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Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*

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Abstract

Piaractus mesopotamicus were fed diets supplemented with three vitamin C and/or E levels (zero, 250, and 500 mg vitamin/kg dry ration) and challenged with *Aeromonas hydrophila*. The fish were fed during the first 60 days with diets without vitamins C and E, in an attempt to reduce vitamin sources. After this period, test diets were offered during 60 days. At the end of the experiment, all fish were intraperitoneally injected with 6×10^6 colony forming units (cfu) of *A. hydrophila* per fish. It was concluded that for pacu, vitamin C and E are essential for protection of erythrocytes. Vitamin C induces an increase in the number of circulating thrombocytes in a dose–response relation. However, just like vitamin deficiency should be avoided, excess vitamins can also cause damage to fish as observed in the hematocrit and hemoglobin values. Based on the hematological responses obtained, the recommended vitamin C and vitamin E levels for *P. mesopotamicus* juveniles are 500 and 250 mg/kg feed, respectively.

Keywords: *Aeromonas hydrophila*; Challenge; Hematology; *Piaractus mesopotamicus*; Vitamin C; Vitamin E

1. Introduction

The occurrence of diseases in fish farms is a consequence of several factors pertaining to the rearing methods and environmental condition variations. Consequently, cultivated fish can become more susceptible not only to pathogenic but also to opportunistic bacteria (Woo and Bruno, 1998). *Aeromonas hydrophila* is a Gram-negative, motile rod recorded as an opportunistic pathogen in a great variety of freshwater fish species, and can be considered to have widespread geographical distribution. The bacterium causes hemorrhagic septicemia, characterized by the presence of small superficial lesions, focal hemorrhages, particularly in the gills and opercula, ulcers, abscesses, exophthalmia, and abdominal distension. Internally, there can be ascitic fluid accumulation, anemia, and lesions in the liver and kidneys (Austin and Austin, 1987).

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The use of antibiotics to treat bacterial infections has resulted in a global increase of resistance. For this reason, studies on immunostimulants are becoming more frequent. Immunostimulants comprise a group of biological or synthetic compounds that rapidly activate nonspecific defense mechanisms such as vitamin combinations, trace minerals, and products derived from either plants or animals that prove effective in the prevention of diseases. However, their mode of action has not been well known. Therefore, substances can be injected or mixed with the food and results can be tested by means of experimental models with suitable animals for this purpose, such as the determination of immune parameters or challenge with pathogens (Siwicki et al., 1994).

Most fish species cannot synthesize vitamin C, and depend on external sources to supply their needs (Chatterrjee et al., 1975). Several authors have reported that vitamin C supplementation in diets for aquatic organisms has prevented the negative effects of stress, stimulated wound healing, minimized toxicity by water contaminants, and increased immune response (Li and Lovell, 1985; Petric et al., 2003; Moraes et al., 2003; Brum, 2003). However, other studies have shown that there was no increase in resistance of fish supplemented with mega doses of vitamin C (Li et al., 1993).

Vitamin E protects cell membranes against lipid peroxidation. Its deficiency produces reductions in survival time and erythrocyte membrane deformations, increased *in vitro* hemolysis (Sau et al., 2004), reduction in the amount of peritoneal macrophages and activity of T and B lymphocytes in rainbow trout challenged with *Yersinia ruckeri* (Blazer and Wolke, 1984). On the other hand, studies have demonstrated that fish given feeds supplemented with vitamin E showed antibody titers statistically higher than non-supplemented fish (Verlhac et al., 1993), and markedly accelerated macrophage accumulation, as well as macrophage polykaryon and connective capsule formation on glass cover slips implanted in the subcutaneous tissue of normal pacus in relation to individuals that did not receive the vitamin (Belo et al., 2005).

The beneficial effects of vitamins C and E are extensively studied by Lim et al. (2000), Xie et al. (2006), Sau et al. (2004) and Belo et al. (2005). However, studies that associate vitamin C and E supplementation in fish diets are scarce, and little is known about the interaction effects of these vitamins *in vivo*. Some studies on this association have demonstrated that there are two interaction mechanisms between vitamins C and E: a synergistic simultaneous protection effect of the lipid and aqueous phases against oxidation, and the action of vitamin C on vitamin E regeneration in the tissues. Data on growth, mortality, hematology, and lipid oxidation in the liver have demonstrated that vitamin C protected fish against vitamin E deficiency (Hamre et al., 1997; Shiau and Hsu, 2002).

