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Challenge of pacu (*Piaractus mesopotamicus*) fed diets supplemented with vitamins C and E by *Aeromonas hydrophila* under different temperature

[*Desafio de pacus (*Piaractus mesopotamicus*) alimentados com dietas suplementadas com vitaminas C e E desafiados por* Aeromonas hydrophila *em diferentes temperaturas*]

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ABSTRACT

Pacu *Piaractus mesopotamicus* fed diets supplemented with three vitamins C and E levels (zero, 250, and 500mg vitamin/kg diet) were challenged with *Aeromonas hydrophila* under different temperatures. Fish were kept in 300L plastic tanks and fed during the first 60 days with diets without vitamins C and E, in an attempt to reduce vitamin sources. After this period, fish were maintained at an initial density of 14 fish/tank and test diets were offered during 60 days. At the end of the experiment, all fish were infected with 6×10^6CFU of *A. hydrophila*/fish, intraperitoneally injected. No interaction of dietary levels of vitamin C *vs.* E on mortality was observed. Supplementation with vitamins C and E did not decrease the mortality rate of fish challenged with *A. hydrophila*. Regardless of vitamin supplementation, after challenge, smaller fish showed higher mortality than larger ones and the fish group maintained in an environment under higher temperatures showed higher mortality rate.

Keywords: fish, *Piaractus mesopotamicus*, vitamin C, vitamin E, *Aeromonas hydrophila*, mortality

RESUMO

Pacus Piaractus mesopotamicus *alimentados com dietas contendo três níveis de vitaminas C e E (zero, 250 e 500mg/kg de ração) foram desafiados, em diferentes temperaturas, com* Aeromonas hydrophila. *Os peixes foram mantidos em caixas plásticas de 300L e, com o objetivo de reduzir as reservas vitamínicas, durante os primeiros 60 dias, foram alimentados com dietas isentas das vitaminas C e E. Após esse período, os peixes foram estocados na densidade inicial de 14 peixes/caixa e as dietas-teste foram oferecidas durante 60 dias. Ao final do experimento, todos os peixes foram infectados com 6×10^6 UFC de* A. hydrophila*/peixe, injetada intraperitonealmente. Não houve interação nível de vitamina C vs. nível de vitamina E quanto à mortalidade. A suplementação com as vitaminas C e E não reduziu a taxa de mortalidade dos peixes desafiados com* A. hydrophila. *Independentemente da suplementação vitamínica, após o desafio, os peixes menores apresentaram maior taxa de mortalidade que os maiores e o grupo mantido em ambiente com temperatura mais alta apresentou maior taxa de mortalidade após o desafio.*

Palavras-chave: peixe, Piaractus mesopotamicus, *vitamina C, vitamina E, Aeromonas hydrophila, mortalidade*

INTRODUCTION

Pacu, *Piaractus mesopotamicus* (Holmberg, 1887), a commercially important serrasalmid fish endemic to the Paraná-Paraguay River basin, is currently one of the most widely cultivated native fish species in the Neotropical region, and its potential for aquaculture has long been recognized (Lowe- McConnell, 1984; Jomori et al., 2003).

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In aquaculture, physiological stress and physical injury are the primary contributing factors of fish disease and mortality. Commercial aquaculture conditions including increased fish density and poor water quality (i.e., low dissolved oxygen; undesirable temperature or pH; and increased levels of carbon dioxide, ammonia, nitrite, hydrogen sulfide, and organic matter); injury during handling (i.e., capture, sorting, and shipping); and inadequate nutrition can result in decreased resistance by the fish, with the spread of disease and parasite infestation (Woo and Bruno, 1998).

Gram-negative *Aeromonas hydrophila* is the causative agent of fatal hemorrhagic septicemia, a systemic disease in fish that causes important yearly loss to the aquaculture (Paniagua et al., 1990). *In vitro* study showed that temperature plays a significant role in regulating the chemistry of the cell surface of *Aeromonas hydrophila* strains, and the changes on the cell surface explained the different growth temperatures for the strain testes (Merino et al., 1992).

Recent studies on the fish immune system indicate that immunostimulants can activate fish nonspecific immune functions, even in stressful situations, and therefore reverse the deleterious effects mediated by stress (Sakai, 1999; Brum, 2003). Vitamins C and E are among the most important nutrients that influence the fish immune system, and the supply of both vitamins can reduce mortality and improve fish performance, while increasing specific and nonspecific immune responses (Shiau and Hsu, 2002; Sau et al., 2004), may prevent the immunosuppression (Belo et al., 2005), aid on the inflammatory response (Petric et al., 2003; Belo et al., 2005; Reno et al., 2005), and can be used in a prophylactic way before transport stress (Okamura et al., 2007).

