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Hematological parameters of Brazilian sardines (*Sardinella brasiliensis* Steinachner, 1879) fed different concentrations of fatty acids in their diet

[*Parâmetros hematológicos de sardinhas brasileiras (*Sardinella brasiliensis *Steinachner, 1879) alimentadas com diferentes concentrações de ácidos graxos em sua dieta*]

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ABSTRACT

This study aimed to evaluate the effect of different levels of omega-3 polyunsaturated fatty acids (n-3 PUFA) in the diet of *Sardinella brasiliensis* on their hematological parameters. A total of 375 fish were distributed in 15 experimental units to evaluate the effects of the diet on their blood for 45 days. The levels of n-3 PUFA added in the diet were 0, 0.3, 0.6, 0.9, and 1.2% of the lipid fraction. These values represent the total percentage of n-3 PUFA in the lipid fraction of the diet, and 0% n-3 PUFA was used as the negative control. Five fish from each experimental unit were sampled at the end of the experiment for hematological analysis, and the parameters measured include the total number of erythrocytes, hematocrit percentage, hemoglobin and glucose concentration and differential leukocyte count. Higher numbers of circulating eosinophils were observed in fish fed 0.3% and 1.2% n-3 PUFA. Although no significant differences were observed in several of the parameters, the results demonstrate that the health of the sardines was not affected by the addition of n-3 PUFA in their diet. It should be emphasized that this is the first study with emphasis on the blood analysis of *S. brasiliensis*.

Keywords: aquaculture, Clupeidae, fatty acids, nutrition, hematology

RESUMO

O objetivo deste estudo foi avaliar o efeito de diferentes níveis de ácidos graxos poli-insaturados ômega-3 (PUFA n-3) na dieta de Sardinella brasiliensis *sobre seus parâmetros hematológicos. Um total de 375 peixes foi distribuído em 15 unidades experimentais para avaliar os efeitos da dieta em seu sangue durante 45 dias. Os níveis de PUFA n-3 adicionados à dieta foram 0, 0,3, 0,6, 0,9 e 1,2% da fração lipídica. Esses valores representam a porcentagem total de PUFA n-3 na fração lipídica da dieta, e 0% de PUFA n-3 foi usado como controle negativo. Cinco peixes de cada unidade experimental foram amostrados no final do experimento para análise hematológica, e os parâmetros medidos incluem o número total de eritrócitos, a porcentagem de hematócrito, a concentração de hemoglobina e glicose e a contagem diferencial de leucócitos. Foram observados números mais altos de eosinófilos circulantes em peixes alimentados com 0,3% e 1,2% de PUFA n-3. Embora não tenham sido observadas diferenças significativas em vários dos parâmetros, os resultados demonstram que a saúde das sardinhas não foi afetada pela adição de PUFA n-3 em sua dieta. Deve ser enfatizado que este é o primeiro estudo realizado com ênfase na análise sanguínea de* S. brasiliensis.

Palavras-chave: aquicultura, Clupeidae, ácidos graxos, nutrição, hematologia

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INTRODUCTION

The Brazilian sardine (*Sardinella brasiliensis* Steinachner, 1879) is a small marine fish belonging to the Clupeidae family. It is found mainly between Cabo de São Tomé, Rio de Janeiro (22°S), and Cabo de Santa Marta, Santa Catarina (29°S) (Jablonski, 2007).

As a result of overfishing, the Brazilian sardine's prevalence in fisheries has been declining over the last 40 years, according to data obtained from the statistical bulletin of the Ministry of Fisheries and Aquaculture (Brasil, 2012). According to Resolution SMAC No. 073 of August 19, 2022, which provides a list of endangered species native to Rio de Janeiro, *Sardinella brasiliensis* is currently threatened with extinction (Rio de Janeiro, 2022).

