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Hematological and phagocytic response of the fat snook, *Centropomus parallelus*, reared in net cages, before and after inoculation with *Sacharomyces ceresivisiae*

Hematologia e resposta fagocítica de robalo, Centropomus parallelus *criado em tanques rede, antes e após inoculação com* Sacharomyces ceresivisiae

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ABSTRACT

The aim of this study was to examine the blood and the macrophagocytic response of fat snook of *Centropomus parallelus* before and after injection with *Saccharomyces cerevisiae*. Ten adults were used, caught using net cages in the lagoon-estuarine region of *Cananéia (SP)*, Brazil. The fish were anesthetized and blood was drawn followed by inoculation with yeast. After 8 hours, the fish were again removed from the cages and anesthetized. A new sample of blood was collected and the animals were then sacrificed by deep sedation. The blood was used for the determination: erythrocyte count, hemoglobin concentration and hematocrit, calculation of Mean Corpuscular Volume and Mean Corpuscular Hemoglobin Concentration, and preparation of smears, utilized for total and differential leukocyte counts white blood cells and thrombocyte count. There was a significant decrease in almost all parameters 8hrs after inoculation ($p \leq 0.05$), with exception of mean corpuscular volume which showed a significant increase ($p > 0.05$). Leukopenia, lymphopenia, neutropenia and monocytosis occurred when comparing the values of the first and second samples, in both inoculated and non inoculated fish. With the white blood cells, lymphocytes and neutrophils, values were significantly higher in the inoculated fish versus

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those which were not inoculated, in both blood samplings. The number of thrombocytes was higher in inoculated than non inoculated individuals, but 8 hours after inoculation the values in the two groups were similar. The phagocytic capacity and index were: mean=56.0 and standard deviation=8.8% and mean= 1.8 standard deviation=0.3, respectively. In conclusion, it was verified that the repeated sampling of blood induces alterations that are harm to the welfare of the animals.

Key words: Phagocytosis. Hematology. Macrophage migration. Fat snook.

RESUMO

O objetivo deste trabalho foi verificar o sangue e a resposta macrofágica de robalo, Centropomus parallelus*, antes e após inoculação com* Sacharomyces cerevisiae*. Foram utilizados 10 exemplares, provenientes de tanques-redes da região estuarino-lagunar de Cananéia (SP), Brasil. De cada peixe foi retirado sangue para as análises hematológicas: número de células, taxa de hemoglobina), hematócrito, cálculo de Volume Corpuscular Médio e Concentração de Hemoglobina Corpuscular Média e contagem diferencial e total de leucócitos* white blood cells *e total de trombócitos antes e após 8h de inoculação com* Saccharomyces cerevisiae*. Cada animal foi medido, pesado, marcado e após a obtenção do sangue, retornou ao tanque. Após 8h foram anestesiados, colhido o sangue e sacrificados por sedação profunda. Ocorreu diminuição significativa na maioria dos valores hematológicos após 8 horas (p≤0.05), com exceção dos valores de Volume Corpuscular Médio que apresentaram aumento significativo (p>0,05). Ocorreu leucopenia, com linfopenia, neutropenia e monocitose comparando os valores iniciais com a segunda colheita, tanto dos indivíduos inoculados como dos não inoculados. Para* white blood cells, *linfócitos e neutrófilos, os valores dos inoculados foram significativamente superiores a aos dos não inoculados, tanto na primeira como na segunda colheita. Foi verificado que o valor de trombócitos dos indivíduos inoculados foi superior aos dos não inoculados e que depois de 8 horas estes valores diminuem e são semelhantes nos dois grupos. A capacidade e o índice fagocíticos foram média de 56,0, e desvio-padrão=8,8% e média=1,8 desvio-padrão=0,3, respectivamente. Em conclusão, verifica-se que a colheita repetida de sangue leva a alterações que prejudicam o bem-estar dos animais.*

***Palavras-chave**: Fagocitose. Hematologia. Migração de macrófagos. Robalo.*

INTRODUCTION

The importance of aquaculture and its utilization as a food resource sustains the fundamental economic basis for research aimed at maintaining homeostasis in fish. Thus, such investigations help achieve better yields to improve the fish industry, both for human food and ornamental fishes, and above all, provide for a better scientific understanding of fundamental biological processes. With regard to its role as a food resource, fish farming is an activity similar to the rearing of any other live organism, to the degree that the occurrence of any disease may render it unviable.

Some studies have been aimed at understanding the physiological processes of fish, principally with the determination of hematological parameters and the levels of biochemical components in plasma (Imagawa *et al.,* 1989; Ranzani-Paiva, 1995a b; Collazos *et al.,* 1998; Ranzani-Paiva & Silva-Souza, 2004; Kang *et al.*, 2005). Others have described the morphology of blood cells in fish and the cytochemistry of the components of circulating

blood cells (Veiga *et al.,* 2000, 2002), but few have dealt with the immunological response of these animals to some pathogens.