South American fish pacu (*Piaractus mesopotamicus*) is increasingly being used in aquaculture due to its good consumer acceptance and high growth rate (Jomori et al., 2003). Thus, the aim of this study was to evaluate the hematological responses of pacus fed diets supplemented with vitamins C and/or E, challenged by *A. hydrophila*.

2. Materials and methods

2.1. Experimental design and procedures

The experiment was conducted in a Randomized Complete Block Design (RBD) with 3 blocks, in a $3 \times 3 \times 2$ factorial combination, in which were tested nine diets: three vitamin C levels (0, 250, and 500 mg vitamin C/kg feed) and three vitamin E levels (0, 250, and 500 mg vitamin E/kg feed) in two periods of sample (before and after the challenge). Each experimental diet was fed to fish in three tanks (blocks).

Juvenile *P. mesopotamicus* with an initial weight of 10.5 ± 1.2 g were distributed in 27 tanks (300 L) with constant flow of water ($45 + 15$ mL s^{-1}). At the beginning of the experiment, 14 fish were stocked in each tank, located in the Aquatic Organisms Pathology Laboratory of Unesp (São Paulo State University), Aquaculture Center, Jaboticabal, SP, Brazil.

In an attempt to decrease their vitamin reserves, the fish were stocked in an environment with low incidence of light, to avoid the occurrence of phytoplankton, a vitamin C food source, and were fed a diet without vitamins C and E for two months prior to the experiment. Experimental feeding was initiated after that period. The fish were fed in two feedings daily at a rate equal to 5% of the weight of the fish, so that no feed leftovers were allowed in the tanks, for two months.

The water quality parameters were maintained within values recommended for fish welfare (Sipaúba-Tavares, 1994) as follows: temperature 30.5 ± 1.8 °C; dissolved oxygen 4.8 ± 0.8 mg L^{-1}; electric conductivity 204.1 ± 28.1 μS cm^{-1} and pH 8.6 ± 1.1.

2.2. Experimental diet

The basal experimental diet was made at the Aquaculture Center of Unesp, so as to meet the requirements of the fish (Table 1).

Table 1
Basic composition of experimental diets for *Piaractus mesopotamicus*

Ingredients	%
Soybean bran	26.22
Corn	31.13
Wheat bran	28.58
Fish meal	11.62
Soybean oil	1.95
Vitamin and mineral supplement*	0.50
Calculated composition	
Crude protein (%)	26.00
Ether extract (%)	7.00
Crude fiber (%)	5.81
Gross energy (kcal/kg feed)	4.150
Nitrogen-free extract (%)	44.00
Mineral matter	6.77

* Vitamin and mineral supplement composition: vitamin A 1,200,000 IU, vitamin B1 4800 mg, vitamin B12 4800 mg, vitamin B2 4800 mg, vitamin B6 4800 mg, vitamin D3 200,000 IU, vitamin K3 2400 mg, Folic acid 1200 mg, Biotin 48 mg, Calcium pantothenate 12,000 mg, Choline chloride 108 g, Niacin 24,000 mg, Selenium 100 mg, Iodine 100 mg, Cobalt 10 mg, Copper 3000 mg, Iron 50,000 mg, Manganese 20,000 mg, Zinc 30,000 mg, vehicle 1000 g, antioxidant 25 g.

The vitamin and mineral mix used did not contain vitamins C and E. The feed was prepared as follows: all ingredients were finely ground and weighed. The vitamin and mineral mixture, as well as the vitamin C and E sources at the appropriate amounts were added into the feed, initially homogenizing them with the ground corn and then mixing them with the dry ingredients. The feed was pelletized at a temperature of approximately 65 °C, using a die that created pellets approximately 2.5-mm-diameter and 7-mm-length.

ROVIMIX STAY C 35 Roche® (ascorbyl polyphosphate 35% activity) was used as the vitamin C source and ROVIMIX E 50 Adsorbate (50% activity) Roche® was used as the vitamin E source. The vitamin concentration calculations for each treatment were made based on vitamin availability in the products used. Pellets were hermetically sealed in flexible pouches and periodically a 1-wk allowance was transferred to a refrigerator (4 °C) and held until fed.

