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Nutritional feed additives reduce the adverse effects of transport stress in the immune system of Tambaqui (Colossoma macropomum)

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ABSTRACT

Here we show that selected nutritional feed additives reduce the adverse effects of transport stress on the immune system and hematology of tambaqui. We formulated a control diet to contain normal dietary levels of vitamin E (21.6 mg/ kg diet) and C (143 mg/kg), then we added supra levels of these vitamins (vit E – 264 mg/kg and vit C – 1000 mg/kg) to a second diet. Finally, a third diet was produced to contain similar levels of vitamins from diet 2 with 0.1% beta-glucan supplementation. Four hundred thirty-two tambaquis (20.91 $g \pm 0.27$ g) were randomly assigned to 12 aquaria and fed the diets for 15 days; then, all fish were transported for five h and then returned to the aquaria. Blood samples were collected before and after the transport and at the end of the trial (60 days). Transportation significantly increased blood glucose that returned to baseline levels at the end of the trial. However, cortisol seemed to be unresponsive to the stress. Surprisingly, the stress significantly increased the immunoglobulin level after transport. Additionally, the transport markedly reduced the red blood cell count and leukocyte and lymphocytes counts while increasing the control group's neutrophil number. These effects lasted until the end of the trial in the control group. Supra levels of the vitamins and glucan supplementation prevented the decrease in red blood cell and leukocyte count after the stress. Additionally, beta-glucan supplementation induced lower cortisol levels in all the sampling points. However, the effect on the immune parameters was limited, increasing only the lysozyme activity and serum protein levels in the beta-glucan supplemented group and the group fed only the supra levels of vitamins, respectively. In sum, our results indicated that transport for five h induced a limited effect on stress biomarkers. The use of supra levels of antioxidant vitamins alone or in combination with beta-glucan could restore or prevent the adverse effects of stress on hematology and the immune system.

1. Introduction

Tambaqui (*Colossoma macropomum*) is an important fish species for the Latin American aquaculture, with emphasis to Brazil. Farmed tambaqui was responsible for 47.7% of total farmed fish production in Brazil, with an annual production of approximately 135.86 thousand tons in 2014 [1]. The rapid growth and meat quality characteristics have driven the expansion of tambaqui farming. However, the growth in tambaqui production has been negatively affected by infectious diseases leading to significant economic loss [2, 3]. Although the use of antibiotics to control and/or prevent diseases is a common practice by the farmers, this approach has several negative impacts on the aquaculture system, such as selecting antibiotics resistant strains, immunosuppression and environmental pollution [4]. Therefore, the development of nutritional strategies might be a sustainable alternative to increase the resistance of tambaqui to the challenges faced during the production cycle and avoid the indiscriminate use of antibiotics.

Vitamins are essential nutrients with multiple roles in the immune system. Among this large group of compounds, vitamin C (vitC) is the most studied in fish nutrition due to the reduced ability of most fish to synthesize ascorbic acid from glucose intermediaries [5]. Thus, vitC is usually supplemented in fish diets to meet metabolic demands. Additionally, earlier studies have shown that supplementation of vitC over the requirement can reduce the effects of different types of stress. However, when the diet is marginally deficient, it might reduce the immune response leading to reduced antibody production, impaired macrophage phagocytic activity and low activity of the complement system [6] [7]. Likewise, vitamin E plays an important role in the

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immune system, improving the immune response by protecting the plasma membrane of immune cells from the cytotoxic effects of their products (NO, H_2O_2 and their derivatives) [8]. Broaden effects of vitamin E include maintaining normal permeability of blood vessels and cardiac muscle and keeping the homeostasis of cell and plasma metabolites [9].

Vitamins C and E are essential nutrients supplemented to fish diets to nurture cells and have specific physiological roles [10]. On the other hand, non-nutritional feed additives, like immunostimulants, are natural compounds that can activate specific innate and adaptive defense mechanisms of the immune system [11]. Beta-glucans are one of the most studied and used immunostimulants in fish. These compounds are polysaccharides with β 1,3, 1,4 and/or 1,6 glucosidic bonds found in the cell wall of yeast, cereals, fungi, bacteria and algae [12]. Although all beta-glucans can activate the immune system to some extent, the type of bonds in the beta-glucan structure dictates the activation of the immune system response [13, 14].

The stress response starts immediately after the perception of a stressor. The first reaction of the neuroendocrine system is the release of catecholamines, followed by corticosteroids, mainly cortisol, which is considered the most important stress indicator in fish. Cortisol, in turn, induces hyperglycemia due to the increase in hepatic gluconeogenesis and glycogenolysis. Additionally, the release of stress hormones induces changes in the permeability of the branchial membranes, increasing the loss of electrolytes to water [15–18].

The use of immunonutrients and immunostimulants can reduce the physiological effects of stress and assist in mounting a better immune response [19]. Fish transport is one of the most severe stress faced by fish during the production cycle. It involves a combination of several stresses during the transportation, such as handling of the fish, hypoxia, and reduced water quality parameters (mainly the increase in ammonia). Therefore, the use of nutritional and non-nutritional feed additives could assist the defense mechanisms to stress in fish submitted to transport [20], reducing the environmental impact of aquaculture and increasing the biosecurity of farming conditions.

