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ORIGINAL ARTICLE

Hematological and histopathological assessment of pacu (*Piaractus mesopotamicus*) after treatment of pathogens with veterinary medicinal products

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Abstract Fish species are impacted by multiple pathogens, and they are exposed to the chemicals used to treat these diseases at several stages during the aquaculture production cycle. This study performed hematological and histopathological evaluations of pacu (*Piaractus mesopotamicus*) that had been naturally infected with *Aeromonas* sp., *Streptococcus* sp., *Ichthyophthirius multifiliis*, *Trichodina heterodentata*, and *Anacanthorus penilabiatus* and treated with enrofloxacin and toltrazuril or with florfenicol and thiamethoxam. After 7 days of treatment from nine fishes were collected blood, via caudal puncture, and samples of the gills, liver, and kidneys. Following toltrazuril and enrofloxacin treatment, fish exhibited leukocytosis with lymphocytosis. With thiamethoxam and florfenicol treatment, the fish showed an increase in hematocrit, hemoglobin level, mean corpuscular volume, and mean corpuscular hemoglobin concentration and a decrease in red blood cells. The infected control fish presented aneurysms and a disruption of the secondary lamellae, which can cause death. The drugs used in this study stimulated the immune systems in the fish or caused electrolyte imbalances, which were temporary.

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Background

Despite advances in aquaculture techniques, the production of pacu (*Piaractus mesopotamicus*), a native fish species from Brazil, is impacted by multiple pathogens. The pacu is one of the most important species used in Brazilian fish farming due to its rapid growth rate, easy adaptation to artificial feeding, and high consumer appreciation, as well as its ecological and commercial value (Biller-Takahashi et al. 2015).

Commercially grown fish, such as the pacu, are often parasitized by members of the Monogenoidea, which results in economic losses, a problem that is especially prevalent in the Neotropics where ecological characteristics facilitate the rapid and constant spread of various parasites (Schalch et al. 2006). Under high-temperature conditions combined with high concentrations of organic matter in the water, the monogenean parasite, *Anacanthorus penilabiatus*, can cause high levels of fish mortality. Another common parasite in aquaculture is *Trichodina heterodentata*. Fish infected by this parasite present abnormal epithelial proliferation in the skin and gills and severe aberrations in their lamellae (Yemmen et al. 2010; Pádua et al. 2012). According to Xu et al. (2007), heavy *Trichodina* infections cause epidermal injuries that lead to an increase in *Streptococcus* infections. Under lower temperatures, *Ichthyophthirius multifiliis*, a ciliated protozoan, causes even greater mortality in larvae and fingerlings, which can become even worse in conjunction with *Aeromonas hydrophila* (Liu and Lu 2004).

Species of the bacterial genera *Aeromonas*, which are gram-negative, and *Streptococcus*, which are gram-positive, are among those that cause the largest losses in fish farming, independent of the species or developmental stage of the fish. *Aeromonas* spp. damage the gills, cause hemorrhagic septicemia, and predispose fish to infection by other pathogens (Carraschi et al. 2012); and *Streptococcus* spp. cause anorexia, lethargy, and erratic swimming behaviors due to its effects on the central nervous system (Azad et al. 2012).

These pathogens are also important to human health; *Aeromonas* spp. are often found in the feces of patients with foodborne diarrhea (Vila et al. 2003), and water is the principal mode for direct human infection, as well as for the contamination of food (Janda and Abbott 2010). *Streptococcus* sp. is a common colonizer of humans that causes septicemia and meningitis and is the leading cause of invasive bacterial disease in neonates and children (Eickhoff et al. 1964; Ragunathan et al. 2009).

In aquaculture, fish are exposed to the chemicals used for disease control at several stages during the production cycle. The use of antimicrobials, such as florfenicol (FFC) (Yáñez et al. 2014) and enrofloxacin (EF) (Bowser et al. 1990), and antiparasitics, such as toltrazuril (TOL) (Jaafar and Buchmann 2011) and thiamethoxam (TH) (Carraschi et al. 2014), has become a common practice. However, before using a chemical in an aquatic system, it is necessary to study its efficacy, toxicity, and the residual of the substance and to understand its possible hematological and histopathological effects in fish following treatment.

Histological techniques are important tools in the evaluation of the sublethal effects of contaminants or pathogens, and pathological changes can serve as biomarkers. Furthermore, histological changes provide a rapid, intermediate methodology to identify stressors and detect their effects on tissues (Bernet et al. 1999; Moon et al. 2006; Figueiredo-Fernandes et al. 2007). In addition, hematological analyses aid in determining the physiological status of an organism (Seriani et al. 2013) and can reflect environmental indicators and the toxic effects of chemicals (Gabriel et al. 2007; Ghaffar et al. 2014). Therefore, it is necessary to identify the effects of both the pathogens and the drugs used to treat them to enable the identification and monitoring of disease and the possible toxic effects in animals following treatment.