Diets were analyzed for vitamins. The concentrations of vitamins C and E of the experimental diets are presented in Table 2:

2.3. A. hydrophila and challenge

The *A. hydrophila* used in this experiment was isolated from muscles of two *Oreochromis niloticus* with clinical signs characteristic of hemorrhagic septicemia, at the Preventive Veterinary Medicine Department of Unesp. Bacteria was isolated on tryptic soya broth (TSB) (Difco) added of ampicillin at a concentration of 10 mg/L incubated for 24 h at 30 °C. Next, this material was plated on Starch–Ampicillin Phenol Red agar and incubated for 24 h at 30 °C. Suspected colonies (smooth) Gram-negatives, motility (+) were selected and plated on Triple Sugar Iron agar (TSI) for the same period and at the same incubation temperature. Cultures that showed acid reaction, both at the base and the beveled edge of the agar, with or without formation of gas, were submitted to biochemical characterization.

Ten fish per each tank were challenged via intraperitoneal injection with 6×10^6 CFU (LD_{50} based on preliminary work) of *A. hydrophila* in 0.2 ml of PBS. The fish were observed daily up to 10 days after the challenge and daily inspected for clinical reactions. Any dead specimens were subjected immediately to gross pathological changes and routine bacteriological examination.

2.4. Hematological analysis

The blood was collected after feeding the fish for 60 days with the test diets and 24 h after challenge, by puncture of the caudal vein of two specimens per tank (six fish per each dietary treatment), by using syringes containing EDTA (10%) for direct determination of erythrocyte (RBC) (Celm Model CC510); total leukocytes (WBC), thrombocytes and erythroblasts according to Martins et al. (2004); total concentration of plasma proteins and globulins; hemoglobin rate according to Collier (1944); hematocrit according to Goldenfarb et al. (1971); mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) calculated according to Wintrobe (1934). The differential

Table 2
Expected and detected vitamin C and vitamin E levels in experimental diets

Treatment	Expected level (mg/kg)		Detected level (mg/kg)	
	Vitamin C	Vitamin E	Vitamin C	Vitamin E
1	0	0	Not detected	Not detected
2	250	0	234	Not detected
3	500	0	461	Not detected
4	0	250	Not detected	223
5	0	500	Not detected	460
6	250	250	233	251
7	250	500	240	482
8	500	250	482	235
9	500	500	484	519

Table 3
Statistics of the hematological data in *Piaractus mesopotamicus* fed diets containing 0, 250, and 500 mg vitamin C and/or E/kg feed, before and after challenge with *Aeromonas hydrophila*

Statistics	Variables				
	Erytrocytes ($\times 10^6$/mm^3)	Hemoglobin (g %)	Hematocrit (%)	Protein (g/dL)	Glucosis (g/dL)
F for BL	3.28*	1.83 ^{ns}	3.94*	0.41 ^{ns}	1.00 ^{ns}
F for VC	1.17 ^{ns}	4.10*	1.25 ^{ns}	1.61 ^{ns}	1.78 ^{ns}
F for VE	0.51 ^{ns}	5.53**	6.97*	0.98 ^{ns}	0.50 ^{ns}
F for VC×VE	1.79 ^{ns}	3.04*	4.97*	0.35 ^{ns}	0.46 ^{ns}
F for PR	4.15*	3.13 ^{ns}	0.39 ^{ns}	64.38**	53.18**
F for VC×PR	1.09 ^{ns}	1.16 ^{ns}	1.58 ^{ns}	0.15 ^{ns}	0.14 ^{ns}
F for VE×PR	0.59 ^{ns}	0.27 ^{ns}	0.16 ^{ns}	0.76 ^{ns}	0.23 ^{ns}
CV	0.57	0.79	0.78	0.86	0.81
CV Plots	7.91	6.65	5.29	11.82	15.79
	MCV (fL)	MCH (%)	MCHC (%)	Thrombocytes ($\times$1000/mm^3)	Erythroblasts ($\times$1000/mm^3)
F for BL	2.48 ^{ns}	1.24 ^{ns}	2.35 ^{ns}	0.82 ^{ns}	4.61*
F for VC	0.84 ^{ns}	0.05 ^{ns}	3.25*	3.02 ^{ns}	3.55*
F for VE	2.15 ^{ns}	2.04 ^{ns}	0.01 ^{ns}	0.21 ^{ns}	0.43 ^{ns}
F for VC×VE	0.92 ^{ns}	0.32 ^{ns}	4.02**	1.62 ^{ns}	0.62 ^{ns}
F for PR	2.44 ^{ns}	6.22*	7.82*	12.62**	5.45*
F for VC×PR	0.44 ^{ns}	0.43 ^{ns}	0.02 ^{ns}	4.33*	1.28 ^{ns}
F for VE×PR	0.81 ^{ns}	0.68 ^{ns}	0.31 ^{ns}	1.76 ^{ns}	0.19 ^{ns}
CV	0.63	0.63	5.74	0.66	28.37
CV Plots	13.22	14.91	6.25	33.73	39.33

BL — block; VC — vitamin C in diet, VE — vitamin E in diet, PR — period.
* $p<0.05$; ** $p<0.01$.
All variables show F Levene for variance homogeneity >0.05 and X^2 for normality (Shapiro Wilks)>0.05.

leukocyte counts were obtained by preparing panchromatically-stained smears.

