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Time-dependent hematological responses of Nile tilapia *Oreochromis niloticus* exposed to an estuarine contaminated water

Respostas hematológicas tempo-dependentes de tilápia do Nilo *Oreochromis niloticus* expostas a água estuarina contaminada

Respuestas hematológicas tiempo-dependientes en tilapia del Nilo *Oreochromis niloticus* expuestas a agua estuarina contaminada

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Abstract
We aimed to study hematological responses of *Oreochromis niloticus* experimentally exposed to the contaminated water of the Santos-São Vicente Estuary, testing hypotheses that exposure time to estuarine water promotes deleterious effects on hematological parameters and evaluating the use of erythrocytes and leukocytes alterations as environmental biomarkers. Estuarine water was collected from Largo da Pompeba. For the biological assay, 28 juveniles of *O. niloticus* (red strain) of both genders were randomly selected from commercial pisciculture. For the biological assay, 28 juveniles of *O. niloticus* of both sexes were randomly selected from commercial fish farms. The juveniles were kept in estuarine water for 72 and 120 hours and, after exposure, blood was collected by puncture of the caudal vein to determine total erythrocytes, hemoglobin concentration, hematocrit, hematimetric indices and total leukocytes, as lymphocyte, neutrophils, monocytes, eosinophils, and basophils were quantified by blood extensions. To test exposure overtime on hematological variables, we performed a two-factor Multivariate Analysis of Variance. Exposure for 72 hours resulted in immunosuppression as seen by the reduced counts of neutrophils, monocytes, and lymphocytes in the bloodstream, whereas after 120 hours the immune system was stimulated with the increase of all leukocyte cell types. Exposure to estuarine water resulted in marked changes in the leukocyte count of *O. niloticus*, demonstrating that alterations in white blood cells might be more sensitive biomarkers than red blood parameters.
Keywords: Biomarkers; Blood; Environmental monitoring; Fish; Hematimetric indices.

Resumo
Objetivamos estudar as respostas hematológicas de *Oreochromis niloticus* expostas experimentalmente à água contaminada do Estuário de Santos-São Vicente, testando as hipóteses de que o tempo de exposição à água estuarina promove efeitos deletérios sobre os parâmetros hematológicos, e avaliando o uso de eritrócitos e alterações leucocitárias como biomarcadores ambientais. Água estuarina foi coletada no Largo da Pompeba. Para o ensaio biológico, 28 juvenis de *O. niloticus* de ambos os sexos foram selecionados aleatoriamente em piscicultura comercial. Os juvenis foram mantidos na água estuarina por 72 e 120 horas e, após a exposição, o sangue foi coletado por punção da veia caudal

para determinação dos eritrócitos totais, concentração de hemoglobina, hematócrito, índices hematimétricos e leucócitos totais, enquanto linfócitos, neutrófilos, monócitos, eosinófilos e basófilos foram quantificados por extensões de sangue. Para testar a exposição ao longo do tempo em variáveis hematológicas, realizamos uma Análise de Variância Multivariada de dois fatores. A exposição por 72 horas resultou em imunossupressão, como visto pela contagem reduzida de neutrófilos, monócitos e linfócitos na corrente sanguínea, enquanto após 120 horas o sistema imunológico foi estimulado com o aumento de todos os tipos de células leucocitárias. A exposição à água estuarina resultou em mudanças significativas na contagem de leucócitos de *O. niloticus*, demonstrando que as alterações nos glóbulos brancos podem ser biomarcadores mais sensíveis do que os parâmetros da série vermelha.
Palavras-chave: Biomarcadores; Sangue; Monitoramento ambiental; Peixe; Índices hematimétricos.