Given this situation, aquaculture of *Sardinella brasiliensis* is a good alternative to meet the industry's demand in a sustainable way and avoid overexploitation of the species in the wild. Since 2009, breeding technologies for sardines have been developed, improving the aquaculture of the species with encouraging results, such as improved reproduction rates (Cerqueira *et al*., 2020).

Currently, the nutritional requirements of *S. brasiliensis* are the focus of research because it has high levels of Omega-3 polyunsaturated fatty acids (n-3 PUFA), which is beneficial to human health (Lee *et al*., 2009). However, fatty acids are also important for fish nutrition as it functions in energy production and homeostatic balance, in addition to being structural components of cell membranes and precursors of hormones and other biologically active compounds (Glencross, 2009).

Sardinella brasiliensis is an omnivorous species that feeds mainly on phytoplankton and zooplankton, which are foods rich in n-3 PUFA (Schneider; Schwingel, 1999). In aquaculture, fish oil is used as a source of n-3 PUFA in the diets of marine fish, but its high price and scarcity in the market make it a limiting option.

Nevertheless, fish farming provides greater control over the rearing of the fish, because they grow under more stable conditions and are fed balanced diets; thus, the number of fatty acids they are fed can be altered to increase the meat quality and nutritional value of the species (Hunter and Roberts, 2000).

In a study conducted by Bandarra *et al*. (2018), the authors observed that sardines had a higher total concentration of n-3 PUFA after being fed with commercial feed for 1 year. These sardines had almost twice the total lipids in their muscles when compared to wild sardines. Therefore, studies focused on fish aquaculture require tools that assist in monitoring animal health, such as hematology.

Hematology is the study of blood elements, decoding their components and analyzing different blood cells in search of altered patterns that indicate possible pathologies (Ranzani-Paiva *et al*., 2013). Changes, in both natural and culture condition as well as their diet leads to a series of alterations in their blood constituents (Lizama *et al*., 2020).

Therefore, using hematological analysis in fish is an important tool for assessing the health of the animals (Tavares-Dias *et al*., 2009), and assists in detecting diseases and hematological changes after experiments (such as the use of new therapies that help fight diseases, water quality changes, or the introduction of new diets) (Fazio, 2019).

Studies conducted on the Brazilian sardine have focused mainly on its use in fishing (Castello, 2006), its ecology (Schneider and Schwingel, 1999), and biology (Braga, 1987), but there are no studies related to blood analysis of *S. brasiliensis* under aquaculture conditions. Therefore, this study aimed to analyze the hematological parameters of sardines fed different levels of n-3 PUFA in their diet.

MATERIAL AND METHODS

The experiment was conducted at the Laboratory of Marine Fish Farming (LAPMAR), at the Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil. The fish were obtained from breeders using natural spawning and were already acclimatized at LAPMAR, following procedures described by Cerqueira *et al*. (2017). The animal handling procedures were approved by the Ethics Committee on Animal Use of the Federal

University of Santa Catarina (CEUA/UFSC PP00861).

The experimental setup included five independent water recirculation systems with a flow rate of 1L min $^{-1}$, and each one was connected to three 500L tanks (15 tanks in total). Each system had a 100L equalizer box, with a motor pump set, heater coupled with a thermostat, ultraviolet (UV) lamp, and biological filtration media (BioBalls).

The water temperature was maintained at 23.2 ± 1.6°C with a natural photoperiod and each experimental unit had constant aeration. The tanks were covered with a screen to prevent predators from killing the fish (birds in the region tended to attack the fish). Water quality parameters were measured daily. Salinity (35 ± 0.0) was measured using a portable refractometer. Dissolved oxygen (5.8 ± 0.4mg L-1) were measured using an oximeter (AT 160 SP Microprocessed), and pH (8.39 ± 0.23) (portable pH meter). Food excess and feces were removed whenever necessary by bottom siphoning.

The initial density per experimental unit was 25 fish with an average weight of 60 g and a biomass of 1.5kg per 0.5m^3 of water. The sardines were fed three times a day at 8:30 am, 12:30 pm, and 4:30 pm, during the entire 45-d experimental period at 4% of their body mass, resulting in 0.06kg of feed per tank per day until the end of the experiment.

