ECOSYSTEMS

Can a biopesticide based on *Bacillus thuringiensis* affect the physiology and histomorphology of *Arapaima gigas*?

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Abstract: The use of biopesticides based on *Bacillus thuringiensis* (Berliner 1915) in agriculture has been considered harmless for non-target organisms such as fish. The present study aimed to investigate the effects of a biopesticide based on *B. thuringiensis* on the physiology and histology of the liver, kidney and intestine of *Arapaima gigas* (Schinz, 1822), via exposure to water (0.13 g/L) and in the diet (0.13 g), after 24 and 48 h. Fish subjected to *B. thuringiensis* in the water of their breeding and feeding tanks exhibited mortality due to changes in erythrogram (hematocrit, hemoglobin, erythrocytes), thrombogram and leucogram blood parameters, and plasma (sodium, chloride, potassium, cholesterol, glucose, triglycerides, cortisol and total proteins) and enzymatic (Aspartate Amino Transferase and Alanine Amino Transferase) biochemistry. Histopathological alterations in the liver and kidney ranged from mild to severe and were characterized by the presence of cytoplasmic vacuolization, nuclear hypertrophy and atrophy, melanomacrophage centers and necrosis, and in the intestine by changes to the number of villi and goblet cells. Therefore, these physiological and histopathological alterations indicate that care should be taken with the dispersion of biopesticides based on agricultural *B. thuringiensis* in fish farming.

Key words: *Bacillus*, biochemistry, contaminant, hematology, histopathology, pirarucu.

INTRODUCTION

The use of pesticides is a vital part of the large-scale production of food to meet the needs of a continually growing global population. However, in general, these pesticides are extremely toxic and contribute to environmental pollution (Wiegand et al. 1999, Chapadense et al. 2009), while also harming aquatic ecosystems. Hence, over the past three decades, efforts have been made to reduce human exposure to and risk from pesticides, especially insecticides. There is great demand for selective and safe pesticides that spare natural enemies and non-target organisms (Oguh et al. 2019). New strategies to minimize the negative effects of pesticides have

emerged, mainly using biopesticides based on *Bacillus thuringiensis* (Berliner 1915) (Mariano et al. 2019).

The first commercial *B. thuringiensis* (Bt) product was produced in France in 1938 (Grisolia et al. 2009). Bt is an aerobic gram-positive and entomopathogenic bacterium, naturally found in soil (Oliveira-Filho 2008). It is responsible for more than 90% of the biopesticides available worldwide. These bioinsecticides are an effective and safe agent against crop pests, as they are highly specific to certain pests (Oguh et al. 2019). However, the exposure of *Piaractus mesopatamicus* (Holmberg 1887) to Bt resulted in blood alterations (Mariano et al. 2019), while

the exposure of *Oreochromis niloticus* (Linnaeus 1758) caused an increase in erythrocyte apoptosis (Grisolia et al. 2009), which can reduce the number of erythrocytes in fish exposed to this biopesticide.

In agricultural environments, the movement of pesticides and biopesticides depends on factors such as volatilization, surface runoff, leaching, preferential flow and relief characteristics, causing the incision of 2 to 90% of these pesticides in the local hydrographic network (Jardim et al. 2009, Kugler 2012). Watersheds and fish breeding ponds located near agricultural areas can be contaminated by Bt-based biopesticides and can affect fish such as pirarucu *Arapaima gigas* (Schinz, 1822) (Osteoglossiformes, Arapaimatidae). Thus, the present study investigated the physiological and histomorphological effects of *A. gigas* following exposure to a Bt-based biopesticide.

MATERIALS AND METHODS

Fish and acclimation

A total of 90 juveniles of *A. gigas* (531.7 ± 153.9g) obtained from a commercial fish farm in Araguaína, in the state of Tocantins (Brazil) were transported to the Laboratory of Zoophysiology and Biochemistry of the Federal University of Tocantins (UFT), in Araguaína (TO), Brazil. These fish were acclimated for 30 days in 500 L water tanks and were given fish feed containing 45% crude protein. A constant water supply was maintained in the tanks, the temperature was kept at 25°C, and the pH was 7.0. The organic matter that accumulated at the bottom of the tanks was removed once a day, and the dissolved oxygen level was 6.2 ± 2.3 mg/L. The present study was developed in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and it complied strictly with the protocols and rules of

the Ethics Committee on Animal Use of Embrapa Amapá (Protocol 002- CEUA-CPFAP).

Preparation of diets with *Bacillus thuringiensis*-based biopesticide

Dipel-WP® biopesticide (Sumitomo Chemical, Brazil) containing spores of *B. thuringiensis* var. kurstaki was used in the diet of *A. gigas*. A total of 0.13 g of the biopesticide was added per kg of commercial carnivorous fish feed (Mariano et al. 2019) containing 45% crude protein. The rations were then allowed to dry in a microbiological oven at 37°C for 12 h to activate the Bt spores. Later, a bacterial growth test was performed to confirm the presence of Bt in the prepared diets. A sterile swab was used in the rations prepared and the material was seeded in Petri dishes with Mueller-Hintom agar medium. After 24 h in a microbiological oven at 37°C, the Gram test was performed to identify Bt. The rations were then used to feed *A. gigas* for 24 or 48 h. These rations were prepared daily to avoid storage.

Experimental design with *Bacillus thuringiensis*-based biopesticide

Two trials using Bt-based biopesticide were performed: one with the biopesticide added to the feed and the other with the biopesticide added to the culture tanks of *A. gigas*. For the latter, 0.13 g/L of biopesticide (Mariano et al. 2019) was added directly to the tank water (250 L). The fish were divided into three groups: (1) control group, (2) group exposed to water with Bt for 24 h and (3) group exposed to water with Bt for 48 h. For each group, a total of 15 fish were used, being 5 fish per replicate and 3 replicates per treatment. The fish were fed ad libitum twice per day, and no ration waste was observed.

Fish fed with ration containing the biopesticide (0.13 g/L) were kept in 250 L tanks and were divided into three groups: (1) control group, (2) group fed with Bt for 24 and (3) group

fed with Bt for 48 h. For each group a total of 15 fish were used, with 5 fish per replicate and 3 replicates per treatment. The fish were fed ad libitum twice per day, and no ration waste was observed. The fish of the control group were fed with commercial ration without the biopesticide, which was moistened only with water and dried in a microbiological oven at 37°C, for 12 h. This control group was used for comparison in both trials, with the addition of the biopesticide in the feed and in the breeding water.

