Aquaculture Reports 17 (2020) 100303

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

# Effect of environmental thermal fluctuations on innate immune responses in pacu *Piaractus mesopotamicus* juveniles

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ARTICLE INFO

*Keywords:*
Stress
Vitamin E
Teleost
Complement
Lysozyme
Leukocytes

ABSTRACT

Immune functions in teleosts are straightly influenced by environmental temperature. Low temperatures usually affect negatively the immune status of fishes favoring the incidence of diseases. Immune cells in fishes are protected from peroxidation by dietary vitamin E. Innate immune defenses of *Piaractus mesopotamicus* were evaluated in fish fed with diets supplemented with vitamin E for 45 days. The fish endured decreases in environmental fluctuations of temperature for 24 and 72 h. The most effects were observed at the 24th hour, in which the level of plasma cortisol increased independently of dietary supplement of vitamin E and a lessening of MNL and a raise of GL counting were observed in fish fed with supplemented diets. The lowest SL variation was observed in fish fed with the maximum level of vitamin E, but the dietary vitamin E decreased the HAC. However, the stressing condition increased HAC. Dietary vitamin E used in the present experimental conditions did not present remarkable effects on the immune frame of pacu to cope with environmental thermal fluctuations but other investigations concerning the vitamin half-life and its association with unsaturated dietary lipids seem to be fundamental.

## 1. Introduction

Many daily culture practices in fish farming are stressful to fish, making the stress a usual condition in aquaculture (Barton and Iwama, 1991). There is a close relationship between teleosts and their aquatic environments electing them a good model to study the many aspects of biological adaptations, especially the environmental temperature, which is close to that observed in the fish body (Fry, 1967). In consequence, their physiological status, including immune functions, are straightly influenced by environmental temperature. Temperatures above the physiological range trigger stress responses that can negatively influence the immune system (Le Morvan et al., 1998). Likewise, low temperatures have a negative impact on the immune status of fishes. For example, the incidence of infections and parasitic infestations are more frequent at suboptimal temperatures. It is known that low temperatures tend to inhibit immune responses in ectotherms, including teleosts. The immunosuppressive effect of low temperatures in fish comes being reviewed for a few decades (Clem et al., 1991). Either low or high temperatures, both are seen to present distinct immune responses in fish. While an increase in environmental temperature is mostly linked to specific immune responses, its decrease is mainly related to non-specific humoral responses (Alcorn et al., 2002; Bowden et al., 2007). The nutritional status of the fish has also been related to animal immunity.

The feeding rate and starvation influence on the immune competence of fish enabling them to cope with infections (Verlhac Trichet, 2010). For example, the protein nutritional status has a strong influence on the immune-competence of shrimps (Pascual et al., 2006). The other

*Abbreviation:* MNL, mononuclear leucocytes; GL, granular leukocytes; SL, serum lysozyme; HAC, hemolytic activity of complement; ROS, reactive oxygen species; OD, oxygen density; TEA, triethanolamine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether) -*N*, *N*, *N′*, *N′*-tetraacetic acid

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https://doi.org/10.1016/j.aqrep.2020.100303
Received 6 October 2019; Received in revised form 19 February 2020; Accepted 23 February 2020
Available online 03 March 2020

macro-components of diets are also reported to influence the fish's immune-competence. Among the micro-nutrients, some vitamins are relevant, particularly vitamin E. Vitamin E plays an important role in protecting cellular lipid from peroxidation, including immune cells. The concentration of vitamin E in immune cells is related to dietary intake in several fish species (Lin and Shiau, 2005). A positive correlation was found with almost all immune responses investigated by Lin and Shiau (2005) in relation to increasing dietary dose of vitamin E. The dietary requirement of vitamin E in fish has been reported of several species (Watanabe et al., 1977; National Research Council (NRC), 1981; Wilson et al., 1984; Hamre and Lie, 1995) ranging from 30 to 300 mg (kg diet)$^{-1}$.

The activity of vitamin E comes from tocopherols and tocotrienols (National Research Council (NRC), 2011) present in many vegetal oil sources such as olive, sunflower, and soy. Since the second decade of the last century, tocopherols were reported for improving the rats' fertility. The lipid-solubility and the chemical structure of tocopherols and tocotrienols (methylated polyphenol molecules) assign to them some biological properties such as one of the most important natural antioxidant of membranes and lipoproteins, particularly preventing methylation of unsaturated fatty acids and the deleterious effects caused by reactive oxygen species (ROS) and free radicals (Mourente et al., 2007; Hamre, 2011). Vitamin E is present in the membranes and is important for their fluidity and the functioning of the immune response receptors based on such membranes. Other physiological effects are reported concerning the tocopherol activity such as enhancement of the red blood cells resistance to hemolysis, the permeability of heart muscle capillaries (Abdel-Hameid et al., 2012), and the enhancement of immunity (Trushenski and Kohler, 2007). Deficiency vitamin E is reported to cause anemia, impaired erythropoiesis, erythrocyte fragility, and muscular dystrophy (Mourente et al., 2007). In contrast, dietary supplementation of vitamin E can improve the growth performance in some fish species (Wilson et al., 1984; Hamre and Lie, 1995; Abdel-Hameid et al., 2012; Tocher et al., 2002), but do not show any evident effect in others (Wilson et al., 1984; Cowey et al., 1983; Gaylord et al., 1998; Bell et al., 2000). Considered the many physiological activities of vitamin E, it has received considerable attention in aquaculture nutrition as a candidate nutraceutical (Raa, 2000). In addition, taking into account the variety of stressors and their action mechanisms it is possible to think about the possibility of using vitamin E as a chemical agent to mitigate the undesirable effects of some stressing conditions.