The aim of this study was to perform a hematological and histopathological evaluation of pacu (*P. mesopotamicus*) using the following chemical treatments for infected fish: (a) fish naturally infected with *Aeromonas* sp., *Streptococcus* sp., *I. multifiliis*, *T. heterodentata*, and *A. penilabiatus* and treated with EF and TOL; and (b) fish naturally infected with *Aeromonas* sp., *Streptococcus* sp., and *A. penilabiatus* and treated with FFC and TH.

Material and methods

There were 20 fish per trial, for a total of 60 fish per treatment, which were maintained under mesocosm conditions in 600-L tanks. Each experiment, there were 60 fish each treatment, totalizing 240 fish each assay. Cohabitation conditions were used for the infection of all fish, and parasites on the surface of the fish and the gills were counted. In addition, the occurrence of bacteria on the epidermal surface before the start of the experiment and after the treatment were also evaluated, with ten fish used as samples for each time period (Carraschi et al. 2014).

Pacus (31.40 ± 2.77 g) that were naturally infected with parasites and bacteria were treated with an antibiotic and a parasiticide in 600-L tanks. In the first experiment, the fish that showed infections with *I. multifiliis* (at least 100 ictio fish^{-1}), *T. heterodentata (*at least 40 *T. heterodentata* fish^{-1}), *A. penilabiatus* (at least 50 monogeneans fish^{-1}), *Aeromonas* sp., and *Streptococcus* sp. (bacterial infections were identified based on biochemical tests) were treated with 3.0 mg TOL L^{-1}, as a parasiticide, for 5 days (Bayer HealthCare©, Sao Paulo, Brazil) and 90 mg EF kg^{-1} (Baytril©, Sao Paulo, Brazil), as an antibiotic in their feed ration, for 7 days.

In this experiment, the treatments were healthy control fish (HC), infected control fish (InC), infected and treated fish with TOL and EF (Tr), and healthy fish exposed to the drugs treatment TOL and EF (ExC).

TOL was directly applied to the water in the tanks (500 L) in which fish were kept, and fish were exposed to the drug for an hour each day, 5 days.

In the second experiment, other fish showing natural infections with *A. penilabiatus* (over 200 parasites fish^{-1}), *Aeromonas* sp., and *Streptococcus* sp. were treated with 75 mg TH L^{-1} (Novartis©, Sao Paulo, Brazil), as a parasiticide, for 4 days, and 10.0 mg FFC kg^{-1} (MSD©, Sao Paulo, Brazil), as an antibiotic in their feed ration, for 7 days.

In this experiment, the treatments were healthy control fish (HC), infected control fish (InC), infected and treated fish with TH and FFC (Tr), and healthy fish exposed to the drugs treatment TH and FFC (ExC).

TH was applied to the water, with 2 h of exposure per day, 4 days. The antibiotics were dissolved in 2 % vegetable oil and added to commercial fish food (containing 40 % protein), which was offered for 7 days (1.5 % body weight).

The concentrations have been chosen accordingly to previous assays in the laboratory conditions where it was evaluated several concentrations in the efficacy for the same pathogens (Carraschi et al. 2014).

In both experiments, the temperature was kept between 25 and 30 °C, the dissolved oxygen was >5.0 mg L^{-1}, the electrical conductivity stayed between 180 and 220 μS cm^{-1}, and the pH ranged from 7.0 to 8.0.

In the end of the treatment, 20 fish from each treatment, from both experiments, were sampled and were evaluated for the parasite and bacterial counts. The samples from skin and gills were evaluated under microscope and counted the parasites. The bacteria were confirmed by the observation of a high bacterial count, visualized under ×1000 magnification.

For the identification of *Aeromonas* sp., samples from skin, liver, and kidney were cultivated in phenol red starch ampicillin media and transferred to nutrient agar media. Subsequently, the samples were Gram stained and subjected to a catalase and oxidase reaction. The colonies were observed to be gram-positive and catalase and oxidase positive, thus providing a positive identification as genre *Aeromonas* sp.

For the identification of *Streptococcus* sp., samples from liver, kidney, and brain were cultivated in blood agar plates, and suggestive colonies were later transferred to dextrin agar. The suggestive colonies had clear color and transparent halos indicating hemolysis. Before, the samples were submitted to Gram staining and subjected to a catalase and oxidase reaction. The colonies were thus identified as gram-positive and catalase and oxidase negative, indicating genre *Streptococcus* sp.

In the first experiment, TOL was 100 % effective at controlling *T. heterodentata* infections and 39.80 % effective at controlling *A. penilabiatus* infections, but it was not possible to evaluate the efficacy of TOL against *I. multifiliis* because there was no infestation found after 7 days in either the treated or the nontreated fish. EF was effective (80 %) in reducing the bacterial density of *Aeromonas* sp. and *Streptococcus* sp. In the second experiment, TH was 81.86 % effective in control of *A. penilabiatus* infections and FFC was effective (60 %) in reducing the bacterial density of *Aeromonas* sp. and *Streptococcus* sp.