The interaction vitamin C x vitamin E has been shown both *in vivo* and *in vitro* (Ortuño et al., 2001; Cuesta et al., 2002; Chen et al., 2004; Garcia et al., 2007), on account of the synergetic effects of the combined action of both on some biological functions. There are two main mechanisms involved in the interaction between vitamins C and E. One is the simultaneous protection of the water and lipid phases against oxidation, and the other is the regeneration of vitamin E from the vitamin E radicals by vitamin C (Hamre et al., 1997; Ortuño et al., 2001).

The purpose of this study was to evaluate the interaction between dietary levels of α-tocopherol and ascorbic acid on external appearance, clinical signs as well as survival of pacu challenged by *Aeromonas hydrophila* under different temperatures.

MATERIAL AND METHODS

The experiment was carried out in a randomized block design (RBD) with three blocks, in a 3×3 factorial combination, in which three vitamins C and E levels (0, 250, and 500mg vitamin/kg feed) were tested. Juvenile pacus (10.5±1.2g) were distributed in 300L capacity tanks with constant flow of water, being 14 fish per tank.

Fish were stocked in an environment with low incidence of light to avoid the occurrence of phytoplankton, a vitamin C food source, and fed a diet without supplementation of vitamins C and E for two months prior to the experiment. After that period, the feeding was offered at a rate of 5% live weight twice a day, for two months.

Before the challenge, the aquatic variables were maintained as follows: temperature 30.5±1.8°C, dissolved oxygen 4.8±0.8mg/L, electric conductivity 204.1±28.1µS/cm, and pH 8.6±1.1. The water renewal rate was maintained at 45±15mL/s. After the challenge, the water temperature in each block was: block 1 - 22$\pm$0.33°C, block 2 - 21.5$\pm$0.21°C, and block 3 - 23$\pm$0.82°C. This temperature naturally occurred, without any equipment utilization.

The feed was formulated and prepared at the UNESP Aquaculture Center, according to requirements of fish (Table 1) without vitamin supplementation. To prepare the ration, all ingredients were finely ground and weighed. The vitamin and mineral supplement, as well as the vitamins C and E sources at the desired levels were incorporated into the feed, initially homogenizing them with the ground corn and then mixing them with the other ingredients. Mixing was performed in a "Y" model mixer. The feed was pelletized (2.5mm diameter and 7mm length) at a temperature of 65°C.

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

Ingredient	%
Soybean bran	26.22
Corn	31.13
Wheat bran	28.58
Fish meal	11.62
Soybean oil	1.95
Vitamin and mineral supplement[1]	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

[1]Composition= vit.A: 1,200,000IU; vit.B1: 4,800mg; vit.B12: 4,800mg; vit.B2: 4,800mg; vit.B6: 4,800mg; vit.D3: 200,000IU; vit.K3: 2,400mg; folic acid: 1,200mg; biotin: 48mg; calcium pantothenate: 12,000mg; choline chloride: 108g; niacin: 24,000mg; selenium: 100mg; iodine: 100mg; cobalt: 10mg; copper: 3,000mg; iron: 50,000mg; manganese: 20,000mg; zinc: 30,000mg; vehicle: 1,000g; antioxidant: 25g.

Rovimix Stay C 35[1] (ascorbyl polyphosphate 35% activity) was used as a source of vitamin C and Rovimix E 50[1] adsorbate (50% activity) was used as a source of vitamin E. The vitamin concentration calculations for each treatment were made based on vitamin availability in the used products. When ready, the feed was stored in plastic bags and frozen. Each week, the portion corresponding to that particular feeding period was stored at 4°C. Feed samples were sent to Labtec Mogiana[2] Laboratory for analysis to determine the vitamins C and E levels in the tested diets.