Although the increase in tambaqui production, few studies on micronutrient requirement and their use as nutritional strategies to improve resistance during the production cycle are available. To the best of our knowledge, only one study with vitamin C and another with beta-glucan evaluated their effects on hematology, growth, and immune response with this species. However, no studies tested the synergistic approach of using vitC and vitE supplementation along with beta-glucan on the stress response of tambaqui. Therefore, based on the hypothesis that immunonutrients (vitC and E) could alleviate the physiological response to stress while immunostimulants could prepare the immune system for a possible infection, we designed a study to evaluate if this nutritional strategy could reduce the impact of transport stress on the resistance of tambaqui.

2. Materials and methods

All experimental procedures with animals were approved by the Ethical Committee on Animal Use (CEUA – Protocol # 078/16) and fit the guidelines for Animal Experimentation of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.1. Experimental diets

All diets were formulated to contain similar macronutrient compositions (Table 1). Basal levels of vitC and E in the control diet were adjusted to meet the minimum requirements for *Piaractus mesopotamicus* juveniles [21] since no requirement data is available for tambaqui. The control diet (diet 1) was formulated to contain 105 and 101.25 mg kg^{-1} of vitC and E, respectively; Diet 2 and 3 were formulated to contain supra levels of these vitamins (1155 and 351.25 mg kg^{-1} vitC and E, respectively) (Table 2); however, diet 3 was already supplemented with 0.1% beta-glucan. The vitamin supplement used in this study was vitC-free, and the vitE level was 75.37 mg kg^{-1} diet. The vitC source used to supplement the diets was L-ascorbate-2-monophosphate (Rovimix Stay-C® 35 DSM), while the vitE source was dl-α-tocopherol acetate (Rovimix E-50® DSM). The glucan source was the β−1.3/1.6 glucan isolated from the cell wall of *Saccharomyces cerevisiae* (Macrogard® Biorigin).

Table 1
Ingredient and proximal composition of experimental diets (%).

	Diets		
	Control	Vit C and E	Vits + β-Glucan
Ingredient			
SPC[a]	32.93	32.93	32.93
Soybean meal	10.00	10.00	10.00
Fish meal	10.39	10.39	10.39
Corn	16.95	16.95	19.65
Wheat middlings	4.90	4.90	4.90
Broken rice	4.58	4.58	4.58
Corn starch	6.86	6.86	6.86
Cellulose	5.65	5.65	5.65
Caulim	0.52	0.17	
Soybean oil	4.45	4.45	4.45
Sodium phosphate	1.70	1.70	1.70
Vitamin C – 35%	0.03	0.33	0.33
Vitamin E – 50%		0.05	0.05
β-Glucan			0.17
Vit./min. mix[b]	0.09	0.09	0.09
Threonine	0.57	0.57	0.57
Methionine	0.32	0.32	0.32
Triptophan	0.05	0.05	0.05
BHT[c]	0.02	0.02	0.02
Proximate composition (dry matter basis)			
Dry matter	98.8	97.3	97.3
Crude protein	35.5	35.0	37.0
Lipid	3.6	3.2	3.0
Total fiber	1.6	2.0	1.8
Calcium	1.62	1.68	1.63
Phosphorus	1.32	1.32	1.44
Ash	7.4	7.7	7.0

[a] Soy protein concentrate.
[b] Mineral and vitamin mix supplied the following per kg $diet^{-1}$: vitamin A, 8000 UI; vitamin D3, 2250 UI; vitamin E, 112 mg; vitamin K, 15 mg; vitamin B1, 16 mg; vitamin B2, 16 mg; pantothenic acid, 40 mg; niacin, 85 mg; biotine, 5 mg; folic acid, 5 mg; vitamin B12, 16 mg; vitamin B6, 16 mg. Cobalt, 0.5 mg; copper, 10 mg; iron, 60 mg; iodine, 1.5 mg; manganese, 50 mg; selenium, 0.35 mg; zinc, 100 mg.
[c] Antioxidant Banox®.

The ingredients were ground to particles smaller than 1.0 mm and thoroughly mixed with water (25%). Then, each diet was extruded at 120 °C using a single screw laboratory extruder (Exteec®, Ribeirão Preto-SP) to obtain 1.5 mm diameter floating pellets. Pellets were then dried in a forced-air oven at 65°C for 24 h and stored at −20 °C until further use.

2.2. Animals and experimental design

Four hundred and thirty-two tambaqui juveniles (20.91 ± 0.27 g) were purchased from a commercial farm (Brejinho de Nazaré-TO, Brazil) and randomly assigned to 12 500L-aquaria (36 fish/aquarium) connected to a recirculation system with a biofilter, UVsterelizer and

Table 2
Analyzed and calculated vitamin C and E levels in experimental diets.

Treatment	Vitamin C (mg ascorbic acid/kg diet)		Vitamin E (mg α-tocopherol /kg diet)	
	Calculated	Analyzed	Calculated	Analyzed
Control	105	143	101.25	21.6
Vit C + E	1155	1000	351.25	264
Vits + β-glucan	1155	1150	351.25	282

thermostatically-controlled temperature. Water temperature (28,0 ± 1,0 °C), dissolved oxygen (> 5,0 mg L^{-1}), ammonia, and pH (7.1 ± 0.3) were monitored throughout the experiment and kept within the optimum range for tambaqui. Photoperiod was maintained at 13 h light and 11 h dark.

