HISTOLOGICAL ANALYSIS OF *Bryconops caudomaculatus* GILLS AND LIVER UNDER DIFFERENT CONCENTRATIONS OF AMMONIA

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ABSTRACT

The objective was to evaluate the effects of different concentrations of ammonia (zero; 0.15; 0.30; 0.50; 1.00 mg L^{-1} NH_3) on histology and morphology of gills and liver of juvenile *Bryconops caudomaculatus*. After 96 hours of exposure, the fishs were anesthetized and necropsied for collect gills and liver, which were embedded in paraffin. Morphological changes were observed on the secondary lamellae of the gills when concentrations greater than 0.15 mg L^{-1}, as hyperplasia, hypertrophy, epithelial displacement, lamellar fusion, swelling, lamellar aneurysm with rupture of the epithelium and increase in width of the lamellae were observed. The liver presented histopathologies (congestion in the capillaries and inflammatory processes) at the presence of 0.30 mg L^{-1} of NH_3, compromising the vital functions, the metabolism, breathing processes, detoxification of the body, hematopoiesis and probably hindered osmoregulation. The results suggest that the changes in gill and liver can be used to monitor fish performance in intensive farming.

Key words: morphological changes; contaminants; histology; toxicity.

ANÁLISE HISTOLÓGICA DAS BRÂNQUIAS E DO FÍGADO DE *Bryconops caudomaculatus* EM DIFERENTES CONCENTRAÇÕES DE AMÔNIA

RESUMO

O objetivo foi avaliar os efeitos de diferentes concentrações de amônia não ionizada (zero; 0,15; 0,30; 0,50; 1,00 mg L^{-1} NH_3) na histologia e morfologia das brânquias e fígado de juvenis *Bryconops caudomaculatus*. Após 96 horas de exposição, os peixes foram anestesiados, submetidos à necropsia, para coleta de brânquias e fígado, que foram incluídos em parafina. Alterações morfológicas nas lamelas secundárias das brânquias foram observadas em concentrações a partir de 0,15 mg L^{-1}, como hiperplasia, hipertrofia, deslocamento epitelial, fusão lamelar, edema, aneurisma lamelar com ruptura do epitélio e aumento na largura das lamelas. O fígado apresentou alterações histológicas (congestão nos capilares, processos inflamatórios e pigmentação endógena) na presença de 0,30 mg L^{-1} de NH_3, e comprometeram as funções vitais, o metabolismo, processos de respiração, desintoxicação do organismo, hematopoese e provavelmente a osmorregulação. Os resultados sugerem que as alterações nas brânquias e fígado podem ser usadas para monitorar o desempenho de peixes em cultivo intensivo.

Palavras-chave: alterações morfológicas; contaminantes; histologia; toxicidade.

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INTRODUCTION

The fluctuations in water quality in the environment are caused by the action of rain and organic and inorganic material. The impact of factors related to agricultural production such as agricultural runoff, fertilizers and decomposition of artificial feeding on the environment could be noxious depending on its type and intensity. Changes in the aquatic environment directly affect fish health and even small changes are enough to trigger stressful stimuli in animals (RANDALL and TSUI, 2002; PEREIRA and MERCANTE, 2005).

Ammonia is a common pollutant on natural water systems (SPENCER *et al.*, 2008) and represents 60-80% of nitrogenous waste excreted by fishes (BENLI and KÖKSAL, 2005, BARBIERI and BONDIOLI, 2013). Considering the variables that affect the water quality, the level of ammonia is described as one of the most limiting factors for the survival and growth of aquatic organisms. If present in high concentrations, it inhibits the growth of aquatic animals, besides promoting changes in their blood and in several vital organs, namely, gills, kidneys and liver (SAHA *et al.*, 2002; WRIGHT and WOOD, 2012; FERREIRA *et al.*, 2013).

The rise on un-ionized ammonia (NH_3) concentration in the environment may be an indicative of toxicity in fish since an excess on retention of metabolic ammonia modifies their physiology (PIEDRAS *et al.*, 2006; MERCANTE *et al.*, 2007; SINHA *et al.*, 2012). High levels of ammonia reduce blood pH due to the accumulation of acidic metabolites, causing swelling and melting of the lamellae in the gills and interfering in the osmoregulation processes (LEASE *et al.*, 2003; LIEW *et al.*, 2013). Also, gill lesions (SPENCER *et al.*, 2008; MIRON *et al.*, 2008) and decrease on liver volume (MILNE *et al.*, 2000) may occur when some species are over exposed to ammonia.