Once the treatments, i.e., feed combined with antibiotics, were complete (7 days), tissue samples were collected from the nine fish per treatment for hematological and histopathological analyses.

Histopathological analysis

From each experiment, nine animals from each treatment (three from each replicate) were euthanized via immersion in a benzocaine bath and used for histopathological analyses. Gill, liver, and kidney samples were collected from fish in both experiments. Samples were immersed in a buffered (0.1 M PBS, pH 7.2) aqueous solution of 10 % formaldehyde for 24 h, and after fixation, they were subjected to dehydration and diaphanization and embedded in Histosec (Merck) wax. The samples were then cut on an automatic microtome (Leica, RM-2155) into 5-mm slices. Staining was performed using hematoxylin–eosin (HE) and periodic acid–Schiff (PAS) methods (Behmer et al. 1998).

Hematological analysis

For hematological analyses, blood samples from the same nine fish (1 ml), each experiment, were collected via caudal puncture with a heparinized syringe and needle.

The blood samples were used to determine the following parameters: hematocrit (Ht) (using a microhematocrit technique), hemoglobin (Hb) content (using the cyanmethemoglobin method), red blood cell (RBC) count (using a Neubauer chamber and an index), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) (Ranzani-Paiva et al. 2013).

Immediately after the blood was collected, the blood smears were performed, and after dry, the slides were stained with May–Grünwald–Giemsa–Wright (MGGW) stain, and total and differential leukocyte (WBC) and thrombocyte (Tromb) counts were conducted using an indirect method (Hrubec and Smith 1998). The total protein content from the plasma was determined using a refractometer.

The mean values were subjected to an analysis of variance (ANOVA) and compared via Tukey's tests at a 95 % significance level using the program Statistica.

Results

Effects of enrofloxacin and toltrazuril treatment

Histopathology

Gills contain four groups of arches, and each is composed of primary lamellae with secondary lamellae (Fig. 1a), which contain chloride, pillar, mucous, and lining cells.

Animals from the InC treatment group exhibited disorders in the secondary lamellae that can compromise survival (Fig. 1c, d), while treated fish presented an increase in the height of the interlamellar epithelium. Fish in the ExC treatment group also exhibited disorders of the secondary lamellae (Fig. 1b).

The liver is composed of sinusoid capillaries and hepatocytes in a coronal arrangement. Hepatocytes have a hexagonal arrangement, a clear cytoplasm (or acidophilic, depending on the state of the cell), glycogen stores, and a nucleus that is slightly displaced to the periphery. Fish from the InC and ExC treatment groups showed an increase in sinusoid capillary diameter and hepatocyte hypertrophy (Fig. 2a). Treated fish exhibited increased glycogen storage (Fig. 2b), and glycogen stores were reduced in the ExC group, which was the opposite of the trend observed for the capillaries, indicating an increase in glycogen metabolism.

The kidney is composed of proximal and distal tubules and glomeruli, which constitute Bowman's capsules, and of fenestrated capillaries. It is surrounded by melano-macrophage

