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Hematological Parameters of Mullet (*Mugil liza*) after Hydrogen Peroxide Immersion Bath

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HIGHLIGHTS

- Mullet (*M.* liza) juveniles were subjected to immersion baths with hydrogen peroxide;
- Hematological parameters were analyzed immediately and 30 days after bath;
- Exposure to H_2O_2 caused reduced MCV, lymphopenia, neutrophilia and monocytosis;
- The effects of H_2O_2 were reversible after 30 days of recovery in clean water.

Abstract: The hematological parameters of fish contribute to the understanding of the health status in response to diseases, treatments, handling, and environmental factors. This study aimed to verify the hematological parameters of mullet *Mugil liza* after one-hour immersion baths in five concentrations of hydrogen peroxide (H_2O_2), an effective chemical treatment for several diseases in fish. A total of 108 fish (35.25 ± 6.09g) was distributed into six treatments with three replicates each: untreated fish (control), and fish treated with five concentrations of H_2O_2 (150, 200, 250, 300 and 350 mg L^{-1}). Blood collection was performed immediately after bathing and 30 days after. Erythrogram, leukogram, thrombocytogram, hematocrit, plasma glucose, hemoglobin concentration and hematimetric indexes were analyzed. There was no significant difference in the erythrocyte and total leukocyte counts. Regarding plasma glucose and hematimetric indexes, the values were increased ($p<0.05$), except for mean corpuscular volume (MCV), that

was decreased ($p<0.05$) after bath, including control. These responses may be associated to stress from handling and bathing procedure. In fish treated with H_2O_2, the number of thrombocytes and lymphocytes was lower ($p<0.05$) immediately after bath, while neutrophils and monocytes showed increased ($p<0.05$) values immediately after bath, which indicates adverse effects due to H_2O_2; however, these effects seem to be reversible after 30 days of recovery. The findings indicate that the exposure of *M. liza* juveniles to baths of 1 hour with up to 350 mg L^{-1} is safe, without significant risks in causing adverse physiological effects.

Keywords: Marine fish farming; lebranche mullet; Mugilidae; treatment; hematology; H_2O_2.

INTRODUCTION

Fish from family Mugilidae, commonly known as "mullets", are among the most widely produced fish species in coastal and marine aquaculture worldwide, with estimated production volume of 291.5 thousand tonnes (estimated revenue of about U$ 1 billion) in 2020, second only to the Atlantic salmon (*Salmo salar*) and milkfish (*Chanos chanos*) [1]. *Mugil liza* is a native species found along the Brazilian coast, known for its potential in aquaculture due to its demonstrated characteristics of hardiness, early growth, euryhalinity, eurythermicity, easy reproduction, and adaptability to feed management, readily accepting inert diets [2].

The expansion of fish production has been primarily facilitated by the intensification of farming systems, which is accompanied by problematic situations related to diseases, directly affecting the productivity and growth of aquaculture sector. Among the main causes of losses are ectoparasites. These pathogens occur naturally in fish and the environment, but their ability to cause disease is intensified under certain conditions inherent to the farming environment, which promote the proliferation and transmission of pathogens while diminishing the host's defenses [3]. To manage these situations, in addition to good management practices, there sometimes the use of therapeutic interventions with chemical products is necessary. Chemical treatments by bath are routinely prescribed for this purpose [4].

Among the numerous chemotherapeutics used for treating diseases in farmed fish, hydrogen peroxide (H_2O_2) is well known for its efficacy. It is a natural metabolite produced by oxidative metabolism in many organisms, and its decomposition results in molecular oxygen and water. Its action against pathogens is based on the formation of hydroxyl radicals, potent and highly reactive oxidants that degrade the lipids of the membrane, DNA, and other cellular components of pathogens [5-6]. H_2O_2 is an accessible and readily available product, among the most widely used substances as therapeutics in aquaculture, showing efficacy against various diseases in different fish species at distinct life stages. Previous studies report effective action against infections and infestations caused by various pathogens in fish, including bacteria, protozoa, fungi, and metazoans [6-8]. Among the pathogens already recorded for mullet *Mugil liza*, ectoparasites from the class Monogenea (Platyhelminthes) predominate, infesting both the gills and the body surface [9-13], and these infections could be controlled using chemotherapeutics, such as H_2O_2. However, there are still no records in the literature of studies verifying the effects of antiparasitic treatments for this species.