The experimental diet was formulated based on the requirements of milkfish (*Chanos chanos*) and Nile tilapia *(Oreochromis niloticus)* (Nutrient, 2011), except for protein, which was determined in a study on juvenile sardines by Sterzelecki *et al*. (2018), in which the juveniles were isoenergetic (4300 kcal) and isoproteic (40%).

Because there is no literature describing the diet requirements for true sardines, the ideal n-3/n-6 ratio of 2:1 used for milkfish (*Chanos chanos*) (Nutrient, 2011) was formulated; thus, the experimental diets contained between 10 - 15% lipids and five levels of n-3 PUFA, using cod liver oil as a source of n-3 and corn oil as a source of n-6. The dietary n-3 PUFA levels used in the experiment were 0, 0.3, 0.6, 0.9, and 1.2% of the lipid fraction of the diet, as shown in Table. 1.

Table 1. Formulation and composition of experimental diets (expressed as percentage of dry matter).

Ingredients	Total n-3 PUFA				
	0%	0.3%	0.6%	0.9%	1.2%
Soybean meal	38.97 mg g^{-1}	38.97 mg g^{-1}	38.97 mg g^{-1}	38.97 mg g^{-1}	38.97 mg g^{-1}
Maize	18.63 mg g^{-1}	18.63 mg g^{-1}	18.63 mg g^{-1}	18.63 mg g^{-1}	18.63 mg g^{-1}
Guts Flour	21.55 mg g^{-1}	21.55 mg g^{-1}	21.55 mg g^{-1}	21.55 mg g^{-1}	21.55 mg g^{-1}
Rice Grits	15 mg g^{-1}	15 mg g^{-1}	15 mg g^{-1}	15 mg g^{-1}	15 mg g^{-1}
Corn Oil	5.09 mg g^{-1}	4.12 mg g^{-1}	2.79 mg g^{-1}	1.22 mg g^{-1}	0 mg g^{-1}
Cod Liver Oil	0 mg g^{-1}	0.86 mg g^{-1}	2.36 mg g^{-1}	3.9 mg g^{-1}	5.4 mg g^{-1}
Premix	0.42 mg g^{-1}	0.42 mg g^{-1}	0.42 mg g^{-1}	0.42 mg g^{-1}	0.42 mg g^{-1}
Bicalcium Phosphate	0.35 mg g^{-1}	0.35 mg g^{-1}	0.35 mg g^{-1}	0.35 mg g^{-1}	0.35 mg g^{-1}
Composition (% DM)					
Crude Energy	4381				
Digestible Energy	3400				
Crude Protein	36.42				
Digestible Protein	31.44				
Lipids	10.66				
Dry Matter	6429.00				

* Vitamin and mineral premix (10 mg g -1) - Vitamin and micromineral premix (Raguife Vaccinar, Belo Horizonte, MG - Brazil), composition kg^{-1} of product: folic acid 1,200 mg, pantothenic acid 10,000 mg, biotin 200 mg, cobalt 80 mg, copper 3,500 mg, choline 100,000 mg, iron 20. 000 mg, iodine 160 mg, manganese 10,000 mg, niacin 20,000 mg, selenium 100 mg, vitamin (vit.) A 2,400,000 IU, vit. B1 4,000 mg, vit. B12 8,000 mg, vit. B2 4,500 mg, vit. B6 3,500 mg, vit. C 60,000 mg, vit. D3 600,000 IU, vit. E 30,000 IU, vit. K 3,000 mg, zinc 24,000 mg, inositol 25,000 mg. Macromineral premix (composition kg^{-1}): bicalcium phosphate 130 g, potassium chloride 120 g, sodium chloride 130 g, magnesium sulphate 620 g.