Resumen
El presente trabajo tuvo como objetivo evaluar las respuestas hematológicas de *Oreochromis niloticus* expuestos experimentalmente a agua contaminada del estuario Santos-São Vicente, evaluando el uso de eritrocitos y cambios leucocitarios como biomarcadores ambientales y, siguiendo la hipótesis de que el tiempo de exposición al agua del estuario promueve efectos deletéreos sobre los parámetros hematológicos. Se colectó agua de estuario en el lago da Pompeba. Para el ensayo biológico, se seleccionaron aleatoriamente 28 juveniles de *O. niloticus* de ambos sexos de piscifactorías comerciales. Los juveniles se mantuvieron en agua de estuario durante 72 y 120 horas y, después de la exposición, se extrajo sangre mediante punción de la vena caudal para determinar eritrocitos totales, concentración de hemoglobina, hematocrito, índices hematimétricos y leucocitos totales, siendo que linfocitos, neutrófilos, monocitos, eosinófilos y basófilos se cuantificaron mediante frotis sanguíneo. Para evaluar la exposición a lo largo del tiempo en variables hematológicas, realizamos un análisis de varianza múltiple de dos factores. La exposición durante 72 horas resultó en inmunosupresión, lo que se refleja en el recuento reducido de neutrófilos, monocitos y linfocitos en el torrente sanguíneo, mientras que después de 120 horas el sistema inmunológico se estimuló con el aumento de todos los tipos de células leucocitarias. La exposición al agua de estuario produjo cambios significativos en el recuento de leucocitos de *O. niloticus*, lo que demuestra que los cambios en los glóbulos blancos pueden ser biomarcadores más sensibles que los parámetros de la serie roja.
Palabras clave: Biomarcadores; Sangre; Monitoreo ambiental; Pescado; Índices hematimétricos.

1. Introduction

In aquatic ecosystems the water quality is strongly linked to fish health and, consequently, to the whole environment. Changes in physical and chemical properties of water, either due to natural events or to anthropogenic actions, might act as a stressor to aquatic organisms and disrupt animal homeostasis. These systemic disturbances might promote a series of physiological responses (Tort, 2011) that in turn can be used as biomarkers (van der Oost, Beyer, & Vermeulen, 2003). The understanding of the cause-effect relationship between pollutant action and physiological responses of fish provides a rapid and efficient diagnosis about the environmental health, as well as a long-term prognosis of possible damages to biodiversity (Vasseur & Cossu-Leguile, 2003; Hamza-Chaffai, 2014).

In this context, alterations in hematological parameters have been seen in several fish species exposed to a wide range of pollutants and were demonstrated to be sensitive biomarkers for environmental monitoring (Javed & Usmani, 2014; Authman, Zaki, Khallaf, & Abbas, 2015; Naqvi, Shaib, & Ali, 2016; Yaghoobi, Safahieh, Ronagh, Movahedinia, & Mousavi, 2017). Once blood parameters rapidly respond to xenobiotic exposure (Alwan et al. 2009), even in reduced concentrations (Ventura, Corsini, & Gabriel, 2015), such alterations stand out other biomarkers because they are simple, low-cost, and non-lethal techniques (Seriani et al., 2013). Experiments have shown that several xenobiotics as cadmium (Parekh & Tank, 2015), silver (Thummabancha, Onparn, & Srisapoome, 2016), *Escherichia coli* toxins (Yacoub, Sabra, & Al-Kourashi, 2018), and pesticides (Harabawy & Ibrahim, 2014; Saravanan et al., 2015) can promote anemia in fish. Anemias arise from marked hemolysis, hemorrhage, and/or damage to hematopoiesis, and through the decrease in both hemoglobin concentration and the number of erythrocytes that might reduce the oxygen transfer to tissues and compromise their physiological functions (Witeska, 2015).

