

Vitamin C and endogenous cortisol in foreign-body inflammatory response in pacus

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Abstract – The objective of this work was to evaluate the effect of food supplementation with vitamin C on macrophage and multinucleated giant cell (MGC) activities of pacus at two stocking densities. The experiment was carried out in a 2x2x3 split-plot factorial arrangement with: 0 and 500 mg kg⁻¹ vitamin C; 5 and 20 kg m⁻³ stocking densities; and evaluation times at 3, 6, and 12 days after the subcutaneous implantation of glass coverslips (DPI). The number of macrophages and MGC, as well as cortisol and glucose plasma levels were determined. The number of macrophages and MGC with two to five nuclei was significantly greater in fish supplemented with vitamin C at 5 kg m⁻³ stocking density at 3 DPI in comparison to nonsupplemented ones. The macrophage and MGC counts were lower in fish with high-plasma cortisol concentration. Supplementation with 500 mg vitamin C benefits macrophage activity on foreign-body inflammation, and high-cortisol concentration has suppressive effects on this response.

Index terms: *Piaractus mesopotamicus*, ascorbic acid, glucocorticoid, inflammation, Langhans cells, multinucleated giant cells.

Vitamina C e cortisol endógeno em resposta inflamatória tipo corpo estranho em pacus

Resumo – O objetivo deste trabalho foi avaliar o efeito da suplementação com vitamina C no aumento da atividade de macrófagos e células gigantes multinucleadas (CGM), em pacus mantidos em duas densidades de estocagem. O experimento foi realizado em parcelas subdivididas, em arranjo fatorial 2x2x3 com: 0 e 500 mg kg⁻¹ de vitamina C; densidade de estocagem de 5 e 20 kg m⁻³; e tempos de avaliação de 3, 6 e 12 dias após o implante subcutâneo (DPI) de lâminulas de vidro. Foram determinados o número de macrófagos e de CGM, bem como os níveis de cortisol e glicose no plasma. O número de macrófagos e de CGM com 2 a 5 núcleos foi significativamente maior nos peixes suplementados com vitamina C, à densidade 5 kg m⁻³, aos 3 DPI, em relação aos não suplementados. Constatou-se menor quantidade de macrófagos e CGM em peixes com baixa concentração plasmática de cortisol. A suplementação com 500 mg de vitamina C por quilograma de ração beneficia a atividade de macrófagos em inflamações do tipo corpo estranho, e a elevada concentração de cortisol circulante tem efeito supressor nesta resposta.

Termos para indexação: *Piaractus mesopotamicus*, ácido ascórbico, glicocorticoide, inflamação, células de Langhans, células gigantes multinucleadas.

Introduction

Fish supplementation with vitamin C prevents the harmful effects of stress, minimizes the toxicity of contaminants in the water and increases the efficiency of defense mechanisms and wound healing (Martins, 1998; Tewary & Patra, 2008). There is evidence that it causes increases in the concentration of serum

antibodies (Kumari & Sahoo, 2006; Nayak et al., 2007) and in phagocytosis, although the mechanisms are unclear (Tewary & Patra, 2008).

Multinucleated giant cells (MGC) have been described in fish with bacteriosis (Olsen et al., 2006; Swaim et al., 2006; Jacobs et al., 2009), parasitosis (Adriano et al., 2005; Hogge et al., 2008), and fungus (Sosa et al., 2007; Oidtmann et al., 2008), with in

vitro (Couso et al., 2002) and in vivo models (Petric et al., 2003; Belo et al., 2005). Inflammatory cell accumulation is the main event that protect organism against foreign elements (Kumar et al., 2004). The macrophage activities are regulated by chemical mediators (Kumar et al., 2004).

Giant cells with nuclei randomly distributed in the cytoplasm are considered foreign-body giant cells. With maturation, the nuclei become organized in the periphery of the cytoplasm, forming Langhans cells (MacLauchlan et al., 2009). Petric et al. (2003) showed that implantation of glass coverslips, in the subcutaneous tissue of *Piaractus mesopotamicus*, induces the formation of MGC, initially as foreign-body giant cells and, later on, as Langhans' type.

