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Deleterious effects of low level of vitamin E and high stocking density on the hematology response of pacus, during chronic inflammatory reaction

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ABSTRACT

The effects of dietary supplementation with 12.6, 58.2 and 310.4 mg of vitamin E/kg dry diet on the hematology response of pacus (*Piaractus mesopotamicus*), submitted to different stocking densities (5 kg/m^3 and 20 kg/m^3), were investigated during chronic inflammatory reaction. After a feeding period of 18 weeks, pacu juveniles were anesthetized for inserting round glass coverslips 13 mm in diameter into the subcutaneous connective tissue. 126 fish from 18 groups (i.e., seven fish per aquaria) were sampled for blood collection 2, 7 and 15 days post-implantation to determine levels of plasma cortisol, erythrocyte, thrombocyte and leucocyte counts. In the presence of chronic inflammation, pacus fed with 12.6 mg/kg of vitamin E and kept in high stocking density (20 kg/m^3) resulted in increased number of circulating red blood cells and hematocrit percentage associated with microcytosis. Thrombocytosis and neutrophilia were observed in the acute phase of pacu defense response. However, it was found that a significant increase in monocyte counts and decrease in thrombocyte, neutrophil and lymphocyte counts in animals were maintained at high stocking density. Pacus fed with 12.6 mg of vitamin E/kg of dry diet presented low number of lymphocytes and LG-PAS+, and these animals showed elevated cortisol levels associated to low counts of monocytes.

1. Introduction

Certain substances added to fish diet favor the defense mechanisms, among them are highlighted vitamins C and E, levamisole, yeast *Saccharomyces cerevisiae* and essential fatty acids n-3 and n-6 (Belo et al., 2005, 2012a; Garcia et al., 2007; Reque et al., 2010; Sakabe et al., 2013; Salvador et al., 2012). The findings observed in these studies provide a promising perspective for prophylactic managements in fish farms (Reque et al., 2010; Salvador et al., 2012).

Vitamin E protects cell membranes against lipid peroxidation and in accordance with Huang et al. (2003) it is essential to maintain the flow of nutrients in phagocytes. In vitamin E deficiency, membrane deformation and anemia have been observed due to oxidative damage to red blood cells with increase of erythrocyte osmotic fragility and impairment of blood viscosity in severe cases (Ambali et al., 2010). In this context, hematological evaluation provides important subsidies for the diagnosis and prognosis of morbid conditions in animal populations (Belo et al., 2009, 2013).

High stocking density in intensive fish rearing represents an important condition for the occurrence of chronic stress, which affects the function of reproduction and growth and depresses the immune response of several species of teleost (Belo et al., 2005; Fujimoto et al., 2005, 2007). According to Tort (2011), cortisol in teleost fish is the main endogenous corticosteroids and this hormone modulates pro and anti-inflammatory cytokine expression in stressful situations.

This study investigated the effect of dietary supplementation with 12.6, 58.2 and 310.4 mg of vitamin E/kg dry diet on the hematology response of pacus, reared in two stocking densities (5 kg/m^3 and 20 kg/m^3), during chronic inflammatory reaction.

2. Material and methods

2.1. Experimental design

810 pacus, *Piaractus mesopotamicus* (96.42 ± 25.23 g SD) from the same spawning, were initially distributed in three ponds of 51 m^3 (270 fish per pond; flow rate of 1 L/s), fed with dry diets supplemented with 12.6, 58.2 and 310.4 mg of vitamin E/kg dry diet for 16 weeks. Afterwards, fish from each pond were randomly redistributed between six aquaria (i.e., 18 aquaria in all) with 200 L (flow rate 1 L/min), three with low stocking density (5 kg/m^3) and three with high stocking

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density (20 kg/m^3). Pacus were allowed to acclimatize to the new environment for 15 days before implantation of coverslips.

2.2. Coverslip implantation

The fish were anesthetized in 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. The glass coverslip (rounded, with a diameter of 13 mm) was placed in the subcutaneous tissue of the lateral–dorsal region, behind the operculum. The skin was sutured using nylon thread and the fish were returned to their original aquariums (Belo et al., 2012a).