Xenobiotics with immunotoxic potential might promote not only the suppression but also the stimulation of the immune system, modifying the extent of immune responses (Burns-Naas, Meade, & Munson, 1996). The cellular apoptosis (Schrek & Tort, 2016), depression of leucopoiesis centers (Seriani et al., 2011), and leukocytes mobilization from the bloodstream to

damaged tissues (Saleh & Marie, 2016; Silva et al., 2018) have been related to the immunosuppression seen in fish exposed to xenobiotics such as the pesticide diazinon (Alishahi, Mohammadi, Mesbah, & Razi, 2016), and metals as lead, copper, cadmium and zinc (Witeska, 2005). Thus, the immunosuppression can make fish living in impacted water bodies immunologically susceptible to infections and diseases (Yada & Tort, 2016). On the other hand, leukocytosis has also been observed in fish exposed to lindane (Saravanan, Kumar, & Ramesh, 2011) and selenium (Seriani, Ranzani-Paiva, Gonçalves, Siqueira, & Lombardi, 2012). Therefore, the kind of immune responses in fish to anthropogenic stressors would depends on the chemical properties of xenobiotics, as well as its concentration and the time of exposure (Tort, 2011).

In addition, the sensitivity of hematological responses to xenobiotic exposure is species-specific (Osman et al., 2018) so that the choice of the target species is determinant in hematological biomarkers effectiveness. Among the most studied fish species as test organisms, Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) stands out with substantial results (Silva, Rocha, Fortes, Vieira, & Fioravanti, 2012; Osman, 2012; Baiomy, 2016; Ndimele, Pedro, Agboola, Chukwuka, & Ekwu, 2017), once reference values for both red and white series have already been determined and blood cell types are well-described in the literature (Ueda, Egami, Sasso, & Matushima, 1997, 2001). For example, the use of hematological responses in *O. niloticus* as environmental biomarkers was demonstrated to be more sensitive than in *Clarias gariepinus*, when animals were collected in both a polluted and non-polluted area from Nile River (Osman et al., 2018).

The region of Santos-São Vicente Estuary is an intensely anthropized area that receives the input of *in natura* industrial, seaport, and domestic effluents from irregular houses (stilts) and the slurry of deactivated dumps (Companhia Ambiental do Estado de São Paulo [CETESB], 2017). Moreover, the water renewal by tides oscillation is slow in this area (Roversi, Rosman, & Harari, 2016), which favors the accumulation of solid residues (Fernandino et al., 2016), as well as the retention of contaminants for long periods through the deposition into the sediment from their aggregation with fine suspended particles (CETESB, 2017).

Previous studies reported high concentration of different types of pollutants in water of the region of Santos-São Vicente Estuary (Azevedo et al., 2009, 2012; Carmo, Abessa, & Machado-Neto, 2012; Albergaria-Barbosa et al., 2017, 2018; Magalhães, Taniguchi, Lourenço, & Montone, 2017; Souza et al., 2018). In this area, the presence of phosphorus, nitrogen, organic carbon, metals (e.g. arsenic, copper and mercury), polycyclic aromatic hydrocarbons (PAHs) and Enterococci were in concentrations above the limits recommended by the Brazilian environmental legislation (Conselho Nacional do Meio Ambiente [CONAMA], 2005), evidencing the quality deterioration in both water and sediments compartments (CETESB, 2017, Capparelli, Gusso-Choueri, Abessa, & McNamara, 2019). Some studies have evidenced that the presence of contaminants in the Santos-São Vicente Estuary region may promote marked changes in hematological parameters in fat snook (*Centropomus paralellus*) (Seriani et al., 2013) and in lined sole (*Achirus lineatus*) (Prado et al., 2015), and that these effects were strongly related to seasonal variations in water quality.

In this context, although the hematological parameters have already been used as biomarkers in different fish species for environmental monitoring, the time-dependent effects on hematological responses following the acute exposure to contaminated water from Santos-São Vicente estuary, as well as their implications for the health status of the animals in natural environment remain uncertain. Thus, we aimed to use the fish species *O. niloticus* as model to test if the acute exposure to the contaminated water from Santos-São Vicente Estuary would promote time-dependent alterations in hematological parameters of fish, contributing to the validation of these responses as biomarkers for environmental monitoring in the region.