Among the blood cells, monocytes-macrophages play a fundamental role in defense, since they participate in practically all immune reactions against diseases and attacks in fish (Zelikoff *et al.,* 1991). These cells also participate in a wide variety of functions such as effector, helper and suppressor cells, in the acute as well as the chronic phase (Rowley & Ratcliffe, 1998). Macrophages are therefore considered, among the teleosts, the most efficient leukocyte type in the phagocytosis of pathogens and cellular debris resulting from the inflammatory process or degenerative processes (Bodamer & Robohm, 1996).

Besides the study of the inflammatory process, either occurring naturally or induced, some *in vitro* assays have been utilized in fish to demonstrate the phagocytic activity of macrophages of different species (Oliver *et al.,* 1986; Bennani *et al.,* 1995). One of the models utilized is the stimulation of phagocytic activity by the injection of yeast into the peritoneal cavity, which leads to phagocytosis by macrophages at this location. Studies indicate that this increase occurs via specific receptors, thanks to the presence of b-glucan, which is a weak antigen in vertebrates; however it is a potent stimulator of non-specific defenses (Iwama *et al.,* 1986).

The fat snook *Centropomus parallelus* is a fish of the family Centropomidae, order Perciformes, class Osteichthyes and is exploited commercially throughout the Americas where this fish occurs. Due to this, the species studied is of great economic importance, since it adapts well to rearing in net cages and serves largely as a source of food (Cerqueira, 2004).

The aim of this study was to examine the macrophagocytic response in blood *C. parallelus* reared in net cages in the lagoon-estuarine region of Cananeia, São Paulo, Brazil, after successive collection of blood from animals with and without injection of yeast into the peritoneal cavity, which served as an inflammatory agent.

MATERIAL AND METHODS

In this study, 800 specimens of *C. parallelus* were stocked in net cages of 5 x 5m and 2.5m in depth, located in the lagoon-estuarine region of *Cananeia*, state of *São Paulo*, Brazil. The fish were fed 3 times a day with fish food containing 45% crude protein.

In July 2005, 10 adult specimens, 14 months of age, were captured from one of the net cages and used for the collection of blood and inoculation of the peritoneal cavity with *Saccharomyces cerevisiae*, at a concentration of $8x10^3$ yeast/mm^3. The fish were anesthetized with 3% benzocaine, weighed (g) and measured (cm), and 0.5mL of blood was drawn by caudal puncture. After the initial drawing of blood, the fish were returned to the cages and, 8 hours after inoculation, they were removed again for a second blood sampling, after which they were sacrificed by deep sedation.

The blood samples drawn were utilized to determine the hematocrit, by the microhematocrit method, hemoglobin level, by the cyanomethemoglobin method, and total cell counts in a Neubauer chamber using 0.7% NaCl as diluent. The red blood cell indices, Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC), were calculated.

Slides of blood smears were made and then stained with May-Grünwald-Giemsa, as per Rosenfeld (1947). In addition to differential leukocyte counts, total leukocytes and thrombocytes were computed by the indirect method according to Hrube & Smith (1998).

After collecting the second blood sample, the fish were opened ventrally and the cavity was washed with saline solution 0.7%. The exudate was collected in an assay tube and centrifuged. The pelleted cells were placed on a slide with a coverslip and examined by phase contrast microscopy for counting macrophages. For each individual, 100 macrophages were counted and examined for engulfed yeast. The phagocytic capacity (PC=number of phagocytes with yeast/100 phagocytes) and phagocytic index

(PI=number of yeast in the phagocyte/number of phagocytes with yeast) were calculated.

Water temperature, transparency and salinity were recorded at the moment the fish were removed from the net cages.

The mean values for each hematological analysis were compared by considering inoculated versus non-inoculated individuals before and 8 hours after inoculation, as well as the total individuals inoculated versus the total not inoculated also before and after inoculation. Comparisons were made using the two-way variance analysis (ANOVA) and Student's *t*-test. The results were considered significant when $p \leq 0.05$ (Zar, 1996).

RESULTS

The fish utilized in this study had a total length ranging between 21.5 and 29.0 cm and a total weight between 104.0 and 276.0 g. The temperature of the water at the moment the fish were removed from the net cage was 210C, transparency 1.2m and salinity 15%, which were considered normal for the region and for fish farming (Cerqueira, 2004).

No mortalities occurred in the net cages during the entire fish-stocking period. The results of the hematological tests for the erythrocyte series are presented in Table 1. It may be noted that alterations occurred in the majority of the hematological parameters when comparing the two blood collections.

