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Long-term organic selenium supplementation overcomes the trade-off between immune and antioxidant systems in pacu (*Piaractus mesopotamicus*)

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ABSTRACT

Selenium (Se) is an essential nutrient for antioxidant defenses in fish because of its role in preventing immunosuppression caused by oxidative stress. In this study it was demonstrated the relation between the oxidative stress and immune status after a long Se supplementation period, as a result of the evaluation of immunological, hematological and antioxidant responses, as well as growth performance of pacu fed diets supplemented with different concentrations of organic selenium (0, 0.3, 0.6, 0.9, and 1.8 mg Se-yeast/kg, but the final analyzed selenium concentrations were 0.72, 0.94, 1.15, 1.57 and 2.51 mg/kg, respectively) for 65 days. Dietary Se supplementation at 1.15 mg Se-yeast/kg (analyzed value) restored the production of antioxidant enzymes (glutathione peroxidase (GPx) and glutathione S-transferase (GST)), and consequently allowed the increased of some immunological parameters (leukocyte respiratory burst activity and lysozyme activity), hematological parameters (red blood cell count (RBC), hematocrit (HTC), mean corpuscular volume (MCV), and white blood cell count (WBC)). Se supplementation in pacu diets at 1.15 mg Se-yeast/kg for 65 days improved immune response and antioxidant defenses, suggesting that oxidative stress impairs immune system response to prevent excessive reactive oxygen species in cells and indicating the occurrence of a physiological trade-off between immune and antioxidant systems. Higher Se levels, such as 1.57 mg Se-yeast/kg increased the leukocyte respiratory burst activity, the WBC and thrombocyte counts, the RBC and HTC, and the GST and GPx enzymes. However, 2.51 mg Se-yeast/kg decreased the lysozyme levels, the WBC and thrombocyte counts, the RBC, HTC and MCV, and the GST and GPx enzymes. Those findings are important to future studies because showed the negative effect of oxidative stress on immunity, and may help to prevent any inhibition of the expected immune response after immunomodulators administration and vaccination. Also it was possible to meet the dietary selenium requirement of pacu, that was estimated to be 1.56 mg/kg.

1. Introduction

Selenium (Se) is an essential mineral that has received considerable attention in animal nutrition. Se is a structural component of the animal enzyme glutathione peroxidase (GPx) [1,2], which plays a key role in regulating reactive oxygen species (ROS) [3]. This selenium-dependent enzyme uses glutathione (GSH) as a substrate in the detoxification of hydroperoxides in extra- and intracellular spaces and of lipid peroxides in cell membranes [1,4,5]. The group of thioredoxin reductases is another selenoprotein subfamily that catalyzes the reduction of thioredoxin (RT) and that is also involved in cellular antioxidant activity, activation of signaling molecules, reduction of ribonucleotides to deoxyribonucleotides for DNA synthesis, and regulation of transcription factors [5].

Recent evidence suggests that Se plays an important role in

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preventing the immunosuppressive action of oxidative stress [6]. Thus, adequate levels of selenoproteins such as GPx and RT are especially important for maintaining proper immune response, mainly because when activated, phagocytes such as macrophages and neutrophils, rapidly release a large amount of ROS. These compounds are harmful and cytotoxic in high concentrations, but if properly regulated, have microbicidal activity in phagocytes and modulate signaling events during cell activation and differentiation [7,5].

Se is an essential nutrient for fish, and required to maintain good immunity primarily in fish under intensive system, which stress could lead to immunosupression, so its dietary requirements have been estimated for rainbow trout (*Oncorhynchus mykiss*) at 0.07 μg Se/g diet [8], channel catfish (*Ictalurus punctatus*) at 0.1–0.5 mg Se/kg diet [9], grouper (*Epinephelus malabaricus*) at 0.7 mg Se/kg diet [10] and at 0.98 mg of organic Se/kg diet or 0.90 of inorganic mg Se/kg diet [3], yellowtail kingfish (*Seriola lalandi*) at 2 mg Se/kg diet [11], and common carp (*Cyprinus carpio*) at 1 mg nano-Se/kg [12]. However, Se requirements have not been quantified for pacu (*Piaractus mesopotamicus*), a Neotropical freshwater species of huge economic importance that is reared in Brazil and most of South America because of its ease of management, rapid growth, and marketability [13]. Thus, this study aimed to evaluate the immunological, hematological, and antioxidant responses and growth performance of pacu fed diets supplemented with different selenium concentrations.