All fish were fed the control diet (diet1) for 15 days. Afterwards, quadruplicate groups of fish were fed diets 2 and 3 for an additional 15 days, while another four groups of fish were kept in a control diet. On the 30th day, all the fish were submitted to transport stress (described in Section 2.6) for 5 h. After the transport, fish returned to the aquaria and were fed the control diet until the 60th day to evaluate the impact of the stress and the nutritional strategies on stress recovery (Fig. 1). Fish were fed *ad libitum* four times a day to ensure maximum feed intake.

2.3. Hematology and serum biochemistry

Blood samplings were performed at 30 (before and after transport) and 60 days of the experiment. Twelve fish per treatment were bled by caudal puncture using 1 mL syringes rinsed with EDTA (3.0%) after fish were anaesthetized with eugenol (1 mL to 100 L water). Red and white blood cells count were performed using the hemocytometer method in Neubauer chamber. Cells were stained in toluidine blue solution (0.01% in Ringer solution at 0.9%) according to a previously reported method with slight modifications [22].

Leukocyte differentiation count was performed in blood smears stained with May-Grünwald Giemsa [22]. Two hundred cells were counted using a microscope at 100X augmentation, and the absolute number was reported. hemoglobin was determined using a commercial colourimetric kit (Labtest Diagnostica®) based on the cyanmethaemoglobin method [23]. Hematocrit was determined by centrifuging a blood sample at 8000 rpm for 5 min. Serum protein was determined using a colourimetric commercial kit (Labtest Diagnostica®) based on the biuret method.

Plasma cortisol was determined using a commercial kit (DRG International®) based on the ELISA assay. Serum chloride concentration was determined using a colourimetric commercial kit (Labtest Diagnostica®), and blood glucose was determined using a glucometer (Roche®).

2.4. Immune parameters

Serum samples of three fish were pooled to compound one replicate due to the reduced volume of blood collected in the samplings. Therefore, the four replicates by treatment are the mean of 12 fish.

2.4.1. Total blood reactive oxygen species (ROS)

Total blood ROS production was determined using NBT (nitro blue tetrazolium) following a previously reported method [24] with slight modifications [25]. Briefly, 100 μL of heparinized blood was immediately incubated with the same volume of NBT buffer (0.2%) for 30 min. Then, 1 mL DMF (dimethylformamide) was added, and the optical density was recorded in a spectrophotometer at 540 nm. All procedures were performed at room temperature.

2.4.2. Lysozyme activity

Lysozyme activity was determined according to Demers and Bayne [15] with slight modifications for tambaqui. Briefly, a standard curve was made with serial dilutions of standard lysozyme from hens chicken egg (Sigma # L6876). Then, 15 μL of serum was mixed with 250 μL of *Micrococcus lysodeikticus* (Sigma # M3770, ATCC No. 4698) suspension in microtiter 96-well plates, and the absorbance was recorded at 450 nm. The mixture was then incubated at 30ºC for 20 min, and the absorbance was recorded. The method is based on the change of absorbance caused by the lysis of *M. lysodeikticus* suspension after incubation using lysozyme from hens' chicken eggs as standard. The analysis was performed in triplicates and expressed in μg mL^{-1}.

2.4.3. Total immunoglobulin (Total ig)

Total immunoglobulin concentration was determined according to Anderson and Siwicki [24]. Firstly, the total protein content of serum was determined using a commercial kit described in Section 2.3. Then, 25 μL of serum was mixed with the same volume of PEG (polyethylene glycol) solution (12%) in a 96-well round-bottomed microtiter plate. Plates were incubated overnight and then centrifuged at 4000 g, 4ºC for 20 min. Five μL of the supernatant was removed, and the protein content was determined. The difference between the protein content of treated and untreated samples with PEG corresponds to total immunoglobulin content. The results were expressed in mg/ mL.

2.5. Vitamin C and E analysis in the diets

Vitamin C and E analysis of the diets were performed according to the method reported by Wang, Liao, Hung and Seib [26] and Huo, Nelis, Lavens, Sorgeloos and De Leenheer [27], respectively. Briefly, diets were ground, and digestion of samples were performed differently for each vitamin.

For vitamin C quantification, 2.0 g of ground samples were incubated with 20 ml of metaphosphoric acid/ dithiothreitol (DTT) solution (6% aqueous solution of reagent grade metaphosphoric acid containing 0.2% DTT) for 10 min at 25 ºC. Then, the mixture was centrifuged at 8160 g for 15 s and an aliquot of the supernatant was removed and treated with acid phosphatase solution for 3 h at 37ºC. An aliquot of the digest was diluted in cold perchloric acid and centrifuged for 2 min at 8160 g. Then, an aliquot of the supernatant was used to determine the ascorbic acid