When organisms are exposed to different types of stressful stimulation, hypersecretion of cortisol occurs, along with changes in homeostasis, which puts health at risk (Mommsen et al., 1999). High-plasma cortisol concentrations can negatively modulate the defense mechanisms (Belo et al., 2005), thereby increasing the susceptibility to diseases (Barton & Iwama, 1991). At high-stocking densities, the cortisol concentration in the plasma is increased, with higher mortality in situations of challenge by *Aeromonas salmonicida* and *Vibrio* sp., in rainbow trout (*Oncorhynchus mykiss*) (Fevolden et al., 1992).

There is little information regarding the influence of vitamin C on the activity of macrophages in chronic inflammation of fish associated to high-density stress in *P. mesopotamicus*.

The objective of this study was to evaluate the effect of food supplementation with vitamin C on macrophage and multinucleated giant cell (MGC) activities of *P. mesopotamicus* at two stocking densities.

Materials and Methods

Pacu juveniles (105.89 ± 15.09 g) from the same spawning were randomly distributed into two cement tanks of 18 m^3 , supplied with running water (1.0 L s^{-1} ; $n = 300$ fish). The experiment was carried out in a split plot $2 \times 2 \times 3$ factorial arrangement: two concentrations of vitamin C – ascorbyl polyphosphate – at 0 and 500 mg kg^{-1} ; two stocking densities (5 and 20 kg m^{-3}); and three evaluation times (three, six and twelve days) after the subcutaneous implantation of glass coverslips.

Fish in each tank started to receive commercial feed, with or without vitamin C, fifteen days after their arrival in two cement tanks, and this continued for 60 days. Feed in one tank was supplemented with 500 mg kg^{-1} vitamin C – ascorbyl polyphosphate, 35% activity –, (Roche, São Paulo, Brazil), the other tank received no supplementation. The pacus in each tank were divided into six aquaria of 500 L each (a total of 12 aquaria). The aquaria were supplied with running water, without chlorine, from an artesian well (1.0 L min^{-1}), with supplementary aeration and natural photoperiods. Three of the aquaria were at 5 kg m^{-3} stocking density, and the other three were at 20 kg m^{-3} stocking density, for each treatment. The groups were maintained under these conditions for 15 days before the implantation of coverslips. After this, seven pacus from each aquarium (42 per treatment) were evaluated at three, six and twelve days. During the experiment, pH (7.16 ± 0.91), dissolved oxygen concentration ($4.27 \pm 0.94 \text{ mg L}^{-1}$), and water temperature ($26.39 \pm 1.51^\circ\text{C}$) in the aquaria were measured daily, in order to keep them suitable for pacu farming (Castagnolli & Pinto, 1992).

The commercial feed (28% of crude protein; $4,000 \text{ kcal kg}^{-1}$ digestible energy) was ground up, and 500 mg of ascorbyl polyphosphate at 35% activity per kg was added (Martins, 1998), using a Y mixer. Feed was pelleted (65°C) at an adequate diameter for fish, and stored in dark plastic bags at -4°C , until the time for use. At the end of the experiment, vitamin C concentration in the feed was determined by titration (Horwitz et al., 1975; Pregnotatto & Pregnotatto, 1985), by which, nonsupplemented feed contained 66.5 mg kg^{-1} of ascorbyl polyphosphate, and the supplemented feed contained 554.4 mg kg^{-1} of ascorbyl polyphosphate.

Fish were anesthetized (aqueous solution of benzocaine 1:10,000) and, then, underwent antiseptic cleansing and removal of scales. A skin incision was made and the subcutaneous tissue was pulled back. A rounded glass coverslip, with 13 mm diameter, was placed in the subcutaneous tissue of the lateral-dorsal region, behind the operculum. Skin was sutured using nylon thread and the fish were returned to their original aquaria (Petric et al., 2003).