Several studies report the effectiveness of hydrogen peroxide for controlling monogenean parasites in other fish species, with doses and times of action varying among different species. Monogeneans from the species *Ligictaluridus floridanus* found in the gills of American catfish (*Ictalurus punctatus*) were effectively controlled with three H_2O_2 baths, every other day, at 570 mg.L^{-1} for 4 minutes [14]. In rainbow trout (*Oncorhynchus mykiss*), baths with H_2O_2 provided significant reduction in the number of monogeneans from the genus *Gyrodactylus* found on the skin. When two baths were applied every other day at 50 mg/L for 30 minutes, there was a 99% reduction in the mean abundance of parasites [15], and when applied three baths on alternate days between (150 to 560 mg.L^{-1}) for 30 minutes, the number of parasites was reduced to zero [16]. Also for fish parasitized by *Gyrodactylus* sp., a reduced mean abundance of parasites was observed after baths with H_2O_2 in brook trout *Salvelinus fontinalis* (150 mg.L^{-1} for 15 minutes), in lake trout *S. namaycush* (100 mg.L^{-1} for 30 minutes), in yellow perch *Perca flavescens* (75 mg.L^{-1} for 60 minutes) and in fathead minnow *Pimephales promelas* (75 mg.L^{-1} for 60 minutes) [17]. However, depending on the species and dose applied, treatment with H_2O_2 may not be effective. In wolf-eel (*Anarrhicthys ocellatus*) parasitized by *Gyrodactylus corti*, there was no significant reduction in the number of parasites after baths with 200mg.L^{-1} for 30 minutes, repeated 3 times on alternate days [18]. These records show that the effective dose for treatment can vary among host and parasite species.

Alongside efficacy tests against pathogens, *in vivo* drug application requires complementary assessments to check the risk of adverse effects and ensure the safety of the treatment protocol to be used. To achieve this, morphological, biochemical, and physiological parameters of the treated animals are evaluated [6,19]. Hematological analysis is a highly useful tool for this purpose as it offers a comprehensive

overview of the fish's health status. Changes in blood parameters can reveal effects related to the toxicity of the chemotherapeutics and/or the stress induced by the treatment [19].

The treatments using hydrogen peroxide baths, despite being widely and successfully, can also lead to adverse effects, which have been reported for various fish species [4,20]. However, there is evidence that sensitivity of fish to H_2O_2 could be species-specific [16] and there are no records of its effects on fish from family Mugilidae (mullets), which hold significant importance in aquaculture and fisheries across various regions worldwide. Therefore, the present study aimed to verify the immediate and prolonged effects of 1-hour immersion baths with hydrogen peroxide at different concentrations on the hematological parameters of juvenile mullets *Mugil liza*.

MATERIAL AND METHODS

Fish and experimental conditions

The juvenile mullets (*Mugil liza*) were raised and maintained at the Laboratory of Marine Fish Farming (LAPMAR) of the Federal University of Santa Catarina (UFSC). The fish were kept in open-air culture in 8000 L circular tanks, with continuous flow and water renewal of 350% per day (19 to 20 L min^{-1}), constant aeration and salinity of 35‰, at natural temperature (25.78 ± 0.65 ºC) and photoperiod, with continuous water supply by pumping seawater from Moçambique beach, Florianópolis, SC, Brazil (27° 34' 02" S, 48° 25' 43" W). All animal procedures were approved by the Ethics Committee on Animal Use (CEUA/UFSC PP00862). For control purposes, pH and dissolved oxygen levels were measured using a multiparameter probe during the experiment in all treatments. Dissolved oxygen levels presented the following values (mean ± standard deviation): 3.43 ± 1.10 mg L^{-1} in the control, 9.10 ± 0.10 mg L^{-1} in the bath with H_2O_2 at 150 mg L^{-1} , 10.50 ± 0.36 mg L^{-1} with H_2O_2 at 200 mg L^{-1}, 9.87 ± 0.49 mg L^{-1} with H_2O_2 at 250 mg L^{-1} , 11.60 ± 0.53 mg L^{-1} with H_2O_2 at 300 mg L^{-1} and 12.53 ± 0.38 mg L^{-1} with H_2O_2 at 350 mg L^{-1}. The values of pH were: 8.00 ± 0.00 in the control, 8.83 ± 0.29 in the bath with H_2O_2 at 150 mg L^{-1} , 8.67 ± 0.29 with H_2O_2 at 200 mg L^{-1}, 8.33 ± 0.29 with H_2O_2 at 250 mg L^{-1} , 8.50 ± 0.00 with H_2O_2 at 300 mg L^{-1} and 8.17 ± 0.29 with H_2O_2 at 350 mg L^{-1}.