2. Methodology

2.1 Animals and Experimental Design

The care and use of experimental animals complied with the Brazilian animal welfare laws, guidelines and policies as approved by the Animal Use Ethics Committee (CEUA) of the Biosciences Institute of Coastal campus (IB/CLP), from the São Paulo State University "Júlio de Mesquita Filho" (UNESP), under protocol n° 09/2018_CEUA IB/CLP.

Estuarine water (approximately 300 l) was collected from Largo da Pompeba (23º55'45'' S, 46º23'16'' W), located in the Santos-São Vicente Estuary, São Paulo, Brazil. For the biological assay, 28 juveniles of *Oreochromis niloticus* (red strain) of both genders were randomly selected from commercial pisciculture. Before the experiment, the fishes were acclimatized in reconstituted salinized water at 20 ppt (Blue Treasure®). Salinization from dechlorinated freshwater occurred gradually, by adding 5 ppt every 48 hours until reach the desired salinity (i.e 20 ppt that correspond to the salinity of the study area), where fish remained for more seven days. After this initial acclimatization, initial biometry (mean weight of 77±25 g and the total length of 16±1.6 cm) was performed and each animal was transferred to an individual experimental glass aquaria covered with black plastic and filled with 12 l of aerated reconstituted salinized water (20 ppt).

After 24h of acclimation on the individualized glass aquaria, fish (n=7) were randomly set to the following treatments: control (reconstituted salinized water at 20 ppt) and contaminated water (from Santos-São Vicente estuary), where fish were sampled after different times of exposure to the treatments (72h and 120h). During the experiments fish were maintained with constant aeration, temperature (28 ± 1 ºC), salinity (20 ppt) and photoperiod (12D: 12L), and 10% of the total water volume was renewed every three days. The animals were not fed during the experimental period. After 72 or 120 hours of exposure, fish were removed from the aquariums and anesthetized in 5% clove oil solution (Ranzani-Paiva, Pádua, Tavares-Dias, & Egami, 2013) for blood collection via caudal vein puncture. Immediately after collection, blood smears were made by loosening a drop of blood on the slide and blood samples were stored in properly identified heparinized tubes.

2.2 Analysis of blood parameters

Total and differential leukocyte counts were performed using blood smears, which were stained with rapid panopticon and analyzed by optical microscopy using immersion objective (X100). Total leukocytes (WBC) were quantified by the indirect method, in which leukocytes (WB) found between approximately 2000 erythrocytes are counted and estimated by the ratio of the number of total erythrocytes (RBC) obtained in the Newbauer Chamber (Hrubec & Smith. 1998), according to the following equation: WBC = WB*RBC/2000.

For the leukocyte differential count, 100 leukocytes were classified according to their cytological characteristics and percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils were determined (Davis, Maney, & Maerz, 2008; Sharma, Chadha, & Borah, 2015). Leukocyte types were quantified by relating percentage values with total leukocytes value. The ratio between the number of neutrophils and lymphocytes was also calculated for each treatment (Davis et al., 2008).

Total erythrocytes (RBC) count was performed in the Newbauer Chamber after dilution at 1:200 in 0.65% of saline solution (Ranzani-Paiva et al., 2013), added with bright cresyl blue. Hematocrit (Ht) and hemoglobin concentration (Hb) values were determined on an automatic analyzer (KX-21N, Sysmex). The hematimetric indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Wintrobe (1934): $MCV = Ht*10/RBC*10^{6}ml^{-1}$; $MCH = Hb*10/RBC*10^{6}ml^{-1}$; and $MCHC = Hb*10/Ht$.