2.3. Blood samples

Two, seven and 15 days post-implantation (DPI), seven fish per aquaria, were anesthetized with benzocaine solution (1:20,000) diluted in ethanol −98° (0.1 g/mL) (Wedemeyer, 1970) and blood samples were collected from the caudal vessel in heparinized tubes and centrifuged at 3000 rpm (1700 *g* at 4 °C) for 10 min, stored at −20 °C for cortisol determination by radioimmunoassay using DPC-kit (Belo et al., 2005). Other set of samples were collected in tubes containing EDTA for erythrocyte count through an automatic blood cell counter (Model CC510, CELM), previously calibrated for counting nucleated red blood cells. Differential leukocyte count was performed on blood smears stained with May–Grünwald–Giemsa–Wright (Tavares-Dias and Moraes, 2003), counting 200 cells. For the quantification of total leukocytes and thrombocytes the number of erythrocytes, leukocytes and thrombocytes in 10 fields for each blood smear were counted. The values for total leukocytes and thrombocytes were determined by the equation: Total number of leukocytes or thrombocytes (μL) =[(number of leukocytes or thrombocytes counted in the smear) ×(erythrocyte global count per μL)] / number of erythrocytes counted in the blood smear.

2.4. Water quality

Water quality parameters were examined daily at 7:00 am and 6:00 pm (pH meter with condutivimeter YSI-63 and oximeter YSI-55), and showed the following value ranges: temperature (28.2–31.4 °C), pH (6.9–7.6), dissolved oxygen (3.1–5.6 mg/L), and electrical conductivity (72.1–80.8 mS/cm). Ammonia levels (60–123.4 μg/L) and nitrite (18.7–23.5 μg/L) present in the water were determined twice a week. All parameters analyzed remained within the comfort range for tropical fish (Ayroza and Scorvo, 2011).

2.5. Diets

Ingredients and nutritional values of the basal diet, formulated without the addition of oil to provide low level of vitamin E, are shown in Table 1. Three dry diets with 12.6, 58.2 and 310.4 mg of alpha tocopherol/kg were prepared from the basal diet. The pelletized diets were kept in a dark bag and stored at −20 °C. The fish were fed (2% of body weight) daily at 08:00 am and 5 pm.

Table 1
Ingredients and nutritional values of basal diet.

Ingredients	%	Composition	Amount
Corn bran	21	Crude protein (%)	27.16
Wheat bran	16	Digestible energy (kcal)	3650.00
Soybean	43	Crude fiber (%)	2.97
Rice bran	10	Ethereal extract (%)	2.29
Yeast, dehydrated	8	Mineral matter (%)	5.35
Methionine[a]	0.2	Calcium (%)	0.21
Vitamin premix[b] (without vits. E and C)	3.0	Phosphorus (%)	0.51
Mineral Premix[b]	4.0	Nitrogen-free extract (%)	41.86
Vitamin C[a]	0.047	Dry matter (%)	79.64

[a] BASF Corporation S.A.—Lutavit® vitamin C 100% and D-methionine.
[b] NUTREMIX Rações Ltda. —Vitamin and mineral premixes for fish.

2.6. HPLC for vitamin E determination

At the end of the feeding period, all diets were evaluated for the amount of alpha-tocopherol by high performance liquid chromatography (HPLC) (Manz and Philipp, 1981). This analysis included the sample saponification and extracting unsaponifiable components, using a mixture of methanol and water (96:4) as mobile phase containing BHT (butyl hydroxy toluene) as an antioxidant and solution of potassium hydroxide in reverse phase chromatography, with fluorescence detection of the alpha-tocopherol. The flow rate was 1.1 mL/min and the wavelength 295–345 nm. The calculations were based on comparing the peaks area of alpha-tocopherol relative to the samples recovered and obtained from the standard solution. The chromatographic analysis showed levels of 12.6, 58.2 and 310.4 mg alpha-tocopherol/kg of dry feed were recovered for diets formulated with 0, 100 and 450 mg of DL-alpha-tocopheryl acetate, respectively.

2.7. Statistical analyses

All data was statistically analyzed using a factorial scheme "split-plot design" [three levels of vitamin E (12.6, 58.2 and 310.4 mg/kg dry diet) × two levels of density (5 and 20 kg/m^3) × three times (2, 7 and 15 days post-implantation)], according to Littell et al. (1998). The analysis of variance for comparing the different experimental groups was carried out by applying a General Linear Model (GLM) procedure (SAS, 2001). Significant differences ($P < 0.05$) were estimated on the basis of T test (Snedecor and Cochran, 1974). Correlation analysis was determined using the Spearman test (SAS, 2001).

