicosis in fish may in part explain the lack of information regarding the incidence of aflatoxicosis in farmed aquatic species. Initial findings associated with aflatoxicosis in fish include pale gills, liver damage, poor growth rates and immune suppression (Chávez-Sánchez et al. 1994; Conroy 2000; Aranas et al. 2002; Lopes et al. 2005; Lopes 2008). The occurrence of mycotoxins in products intended for animal feed may affect not only animal performance but also our health.

Several reports have documented evidence of the negative impact of mycotoxins in fish species that were informed in the south of Brazil (Lopes et al. 2005; Vieira et al. 2006; Lopes et al. 2010). However, no information is available about the negative effects of mycotoxins in the fish farmed in the Northeast of Brazil.

The aim of this study was to evaluate the effects of aflatoxin B_1 on the performance of tambaqui fingerlings (*Colossoma macropomum*) simulating the appropriate management in fish farming.

Materials and methods

Aflatoxin production

Aflatoxin production was performed according to Magnoli et al. (2011). Briefly, aflatoxins were produced via fermentation of rice by *A. parasiticus* NRRL 2999 (USDA, Agricultural Research Service, Peoria, IL). The sterile substrate, placed in Erlenmeyer flasks, was inoculated with 2 mL of an aqueous suspension of the mold containing 10^6 spores.mL^{-1}. Cultures were allowed to grow for 7 days at 25 °C in darkness. On the seventh

day, the Erlenmeyer flasks were autoclaved, and the culture material was dried for 48 h at 40 °C in a forced-air oven and then ground to a fine powder. The AFB_1 levels in the rice powder were measured by HPLC (SHIMADZU model Prominence) connected to a fluorescence detector (RF-10AXL SUPER model) (Trucksess et al. 1994; AOAC 2019). This AFs concentrate was used to contaminate feed.

Determination of aflatoxin B_1

For the detection and quantification of AFB_1, a Shimadzu® high-performance liquid chromatograph (HPLC model Prominence) was used with fluorescence detector model RF-10AXL Super, loop of 20 µL, with excitation and emission of 360 nm and 460 nm, respectively. Chromatographic separation was performed using a reversed-phase column (150 × 4.6 mm id., 5.0 µm particle sizes, VARIAN®, Inc. Palo Alto, USA), connected to a Supelguard LC-ABZ precolumn (20 × 4.6 mm, 5.0 µm particle size, Supelco). For analysis, an aliquot of 100 µL of the sample was derivatized with 350 µL of derivatizing solution (trifluoroacetic acid/ glacial acetic acid/ water, 20:10:70, v/v). The mobile phase consisted of an isocratic system of acetonitrile/ methanol/ water (17:17:66 v/v) at a flow rate of 1.5 mL min^{-1}. The quantification was performed by measuring the heights and their interpolation to a calibration curve constructed with different concentrations of AFB_1 standard, dissolved in acetonitrile, from which the limits of detection and quantification of the technique were extracted. The limit of detection of the analytical method used was 0.4 ng g^{-1}. The adsorbed AFB_1 quantifications were performed by the following equation, where: $A = [(B - C) \times D]/E$, where A = amount (ng mL^{-1}) of toxin adsorbed by the commercial product, B = height of the peak chromatographic peak of the sample, C = peak of the negative control chromatographic peak, D = concentration (ng mL^{-1}) of the positive control, E = peak of the positive control chromatographic peak.

Animals and installations

A total of 400 fingerlings (about 0.2 g) for the in vivo assay were selected. Previously, fingerlings were submitted to an adaptation in a tank with control feed. Groups of 25 fingerlings were randomly transferred to 16 experimental units. The assay was carried out according to international health standards and ethical guidelines (The Ethics Committee for Animal Experimentation of the Universidade Federal de Piauí—number 033/13).

The in vivo assay was performed in 16 polyethylene fish water tanks (250 L) using a continuous water flow system. The effluent generated in the experiment was decontaminated before being thrown into the environment using sodium hypochlorite 2–2.5% for 30 min and acetone for another 30 min (Calvet 2012; Albuquerque 2013).