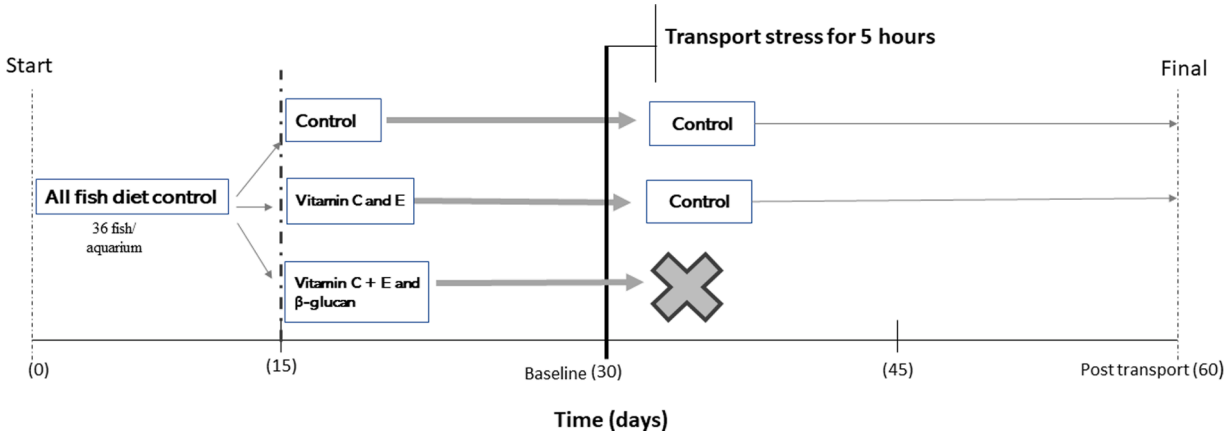

Fig. 1. Schematic overview of the experimental design.

concentration using an electrochemical detector.

Vitamin E was quantified in samples of ground diets as total tocopherol. Briefly, tocopherol was extracted in the samples with methanol containing butylhydroxytoluene (BHT). Then, total tocopherol was measured using reversed-phase chromatography with consecutive fluorescence detection.

2.6. Transport stress

On the 30th day of the feeding trial, 36 fish were collected and used for sampling. The remaining fish were transferred to a 400 L fish transport tank. The tank was divided into three similar sizes with a plastic net (10 mm mesh diameter) to separate the fish from different treatment groups. Then, fish were transported for 5 h at the stocking density of 38 g L^{-1} to mimic the transport conditions commonly performed in commercial fish production. Dissolved oxygen, total ammonia and temperature were monitored during the transport to be similar to the raising conditions in the laboratory.

2.8. Data analysis

We used a completely randomized design with two factorial arrangements to test our hypothesis: 1) a 3 × 2 factorial scheme considering the three diets as factor 1 and two sampling points as the factor 2 (before and after transport); 2) a 2 × 3 scheme considering only two diets (control and VitC + E) as factor 1 and 3 sampling points (before and after transport, and at the end of the experiment) as factor 2. The factorial scheme 2 × 3 was used because the fish fed with diet 3 died after the transport. The reason for death was unrelated to the diet or the stress during transport.

Data were analyzed using the GLM procedure of SAS software. The significance level was 0.05, and Bonferroni's multiple range test was used to compare the treatments.

3. Results

Nutritional strategies did not affect hemoglobin concentration, hematocrit (HTC), and total leukocyte count in each sampling point, however, both nutritional strategies prevented the decrease on total leukocyte count after the stress observed only on fish fed control diet (Fig. 2). However, the transport stress decreased the hemoglobin concentration only at 30 days after the stress (Fig. 2a, $p = 0.0072$). Only the fish fed the control diet had a significant decrease in total leukocyte count at 30 days after stress (Fig. 2d, $p = 0.0015$). Nutritional strategies significantly increased the RBC count of tambaqui (Fig. 2b, $p = 0.0073$). The transport stress increased the HTC on the control and the vitamin and glucan-supplemented groups (Fig. 2c, $p = 0.0496$); however, HTC of fish fed the diet supplemented only with supra levels of antioxidant vitamin was unaffected by the transport stress.

Transport stress significantly reduced the number of circulating lymphocytes in the control and vitamins-supplemented groups. However, only the fish fed the control diet significantly decreased the number of lymphocytes at 30 days post-transport, while the groups fed the supra levels of antioxidant vitamins had a significant decrease only soon after transport (Fig. 3a, $p = 0.0008$). Transport stress increased the number of circulating neutrophils in fish fed the control diet and the supra levels of antioxidant vitamins (Fig. 3b, $p < 0.0001$). Fish fed supra levels of vits + glucan supplemented diet showed the lowest number of circulating neutrophils soon after transport ($p = 0.0078$). The neutrophil number reduced to values similar to the baseline at 30 days post-transport. Transport stress and/or nutritional strategies did not affect the number of monocytes (Fig. 3c).

Stress biomarkers of tambaqui juveniles were differently affected by the transport stress and nutritional strategies (Fig. 4). Blood glucose increased significantly after the stress and returned to baseline levels (Fig. 4a, $p = 0.0002$). However, the nutritional strategies did not affect the blood glucose level of tambaqui ($p = 0.3409$). Blood cortisol of