2.5. Statistical analysis

Assumptions of ANOVA were assessed by the Shapiro–Wilk test for normality on residuals and Levene's test for homogeneity of variance. A 3×3×2 factorial design was used to test the influence of the main effects (three vitamin C levels, three vitamin E levels and two periods) and the interaction between them (vitamin C×vitamin E, vitamin C×period and vitamin E×period) by two-way factorial ANOVA (Steel and Torrie, 1960). The means of analyses that showed

Table 4
Statistics of the hematological data in *Piaractus mesopotamicus* fed diets containing 0, 250, and 500 mg vitamin C and/or E/kg feed, before and after challenge with *Aeromonas hydrophila*

Statistics	Variable					
	Leucocytes ($\times$1000/mm^3)	Lymphocytes ($\times$1000/mm^3)	Monocytes ($\times$1000/mm^3)	Neutrophils ($\times$1000/mm^3)	Eosinophils ($\times$1000/mm^3)	SGC ($\times$1000/mm^3)
F for BL	1.10 ^{ns}	1.95 ^{ns}	1.54 ^{ns}	4.40**	0.62 ^{ns}	0.81 ^{ns}
F for VC	0.20 ^{ns}	0.30 ^{ns}	0.12 ^{ns}	0.52 ^{ns}	0.82 ^{ns}	1.61 ^{ns}
F for VE	0.18 ^{ns}	0.39 ^{ns}	0.74 ^{ns}	0.94 ^{ns}	0.30 ^{ns}	2.22 ^{ns}
F for VC×VE	1.16 ^{ns}	1.03 ^{ns}	0.82 ^{ns}	0.72 ^{ns}	1.50 ^{ns}	2.91*
F for PR	45.31**	63.12**	7.46*	16.94**	6.21*	3.80 ^{ns}
F for VC×PR	0.62 ^{ns}	0.61 ^{ns}	0.91 ^{ns}	0.10 ^{ns}	0.34 ^{ns}	2.88 ^{ns}
F for VE×PR	2.36 ^{ns}	1.72 ^{ns}	3.59 ^{ns}	2.39 ^{ns}	0.76 ^{ns}	5.51**
CV	65.51	0.74	63.78	91.76	0.63	0.72
CV Plots	51.72	56.69	64.87	62.81	166.43	192.25

SGC: Special granulocytic cell; BL — block; VC — vitamin C in diet, VE — vitamin E in diet, PR — period.
* $p<0.05$; ** $p<0.01$.
All variables show F Levene for variance homogeneity >0.05 and X^2 for normality (Shapiro Wilks)>0.05.

significant differences between factors were compared by Tukey's test ($p<0.05$).

3. Results

The treatments did not interfere with fish mortality values after challenge ($p>0.05$). Regardless of treatment, mortality rates were increasingly higher until 72 h. After that period, mortality gradually declined until stabilizing after 168 h.

Tables 3 and 4 show the statistics obtained in the analysis of variance of hematology of the fish before and after the challenge. Adding vitamin C and E to the diet had no effect on total erythrocyte counts, total protein, and globulins in the fish blood during this period. However, the number of erythroblasts was smaller for fish supplemented with 500 mg vitamin C in the diet.

Vitamin E deficiency (0 mg/kg) caused an increase in hematocrit values when the levels of vitamin C were low (0 or 250 mg/kg). The same response in fish that received 500 mg vitamin C/kg associated with the highest vitamin E level (500 mg/kg) was also observed (Table 5). Fish fed the diet with no vitamin C and E (0 mg/kg) showed the highest hemoglobin values (Table 5). In turn, supplementation with 500 mg vitamin C/kg caused an increase in MCHC (Table 5).