3. Results and discussion

In the presence of chronic inflammation, a significant increase ($P < 0.05$) in erythrocyte count and hematocrit percentage was observed in pacus fed 12.6 mg of α-tocopherol/kg of dry diet and kept in high stocking density when compared to fish reared in low density and fed with 310.4 mg vitamin E/kg diet in the second and seventh DPI (Table 2). Hemoconcentration could be related to changes in fluid–electrolyte balance. Under stress conditions release of endogenous cortisol, steroid with glyco and mineralocorticoid functions in teleost fish is increased, resulting in electrolyte imbalance and changes in the volume of water present in the blood extracellular fluid (Alsop and Vijayan, 2008). In accordance with Weendelar-Bonga (1997), disturbance of water and ion homeostasis is one of the most characteristic aspects of stress in teleost fish. Cases of relative polycythemia associated with high cortisolemia have been described in mammals; there is the hypothesis that glucocorticoids act by stimulating erythropoiesis (Randolph et al., 2010).

Spleen contraction in stress response leads to an increase in the number of circulating red blood cells, as a consequence of the release of catecholamines that interact with alpha-adrenergic receptors present in the splenic capsule (Belo et al., 2012b; Randolph et al., 2010). In the seventh DPI, pacus maintained in high stocking density showed a significant decrease ($P < 0.05$) in mean corpuscular volume of red blood cells (Table 2). The microcytosis observed may be due to the deleterious effects of stress, since cortisol has a broad activity spectrum in fish (Weendelar-Bonga, 1997), and regulates both the hydromineral balance (mineralocorticoid activity) and energy metabolism (glucocorticoid activity). In accordance with Tvedten (2010), in mammals, microcytosis is common in animals with hyponatremia, an electrolyte disturbance in which the sodium ion concentration in the plasma is lower than normal. In freshwater teleost, stress-induced release of catecholamines causes an increase in blood pressure resulting in an

Table 2
Mean values[a] (±SE) and ANOVA[b] observed in the hematologic[c] study of pacus during foreign body inflammatory response.