A. hydrophila used in this experiment was isolated from two Nile tilapia *Oreochromis niloticus* specimen with clinical signs, characteristics of hemorrhagic septicemia. The following procedures were performed: 10g of fish muscle samples collected at random were weighed; the samples were ground and incubated in 100mL tryptone soy broth (TSB) added of ampicillin at a concentration of 10mg/L and incubated for 24 hours at 30°C. Next, this material was plated on Starch-Ampicillin Phenol Red agar and incubated for 24h at 30°C. The colonies were selected and plated on triple sugar iron agar (TSI) for the same period and at the same incubation temperature. Colonies that showed acid reaction both at the base and the beveled edge of the agar, with or without formation of gas, were submitted to tests for oxidase, mannitol fermentation, indole production, esculin and arginine hydrolysis, lysine and ornithine decarboxylation, and acetoin production from glucose, in order to characterize the species.

According to the results obtained in preliminary tests, the challenge was carried out after fish fed with test diets for 60 days, by intraperitoneal injection of *A. hydrophila* at a concentration of 6×10^6CFU of *A. hydrophila*/fish, diluted in 0.2mL sterile saline solution, using the MacFarland's scale.

After the challenge, mortality and the occurrence of clinical signs were observed at every 12 hours for 10 days. Soon after death, the fish were weighed, identified, and immediately subjected to gross pathological changes and routine bacteriological examination (reisolation of *A. hydrophila*, following the procedure described for bacterial isolation).

Assumptions of ANOVA were assessed by the Levene´s test for homogeneity of variance and Shapiro-Wilk test for normality on residuals. A 3x3 factorial design was used to test the influence of the main effects (three vitamin C levels and three vitamin E levels) and the interaction between them on total number of dead fish at final time (240h) by two-way factorial ANOVA (Steel and Torrie, 1960). The means were compared by Tukey test at 5% confidence level. Number of dead fish was correlated with fish weight and water temperature (Pearson correlation).

RESULTS

The results for detected vitamin in each treatment at the finish of the essay are shown in Table 2. After the challenge with the bacterium, fish mortality was observed for 10 days, every 12h. Table 3 presents the statistical results obtained in the analysis of variance of mortality data of pacu fed diets supplemented with different vitamins C and E levels (0, 250, and 500mg/kg), submitted to challenge. The treatments did not interfere in fish mortality after the challenge. The cumulative mortality was higher until 72 hours after the challenge. After that period, mortality gradually declined until stabilizing after 168 hours.

[1]Roche do Brasil – Rio de Janeiro, Brazil.
[2]Labtec Mogiana Alimentos S.A. – Campinas, Brazil.

Table 2. Expected and detected levels of vitamin C and E in experimental diets for *Piaractus mesopotamicus*

	Expected level (mg/kg)		Detected level (mg/kg)	
Treatment	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 obtained in the analysis of variance and mean comparisons by Tukey test for mortality data of *Piaractus mesopotamicus* fed diets supplemented with different levels of vitamins C and E, submitted to challenge by *Aeromonas hydrophila* under different temperature (blocks) after 240h

Statistics		Mortality (Number of dead fish)
F for BL		18.72
F for VC		2.04ns
F for VE		0.17ns
F for the VC × VE interaction		1.35ns
CV (%)		61.23
Means of blocks	21°C	1,11c
	22°C	16,67b
	23°C	40,00a

BL: block; VC: vitamin C in diet; VE: vitamin E in diet.
All variables show F Levene for variance homogeneity >0.05 and X^2 for normality (Shapiro Wiks) >0.05.

There were significant mortality rate differences among different blocks (Table 3). It is noteworthy to point out that the blocks were in distinct environments, at different water temperature values. Block 3 corresponded to tanks placed in a closed environment with the highest temperature and mortality rates when compared to other environments with lower temperature (Fig. 1). A positive linear correlation ($P<0.01$) ($y=17.564x-372.28$, $r=0.87$) between number of dead fish and water temperature was observed (Fig. 2a).

A positive linear correlation between dead fish weight after the challenge and time elapsed for death was found ($P<0.01$) ($y=0.239x+68.524$ ($r=0.93$) (Fig. 2b). This correlation indicated that fish susceptibility to the infection was related to animal size. In the first days after the challenge, death occurred in fish with lower weight (Fig. 2b), demonstrating that heavier fish were more resistant to the disease, in spite of being the same age.

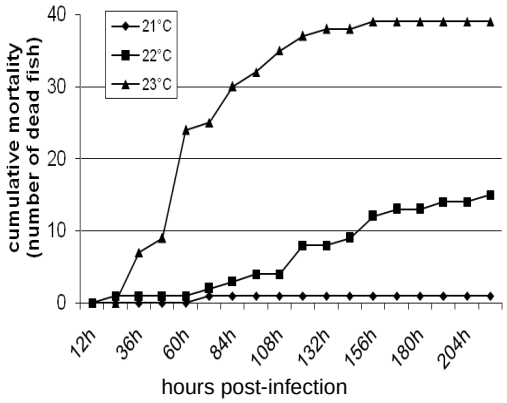

Figure 1. Cumulative mortality of *Piaractus mesopotamicus* under different water temperature.