The behavior (erratic swimming, lethargy, loss of balance and loss of appetite) of the fish was analyzed during all the trials.

A constant water supply was maintained in the tanks and the temperature was kept at 25.0 ± 1.1°C and pH 7.0 ± 1.0. The organic matter that accumulated at the bottom of the tanks was removed once a day and dissolved oxygen was 6.0 ± 2.1 mg/L.

Blood collection and analysis

Samples of blood were collected by the puncture of the caudal vessel with heparinized syringes (5,000 IU), and these were divided into two aliquots for the determination of the blood parameters. One aliquot was used for the determination of the hematocrit by the microhematocrit method, hemoglobin concentration by the cyanometahemoglobin method and total erythrocyte count was calculated in a Neubauer chamber. From these data, the hematimetric indices were determined: Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC). Blood smears were confectioned and stained with panoptic staining (Laborclin®) and used to determine the number of leukocytes and total thrombocytes (Ranzani-Paiva et al. 2013). The nomenclature of the leukocytes followed the recommendations of Tavares-Dias et al. (2007).

Another blood aliquot was centrifuged to obtain the plasma, which was frozen at -20°C and used for biochemical analysis. Cortisol was determined using the DBC Kit (CAN-E-270®), which allowed the direct quantitative determination of this enzyme-immunoassay and a reading was taken at 450 nm in a microplate reader. Concentrations of potassium (K^+), sodium (Na^+), chloride (Cl^-), glucose, lactate, total protein, total cholesterol, triglycerides, and enzymes Aspartate Amino Transferase (AST) and alanine Amino Transferase (ALT) were determined using kits from Labtest (MG, Brazil) for each.

Histopathological analyzes

At the end of 24 and 48 hours of exposure to the Bt-based biopesticide, three fish from each repetition (nine per treatment) were collected randomly, anesthetized in water and ice (5 min), for subsequent spinal section and the collection of fragments of the midgut, liver and kidney. The organ fragments were fixed in Bouin's solution for 24 h, dehydrated in an alcoholic series and diaphanized for inclusion in paraffin, then sectioned into 3 μm thick histological sections and mounted on histological slides. The histological sections were stained with Hematoxylin and Eosin (HE) and examined under a light microscope with a digital camera (LEYCA DM 500). The remaining fish from each treatment remained in the tanks for post-experiment observation.

The analysis of histopathological alterations in the liver and kidney were classified in progressive stages in relation to the impairment of organ functions: stage I alterations (do not compromise the functioning of the organ), stage II alterations (severe and which impair the normal functioning of the organ) and stage III alterations (extremely severe and irreversible). Histopathological analyzes were performed in a semi-qualitative manner using the mean

alteration value (MAV) (Schwaiger et al. 1997) and histopathological alteration index/HAI (Poleksic & Mitrovic-Tutundzic 1994).

For the analysis of histopathological alterations in the middle intestine, the total number of vertical and horizontal villi of fish, goblet cells and lymphocytes were counted after 24 and 48 h exposure to the Bt-based biopesticide.

Statistical analysis

All the data were initially assessed with regard to the assumptions of normal distribution and homoscedasticity, using the Shapiro–Wilk and Bartlett tests, respectively. Data were analyzed using ANOVA-One way followed by the Tukey's test, to enable comparisons between the means (Zar 2010).

RESULTS

In *A. gigas* exposed to water or diet containing Bt-based biopesticide, no fish died within 24 h of the experiment. However, after 48 h, death began, with 100% mortality reached in 168 h (Table I). Before death, the fish exhibited slow and erratic swimming.

Table I. Mortality of *Arapaima gigas* during and after exposure to biopesticide based on *Bacillus thuringiensis*.

Period	Control	Water	Diet
During trials	0	0	0
After 24 h	0	0	0
After 48 h	0	0	1
After 72 h	0	1	0
After 96 h	0	2	1
After 120 h	0	0	2
After 144 h	0	1	1
After 168 h	0	1	0
Total	0	5	5

Exposure of *A. gigas* to water containing biopesticide decreased glucose levels (p <0.05), as well as total protein and sodium levels after 24 h, while cholesterol, triglycerides and potassium increased (p <0.05) after this exposure period. The levels of chloride and AST also increased in fish exposed to water containing the biopesticide after 48, when compared to the other groups. In fish fed a Bt-based diet, plasma levels of total protein and chloride increased (p <0.05) after 24h and decreased (p<0.05) after 48h of exposure, while levels of triglycerides, ALT and sodium decreased (p <0.05) after 24 and 48h, when compared to the control. However, AST levels increased (p<0.05) after 48 h in fish fed a diet containing the biopesticide, when compared to the other groups (Table II).

In fish exposed to water or a diet containing a Bt-based biopesticide, the hemoglobin concentration did not change (p>0.05), but the hematocrit levels declined (p<0.05) after 24 and 48 h, when compared to control. The number of total erythrocytes increased (p<0.05) after 24 h in fish fed a diet containing *B. thuringiensis*-based biopesticide, in comparison with the other groups. MVC declined (p<0.05) after 24 and 48 h after feeding with a diet containing biopesticide, while MCHC increased (p<0.05) after 24 h in fish exposed to biopesticide in their tank water, when compared to the other groups. In fish exposed to water containing Bt-based biopesticide, the number of total thrombocytes and lymphocytes increased (p<0.05) after 24 h, but declined (p<0.05) after 48 h of exposure. At 48 h after the exposure of fish to water with biopesticide there was a reduction (p<0.05) in the number of total leukocytes, as there was in fish that received a diet containing the biopesticide, after 24 and 48 h. The number of neutrophils decreased (p<0.05) after 24 and 48 h in fish exposed to water with the biopesticide and in fish fed with the biopesticide after 48 h. The

Table II. Biochemical parameters of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis*.