Period	Density[d]	Vitamin E[e]	Erythrocytes (10^6/μL)	Hematocrit (%)	MCV (fL)	Leukocytes (μL)	Thrombocytes (μL)
Day 2	LD	12.6 mg	2.96 ± 0.12^{ABa}	34.64 ± 0.45^{ABa}	118.28 ± 5.00^{Aa}	23898 ± 2597^{Aa}	48026 ± 4848^{Aa}
		58.2 mg	3.15 ± 0.22^{ABa}	37.50 ± 0.72^{Aa}	121.90 ± 7.79^{Aa}	29571 ± 4008^{Aa}	39038 ± 6982^{ABa}
		310.4 mg	2.74 ± 0.08^{Ba}	33.07 ± 1.20^{Bab}	121.45 ± 6.04^{Aa}	32554 ± 3717^{Aa}	42432 ± 3711^{ABa}
	HD	12.6 mg	3.26 ± 0.22^{Aa}	37.14 ± 1.15^{Aa}	116.29 ± 6.62^{Aa}	22036 ± 3883^{Aa}	35389 ± 4013^{BCa}
		58.2 mg	2.83 ± 0.06^{ABa}	33.21 ± 0.96^{Ba}	117.59 ± 2.55^{Aa}	27924 ± 4060^{Aa}	30335 ± 5592^{Ca}
		310.4 mg	2.86 ± 0.08^{ABb}	35.36 ± 0.95^{ABa}	123.61 ± 1.22^{Aa}	32278 ± 4684^{Aab}	28821 ± 2812^{Ca}
Day 7	LD	12.6 mg	2.64 ± 0.15^{Ca}	33.29 ± 2.65^{Aa}	124.94 ± 5.92^{Aa}	28367 ± 4810^{Aa}	26484 ± 1790^{BCb}
		58.2 mg	3.11 ± 0.26^{ABa}	34.93 ± 0.59^{Aab}	116.77 ± 8.78^{ABa}	37001 ± 6451^{Aa}	31925 ± 4233^{ABCa}
		310.4 mg	2.65 ± 0.14^{Ca}	28.79 ± 1.41^{Bb}	108.95 ± 4.13^{BCa}	18810 ± 2352^{Bb}	29469 ± 3684^{ABCb}
	HD	12.6 mg	3.35 ± 0.24^{Aa}	32.64 ± 1.14^{Ab}	99.6 ± 5.42^{Cb}	27451 ± 7885^{ABa}	35578 ± 5206^{ABa}
		58.2 mg	2.90 ± 0.16^{BCa}	27.86 ± 1.39^{Bb}	96.43 ± 3.92^{Cb}	24446 ± 3739^{ABa}	20709 ± 4348^{Ca}
		310.4 mg	3.44 ± 0.20^{Aa}	33.07 ± 1.29^{Aa}	97.29 ± 4.81^{Cc}	38061 ± 6905^{Aa}	40075 ± 5870^{Aa}
Day 15	LD	12.6 mg	2.97 ± 0.14^{Aa}	34.03 ± 0.95^{Aa}	115.13 ± 3.23^{ABa}	22671 ± 2934^{Ba}	25588 ± 3888^{Bb}
		58.2 mg	3.23 ± 0.15^{Aa}	32.93 ± 0.63^{ABb}	103.15 ± 4.09^{Bb}	36886 ± 4517^{Aa}	30744 ± 3629^{ABa}
		310.4 mg	2.92 ± 0.07^{Aa}	34.14 ± 0.32^{Aa}	117.43 ± 3.59^{Aa}	23733 ± 2365^{Bab}	33824 ± 4331^{ABab}
	HD	12.6 mg	3.03 ± 0.11^{Aa}	32.93 ± 0.94^{ABb}	108.94 ± 3.10^{ABa}	27758 ± 3389^{ABa}	37726 ± 3824^{Aa}
		58.2 mg	2.87 ± 0.17^{Aa}	30.07 ± 1.63^{Bab}	105.22 ± 3.87^{ABab}	28111 ± 2318^{ABa}	31069 ± 4470^{ABa}
		310.4 mg	3.20 ± 0.19^{Aab}	34.43 ± 1.49^{Aa}	108.41 ± 4.21^{ABb}	22479 ± 3709^{Bb}	29946 ± 4475^{ABa}
V[f]			0.25^{NS}	1.95^{NS}	0.86^{NS}	2.08^{NS}	1.43^{NS}
SD			3.93^{*}	1.60^{NS}	12.26^{**}	0.02^{ns}	0.87^{NS}
T			0.30^{NS}	11.72^{**}	10.52^{**}	0.32^{NS}	3.91^{*}
V × SD			8.42^{**}	13.09^{**}	0.40^{NS}	3.47^{*}	1.62^{NS}
V × T			0.83^{NS}	1.64^{NS}	1.60^{NS}	1.44^{NS}	0.88^{NS}
SD × T			3.24^{*}	0.61^{NS}	5.36^{*}	0.28^{NS}	5.22^{*}
V × SD × T			0.56^{NS}	1.77^{NS}	0.71^{NS}	2.08^{NS}	1.88^{NS}
C.V.[f]			14.44	9.71	11.83	41.64	35.36

[a] Means (n = 7) followed by the same letter do not differ by the T test (P < 0.05).
[b] Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment in the different experimental days.
[c] MCV = Mean corpuscular volume.
[d] LD = Low stocking density (5 kg/m^3), HD = High stocking density (20 kg/m^3).
[e] Levels of alpha tocopherol/kg of dry diet.
[f] V = Vitamin E; SD = Stocking density; T = Time; NS = not significant; * = significant (P < 0.05); ** = significant (P < 0.01); C.V.: Coefficient of variation.

increased electrolyte permeability of the gill which results in a rapid decrease in chloride and sodium plasma levels (Campbell, 2012).

The thrombocyte counts of pacus expressed in Table 2 show a higher number of circulating cells in the initial phase of inflammation (2 DPI), corroborating the findings of Reque et al. (2010) and Claudiano et al. (2013). Besides participating in the process of blood clotting, piscine thrombocytes represent a link between innate and adaptive immunity, and these cells could be mobilized to contribute in the organic defense mechanisms (Claudiano et al., 2013; Reque et al., 2010; Tavares-Dias et al., 2007). However, pacus reared in high stocking density presented significant decrease (P < 0.05) in the counting of thrombocytes compared to fish maintained in low stocking density (2° DPI). According to Clauss et al. (2008), thrombocytopenia has been described in fish with high levels of glucocorticoids. During the acute phase of inflammatory reaction, decrease of thrombocytes was observed in the exudate of pacus treated with dexamethasone, which is classified as a glucocorticoid of long action (Claudiano et al., 2013).