Three, six and twelve days after the implantation, seven fish from each group were randomly chosen for euthanasia by means of deep anesthesia (aqueous solution of benzocaine 1:500). The coverslips were

then removed and washed with a 0.65% saline solution. They were fixed in a Bouin solution for 5 min and stained with hematoxylin-eosin. The macrophages thus isolated, along with the polykaryons (giant cells) with their nuclei, were counted using an image analyzer Videoplan KS100, (Carl Zeiss, São Paulo, SP Brazil), in ten randomly chosen fields, using a 400X magnification.

For the collection of blood samples at the post-implantation evaluation times, fish were briefly removed from the aquaria and blood was withdrawn into a syringe from a caudal vessel by needle puncture. Blood was immediately dispensed into two sets of tubes with EDTA for separate analysis of cortisol and glucose concentrations in the plasma. Blood was centrifuged at 1,700 g, at 4°C, for 10 min, and plasma was either stored at -20°C, for cortisol analysis, or processed immediately for glucose determination. Cortisol concentrations were determined by radioimmunoassay using a DPC Coat-a-Count Kits, (Diagnostic Products Corporation, Los Angeles, CA, USA) (Gazola et al., 1996). Glucose concentrations were assessed by colorimetric assay, as described by King & Garner (1947).

All data were transformed by $\log(x + 1)$ and statistically studied using a completely randomized design. Means were compared by Tukey's test, at 5% probability, using the general linear model (GLM) procedure (SAS Institute, 2001). Correlations between cortisol values and cell counts on the coverslips were made using Spearman's test (GraphPad Software, 2005).

Results and Discussion

Macrophage recruitment through MGC formation was clearly identified along the evaluation times. Macrophage accumulation and MGC formation showed that the number of cells increased significantly over time. MGC formation depends on daily recruitment of monocytes from the reserve compartments of blood. After diapedesis and transformation into macrophages, these cells reach the inflammation focus by means of chemotaxis, in which interleucines, such as IL-4 and IL-13, induce macrophage fusion (Brodbeck & Anderson, 2009) and interferon-gamma participate on Langhans giant cell formation (Anderson et al., 2008).

As a result of the substantial accumulation of macrophages isolated on the coverslips, formation of

foreign-body MGC, with more than ten nuclei, and of Langhans polykaryons was observed on the 12th day (Figure 1). There was a prominent increase in the number of MGC between the 6th and 12th days, thus showing the evolution of the chronic inflammation (Table 1). These findings are in accordance with results described by Petric et al. (2003) and Belo et al. (2005).

There was no difference in the counts of Langhans cells between the different times, except for the treatments with 5 kg m⁻³ stocking density, which had higher counts at 12 DPI (Table1).