These values represent the total percentage (%) of n-3 PUFA of the diet's lipid fraction, and 0% n-3 PUFA was used as the negative control. For the diets containing different levels of n-3 PUFA, increasing total n-6 caused an increase in PUFA levels and a decrease in monounsaturated fatty acids (MUFA) levels. The mixtures were pelleted and dried in an oven at 50°C for 6 h, then they were broken apart and sieved (0.8-mm). All diets were color coded (0% = orange; 0.3% = green; 0.6% = pink; 0.9% = blue; and 1.2% = purple) and stored in plastic bags at –20°C until use.

For hematological analyses, five fish from each experimental unit were anesthetized with eugenol solution (75mg L^{-1}) and a 3mL syringe containing an anticoagulant solution, ethylenediaminetetraacetic acid (EDTA 3%) (Ranzani-Paiva *et al*., 2013), was used for blood collection by puncturing the caudal vessel. A total of 15 fish were sampled.

Glucose analysis was conducted using a Glucose Liquiform Kit (Labtest) according to the manufacturer's instructions. Briefly, blood plasma samples from the fish from the same experimental unit was used, and 1mL of reagent was applied to every 10µL of blood plasma.

A blood aliquot was used to determine the hematocrit using the microhematocrit method (Goldenfarb *et al*., 1971), and the analysis of the hemoglobin concentration was performed using the cyanmethemoglobin method described by Ranzani-Paiva *et al*. (2013).

A blood aliquot was used to determine the total erythrocyte count. The count was performed in a Neubauer chamber in DACIE solution at a dilution ratio of 1:200. The total and differential leukocyte counts were determined using the indirect method. Blood samples were collected in duplicate and stained with May-Grünwald-Giemsa-Wright, according to the method described by Ranzani-Paiva *et al*., (2013).

A Shapiro-Wilk test was used to determine the normality of the data, and Levene's test was used to assess the homoscedasticity of variances. Analysis of variance (ANOVA) was used once the prerequisites were confirmed. Duncan's test was used in cases where differences between treatments were observed, to verify the data. STATISTICA software (version 10.0) was used for all statistical analyses performed and a significance level of 95% was applied (p<0.05).

RESULTS

From the total and differential leukocyte counts, we identified monocytes, neutrophils, lymphocytes, thrombocytes, and eosinophils, but there was an apparent absence of basophils, as shown in Fig 1 A-F.