Statistical analysis showed that there was a significant decrease in almost all the hematological parameters 8 hours after inoculation ($p \leq 0.05$) and second blood sampling, with the exception of MCV which showed a significant increase at 8 hours after inoculation ($p \geq 0.05$). Hemoglobin level and erythrocyte count, at 8 hours after inoculation, were about 50% lower than initial values, regardless of inoculation status.

The numbers of total leukocytes, lymphocytes, neutrophils and monocytes in the peripheral blood of the fat snook are displayed in Table 2. It can be seen that leucopenia occurred, mainly due to lymphopenia and neutropenia, but also monocytosis, when comparing the second blood sampling to the initial one, both in inoculated and non- inoculated individuals.

At 8 hours after inoculation, there was a significant difference in values between the inoculated and non inoculated individuals, for total leukocytes, lymphocytes and monocytes ($p \leq 0.05$). With regard to the number of total leukocytes, lymphocytes and neutrophils, values for the inoculated individuals were significantly higher than those of the non-inoculated animals, in both blood samplings.

Table 1. Means (x) and standard-error of the mean (SEM) for hematological parameters of *C. parallelus*, before and at 8hours after inoculation with *S. cerevisiae,* and the total number of specimens.

		Ht	Ht 8h	Hb	Hb h	RBC	RBC 8h	MCV	MCV 8h	MCHC	MCHC 8h
Inoc	x	36.30^{A}	24.00^{B}	5.98^{A}	3.42^{D}	345.00^{A}	168.60^{B}	106.40^{A}	152.20^{B}	16.48^{A}	15.06^{AB}
n=5	SEM	0.66	0.54	0.18	0.20	21.00	20.23	5.08	27.72	0.48	0.45
Non inoc	x	33.50^{AD}	25.60^{BC}	6.17^{AC}	4.53^{B}	375.00^{AC}	233.00^{BD}	90.56^{AC}	112.13^{BC}	18.47^{AC}	17.96^{ACD}
n=5	SEM	2.46	5.54	0.41	0.30	20.53	25.66	5.05	10.80	0.52	0.67
		sig		sig		sig		sig		sig	
Total	x	34.90	24.90	6.07	3.97	360.00	204.40	98.48	129.98	17.48	16.67
n=10	SEM	1.28	1.35	0.21	0.80	11.98	19.47	4.29	14.50	0.47	1.94

* values in columns and lines followed by the same letters do not differ statistically.

Inoc: inoculated; n inoc: non-inoculated; Ht: hematocrit (%); Ht 8hrs: hematocrit 8 hours after inoculation; Hb: hemoglobin level (g/100L); Hb 8hrs: hemoglobin level 8 hours after inoculation; RBC: number of erythrocytes (104/mm^3); RBC 8hrs: number of erythrocytes 8 hours after inoculation; MCV: mean corpuscular volume (fL); MCV 8hrs: mean corpuscular volume 8 hours after inoculation; MCHC: mean corpuscular hemoglobin concentration (%); MCHC: mean corpuscular hemoglobin concentration 8 hours after inoculation, p=0.05; sig: statistical significance, p=0.05.

Table 2. Means (x) and standard error of the mean (SEM) for the total and differential leukocyte count and absolute number of thrombocytes of *C. parallelus*, before and after 8 hours inoculation of animals with *S. cerevisiae* and of the total specimens.

		Lc	Lc 8h	Lph	Lph 8h	Nt	Nt 8h	Mn	Mn 8h	Thr	Thr-8h
Inoc	x	75980.85^{A}	16340.38^{AB}	68437.34^{A}	9784.84^{AB}	6423.39^{A}	3527.20^{A}	1055.80^{A}	3028.35^{B}	35090.26^{A}	2629.05^{A}
n=5	SEM	1561.40	1773.20	1502.20	2582.60	911.53	1215.10	666.54	831.48	2197.30	1113.40
Non inoc	x	51213.24^{ABC}	16762.29^{BC}	42335.16^{AB}	13555.27^{AB}	6998.55^{A}	1949.99^{BC}	2014.80^{ABC}	1194.54^{A}	11092.82^{A}	6101.17^{A}
n=5	SEM	16240.00	5416.10	1364.30	4254.10	3375.00	733.16	953.60	604.55	6982.80	3341.90
		sig		sig		ns		sig		ns	
Total	x	63597.05	16551.34	55386.25	11670.05	6660.97	2738.60	1535.30	2111.45	2309.54	4365.11
n=10	SEM	11394.00	2687.40	1050.90	2428.70	1649.90	718.77	571.29	572.95	1158.10	1758.50

* Values in columns and lines followed by same letter did not differ statistically.

inoc.: inoculated; n inoc: non-inoculated; Lc: total leukocyte count; Lc 8hrs: total leukocyte count 8 hours after inoculation; Lph: number of lymphocytes; Lph 8hrs: number of lymphocytes 8 hours after inoculation; Nt: number of neutrophils; Nt 8hrs: number of neutrophils 8 hours after inoculation; Mn: number of monocytes; Mn 8hrs: number of monocytes 8 hours after inoculation; Thr: number of thrombocytes; Thr 8hrs: number of thrombocytes 8 hours after inoculation, sig: statistical significance; ns: non-significant, p=0.05.