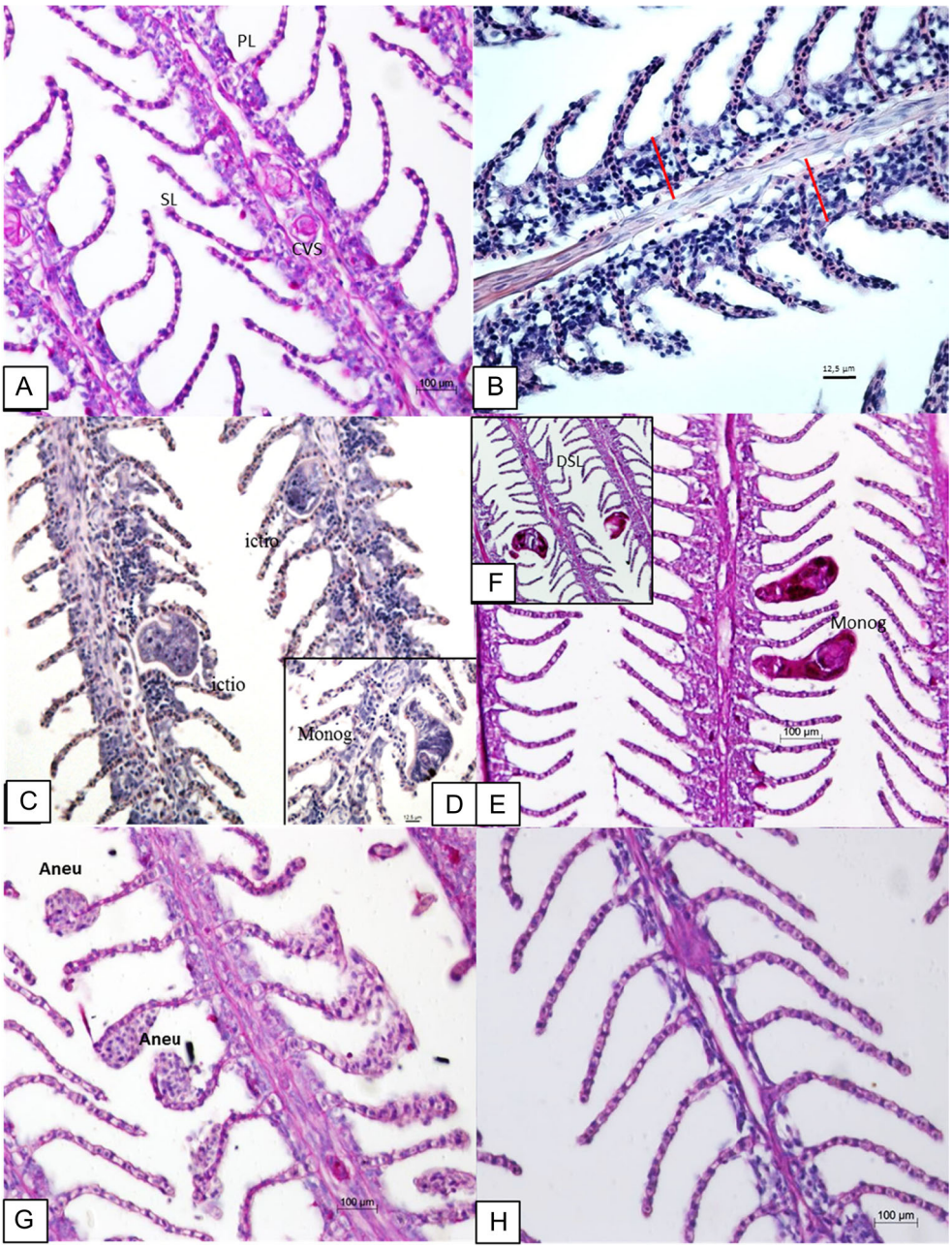

Fig. 1 Gills of *P. mesopotamicus*. **a** HC. *CVS* central venous sinus, *PL* primary lamellae, *SL* secondary lamellae. **b** Tr with TOL + ENRO. *Lines*: increase in interlamellar epithelium. **c**, **d** InC group treated with TOL + ENRO. *Monog* monogenetic, *Ictio I. multifiliis*. **e**, **f** InC group treated with THI + FFC. *DSL* disruption of secondary lamellae. **g** ExC group treated with TH + FFC. *Aneu* aneurism. **h** Tr with TH + FFC. All staining is with HE except in **e** (PAS staining)

centers and hematopoietic and lymphoid tissues. Fish from all treatment groups showed histological features that were similar to those of the control (Fig. 2d).

Hematology

The Ht, MCV, and MCHC values were not significantly different among the treatment groups. Fish from the ExC treatment group had significantly higher RBC counts than those of the treated and InC fish. Additionally, the InC group showed a 19.0 % reduction in Hb compared with that of the HC group (Table 1).

WBC and lymphocyte levels were significantly higher in treated fish. Eosinophil levels were lower in the ExC treatment group and higher in the HC group, while the other variables remained unchanged (Table 2).

After treatment with thiamethoxam and florfenicol

Histopathology

Fish in the InC treatment group exhibited aneurysms in their gills and increased thickness of the interlamellar epithelium (Fig. 1e–g). The ExC treatment group showed less intensive aneurysms and a disruption of the secondary lamellae