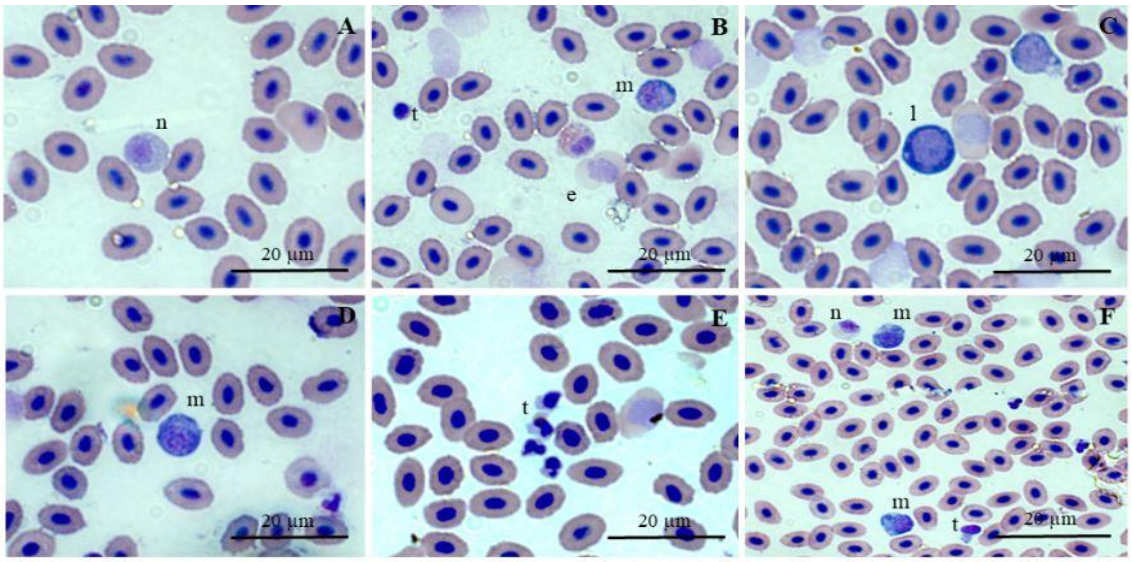

Figure 1. Blood cells of true sardine, *Sardinella brasiliensis*. A: neutrophil (n). B: eosinophil (e), monocyte (m) and thrombocyte (t). C: lymphocyte (l). d: monocyte (m). E: thrombocyte (t). F: showing two monocytes (m), a neutrophil (n) and a thrombocyte (t).

Hematological parameters measured among the different concentrations of fatty acids in the sardine's diet are shown in Tables 2 e 3. The number of eosinophils (E) were significantly different among the treatments, with greater numbers of eosinophils presenting in the blood of fish fed the diets containing 0.3% and 1.2% fatty acid concentrations, compared to that of the fish fed the 0.6% and 0.9% fatty acid concentrations diet. The sardines of the control group (0% n-3 PUFA) presented several eosinophils statistically equal to the other treatments. In the case of eosinophils, significant difference among treatments regarding the sex of the sardines were not observed.

Table 2. Hematological parameters (mean ± standard deviation) of true sardines (*Sardinella brasiliensis*) after addition of n-3 PUFA at concentrations of 0%, 0.3%, 0.6%, 0.9% and 1.2% of the dietary lipid fraction

Treatments	Sex	Parameters					
		T (x 10^3 µL^{-1})	LT (x 10^3 µL^{-1})	L (x 10^3 µL^{-1})	M (x 10^3 µL^{-1})	N (x 10^3 µL^{-1})	E (x 10^3 µL^{-1})
0%	M	31.04±9.67	302.92±49.80	249.94±62.07	4.23±2.08	1.71±2.08	47.04±13.50^{ab}
	F	23.29±13.18	318.62±49.77	274.50±64.60	4.83±3.13	1.14±1.31	38.20±11.24^{ab}
0.3%	M	22.67±8.85	282.08±59.51	229.85±42.95	4.37±2.93	1.41±1.78	46.50±41.16^{a}
	F	28.34±19.59	343.71±90.50	257.87±70.94	9.31±5.93	6.11±9.61	70.43±67.10^{a}
0.6%	M	12.28±5.93	284.43±31.33	243.04±25.56	4.43±3.70	4.98±3.51	32.18±11.78^{b}
	F	14.34±9.72	321.38±71.40	289.80±66.93	3.28±4.11	2.60±3.63	25.71±5.46^{b}
0.9%	M	22.02±9.00	310.79±44.22	269.18±54.54	6.41±5.18	5.80±4.54	29.56±15.66^{b}
	F	32.51±3.61	314.38±123.40	271.04±131.68	4.83±5.60	3.95±3.96	34.56±20.17^{b}
1.2%	M	20.75±6.20	277.95±68.50	218.21±68.24	3.50±3.16	4.64±4.44	51.60±15.67^{a}
	F	27.4±10.41	324.25±12.37	251.25±7.30	5.61±3.22	1.60±2.23	65.81±10.53^{a}
p fatty acids		0.22	0.992	0.728	0.433	0.51	0.006
p sex		0.252	0.087	0.15	0.398	0.636	0.341
p interaction		0.283	0.845	0.956	0.307	0.288	0.287

*Lowercase letters (a.b) represent significant difference between treatments;
**Lowercase letters (x.y) represent significant difference between the interaction (treatment x sex).
Legend of parameters: (RBC) Red blood cells; (T) Thrombocyte; (LT) Total leukocytes; (L) Lymphocyte; (M) Monocyte; (N) Neutrophil; (E) Eosinophil; (M) male, (F) female.