Pacu fed with 310.4 mg of vitamin E and kept at low stocking density presented a better leukocyte response in the presence of chronic inflammatory reaction. White blood cell increment was observed promptly to the stimulus exerted by the foreign body (2° DPI), followed by a significant decrease (P < 0.05) in circulating leukocyte counts with the evolution of the inflammatory response (7° and 15° DPI) (Table 2).

The leukocyte differential study revealed significant (P < 0.05) increase in the absolute number of neutrophils in the 2° DPI (Table 3). As shown in mammals, neutrophilia in the initial phase of inflammation is common among teleost fishes, and these granulocytes play an important role in the innate immunity (Reque et al., 2010). On the other hand, during the acute phase of the inflammatory reaction (2° DPI), the absolute neutrophil counts were favored by low stocking density and vitamin E supplementation with 310.4 mg/kg of diet, when compared to animals kept in crowding (20 kg/m^3). On the inflammatory reaction in pacus, neutrophils showed a positive correlation with thrombocytosis (Table 4), and such results were more meaningful in fish fed a diet deficient in vitamin E (12.6 mg/kg diet).

In the initial phase of inflammation (2 DPI), pacu kept in high density showed (P < 0.05) significant lymphopenia when compared to fish fed 58.2 mg of vitamin E/kg diet and kept in low stocking density, except for fish fed 310.4 mg vitamin E/kg diet (Table 3). The populations of lymphocytes assume essential role in the initiation and regulation of inflammatory responses, since they participate in the activation of other cells by releasing cytokines that act on specific receptors, transmitting signals to determine their activity, proliferation, differentiation, chemotaxis and apoptosis (Lieschke and Trede, 2009).

In the 7 DPI, there was a significant increase (P < 0.05) in absolute counts of lymphocytes in fish kept at low stocking density (Table 3), except in pacu fed 310.4 mg vitamin E/kg diet which lymphocyte counts followed the low number of total leukocytes (Table 2). Pacus reared in high density showed a significant lymphocytopenia (2 and 7 DPI), probably due to stress, as described by Fujimoto et al. (2005, 2007). According to Padgett and Glaser (2003), low lymphocyte counts can be observed in animals with increased cortisolemia. Moreover, the lymphocyte counts showed a negative correlation when compared to neutrophil counts in pacus fed a diet deficient in vitamin E (12.6 mg/kg diet) (Table 4), characterized by a significant suppression of circulating lymphocytes in the initial inflammatory reaction and increased in the late phase.

Pacus reared in high stocking density showed an increase in monocyte counts 2 and 7 DPI (Table 3), being statistically higher in animals supplemented with 310.4 mg of vitamin E/kg of dry diet when compared to fish fed with 58.2 mg/kg of dry diet and maintained at low stocking density. During foreign body chronic inflammation, monocytes migrate to inflamed focus and after diapedesis these cells differentiate into macrophages. According to Belo et al. (2005, 2012a), pacu kept in high density presented elevated cortisol levels, resulting in less accumulation of macrophages on coverslips implanted in the subcutaneous tissue. This effect results from the decrease in adhesion to vascular

Table 3
Mean values[a] (±SE) and ANOVA[b] observed in the leukocyte differential counts (absolute numbers) of pacus during foreign body inflammatory response.