The *Bryconops caudomaculatus* (Characidae) is known as tetra, lambari, piaba or tambiú among riverine communities, where it is very popular for its notorious jumping ability (COSTA PEREIRA and SEVERO-NETO, 2012). *B. caudomaculatus* is found in small streams, lakes and large rivers in the tropical watersheds of northern coastal regions of Brazil and Guyana, especially in the Amazon basins, Tocantins, Paraguay and São Francisco Rivers (WINGERT and MALABARBA, 2011). This species is considered insectivorous, but also feeds on fruits (SILVA *et al.*, 2008). The objective of this study was to evaluate the effects of different concentrations of ammonia on histology and morphology of gills and liver of juvenile *Bryconops caudomaculatus*.

METHODS

Juvenile *B. caudomaculatus* were collected in Araguaína region, State of Tocantins, Brazil (7°11'31"S and 48°12'28"W) and transported to the Laboratory of Morphophysiology and Biochemistry of Neotropical Fish of the Federal University of Tocantins. Prior to the experiment, fish were acclimated to the experimental condition for 30 days in 250 L boxes with constant aeration and feeding twice a day. After acclimation, 150 animals (Weight 16.3 ± 1.35 g and total length 11.4 ± 0.3 cm) were randomly assigned (n = 10) to fifteen polyethylene boxes (250 L) with constant aeration and subjected to five treatments (in triplicate) with different concentrations of NH_3 (mg L^{-1}): 0.003 ± 0.04 (control); 0.15 ± 0.5; 0.30 ± 0.3; 0.50 ± 0.5; 1.00 ± 0.4 for 96 hours. The un-ionized ammonia concentration was maintained by addition of a concentrated solution of NH_4Cl as described in MIRON *et al.* (2011). Dissolved oxygen and water temperature were measured twice a day with a digital oximeter (ITT 71440). Also, the pH was verified with a pH meter (Digimed). The total ammonia concentrations were determined daily according to VERDOUW *et al.* (1978) and un-ionized ammonia concentrations were calculated according to COLT (2002) at 06:00, 14:00 and 20:00 hours. Water hardness was analyzed by the EDTA titrimetric method (EATON *et al.* 2005), and the nitrite concentration by the method of BOYD and TUCKER (1992), at the beginning and the end of experiment.

The boxes were cleaned daily by siphoning and 20 to 40% of the water was replaced to maintain desired concentrations. During the experiment the animals were fasted.

After 96 hours of exposure, 12 animals from each treatment were randomly selected, anesthetized using clove oil (30µL L^{-1} water) (INOUE *et al.*, 2003), submitted to section spinal cord and autopsy to gather gills and liver. Afterwards, tissue samples were quickly removed and fixed in Bouin. They were cut into small pieces and dehydrated for embedding in paraffin. Serial sections of 3 µm were made for preparing the slides, which were

stained with hematoxylin and eosin to the gills and liver (ROUMIEH *et al.*, 2013) and observed using a light microscope. Observed changes were ranked by occurrence and extent of the injuries. The analysis were performed by digital photographs in light microscope LEICA DM 500 and the camera ICCHD50 with LAS 2.0.0 image software. For the histopathologic analysis in the gills, five primary lamellae (PL) from each blade were randomly selected for the analysis, and observed 10 secondary lamellae (SL) in 12 animals of each treatment (100x magnification). The changes were classified from the occurrence and the graduation of injuries following the key: 0 for no visible change; + slight; ++ moderate; and +++ accentuated. In liver, the changes were classified according to the level of impact: 0 for no visible change; + slight modifications, affecting up to 30 injured hepatocytes; ++ incidence between 30 and 60 fields; +++ incidence higher than 60 fields with changes (GAD and ROUSSEAUX, 2002). For the analysis of glycogen, five sections of each individual (n=12) were analyzed after the PAS reaction, in which five fields were photographed and analyzed (400x magnification).