2.3 Data analysis

To test the effects of contaminated estuarine water (A_i) and time of exposure on contaminated estuarine water (B_i) on the set of *m* hematological variables (Y_{ijkm}) of *O. niloticus*, we performed a two-factor Multivariate Analysis of Variance (two-way-MANOVA), as follows: $Y_{ijkm} = m + A_i + B_j + AB_{ij} + e_{ijkm}$

As the multivariate normality assumptions were not met, treatments and interaction effects were tested by permutation (9999 randomizations). The homogeneity assumption of the variance-covariance matrix was tested by the *betadisper* function (Oksanen et al., 2016).

3. Results

Hematological responses related to exposure to contaminated estuarine water were predominant in leucograms (Table 1). During the first 72h, significant reductions of about 55%, 38%, and 35% in monocyte, neutrophil, and total leukocyte counts, respectively, were seen in fish exposed to estuarine water, compared to the control group. On the other hand, there was a significant increase ($p < 0.05$) of 2.76 times in the number of total leukocytes in *O. niloticus* after 120 h of exposure to contaminated estuarine water, characterized by the increase in the cellular types of lymphocytes (2.78 times), neutrophils (2.67 times), monocytes (3.55 times), and eosinophils (1.82 times), compared to the control group. Basophils were observed in only one specimens of each treatment tested and therefore were not included in the analyses. The erythrogram showed no significant changes in any of the evaluated variables between the animals exposed to contaminated estuarine water and the control group (Table 1).

Table 1. Mean values (±SD, n=7) of hematological parameters of *Oreochromis niloticus* exposed to contaminated estuarine water for 72 h and 120 h and their respective controls in reconstituted salinized water at 20 ppt.

Parameters	**72 h**		**120 h**	
	Control	**Estuarine water**	**Control**	**Estuarine water**
WBC (10^3 mL^{-1})	17.96±5.3^{A}	11.67±3.7^{B}	11.00±2.9^{b}	30.37±10.7^{a}
Neutrophils (10^3 mL^{-1})	7.74±2.5^{A}	4.73±1.3^{B}	3.96±1.7^{b}	10.57±5.8^{a}
Lymphocytes (10^3 mL^{-1})	8.59±2.6^{A}	6.12±2.1^{A}	6.20±1.3^{b}	17.25±5.3^{a}
Monocytes (10^3 mL^{-1})	1.34±0.8^{A}	0.60±0.4^{B}	0.60±0.6^{b}	2.13±1.2^{a}
Eosinophils (10^3 mL^{-1})	0.25±0.4^{A}	0.22±0.2^{A}	0.23±0.1^{b}	0.42±0.2^{a}
N:L	0.90±0.2^{A}	0.79± 0.1^{A}	0.65 ±0.3^{a}	0.60±0.2^{a}
RBC (10^6 mL^{-1})	1.61± 0.4^{A}	1.37±0.3^{A}	1.39±0.4^{a}	1.42±0.2^{a}
Hemoglobin (g dL^{-1})	9.29±0.9^{A}	9.13±1.2^{A}	9.39±1.0^{a}	10.0±0.9^{a}
Hematocrit (%)	27.86±1.8^{A}	25.86±3.2^{A}	28.71±3.6^{a}	29.71±3.1^{a}
MCV (fL)	183.71±43.7^{A}	195.00±46.6^{A}	217.86±59.5^{a}	213.00±38.4^{a}
MCH (pg)	60.57±16.7^{A}	68.86±17.1^{A}	71.00±18.6^{a}	71.86±13.1^{a}
MCHC (g dL^{-1})	33.29±1.6^{A}	35.14±2.0^{A}	32.86±1.2^{a}	33.71±1.6^{a}

Means followed by the same letters within a row (upper case for 72 h and lowercase for 120 h) do not statistically differ from each other ($p > 0.05$), when treatment and its respective control were compared. Abbreviations: SD, Standard Deviation; h, hours; WBC, Total leukocytes; N:L, ratio between the number of Neutrophils and Lymphocytes; RBC, Red Blood Cells; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, and Mean Corpuscular Hemoglobin Concentration. Source: Authors.