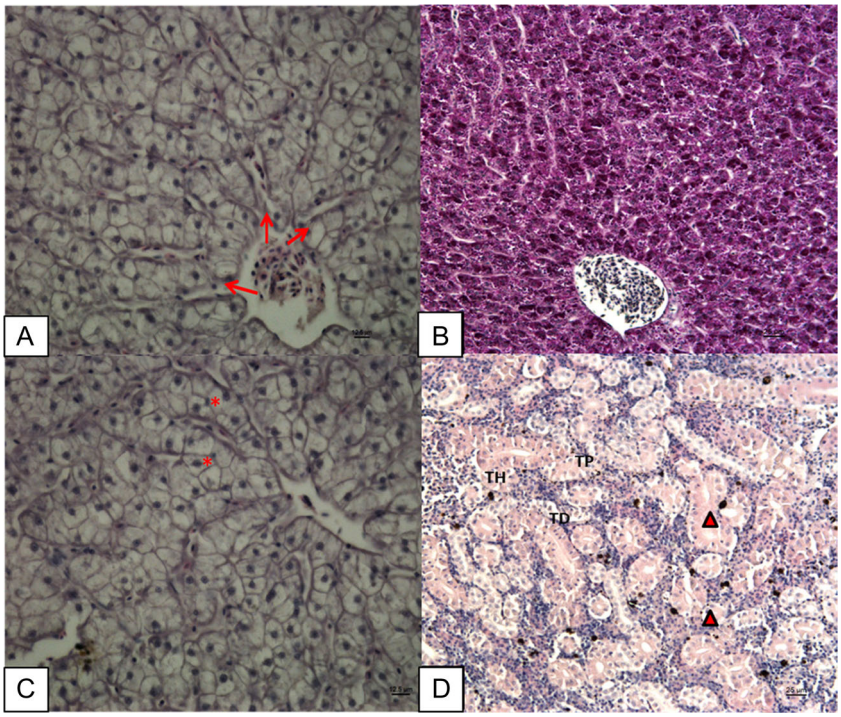

Fig. 2 **a** Liver samples from the InC group treated with TOL + ENRO. *Arrows* indicate increase in sinusoid capillary diameter. **b** Liver samples from Tr with TOL + ENRO. Hepatocytes with glycogen. **c** Liver samples from the group treated with TH+FFC. *Asterisk*: hypertrophy **d** Kidney samples from the group treated with TH+FFC. *TH*: tissue hematopoietic. TD: tubule distal. TP: tubule proximal: *Arrowhead*: melano-macrophages. Staining is with PAS in B and HE in A, C, and D

(Fig. 1g). In contrast, there were no changes in the treated fish (Fig. 1h).

The ExC, InC, and treated fish showed low glycogen levels, and glycogen stores were polarized in the hepatocytes: they were located in the region of the cells away from the capillaries. The treated fish also showed congestion in their sinusoid capillaries and hepatocyte hypertrophy, which indicates an increase in the organelles involved in metabolism (Fig. 2d).

There was no change in the histomorphology of the kidneys from all treatments.

Hematology

The treated fish showed a 19.0 % increase in Ht, a 35.0 % increase in Hb, and an 18 % decrease in RBC, all of which differed significantly from the values in the HC group, and the MCV and MCHC values were significantly higher in the treated fish. The increase in Ht, Hb, and MCV in the treated fish and ExC groups (Table 3) suggests an electrolytic imbalance.

The ExC fish showed a 30.0 % decrease in thrombocytes, and in the InC group, leukopenia, lymphocytopenia, eosinopenia, and neutrophilia were observed (Table 4).

Discussion

Histopathology

Gills are sensitive to changes in the water caused by xenobiotics because they have a large surface area and a short diffusion distance (Nero et al. 2006; Brunelli et al. 2011).

The pathogens *I. multifiliis*, *T. heterodentata*, *A. penilabiatus*, and bacteria can cause changes in the gills that can impact oxygen uptake, metabolism, and the intake

Table 1 Hematological variables of *Piaractus mesopotamicus* after treatment with toltrazuril and enrofloxacin (average ± standard error)

Treatments	Ht (%)	Hb (g dL^{-1})	RBC (10^6 mm^{-3})	MCV (fL)	MCHC (g dL^{-1})
InC	26.44 ± 0.66	7.60 ± 0.15B	1.64 ± 0.16B	172.11 ± 8.29	28.87 ± 0.78
Treated	27.11 ± 1.04	8.31 ± 0.25AB	1.56 ± 0.21B	166.05 ± 6.78	30.89 ± 1.05
HC	27.94 ± 1.05	9.27 ± 0.26A	1.83 ± 0.25AB	155.33 ± 10.11	33.56 ± 1.59
ExC	27.66 ± 0.74	9.01 ± 0.35A	1.92 ± 0.22A	145.13 ± 5.53	32.72 ± 1.45

Different letters indicate significant differences in the column according to Tukey's test ($P < 0.05$)

Ht hematocrit, *Hb* hemoglobin content, *RBC* red blood cells, *MCV* medium corpuscular volume,;*MCHC* medium corpuscular hemoglobin concentration, *InC* infected control, *ExC* exposed control, *HC* healthy control

Table 2 Absolute number of leukocytes in *Piaractus mesopotamicus* treated with toltrazuril and enrofloxacin (average ± standard error)

Cells (μL^{-1})	Infected control	Treated	Healthy control	Exposed control
Tromb	24,690.64 ± 2795.03	23,275.89 ± 3792.84	22,923.29 ± 1835.32	31,552.28 ± 3474.33
WBC	15,498.46 ± 1969.61B	25,031.90 ± 3921.9A	12,859.73 ± 1284.0B	10,247.5 ± 1558.5B
Mono	814.37 ± 134.90A	519.16 ± 124.95AB	464.59 ± 135.91AB	278.22 ± 82.51B
Neut	283.59 ± 123.13	218.80 ± 81.95	346.22 ± 108.10	172.21 ± 70.24
Eos	155.16 ± 65.13AB	134.36 ± 41.55AB	330.49 ± 118.78A	10.55 ± 5.31B
Lymph	13,924.39 ± 1818.46B	23,720.49 ± 3572.9A	11,541.87 ± 1282.1B	9723.78 ± 1472.2B
EGC	57.98 ± 26.40	124.16 ± 58.09	77.77 ± 51.94	0.00 ± 0.00
ImLeu	262.97 ± 92.53	338.70 ± 193.85	98.78 ± 45.94	62.78 ± 34.89

Different letters indicate significant differences in the line according to Tukey's test ($P < 0.05$)

WBC white blood cells (total leukocytes), *Mono* monocytes, *Neut* neutrophils, *Eos* eosinophils, *Lymph* lymphocytes, *SGC* special granulocytic cells, *ImLeu* immature leukocytes, *Tromb* thrombocytes

and excretion of various molecules. *I. multifiliis* has been shown to cause hyperplasia and hypertrophy of the secondary lamellae in *Oreochromis mossambicus* (Subasinsghe 1990), *Ictalurus punctatus* (Maki et al. 2001), and *Rhamdia quelen* (Carneiro et al. 2006), and *A. hydrophila* can cause these same conditions in pacu (Carraschi et al. 2012).