Preparation of a naturally contaminated substrate and experimental design

The chemical composition (kg) of commercial fish feed used in the preparation of the experimental diets was: moisture—120 (g) max., crude protein—400 (g) min., crude fat—75 (g) min., crude ash—130 (g) max., fibrous matter—50 (g) max., calcium—10–35 (g) min–max., phosphorus—7000 (mg) min., vitamin A—10,000 (UI) min., vitamin C—400 (mg) min., vitamin E—120 (UI) min., and inositol—80 (mg). Aflatoxin B_1 was analyzed in the samples, and those that presented 3.48 µg.kg^{-1} AFB_1 or below (amount allowed according to Brazil legislation—Ministério da Agricultura do Brasil 1988) were used as control (T1) in the diets. Fish feed samples were grounded and mixed with different amounts of milled rice contaminated with AFB_1 in order to obtain 500 µg AFB_1 kg^{-1} (T2), 1000 µg AFB_1 kg^{-1} (T3) and 2000 µg AFB_1 kg^{-1} of feed (T4). After homogenization, all samples were re-pelletized as follows: addition of water (30 mL/100 mL), manual homogenization, pelletization by mincer and selection of pellets using a sieve (0.84 mm). Pellet was dried in an oven (Secagen—NOVA ETICA) at 50 °C for 48 h and stored at room temperature until the beginning of the assay.

The assay consisted of four treatments and four repetitions using 25 fingerlings by an experimental unit. The substrate was used to contaminate the feed with aflatoxins. Treatments were classified as treatment 1 (T1): $\leq$ 3.84 µg kg^{-1} de AFB_1, being the control; treatment 2 (T2) with 500 µg kg^{-1} of AFB1; treatment 3

(T3) with 1000 μg kg^{-1} of AFB_1 and treatment 4 (T4) with 2000 μg kg^{-1} of AFB_1. Selection of the commercial fish food to be used in the assay was based on the lowest levels of contamination.

Water physicochemical parameters

The parameters (pH, dissolved oxygen, temperature and ammonium) were weekly measured using an ALFAKIT (Florianópolis, Brasil), according to manufacturer manual.

In vivo assay

In vivo assay was performed from December 2013 to January 2014 for 39 days, still within the raising stage. After adaptation time, fingerlings were transferred into experimental units and fed three times a day (8 am, 12 am and 17 pm). Body weight and biometric data of fingerlings were recorded at 10, 24 and 39 days. Biometric parameters were measured using a digital caliper with a resolution of 0.01 mm (DIGIMESS, São Paulo, Brazil). Fingerlings were fasted for 24 h to obtain from each experimental unit average of weight gain, food intake and food conversion. Samples were randomly collected using a manual fishing net.

Residual aflatoxin B_1 detection from fish

At the end of the experiment, each fingerling was submerged in 20 L of water added with 10 mL of eugenol solution (5 mL of eugenol + 95 mL of alcohol) for its sensibilization. Then, they were weighted, measured and killed. Liver, kidneys and muscle were collected and transferred into individual microtubes for AFB_1 detection and quantification. Mycotoxin extraction was performed using multifunctional MycoSep 228 columns (MFC, Romer Labs®, Inc., MO, USA), and detection and quantification according to Trucksess et al. (1994) as mentioned above.

Histopathology

For the histopathological analyses, four fingerlings of each treatment were collected at 39 days. Visual analysis of the fish was done to observe external changes. Samples of the musculature, liver and kidneys were then taken.

The samples were transferred to microtubes, fixed in 10% buffered formalin and sent to the Histopathology Laboratory of the Agricultural Sciences Center of the Federal University of Piauí (UFPI) for further histological processing. The fixed tissues were processed according to routine technique and cut into microtome (4.0 μm) and stained by the hematoxylin and eosin method (LUNA 1968). The processed slides were sent to the Histology Laboratory of the Health Sciences Center (UFPI) for analysis. The tissues were examined under binocular light microscope (Olympus, Tokyo, Japan) with magnification of 100, 200 and 400 times and photographed with a digital photomicrographic system.