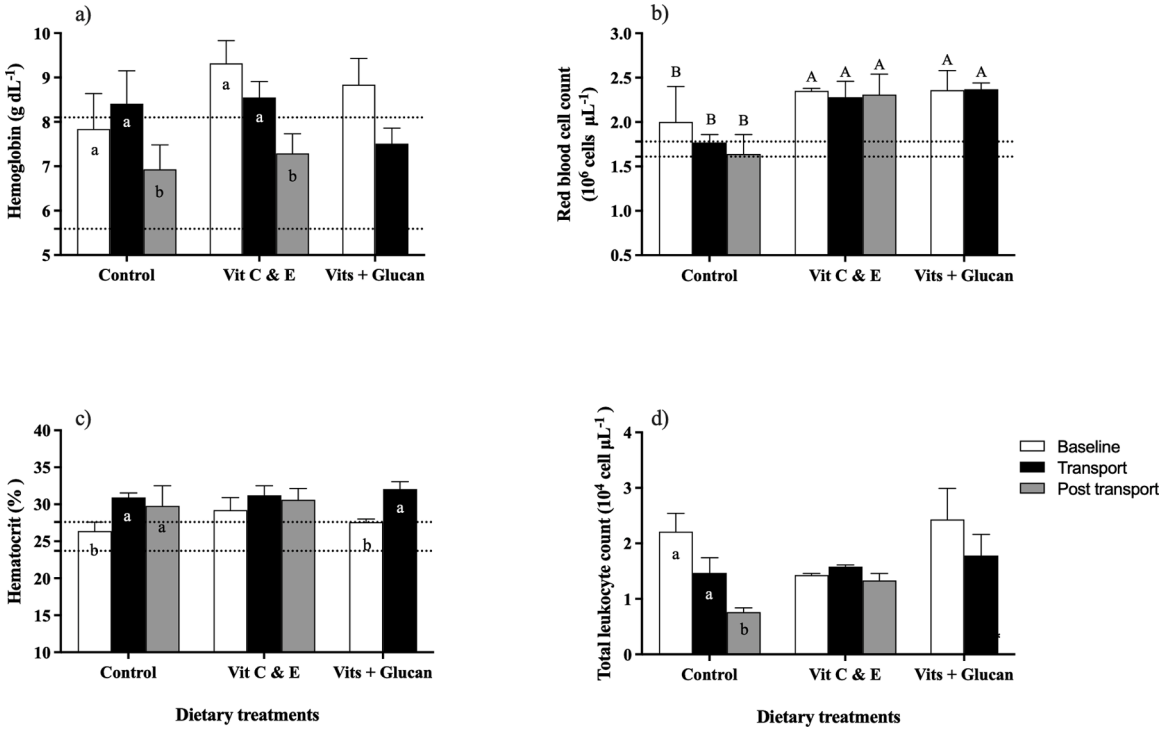

Fig. 2. Hemoglobin concentration (a), red blood cell (RBC) count (b), hematocrit (c), total leukocyte count (TLC) (d) of juvenile tambaqui fed the experimental diets before and after transport stress and at 30 days post-transport. Capital letters on the top of bars compare the treatments in each time point, while small letters inside the bars evaluate the effect of the sampling points in each dietary treatment using Bonferroni's multiple range test ($p<0.05$). Values are means of two determinations per fish, two fish per tank and four tanks per treatment. Space between the dotted lines indicates the reference interval for healthy tambaqui (Ranzani-Paiva et al. 1998, 1999; Tavares-Dias & Sandrin 1998; Tavares-Dias et al. 1998).

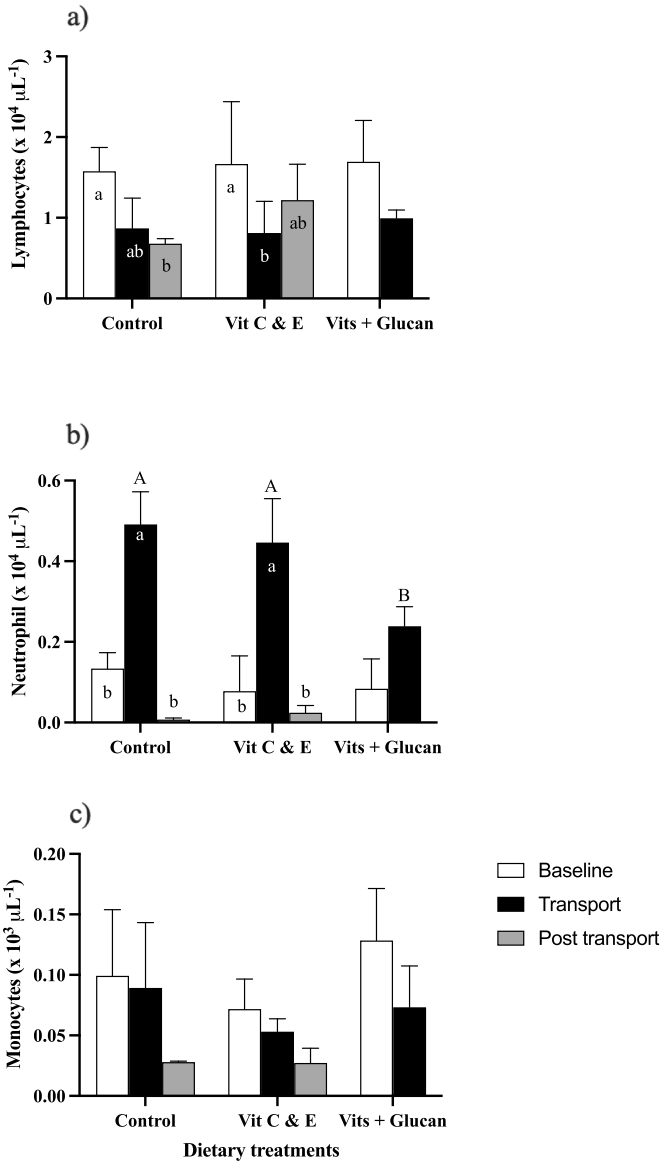

Fig. 3. Lymphocyte (a), neutrophil (b), and monocytes (c) counts of juvenile tambaqui fed the experimental diets before and after transport stress and at 30 days post-transport. Capital letters compare the treatments in each time point, while small letters inside the bars evaluate the effect of the sampling points in each dietary treatment using Bonferroni's multiple range test ($p<0.05$). Values are means of two determinations per fish, two fish per tank and four tanks per treatment.