Experimental design

Initially, fish biometry was performed for subsequent distribution in 500 L with 350% daily renewal, constant aeration, and feeding three times a day until apparent satiation with commercial Nutripiscis Starter® feed with 45% protein and size of 1.3 mm, at a constant salinity of 35‰. Fish feeding was ceased 24 hours prior to the start of the immersion baths for all fish. The feeding was also ceased 24 hours prior to the second blood sampling, 30 days after baths. The treated fish were exposed to five concentrations of 35% hydrogen peroxide (H_2O_2): 150, 200, 250, 300 and 350 mg L^{-1} H_2O_2 and there was a control with non-treated fish (culture tank water without hydrogen peroxide). A total of 108 *M. liza* juveniles with a mean weight of 35.25 ± 6.09 g were used, divided into 6 fish per tank in triplicate with continuous aeration. The immersion bath was administered for 1 hour, consisting of fish capture using a dip net and transfer to buckets, where H_2O_2 was added at the tested concentrations mentioned above. The fish in the control group underwent the same procedures, but without the addition of hydrogen peroxide to the bucket. After the bathing period and blood sampling for hematological analysis, the fish were returned to the 500-liter tanks, where they continued to be fed twice a day with the same feed as the initial period until apparent satiety.

Hematological analysis

Immediately after baths and 30 days later, nine fish per treatment were anesthetized with 50 mg L^{-1} benzocaine [21] and blood was collected by puncture of the caudal vessel using 3 mL syringes containing ethylenediaminetetraacetic acid (EDTA 10%) anticoagulant [19]. In the first collection, for all treated fish feeding was ceased 24 hours prior to the start of the immersion baths and in the 30th day the feeding was ceased 24h prior to the blood collection for all fish. The collected blood was destined to hematocrit [Ht] percentage analysis by the microhematocrit method [22], determination of hemoglobin concentration [Hb] by the cyanometahemoglobin technique [23] using a commercial kit (Labtest Diagnóstica® MG, Brazil), erythrocyte count [RBC] in Neubauer chamber after dilution 1: 200 in Dacie's solution [24], and hematimetric indexes (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH] and mean corpuscular hemoglobin concentration [MCHC]) according to [25]. After blood centrifugation for Ht, plasma was used for glucose determination with an Accu-Chek® Advantage portable glucometer kit (Roche Diagnóstica, Brazil). Blood smears were made for total leukocyte and thrombocyte counts, as well as for differential leukocyte counts, and stained with May-Grünwald-Giemsa-Wright (MGGW) [19].

Statistical analysis

The data were submitted to statistical analysis using the Statistica® 10 software (StatSoft, USA) with a 5% significance level. Initially, the Shapiro-Wilk test was performed to verify the normality of the data and the Levene test to assess homoscedasticity. The data that did not present homogeneity were submitted to standardization by means of logarithmic transformation. After the data were submitted to two-way ANOVA (time and concentration), if significant differences were found between at least one of the factors, the means were compared using Tukey's test.

RESULTS

Throughout the experimental period, no mortality was recorded. The hematological parameters related to erythrocytes (RBC, Ht, Hb and hematimetric indexes) and plasma glucose are shown in Table 1. Regarding erythrocyte counts [RBC], for all treatments, there was no significant difference between the sampling times (p>0.05) and no significant difference among treatments at the same sampling time (p>0.05;). The hematocrit percentage [Ht] showed no significant difference (p>0.05) among treatments at the same time. However, for all treatments, there was significant difference (p<0.05) between samplings times, with higher values at 30 days, except for H_2O_2 at 300 mg L^{-1}. Regarding hemoglobin concentration [Hb], there was significant difference between sampling times (lower values at 30 days) and among treatments at the same time, with significant interaction between concentration of H_2O_2 and sampling time(p<0.05). This interaction was observed at the concentration of 250 mg L^{-1} H_2O_2 between 1 hour of exposure and 30 days.

Regarding plasma glucose levels, there was significant difference among treatments at the same sampling time, both immediately and 30 days after bath. After bath, none of the treated groups differed significantly from the control. However, plasma glucose was significantly higher in fish treated with the lower dose (150 mg L-1) of H_2O_2, compared to those treated with 250 and 300 mg L^{-1}. At 30 days after baths, the same effect was observed (fish treated with 150 mg L-1 H_2O_2 presented higher levels of plasma glucose when compared to 250 and 300 mg L^{-1}, but all treatments were similar to control). In all treatments, except for 300 mg L-1, plasma glucose levels were significantly higher (p<0,05) immediately after bath, compared to 30 days. Fish treated with H_2O_2 at 300 mg L^{-1} showed no significant difference in plasma glucose between sampling times (p>0.05).

The mean corpuscular volume [MCV] of erythrocytes was significantly influenced by time, with higher values at 30 days (p<0.05), except for H_2O_2 at 300 mg L^{-1}. However, at the same sampling time, there was no significant difference in MCV among treatments (p>0.05). The values of mean corpuscular hemoglobin [MCH] and mean corpuscular hemoglobin concentration (MCHC] were significantly reduced after 30 days in all treatments (p<0.05), compared to 1h after baths. However, for both parameters, there was no significant difference (p>0.05) among treatments at the same time. Regarding MCH, there was significant interaction (p<0.05) between the factors “sampling time” and “treatment”, when comparing control to 200, 250, 300 and 300 mg L^{-1}.