This study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Tocantins/TO (protocol number: 23101.000282/2014-16). The specimens were deposited in the scientific collection of the Laboratory of Ichthyology at the Federal University of Rio Grande do Sul (UFRGS 20239).

The results of histologic changes were submitted to the Kruskal Wallis test via statistical program GraphPad Instat v 3.00 for Windows 95® (GRAPHPAD INSTAT, 1998) followed by the Dunn test, when applicable, considered significant $P \leq 0.05$.

RESULTS

The gills of fish exposed to 0.30, 0.50 and 1.0 mg L^{-1} of NH_3 showed changes as hypertrophy of the lamellar epithelium, hyperplasia, fusion of secondary lamellae, edema and necrosis (Table 1 and Figure 1). Thickness of secondary lamellae changed when NH3 concentrations were above 0.5 and 1.0 mg L^{-1} (Table 2). Histopathological evaluation of the liver of fishes exposed to NH_3, showed vacuolar degeneration, congestion, necrosis of hepatocytes and inflammatory infiltrate (Figure 2; Table 3).

Table 1. Histopathologic changes in gills of *B. caudomaculatus* exposed to un-ionized ammonia.

STAGE I	0.003 mg L^{-1}	0.15 mg L^{-1}	0.30 mg L^{-1}	0.50 mg L^{-1}	1.00 mg L^{-1}
Hypertrophy	+	+	++	++	+++
Hyperplasia	+	+	+	+	++
Vascular Congestion	+	+	+	++	++
Capillary Dilatation	+	+	++	++	++
Epithelial Displacement	0	+	+	++	++
Capillary Constriction	0	0	+	+	+
Lamellar Fusion	0	+	+	+	++
Edema	0	+	+	+	+
STAGE II					
Lamellar Aneurysm	0	+	+	+	++
Epithelial Rupture (hemorrhage)	0	+	+	++	++
STAGE III					
Focal Necrosis	0	0	+	+	+