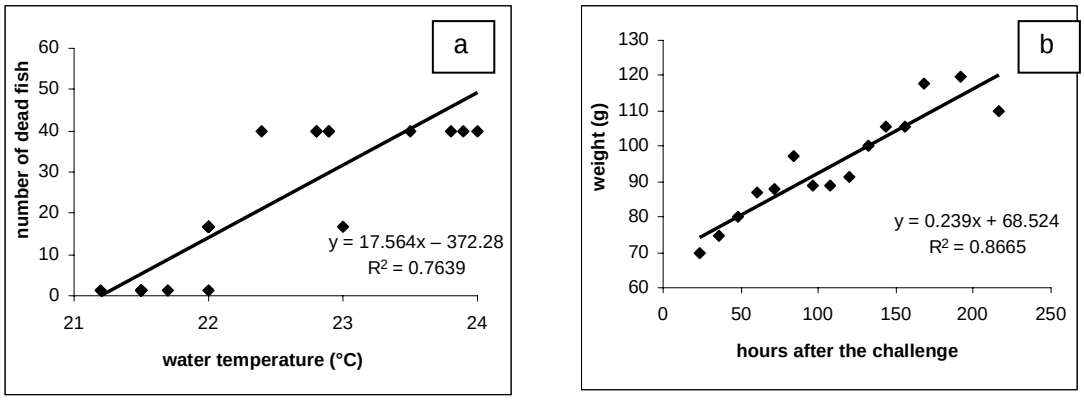

Figure 2. Pearson correlation between: a - number of dead fish after the challenge and water temperature (°C). b - weight (g) of *Piaractus mesopotamicus* that died after the challenge and time (h) until death occurred.

Regarding to the occurrence of clinical-pathological characteristics, 24 hours after inoculation, petechiae and suffusions appeared on the body surface. The abdominal cavity was distended, with transparent and clear ascite content (2.0 to 3.0mL). From the second day after the challenge, some fish became isolated from the shoal, showed a blackish color, a hematoma at the injection site, anal hemorrhage, hemorrhagic and tattered pelvic fins, with death ensuing in a few hours. Internal examination revealed a marked congestive-hemorrhagic scenario in the liver, cephalic kidney, and spleen.

DISCUSSION

In the present study, vitamin C levels in the diet did not influence the mortality rate of fish infected with *A. hydrophila* (Fig. 3). There has been divergence with respect to the beneficial effects of vitamins C and E supplementation. Many authors have showed the beneficial effects of vitamins C and E (Chagas and Val, 2003; Azad et al., 2006; Menezes et al., 2006; Garcia et al., 2007). However, controversial reports on the effect of vitamins C and E on protection have also been described (Li et al., 1998) and some studies show that feeding of either vitamin to fish did not enhance protection (Sealey and Gatlin, 2002; Xie et al. 2006).

The challenge method by intraperitoneal injection may be inadequate for research on resistance against infection induced by vitamins supplement. According Pressley et al. (2005), the absence of lesions in moribund zebrafish *Brachydanio rerio* intraperitonelly injected with *Edwardsiella tarda* may be due to the rapid progression of infection, which would not allow sufficient time for abscess formation. However, the challenge method of abrasion followed by immersion is used to simulate natural infection of injured fish. Intraperitoneal injection of *E. tarda* in adult fish allowed the bacteria immediate access to the vasculature. In fish that were scraped and then immersed in *E. tarda*, more time was needed for the bacteria to invade and establish infection. By 12 hours post-infection, these fish launched a very strong inflammatory response, producing cytokines transcripts at much higher levels than in intraperitoneally injected fish (Pressley et al., 2005).

Studies involving channel catfish indicated that the benefits from feeding on high vitamin C levels to increase resistance against infection by *E. tarda* are minimal when the fish is at an optimal temperature for its defense mechanisms. In addition, at unfavorable temperatures, the fish favorably responses to high vitamin C levels (Durve and Lovell, 1982). In this study, water temperature seems to have had an influence on the mortality rates caused by *A. hydrophila*. However, another fact that must be commented is the ideal temperature for bacterial development. It is known that hemorrhagic septicemia outbreaks caused by *A. hydrophila* are associated with high water temperatures. In this assay, in spite of the small temperature range, higher mortality rate occurred at 23°C (block 3), while fish maintained at 21°C (block 2) showed almost null mortality rates. Probably, the last temperature corresponds to the lethal point for the used strain.