The values regarding total leukocytes or white blood cells [WBC], thrombocytes and differential leukocytes count are shown in Table 2. In al treatments, the number of WBC was similar (p>0.05) between sampling times and among the treatments. The counting of thrombocytes was significantly lower (p<0.05) immediately after bath in fish treated with H_2O_2 at 150 and 350 mg L^{-1}, when compared to 30 days after bath. In the other treatments, there was no significant difference (p>0.05) in thrombocytes count between sampling times. Regarding differences among treatments in the same sampling times, at 30 days the number of thrombocytes in fish treated with H_2O_2 at 150 mg L^{-1} was significantly higher (p<0.05) compared to those treated with 200 and 300 mg L^{-1}; however, none of the treatments differed from the control. Immediately after bath, no significant difference among the treatments was observed (p>0.05).

Regarding differential counting of leukocytes, the number of lymphocytes immediately after bath was significantly lower (p<0.05) in fish treated with H_2O_2 at the three higher doses (250, 300 and 350 mg L^{-1}), when compared to control. At 30 days, this effect persisted in the fish treated with the higher dose (350 mg L^{-1}), with these values being lower than control (p<0.05) and lower than those found in fish treated with 250 mg L^{-1}. Comparing the sampling times, in the fish treated with H_2O_2 at the four higher doses (200, 250, 300 and 350 mg L^{-1}) the number of lymphocytes was significantly lower (p<0.05) immediately after bath, when compared to 30 days after. This effect did not occur in control and 150 mg L^{-1}. The number of neutrophils and monocytes was not significantly influenced by treatment, neither immediately after the bath nor 30 days later (p>0.05), but significantly higher values (p<0.05) were observed in fish treated with 150, 200, 300 and 350 mg L^{-1} immediately after bath, compared to 30 days after.

Table 1. Hematological parameters related to red blood cells and plasma glucose levels in juvenile mullet (*Mugil liza*) immediately (Imme) and 30 days (30 d) after immersion bath at different concentrations of hydrogen peroxide (H_2O_2). Legend: Control, without exposure to H_2O_2 (C); Fish treated with 150 mg L^{-1} of H_2O_2 (H_2O_2 150); Fish treated with 200 mg L^{-1} of H_2O_2 (H_2O_2 200); Fish treated with 250 mg L^{-1} of H_2O_2 (H_2O_2 250); Fish treated with 300 mg L^{-1} of H_2O_2 (H_2O_2 300); and Fish treated with 350 mg L^{-1} of H_2O_2 (H_2O_2 350). Data presented as means ± standard deviation.