Histopathological alterations were classified as scores of 0–3, where 0 = no alteration, 1 = slight alteration, 2 = moderate alteration and 3 = severe alteration (Hose et al. 1996). The definitions slight, moderate and severe alterations were modified from Poleksic and Mitrovic-Tutundzic (1994) and are characterized in the following manner. Slight alteration (1) involves changes that do not damage tissues. Moderate alteration (2) involves changes that are more severe and that lead to effects in tissues associated with the functioning of the organ. Severe alteration (3) is where recovery of the organ structure is not possible, even with the improvement in water quality or no. The presence of histopathological alterations in the tissues was determined semiquantitatively by the degree of tissue alteration which is based on the severity of the lesions: degree 1 = no pathologic alterations; degree 2 = moderated alterations; degree 3 = high distributed and severe pathologic alterations.

Statistical analysis

For animal performance evaluation, the data analyses were performed by analysis of variance using Tukey test, and SNK test to determine differences between means ($p \leq 0.05$). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC).

Results

Water physicochemical parameters

During the whole experimental period, the physical–chemical parameters of the water were checked weekly (Table 1). The environmental conditions were uniform between the treatments, and the measured parameters were within the values recommended by the water quality manual for aquaculture and by the current legislation. The measured variables did not influence the experiment. The pH in all treatments was 8.

Effects of AFB_1 on productive parameters

At the end of the experiment (day 39), the mean length of fingerling from T2, T3 and T4 was significantly smaller than the control treatment. These results could also be observed since day 10, comparing the mean length of fingerling and their length at the beginning of the experiment. Statistical differences ($p < 0.05$) were found along the treatments with respect to weight gain (g), food intake (g) and conversion (g/g) of fingerlings. Comparing to T1, weight gain was reduced from 32% in T2 to a 78% in T3 and T4, while the feed intake (%) decreases from 26% in T2 to 50 in T3 and T4. On the other hand, the conversion increases in relation to the increase in AFB_1 levels in the diet (Table 2).

Residual aflatoxin B_1 detection from fish

Aflatoxin B_1 was detected in muscle (3.28 μg kg^{-1}), kidneys (8.8 μg kg^{-1}) samples in T3 and liver (4.45 μg kg^{-1}) and kidneys (4.08 μg kg^{-1}) samples in T4, at the end of the experiment.

Histopathology

With respect to histopathological alterations, liver and renal tissues of fingerlings in T3 and T4 presented the higher severity and compromise of the organs (Table 3). The muscle was the tissue that did not show histological lesions (Fig. 1). All analyzed tissue samples of T1 and T2 presented normal structures. Histopathological changes observed in T3 and T4 treatments, such as vacuolization and degeneration of

Table 1 Physical–chemical parameters of the water measured in the experiment

Water parameter	T1		T2		T3		T4		Legislation
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	
Temperature (°C)	24.5	26.0	24.5	26.3	25.0	26.2	25.0	26.3	20–29[a]
Dissolved oxygen (mg/L)	8.0	8.0	8.0	8.0	7.0	9.0	8.0	8.0	Not less than 5 mg/L
Ammonia (mg/L)	0.00	0.25	0.00	0.25	0.00	0.25	0.00	0.25	2 mg/L for pH ≤ 8.0
Nitrite (mg/L)	0.00	0.05	0.00	0.50	0.00	0.50	0.00	0.40	1 mg/L
pH	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	6.0–9.0

[a]Value recommended by aquaculture water quality manual (Alfakit) experiment

Table 2 Mean length (mm) and average yield of fingerling subjected to diets with different levels of AFB_1