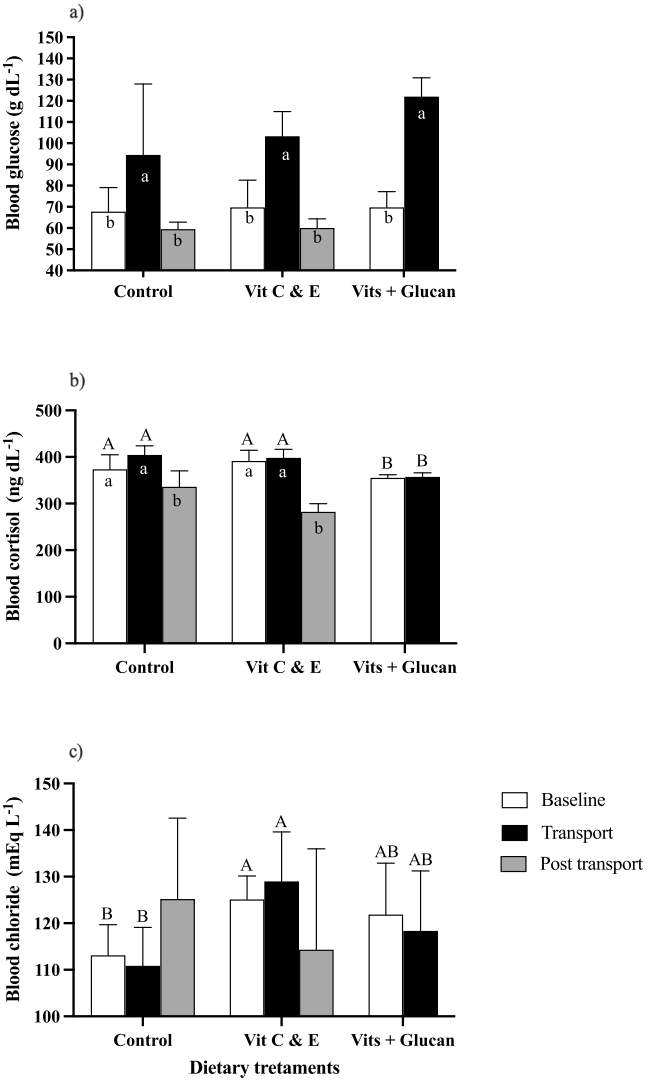

Fig. 4. Blood glucose (a), blood cortisol (b) and chloride level (c) of juvenile tambaqui fed the experimental diets before and after transport stress and at 30 days post-transport. Capital letters compare the treatments in each time point, while small letters inside the bars evaluate the effect of the sampling points in each dietary treatment using Bonferroni's multiple range test ($p<0.05$). Values are means of two determinations per fish, two fish per tank and four tanks per treatment.

tambaqui significantly reduced only at 30 days post-transport ($p < 0.0001$), and no differences were observed between the baseline and soon after transport stress (Fig. 4b). Irrespective of the transport stress, fish fed diets supplemented with vits and glucan had lower blood cortisol than the other groups ($p = 0.0115$). Fish fed the control diet showed lower blood chloride concentration than fish fed the supra levels of antioxidant vitamins at baseline and soon after transport (Fig. 4c, $p = 0.0178$). However, no effect of transport stress was observed for this parameter (Fig. 4c, $p = 0.8749$).

A significant increase in total blood ROS production of tambaqui soon after the transport stress was observed in all dietary groups (Fig. 5a, $p = 0.0496$). However, the nutritional strategies did not affect this parameter ($p = 0.5784$). Lysozyme activity was higher than the control group in fish fed the vits + glucan diet (Fig. 5b, $p = 0.0246$), but no effect of the transport stress was observed for this parameter. Soon after the transport, a markedly increase in total Ig content was observed in tambaqui, irrespective of the dietary groups (Fig 5c, $p < 0.0001$). Total Ig values returned to baseline levels in the control and the vits supplemented groups after 30 days of transport. Total serum protein concentration significantly increased after the transport only in fish fed the supra levels of antioxidant vits (Fig 5d, $p < 0.0001$) and then returned to baseline levels. However, no differences between the experimental groups were observed for total serum protein.