Table 5
Means hematological data of *Piaractus mesopotamicus* fed diets supplemented with different vitamin C and/or vitamin E levels

	Vitamin E		
Vitamin C	0	250	500
	Hematocrit (%)		
0	34.00 ^{Aa}	31.67 ^{b}	31.17 ^{b}
250	32.00 ^{ABa}	29.67 ^{b}	31.08 ^{ab}
500	29.58 ^{Bb}	29.42 ^{b}	32.42 ^{a}
	[Hemoglobin] (dL mm^{-3})		
0	9.62 ^{A}	9.01	8.61
250	8.56 ^{B}	8.50	8.71
500	9.50 ^{ABa}	8.38 ^{b}	9.11 ^{ab}
	MCHC (%)		
0	28.37 ^{AB}	28.42	29.14
250	26.77 ^{B}	28.72	28.13
500	30.50 ^{A}	28.52	28.20
	SGC (×1000 mm^{-3})		
0	0.43 ^{b}	2.02 ^{Aa}	0.34 ^{b}
250	0.66	0.40 ^{B}	0.25
500	0.53	0.57 ^{B}	0.75

SGC: Special granulocytic cells; equal letters in rows or columns: no significant difference ($p>0.05$).
Lower case letters compare rows and upper case letters compare columns.

Table 6
Means hematological data of *Piaractus mesopotamicus* fed diets supplemented with different vitamin C and/or vitamin E levels, before and after challenge with the bacterium *Aeromonas hydrophila*

	Challenge	
	Before	After
Vitamin C	Thrombocytes (×1000 mm^{-3})	
0	62.21 ^{B}	52.61
250	74.53 ^{AB}	68.68
500	90.40 ^{Aa}	50.25 ^{b}
Vitamin E	Monocytes (×1000 mm^{-3})	
0	1.27 ^{b}	3.63 ^{a}
250	1.70	2.13
500	2.08	2.26
Vitamin E	SGC (×1000 mm^{-3})	
0	0.39 ^{B}	0.69
250	1.74 ^{Aa}	0.25 ^{b}
500	0.52 ^{B}	0.37

SGC: Special granulocytic cells; equal letters in rows or columns: no significant difference ($p>0.05$).
Lower case letters compare rows and upper case letters compare columns.

The highest number of special granulocytic cells was observed in fish fed the diet no vitamin C with 250 mg vitamin E/kg (Table 5). The higher the vitamin C level in the diet, the higher the number of circulating thrombocytes. However, after the challenge, this number decreased in animals that received 500 mg vitamin C/kg (Table 6).

The fish that received diet without vitamin E supplementation (0 mg/kg) showed a smaller number of monocytes before challenge (Table 6). However, after challenge, fish that received 250 mg vitamin E/kg showed a reduction in the numbers of this cell type (Table 6).

The number of erythrocytes increased, while the concentrations of total protein and globulins in the fish blood of all treatments decreased, after challenge. In addition, vitamin supplementation did not interfere with blood mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values. However, regardless of treatment, after challenge with *A. hydrophila* there was a reduction in mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration values (MCHC) and erythroblast counts.

Total leukocyte counts, as well as the numbers of lymphocytes, monocytes, neutrophils, and eosinophils in the fish blood were not changed by the diet during that period. However, after challenge the numbers of total leukocytes, lymphocytes, and eosinophils decreased, while the numbers of neutrophils and monocytes increased.

4. Discussion

Neither dietary levels of vitamin C and E influences survival of *P. mesopotamicus* challenged by *A. hydrophila.* Li et al. (1993), Lim et al. (2000) and Xie et al. (2006) showed the same result with channel catfish *Ictalurus punctatus* and Siberian sturgeon *Acipenser baerii* Brandt.

In fish fed the highest vitamin C level (500 mg/kg) a smaller number of erythroblasts was observed. The antioxidant activity of ascorbic acid is based on its ability to react with free radicals. This reaction is likely to be of fundamental importance in all aerobic cells, which must defend against the toxicity of the very element depended upon as the terminal electron acceptor for energy production via the respiratory chain enzymes. It is this type of reaction that appears to be the basis of most, if not all, of the essential biological function of ascorbic acid. One of these is important in extending the antioxidant protection to the hydrophobic regions of cells: ascorbic acid appears to be able to reduce the semi-stable chromanoxyl radical, thus, regenerating the metabolically active form of the lipid antioxidant vitamin E (Combs, 1992). These evidences show that in fish fed with 500 mg of vitamin C the antioxidant protection of cells increases their time of life and decreases the necessity of new erythrocytes production (erythroblasts).