It is reported from several decades that diets supplemented with α-tocopherol affect disease resistance in a number of animal species (Heinzerling et al., 1974b; Nockels, 1979). The increased resistance is attributed to the enhancement of humoral response and the phagocytic ability, modulating different parts of the immune system during disease outbreaks (Verlhac Trichet, 2010; Waagbø et al., 1993). It is well known that many disease outbreaks often occur after handling, transport, spawning, and water temperature oscillation. We have studied the hypothesis of supplementation of vitamin E in the feeding of pacu *Piaractus mesopotamicus* enhances the fish's immune potential to cope with the natural changes of temperature, able to occur in its rearing conditions. This freshwater fish is warm-water teleost widely reared in South America and other places. In spite of being able to tolerate the wide range of environmental temperatures between 15–35 °C, values lower than 24 °C result in cardiorespiratory alterations and decrease of food intake changing the immune status (Milstein et al., 2000; Borghetti and Canzi, 1993). In the native sites, the species is able to refuge from harmful temperatures but as a captive fish, it is usually exposed to daily thermal oscillations, particularly from the autumn to the spring, in the fish farms in the south of South America.

## 2. Material and methods

Pacu juveniles (*Piaractus mesopotamicus*) were supplied by São Geraldo Fish Farm (Sertãozinho, SP, Brazil), transported to the Lab of Adaptive Biochemistry (DGE-UFSCar) and maintained outdoor in a closed system of 2000 L tanks. The water, under continuous flow, was filtered with bio-beds, kept at 27 °C, and air saturated with porous stones. The fish were fed to satiety with fish commercial pellets 35 % CP and let to reach the optimal size for the experiments.

### 2.1. Experimental design

Two-hundred-and-twenty-eight acclimated pacu (N = 228), ranging 63.8 ± 9.9 g and 13.6 ± 0.7 cm, were randomly and equally distributed in twelve 250 L tanks (n = 19). The water quality was monitored and controlled to keep temperature 27 °C; pH - 7.0; OD - 5.7 mg/L; conductivity - 74.5 μS; and ammonia [$NH4^+$] less than 1 mg/L. The fish were acclimated for 5 days in the new environment and fed with the same diet twice a day, until satiety. Following the acclimation period, one fish from each tank was sampled to attest to the homogeneity of the groups through a couple of biochemical stress-parameters. After verified the homogeneity ($P < 0.05$), each experimental tank was considered an experimental unit. The twelve tanks were equally divided into three groups (treatments) of four tanks and assigned as Ci-0, Ci-100, and Ci-300. Three diets containing 0, 100 and 300 mg of vitamin E per kg of feed were made in a lab extruder and their composition is shown in Table 1. These diets fed the fish in Ci-0, Ci-100, and Ci-300, respectively. After 45 days of the feeding, two fish from each tank were randomly netted (eight fish per treatment). These fish were the initial control (Ci) of each treatment. Thereupon, eight fish from each tank were netted, transferred to a new corresponding tank of 250 L, and a new set of twelve tanks were performed. Such a new set was let at the natural fluctuations of the environmental temperature without any control except by the other water quality parameters, which were maintained and monitored. These twelve tanks were sampled at the 24th and 72nd hour after the natural thermal shock; these samples were assigned as S24 and S72 followed by 0, 100 or 300, according to the respective feeding condition. The remaining fish in the provider tanks were kept at the initial, controlled conditions (Ci) and were also sampled at the 24th and 72nd hour; these samples were assigned as Ci24 and Ci72 followed by 0, 100 or 300 as explained above. Two fish per tank were randomly netted at the 24th and 72nd

**Table 1**
Base formulation and nutrient composition of the experimental diets.

| Ingredient | Diet 1 | Diet 2 | Diet 3 |
|---|---|---|---|
| Corn (%) | 34,5 | 34,5 | 34,5 |
| Wheat bran (%) | 12,0 | 12,0 | 12,0 |
| Rice bran (%) | 10,5 | 10,5 | 10,5 |
| Soybean bran (%) | 22,0 | 22,0 | 22,0 |
| Fish flour (%) | 18,0 | 18,0 | 18,0 |
| Soybean oil [1] (%) | 2,5 | 2,5 | 2,5 |
| Vitamin and Mineral Supplement [2] (%) | 0,5 | 0,5 | 0,5 |
| Vitamin E (mg/kg) | 0 | 100 | 300 |
| TOTAL (%) | 100 | 100 | 100 |
| **Analyzed composition** | | | |
| Dry matter (%) | 88,14 | 87,63 | 88,09 |
| Crude protein (%) | 27,23 | 27,44 | 26,42 |
| Lipids (%) | 6,67 | 7,29 | 6,36 |
| Crude fiber (%) | 2,03 | 1,92 | 3,40 |
| NNE (%) | 46,85 | 45,54 | 46,49 |
| Minerals (%) | 5,36 | 5,44 | 5,42 |
| Gross energy (kcal/g) | 4184,31 | 4181,94 | 4210,76 |
| Vitamin E (mg/kg) | < 0,005 | 101 | 290 |