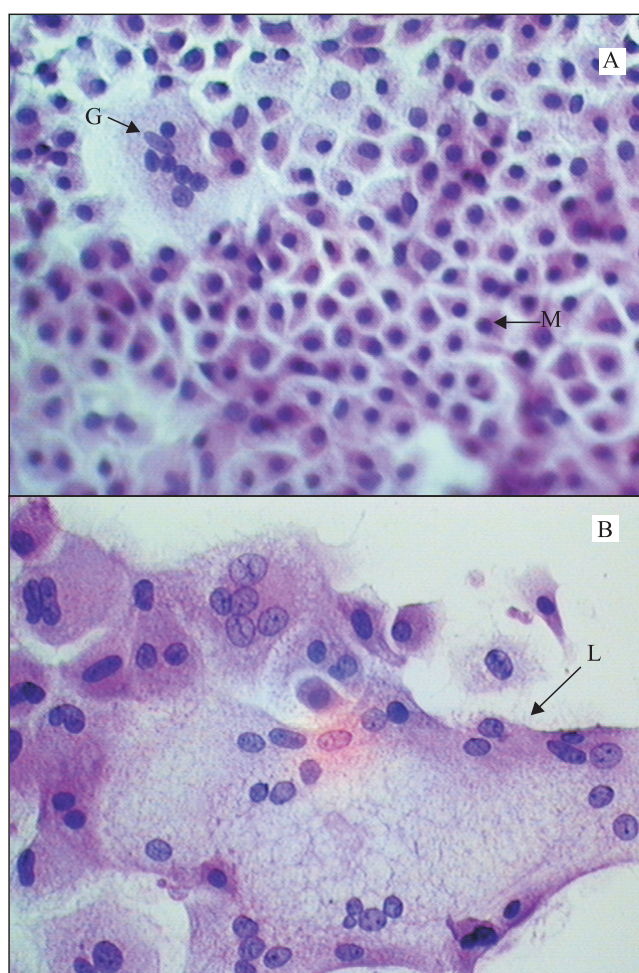


Figure 1. Photomicrograph of: A, macrophages (M) and foreign-body giant cells (G) on a glass coverslip, six days after implantation – staining with hematoxylin-eosin; objective-magnification 40X –; and B, photomicrograph of Langhans cell (L) on a glass coverslip, 12 days after sacrificing the fish, at 100X magnification. Note the beginning of nucleus organization at the periphery of the cell.

There was a clear stimulating effect of ascorbyl polyphosphate on macrophage accumulation and MGC formation (Table 1). Three days after implantation, although not significantly different from the other treatments, the greatest number of macrophages and polykarions with 2–5 nuclei occurred among fish supplemented with ascorbyl polyphosphate at a 5 kg m⁻³ density. Moreover, only this treatment showed any MGC with 6–10 nuclei. On the 6th day, the smallest accumulation ($p < 0.001$) of MGC was observed in the treatment supplemented with ascorbyl polyphosphate, at 20 kg m⁻³ stocking density, thus showing an inhibition of the inflammatory response because of the greater density.

Fish supplemented with ascorbyl polyphosphate at 5 kg m⁻³ density showed greater ($p < 0.05$) formation of Langhans multinucleated giant cells, in comparison with the other treatments, on the 12th day (Table 1). Macrophage accumulation and MGC formation suggests that feed supplementation with 500 mg kg⁻¹ ascorbyl polyphosphate increased the macrophage activity on the inflammation, thereby speeding up the process, and making it easier and quicker for Langhans giant cell formation to occur. The macrophage accumulation and MGC formation increased

progressively until the 12th day after the implantation, thus corroborating the findings of Petric et al. (2003). Some authors have studied the effect of vitamin C ingestion on the defense mechanism of fish. *Sparus aurata*, fed a vitamin C-supplemented diet, exhibited an increased hemolytic activity of complement, an increased phagocytic leucocyte function, an enhancement of acidophilic granulocyte activity, and migration of leucocytes from the head kidneys (Mulero et al., 1998). According to Tewary & Patra (2008), different haematological and serological parameters, along with nonspecific immune parameters, were influenced by vitamin C supplementation. Among the nonspecific immune parameters, phagocytic and respiratory burst activities were significantly enhanced by increasing doses of vitamin C supplementation.

There was no significant difference between the plasma cortisol concentration and blood glucose from the different treatments and evaluation times. Also, no correlation between cortisol and blood glucose values was observed, and hyperglycemic effect of the hormone was not observed in the present study. According to Tort (2011), the glucocorticoid effects of cortisol are more easily observed under conditions of acute stress because, in chronic stress, the drawdown

Table 1. Mean values⁽¹⁾ and results from variance analysis⁽²⁾ on the counts of mononuclear, multinuclear, and Langhans inflammatory cells in pacus (*Piaractus mesopotamicus*).