Hypertrophy and hyperplasia of the lamellae were observed in the treated fish (TH + FFC and EF + TOL) and have also been observed in *Cyprinus carpio* exposed to carbamazepine (40 at 80 mg L^{-1}) (Malarvizhi et al. 2012) and *Gambusia holbrooki* exposed to sublethal doses of tetracycline (5, 50, and 500 ng L^{-1}) (Nunes et al. 2015). These changes are reversible and act as an adaptive defense response because the decrease in gas exchange area also acts to prevent the intake of chemicals (Fernandes and Mazon 2003; Lupi et al. 2007).

Powell et al. (1995) suggested that the thickening of the interlamellar epithelium in trout (*Oncorhynchus mykiss*) exposed to chloramine-T occurs via ionic regulation due to decreases in Na^+, Cl^-, and Ca^{2+} in the bloodstream. These types of changes manifest as an innate defense mechanism that organisms use to survive in adverse conditions.

According to Mallatt (1985), the hyperplasia of mucous cells provokes the hypersecretion of mucous, which protects tissue structure under adverse environmental conditions and during exposure to toxic agents. Mucosubstances have polyanions that can act as a protective barrier against the absorption of xenobiotics or invasion by pathogens. These changes were observed in the InC and treated fish, and they protect the gill epithelium from exposure to pathogens and drugs.

The disruption of the secondary lamellae observed in the ExC and InC groups in both experiments has been previously shown to be caused by drug exposure or by pathogens, such as *A. hydrophila*, in *P. mesopotamicus* (Carraschi et al. 2012). The changes observed in the InC treatment (*T. heterodentata*, *I. multifiliis*, *A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) can cause hypoxia, respiratory failure, and ionic and acid base imbalances. Furthermore, after exposure, organisms can remain more susceptible to secondary infections and can die (Hawkins et al. 1984; Yasser and Naser 2011).

The aneurysms observed in the InC (*A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) and ExC (TH + FFC) fish occurred due to an increase of blood in the lamellae, which can cause the damage to the pillar cells and the loss of vascular integrity (Nunes et al. 2015). Hyperplasia, epithelial hemorrhaging, and increased mucous production can disturb the respiratory function of the gills, and these changes can be caused by monogeneans (Hayes and Ferguson 1989).

Aneurysms have also been observed by Nunes et al. (2015) in *G. holbrooki* exposed to tetracycline and in *Salmo trutta*

Table 3 Hematological variables of *Piaractus mesopotamicus* after treatment with thiamethoxam and florfenicol (average ± standard error)

Treatments	Ht (%)	Hb (g dL^{-1})	RBC (10^6 mm^{-3})	MCV (fL)	MCHC (g dL^{-1})
InC	27.94 ± 1.06B	9.27 ± 0.27D	1.83 ± 0.08AB	155.32 ± 10.12BC	33.56 ± 1.59C
Treated	33.22 ± 0.62A	15.91 ± 0.53A	1.56 ± 0.07B	216.80 ± 11.40A	47.82 ± 0.86A
HC	27.83 ± 0.71B	11.70 ± 0.60C	1.92 ± 0.08A	146.99 ± 7.85C	41.94 ± 1.54B
ExC	30.88 ± 0.89AB	13.77 ± 0.55B	1.64 ± 0.05B	189.97 ± 8.56AB	44.69 ± 1.63AB

Different letters indicate significant differences in the column according to Tukey's test ($P < 0.05$)

Ht hematocrit, *Hb* hemoglobin content, *RBC* red blood cells, *MCV* medium corpuscular volume, *MCHC* medium corpuscular hemoglobin concentration, *InC* infected control, *ExC* exposed control, *HC* healthy control

Table 4 Absolute number of leukocytes in *Piaractus mesopotamicus* treated with thiamethoxam and florfenicol (average ± standard error)