(0) for no visible change; (+) slight; (++) moderate; (+++) accentuated

**Descriptive Statistics, Kruskal-Wallis test.*

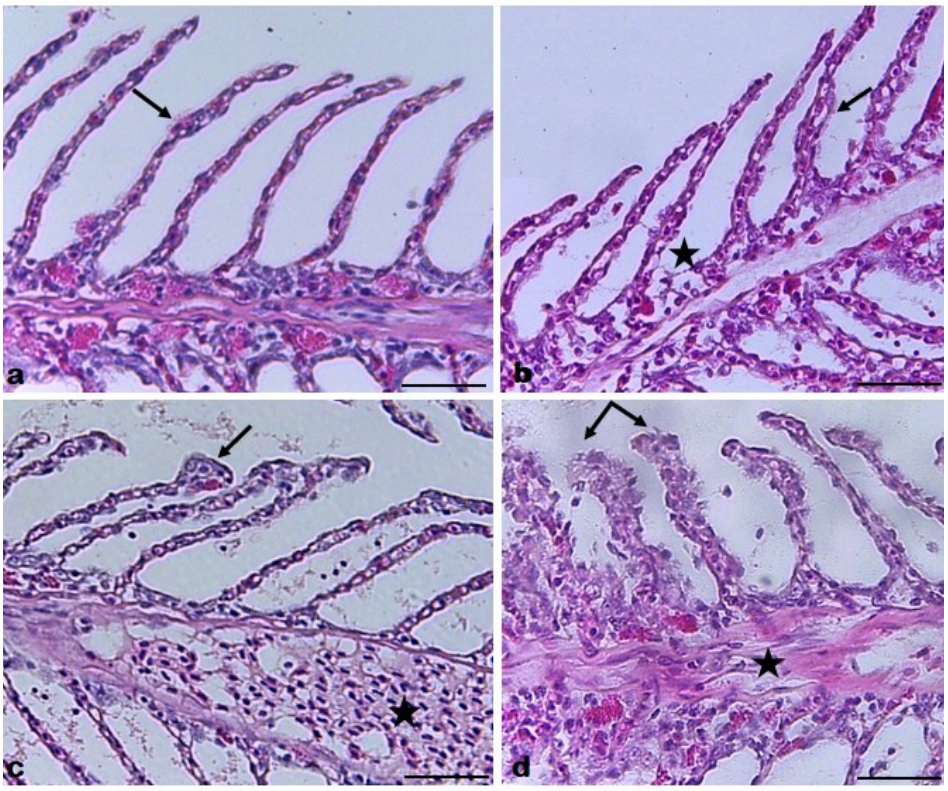

Figure 1. Photomicrograph of secondary lamellae (SL) of *Bryconops caudomaculatus*. (a) Control (0.003 mg L^{-1}): normal LS (arrow); (b) Treatment with NH_3 0.15 mg L^{-1}: SL with epithelial displacement (arrow), edema (star); (c) Treatment with NH_3 0.50 mg L^{-1}: aneurysm (arrow), congestion (star); (d) Treatment with NH_3 1.00 mg L^{-1}: SL hyperplasia with foci of necrosis (arrow), constriction (star). Scale bar 10 mm, H.E. 100x.

Table 2. Width (µm) of secondary lamellae of the gills of *B. caudomaculatus* exposed to NH_3.

0.003 mg L^{-1}	0.15 mg L^{-1}	0.30 mg L^{-1}	0.50 mg L^{-1}	1.0 mg L^{-1}
4.1 ± 0.20[a]	4.3 ± 0.14[a]	5.2 ± 0.29[a]	5.5 ± 0.28[b]	5.8 ± 0.27[b]

**Mean ± estimated standard error. Values followed by the same letter do not differ according to Dunn test ($p < 0.05$).*

Table 3. Frequency of histopathology in liver of *B. caudomaculatus* exposed to un-ionized ammonia.

*Histopathology	0.003 mg L^{-1}	0.15 mg L^{-1}	0.30 mg L^{-1}	0.50 mg L^{-1}	1.00 mg L^{-1}
Cholestasis	+	+	++	++	++
Hepatic Disorders	+	+	+	++	++
Vacuolar Degeneration	+	++	++	++	++
Glycogen Accumulation	+++	++	++	++	++
Dilation in the Capillaries	+	+	++	+++	+++
Congestion	+	+	++	+++	+++
Kupffer's Cell Hyperplasia	+	++	++	++	++
Dilation in the Sinuses	+	++	++	++	++
Inflammatory Infiltrate	+	++	++	++	+++
Necrosis	0	+	+	++	++

(0) for no visible change; (+) slight; (++) moderate; (+++) accentuated.