Vitamin C (mg kg ⁻¹)	Stocking density (kg m ⁻³)	Number of nuclei				Langhans cells
		1	2–5	6–10	>10	
3 days						
0	20	0.51Bb	0.15Ab	0.00Ab	0.00Ab	0.00Aa
0	5	0.75Bb	0.04Ac	0.00Ab	0.00Ab	0.00Ab
500	20	0.28Bb	0.09Ac	0.00Ab	0.00Ab	0.00Aa
500	5	2.39Aa	0.71Ab	0.14Ab	0.00Ab	0.00Ab
6 days						
0	20	3.37Aa	2.00Aa	0.84Aa	0.55Aa	0.00Aa
0	5	2.59ABa	1.40ABb	0.47Aa	0.29Aa	0.00Ab
500	20	2.03Bab	0.84Bb	0.37Aab	0.11Aab	0.00Aa
500	5	3.19ABa	1.40ABb	0.23Ab	0.36Aa	0.00Ab
12 days						
0	20	3.05Aa	2.25Aa	0.73Aa	0.22Aab	0.26Ba
0	5	3.39Aa	2.53Aa	0.45Aa	0.26Aa	0.39Ba
500	20	3.44Aa	2.18Aa	0.93Aa	0.59Aa	0.11Ba
500	5	3.48Aa	2.93Aa	0.97Aa	0.47Aa	0.90Aa
F		4.91 **	2.12 *	4.91 **	2.35 *	5.98 **
CV (%)		30.97	42.89	88.51	124.36	154.32

⁽¹⁾Mean values (n = 7) transformed log (x + 1). ⁽²⁾Lowercase letters compare evaluation times within treatments; and capital letters compare treatments within evaluation time. ⁽³⁾Means followed by equal letters, in the columns, do not differ, by Tukey's test, at 5% probability. * and **Significant at 5 and 1% probability, respectively.

and consumption of glucose reserves reduce the effects of their mobilization in neoglycogenic and glycogenic processes.

However, pacus that showed low-plasma cortisol concentration had greater macrophage and polykaryon counts, showing a negative correlation between these traits (Figure 2). Belo et al. (2005) observed a similar effect among pacus supplemented with vitamin E and maintained at 20 kg m⁻³ stocking density; a significant reduction in macrophage count and MGC formation were observed in some of these animals with high concentrations of corticosteroid in the plasma. These results confirm that cortisol has an inhibitory effect on chronic inflammation, caused by foreign giant cells in pacus. In the present study, among fish maintained at 20 kg m⁻³ without supplementation, the highest cortisol concentrations coincided with the lowest level of MGC formation. In fish supplemented with the ascorbyl polyphosphate and at 20 kg m⁻³ stocking density, the plasma cortisol concentration was lower, which, at least partly, favored cell accumulation on the coverslip. In teleost fish, the glucocorticoid activity of cortisol has proved to suppress immune defense responses, acting mainly on the lymphocyte populations which coordinate the release of inflammatory mediators such as cytokines (Reque et al., 2010). However, pacus supplemented with ascorbyl polyphosphate at 5 kg m⁻³ stocking density showed a greater formation of Langhans cells, which have greater activity of acid phosphatase and succinic hydrogenase, a better developed Golgi system, and more orderly distribution of cell organelles (Anderson et al., 2008).

Increases in plasma cortisol concentration are an indicator of stress among teleost fish (Alsop & Vijayan, 2009). In several species of fish, crowding has been reported to be an aquaculture-related chronic stress factor that reduces growth and affects the inflammatory responses (Belo et al., 2005). However, in situations of chronic stress due to high density, adaptation is possible (Tort, 2011). Differences in time and quantity of cortisol release between different fish species, which were administered different stimuli, have been shown (Alsop & Vijayan, 2009). Differences in the basal values of cortisol were observed among pacus in the present study, in comparison to the plasmatic concentration described by Belo et al. (2005). In culture systems, fish are subjected to environmental variation, different diets and stressful procedures, which may affect their physiological status. All environmental

parameters regarding water in the fish tanks used in these studies varied within normal limits for pacus (Ayroza & Scorvo, 2011).