2. Material and methods

2.1. Animals and experimental diets

A total of 260 pacu (mean initial weight: 39.8 ± 6.0 g and mean initial length: 12.6 ± 0.7 cm; mean ± SD) were fasted for approximately 24 h and randomly distributed into 20 100-L polyethylene tanks at a stocking rate of 13 fish per tank. The tanks were provided with continuous water flow at 2–2.5 L per min (approximately 3.000 L per day) and fitted with a recirculating system comprising a biological filter and forced aeration system using radial air compressors and porous stones.

Periodically, the organic matter deposited in the tanks was removed by siphoning. The water quality parameters measured during the experiment were within the adequate range for the species [14]: dissolved oxygen (6.21 ± 0.11 mg/L) and water temperature (27.3 ± 0.2 °C) with a YSI 55 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH, USA), and total ammonia (0.05 ± 0.02 N-NH4 mg/L) with Nessler reagent.

An isoprotein and isoenergetic basal diet was formulated with complete vitamin and mineral supplementation, except for the source of selenium. The formulation and proximate composition of the basal diet was shown in Table 1. The basal diet was supplemented with 0.0.3, 0.6, 0.9 and 1.8 mg Se/kg dry diet from Se-yeast (Selplex®, Alltech, Nicholasville, KY, USA). The final selenium concentrations in the experimental diets were 0.72, 0.94, 1.15, 1.57 and 2.51 mg/kg, respectively, as determined by hydride generation atomic absorption spectrophotometer (HG-AAS), briefly, the assay was done with the diet, and was based on hydride generation of the selenium, which was reduced to hydride in the liquid phase, and therefore transformed into the vapor phase in atomic absorption spectrometer.

Diet ingredients were ground in a mill through a 0.8 mm mesh and mixed in the proportions above. The diets were extruded with water and the granules produced were oven dried at 45 °C for 12 h, stored in plastic bins at 8–10 °C until used.

This study was approved by the Ethics Committee on animal use of Universidade Estadual Paulista "Julio de Mesquita Filho" - UNESP (protocol number 13/2014).

2.2. Feeding trials and experimental design

Tanks were randomly assigned to each diet (0.72, 0.94, 1.15, 1.57 and 2.51 mg Se-yeast/kg) with four replicates each. Fish were fed experimental diets twice daily to apparent satiation for 65 days. Next, fish were starved for 18–24 h and then four fish from each tank (16 fish per treatment) were gently netted from the tanks and anesthetized (benzocaine dissolved in ethanol, 0.1 g/L), weighed, and had their length measured. After biometrics, whole blood samples were taken from the caudal vasculature of each fish for hematological assays and to determine leukocyte respiratory burst activity. The remaining blood was allowed to clot and serum was separated by centrifugation (3000 × g for 5 min) and stored at −80 °C until lysozyme activity and hemolytic activity of the alternative complement pathway (ACP) were determined. After blood sampling, liver samples were collected, frozen, and stored at −70 °C until catalase (CAT) and glutathione S-transferase (GST), glutathione peroxidase (GPx), and reduced glutathione (GSH) levels were determined.

2.3. Immunological and hematological assays

Lysozyme activity was determined by turbidimetric assay as described by Ellis [15] modified by Abreu et al. [16] and Biller-Takahashi et al. [17]. Briefly, 125 μL of a *Micrococcus lysodeikticus* (0.2 mg/mL) suspension (pH 6.2) was mixed with 50 μL of serum and was reacted at 30 °C for 1 h followed by measuring absorbance at 450 nm. Lysozyme activity was expressed as units/mL, with 1 unit indicating a decrease in absorbance of 0.001/min.

Hemolytic activity of the alternative complement pathway (ACP) was measured by kinetic assay as described by Ferriani et al. [18] modified by Biller-Takahashi et al. [19], for determining the time required for serum proteins to lyse 50% of target erythrocytes.

Leukocyte respiratory burst activity, as measured by nitroblue tetrazolium (NBT) reduction into formazan by ROS in phagocytes [20], was determined as described by Biller-Takahashi et al. [21]. To obtain the precipitate, 0.1 mL of heparinized whole blood was added to 0.1 mL of NBT (Sigma, St. Louis, MO, USA), homogenized, and incubated for 30 min at 25 °C. Following incubation, 50 μL of the homogenized suspension was mixed with 1 mL of N,N-dimethylformamide (DMF, Sigma, St. Louis, MO, USA) in a glass tube and centrifuged at 3000 g for 5 min. DMF lyses leukocyte cell walls to release formazan granules into the solution. The optical density of the solution was determined in a spectrophotometer at 540 nm.