Table 3. Hematological parameters (mean ± standard deviation) of true sardines (*Sardinella brasiliensis*) after addition of n-3 PUFA at concentrations of 0%, 0.3%, 0.6%, 0.9% and 1.2% of the dietary lipid fraction

Treatments	Sex	Parameters					
		RBC (x 10^6 µL^{-1})	Ht (%)	Hb (g dL^{-1})	MCV (fL)	MCH (g dL^{-1})	MCHC (g dL^{-1})
0%	M	3.03±0.50	39.00±6.29	10.79±1.61^{xy}	129.93±20.05	35.85±3.81	28.03±4.42
	F	3.18±0.60	42.83±6.35	11.57±1.62^{xy}	139.85±40.99	36.85±4.77	27.54±5.42
0.3%	M	2.82±0.60	35.58±4.34	10.56±1.86^{xy}	128.72±18.35	37.88±5.14	29.51±1.94
	F	3.44±0.90	41.36±4.52	11.75±1.20^{xy}	126.03±28.26	35.83±7.76	28.48±1.80
0.6%	M	2.85±0.31	40.38±5.37	12.77±1.22^{xy}	142.51±16.91	40.53±3.97	28.56±2.05
	F	3.21±0.71	42.13±7.98	1.56±1.65^{x}	133.03±23.99	40.43±5.64	30.68±3.74
0.9%	M	3.11±0.44	43.64±8.24	9.97±1.39^{x}	143.98±39.01	40.41±4.78	29.29±5.91
	F	3.14±1.23	41.88±4.87	11.48±2.15^{y}	144.95±43.82	33.06±5.20	23.95±5.31
1.2%	M	2.78±0.69	37.70±4.45	10.60±1.54^{xy}	143.88±42.63	39.06±5.19	28.58±6.12
	F	3.24±0.12	42.25±7.42	11.61±0.18^{xy}	130.83±27.89	36.76±0.84	28.68±5.47
p fatty acids		0.992	0.512	0.537	0.797	0.349	0.592
p sex		0.088	0.095	0.336	0.757	0.149	0.471
p interaction		0.843	0.613	0.032	0.942	0.407	0.446

*Lowercase letters (a.b) represent significant difference between treatments;
**Lowercase letters (x.y) represent significant difference between the interaction (treatment x sex).
Legend of parameters: (Ht) Hematocrit; (Hb) Hemoglobin; (MCV) Mean Corpuscular Volume; (MCH) Mean Corpuscular Hemoglobin; (MCHC) Mean Corpuscular Hemoglobin Concentration. (M) male, (F) female.

The concentration of hemoglobin (Hb) in the blood of *S. brasiliensis* was significantly different among treatments regarding sex. The females fed the diet containing a 0.6% fatty acids concentration had significantly lower hemoglobin concentrations than males in the same treatment. Males fed the diet containing a 0.9% fatty acids concentration showed hemoglobin concentrations statistically equal to those of females in the 0.6% group but showed significantly lower hemoglobin concentrations than females in their own treatment group. The other treatments showed no significant difference between treatments regarding sex.

The other hematological parameters analyzed, which include the total number of erythrocytes (RBC), percentage of hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCH), thrombocytes (T), total leukocytes (LT) and differential leukocyte count (lymphocytes, monocytes, and neutrophils) showed no significant difference among the treatment groups and sexes. Table. 4 shows the glucose analysis results of the specimens after the addition of different concentrations of n-3 PUFA in the diet. No statistically significant differences in glucose levels were observed between treatments.