In the present study, fish that were not supplemented with ascorbyl polyphosphate and which were not kept at 20 kg m⁻³ stocking density showed greater variations for cortisol concentrations in the plasma, and the effect of this steroid on their cell response was clear. Fish at 5 kg m⁻³ stocking density showed a more

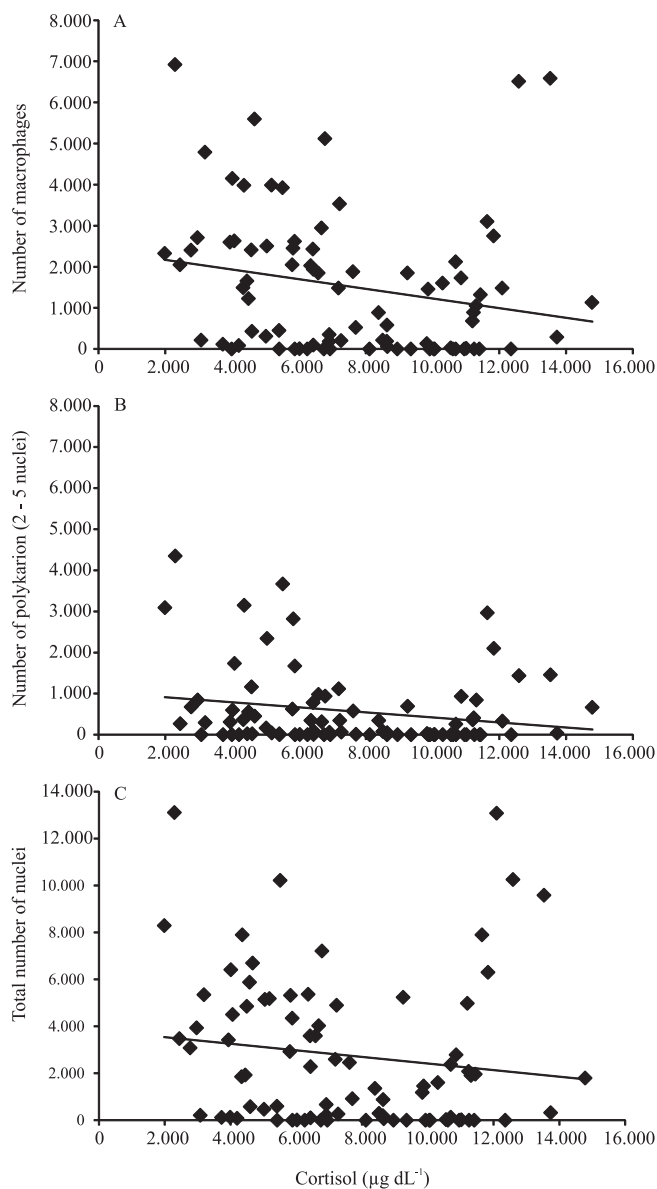


Figure 2. Spearman's linear correlation between plasma cortisol concentration and number of: A, macrophages ($r = -0.29$, $n = 84$, $p = 0.0066$); B, polykaryon of 2–5 nuclei ($r = -0.21$, $n = 84$, $p = 0.0585$); and C, total number of nuclei ($r = -0.23$, $n = 84$, $p = 0.0340$) counted on the coverslips.

constant plasma-cortisol concentration. However, in supplemented fish maintained at 5 kg m⁻³, there was a greater formation of Langhans cells, in comparison to the other groups, suggesting that the supplementation had, at least partly, contributed towards the increase in macrophage response associated with lower-plasma cortisol concentration. Moreover, there was a greater accumulation of MGC in a shorter time, which corroborates the results of Petric et al. (2003).

Conclusion

Dietary supplementation with 500 mg kg⁻¹ of ascorbyl polyphosphate in the feed benefits the macrophage activity on foreign-body inflammation; and high-plasma cortisol concentration has an inhibitory effect on this response.

Acknowledgements

To Francisca de Assis Ardisson and Maria Inês Yamazaki de Campos, for technical services.