Parameters	Exposure to water with *Bacillus thuringiensis*		
	Control	**24 h**	**48 h**
Lactate (µg/dL)	28.9 ± 0.85[a]	32.8 ± 5.0[a]	36.6 ± 4.3[a]
Cortisol (µg/dL)	63.5 ± 0.6[a]	68.4 ± 3.0[a]	48.9 ± 3.4[b]
Glucose (µg/dL)	141.3 ± 7.7[a]	103.7 ± 7.1[b]	92.1 ± 7.4[b]
Total protein (g/dL)	7.5 ± 0.7[a]	4.9 ± 0.8[b]	6.8 ± 0.7[a]
Cholesterol (mg/dL)	168.1 ± 13.8[a]	201.7 ± 23.5[b]	176.2 ± 17.6[a]
Triglycerides (mg/d	92.9 ± 15.0[a]	121.2 ± 14.1[b]	115.4 ± 15.1[a]
Sodium (mmol/L)	182.1 ± 9.5[a]	196.2 ± 14.3[a]	174.6 ± 16.1[b]
Potassium (mmol/L)	14.5 ± 1.3[a]	20.4 ± 4.5[b]	12.4 ± 1.6[a]
Chloride (mmol/L)	132.3 ± 9.6[a]	129.5 ± 14.8[a]	161.9 ± 5.0[b]
AST (U/L)	15.8 ± 6.6[a]	22.3 ± 10.7[a]	43.4 ± 17.4[b]
ALT (U/L)	35.6 ± 17.5[a]	23.4 ± 28.5[a]	21.0 ± 10.0[a]
Parameters	Feeding with diets containing *Bacillus thuringiensis*		
	Control	**24 h**	**48 h**
Lactate (µg/dL)	28.9 ± 0.85[a]	35.6 ± 2.0[a]	27.5 ± 2.0[a]
Cortisol (µg/dL)	63.5 ± 0.6[a]	58.5 ± 2.8[a]	57.9 ± 3.1[a]
Glucose (µg/dL)	141.3 ± 7.7[a]	143.0 ± 3.4[a]	139.7 ± 7.9[a]
Total protein (g/dL)	7.5 ± 0.7[a]	7.9 ± 1.1[b]	4.8 ± 0.6[b]
Cholesterol (mg/dL)	168.1 ± 13.8[a]	182.2 ± 3.8[a]	138.6 ± 18.1[b]
Triglycerides (mg/d	92.9 ± 15.0[a]	135.7 ± 12.5[b]	138.8 ± 24.7[b]
Sodium (mmol/L)	182.1 ± 9.5[a]	150.4 ± 17.3[b]	148.4 ± 7.0[b]
Potassium (mmol/L)	14.5 ± 1.3[a]	15.8 ± 2.5[a]	13.5 ± 0.3[a]
Chloride (mmol/L)	132.3 ± 9.6[a]	168.3 ± 13.8[b]	113.3 ± 3.4[b]
AST (U/L)	15.8 ± 6.6[a]	19.7 ± 8.14[a]	39.6 ± 20.5[b]
ALT (U/L)	35.6 ± 17.5[a]	15.3 ± 12.0[b]	12.3 ± 11.1[b]

Values express means ± standard deviation. AST: Aspartate Amino Transferase, ALT: Alanine Amino Transferase. **Different letters, in the same line, indicate differences by the Tukey test (p<0.05).**

number of monocytes decreased (p<0.05) in fish exposed to water containing the biopesticide after 24 h and increased (p<0.05) after 48 h, while in fish that received a biopesticide diet there was a reduction (p<0.05) after 24 and 48 h, when compared to the control (Table III).

Hepatic histopathological alterations were observed in *A. gigas* exposed to tank water and fed with a diet containing Bt-based biopesticide after 24 and 48 h (Figure 1). These hepatic alterations were classified, in their majority, as stages I, II and III, with lesions varying from mild to severe (Table IV), and there was an increase (p

<0.05) in the AHI and AMV values when compared to control (Figure 2).

Renal histopathological alterations in *A. gigas* exposed to water and a diet containing biopesticide were classified as stages I, II and III, showing mild to severe lesions (Table V and Figure 3). Furthermore, in fish exposed to the biopesticide in tank water and fed with a diet containing biopesticide, an increase (p<0.05) in the values of AHI and VMA was observed after 24 and 48 hours, other than after 24 h in fish fed with a diet containing a Bt-based biopesticide (Figure 4).

Table III. Blood parameters of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis.*

Parameters	Exposure to water with *Bacillus thuringiensis*		
	Control	24 h	48 h
Hematocrit (%)	31.5 ± 2.1[a]	24.5 ± 2.1[b]	27.7 ± 1.9[b]
Hemoglobin (g/dL)	5.9 ± 0.3[a]	6.3 ± 0.5[a]	6.0 ± 0.5[a]
Erythrocytes (x 106/µL)	1.7 ± 0.3[a]	1.6 ± 0.04[a]	1.7 ± 0.1[a]
MCV (fL)	160.3 ± 7.5a	161.5 ± 7.5[a]	174.5 ± 13.8[a]
MCHC (g/dL)	18.7 ± 1.8[a]	25.8 ± 2.5[b]	19.7 ± 1.7[a]
Thrombocytes (µL)	191,439 ± 26,101[a]	252,176 ± 29.892[b]	131,998± 128,878[b]
Leukocytes (µL)	257,939 ± 25,733[a]	264,671 ±23.034[a]	75,936 ± 32,303[b]
Lymphocytes (µL)	81,684 ± 20.603[a]	152,489 ± 15.910[b]	48,419±19,303[b]
Neutrophils (µL)	105,090 ± 2.413[a]	64,712 ±15,309[b]	31,558 ± 14.155[b]
Monocytes (µL)	48,817 ±7.345[a]	24,864 ±7.367[b]	52,783 ± 2.598[b]
Eosinophils (µL)	4.311±1.546[a]	4.107±1.223[a]	3.929 ± 2.523[a]
Parameters	Feeding with diets containing *Bacillus thuringiensis*		
	Control	24 h	48 h
Hematocrit (%)	31.5 ± 2.1[a]	25.8 ± 2.9[b]	27.2 ± 1.3[b]
Hemoglobin (g/dL)	5.9 ± 0.3[a]	5. 9 ± 0.3[a]	6.3 ± 0.3[a]
Erythrocytes (x 10^6/µL)	1.7 ± 0.3[a]	2.0 ± 0.2[b]	1.8 ± 0.1[a]
MCV (fL)	160.3 ± 7.5[a]	136.9 ± 114.3[b]	150.9 ± 12.3[b]
MCHC (g/dL)	18.7 ± 1.8[a]	19.4 ± 2.1[a]	19.7 ± 1.6[a]
Thrombocytes (µL)	191,439 ± 26,125[a]	225,571 ± 49,123[a]	196,332 ± 47.401[a]
Leukocytes (µL)	257,939 ± 25,734[a]	199,639 ± 54,724[b]	105,371 ± 42,980[b]
Lymphocytes (µL)	81,684 ± 20.634[a]	75,510 ± 19.245[a]	68,620 ± 11,902[a]
Neutrophils (µL)	105,429 ±24,414[a]	127,097 ± 25,675[a]	41,219 ± 21,477[b]
Monocytes (µL)	48,817 ±7.363[a]	32,241 ± 6.789[b]	21,868 ± 3.089[b]
Eosinophils (µL)	4.311 ± 1.567[a]	5.334 ±3.229[a]	4.170 ± 1.940[a]

Values express means ± standard deviation. MCV: Mean corpuscular volume, MCHC: Concentration of mean corpuscular hemoglobin. Different letters, in the same line, indicate differences by the Tukey test (p<0.05).