Parameter measured			Treatments				CV (%)
			T1	T2	T3	T4	
Mean length (mm) of fingerling	Days	10	27.5^a	25.0^b	20.6^c	18.9^c	5.84
		24	40.3^a	35.7^b	30.3^c	28.0^c	8.61
		39	52.3^a	44.5^b	34.2^c	31.1^c	7.69
Average yield of fingerlings	Variable	Weight gain (g)	4.1^a	2.8^b	0.9^c	0.9^c	11.3
		Food intake (g)	3.4^a	2.5^b	1.7^c	1.7^c	6.8
		Conversion (g/g)	0.9^b	0.9^b	2.1^a	2.0^a	29.5

CV coefficient of variation. The statistic was performed for each parameter measured by separate

Means followed by different letters differ by SNK test (< 0.05)

Table 3 Histopathological changes observed in tissue fingerlings submitted to diets with different levels of AFB_1

Treatments	Tissue	Histopathological changes
T1	Muscle	No remarkable change—normal fibers, cores preserved
	Liver	Hepatocytes preserved, vessels and conjunctive tissue normal
	Kidney	Kidney tissue with preserved and preserved connective epithelium, vessels and ducts unchanged
T2	Muscle	Muscle tissue preserved with visible fibers and preserved cores
	Liver	Hepatic parenchyma preserved with hepatocytes arranged in cords with capillary sinusoids
	Kidney	Parenchyma preserved with tubular space and Bowman space preserved
T3	Muscle	Preserved fibers, vessels and conjunctive tissue preserved
	Liver	Discrete vacuolization and degeneration of hepatocytes, vacuolization and discrete nuclear atrophy
	Kidney	Discrete occlusion of the tubular lumen, necrosis and inflammatory infiltration, decreased Bowman space
T4	Muscle	Preserved fibers, vessels and conjunctive tissue preserved
	Liver	Discrete vacuolization and degeneration of hepatocytes, cytoplasmic vacuolization and discrete nuclear atrophy
	Kidney	Discrete occlusion of the tubular lumen, necrosis and inflammatory infiltration, decreased Bowman space

hepatocytes, necrosis and inflammatory infiltrate, decreased Bowman's space, characterizing nephritis. The histopathological alterations are registered in Figs. 2 and 3.

Discussion

The effects of AFB_1 on the performance of tambaqui fingerlings (*Colossoma macropomum*) simulating the appropriate management in fish farming were studied.

The water physicochemical parameters were analyzed weekly during the experiment. Environmental conditions were uniform through the treatments, and measured parameters were within those recommended by the legislation (Diário Oficial da União, 2005), so the registered variables did not have an influence on zootechnical parameters (weight gain, conversion food and food intake).

Fish survive and grow best in water with pH between 6 and 9. If the pH leaves this range, its growth will be affected, for example, if values below 4.5 or above occur, mortality may occur (SILVA 2003). In the study, the pH remained at 8.0, proving that this parameter was not responsible for fish mortality.

In this study, we observed the adverse effects of the addition of 500 $\mu g\ kg^{-1}$ of AFB_1 in the diet. In another study, the incorporation of more than 203 $\mu g\ kg^{-1}$ of AFB_1 into the experimental diet of jundiá (*Rhamdia quelen*) decreased the growth rate, proving similar effects. (Lopes et al. 2005). Conroy et al. (2000) in a study using tetrahybrids of red tilapia (*Oreochromis mossambicus* × *O. urolepis* × *O. niloticus* × *O. aureus*) reported the same behavior after the ingestion of feed contaminated with levels of AFB_1 over 5 $\mu g\ kg^{-1}$ in a period of 135 days. Tuan et al. (2002) investigated the effect of different concentrations of AFB_1 (0, 250,