References

- ADRIANO E.A.; ARANA, S.; CORDEIRO, N.S. An ultrastructural and histopathological study of *Henneguya pellucida* n. sp. (Myxosporea: Myxobolidae) infecting *Piaractus mesopotamicus* (Characidae) cultivated in Brazil. **Parasite**, v.12, p.221-227, 2005.
- ALSOP, D.; VIJAYAN, M. The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. **General and Comparative Endocrinology**, v.161, p.62-66, 2009.
- ANDERSON, J.M.; RODRIGUEZ, A.; CHANG, D.T. Foreign body reaction to biomaterials. **Seminars in Immunology**, v.20, p.86-100, 2008.
- AYROZA, D.M.M.R.; SCORVO, C.M.D.F. A qualidade da água para fins de piscicultura. In: AYROZA, L.M. da S. (Coord.). **Piscicultura**. Campinas: CATI, 2011. 246p. (CATI. Manual técnico, 79).
- BARTON, B.A.; IWAMA, G.K. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. **Annual Review of Fish Disease**, v.1, p.3-26, 1991.
- BELO, M.A.A.; SCHALCH, S.H.C.; MORAES, F.R.; SOARES, V.E.; OTOBONI, A.; MORAES, J.E.R. Effect of dietary supplementation with vitamin E and stocking density on macrophage recruitment and giant cell formation in the teleost fish, *Piaractus mesopotamicus*. **Journal of Comparative Pathology**, v.133, p.146-54, 2005.
- BRODBECK, W.G.; ANDERSON, J.M. Giant cell formation and function. **Current Opinion of Hematology**, v.16, p.53-57, 2009.
- CASTAGNOLLI, N.; PINTO, M.L.G. **Piscicultura**. 3.ed. Jaboticabal: Funep, 1992. 117p.
- COUSO, N.; CASTRO, R.; NOYA, M.; OBACH, A.; LAMAS, J. Formation of short-lived multinucleated giant cells (MGCS) from cultured gilthead seabream macrophages. **Anatomical Record**, v.267, p.204-212, 2002.
- FEVOLDEN, S.E.; REFSTIE, T.; ROED, K.H. Disease resistance in rainbow trout (*Oncorhynchus mykiss*) selected for stress response. **Aquaculture**, v.104, p.19-29, 1992.
- GAZOLA, R.; BONELLA, M.I.; DONALDSON, E.M.; VALSELLA, M.V.; FAVA DE MORAES, F.; BERNARDINO, G. Plasma steroid and corticosteroid levels in female pacu *Piaractus mesopotamicus* Teleostei-Characidae. **Brazilian Journal of Medical Biology and Research**, v.29, p.659-664, 1996.
- GRAPHPAD SOFTWARE. **GraphPad Prism for Windows**. Version 5.0. San Diego: GraphPad Software, 2005.
- HOGGE, C.I.; CAMPBELL, M.R.; JOHNSON, K.A. A new species of myxozoan (Myxosporea) from the brain and spinal cord of rainbow trout (*Oncorhynchus mykiss*) from Idaho. **Journal of Parasitology**, v.94, p.218-222, 2008.
- HORWITZ, W.; SENZEL, A.; REYNOLDS, H.; PARK, D.L. (Ed.). **Official methods of analysis of the Association of Official Analytical Chemists**. 12th ed. Washington: Association of Official Analytical Chemists, 1975. 1094p.
- JACOBS, J.M.; RHODES, M.R.; BAYA, A.; REIMSCHUESSEL, R.; TOWNSEND, H.; HARRELL, R.M. Influence of nutritional state on the progression and severity of mycobacteriosis in striped bass *Morone saxatilis*. **Diseases of Aquatic Organisms**, v.87, p.183-197, 2009.
- KING, E.J.; GARNER, R.J. The colorimetric determination of glucose. **Journal of Clinical Pathology**, v.1, p.30-44, 1947.
- KUMAR, V.; ABBAS, A.K.; FAUSTO, N. **Robbins and Cotran: pathologic basis of disease**. 7th ed. Milano: Elsevier, 2004. 1552p.
- KUMARI, J.; SAHOO, P.K. Dietary immunostimulants influence specific immune response and resistance of healthy and immunocompromised Asian catfish *Clarias batrachus* to *Aeromonas hydrophila* infection. **Disease of Aquatic Organisms**, v.70, p.63-70, 2006.
- MACLAUHLAN, S.; SKOKOS, E.A.; MEZMARICH, N.; ZHU, D.H.; RAOOF, S.; SHIPLEY, J.M.; SENIOR, R.M.; BORNSTEIN, P.; KYRIAKIDES, T.R. Macrophage fusion, giant cell formation, and the foreign body response require matrix metalloproteinase 9. **Journal of Leukocyte Biology**, v.85, p.617-626, 2009.
- MARTINS, M.L. Evaluation of the addition of ascorbic acid to the ration of cultivated *Piaractus mesopotamicus* (Characidae) on the infrapopulation of *Anacanthorus penilabiatus* (Monogenea). **Brazilian Journal of Medical and Biological Research**, v.31, p.655-658, 1998.
- MOMMSEN, T.P.; VIJAIAN, M.M.; MOON, T.W. Cortisol in teleost: dynamics, mechanisms of action, and metabolic regulation. **Reviews in Fish Biology and Fisheries**, v.9, p.211-268, 1999.
- MULERO, V.; ESTEBAN, M.A.; MESEGUER, J. Effects of in vitro addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. **Veterinary Immunology and Immunopathology**, v.66, p.185-199, 1998.