The main histopathological alterations found in the intestine of *A. gigas* exposed to water and feed with a Bt-based biopesticide, after 24 and 48 h, are shown in Figure 5. The number of vertical villi and goblet cells decreased (p <0.05), after 48 h, in the intestine of fish exposed to water containing Bt and the number of vertical villi increased (p<0.05), after 24 h, in fish that received a biopesticide diet, as well as in the number of horizontal villi after 24 and 48 h, when compared to the other groups. The number of lymphocytes in the intestine of fish exposed to water containing biopesticide showed a reduction (p<0.05) after 24 h, when compared to the other groups, as well as the number of goblet cells after 24 and 48 h in fish fed with Bt-based diet (Table VI).

DISCUSSION

The contamination of water resources by pesticides is a current global problem, with some countries, such as Brazil, the largest pesticide consumers. The most widely used pesticides and biopesticides are potentially major contaminators of hydric resources. Studies have reported that Bt has no toxicity to non-target species, such as fish (Jackson et al. 2002, Meher et al. 2002). However, in *A. gigas* exposed to water and a diet containing Bt-based biopesticide, mortality occurred after 48 h of exposure and reached 100% in 168 h, due to toxicity. Oliveira-Filho (2008) cited 20% mortality in trout exposed to Bt after 32 days, attributed to excessive competition for food
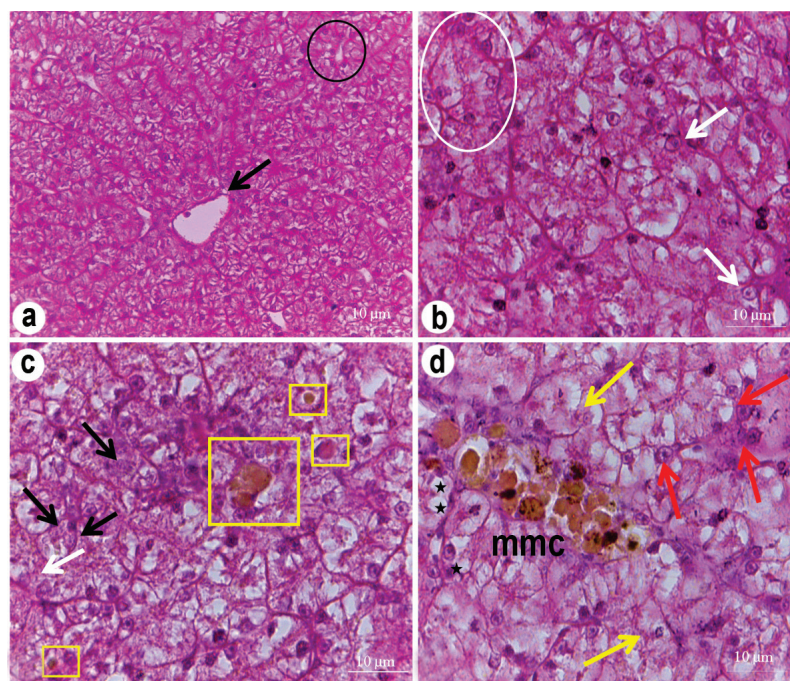
Figure 1. Histopathological changes in the liver of *Arapaima gigas* exposed to a biopesticide based on *Bacillus thuringiensis*. (a) Control showing sinusoidal (circular area) and central vein (arrow). (b) Fish exposed to water containing biopesticide, after 24 h, had nuclei located on the periphery of the cell (circular area) and cells with nuclear vacuolization (arrow). (c) Fish fed with diet containing biopesticide, after 48 h, showing cells with no nucleolus and biliary stagnation (checkered area). (d) Fish fed with diet containing biopesticide, after 48 h, showing cells with nuclear hypertrophy (red arrows), nuclear atrophy (yellow arrows), melanomacrophage center (MMC) and cytoplasmic vacuolization in hepatocytes (¬). Staining: Hematoxylin and Eosin.

in the water and high concentrations of the microorganism. Snarski (1990) also observed mortality in *Pimephales promelas* (Rafinesque 1820) exposed to 10^6 CFU / mL Bt. For *O. niloticus*, Ahmad et al. (2011) reported 30% mortality after four days of exposure to 128 mg/L of a Bt-based biopesticide. In contrast, Grisolia et al. (2009) observed that exposure to 1 x 10^6 of Bt, over 30 days, did not affect the survival of *O. niloticus*. Therefore, these results indicate the toxicity of Bt, depending on the species of fish, concentration and form of administration of Bt.

Studies of blood parameters have been shown to be a valuable approach for analyzing the health status of a fish population, as these indices provide reliable information on metabolic disorders, deficiencies, and chronic stress status, and thus help in understanding the relationship of blood characteristics to habitat and the adaptability of the species to the environment, as well as exposure to toxicant substances (Ranzani-Paiva et al. 2013, Fazio 2019, Mariano et al. 2019, Li et al. 2020). Hence, there is growing interest in the study

of hemato-biochemical parameters, which are considered important for fish aquaculture.

In *A. gigas* exposed to water containing Bt-based biopesticide, there was a reduction in plasma cortisol and sodium levels after 48 h and glucose levels after 24 and 48 h), and an increase in chloride, AST, cholesterol, triglycerides and potassium in 24h. However, in fish fed with Bt-based diets, there was an increase in total protein and chloride levels after 24 h and a reduction after 48 h, and in cholesterol levels in 48 h. In addition, there was an increase in triglyceride levels and a decrease in sodium and ALT levels after 24 and 48 h, as well as an increase in AST levels. In *P. mesopotamicus* exposed to water containing Bt-based biopesticide, an increase in plasma cholesterol, sodium, chloride, AST and ALT was reported, as well as a reduction in cortisol, glucose and lactate. However, in fish fed with Bt-based diets there was a decrease in cortisol, glucose, total protein, cholesterol and potassium, as well as increased levels of AST, ALT and triglycerides (Mariano et al., 2019). Therefore, these results indicate a dysfunction

Table IV. Frequency of histopathological alterations in liver of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis*.