Table 4. Glucose analysis (mean ± standard deviation) by plasma of sardines (*Sardinella brasiliensis*) after addition of n-3 PUFA addition at concentrations of 0%, 0.3%, 0.6%, 0.9% and 1.2% of the dietary lipid fraction

Treatments	0%	0.3%	0.6%	0.9%	1.2%
Glucose (mg dL^{-1})	0.26 ± 0,02	3.23 ± 0.03	0.22 ± 0.01	0.25 ± 0,03	0.24 ± 0.04

DISCUSSION

Sardinella brasiliensis is an important species for commercial fisheries in the south and southeast regions of Brazil, but they are at risk of extinction due to overfishing. Thus, studies focusing on the aquaculture of this species are important, as they contribute to the maintenance and monitoring of *S. brasiliensis* under aquaculture conditions. Hematological parameters are commonly used as a health indicator in fish aquaculture. However, there are no studies on the hematological parameters of *S. brasiliensis* that indicate this study is as novel.

Among all the hematological parameters analyzed, only the number of eosinophils showed significant difference among treatments used in the current study. Although the function of these cells in fish are not fully understood, according to the previous literature, they participate in the immune defense system of fish, especially against parasitic infestations and infections by pathogens (Ranzani-Paiva *et al*., 2013).

A study conducted by Subhadra *et al*. (2006), in which the hematological parameters of *Micropterus salmoides* were measured after they were fed diets containing different concentrations and types of lipids, showed that the fish fed diets containing more than 4% n-3 fatty acids had a greater number of lymphocytes in their blood, which is not consistent with the present study, because there was no significant difference in the concentration of lymphocytes between treatments. In another study, in which the nutritional value of pigs fed a diet enriched with fish oil with 8% n-3 PUFA was evaluated, and a significant increase in the number of eosinophils was observed when compared to the control group of the study (Komprda *et al*., 2020). Although the present study demonstrated a higher number of eosinophils in the blood of sardines fed diets containing 0.3% and 1.2% n-3 PUFA, this increase was compared with the blood of sardines fed diets containing 0.6% and 0.9% n-3 PUFA. Even though control group did not show a significant difference in the number of eosinophils when compared to the other groups, it was not clear the association between the increase in the eosinophils number and the addition of n-3 PUFA to the diet.

To date, eosinophils are sensitive cells to the environment of an organism, and their concentration can vary in fish when they are exposed to different stimuli, as shown in the studies that observed greater numbers of eosinophils in the blood of fish during summer compared to that during winter (Chen and Luo, 2022), or when the fish were exposed to intense stimuli compared to normal conditions (Hosoki *et al*., 2012). Therefore, it should be noted that the experiment of the present study was conducted from November to December, when Brazil has higher average temperatures, which may have induced the increase in the number of eosinophils in the sardines' blood. Furthermore, it may be related to a type of eosinophilic immune response to the different concentrations of fatty acids in the experimental diet, which could be considered an unusual stimulus.

However, increased number of eosinophils does not always indicate an immunological response to an infection by a pathogen or a physiological response to an adverse situation, as shown in the case of *Megalancistrus acuelatus*, which has a naturally high number of eosinophils (Ranzani-Paiva and Eiras, 1992). Therefore, we can assume that this is also the case for the blood composition of sardines, as it is normal for this species to have a greater number of eosinophils in its differential leukocyte count.

Hemoglobin is a protein that transports oxygen, carbon dioxide, and other nutrients throughout the body (Bernard *et al*., 2000). The concentration of hemoglobin (Hb) in the blood of *S. brasiliensis* was lower in females, which may be because the fish already reached sexual maturity, but there are no conclusive results regarding this parameter.

CONCLUSION

The results obtained in this study suggest that the addition of n-3 PUFA at 0, 0.3, 0.6, 0.9, and 1.2% concentrations of the lipid fraction in Brazilian sardines did not negatively affect the health of the animals or their hematological parameters, because no significant differences between the treatments, compared to the control group (0% n-3 PUFA) were observed.

Considering the importance of *S. brasiliensis* as live bait for fishing, its current risk of extinction caused by overfishing, and the fact that the present study is the first to report the hematological analysis of the species. Further studies must be performed on the hematological parameters of the Brazilian sardine for more comprehensive and satisfactory conclusions.

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