Blood parameters including hematocrit, red blood cell count (RBC) and mean corpuscular volume (MCV) of erythrocytes were determined using an automated cell counter (Celm CC550, São Caetano, SP, Brazil). White blood cell count (WBC) and differential leukocyte count were determined from blood smears stained with May-Grünwald-Giemsa stain [22]. WBC count was estimated using the following formula (Ishikawa et al., 2008): leukocytes/μL = (leukocyte number in the smear x erythrocyte number/μL)/2000 erythrocytes counted in the smear [23].

2.4. Antioxidant enzymes

Liver samples were used for determination of CAT, GST, GPx, and GSH activity. The specific enzymatic activities were expressed according to tissue protein levels determined by Biuret reaction [24] with bovine serum albumin as standard.

The liver fragment used to assess CAT and GST activity was weighed, macerated in a 10 mM potassium phosphate buffer, pH 7.0

Table 1
Nutritional and chemical composition of the experimental diets.

Ingredients	Diets (mg Se/kg)				
	0.72	0.94	1.15	1.57	2.51
Fish meal	10.0	10.0	10.0	10.0	10.0
Soybean meal	31.8	31.8	31.8	31.8	31.8
Corn	25.2	25.2	25.2	25.2	25.2
Wheat bran	29.2	29.2	29.1	29.1	29.0
Soy oil	2.60	2.60	2.60	2.60	2.60
Dicalcium phosphate	0.50	0.50	0.50	0.50	0.50
Premix[a]	0.50	0.50	0.50	0.50	0.50
Antioxidant	0.10	0.10	0.10	0.10	0.10
Antifungal	0.10	0.10	0.10	0.10	0.10
Selplex®[b]	0.00	0.03	0.06	0.09	0.18
Total	100	100	100	100	100
Composition					
Dry matter %	88.5	88.49	88.46	88.43	88.36
Crude protein %	27.3	27.28	27.28	27.27	27.26
Lipid %	5.70	5.70	5.70	5.70	5.70
Crude fiber %	4.68	4.68	4.68	4.68	4.68
Ash %	6.82	6.82	6.82	6.82	6.82
Nitrogen free-extract[c] %	44.00	44.00	43.99	43.97	43.92
Selenium mg/kg	0.72	0.94	1.15	1.57	2.51
Gross energy kcal/kg	4.08	4.08	4.08	4.08	4.08

[a] Enrichment per kilogram of feed: Vit.-A 3000 UI; Vit.-D3 3000 UI; Vit. E-200.00 mg; Vit. B1-6.00 mg; Vit. B2-8.00 mg; Vit. B6-3.00 mg; Vit. B12-20.00 mg; Vit. C-350.00 mg; Vit. K-6.00 mg; Folic acid-1.00 mg; Pantothenic acid-20.00 mg; Biotin-0.10 mg; Copper-10.00 mg; Iron-100.00 mg; Iodine-5.00 mg; Manganese -70.00 mg; Niacin- 100.00 mg; Zinc -150.00 mg; B.H.T.-125.00 mg; Colin -150.00 mg.
[b] Selplex®, (ACT) Alltech, Nickolasville, KY, USA.
[c] Nitrogen free-extract = Dry matter − Crude protein − Lipid − Crude fiber − Ash.

(1:10 w/v), and centrifuged at 5000 g at 4 °C for 20 min. Next, the supernatant was transferred to a microtube and stored at −80 °C until analysis. CAT activity was determined as described by Beutler [25]. Briefly, the initial rate of exogenous hydrogen peroxide (H_2O_2) decomposition is determined at 240 nm using a spectrophotometer. The assay was done at 17 s intervals in a quartz cuvette containing 15 mM of H_2O_2 solution in 50 mM sodium phosphate buffer pH 7.0 and the sample extract.

GST activity was determined as described by Keen and Williams [26]. The GST assay uses the increase in absorbance at 340 nm that accompanies the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (GSH) to form glutathione-2,4-dinitrobenzene. The reaction mixture consisted of 1 mM CDNB in ethanol, 1 mM GSH in 100 mM potassium phosphate buffer pH 7.0, and the sample extract.

To determine GPx and GSH levels, liver fragments were homogenized in an ice bath using a Potter-Elvehjen homogenizer (2 replicates x 3 times at 1-min intervals) with 0.1 M sodium phosphate buffer pH 7.0 (1 mL/100 mg tissue). After centrifugation at 3000 g at 4 °C for 30 min, the supernatant was collected for determination of GSH and GPx activity.