Stages	Alterations	Control	Water 24 h	Water 48 h	Diet 24 h	Diet 48 h
I	Vacuolization of cytoplasm	+	++	++	++	++
	Hypertrophy of nucleus	0+	+	+	++	++
	Nucleus on the periphery of the cell	0+	++	+	++	++
	Hepatic disorganization	+	+	0+	++	+
II	Nuclear atrophy	0+	0+	0+	+	+
	Nuclear vacuolization	0+	+	0+	0+	+
	Biliary stagnation	0+	+	+	+	+
	Nuclear degeneration	0+	+	+	+	+
	Absence of nucleolus	0+	0+	+	+	+
III	Necrosis	0	0	0	0+	0+

0: No changes, 0+: Little frequent changes, +: Frequent changes. ++: Very frequent changes.

in the osmoregulation of fish exposed to Bt and liver impairment. Elevated levels of ALT and AST are bioindicators of tissue damage or liver dysfunction (Mariano et al. 2019, Li et al. 2020). Therefore, these results indicate that exposure to Bt had adverse effects on the liver physiology and histology of *A. gigas*.

Erythrocyte and leukocyte parameters enable the rapid detection of possible blood disorders in the fish population (Ranzani-Paiva et al. 2013, Fazio 2019, Mariano et al. 2019, Li et al. 2020). In *A. gigas* exposed to water or a diet containing Bt-based biopesticide, the hematocrit decreased (24 and 48 h), while the number of total erythrocytes increased (in 24 h) in fish fed with Bt. The MVC and number of neutrophils decreased after 24 and 48 h of a biopesticide-containing diet, while the MCHC increased (in 24 h) in fish exposed to water containing Bt-based biopesticide. In fish exposed to water containing the biopesticide the number of total thrombocytes and lymphocytes increased (p<0.05) after 24 h, decreasing (p<0.05) after 48 h of exposure. At 48 h after fish exposure to water with biopesticide there was a reduction (p <0.05)

in the number of total leukocytes, while in fish that received a diet containing the biopesticide this reduction occurred after 24 and 48 h. In addition, there was a reduction in the number of monocytes in fish exposed to water containing biopesticide, after 24 h, and an increase after 48 h, while in fish that received a biopesticide diet, there was a reduction in all the studied periods. In a similar study with *P. mesopotamicus* exposed to Bt-based biopesticide, an increase in hematocrit, hemoglobin, MVC, number of leukocytes, lymphocytes and neutrophils was reported, as well as a reduction in the number of total erythrocytes (Mariano et al. 2019).

In Bt-based biopesticides, the bacterium causes lesions in the gastrointestinal tract of the target insect, leading to nutrient absorption problems and death due to starvation (Chen et al. 2013, Portugal et al. 2017). The number of intestinal villi, permanent structures that increase the surface area of the nutrient absorption organ, increased in fish fed with Bt-based diets, due to the increase in the mucous layer in the endothelial and goblet cells. The presence of a thicker mucous layer hinders the

absorption of nutrients by the endothelial cells, making it necessary to increase the absorption surface, as it is being impaired by the intense action of the goblet cells. In fish, the presence of goblet cells in the intestine is related to different feeding conditions and protection against bacterial activities, the protection of the epithelium against food from the stomach, rich in digestive enzymes with a markedly acidic pH (Honorato et al. 2011). In *A. gigas*, exposure to Bt-based biopesticide seems to have promoted adjustments in the intestine, characterized by a decrease in goblet cells. However, an increase in goblet cells is related to the good quality of the intestinal microbiota and an increase in mucus production that occurs after aggression by pathogens, thus playing an important role in protecting against infections (Melo et al. 2013, Kalhoro et al. 2018). In addition, among the enterocytes of *A. gigas*, we also observed the presence of lymphocytes, indicating an immune defense cell barrier. Intraepithelial lymphocytes (cytotoxic T cells) may be involved in the immune response of the intestinal mucosa of fish (Kalhoro et al. 2018). B-lymphocytes appear late in the intestinal mucosa, while the early appearance of T-lymphocytes suggests that this may be a place for the extra-thymic differentiation of these leukocytes (Cain et al. 2000, Rombout et al. 2010). Therefore, the integrity of the intestine is assumed to be a key factor for the growth and welfare of farmed *A. gigas*.

In fish, histomorphological studies help in understanding the relationship between physiological and biochemical functions and molecular mechanisms (Kalhoro et al. 2018, Li et al. 2020). In *A. gigas* exposed to Bt-based biopesticides there were liver injuries such as cytoplasmic vacuolations, nuclear hypertrophies and atrophies and necrosis, with the presence of melanoma-macrophage centers, indicating the harmful effects of this biopesticide. However, it

was not possible to determine the mechanism that led to these liver lesions, that is, whether it was mediated by Cry toxins or Bt spores, as this bacterium produces and secretes dozens of chemical substances, among them the Cry proteins that are used as entomotoxins (Praça et al. 2007). The liver performs numerous vital metabolic functions such as the metabolism of endogenous compounds, carbohydrates and lipids, in addition to acting on hematopoiesis and playing a fundamental role in the synthesis and oxidation of fatty acids. Liver cells also store glycogen and synthesize plasma proteins (Hilton et al. 1992, Evans 1993).