Cells (μL^{-1})	Infected control	Treated	Healthy control	Exposed control
Trombo	28,273.22 ± 2704.29A	25,067.26 ± 1536.21AB	28,156.93 ± 1304.94A	19,506.04 ± 1892.06B
WBC	6214.98 ± 983.82B	8049.44 ± 1106.8AB	13,138.433 ± 2642A	10,749.83 ± 1009.4AB
Mono	500.66 ± 114.42	493.73 ± 104.69	331.93 ± 65.72	636.23 ± 82.91
Neut	678.29 ± 193.11A	384.76 ± 8060AB	146.16 ± 34.67B	496.35 ± 96.75AB
Eos	132.26 ± 44.43B	202.48 ± 46.46AB	594.26 ± 189.81A	398.954 ± 79.42AB
Lymp	4859.37 ± 728.37B	6783.04 ± 943.5AB	11,760.77 ± 2418.9A	7961.35 ± 1358.82AB
EGC	0.00 ± 0.00B	80.26 ± 23.03AB	86.773 ± 31.33AB	108.67 ± 34.02A
ImLeu	83.00 ± 19.83	164.34 ± 71.21	139.28 ± 28.75	142.47 ± 30.71

Different letters indicate significant differences in the line according to Tukey's test ($P < 0.05$)

WBC white blood cells (total leukocytes), *Mono* monocytes, *Neut* neutrophils, *Eos* eosinophils, *Lymph* lymphocytes, *EGC* especial granulocytic cells, *ImLeu* immature leukocytes, *Tromb* thrombocytes

exposed to salicylic acid. However, this disruption of the secondary lamellae is reversible, and after water purification, the morphology and function of the tissues return to normal (Lupi et al. 2007).

The liver is the primary detoxifying organ in fish, and hepatic changes suggest a type of defense mechanism and that the substances used for chemical storage can be used for detoxification (Olsson et al. 1996; Nunes et al. 2015). Hypertrophy in the hepatocytes indicates an increase in the organelles responsible for metabolism, as verified in *S. trutta* exposed to salicylic acid (Nunes et al. 2015) and *P. mesopotamicus* infected with *A. hydrophila* and treated with 10 mg FFC kg^{-1} (Carraschi et al. 2012).

No critical or irreversible damage to the liver was found. This is consistent with the observations of Maki et al. (2001) in *I. punctatus* infested with *I. multifiliis*, which suggested that this parasite does not damage the kidney, liver, or spleen.

Low levels of glycogen were observed in the fish in the ExC treatment in both experiments that can be used as a histopathological indicator of environmental quality (Teh et al. 1997); the results observed in the treated (TH + FFC) and InC fish (*A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) may reflect the results of environmental stress caused by pathogens or xenobiotics.

The changes observed in the liver are reversible and nonspecific and suggest an initial effect of the drug treatment on metabolism. The histopathological changes in the treated fish did not compromise their development, and the use of drugs during the production cycle may successfully treat disease.

Hematology

Normal variations due to intrinsic or extrinsic factors or diseases affecting blood cell function and number may be evaluated via clinical hematology. Obtaining even a small blood sample may reveal information helpful in guiding treatment options (Grant 2015).

The anemia observed in the InC group (*T. heterodentata*, *I. multifiliis*, *A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) is a characteristic of the presence of the pathogens, primarily that of the monogenean *A. penilabiatus*, which enters the mouth and affixes to the gill epithelium, causing disruption of the vessels (Jerônimo et al. 2014). This change and the low RBC levels observed in the InC group compromise the transportation of oxygen via Hb to the tissues. Without treatment and under chronic exposure, fish will experience respiratory deficiencies that can cause death.

The reduction in RBC and Hb contents in the InC group (*T. heterodentata*, *I. multifiliis*, *A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) indicates that bacteria are using the iron from the hemoglobin of the erythrocytes. Bacteria cause erythrocyte hemolysis, which releases more hemoglobin and heme groups to the bloodstream (Wooldridge and Williams 1993). This ability is associated with a bacterial virulence factor and involves siderophores, which are iron-transport cofactors secreted by bacteria (Massad et al. 1991). A low iron concentration is the principal change that occurs when bacteria invade a host and produce virulence factors (Litwin and Calderwood 1993).

Anemia occurs due to the inhibition of erythropoiesis or an increase in the erythrocyte destruction rate in hematopoietic organs or in circulation. A decrease in RBC and Hb content in *C. carpio* following exposure to diazinon has also been reported by Svoboda et al. (2001).

TOL, a derivative of triazinetrione, and EF, a quinolone, cause lymphocytosis in *P. mesopotamicus*, similar to the effects of trichlorfon in *C. carpio* parasitized by *Argulus* (Ranzani-Paiva et al. 1987) and of endosulfan in *Clarias gariepinus* (Yekeen and Fawole 2011). Sublethal doses of cypermethrin (0.30 mg L^{-1}) and carbofuran (0.8 μg L^{-1}) also cause leukocytosis in *Labeo rohita* (Adhikari et al. 2004), but 1.0 mg L^{-1} diflubenzuron, a derivative of benzoylurea, has been shown to not change hematological parameters in

R. quelen infected with *Lernaea cyprinacea* (Mabilia and Souza 2006).

The effects of the drugs (ExC: TOL + EF) or the pathogens alone (InC: *T. heterodentata*, *I. multifiliis*, *A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) did not stimulate leukocyte formation, but the infected fish that were treated with TOL and EF exhibited leukocytosis and lymphocytosis. The main function of leukocytes is in the defense against bacteria or foreign bodies entering the tissues (Ranzani-Paiva and Silva-Souza 2004). Jerônimo et al. (2014) observed leukocytosis and lymphocytosis in *P. mesopotamicus* with >2200 *A. penilabiatus*. Thus, the organism's defenses are stimulated when the exposure of the fish to drugs and pathogens decreases.