GPx activity was measured indirectly by the reaction of GSH with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), as described by Hafeman et al. [27]. The reaction mixture consisted of 20 μL of sample (supernatant), 137 μL of milli-Q H_2O, 100 μL of 2 mM GSH, 100 μL of 0.4 mM sodium phosphate buffer, and 50 μL of 0.01 M sodium azide. After homogenization, the mixture was incubated at 37 °C for 5 min. Next, 100 μL of 1.25 mM H_2O_2 solution was added and the mixture was incubated for 3 min at 37 °C. After incubation, 200 μL of mixture was removed and 800 μL of precipitating reagent (600 μL of 70% perchloric acid [PCA] + 200 μL of 100 mM ethylenediaminetetraacetic acid [EDTA]) was added. After centrifugation at 300g at 4 °C for 10 min, 200 μL of supernatant was added to 1780 μL of 0.1 M phosphate buffer pH 8.0 with 5 mM EDTA, and 20 μL of 10 mM DTNB. After homogenization, the solution was incubated at room temperature for 10 min in a dark room, and GPx levels were determined in a spectrophotometer (Genesys 10UV, Thermo Spectronic, Rochester, NY, USA) at 412 nm.

Hepatic GSH levels were determined as described by Beutler et al. [28]. An aliquot of the homogenate and 0.1 M sodium phosphate buffer pH 7.0 (1 g protein/mL) was added to 1 mL of reaction medium containing 125 mM/L sucrose, 65 mM/L KCl, 10 mM/L HEPES-KOH pH 7.0, and 500 μL of 13% TCA, and centrifuged at 9000 g at 4 °C for 3 min. Next, 200 μL of the supernatant was added to 1780 μL of 0.1 M/L phosphate buffer pH 8.0 with 5 mM EDTA and 20 μL of DTNB. After incubation at room temperature for 15 min in a dark room, GSH levels were determined in a spectrophotometer (Genesys 10UV, Thermo Spectronic, Rochester, NY, USA) at 412 nm.

2.5. Growth performance

At the end of the 65-day feeding trial, all fish of each tank were anesthetized with benzocaine (1 g 15 L^{-1} water), individually weighed and measured to determine growth performance. The growth performance parameters assessed were: body weight BW (g); weight gain (WG) (g) = final weight (FW) (g) - initial weight (IW) (g); feed intake; and feed conversion (FC) = food provided (g)/WG (g).

2.6. Statistical analysis

Treatments were arranged in a completely randomized design including five treatments (Se concentrations) with four replicates each. After tested for normality (Cramer Von Mises) and homoscedasticity (Brown-Forsythe), data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for means comparisons. Treatment effects were considered at $P < 0.05$ level of significance. Polynomial regression analysis was used to estimate the optimum dietary Se supplementation based on GPx activity. All analyses were performed using SAS 9.0 software (SAS Inst. Inc., Cary, NC, USA). Differences were considered significant at $P < 0.05$.

3. Results

No fish mortality or signs of selenium deficiency or toxicity were observed during the experimental period. And polynomial regression analysis of GPx indicated that the optimum level of Se could be approximately 1.56 mg Se/kg of diets for pacu ($R^2 = 0.6844$; $y = -4.9316 \times ^2 + 15.353x + 3.9448$; $p < 0.05$).

Lysozyme activity was highest in fish fed diets supplemented with 1.15 mg Se-yeast/kg ($p = 0.0254$). Conversely, the hemolytic activity of ACP was not significantly different across treatments ($p = 0.3379$). Additionally, leukocyte respiratory burst activity was significantly higher in fish fed 1.15, 1.57 and 2.51 mg Se-yeast/kg ($p = 0.0015$) than in fish fed 0.72 (control) and 0.94 mg Se-yeast/kg (Fig. 1).

Blood parameters were significantly different across treatments, hematocrit ($p = 0.0354$) and mean corpuscular volume ($p = 0.0331$) were higher in fish fed diets supplemented with 1.15 mg Se-yeast/kg (Table 2). Mean RBC count was highest in fish fed 1.15 mg Se-yeast/kg and was significantly higher in fish fed 0.72, 1.15, and 1.57 mg Se-yeast/kg than in fish fed diets supplemented with 0.94 and 2.57 mg Se-yeast/kg ($p = 0.0468$). (Table 2).

WBC count was significantly higher in fish fed diets supplemented with 1.15 mg Se-yeast/kg ($p = 0.0088$) (Fig. 2), whereas thrombocyte count was significantly higher in fish fed diets supplemented with 1.57 mg Se-yeast/kg ($p = 0.0021$) (Fig. 2). Conversely, lymphocyte, monocyte, and eosinophil counts were not significantly affected ($p = 0.2031$; $p = 0.5349$; $p = 0.9731$) by Se-yeast supplementation (Table 3).