Parameter	C	H_2O_2 150		H_2O_2 200		H_2O_2 250		H_2O_2 300		H_2O_2 350		p-value			
	Imme.	30 days	Imme.	30 days	Imme.	30 days	Imme.	30 days	Imme.	30 days	Imme.	30 days	Treat.	Time	Inter.
RBC ($\times 10^6 \mu L^{-1}$)	3.08 ± 0.28	3.16 ± 0.24	3.19 ± 0.58	3.03 ± 0.17	3.08 ± 0.17	3.07 ± 0.18	3.11 ± 0.24	3.15 ± 0.31	2.99 ± 0.23	3.11 ± 0.27	3.17 ± 0.26	3.34 ± 0.23	0.450	0.488	0.662
Ht (%)	24.00 ± 5.63 ^{A}	30.63 ± 2.97 ^{B}	23.89 ± 3.18 ^{A}	30.88 ± 2.75 ^{B}	26.00 ± 4.44 ^{A}	31.00 ± 4.14 ^{B}	25.22 ± 2.68 ^{A}	32.86 ± 3.34 ^{B}	25.00 ± 3.87 ^{A}	28.38 ± 3.81 ^{A}	26.22 ± 3.07 ^{A}	30.25 ± 2.49 ^{B}	0.418	0.000*	0.451
Hb (g dL^{-1})	12.79 ± 1.44 ^{Aax}	9.05 ± 0.70 ^{Bbxy}	14.29 ± 1.56 ^{Aax}	9.11 ± 0.47 ^{Bbxy}	16.35 ± 1.56 ^{Aax}	9.38 ± 0.56 ^{Bbxy}	17.71 ± 2.21 ^{Aax}	8.75 ± 0.65 ^{Bby}	17.39 ± 2.21 ^{Aax}	9.92 ± 1.12 ^{Bbxy}	16.26 ± 1.03 ^{Aax}	9.40 ± 0.90 ^{Bbxy}	0.000*	0.000*	0.000*
Glu (mg dL^{-1})	129.11 ± 41.49 ^{Aab}	88.00 ± 19.40 ^{Bab}	166.67 ± 47.41 ^{Aa}	97.13 ± 27.24 ^{Ba}	146.67 ± 29.05 ^{Aab}	57.13 ± 25.32 ^{Bab}	118.78 ± 48.23 ^{Ab}	74.00 ± 14.20 ^{Bb}	104.33 ± 34.51 ;^{Ab}	77.88 ± 31.06 ^{Bb}	128.78 ± 37.04 ^{Aab}	80.75 ± 24.42 ^{Bab}	0.011*	0.000*	0.053
MCV (fL)	81. 65± 14.26 ^{A}	95.99 ± 7.50 ^{A}	75.55 ± 6.77 ^{A}	99.57 ± 7.99 ^{B}	85.18 ± 12.03 ^{A}	103.96 ± 10.14 ^{B}	81.32 ± 7.51 ^{A}	104.21 ± 4.74 ^{B}	83.32 ± 8.90 ^{A}	91.15 ± 8.44 ^{A}	83.00 ± 8.31 ^{A}	90.80 ± 5.90 ^{B}	0.082	0.000*	0.458
MCH (pg)	0.44 ± 0.06 ^{Ax}	0.28 ± 0.03 ^{Bxy}	0.48 ± 0.05 ^{Axy}	0.29 ± 0.02 ^{Bxy}	0.53 ± 0.05 ^{Ay}	0.29 ± 0.03 ^{Bxy}	0.57 ± 0.07 ^{Ay}	0.28 ± 0.03 ^{Bxy}	0.58 ± 0.09 ^{Ay}	0.31 ± 0.05 ^{Bxy}	0.53 ± 0.06 ^{Ay}	0.28 ± 0.02 ^{Bxy}	0.087	0.000*	0.003*
MCHC (g dL^{-1})	55.12 ± 9.97 ^{A}	29.38±2.99 ^{B}	60.79 ± 10.21 ^{A}	29.65 ± 2.44 ^{B}	63.35 ± 9.82 ^{A}	28.39 ± 2.93 ^{B}	71.08 ± 12.93 ^{A}	26.86 ± 3.41 ^{B}	70.95 ± 13.13 ^{A}	32.46 ± 6.95 ^{B}	62.86 ± 8.86 ^{A}	31.07 ± 1.37 ^{B}	0.141	0.000*	0.785

RBC: Erythrocytes; Ht: Hematocrit; Hb: Hemoglobin; Glu: plasma glucose; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

Uppercase letters (A, B) represent significant differences between sampling times within the same treatment, according to the Tukey test ($p < 0.05$). Lowercase letters (a - c) represent significant differences among treatments within the same sampling period by the Tukey test ($p < 0.05$). Lowercase letters (x-z) represent significant interaction between factors (time after immersion bath and H_2O_2 concentrations) by the Tukey test ($p < 0.05$).

Table 2. Hematological parameters related to leukocytes and thrombocytes of juvenile mullet (*Mugil liza*) immediately (Imme) and 30 days after immersion bath at different concentrations of hydrogen peroxide (H_2O_2). Legend: Control, without exposure to H_2O_2 (C); Fish subjected to 150 mg L^{-1} of H_2O_2 (H_2O_2 150); Fish treated with 200 mg L^{-1} of H_2O_2 (H_2O_2 200); Fish treated with 250 mg L^{-1} of H_2O_2 (H_2O_2 250); Fish treated with 300 mg L^{-1} of H_2O_2 (H_2O_2 300); and fish treated with 350 mg L^{-1} of H_2O_2 (H_2O_2 350). Data presented as means ± standard deviation.