The kidney is an important hematopoietic organ in fish, that receives a large flow of blood from the gills and, consequently, can be a target organ of aquatic contamination. The main renal histopathological alterations in *A. gigas* exposed to a Bt-based biopesticide were: nuclear hypertrophy, reduction of the space of Bowman's capsule, occlusions and the narrowing of the tubular lumen and necrosis. Such degenerative alterations may be due to the presence of toxic products in the glomerular filtrate (Rand 1995, Takashima & Hibiya 1995). Similar histopathological alterations have been described in the kidney of *Prochilodus lineatus* (Valenciennes 1837) exposed to sublethal concentrations of the trichlorfon pesticide (Veiga et al. 2002). In addition, in the kidney of *A. gigas* exposed to Bt-based biopesticide there was the presence of melanomacrophage centers, which are responsible for the removal of foreign particles or products of cell degradation by phagocytosis. Melanomacrophage centers may be associated with inflammatory lesions (Wolke et al. 1995, Agius & Roberts 2003, Steinel & Bolnick 2017) and, therefore, such pigments can be considered indicators of a response to the Bt-based biopesticide in *A. gigas*.
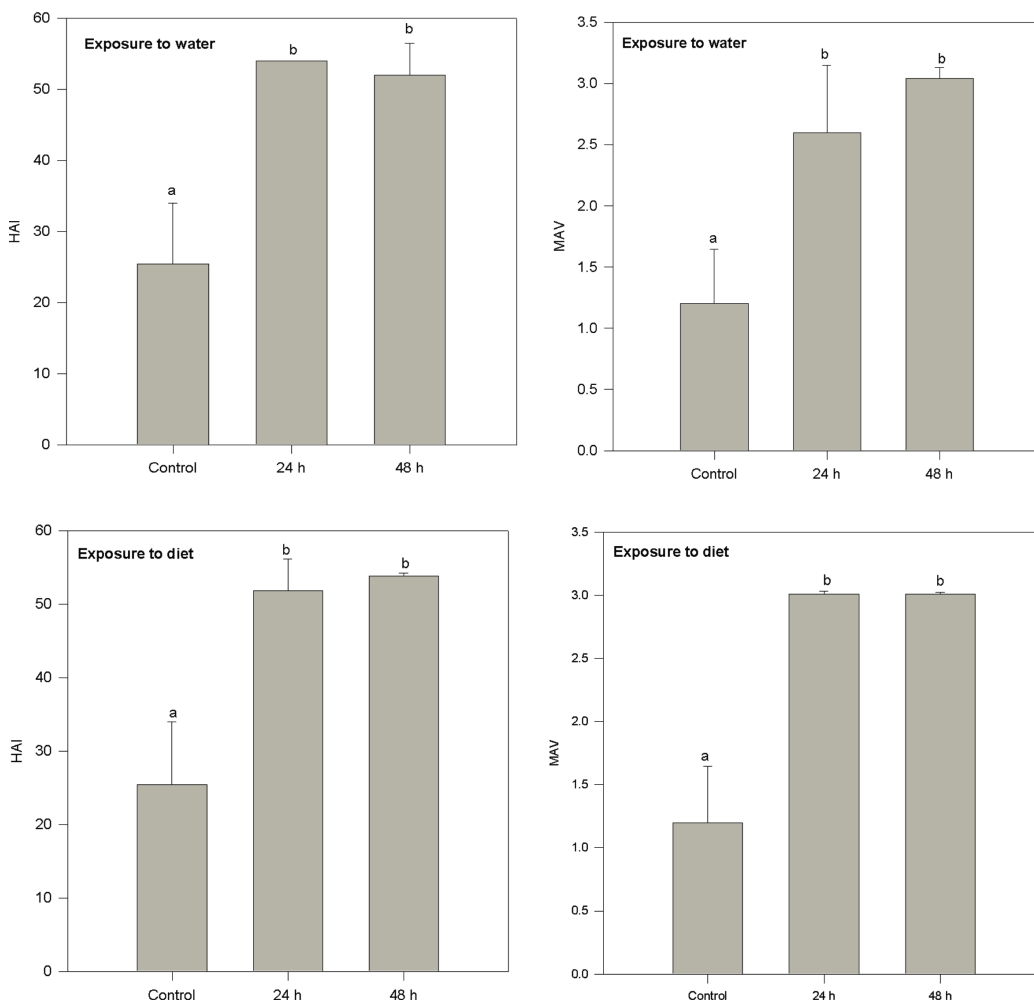
Figure 2. Values of histopathological alteration index (HAI) and mean assessment values (MAV) for liver of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis* in water and diet. Different letters indicate differences by the Tukey test (p<0.05).

Table V. Frequency of histopathological alterations in kidney of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis*.

Stages	Alterations	Control	Water 24 h	Water 48 h	Diet 24 h	Diet 48 h
I	Atrophy of the glomerulus	0	0	0+	**+**	**+**
	Hyaline degeneration	0	0	0	0	+
	Capillaries dilation of the glomeruli	0+	+	+	0+	+
	Narrowing of the tubular lumen	0+	+	+	0+	+
	Cellular hypertrophy	0+	+	+	0+	+
	Nuclear hypertrophy	0+	+	+	+	+
	Melanomacrophages	+	++	++	+	+++
	Cellular vacuolization	0	0+	+	+	+
II	Tubular degeneration	0	0	+	+	+
	Tubular lumen occlusion	0+	+	++	+	++
	Bowman capsule space reduction	0	0+	0+	0+	+
III	Necrosis	0	0+	0+	0	+