[1] Vitamin E was diluted in soybean oil and added after extrusion;.
[2] Without vitamin E- Ingredient (kg of ration)$^{-1}$. Vitamins: A = 160.0000 IU; D3 = 30.0000 IU; K3 = 1000 mg; B12 = 5 mg; B1 = 1,600 mg; B2 = 2,000 mg; B6 = 1,600 mg; Biotin = 200 mg; Folic Acid = 800 mg; Pantothenic Acid = 31,500 mg; Niacin = 14,000 mg; Choline = 120 mg; C = 40,000 mg; Iron = 30,000 mg; Copper = 600 mg; Manganese = 2,600 mg; Iodine = 220 mg; Selenium = 60 mg; Zinc = 6,000 mg. Nova America - Produtos Agropecuarios LTDA, Brazil. 0.005 g of premix per kg of feed.

hours in every condition. During the 45 days of the experimental period, the fish were fed to satiety and the feed consumed was gauged to calculate the growth performance. From the thermal shock moment, the feeding was discontinued for all the fish. The environmental temperature fluctuation ranged between 19 − 26 °C, over the 72 h of the experimental, thermal stress-period.

### 2.2. Procedures with the fish sampled

Following the sampling, the fish were quickly anesthetized in eugenol solution (1:20) (Inoue et al., 2003) and a blood sample was withdrawn from the caudal vein in two steps, with heparinized and non-heparinized syringes. The heparinized blood was centrifuged at 13,400 × g for 3 min, and plasma was used to cortisol determinations. Blood extensions and serological tests were performed with non-heparinized blood samples and serum, respectively. Serum was obtained after blood centrifugation at 600 × g for 15 min and used to determine complement and lysozyme activity. The fish final weight and the size were determined for the growth performance calculation.

### 2.3. Serological tests

The serum samples were used to the following evaluations: *i)* Differential leukocyte counting. Blood smears were stained with Rosenfeld dye (Faccioli et al., 1996) and 100 cells were counted under light microscopy at 1000 × . *ii)* Serum lysozyme. This parameter was determined by turbidimetric assay (Ellis et al., 1990; Abreu et al., 2009) with 1 mg/mL of chicken egg lysozyme (L6876, Sigma) as control. A sample of 0.2 mg $ml^{-1}$ of *Micrococcus lysodeikticus* (M3770, Sigma) was suspended in 0.05 M phosphate buffer pH 6.2. The final lysozyme solution contained 1 ng $\mu L^{-1}$ diluted in $NaH_2PO_4$, 0.05 M, pH 6.2. Serum samples were preheated at 56 °C for 30 min to inactivate the complement system proteins. The method was adapted for microplate reading at 450 nm for 10 min at 25 °C, against phosphate buffer as blank (Molecular Devices Reader), and the lysozyme concentration was inferred from a linear reference curve and expressed in µg $mL^{-1}$. *iii)* Hemolytic activity of the complement system. The serum complement activity was determined (Ferriani et al., 1990) after adjustments to the pacu serum according to a method just reported (Biller, 2008). Briefly, rabbit erythrocytes eluted in Alsever's solution pH 6.1 v/v, free of suspension by gauze filtering, chelated by TEA-EDTA pH 7.4 in 0.1 % gelatin solution, and incubate at 37 °C for 15 min were re-suspended in TEA 2 mM pH 7.4 and washed 3 times before use. These erythrocytes were suspended in TEA-EGTA 1 % buffer to an OD between 0.7 and 0.8 at 700 nm and adjusted to pacu serum methodology. The hemolytic activity of complement was performed with serum dilution added to the erythrocyte suspension and read at 700 nm for 10 min at 37 °C (Hitachi U-2910). Serum pre-heated at 56 °C for 30 min was used as a negative control. The hemolytic activity was directly proportional to the OD variations and is expressed in Δ OD. *iv)* Plasma cortisol. Cortisol was quantified in heparinized serum samples by radioimmunoassay in a beta particle counter (Laboratory of Endocrinology - Clinics Hospital - Medicine School of the University of São Paulo-RP, São Paulo, Brazil).

### 2.4. Statistics

The experimental design was completely randomized, in a multifactor scheme (2 conditions x 3 diets x 3 periods), consisting of two temperature conditions (controlled and variable), three levels of vitamin E in the diet (0, 100 and 300 mg (kg of diet)$^{-1}$) and three sampling periods 0, 24 and 72 h. The homogeneity was assessed with the Levene's test at a significance level $P < 0.05$ and a difference among the groups was evaluated with ANOVA. Different means were compared by the posthoc Tukey's test accepted a confidence level $P < 0.05$. The SAS v.8.0 program (SAS, 2020) was used in the statistical analysis of the data. The graphs were generated through the software Origin 5.0, based on means and SD.