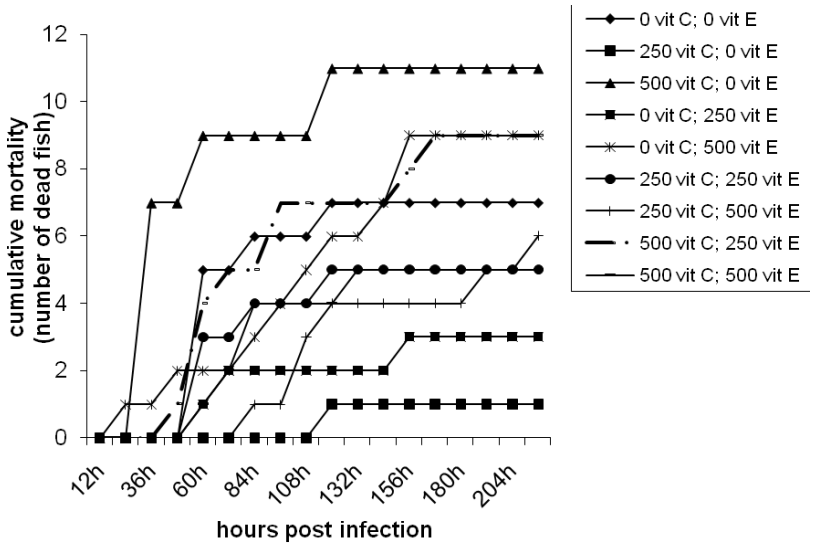

Figure 3. Cumulative mortality of *Piaractus mesopotamicus* fed different vitamins C and E levels, and challenged by *Aeromonas hydrophila*.

The positive linear correlation between fish weight that died after the challenge and time elapsed for death indicates that fish susceptibility to the infection was related to animal size. In the first days after challenging, death occurred in fish with lower weight, showing that heavier fish were more resistant to the disease, although all animals came from the same original stock and had the same age. A dominance behavior is well known among fish and can explain the lack of development uniformity in different lots. The underlying mechanisms have not yet been completely clarified, so it is not known whether dominant fish stand out in the competition for food and show better performance than dominated ones, or greater energy expenditure occurs due to the escape movements of subordinate fish. Anyhow, in this study, it was verified that this hierarchy directly influences resistance of these animals to diseases. Iida and Kurogi (2001) investigated the stress effect on the non-specific defense activity of Nile tilapia and reported that cortisol secreted under stress conditions directly impairs non-specific cell defense, making the fish more susceptible to invasion by a microorganism.

Another behavior described in this study was the blackish color of diseased fish. Secombes (1996) reported dermal darkening in diseased fish related to an increase in dermal cellularity associated with an increase in melanophores in an attempt to regenerate damaged tissues. There is also evidence that dermal darkening in fish is related to social hierarchy determination and stress conditions: subordinate fish, considered to endure greater stressor stimulus, show darker colors than dominant individuals (Beeching, 1995; Falter, 1987), and a diseased condition can be considered a stress stimulus, as here observed.

Regarding to the occurrence of clinical-pathological characteristics, fish infected with *A. hydrophila* showed petechiae and hemorrhagic suffusions on the body surface, the abdominal cavity was distended, with clear ascites content (2.0 to 3.0mL), in addition to a hematoma on the injection site, anal hemorrhage, and hemorrhagic and tattered pelvic fins. Internal examination revealed a marked congestive-hemorrhagic scenario in the liver, cephalic kidney, and spleen. Secombes (1996) reported that neutrophil extracellular enzymes caused damage to the tissues of the host, possibly contributing toward the hemorrhagic liquefaction of tissues commonly seen in bacterial infections.

CONCLUSION

There was not interaction between dietary levels of vitamin C x vitamin E on number of dead fish and supplementation with vitamins C and E did not decrease this parameter in fish challenged with *A. hydrophila.* Regardless of vitamin

supplementation, after the challenge, smaller fish showed higher mortality than larger ones and the fish group maintained in an environment under higher temperature showed greater mortality rate after the challenge.

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