0: No changes, 0+: Little frequent changes, +: Frequent changes, ++: Very frequent changes, +++: Extremely frequent changes.
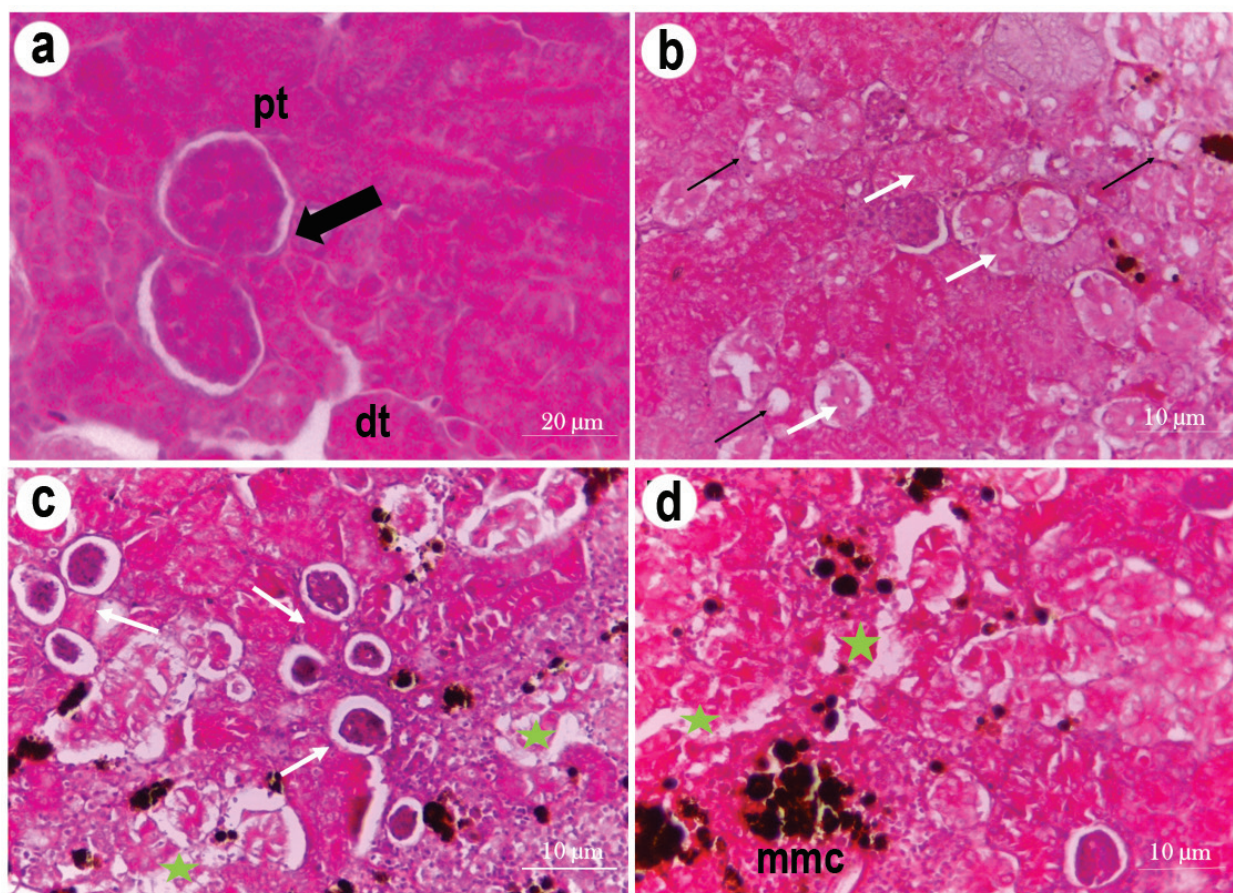
Figure 3. Histopathological changes in the kidney of *Arapaima gigas* exposed to a biopesticide based on *Bacillus thuringiensis*. (a) Control fish showing glomeruli (arrow), Distal tubule (DT) and Proximal tubule (PT). (b) Narrowing of the tubular lumen (white arrow) and vacuolization of the tubular cells (black arrow) in fish exposed to biopesticide in the water, after 24 h. (c) area with presence of aggregates of melanomacrophages and glomeruli with an increase in Bowman's capsule and focal necrosis (stars) in fish exposed to biopesticide in the diet, after 48 h. (d) Area with melanomacrophage centers (CMM) and focal necrosis (¬) in fish exposed to the biopesticide in the water after 48 h. Staining: Hematoxylin and Eosin.