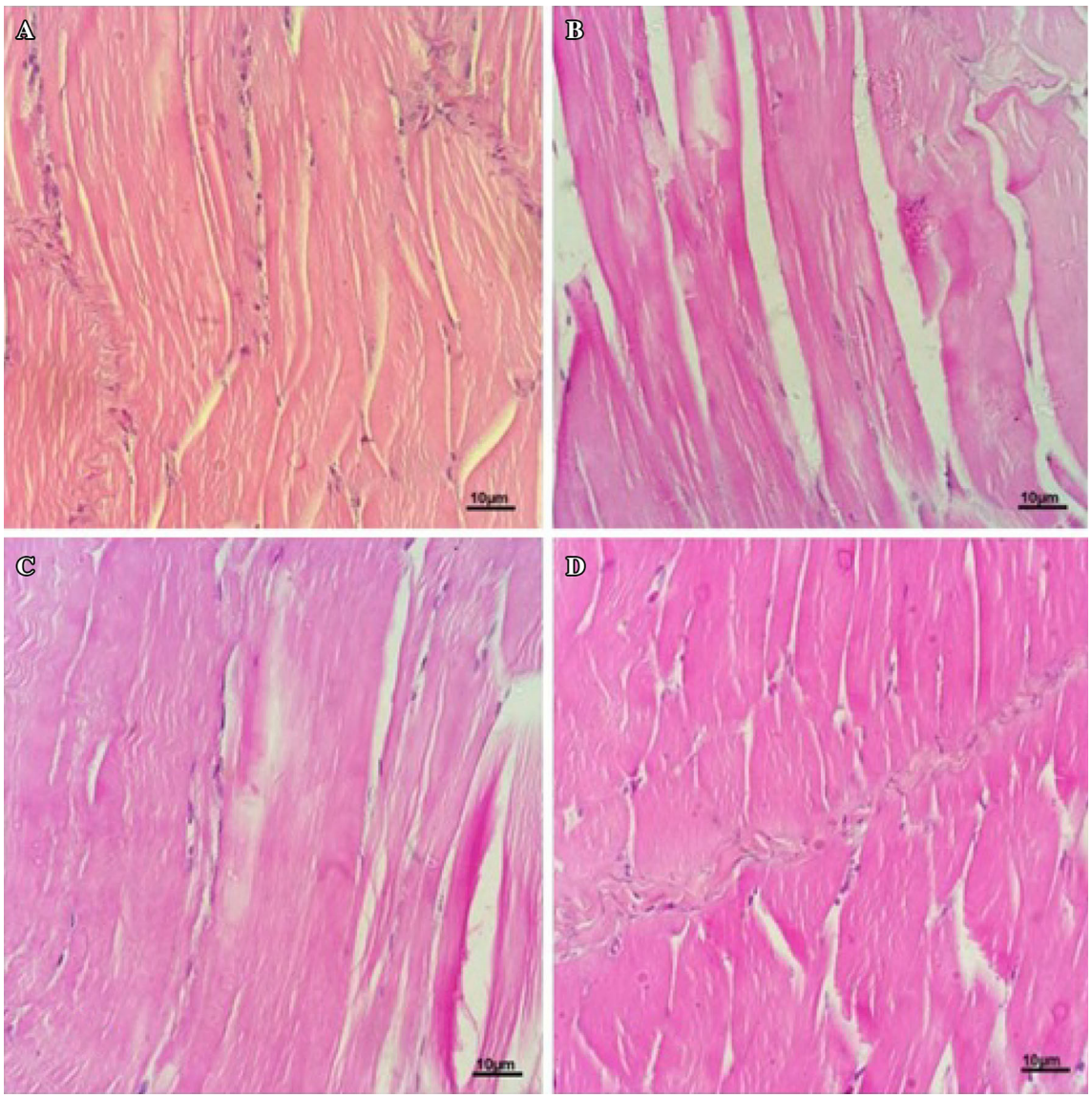

Fig. 1 Photomicrograph of muscle tissue of fingerlings of *Colossoma macropomum* stained with HE (40 ×). **a** (T1), **b** (T2); **c** (T3) and **d** (T4). Smooth muscle tissue with preserved fibers and nuclei arranged in the periphery of the normal cell

2500, 10,000 and 100,000 µg kg^{-1}) on Nile tilapia fingerlings (*Oreochromis niloticus*) and informed that 2500 and 10,000 µg kg^{-1} of the toxin led to the reduction of weight gain and 100,000 µg kg^{-1} caused severe weight loss and dead of the 60% of the assayed animals; it can be said that the effects of AFB_1 in Nile tilapia are dose dependent.