No evidence of variance heterogeneity was found between the groups ($p > 0.05$) and some variables were highly correlated with each other (e.g. Lymphocytes, Neutrophils and WBC or Hematocrit, Hemoglobin and RBC). Multivariate distances between treatment and control were evidenced after 120h of exposure, resulting in a significant interaction between exposure and time of exposure to contaminated estuarine water in MANOVA ($F_{1,21} = 4.13$; $p = 0.007$).

4. Discussion

Fish immune responses to stressors directly depend to the chemical properties of the xenobiotic, on its intensity/concentration and the time of exposure (Yada & Tort, 2016). In the present study, we demonstrated that the exposure time exerted a differential effect on hematological response of white series in *O. niloticus* exposed to the contaminated water of the Santos-São Vicente Estuary. Although a discrete initial immunosuppression was seen in *O. niloticus* after 72h of exposure, fish exposed for 120h to the estuarine contaminated water displayed a markedly increase in all white blood cell types, indicating a severe leukocytosis.

The slight leukopenia observed in *O. niloticus* specimens after 72h of exposure to contaminated estuarine water was related to a reduction in the number of neutrophils, monocytes and lymphocytes in the bloodstream, a different result from the triad lymphopenia, neutrophilia and monocytopenia that is expected for an acute immune response in fish (Tort, 2011). Fish immunosuppression can be caused by several aquatic contaminants, such as pesticides (Li et al., 2011), PAHs (Dunier & Siwick, 1993), and heavy metals (Witeska, 2005; Authman et al., 2015), as well as the toxins of *Escherichia coli*, a bacteria that is usually found in the vertebrate gut and that is taken as a typical indicator of domestic sewage aquatic contamination (Yacoub et al., 2018). Earlier studies have demonstrated that the study area is highly impacted by domestic and industrial sewage, resulting in alterations of microbiological parameters and serious contamination in water, which is aggravated by the entrapment of these compounds in the place for long periods because of the estuary's hydrodynamics (Roversi et al., 2016), as well as for the retention potential to xenobiotic particles from sediments (CETESB, 2017; Capparelli et al., 2019).

Through the use of Water Quality Index (WQI), the quality of water in the study area was classified as highly eutrophic and ranked as "bad" and "poor", with a high level of ammonia/nitrogen, phosphorus and total organic carbon and lowered levels of dissolved oxygen, while the presence of some metals as borium, cadmium, lead and cooper have also been reported (CETESB, 2015, 2017). In addition, thermotolerant coliforms and *Clostridium perfringens* have been evidenced in sediments, which demonstrated high acute and chronic toxicity in tests with larvae of *Leptocheirus plumulosus* and *Lytechinus variegatus*, respectively. It was also observed enrichment of sediment with metals (as arsenic, lead and copper) and PAHs, where some sampling points displayed higher levels than the Threshold Effect Level (TEL) stablished by the Environmental Sanitation Technology Company (CETESB) from São Paulo State (CETESB, 2017). In fact, the historical deterioration in environmental quality in Santos-São Vicente Estuary has impacting the resident aquatic biota, resulting in bioaccumulation of several toxic compounds (e.g. copper, nickel, zinc, benzo[*a*]pyrene, dibenzo[*a*]antracene, PCBs and dioxins) in both fish (manly in *Diapterus rhombeus* and *Centropomus paralellus*) and crabs (*Callinectes sapidus*) that are usually consumption by local population (Lamparelli et al., 2001).

In addition, many studies have been demonstrated that several stress conditions, as maintenance in laboratory (Ishikawa, Ranzani-Paiva, Lombardi, & Ferreira, 2007), farm handling condition (Ghiraldelli, Martins, Yamashita, & Jerônimo, 2006), acclimation to higher salinities (Pereira, Guerra-Santos, Moreira, Albinati, & Ayres, 2016) and air exposure (Silva et al., 2012) might also promote a reduction in leukocytes and their cell types in *O. niloticus*. For example, a marked reduction in total leucocytes count was reported to *O. niloticus* following three (62.11%), seven (52.48%) and 10 days (37.64%) of acclimation to laboratory conditions (Ishikawa et al., 2007). Thus, the slight leukopenia seen in *O. niloticus* after 72h of exposure might be

related to the sudden contact to different contaminants found in water of Santos-São Vicente Estuary, as well as unspecific stress response to the acclimation to laboratory conditions.