### 2.5. Ethics

This work was approved by the Animal Experiments Ethics Committee of the Federal University of São Carlos, under the protocol 003/2010.

## 3. Results

### 3.1. Growth performance

At the end of the experimental period, no difference was observed in the growth performance of pacu at any level of dietary vitamin E. The final weight means and size were 144.2 g ± 29.1 g and 17.4 cm ± 1.6 cm, respectively; and the relative weight gain of pacu was 55.75 %. During the experimental span, no fish mortality was observed.

### 3.2. Plasma cortisol

The plasma cortisol levels just before the thermal stress experiments were equal (7.55 ± 2.2) in all fish independently of being previously supplemented or not with vitamin E. The fish submitted to thermal stress increased the plasma cortisol until the 24$^{th}$ hour of the stress period decreasing from this point (Fig. 1A). Only the fish fed without supplement of vitamin E presented a significant decrease of cortisol at the 72$^{nd}$ hour ($P < 0.05$); this variation was the highest one. In this sampling point, the fish fed with the maximum amount of vitamin E depicted the lowest change in cortisol (Fig. 1B).

### 3.3. Mononuclear leukocytes and granular leukocytes

In the initial condition (Ci) before the stress, the MNL values were similar among all the fish (Table 2). The thermal stress reduced the relative counting of MNL in fish fed with 100 or 300 mg of vitamin E at the 24$^{th}$ hour. The fish stressed for 72 h presented a significant decrease

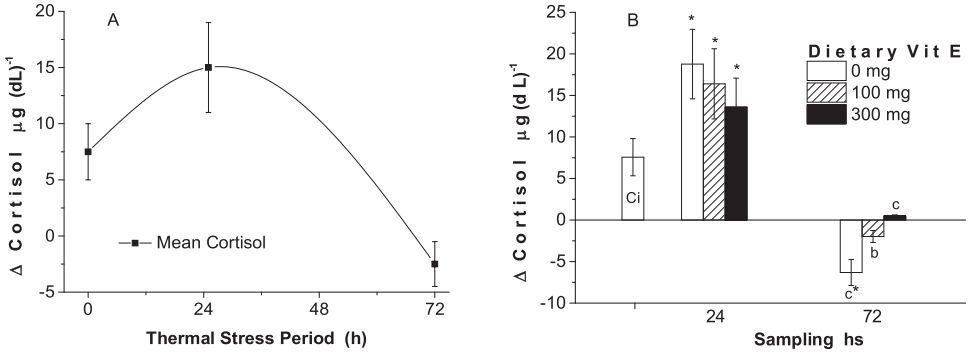


**Fig. 1. (A)** General profile of plasma cortisol from the mean values from each sampling point. **(B)** Plasma cortisol variation in *Piaractus mesopotamicus* fed with three levels of dietary vitamin E (0, 100 and 300 mg) and submitted to a thermal stress exposure for 24 and 72 h. Each point comes from the stressing value minus the respective control. Ci is the mean cortisol just before the beginning of the stress exposure (Ci). Super script letters means significantly different compared within the same sampling point; (*) means significantly different compared with the respective control; Differences were accepted for $P < 0.05$.

**Table 2**
Mononuclear Leucocytes (MNL), Granular Leucocytes (GL), Serum Lysozyme (SL), Hemolytic Activity of Complement (HAC) values in pacu (*Piaractus mesopotamicus*) fed with diets supplemented with three levels of vitamin E for 45 days and submitted to thermal stress at environmental temperature for 24 and 72 h.

| Condition | **Mononuclear Leucocytes (MNL)** | | | | | |
|---|---|---|---|---|---|---|
| | Vitamin E (mg) | | | | | |
| | 0 | | 100 | | 300 | |
| Ci | 98.25 ± | 0.71 | 96.75 | ± 1.28 | 98.28 | ± 0.76 |
| C24 | 90.25 ± | 1.91 | 91.13 | ± 3.48 | 93.63 | ± 2.93 |
| S24 | $85.25^{B}$ ± | 5.04 | $74.88^{A}$* | ± 6.47 | $83.13^{AB}$* | ± 4.85 |
| C72 | 95.57 ± | 0.79 | 95.29 | ± 1.60 | 95.00 | ± 1.83 |
| S72 | 83.00* ± | 4.04 | 82.63* | ± 3.40 | 79.38* | ± 4.85 |
| | **Granular Leucocytes (GL)** | | | | | |
| | Vitamin E (mg) | | | | | |
| | 0 | | 100 | | 300 | |
| Ci | 1.75 | ± 0.71 | 3.25 | ± 1.28 | 1.71 | ± 0.76 |
| C24 | 9.75 | ± 1.91 | 8.88 | ± 3.48 | 6.38 | ± 2.92 |
| S24 | $14.25^{B}$ | ± 5.04 | $25.13^{A}$* | ± 6.47 | $16.88^{A}$* | ± 4.85 |
| C72 | 4.43 | ± 0.79 | 4.71 | ± 1.60 | 5.00 | ± 1.83 |
| S72 | 17.00* | ± 4.04 | 17.38* | ± 4.00 | 20.63* | ± 4.66 |
| SL | **Serum Lysozyme (SL)** | | | | | |
| | Vitamin E (mg) | | | | | |
| | 0 | | 100 | | 300 | |
| Ci | 8.08 | ± 1.93 | 8.44 | ± 1.83 | 6.98 | ± 1.78 |
| C24 | 6.32 | ± 1.49 | 8.44 | ± 1.83 | 6.98 | ± 1.78 |
| S24 | $5.95^{A}$ | ± 1.12 | $6.33^{A}$ | ± 1.20 | $6.32^{B}$ | ± 1.60 |
| C72 | 8.05 | ± 1.59 | 7.25 | ± 1.94 | 11.87 | ± 1.97 |
| S72 | $7.81^{B}$ | ± 0.83 | $10.63^{A}$* | ± 0.81 | $8.37^{C}$* | ± 1.89 |
| HAC | **Hemolytic Activity of Complement (HAC)** | | | | | |
| | Vitamin E (mg) | | | | | |
| | 0 | | 100 | | 300 | |
| Ci | 0.73 | ± 0.02 | 0.65 | ± 0.03 | 0.42 | ± 0.10 |
| C24 | 0.37 | ± 0.07 | 0.60 | ± 0.11 | 0.36 | ± 0.03 |
| S24 | $0.47^{A}$* | ± 0.07 | $0.37^{B}$* | ± 0.14 | $0.43^{A}$ | ± 0.07 |
| C72 | 0.19 | ± 0.08 | 0.60 | ± 0.17 | 0.07 | ± 0.03 |
| S72 | $0.37^{A}$ | ± 0.15 | $0.68^{B}$* | ± 0.06 | $0.22^{A}$* | ± 0.08 |