Feed conversion was better in T1 and T2, and there was no significant difference between them. It was further observed that the higher the AFB_1 levels in the feed, the greater the feed conversion and greater would be the period of growth to reach the desired size. These factors may lead to an increase in production costs, causing economic losses for the producer.

The levels of AFB_1 used in the treatments interfered significantly ($p < 0.05$) in weight gain, growth rate and feed conversion of tambaqui fingerlings; this indicate that the ingestion of food contaminated with AFB_1 may cause economic harm to producers at the beginning of fish farming. Therefore, it is necessary to use quality ingredients (free from fungal and mycotoxins contamination) to obtain safe fish feed.

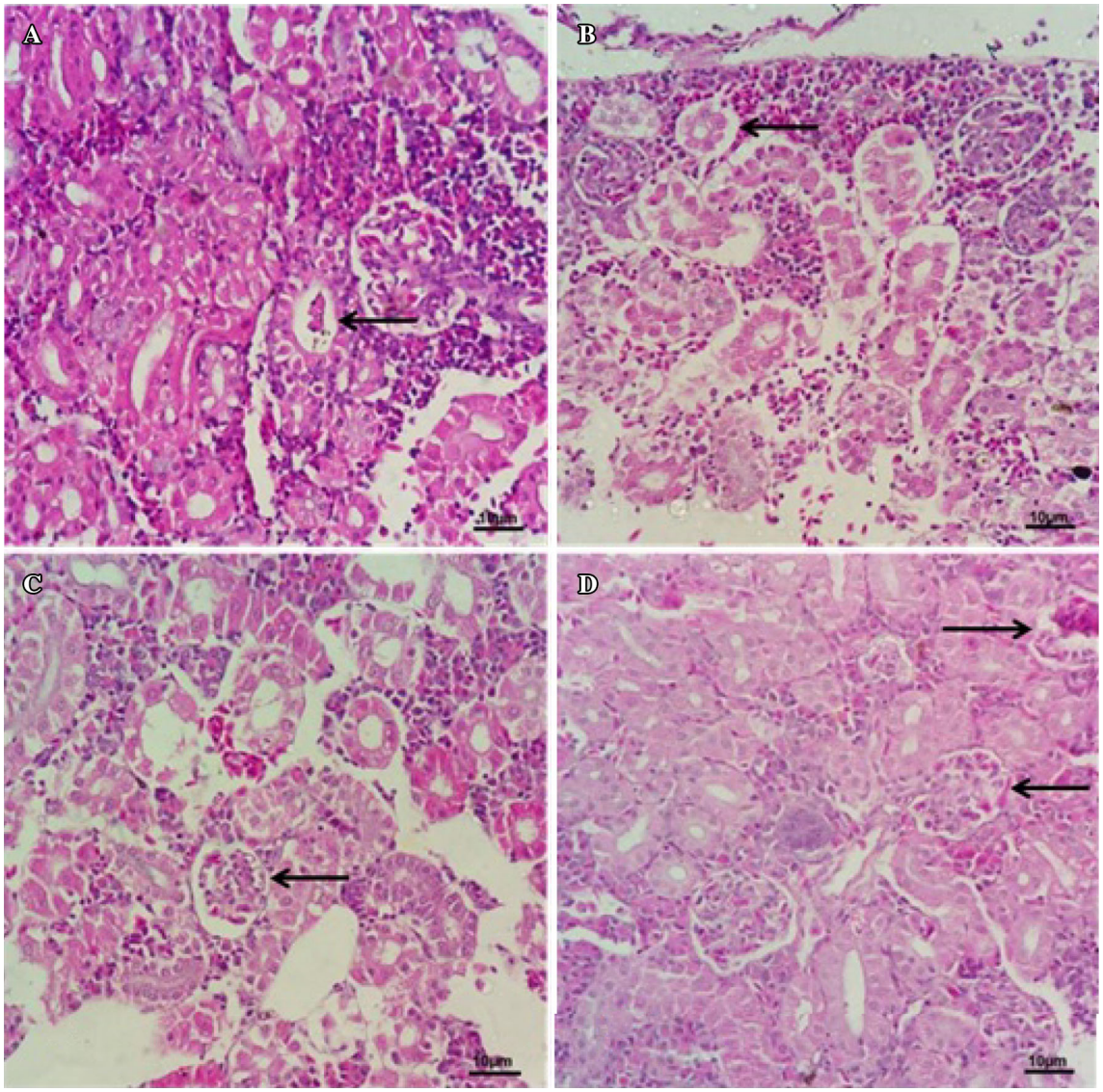

Fig. 2 Photomicrograph of renal tissue of fingerlings of *Colossoma macropomum* stained with HE (40 ×). **a** (T1) Preserved renal parenchyma, preserved conjunctival epithelium, preserved Bowman space and tubular; **b** (T2) preserved renal parenchyma; **c** (T3) and **d** (T4) focal tubular necrosis, reduction of Bowman space