Some authors have demonstrated that there is a relation between vitamin C and E supplementation and hematocrit increase in fish (Andrade et al., 2007; Menezes et al., 2006). Others have established a relationship between vitamin C deficiency and a reduction in hematocrit (Lim and Lovell, 1978; Chagas and Val, 2003). In the present study, an interaction effect between vitamins C and E on hematocrit was observed. This parameter increased both in fish with vitamin E deficiency (0 mg/kg) associated with feeding under low vitamin C levels (0 or 250 mg/kg) and in fish that received the highest levels of both vitamins (500 mg/kg), suggesting that this hematological response might be linked to either vitamin deficiency or hypervitaminosis, and that supplementation with 500 mg/kg vitamin C in association with the same level of vitamin E could be considered excessive for the species under study.

Just like the hematocrit, hemoglobin concentration increased under vitamin deficiency. Studies by Tavares-Dias et al. (2000a,b) demonstrated a positive correlation between hemoglobin concentration and hematocrit percentage.

Although there was a reduction in blood total protein and globulin levels after challenge, supplementation with vitamins C and E did not change these hematological parameters. Results from the literature have revealed that fish attacked by either bacteria or parasites showed reduction in blood protein levels (Boon et al., 1990), and that supplementation with vitamins C and E did not have an influence on these hematological parameters (Sealey and Gatlin, 2002).

Cases of acute inflammatory response are characterized by blood neutrophilia and monocytosis and accumulation of neutrophils and macrophages in the injured or infected site (Roberts, 1989; Secombes, 1996). In the present study, although no fluid analyses were performed, an increase in the number of neutrophils and monocytes in the blood was observed 24 h after challenge with *A. hydrophila*, corroborating studies with rainbow trout infected with *Vibrio anguillarum* (Lamas et al., 1994) and Atlantic salmon infected with *Renibacterium salmoniarum* (Bruno and Munro, 1986), in which an increase of these blood cells also occurred.

We observed a reduction in the number of thrombocytes and lymphocytes in fish blood 24 h after challenge, suggesting the migration of these cells to the inflammation focus, as verified by Lamas et al. (1994) in the blood of rainbow trout infected with *V. anguillarum*. Therefore, the hypothesis of cell migration to inflammation sites can be confirmed by cell composition studies in the inflammatory exudate, induced in the swim bladder by thioglycolate, *A. hydrophila*, and (LPS) *Escherichia coli* endotoxin in *P. mesopotamicus*, for which Bozzo et al. (2007) demonstrated that predominant cells in the inflamed site were thrombocytes, accompanied by a smaller quantity of lymphocytes, while macrophages and granulocytes were present in small numbers since the initial evaluations, regardless of the inflammatory stimulus considered. Thus, the authors suggest that thrombocytes, along with leukocytes, besides presenting haemostatic functions, also act as defense cells in fish.

There have been reports that nutritional supplementation with 500 mg vitamin C per kg feed increased macrophage accumulation as well as macrophage polykaryon formation on glass coverslips implanted in the subcutaneous tissue of *P. mesopotamicus* (Petric et al., 2003). In the present study, we observed that vitamin C supplementation interfered with the number of thrombocytes in fish blood. Before challenge, the number of blood thrombocytes increased with increasing vitamin C levels in the diet. However, after challenge, there was a reduction in thrombocyte values only in the fish group that received the highest vitamin C level, and a faster migration of these cells to inflammation foci can be suggested.

When testing the addition of 500 mg vitamin E/kg feed, Belo et al. (2005) observed an increase in

macrophages as well as macrophage polykaryon formation on glass cover slips implanted in the subcutaneous tissue of *P. mesopotamicus*. In the present study, it was observed that the vitamin E-deficient (0 mg/kg) and supplemented fish did not show statistical difference in the number of circulating monocytes before and after the challenge. However this fact does not demonstrate what is happening in the inflammatory site in relation to the type of accumulated cells.

A higher number of special granulocytic cells were observed in the blood of animals that received 0 mg vitamin C and 250 mg vitamin E/kg feed. This group showed a reduction in this value after challenge, probably because cells migrated toward the inflammation focus.

Based on the results obtained in this study it can be concluded that for pacu, supplementation with vitamins C and E is essential for the protection of erythrocytes. Vitamin C induces an increase in the number of circulating thrombocytes in a dose–response relation. However, just like vitamin deficiency should be avoided, excess vitamins can also cause damage to fish as observed in the hematocrit and hemoglobin values. Based on the hematological responses obtained, the recommended vitamin C and vitamin E levels for *P. mesopotamicus* juveniles are 500 and 250 mg/kg feed, respectively.

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