Ci- initial control previously the stress condition; C24- stress control at the 24$^{th}$ hour; S24- stress at the 24$^{th}$ hour; C72- stress control after 72$^{nd}$ hour; S72- stress at the 72$^{nd}$ hour. MNL and GL are expressed in Δ%; SL is expressed in μg $(mL)^{-1}$; HAC is expressed in Optical Density (OD); the values are followed by ± SD. Super script letters means significantly different compared within the same sampling point; (*) means significantly different compared with the respective control; Differences were accepted for $P < 0.05$.

($P < 0.05$) in MNL independently of the supplement of vitamin E (Fig. 2a). The relative counting of GL in the fish just before the beginning of the stress (Ci) was similar in all the vitamin E levels (Table 2). In all stressing conditions, it was observed a significant increase ($P < 0.05$) of the GL percentage except in fish deprived of dietary vitamin E and exposed to thermal stress for 24 h. In spite of such a significant increase of GL in the most conditions, the amplitude of variation was significantly different only at the 24$^{th}$ hour between the fish fed with vitamin E (100 and 300 mg) and the non-vitamin supplemented fish (Fig. 2b) ($P < 0.05$).

### 3.4. Lysozyme and hemolytic activity of complement

The amounts of SL among the fish supplemented with the three levels of vitamin E were closely the same (Table 2) at the initial conditions (Ci). The fish fed with 300 mg of vitamin E presented the lowest SL variation at the 24$^{th}$ hour of thermal stress. After the thermal stressing exposure, the only SL positive variation was observed at the 72$^{nd}$ hour in fish fed with diets supplemented with 100 mg of vitamin E (Fig. 2c) ($P < 0.05$). In the initial control (Ci) it was observed a decrease in the HAC with the increase of vitamin E (Table 2). In general, the thermal stressing conditions increased positively the HAC variation in pacu (Fig. 2d). The only negative HAC value observed was in fish fed with 100 mg of vitamin E at 24$^{th}$ hour of stressing condition ($P < 0.05$).

## 4. Discussion

To the present time, there are no data concerning the ideal range of vitamin E in the feeding of *P. mesopotamicus*. The dietary requirements of this fatty-soluble vitamin are reported to be dependent on the dietary levels of polyunsaturated fatty acids (Hamre and Lie, 1995; Schwarz et al., 1988; Shiau and Shiau, 2001; Lin and Shiau, 2003). There are a number of fishes responsive to dietary vitamin E, however, some others are not (Peng and Gatlin, 2009; Lozano et al., 2017). This vitamin is usually offered in the diets of the most freshwater Teleostei into a range of $50-100$ mg (kg of ration)$^{-1}$. Then, to prevent sub-optimized concentrations, the amount added to the diets can be considered high. However, even the diet without vitamin E supplementation should contain low concentration, or traces of tocopherols, usually present in fatty-soluble dietary components. A thorough evaluation of growth performance in pacu concerning vitamin E was not intended in the present study. In fact, its use was mainly as a nutraceutical instead of a dietary trace element. Therefore, the same weight gain, observed in the fish from all feeding conditions, was predictable. Other experiments with grading dietary macronutrients, particularly total lipids, would be performed before concluding about the role of vitamin E in the growth performance of pacu.