**Descriptive Statistics, Kruskal-Wallis test.*

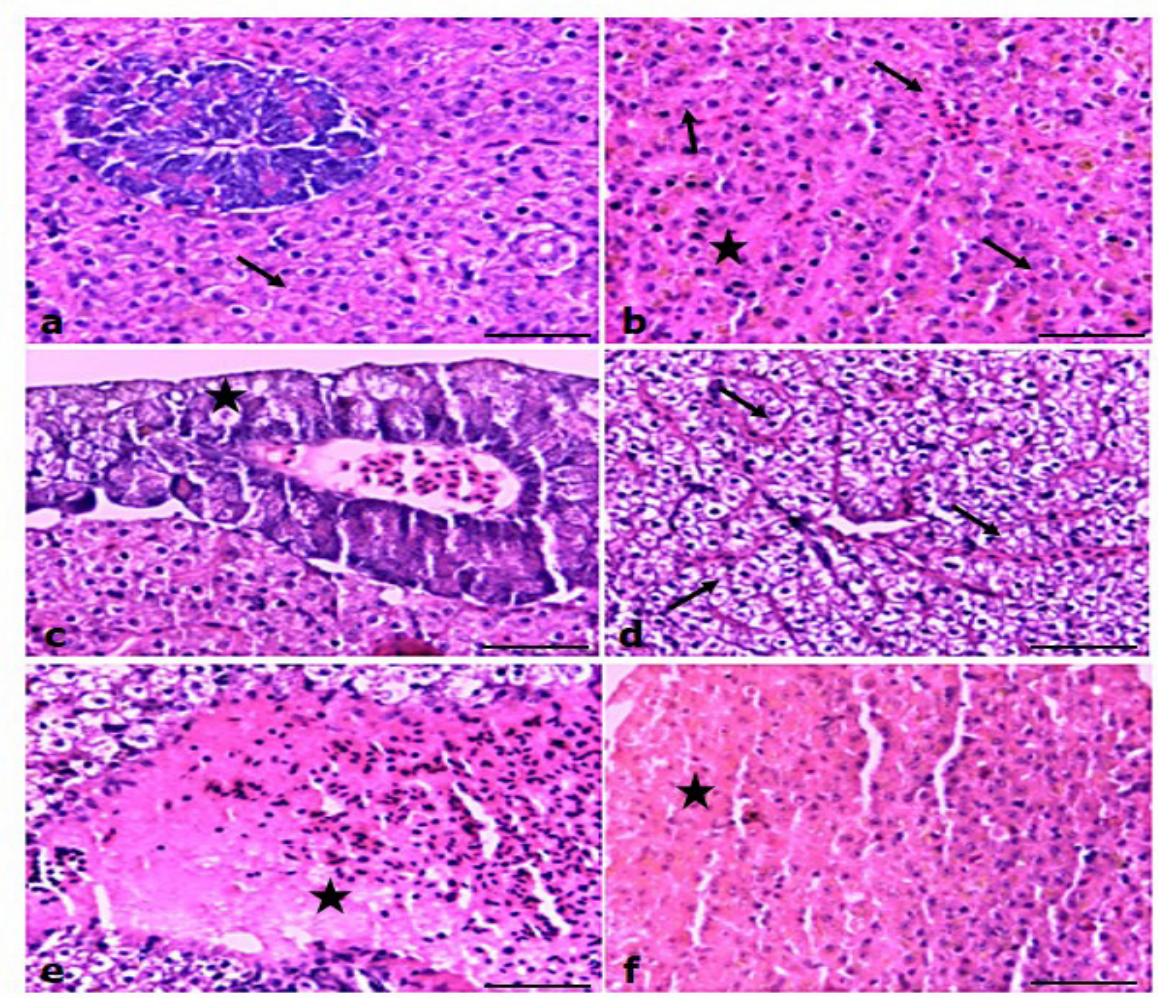

Figure 2. Photomicrograph of liver from *B. caudomaculatus*. (a) Control (0.003 mg L^{-1}) without significant changes; (b) Treatment with NH_3 0.15 mg L^{-1}: area with inflammatory infiltrate (arrow); (c) Treatment with NH_3 0.30 mg L^{-1}: indications vacuolar degeneration (arrow); (d) Treatment with NH_3 0.50 mg L^{-1}: region with cells of Kupffer, dilation of the sinusoids (arrow); (e) Treatment with NH_3 1.00 mg L^{-1}: capillary congestion (star); (f) Treatment with NH_3 1.00 mg L^{-1}: necrosis of hepatocytes. Scale bar 10 mm, H.E. 400x..

A reduction in liver glycogen accumulation was in individuals exposed to higher levels of ammonia, when compared to the control (Table 3). The treatment with 0.50 mg L^{-1} of NH_3 resulted in concentration of lymphocytes around the vessels in the liver and vacuolization of cells closest to the major vessels. The congestion in the capillaries occurred in fish exposed to 0.30 mg L^{-1} NH_3, although more frequently in treatment with 0.50 and 1.0 mg L^{-1} of NH_3.

DISCUSSION

In fish, the toxic effects of ammonia present in the water are mainly related to its un-ionized form. The NH_3 may be toxic to fish, depending on factors such as pH, and, to a lesser extent, on water temperature (SPENCER *et al.*, 2008) and concentration of ions (WEE *et al.*, 2007). In this study, pH, dissolved oxygen and temperature were controlled, so that NH_3 levels in the water remained stable.

Bryconops caudomaculatus presents tolerance to high concentrations of NH_3, when compared to other Neotropical species. MARTINEZ *et al.* (2004) reported that the mean LC50 values (24 h) of NH_3 to *Astyanax altiparanae*, *Piaractus mesopotamicus* and *Prochilodus lineatus* are 0.66, 0.85 and 0.74 mg L^{-1}, respectively. In Rhandia quelen, LC50 in 96 h and pH 6.0 was 0.44 mg L^{-1} of NH_3 (MIRON *et al.*, 2008). BENLI and KÖKSAL (2005) reported that the lethal concentration of NH_3 (48 hours) to juvenile *Oreochromis niloticus* was 7.4 mg L^{-1} and, therefore, that tilapia is very tolerant to

ammonia. For other species of the family Characidae (PORTO-FORESTI *et al.*, 2010), may occur mortality in ammonia concentrations above 1.00 mg L^{-1}.