- NAYAK, S.K.; SWAIN, P.; MUKHERJEE, S.C. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). **Fish and Shellfish Immunology**, v.23, p.892-896, 2007.
- OIDTMANN, B.; STEINBAUER, P.; GEIGER, S.; HOFFMANN, R.W. Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. **Disease of Aquatic Organisms**, v.82, p.195-207, 2008.
- OLSEN, A.B.; MIKALSEN, J.; RODE, M.; ALFJORDEN, A.; HOEL, E.; STRAUM-LIE, K.; HALDORSEN, R.; COLGUHOUN, D.J. A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. **Journal of Fish Disease**, v.29, p.307-311, 2006.
- PETRIC, M.C.; MORAES, F.R. de; MORAES, J.R.E. de. Kinetics of polycarion macrophage formation in granulomatous inflammatory response of *Piaractus mesopotamicus* Holmberg, 1887 (Osteichthyes: Characidae). Experimental model. **Boletim do Instituto de Pesca**, v.29, p.95-100, 2003.
- PREGNOLATTO, W.; PREGNOLATTO, N.P. (Coord.). **Normas analíticas do Instituto Adolfo Lutz: métodos químicos e físicos para análise de alimentos**. 3.ed. São Paulo: Instituto Adolfo Lutz, 1985. 533p.
- REQUE, V.R.; MORAES, J.R.E. de; BELO, M.A.A.; MORAES, F.R. de. Inflammation induced by inactivated *Aeromonas hydrophila* in Nile tilapia fed diets supplemented with *Saccharomyces cerevisiae*. **Aquaculture**, v.300, p.37-42, 2010.
- SAS INSTITUTE. **SAS/STAT user's guide**. Version 8.2. Cary: SAS Institute, 2001. 3809p.
- SOSA, E.R.; LANDSBERG, J.H.; KIRYU, Y.; STEPHENSON, C.M.; CODY, T.T.; DUKEMAN, A.K.; WOLFE, H.P.; VANDERSEA, M.W.; LITAKER, R.W. Pathogenicity studies with the fungi *Aphanomyces invadans*, *Achlya bisexualis*, and *Phialemonium dimorphosporum*: induction of skin ulcers in striped mullet. **Journal of Aquatic Animal Health**, v.19, p.41-48, 2007.
- SWAIM, L.E.; CONNOLLY, L.E.; VOLKMAN, H.E.; HUMBERT, O.; BORN, D.E.; RAMAKRISHNAN, L. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. **Infection and Immunity**, v.74, p.6108-6117, 2006.
- TEWARY, A.; PATRA, B.C. Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of *Labeo rohita* (Ham.). **Fish Physiology and Biochemistry**, v.34, p.251-259, 2008.
- TORT, L. Stress and immune modulation in fish. **Developmental and Comparative Immunology**, v.35, p.1366-1375, 2011.

Received on April 11, 2011 and accepted on June 4, 2012