Based on the literature, particularly on marine fish species, growing levels of dietary tocopherol leads to vitamin E gradation in the liver of turbot *Scophthalmus maximus*, Atlantic halibut *Hippoglossus hippoglossus*, sea bream *Sparus aurata*, (Tocher et al., 2002), and muscle and liver of grouper *Epinephelus malabaricus* (Lin and Shiau, 2005). The increase of vitamin E contents in cell membranes, proportionally to the dietary amounts, is also reported (Hamre, 2011). These facts make reasonable to assume that the high experimental ranges of vitamin E in the feeding of pacu were enough to soak the cell medium with it; and consequently the vitamin content in the lipid cell components, such as membranes. Such a premise was assumed to set up any physiological contrast between the responses from fish regularly fed with vitamin E *versus* fish unfed with it.

The same concentrations of plasma cortisol observed at the 45$^{th}$ day in all experimental conditions ensure that the fish stress degree was quite similar at the beginning of the thermal stress experiments, independently of the dietary vitamin E levels. Likewise, the difference between the dietary levels of vitamin E did not interfere in the stressing effects of temperature twenty-four hours after thermal stress in pacu, in spite of an increase in plasma cortisol was observed in all fish. This is a usual physiological response of fish to cope with the temperature at the border of the tolerance range (Arends et al., 1998; Tanck et al., 2000). However, seventy-two hours of environmental temperature fluctuation were enough to trigger some physiological adaptations. Cortisol is reported to attend directly in such an adaptive response (Jaxion-Harm and Ladich, 2014). This adaptation is inferred from the concentrations of plasma cortisol in fish from the experimental conditions, either those from control or those fed with vitamin E; no differences were observed among the plasma concentrations at the 72$^{nd}$ hour under thermal fluctuations, and the cortisol values recorded were close to the basal levels. The harsher environmental condition imposed on pacu during the thermal experiments were mostly the drops of temperature at night. However, these were not abrupt but gradual. This particular made the thermal changes closer to a long-term acclimation than cold shock stress.

The primary function of cortisol is to induce physiological changes that help animals either protecting themselves or adapting to the stressor (Jaxion-Harm and Ladich, 2014). Continuous high levels of cortisol can become maladaptive by impairing the immune system and the animal's health (Houghton and Matthews, 1990). Nevertheless, the environmental condition imposed on pacu worked as chronic stress, which normally involves longer periods of action of the stressor, switching the energetic metabolism to allocate resources to the stress process. This mechanism implies continuous energy availability by the

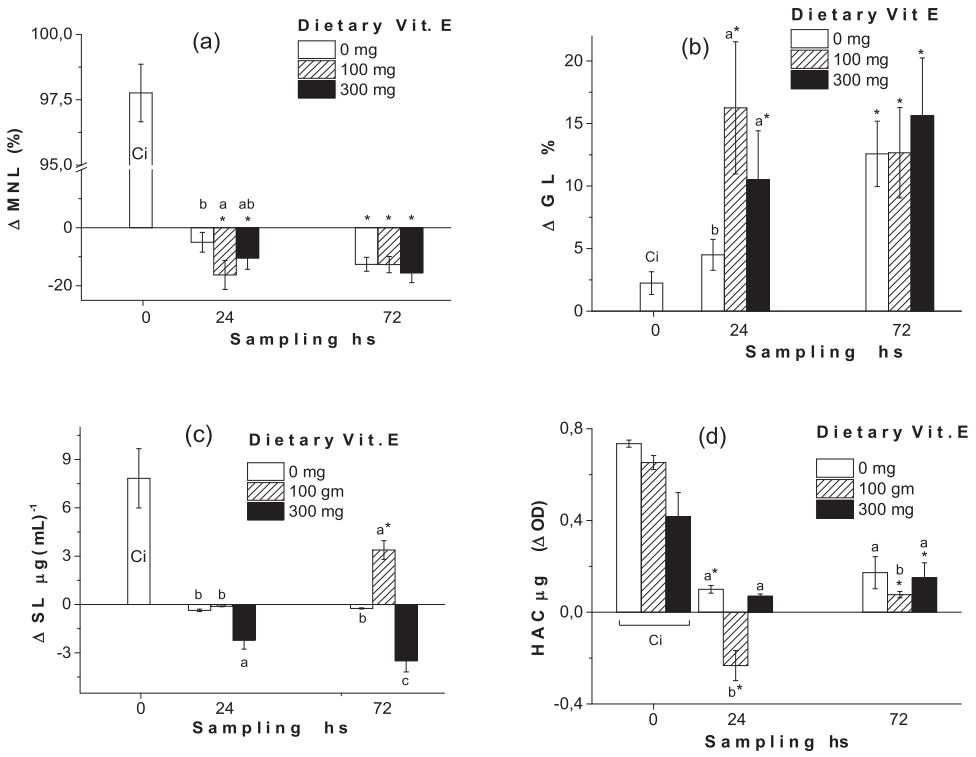


**Fig. 2.** **(a)** Mononuclear Leucocytes (MNL), **(b)** Granular Leucocytes (GL), **(c)** Serum Lysozyme (SL), **(d)** Hemolytic Activity of Complement (HAC) variations in pacu (*Piaractus mesopotamicus*) fed with diets supplemented with three levels of vitamin E for 45 days and submitted to thermal stress at environmental temperature for 24 and 72 h. The values express the difference between the thermal stressed fish and the respective sampling control at the 24$^{th}$ and 72$^{nd}$ hour of the stress. The sampling (0) correspond to the initial moment just before the thermal stress. Super script letters means significantly different comparing the variation (Δ) within the same sampling point; (*) means significantly different compared with the respective control. Differences were accepted for $P < 0.05$.

immune system, such as antibodies production, protein synthesis such as complement, and production and differentiation of leukocytes (Tort, 2011).