Function and morphology of gills are intrinsically related and are constantly changing during the adaptation of fish to environmental changes, so that the optimization of a function can compromise other (FERNANDES and MORON, 2014; BARBIERI *et al.*, 2016). The morphological changes observed in gill epithelium as hyperplasia, lamellar fusion and hypertrophy hinder factors such as gas exchange, acid-base balance and excretion of nitrogenous compounds. These lesions in gills were similar to those observed by other authors for other species exposed to NH_3 (SPENCER *et al.*, 2008; DONG *et al.*, 2013; ROUMIEH *et al.*, 2013). MIRON *et al.* (2008) reported severe damage to the gills in R. quelen, as fusion of secondary lamellae when animals were exposed to changes in pH and different concentrations of ammonia.

Hyperplasia or fusion of the lamellar epithelium observed in treatments 0.5 and 1.0 mg L^{-1} of NH_3 may have caused the fusion of two or more secondary lamellae, hindering gas exchange, reducing the area and respiratory efficiency. Other studies have also shown that ammonia may cause injuries that diminish gill surface area. This may reduce the capacity of gas exchange and the diffusion capacity of the gills, which consequently hamper the vital respiration process, the acid-base balance, osmoregulation and the excretion of nitrogen compounds (FRANCES *et al.*, 2000; RANDALL and TSUI, 2002). MARTINEZ *et al.* (2004) observed the effects of ammonia in the gills of *Astyanax fasciatus*, *Piaractus mesopotamicus* and *Prochilodus lineatus*. The authors verified detachment of the branchial epithelium, cell hyperplasia and small amount of aneurysm, characterized by the extravasation of blood inside the lamellae and subsequent congestion and dilation of blood channels.

The liver is another targeted organ where high NH_3 concentrations caused damage, such as vacuolization of hepatocytes, decreased glycogen storages and other changes, affecting its vital functions. Ammonia can be carried by the hepatic portal vein to the liver as a nutrient and enter liver metabolic pathways (BENLI *et al.*, 2008). The degeneration found in the liver of *B. caudomaculatus* exposed to higher levels of un-ionized ammonia (0.30, 0.50 and 1.00 mg L-1) is a stage of degeneration characterized due to degradation and cytoplasmic vacuolization (POLEKSIC and MITROVIC-TUTUNDZIC, 1994). The area occupied by liver glycogen granules of fish subjected to treatment with 0.5 mg L^{-1} was lower than that found for the control treatment. In the treatment with the highest concentration of NH_3, there was a high incidence of necrosis, which prevented evaluations.

Hepatic necrosis was moderate observed in fish exposed to higher concentrations of NH_3 (0.5 and 1.0 mg L^{-1}). There was also the presence of congestion of blood vessels, suggesting that the blood flow that drains an area are clogged and, consequently, the blood accumulates in the venous circulation. It may be caused by physical occlusion of small or large vessels or by failure of the normal flow (JONES *et al.*, 2000). MIRON *et al.* (2008) reports a reduction in liver glycogen in catfish after exposure to NH_3 caused by stress due to its toxicity. The glycogen is therefore used in response to stress conditions (MILNE *et al.*, 2000; VIJAYAVEL *et al.*, 2006).

Un-ionized ammonia concentrations above 0.15 mg L^{-1} promoted significant changes in the morphological structure of the gills of *B. caudomaculatus*, hindering vital functions such as breathing processes and, probably, osmoregulation. Concentrations above 0.30 mg L^{-1} also promoted histologic changes in the liver, related to the intoxication processes, causing an increase in the degree and intensity of these injuries.

CONCLUSIONS

The results suggest that un-ionized ammonia concentrations above 0.15 mg L^{-1} promoted significant changes in the morphological structure of the gills and concentrations obove 0.30 mg L^{-1} promoted histologic changes in the liver of *Bryconops caudomaculatus*. The observation of these organs and the monitoring of un-ionized ammonia concentration can be a complementary tool and to support the fish monitoring.

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