Parameter	C		H_2O_2 150		H_2O_2 200		H_2O_2 250		H_2O_2 300		H_2O_2 350		p-value		
	Imme	30 days	Imme	30 days	Imme	30 days	Imme	30 days	Imme	30 days	Imme	30 days	Treat	Time	Inter.
WBC ($x\ 10^3 \mu L^{-1}$)	307.57 ± 28.05	315.88 ± 24.31	318.88 ± 58.51	302.85 ± 16.68	308.33 ± 18.80	307.00 ± 16.60	310.55 ± 23.48	315.42 ± 30.60	299.11 ± 23.45	311.00 ± 26.65	316.55 ± 25.65	333.50 ± 22.93	0.450	0.488	0.662
Thr ($x\ 10^3 \mu L^{-1}$)	7.41 ± 3.24 ^{Aa}	26.38 ± 24.23 ^{Aab}	4.81 ± 3.76 ^{Aa}	42.84 ± 20.72 ^{Ba}	9.20 ± 4.05 ^{Aa}	14.21 ± 12.08 ^{Ab}	8.20 ± 7.21 ^{Aa}	26.53 ± 15.21 ^{Aab}	6.57 ± 5.73 ^{Aa}	15.28 ± 15.00 ^{Ab}	6.79 ± 2.99 ^{Aa}	30.98 ± 13.94 ^{Bb}	0.023*	0.000*	0.266
Lym ($x\ 10^3 \mu L^{-1}$)	259.19 ± 34.56 ^{Aa}	267.38 ± 48.98 ^{Aa}	206.71 ± 36.72 ^{Aab}	290.41 ± 65.45 ^{Aab}	183.48 ± 38.53 ^{Aabc}	286.84 ± 26.65 ^{Bab}	153.88 ± 71.27 ^{Abc}	259.04 ± 48.78 ^{Ba}	138.59 ± 68.97 ^{Ac}	296.35 ± 27.34 ^{Bab}	149.56 ± 73.39 ^{Abc}	330.27 ± 20.37 ^{Bb}	0.019*	0.000*	0.258
Mon ($x\ 10^3 \mu L^{-1}$)	5.20 ± 5.23 ^{A}	9.57 ± 9.30 ^{A}	14.25 ± 8.58 ^{A}	3.79 ± 6.91 ^{B}	11.44 ± 5.89 ^{A}	0.64 ± 0.81 ^{B}	10.78 ± 6.46 ^{A}	4.20 ± 1.59^{A}	14.88 ± 9.24 ^{A}	1.15 ± 1.05 ^{B}	12.62 ± 7.86 ^{A}	0.84 ± 1.29 ^{B}	0.797	0.000*	0.068
Neu ($x\ 10^3 \mu L^{-1}$)	38.56 ± 27.64 ^{A}	41.92 ± 28.08 ^{A}	81.64 ± 24.13 ^{A}	16.91 ± 52.26 ^{B}	113.40 ± 33.74 ^{A}	9.47 ± 8.52 ^{B}	128.10 ± 60.69 ^{A}	50.57 ± 28.23 ^{A}	145.63 ± 64.34 ^{A}	13.49 ± 4.12 ^{B}	118.73 ± 83.23 ^{A}	6.88 ± 3.34 ^{B}	0.138	0.000*	0.124

Uppercase letters (A, B) represent significant differences between sampling times within the same treatment, according to the Tukey test ($p < 0.05$). Lowercase letters (a - c) represent significant differences among treatments within the same sampling period by the Tukey test ($p < 0.05$). Lowercase letters (x-z) represent significant interaction between factors (time after immersion bath and H_2O_2 concentrations) by the Tukey test ($p < 0.05$).

DISCUSSION

Hematological analysis plays a crucial role in assessing potential adverse physiological effects of a treatment in fish. By examining blood parameters such as red blood cell count, hemoglobin, hematocrit, and counting of leukocytes, it is possible to detect alterations that may indicate stress, toxicity, or immune responses to chemical substances [19]. These changes can provide valuable insights into the health and well-being of the animals, enabling early detection of risks and the implementation of preventive measures, thus contributing to ensure more effective and safer management practices. In the present study, no mortality was recorded. In a trial conducted with nine different fish species, the bath with H_2O_2 at 100 mg L^{-1} for one hour resulted in mortalities, primarily concentrated within the first 30 hours after the bath [20]. Regarding this issue, the juvenile mullets showed considerable resistance to the applied treatments, with higher concentrations of H_2O_2 and same bath duration.

For species from the genus *Mugil*, despite their significant commercial relevance, information regarding the behavior of their hematological parameters in response to therapeutic chemicals is still scarce. In the present study, regarding erythrocyte counts [RBC], it was observed that the treatments with hydrogen peroxide (H_2O_2) did not produce a significant alteration in any of the sampling periods. An increase in the number of circulating erythrocytes after bath could be interpreted as an indicator of acute stress response due to spleen contraction for releasing these cells in circulation [26]. However, the opposite can also occur, as seen in African catfish (*Clarias gariepinus*) juveniles subjected to baths with H_2O_2 at 150 mg L^{-1}, in which a reduction in the number of erythrocytes was observed, and was attributed to a possible impairment in the erythropoietic process [27]. Reduction in RBC may also be caused by oxidative damage and subsequent hemolysis in erythrocytes, a known effect from H_2O_2 exposure [28]. In the present study, RBC did not undergo any of these effects, suggesting increased resistance of juvenile mullet (*M. liza*) to the effects of peroxide and to the bath stress on this parameter compared to other species.