Period	Density[c]	Vitamin E[d]	LG-PAS+[e] (μL)	Lymphocytes (μL)	Monocytes (μL)	Neutrophils (μL)	Eosinophils (μL)
Day 2	LD	12.6 mg	360 ± 135^{Ca}	8634 ± 2211^{ABb}	6689 ± 504^{ABa}	8214 ± 717^{Ba}	0 ± 0^{Aa}
		58.2 mg	1336 ± 277^{Ca}	10763 ± 2677^{Ab}	6562 ± 1241^{Bb}	10910 ± 2345^{ABa}	0 ± 0^{Ab}
		310.4 mg	5237 ± 1420^{Aa}	5180 ± 605^{ABa}	8804 ± 1099^{ABa}	13333 ± 2392^{Aa}	0 ± 0^{Ab}
	HD	12.6 mg	956 ± 290^{Cb}	3425 ± 302^{Bb}	8802 ± 1616^{ABb}	8854 ± 2171^{Ba}	0 ± 0^{Aa}
		58.2 mg	3499 ± 682^{ABa}	3986 ± 1113^{Bb}	11240 ± 1723^{ABb}	9199 ± 2282^{Ba}	0 ± 0^{Aa}
		310.4 mg	2688 ± 821^{BCab}	9041 ± 2317^{ABa}	13678 ± 2418^{Aab}	6871 ± 2099^{Ba}	0 ± 0^{Aa}
Day 7	LD	12.6 mg	1108 ± 744^{Ba}	15907 ± 3925^{Aa}	9494 ± 1990^{Ca}	1860 ± 611^{Ab}	0 ± 0^{Ba}
		58.2 mg	1153 ± 320^{Ba}	22258 ± 5246^{Aa}	11600 ± 1852^{BCab}	1787 ± 570^{Ab}	203 ± 101^{Aa}
		310.4 mg	1144 ± 528^{Bb}	5533 ± 835^{Ba}	10333 ± 1499^{BCa}	1733 ± 427^{Ab}	67 ± 66^{Aa}
	HD	12.6 mg	946 ± 341^{Bb}	8807 ± 3052^{Bab}	15372 ± 4491^{Ba}	2326 ± 914^{Ab}	0 ± 0^{Ba}
		58.2 mg	942 ± 291^{Bb}	5920 ± 918^{Bb}	17007 ± 2687^{ABa}	577 ± 240^{Ab}	0 ± 0^{Ba}
		310.4 mg	5714 ± 1313^{Aa}	6600 ± 1079^{Ba}	22015 ± 4584^{Aa}	4363 ± 1225^{Aab}	0 ± 0^{Ba}
Day 15	LD	12.6 mg	347 ± 116^{Ca}	12852 ± 2286^{Aab}	8954 ± 1339^{ABa}	571 ± 154^{Ab}	0 ± 0^{Aa}
		58.2 mg	2844 ± 495^{ABa}	18253 ± 2596^{Aa}	14432 ± 2335^{Aa}	1357 ± 253^{Ab}	0 ± 0^{Ab}
		310.4 mg	2433 ± 686^{Bb}	9354 ± 2152^{Aa}	10865 ± 1490^{ABa}	899 ± 269^{Ab}	0 ± 0^{Ab}
	HD	12.6 mg	4544 ± 1419^{Aa}	12208 ± 1588^{Aa}	8401 ± 1209^{Bb}	2441 ± 579^{Ab}	0 ± 0^{Aa}
		58.2 mg	3060 ± 1094^{ABa}	13284 ± 1493^{Aa}	9698 ± 1084^{ABb}	2069 ± 496^{Ab}	0 ± 0^{Aa}
		310.4 mg	1106 ± 313^{BCb}	10178 ± 1979^{Aa}	8673 ± 1351^{ABb}	2523 ± 611^{Ab}	0 ± 0^{Aa}
V[f]			7.22^{**}	5.92^{*}	2.66^{NS}	0.77^{NS}	2.06^{NS}
SD			5.28^{*}	12.07^{**}	8.43^{*}	0.06^{NS}	4.75^{*}
T			0.95^{NS}	9.55^{**}	8.65^{**}	8.31^{**}	4.65^{*}
V × SD			1.14^{NS}	8.29^{**}	0.76^{NS}	0.88^{NS}	2.06^{NS}
V × T			4.90^{**}	1.82^{NS}	0.69^{NS}	0.56^{NS}	1.99^{NS}
SD × T			1.20^{NS}	2.49^{NS}	8.09^{**}	3.75^{*}	4.65^{*}
V × SD × T			8.61^{**}	0.94^{NS}	0.61^{NS}	2.03^{NS}	1.99^{NS}
C.V.[f]			90.99	61.47	51.67	77.20	501.28