Table 2 shows the number of thrombocytes in the peripheral blood of the fat snook for the two sampling times. It is seen that the value for inoculated individuals is higher than that for non-inoculated fish and that at 8 hours after inoculation, the values for the two groups are similar.

With regard to the migration and phagocytic activity of phagocytes, the peritoneal cavity of *C. parallelus* was found to be infiltrated with phagocytes, mainly neutrophils, 8 hours after inoculation when compared to the control group.

The results for mean capacity and the phagocytic index of phagocytes were: mean 56.0, standard deviation 0.8% and mean1.8, standard deviation 0.3 yeast/macrophages (Table 3). When comparing capacity and phagocytic index between stimulated individuals, proportionality between both can be noticed.

Table 3. Mean (x) and standard-error (SEM) of phagocytic activity of phagocytes of *C. parallelus*, 8 hours after inoculation with *S. cerevisiae*.

Fish	Phagocytic capacity (%)	Phagocytic index
1	66	2.1
2	47	1.5
3	55	2.0
4	50	1.6
5	69	2.0
X	56.0	1.8
SEM	8.8	0.3

DISCUSSION

The time of 8 hours after inoculation was chosen for this study because it was the duration which showed the highest phagocytic activity of macrophages and neutrophils in the peritoneal cavity of the fat snook.

The studies were found in the literature related to hematological analyses of fish undergoing two successive blood collections, yet the results presented here demonstrate that there were profound changes in the physiology of the animals, with a decrease in the number of blood cells and hemoglobin content. The significant increase in the MCV values indicates that there was a release of juvenile cells, with greater diameters but with the same hemoglobin concentration.

Soivio *et al.* (1974) demonstrated that the response of organisms to conditions of anoxia, as was the case here, is an increase in hematocrit, which can, after a certain period of time, return to initial values. These authors considered that the high Ht observed was due to cellular intumescence caused by the anoxia conditions resulting from the stress of handling.

In this study, where yeast cells were injected into the peritoneal cavity of the fat snook, there was a decrease in the number of leukocytes, particularly the lymphocytes and neutrophils, and also an

occurrence of monocytosis. This result conflicts with those found in literature that indicates that the acute inflammatory response is characterized by neutrophilia and monocytosis (Silva *et al.,* 1998; Ranzani-Paiva *et al.*, 2004). The decrease in the number of neutrophils shown in the present work could have been the result of a greater and more rapid migration of these cells toward the peritoneal cavity. Because of their physiological characteristics, the monocytes, on the other hand, could have had a slower migration toward the site of the injury, thereby inducing or increasing their production in the hematopoietic tissue. Kodama *et al.* (2002) demonstrated that inoculation of animals with Lipopolysaccharide (LPS) and Muramyl Dipeptide (MDP) can stimulate the formation of colonies of leukocytes in the cephalic kidney, especially of monocytes/macrophages.

The decrease in the number of thrombocytes in circulating blood was probably due to their migration to the site of the injury, according to the findings of Matushima & Mariano (1996) that these cells participate actively in the inflammatory process in fish.

These alterations, however, may be due to stress from handling the animals. Stress plays an important role in the suppression of inflammatory cells, with deleterious effects on resistance to infections (Wedemeyer, 1970; Saad *et al.*, 1973). Bouck & Ball (1966) reported a high mortality in *Salmo gairdneri* after catching and handling. These authors, however, attributed the deaths to shock and peripheral coagulation, after the apparent diminution of fibrinogen in the blood and increase in the number of thrombocytes, 2 or 3 days after the incidence of stress.

The data on the migration and phagocytic activity of macrophages, corroborate the findings from hematological assays demonstrating neutrophilia and monocytosis, thereby characterizing a dual cellular response.

When comparing capacity and the phagocytic index of phagocytes between stimulated individuals, it is noted that phagocytic activity is proportional to the phagocytic index. The proportionality between capacity and phagocytic index of phagocytes of stimulated individuals suggests that the pattern of phagocytic activity of phagocytes in fish should be considered individually (Pulsford *et al.,* 1984; Blazer *et al.,* 1987; Blazer, 1991).

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