4. Discussion

In this study, we tested if the use of two different nutritional strategies commonly used in fish nutrition could improve the resistance of tambaqui to transport stress. Here, we found evidence that using supra levels of antioxidant vitamins (C and E) associated with β-glucan supplementation could prepare the fish to cope with the transport stress. To the best of the authors' knowledge, this is the first study to demonstrate the associated effect of using supra levels of antioxidant vitamins and

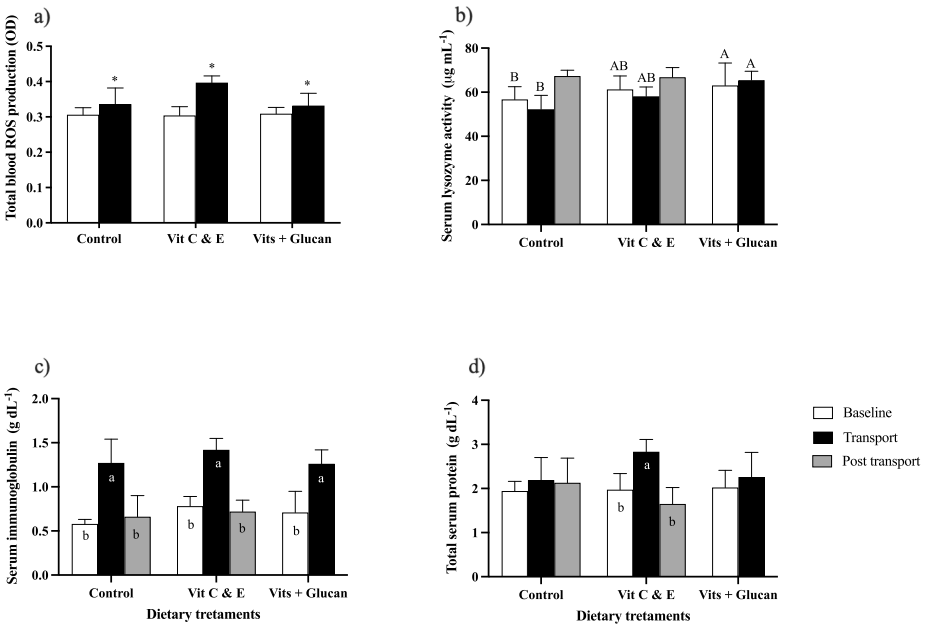

Fig. 5. Total blood ROS production (a), lysozyme activity (b), total immunoglobulin concentration (c) and total serum protein (d) of juvenile tambaqui fed the experimental diets before and after transport stress and at 30 days post-transport. Capital letters compare the treatments in each time point, while small letters inside the bars evaluate the effect of the sampling points in each dietary treatment using Bonferroni's multiple range test ($p<0.05$). Values are means of two determinations per fish, two fish per tank and four tanks per treatment.

β-glucan on the response to stress, hematology, and immunity of tambaqui. Additionally, although the vitE content of the control diet was 78% lower than the theoretical values (Table 2), this level was satisfactory to maintain normal growth as no difference between the groups was observed in the weight gain recorded (data not shown). On the other hand, the nutritional strategies formulated to contain supra levels of the antioxidant vitamins showed a decrease of 22% in vitE concentration compared to the theoretical levels. This result reinforces the nutritional recommendation differences in this phylogenetic group, and care should be exercised when using nutrient requirement data interchangeably between fish species [28].

Nutritional strategies are commonly used to improve the health status of fish, and consequently, improve fish growth. Although the nutritional strategies used in this study did not affect the growth of tambaqui, we observed a positive effect on improving the resistance to stress . This effect has been previously reported for other fish species [8, 12, 29-33].

Fish transport is one of the most stressful events during the fish production cycle. It involves several types of stress, including crowding, handling, and hypoxia, leading to high mortality rates during and after transport. Glucose, cortisol, and chloride levels in the blood are commonly used biomarkers to assess the effect of stress in fish. Here, we observed that blood glucose was the most sensitive stress biomarker to evaluate the impact of transport stress in tambaqui, increasing soon after the transport and returning to baseline levels 30 days after transport. However, none of the nutritional strategies used could prevent the increase in blood glucose after the transport stress. Generally, the rise in blood glucose after the stress is driven by hepatic glycogenolysis and/or gluconeogenesis induced by cortisol, leading to hyperglycemia [34]. Therefore, the increase in blood glucose is generally linked to increased blood cortisol levels. However, failure on blood cortisol response might occur due to negative feedback of this hormone in the hypothalamus, leading to suppression of ACTH release. This mechanism is responsible for unchanged blood cortisol levels in fish exposed to chronic stress [35]. Additionally, we might have lost the peak of cortisol secretion during blood sampling. Altogether, this might explain the high cortisol levels at baseline in this study.

Here, we observed that using dietary supra levels of antioxidant vitamins in combination with beta-glucan supplementation could reduce cortisol levels. Once the fish fed only the supra levels of the antioxidant vitamins did not differ from the control, we concluded that this effect was mediated by beta-glucan supplementation. Conflicting and limited results of the effect of beta-glucan supplementation on blood cortisol levels in fish have been reported. For instance, dietary beta-glucan supplementation to *Piaractus mesopotamicus* reduced blood cortisol levels, while no effect of glucan supplementation was observed in rainbow trout [36]. On the other hand, the dietary supplementation of beta-glucan to *Brycon amazonicus* induced an increase in blood cortisol levels [37]. Although the view that stress has a suppressive effect on the immune system modulated by the cortisol blockage on pro-inflammatory cytokine synthesis is well-established [18], recent findings have indicated that a stimulatory effect could be observed as well [38]. Based on these recent findings, beta-glucan might affect the neuroendocrine system probably by modulating the synthesis of specific cytokines that control cortisol secretion. However, further studies in fish should be performed to confirm this hypothesis.