[a] Means (n = 7) followed by the same letter do not differ by the T test (P < 0.05).
[b] Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment in the different experimental days.
[c] LD = Low stocking density (5 kg/m^3), HD = High stocking density (20 kg/m^3).
[d] Levels of alpha tocopherol/kg of dry diet.
[e] LG-PAS+: Leukocyte granular-periodic acid Schiff positive.
[f] V = Vitamin E; SD = Stocking density; T = Time; NS = not significant; * = significant (P < 0.05); ** = significant (P < 0.01); C.V.: Coefficient of variation.

endothelium, as well as diapedesis and chemotaxis of monocytes to inflamed focus due to inhibition of the synthesis of eicosanoids derived from arachidonic acid (FAST et al., 2005) and the release of IL-1, IL-2, IL-6 and TNF-α (SONG et al., 2005). In these cases, there is an increase of monocyte number in the intravascular compartment, since they are prevented from moving out of the blood vessels.

Pacus fed a diet deficient in vitamin E (12.6 mg/kg) showed a negative correlation between monocyte counts and cortisol levels (Table 4), which indicates the susceptibility of these animals to the suppressive effect of glucocorticoids during the entire experimental period, corroborating the findings of Belo et al. (2005) that verified the beneficial effect of vitamin E supplementation on the activity of macrophages in chronic inflammation.

Pacus fed with 12.6 mg vitamin E/kg diet showed significant (P < 0.05) decrease on leukocyte granular PAS+ (LG-PAS+) counts in the 2 DPI (Table 3), while the highest counts were observed in fish fed 310.4 mg of vitamin E and reared in low stocking density. Martins et al. (2000) observed a significant increase in LG-PAS+ of pacus during acute phase of carrageenan-induced inflammation in the swim bladder. According to Reite and Evensen (2006), the LG-PAS+ shows granulation rich in glycogen factor that determines strong cytochemistry reaction with periodic acid Schiff (PAS-positive), whose functions are not well established although there is evidence that shares morphological and functional characteristics with basophils and mast cells. In Table 4, a positive correlation between LG-PAS+ counts and monocytes was observed in pacus reared in high stocking density, these findings could have been influenced by high cortisol levels that inhibit adhesion, diapedesis and chemotaxis as described above for monocytes.

Eosinophil counts were only observed in pacus supplemented with vitamin E and kept in low stocking density at 7 DPI (Table 3). However due to its scarcity probably these cells would not have an important role in chronic inflammation induced by foreign body.

Table 4
Correlation analysis between different hematological parameters of pacus during foreign body inflammatory response.

Experimental	Correlated parameters[b]	Correlation analysis[c]	
Sampling[a]		ρ^2	Prob > $\lvert\rho\rvert^2$
All animals	Thrombocytes × neutrophils	0.38492	<.0001
	LG-PAS × monocytes	0.26400	0.0030
	LG-PAS × neutrophils	0.27107	0.0023
12.6 mg of vitamin E	Thrombocytes × neutrophils	0.49575	0.0008
	Lymphocytes × neutrophils	−0.41762	0.0059
	Cortisol × monocytes	−0.31691	0.0409
High density (20 kg/m^3)	Thrombocytes × neutrophils	0.26400	0.0030
	LG-PAS × monocytes	0.27107	0.0023
	LG-PAS × neutrophils	0.21008	0.0192

[a] Correlation between all animals (n = 126), only animals fed with 12.6 mg of vitamin E/kg of dry diet (n = 42); only animals reared in high stocking density (n = 63).
[b] LG-PAS+: Leukocyte granular-periodic acid Schiff positive.
[c] ρ^2 = Coefficient of Spearman correlation; Prob > $\lvert\rho\rvert^2$ —significance probability of ρ value.

4. Conclusions

In chronic inflammation of pacus, deficiency of vitamin E and high stocking density resulted in increased number of red blood cells associated with microcytosis. Thrombocytosis and neutrophilia were observed in the initial reaction stage and the comparative study among treatments demonstrated a significant increase of monocyte counts and decrease in the thrombocyte and neutrophil counts of pacus reared in high stocking density during the acute phase of the inflammatory reaction. Low counts of lymphocytes were initially observed in pacus supplemented with low levels of vitamin E and maintained in high stocking density, but with the evolution of the inflammatory process, the lymphopenia persisted in pacus maintained in crowding. Pacus fed with diet deficient in vitamin E presented decrease in LG-PAS+ counts, as well as, a negative correlation between high cortisol levels and low monocyte counts.

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