The immune system of bony fish such as rainbow trout *Oncorhynchus mykiss* and common carp *Cyprinus carpio* has long since been investigated and, in this particular, these species are likely the most studied among the farmed fish. In Teleostei, the innate immune system is accepted more relevant than the adaptive system, which is not as elaborate as in higher vertebrates (Warr, 1995). In the current study, the innate immune responses were investigated through GL counting and the humoral variables: SL and HAC. In addition, the counting of MNL, usually involved in adaptive immune responses, was performed. Decreases in the number of MNL were observed in all experimental conditions and, in general, such decreases were similar among the samples. Then, considering these cells, the dietary vitamin E was apparently ineffective to cope with natural thermal oscillation over seventy-two hours.

Mononuclear leukocytes are basically the adaptive arm of the vertebrate immune system. Composed of B and T lymphocytes, and monocytes, the MNL counting is responsive to temperature decreasing in some fish species. In channel catfish, the *in vitro* cell proliferation of leukocytes is inhibited when fish is maintained at 11 °C (Bly and Clem, 1991). Suboptimal temperatures adversely impact B lymphocytes in rainbow trout (Kollner and Kotterba, 2002). Temperature decrease from 25° to 16 °C over 3 h reduces significantly the B-lymphocytes counting in common carp (Engelsma et al., 2003). In spite of particular differences in the responsiveness, T-lymphocytes are also reactive to temperature decrease in channel catfish. The counting of T-cells in fish maintained at 11 °C is lower compared to 24 °C (Bly and Clem, 1991). The observed differences between T and B responses to low temperatures are attributed to the membrane fluidity which is associated with the content of oleic acid in the lipid-soluble component of both cell membranes (Vallejo et al., 1992). Therefore, large decreases in temperature impact lymphocytes and the teleost immune system as a whole. In spite of the temperature fluctuations in the wild do not occur as quickly as those imposed in several experimental conditions, it is important to be aware of the potential for large temperature decreases to act as acute stressors and immunosuppressors in artificially reared fish. Leukocytes counting is an important indicator of immunocompetence particularly when the relative proportion in the blood, kidney, and spleen is available.

The number of GL in pacu was indistinctly affected by both experimental levels of dietary vitamin E. Oscillation of environmental temperature increased the GL at the 24$^{th}$ hour of stressing condition. However, the longest period (72 h) equalized the physiological responses in all fish. The effects of hypothermic temperatures on fish are contrasting; whereas low temperatures have no effects on the volume or percentage of leukocytes in Atlantic halibut *H. hippoglossus* L (Langston et al., 2002), head kidney neutrophils of channel catfish (Ainsworth et al., 1991), and the peripheral blood leukocytes of rainbow trout (Kollner and Kotterba, 2002), the number of granulocytes double in the blood of carp *C. carpio* (Engelsma et al., 2003); and the peripheral blood leukocytes decrease in sockeye salmon (Alcorn et al., 2002). At first sight, a couple of traits discern both sampling (24 and 72 h); the plasma cortisol concentrations and the span from the sampling moments to the last feeding.

Plasma cortisol concentrations at the 72$^{nd}$ hour were at the basal levels, and this fact should be interpreted as an absence of stressing condition, which makes reasonable the similarity between the GL counting at the 72$^{nd}$ hour. However, the variation between each stress condition and the respective control is high enough to ensure more credibility to such an assumption. The span from the sampling and the initial condition can be fundamental whether considered the half-life of vitamin E. This period is distinct to the many vitamin E isoforms in humans; α-tocopherol acetate has the longest half-life (44 h) while tocotrienol varies between 4.4 to 2.3 h (Singh et al., 2012). The half-life and pharmacokinetics of vitamin E are scarcely reported; in pig, it is 2.6 h to α-tocopherol acetate taken orally (Van Kempen et al., 2016). The authors found no reports about vitamin E half-life in fish but this information precedes several conclusions upon *in vivo* experiments. In any case, the variation of GL counting between the three experimental conditions and the respective control, at the 72$^{nd}$ hour, does not allow discarding environmental stress and calls into question the effect of vitamin E in pacu after three days of starvation. This period without

food could be enough to deplete the vitamin E levels, although it is not large enough to cause significant stress caused by fasting. Pacu is known to withstand long periods of fasting, and significant food residues are found in its digestive tract after a week without food (personal observation), and an energetical deficit does not affect its immune response (Gimbo et al., 2015). Even so, in face of the unpredictable granular leukocytes behavior to cope with thermal stress in fishes, it is reckless to ascribe the same level of GL variation observed at the 72nd hour to the inefficacy of vitamin E to avoid immunosuppressive effects of temperature on these cells.