Our results showed that the use of supra levels of antioxidant vitamins increased the serum chloride levels in tambaqui, which could affect the osmoregulation of fish. During stress events, released catecholamines increase blood flow and gill permeability, leading to loss of electrolytes (mainly Na and Cl) and increasing water influx. All these processes culminate with osmoregulatory disturbances in freshwater fish [17, 39]. Feeding tambaqui with high levels of antioxidant vitamins probably improved cell membrane permeability, avoiding the leakage of chloride to the water, increasing the serum chloride levels. This synergistic effect of vit C and E on the cell membrane is well described in fish and mammals [40]. Additionally, the transport stress had a limited impact on blood chloride levels of tambaqui. This result is consistent with a recent study with the same species that evaluated the effect of anaesthetics on transport stress [41]. However, previous reports with tilapia and *Brycon cephalus* showed impairment in maintaining blood chloride levels after stress [42, 43]. These differences among studies might be related to the different abilities of fish species to cope with osmoregulatory disturbances.

The protective effect of the antioxidant vitamins was consistent with the results of the RBC count in this study. The high RBC count of the vitamin supplemented groups is probably a direct effect of the protection of the RBC membrane from the free radicals produced during the stress [40]. Similar results have been previously reported for other fish species, including tambaqui [44], *Piaractus mesopotamicus* [45], *Cirrhina mrigala* [46]. Additionally, vitamin C participates in erythropoiesis by stabilizing the heme group in the hemoglobin synthesis and is already

involved in preventing methemoglobin formation, which is associated with RBC hemolysis. Therefore, the combining effect of the antioxidant vitamins might have prevented the hemolysis of RBC and improved the differentiation of new RBCs leading to a significant increase in their number. Here, the effect of supra levels of antioxidant vitamins was so beneficial that it kept the RBC count unchanged even at 30 days post-stress, while the fish fed conventional levels of vitamins showed a trend to reduced RBC counts even after 30 days post transport. The effect of stress was so intense that it almost induced anemia in control fish at 30 days post-stress, reaching the inferior limit of the reference interval for tambaqui (Fig. 2b).

Generally, stress-induced effects in hematology include a decrease in RBC counts, higher hematocrit values and lower Hb content. All these changes are caused by the release of immature RBCs, which are larger cells with reduced hemoglobin content [34, 42]. This effect is consistent with our results, where the transport stress significantly affected those parameters in fish fed the control diet. However, the supplementation of supra levels of the antioxidant vitamins prevented some of these effects, at least keeping normal hematocrit values throughout the experiment. Although this mild effect has been observed in the red blood cells, a clear trend of protection was observed for the white blood cells (WBC). While the fish fed the control diet showed a significant decrease of the WBC count after the stress, the fish fed diets with supra levels of vitamins and/or beta-glucan did not show any changes after the transport.

The reduced number of lymphocytes after the stress could induce a low resistance to infection in fish due to their role in antigen-presenting and mounting an immune response [47]. Additionally, the transport stress caused a significant increase in neutrophils. The increased neutrophil number after stress is probably the reason for the significantly higher total ROS production observed post-stress. Activated neutrophils produce high amounts of ROS generated by the increased activity in NADPH oxidase [48]. Probably, the handling procedures might have induced the release of DAMPs (damage-associated molecular patterns) in peripheral blood vessels, leading to the neutrophil activation cascade. These events culminate with G-CSF secretion (granulocyte colony-stimulating factor), which increases the number of circulating neutrophils and, as they reach the peripheral tissues, undergo activation, and release significant amounts of ROS [49, 50].

The nutritional strategies used in this study have limited effects on the immune parameters assessed in tambaqui. Fish fed supra levels of antioxidant vitamins with beta-glucan showed an increase in serum lysozyme activity compared to the control group. In contrast, a significant increase in total serum protein was observed only in fish fed the supra levels of antioxidant vitamins after the transport. Usually, dietary beta-glucan supplementation induces an increase in innate immune proteins such as lysozyme, complement and cytokines [51] [62 [52], therefore, increasing the total serum protein level. However, here we did not observe a consistent effect of beta-glucan supplementation. In fact, the antioxidant vitamins seem to have a more prominent impact on serum protein than the diet with beta-glucan. Although we expected a better condition of fish fed the supra levels of vitamins due to their reducing effect on ROS production during the stress response, the beta-glucan supplementation seems to have repressed these effects. Beta-glucan supplementation might have activated neutrophils and induced additional ROS production; the association of ROS production by the immune cells and the stress response might have impaired the synthesis of immune proteins and therefore reduced total serum protein content to levels similar to the control group.

Immunoglobulins are proteins secreted by lymphocytes. Although they are involved mainly with the adaptive immune system, they are potent inductors of innate immune responses [50]. As previously mentioned, immune responses are generally depressed in stressed fish [18, 53]. However, the significant increase immediately after the transport stress of tambaqui in all dietary groups challenges this assumption. Previous reports with Atlantic cod [54] and Persian sturgeon [55] have observed similar results, and no conclusive mechanisms have been proposed for these effects. These authors attributed the results to the nature of the stress (acute stress), which can be the case in our study once a limited effect of the transport stress was observed on blood cortisol levels.

In conclusion, our results indicated that transport for 5 h induced a limited effect on stress biomarkers. The use of supra levels of antioxidant vitamins alone or in combination with beta-glucan could restore or prevent the adverse effects of stress on hematology and the immune system. Additionally, beta-glucan supplementation to tambaqui diets reduced the cortisol levels and further research on the mechanism that beta-glucan modulate the neuroendocrine system is warranted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author statement

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