Similarly to the number of circulating erythrocytes, hematocrit is also an important indicator of stress, often showing increased values after the stress stimulus, as catecholamines induce an increase in the volume of erythrocytes [29]. In this study, in both sampling times, RBC in fish treated with H_2O_2 did not differ from control. Corroborating this result, in tambaqui (*Colossoma macropomum*) the treatment with H_2O_2 up to 126 mg L^{-1} for 30 minutes, produced no significant changes in RBC counts [7]. In the present study, all groups showed decreased hematocrit immediately after baths, when compared to 30 days. This decrease after bath was also present in the control; therefore, it cannot be attributed to H_2O_2. Although all fish underwent handling relative to bath procedure, it is unlikely that this effect occurred due to stress, since the stress response typically produces the opposite effect, increasing hematocrit values [29]. It is possible that the higher values after 30 days occurred in response to conditions inherent to experimental environment or to the growth of juveniles over the experimental period, as it is known that the growth process can naturally be accompanied by an increase in certain hematological parameters. Intrinsic factors, like size and weight, could potentially be the reason for changes in quantitative and morphological aspects of blood elements in fish [19].

In this study, hemoglobin concentrations were significantly higher one hour after the bath compared to the 30 days. The mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values behaved similarly, with higher values immediately after the bath and lower values after 30 days and no significant differences among treatments. However, these effects were also observed in the control group, indicating no causal relationship with H_2O_2 exposure. Reinforcing this hypothesis, there were no changes in hemoglobin concentration in tambaqui (*C. macropomum*) treated with H_2O_2 up to 126 mg L^{-1} for 30 minutes [7]. However, these responses may be dose-dependent, showing a specific dose-response relationship for each species. For instance, in Atlantic salmon exposed to H_2O_2 at 300 mg L^{-1} for 20 minutes, an increase in hemoglobin and hematocrit was observed, whereas the bath with 100 mg L^{-1} did not cause changes in these parameters [30]. The same authors report that the increase lasted more than an hour after the bath at 300 mg L^{-1} and attribute it to adrenergic activation as acute stress response. This allows us to infer that, in the present study, the species *M. liza* showed higher tolerance to the stress of the H_2O_2 treatment.

The mean corpuscular volume (MCV) values were significantly lower 1h hour after bath when compared to 30 days. This was also observed in the control group, indicating that this difference in MCV values is not attributed to H_2O_2. The MCV decrease in fish may indicate a release of smaller immature erythrocytes produced by splenic contraction due to stress response [26]. In a previous study, values of MCV ranging from approximately 74 to 157 fL were observed as normal values for wild mullet (*M. liza*, formerly named *M. platanus*) [31]; and in wild white mullet (*M. curema*) the values of MCV ranged from approximately 70 to 200 fL [32]. Considering these findings, the mean MCV values observed in this study for *M. liza* in both sampling periods demonstrate a reasonable condition for this parameter, despite changes between sampling times,

falling within the range considered as normal for healthy mullets, as indicated by previous results. In general, regarding red blood cell parameters, H_2O_2 toxicity is often associated with anemia due to erythrocyte membrane destruction and hemoglobin oxidation [33]. However, in this study, the results of RBC, hemoglobin, and hematimetric indices indicate that the tested concentrations do not pose this risk to juvenile mullets. The evaluation of stress effects through blood parameters is more effective when H_2O_2 is used to treat species sensitive to the product [33], which seems not to be the case for *M. liza*.

The plasma glucose concentrations showed increased values immediately after bath, including the control, when compared to 30 days. In healthy adult mullets (*M. liza*), formerly named *M. platanus*) collected from an estuary in the Southeast region of Brazil, the mean values of plasma glucose concentration were around 99 mg dL^{-1} [34] and mean values close to 82 mg dL^{-1} were observed in juvenile mullets *M. liza* kept in tanks with salinity 15 for 50 days [35]. The values were similar to those found at 30 days in the present study (means ranging from 57.13 to 97.13 mg dL^{-1}), indicating that the values immediately after the bath were indeed above normal. Considering that this increase also occurred in the control group, it is not possible to assume that this response was a direct effect of hydrogen peroxide, but rather a result of the stress to which the animals were subjected during transfer and permanency in the bath containers.

Exposure to stress typically results in two types of endocrine responses: the response via the hypothalamus-pituitary-adrenal axis and inter-renal cells, culminating in increased plasma cortisol; and the adrenergic response, resulting in increased plasma catecholamines [36]. Fish exhibit a general pattern of physiological responses to stress, which includes increase in plasma glucose because of cortisol release. In a study involving seven species of marine fish belonging to different families, it was observed that the increase in plasma glucose begins approximately 30 minutes after the stressful stimulus (chase and exposure to air). However, the duration and magnitude of the response follow species-specific patterns, and it may take up to 24 hours for glucose levels to return to baseline [37], corroborating the present result, with increased values immediately after 1 hour of stress stimulus.