CONCLUSIONS

The present study measured the hematological and histopathological parameters of *A. gigas* exposed to a biopesticide based on Bt and found that this exposure caused alterations in both parameters. Blood and histopathological analyzes proved to be useful tools for the detection of the effects of this contaminant in *A. gigas*. Fish mortality was due to blood and structural alterations in the liver and kidney. Therefore, knowing the effects of biopesticides on the health of exposed fish is essential for aquaculture. Bt-based biopesticide has been shown to be harmful to fish health in the concentrations and media tested, so contamination of the aquatic environment by agriculture must be considered. Lastly, the goal of this study was to lay the foundations for a healthy aquaculture and provide information for further research using Bt.
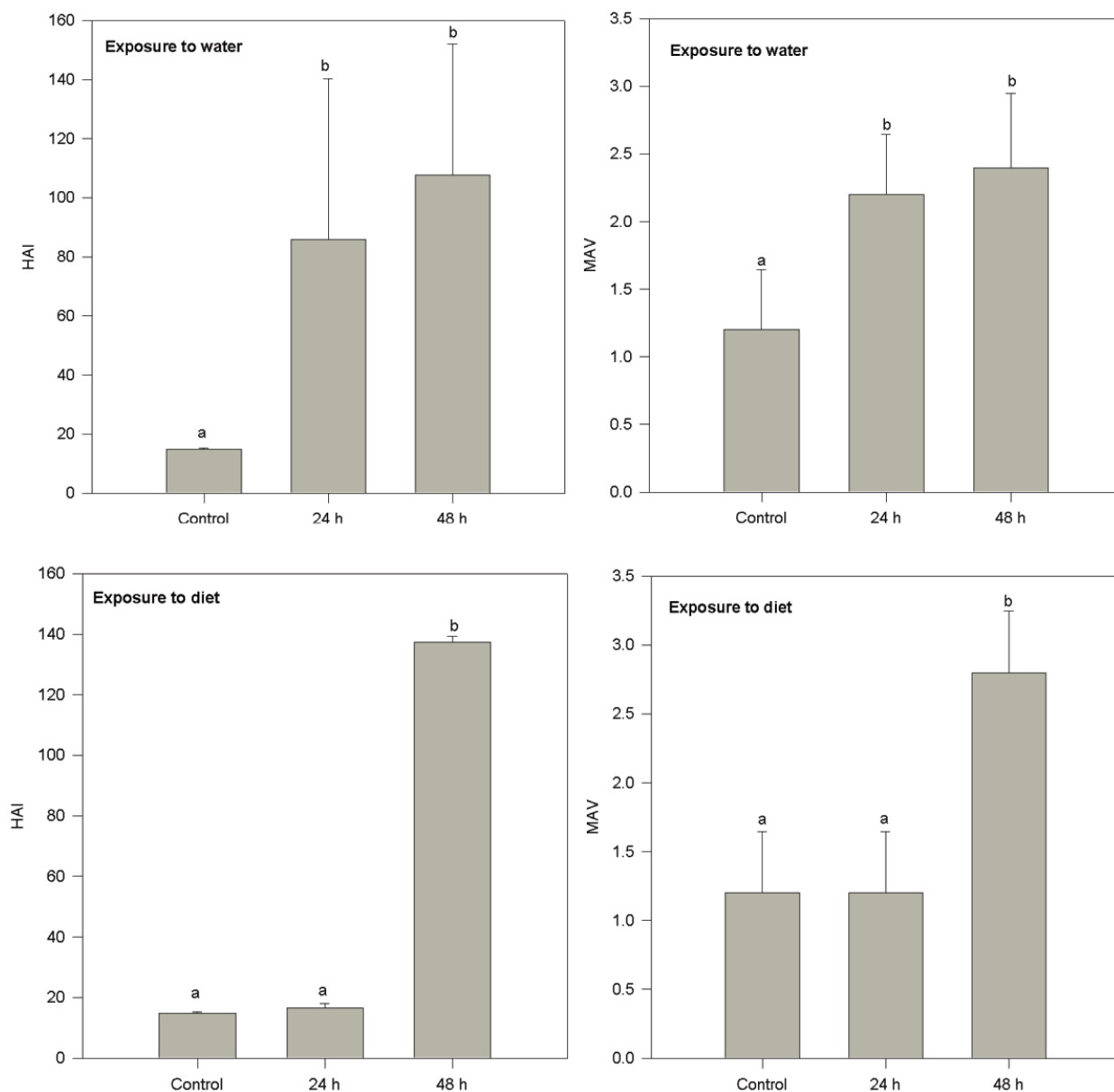
Figure 4. Values of histopathological alteration index (HAI) and mean assessment values (MAV) for kidney of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis* in water and diet. Different letters indicate differences by the Tukey test (p<0.05).
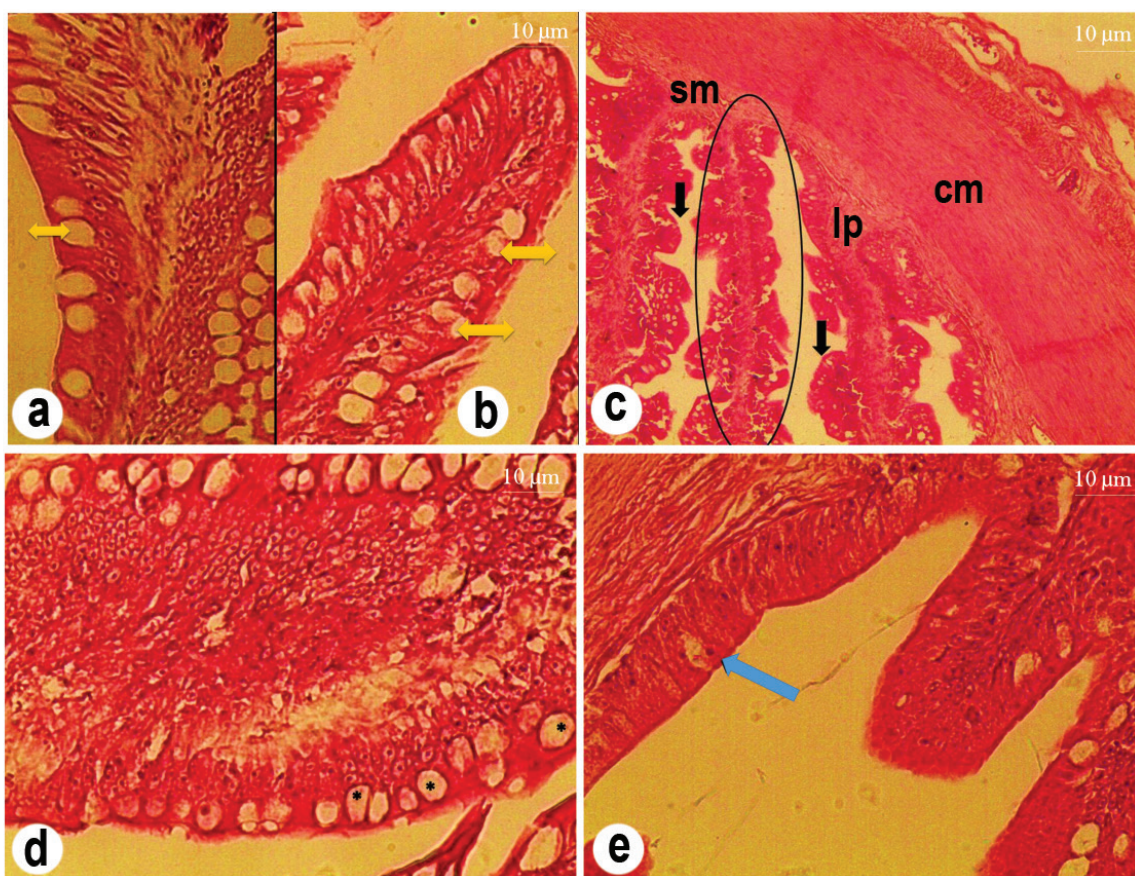
Figure 5. Histopathological changes in the intestine of *Arapaima gigas* exposed to a biopesticide based on *Bacillus thuringiensis*. A-b. Control fish showing intestinal epithelium, vertical villi, vertical villi and changes in the thickness of the intestinal mucosa (arrows). c. Fish fed diets containing biopesticide, after 48 h, showing muscle layer (CM), submucosa (SB), lamina propria (LP), horizontal villi (arrow) and vertical villi (circle). d. Fish fed with diets containing biopesticide, after 48 h, showing intestinal epithelial cells and the presence of goblet cells. e. Fish exposed to water containing biopesticide, after 24 h, showing intestinal epithelium with the presence of lymphocyte (arrow). Staining: Hematoxylin and Eosin.

Table VI. Measures of intestine structures of *Arapaima gigas* exposed to biopesticide based on *Bacillus thuringiensis*.

Parameters	Exposure to water with *Bacillus thuringiensis*		
	Control	**24 h**	**48 h**
Vertical villi	22.6 ± 2.5[a]	19.2±1.5[a]	15.0±1.1[b]
Horizontal villi	40.2 ± 7.8[a]	40.6 ± 5.7[a]	57.7 ± 3.9[a]
Caliciform cells	293.4 ± 43.0[a]	264.4 ± 42.1[a]	173.6 ± 6.8[b]
Lymphocytes	10.7 ± 1.1[a]	6.0 ± 0.5[b]	9.6 ± 0.7[a]
Parameters	**Feeding with diets containing *Bacillus thuringiensis***		
	Control	**24 h**	**48 h**
Vertical villi	22.6 ± 2.5[a]	29.6 ± 2.6[b]	20.5 ± 1.1[a]
Horizontal villi	40.2 ± 7.8[a]	151.4 ± 14.3[b]	165.3 ± 19.5[b]
Caliciform cells	293.4 ± 43.0[a]	217.5 ± 31.4[a]	129.8 ± 7.4[b]
Lymphocytes	10.7 ± 1.1[a]	9.0 ± 0.6[a]	8.7 ± 0.7[a]

Different letters, in the same line, indicate differences by the Tukey test (p<0.05).

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