On the other hand, longer exposure to estuarine water (120h) stimulated the immune system, increasing the leukocyte counts and their cell types (specially the monocytes, lymphocytes and neutrophils) in comparison to the control. Similar results were reported by Seriani et al. (2012), where *O. niloticus* exposed for 10 days to different selenium concentrations presented increased leukocyte types compared to the collection performed on the third day. Also, samples of *O. niloticus* collected in a polluted artificial lake showed increased total leukocyte number, associated with increased monocytes, eosinophils, and the presence of erythrocyte nuclear abnormalities, which was accompanied by the reduction of neutrophils, lymphocytes, and erythrocytes (Silva et al., 2018). Leukocytosis was also observed in fish collected in polluted areas, relative to reference values, as detected in *Arius thalassinus* species from the Red Sea Coast of Hodeida, Republic of Yemen (Saleh & Marie, 2016), *Centropomus parallelus* (Seriani et al., 2013), *Mugil curema* (Cicero, Souza, Rotundo, Pereira, & Sadauskas-Henrique, 2020) from the Santos-São Vicente Estuary and *Clarias gariepinus* from El-Rahawy Drain, Egypt (Gaber, El-Kasheif, Ibrahim, & Authman, 2013). These results corroborate those observed in this study and demonstrate the feasibility of carrying out experimental tests with estuarine water from highly impacted environments, and those marked effect on immune system response in fish.

The significant increase observed in neutrophil counts indicates activation of the inflammatory process, which is the first response of body to injury in attempting to eliminate pathogens (Kumar, Clermont, Vodovotz, & Chow, 2004). One of the characteristics of this response is the presence of neutrophils and monocytes in the blood (Rowley, 1996; Xu et al. 2018), mainly because of its phagocytic capacity (Fánge, 1992). Xu et al. (2018) demonstrated that neutrophilia in larval zebrafish *Danio rerio* can be modulated by different pollutants such as metals, endocrine disrupters, organic nitrogen compounds, and organochlorine pesticides after 24h of exposure. The herein observed monocytosis may arise from the presence of urban sewage in the water, as it was observed for *O. niloticus* collected in a degraded reservoir with high concentrations of organic matter from urban effluents and outflow of agricultural activities (Corrêa, Abessa, Santos, Silva, & Seriani, 2016).

In general, neutrophilia is accompanied by a reduction in lymphocyte counts (Davis et al., 2008; Tort, 2011). However, in this study, contaminated estuarine water promoted the increase of lymphocytes in the bloodstream, possibly indicating a leukemic condition accompanied by cell degeneration, immature lymphocytes or malignant cells (Clauss, Dove, & Arnold, 2008). Similar results were observed in *O. niloticus* exposed to sub-lethal sodium selenite concentrations where leukocytosis was also associated to an increased lymphocyte, neutrophil, and monocyte counts (Ranzani-Paiva, Lombardi, Maiorino, Gonçalves, & Dias, 2014). The increase in cell types of the white series has been reported in other studies where fish were exposed to water contaminated with heavy metals, like in *Anguilla anguilla* exposed to water contaminated with lead (Santos & Hall, 1990), *O. niloticus* exposed to metals such copper, borium and lead in the aquatic environment (Garcia, Miguel, Gabriel, & Mingala, 2016), and *Ctenopharyngodon idella* exposure to copper and chromium (Shah et al., 2020).