Non-specific humoral defenses in Teleostei are considerably similar to those in mammals (Yano, 1996). Lysozyme and complement are relevant biomarkers of the innate immune response in fish. The activity (amount) of plasma lysozyme has been reported in plasma and many other physiological compartments in several fish species (Saurabh and Sahoo, 2008). Physiological responses to cope with stress, subsequently to the primary cortisol response, include elevation of glucose and lysozyme. It is well established that the immune system of fish can be severely affected by various stress conditions. Some stressing environmental factors influence the activity of lysozyme, for example, the water temperature seems to affect negatively the lysozyme concentration in plasma (Langston et al., 2002). It reduces drastically the level of serum lysozyme in plaice (Fletcher and White, 1973) and carp (Studnicka et al., 1986) among other species. However, the effect of low water temperature varies even into different strains of the same species (Langston et al., 2002).

Usually, several stress sources are immunosuppressive (Saurabh and Sahoo, 2007). The plasma decrease of lysozyme was also observed in pacu fed with supplemented diets with 300 mg of vitamin, either at 24 or 72 h of stressing condition. Nevertheless, the fish fed with 100 mg of vitamin E seem to have no suppressive effect in the levels of lysozyme at the 24th hour. Moreover, in the course of the experimental span, the lysozyme activity goes increasing to positive values at the 72nd hour. Therefore, the lysozyme profile in pacu was apparently incoherent making difficult to draw a conclusion from the current data. The similar responses between fish fed with 100 mg of vitamin E, and without it, lead to thinking that such vitamin is ineffective in the immune responses of pacu concerning the role of lysozyme. Since the inclusion of vitamin E in the feeding of numerous fish stimulates the lysozyme activity (Waagbø et al., 1993; Sahoo and Mukherjee, 2002a, b), it is reckless attributing the increase of lysozyme in pacu fed with 100 mg of vitamin E at the 72nd hour to any stress mitigating effect, not least since at this point some adaptation has just been suggested.

There is a bridge between innate and adaptive immune responses in fish fulfilled by the complement system. This assembly of circulating and membrane-associated proteins play a central role in the many defense mechanisms. The three complement pathways: the classic, alternative, and lectin have been described in several teleost groups (Zardakis et al., 2001) in spite of the fish preference is reported to the alternative pathway (Sunyer and Tort, 1995). This is likely due to the typical presence of many C3 isoforms, present in fish but absent in mammals (Sunyer and Tort, 1995), which makes the hemolytic and bacteriolytic activity higher in fish than in mammals (Sunyer and Tort, 1995). The effect of environmental temperature on innate and specific immune activity has been reviewed (Le Morvan et al., 1998; Tort et al., 2003), and the effect of such environmental parameter on the complement activity remains inconclusive. While in some fish species, such as *Ictalurus punctatus*, low environmental temperatures decrease the activity of complement (Hayman et al., 1992) in some others, such as *Tinca tinca*, there is an opposite effect (Collazos et al., 1994). The effect of the thermal stress in pacu was transient. Viewed the complement activity in its entire frame, it is possible to say that it was reduced under the thermal stress and, at the 72nd hours, this effect was attenuated, as discussed above. Even so, the negative decrease in the fish at the 24th hour and fed with 100 mg of vitamin E is a controversial point.

In conclusion, on the basis of plasma cortisol concentrations, pacu let in outdoor tanks was affected by the natural thermal fluctuation and seemed to become adapted in the course of 72 h. The number of lymphocytes was reduced by the effect of lower environmental temperature, which seemed more effective in the short term exposure. Conversely, the counting of granular leucocytes increased with lower temperatures. The non-specific humoral responses, lysozyme level, and complement activity declined with low environmental temperatures. The level of supplementary, dietary vitamin E used in the present experiments did not present some expected effects but other investigations concerning its half-life and association with unsaturated dietary lipids seem to be fundamental.

## Authors contribution

The authors of the present work were fundamental to the final format of the manuscript. Their roles were summarized below:

1 Lívia Maria Gruli Barbosa: Project performer (conceptualization, analyses performer, data collection and organization, wrote the original draft)
2 Gilberto Moraes: Supervisor (conceptualization, funding acquisition, project administration, data reviewer, writing reviewer)
3 Fernanda de Freitas Anibal: Investigator, co-advisor.
4 Cleni Mara Marzocchi-Machado: Investigator, co-advisor, monitoring the experimental work concerning immune techniques.

## Submission declaration

I hereby declare that this manuscript has not been submitted or reproduced to date, in whole or in part, to any scientific journal.

## Declaration of Competing Interest

None.

## Acknowledgments

The authors are thankful to the National Council for Scientific and Technological Development (CNPq) for the MSc fellowship of Lívia Maria Gruli Barbosa; to the Federal University of São Carlos-SP (UFSCAR) for the scientific and lab devices; to Prof. Dr. Yara Maria Lucisano Valim (School of Pharmaceutical Sciences - Ribeirão Preto - FCFRP-USP), for kindly allowing the use of her laboratory; to Ana Elisa C.S. Azzolini (FCFRP-USP) for her technical assistance; to Prof. Dr. Margaret de Castro and Mr. José R. da Silva (FMRP-USP) for technical support. We are still in debt with the technician Antonio Aparecido Donizetti from the Laboratory of Adaptive Biochemistry - DGE - UFSCar.

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