There are no previous reports about effects of AFB_1 on productive parameters; from what has been exposed about the significance of tambaqui (*Colossoma macropomum*) production, the assessment of food quality must include mycotoxicological aspects that also interfere in fingerlings performance.

In this study, different concentrations of toxin were detected in different organs. Lopes et al., (2005) informed AFB_1 residual deposition on liver (1.6 μg kg^{-1}, 4.0 μg kg^{-1} and 12.9 μg kg^{-1}) of Jundiá fingerlings (*Rhamdia quelen*) that has been feed with diets containing different levels of toxin (350 μg kg^{-1}, 757 μg kg^{-1} and 1177 μg kg^{-1}) along 5 weeks. The toxin deposition of AFB_1 was higher in jundiá fingerlings than that in tambaqui fingerlings used in this study.

Hepatotoxic and nephrotoxic effects of AFB_1 on animals (Santacroce et al. 2008; Williams et al. 2004; 2003; Creppy 2002) can increase tambaqui fingerlings susceptibility to acquiring certain pathologies as other aquatic animals assayed (Calvet 2012; Albuquerque 2013).

Along the experiment, fingerlings died in a 1% in T1 and T2, 48% in T3 and 24% in T4 where the mortality rate was higher than the expected (5–10%). This could be related to the histopathological alterations observed