The larger number of eosinophils found in the HC group from both experiments is an inherent feature of *P. mesopotamicus* because they are resident cells (Ranzani-Paiva et al. 1999). Eosinopenia occurred in the ExC group (TOL + EF), which showed a 96.0 % decrease in cells, suggesting a disturbance caused by the drugs and in the InC treatment group (*A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.), which showed a 77.8 % decrease caused by the pathogens. Eosinopenia is a decrease in the production and release of eosinophils from the bone marrow (Savari et al. 2011), such as that caused by cortisol in *C. carpio* (Wojtaszek et al. 2002).

Hematocrit percentages reflect the proportion of RBCs in the blood relative to the white blood cells (WBCs) and plasma. Because RBCs are responsible for the transport of oxygen and carbon dioxide (Ranzani-Paiva et al. 2013), the greater Ht and lower RBC levels in the treated fish (TH + FFC) suggest that some stressor and/or respiratory dysfunction was present that suppressed RBCs. This has also been observed by Hashimoto et al. (2016) in Nile tilapia infected with a monogenean and treated with an essential oil from *Lippia sidoides*.

The increases in MCV and MCHC observed in the treated fish (TH + FFC) have also been found in *L. rohita* exposed to cypermethrin (Adhikari et al. 2004) and in *C. carpio* exposed to dichlorvos (Svobodova 1975) and diazinon (Svobodova et al. 2001).

The increase in MCHC and the decrease in RBCs in the treated fish (TH + FFC) indicate hemolysis, according to Thrall et al. (2006). Cypermethrin causes hemolysis in *L. rohita* (Adhikari et al. 2004), which is also caused by diazinon in *C. carpio* (Banaee et al. 2008) and by potassium permanganate in *Oreochromis niloticus* (Salawu et al. 2013), and *C. carpio* fed with praziquantel have been shown to have decreased RBC, Hb, and MCV values (Sudová et al. 2008). Increases in Hb and MCV are related to a higher capacity of the blood to transport oxygen to meet energy demands (Labarrére et al. 2012).

Reduced thrombocyte counts indicate damage to the coagulation capacity and defense mechanisms of an organism (Martins et al. 2008). It can be inferred that the low thrombocyte counts found in the fish exposed at TH and FFC (ExC) may have been associated with the migration of these cells, as verified by Hashimoto et al. (2016) in Nile tilapia infected with a monogenean and treated with an essential oil from *L. sidoides*.

According to Tavares-Dias et al. (2001), lymphocytopenia is an indicator of stress. Lymphocytes are white blood cells that remain in circulation for a long time, and lymphocytopenia most often occurs when there is stress or in response to steroids (Gad 2007).

Neutrophils are granulocytes that are responsible for organismal defense. They phagocytose small objects (e.g., bacteria) and participate in inflammatory processes (Savari et al. 2011). The presence of neutrophilia observed in the InC group (*A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) has also been shown to be caused in situations involving infection (Martins et al. 2008) or stress management (Jerônimo et al. 2011). Hashimoto et al. (2016) also reported the presence of neutrophilia in Nile tilapia infected with a monogenean and treated with an essential oil from *L. sidoides*.

The immunosuppression observed in the InC (*A. penilabiatus*, *Aeromonas* sp., and *Streptococcus* sp.) group caused by pathogens has also been verified by Achuthan Nair and Balakrishnan Nair (1983) in *Channa striatus* infested with *Alitropus typus*, by Höglund et al. (1992) in *Anguilla anguilla* infested with *Anguillicola crassus*, and by Tavares Dias et al. (2002) in *O. niloticus* with ichthyophthiriasis.

TOL, EF, FFC, and TH caused reversible histopathological changes; thus, these drugs have the potential for use in Brazilian aquaculture. However, the changes observed in the InC fish can lead to death, so more detailed studies of dosage adjustments and residue quantification must be performed to inform the regulatory process. The use of drugs at standardized concentrations, exposure times, and number of treatment days will improve the success of the production cycle and decrease the losses caused by pathogens.

TOL, TH, FFC, and EF caused reversible and minor hematological and histopathological changes, while the pathogens caused more serious hematological and histopathological changes that can compromise *P. mesopotamicus* survival. The drug-related data presented here can be used in viability studies as part of the registration process for their use in commercial aquaculture.

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Ethical approval All procedures involving animals were in accordance with the ethical standards of the institution at which the studies were conducted and were approved by the University's Institutional Animal Care and Use Committee under approval number 017335/10.

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

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