Regarding thrombocytes counts, fish treated with 150 and 350 mg L^{-1} of H_2O_2 showed reduced values immediately after the bath compared to the sampling after 30 days. It is possible that H_2O_2 has produced injuries on tissues exposed to the treatment (gills, mouth cavity and body surface), and these cells may have migrated from circulation to these tissues to perform reparation and defense functions [38-39]. In tambaqui (*C. macropomum*) treated with immersion baths in acetonic extract of annatto (*Bixa orellana*) [40], as well as in Nile tilapia (*Oreochromis niloticus*) juveniles treated with baths in essential oil of pepper-rosmarinus (*Lippia sidoides*) [41], significant reduction in the number of circulating thrombocytes was also observed, supporting the hypothesis of migration as a result of the action of irritating substances present in the water. In the present study, at 30 days after treatment, fish treated with H_2O_2 at 200 and 300 mg L^{-1} showed significantly reduced thrombocytes counts compared to those treated with 150 mg L^{-1}, suggesting that this migration response may be persistent and dose-dependent.

The number of total leukocytes (WBC) was not influenced by the treatments and showed no significant changes between the time immediately after the bath and 30 days later. Regarding this parameter, it is not possible to detect signs of stress or physiological impairment related to the treatment with H_2O_2 and/or the handling associated with the baths. Stress hormones may act on hematopoietic tissues resulting in the suppression of lymphocyte production, monocytopenia, and neutrophilia, as an immunosuppressive response [29]. Thus, even though the total leukocyte count remained unchanged, effects on the differential count can be observed, as was the case in the present study. In fish treated with higher doses of H_2O_2 (200 mg L^{-1} and above), the lymphocyte count was reduced immediately after bath when compared to 30 days later. This effect can be attributed to H_2O_2, as it was not observed in the control group or at the lower dose of H_2O_2, indicating a potentially dose-dependent response. This hypothesis is reinforced by the fact that 30 days after treatment, this effect persisted in fish treated with the highest dose (350 mg L^{-1}). This reduction in lymphocytes may be attributed to immunosuppression as a part of the stress response [29] and/or to migration to injured tissues as part of an inflammatory response induced by exposure to H_2O_2 [42]. In amberjack (*Seriola dumerilii*) exposed to H_2O_2 at 300 ppm for 60 minutes, infiltration of mononuclear cells into the gill tissue was observed, with this effect being noticeable as early as 15 minutes into the exposure [43]. However, considering that the method used in this present study for the differential count is based on the proportions among cell types, this reduction in lymphocytes may also simply be a consequence of an increase in the proportion of other counted cell types (neutrophils and monocytes), discussed below.

Both neutrophils and monocytes number showed similar response, with no differences among treatments at the same sampling time and increased values immediately after bath, when compared to 30 days, in fish treated with H_2O_2 at 150, 200, 300, and 350 mg L^{-1}. The increase in neutrophils combined to decrease in lymphocytes is a typical sign of stress response in fish [29] and, in the present study, it may be attributed to

the exposure to H_2O_2, even at the lowest dose. Similarly, a previous study analyzing stress response in pacu (*Piaractus mesopotamicus*) after capture and air exposure, found increase in the number of neutrophils and decrease in the number of lymphocytes [44]. Considering that this effect did not occur in the control group, it can be attributed not to the stress of the bathing procedure but to the exposure to hydrogen peroxide, since substances that have irritant effects on tissues can trigger similar responses.

There are no previous studies reporting the effects of hydrogen peroxide on the differential leukocyte count in fish; however, similar effects to those observed in the present study have been reported after exposure to other therapeutic chemicals applied as immersion bath. The present result is corroborated by assays with Nile tilapia juveniles subjected to bath treatments with essential oils of pepper rosemary (*L. sidoides*) and peppermint (*Mentha piperita*), which caused significant increase in neutrophils and monocytes, respectively [41]. Increased number of neutrophils and monocytes also occurred simultaneously in tambaqui (*Colossoma macropomum*) juveniles exposed to *L. origanoides* essential oil [45], corroborating the present result. The same effect occurred in common carp (*Cyprinus carpio*) exposed to malachite green baths for 1 hour, occurring increased number of neutrophils and monocytes accompanied by leukopenia, as observed in the present study [46]. Further analyses are necessary to elucidate the mechanisms by which exposure to hydrogen peroxide may affect hematopoiesis and function of different leukocyte types in fish. Toxicity tests and the determination of hydrogen peroxide metabolites, especially in fish muscle, will be determining parameters for the safe consumption of fish by humans.

CONCLUSIONS

At the doses tested in the present study, besides some adverse effect immediately after bath, there was an evident capacity for recovery 30 days after exposure to H_2O_2. Considering the effects on hematological parameters, we conclude that immersion baths with H_2O_2 up to 300 mg L^{-1} are safe as a treatment for *M. liza* juveniles. Future tests to assess the toxicity of this compound to this species, including biochemical assays and histopathological analysis of the exposed tissues, may be useful to deepen the understanding of the physiological effects of these treatments in fish.

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