Eosinophils play an important role in parasite phagocytosis (Lataretu, Furnaris, & Mitrãnescu, 2013), and eosinophilia of *O. niloticus* specimens after 120h of exposure to contaminated estuarine water indicates that initial immunosuppression may have led to increased susceptibility to opportunistic diseases (Burnett, 2005), as well as the possible rupture of the physical barrier due to tissue damages. On the other hand, basophils are cellular elements rarely found in fish blood (Ishikawa et al., 2007; Cazenave et al., 2014) and that are therefore less studied – in this study, basophils were observed in only two animals. The energy cost of these immune responses may interfere with an organism's long-term vital functions and increase its vulnerability to pathogens, which might result in alterations of growth, reproduction, behavior (Schreck, 2010; Schreck & Tort, 2016) and disease incidence. These immune responses, in turn, may negatively affect fish populations (Arkoosh et al., 1998) and, consequently, the local biodiversity.

Studies have shown that exposure to polluted water causes anemia in fish with a decrease in hemoglobin concentration, hematocrit, and total circulating erythrocytes (Seriani et al. 2010, Saleh & Marie 2016, Silva et al. 2018). However, contrary to previous studies that collected fish *in situ* (Seriani et al., 2010, 2013; Silva et al., 2018), *O. niloticus* experimentally exposed for 72h and 120h to the contaminated water from Santos-São Vicente Estuary did not present an anemic picture. Once red blood cells account for gas transport in blood, fish erythrocytes are quite sensitive to changes in dissolved oxygen concentration, as a compensatory response to hypoxic environments (Sweilum, 2006; Cruz, Prado, Maciel, & Couto, 2015), a condition usually found in polluted waters by domestic organic effluents. Temperature fluctuations also affect blood parameters (Shahjahan, Uddin, Bain, & Haque, 2018), especially through controlling both the metabolism and activity of ectothermic organisms. Cicero et al. (2020) observed an increase in both the hematocrit and the number of erythrocytes (RBC) in *M. curema* collected in the Santos-São Vicente Estuary, which seems to be a physiological adjustment against the stress promoted by the contaminated environment, in order to supply a higher tissue demand for oxygen, as previously noted by Seriani et al. (2013) with *C. parallelus* in the same area. The maintenance of oxygen availability and strict control of temperature at laboratory conditions in the present study may justify the absence of anemic responses.

Previous studies have been able to demonstrate that red series in fish are directly affect by environmental factors, parasitism, nutritional status (Witeska, 2015) and animal size (Santos, Cavalcante, Hauser-Davis, Lopes, & Mattos, 2016). Experimentally, these conditions are less likely to be observed, especially in pisciculture animals, such as those used in this study, due to the provision of nutritionally balanced feed and quality control of culture water. On the other hand, in the natural environment, fishes are more susceptible to changes in these blood parameters, especially in contaminated places where the immunity response is impaired (Schreck & Tort, 2016), as well as food shortages due to reduction of phyto and zooplankton abundance (Swelium, 2006). However, it has been recently demonstrated that fish erythrocytes also express genes related to immune responses (Shen, Wang, Zhao, & Chen, 2018), which may open up possibilities for future studies on the physiology of erythrocyte and leukocyte responses, and their overall role in fish immunity, which possibly bringing new blood biomarkers and applications in environmental monitoring.

5. Conclusion

We observed that the acute exposure of *O. niloticus* to the contaminated water of the Santos-São Vicente Estuary resulted in marked alterations in hematological responses of fish, indicating that the deterioration in water quality, due to degradation and environmental pollution, might disturb fish health in the region. At laboratory conditions, the alterations of *O. niloticus* leukocytes were demonstrated to be biomarkers with higher sensitivity to contaminated water from Santos-São Vicente Estuary than red series parameters. Also, the leukopenia and leukocytosis observed in *O. niloticus* after 72h and 120h, respectively, suggest a differential time-dependent effect on the animals' white series following the acute exposure to the contaminated estuarine water.

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