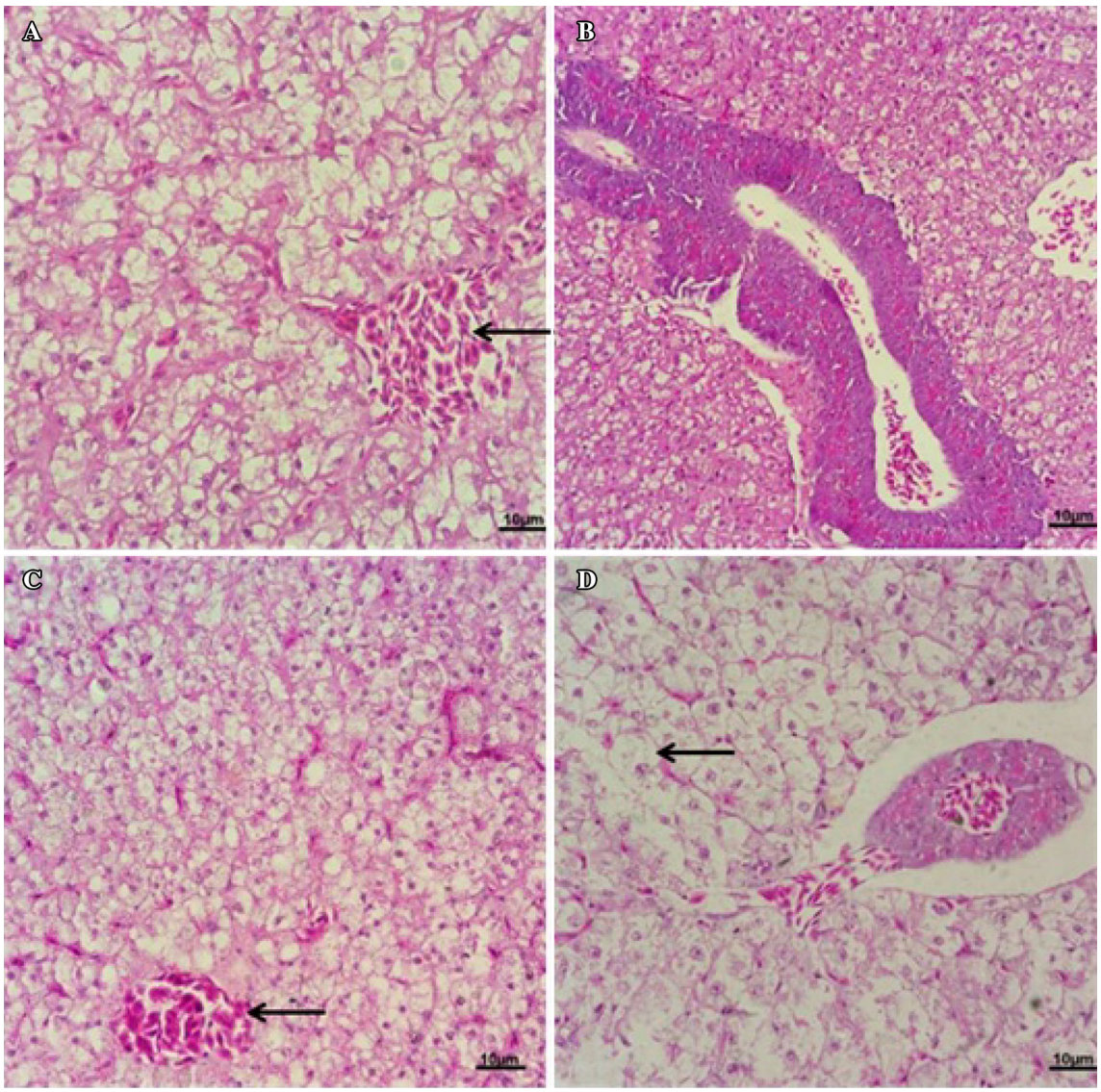

Fig. 3 Photomicrograph of hepatic tissue of fingerlings of *Colossoma macropomum* stained with HE (40 ×). **a** (T1) hepatocytes in cords, large-caliber vessel; **b** (T2) hepatocytes cords; **c** (T3) vacuolization; **d** (T4) intense vacuolization greasy, degeneration of hepatocytes, nuclear atrophy

on T3 and T4 as hepatocytes vacuolization and degeneration necrosis and inflammatory infiltrate and reduction of Bowman space representing a nephritis situation.

In conclusion, fingerlings that consume feed contaminated with AFB_1 in concentrations higher than 500 μg kg^{-1} present decreases in growth, reduction in weight gain and feed intake with increased feed conversion. The consumption of feed contaminated with AFB_1 (1000 μg kg^{-1} and 2000 μg kg^{-1}) causes renal impairment and severe hepatic tissues of fingerlings.

The number of studies addressing the effects of aflatoxins in aquatic species is very limited. This report provides new studies in Brazil, since there are differences between the aquaculture practices of the south and north of Brazil, the cultivated species are different, as well as the food provided.

In this study, effects of aflatoxin B_1 on the performance of fingerlings of tambaqui (*Colossoma macropomum*) were found. The AFB_1 levels used in the treatments interfered significantly on weight gain, growth rate and food conversion; this could indicate that the intake of contaminated food with AFB_1 may cause economic damage to the producers at the beginning of fish farming. The assessment of food quality must include mycotoxicological aspects that also interfere in fingerlings performance.

Acknowledgements This work was carried out with grants from CAPES, Brazil.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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