CAT activity was not significantly affected by Se supplementation ($p = 0.2742$; Fig. 3). In addition, GSH levels were not significantly affected by Se supplementation ($p = 0.2950$; Fig. 3). Conversely, GPx activity was highest in fish fed diets supplemented with 1.15 mg Se-yeast/kg ($p = 0.001$; Fig. 3). GST activity was significantly higher in fish fed 0.94 mg Se-yeast/kg ($p = 0.0012$) than in fish fed 0.72 (controls), 1.57 and 2.51 mg Se-yeast/kg (Fig. 3).

Feed intake was significantly higher in fish fed diets supplemented with 1.15 mg Se-yeast/kg ($p = 0.0879$). However, weight gain and feed conversion were not significantly affected by Se supplementation ($p = 0.3848$; $p = 0.3369$) (Table 4).

4. Discussion

The dietary selenium requirement of pacu was estimated to be 1.56 mg/kg and the dietary Se supplementation in pacu diets at 1.15 mg/kg for 65 days increased the production of immunological, hematological, and antioxidant factors, supporting the immunosuppressive role of oxidative stress, which impairs immune system response and thus prevent excessive reactive oxygen species in cells, and indicating the occurrence of a physiological trade-off between the immune and antioxidant systems [6]. The negative effect of oxidative stress on immunity observed in our results may help explain several unsuccessful immunomodulatory protocols in use, because fish usually are in a suboptimal antioxidative status, due to several biological processes that lead to ROS production, with consequently unsatisfied immunity response [29]. Altogether, our findings indicate that supplementation with 1.15 mg Se-yeast/kg is adequate to prevent any inhibition of the expected immune response after immunomodulators administration, vaccination or even to maintain a healthy production.

The beneficial action of selenium on innate defense against microbial invasion was supported by the elevated serum lysozyme activity in pacu fed 1.15 mg Se-yeast/kg. Some nutrients supplemented in the diet can modulate lysozyme activity in fish, and lysozyme activity has been used to determine innate immune status in fish. A similar result was observed for leukocyte respiratory burst activity even in the absence of antigenic stimulation, demonstrating the protective role of Se and the immunosuppressive action of oxidative stress caused by antioxidant deficiency [30].

The hemolytic activity of ACP was not significantly affected by Se supplementation. Similar results were reported by Biller-Takahashi et al. [6] for pacu fed selenium-supplemented diets for 10 days, indicating that ACP activity is not affected by oxidative stress. Additionally, elevated ACP activity has been observed in pacu challenged with *Aeromonas hydrophila* [17]. *In vivo*, the ACP is activated directly by carbohydrates or proteins on a foreign cell surface [31], even though increased ACP activity has been demonstrated in fish fed diets supplemented with vitamin C and E [32].

Dietary Se supplementation at 1.15 mg/kg for 10 days resulted in a significantly higher RBC, WBC and thrombocyte counts in pacu, and these changes were attributed to a possible antioxidant role of selenium and increased lifespan of blood cells [6]. In our study, the same effect of Se supplementation on blood parameters was observed, (higher RBC, WBC and thrombocyte counts), which were observed in fish fed the same amount of selenium in the diet. In fact, long-term Se supplementation positively affected cell-mediated immunity, as shown by the increased WBC and thrombocyte counts in fish fed diets supplemented with 1.15 and 1.57 mg Se-yeast/kg. The immunomodulatory and antioxidant effects of selenium have also been demonstrated in the elevated WBC count of rainbow trout exposed to 4.0 and 6.0 ppm sodium selenite [33].

Se plays a significant physiological role in fish antioxidant defenses. In our study, CAT activity was not significantly affected by increasing dietary Se concentrations, and similar results were reported for gibel carp (*Carassius auratus gibelio*) by Han et al. [34]. Once there is a lack of CAT, the reduction reaction of H_2O_2 into H_2O and O_2 is accomplished by GPx [35]. In our study, GPx activity was elevated and this enzyme probably performed the same role. Conversely, GST activity was significantly higher in pacu fed diets supplemented with 0.94 and 1.15 mg Se-yeast/kg. Similarly, elevated GST activity was also observed in common carp fed diets containing 0.15 ppm Se-yeast for four months [36].

Se is a cofactor and structural component of GPx [4, 37], and thus GPx activity can be indicative of dietary Se supplementation and be involved in detoxification of lipid hydroperoxides [4,38]. In this study, Se supplementation at 1.15 and 1.57 mg Se-yeast/kg promoted GPx activity, in spite of the glutathione to be a

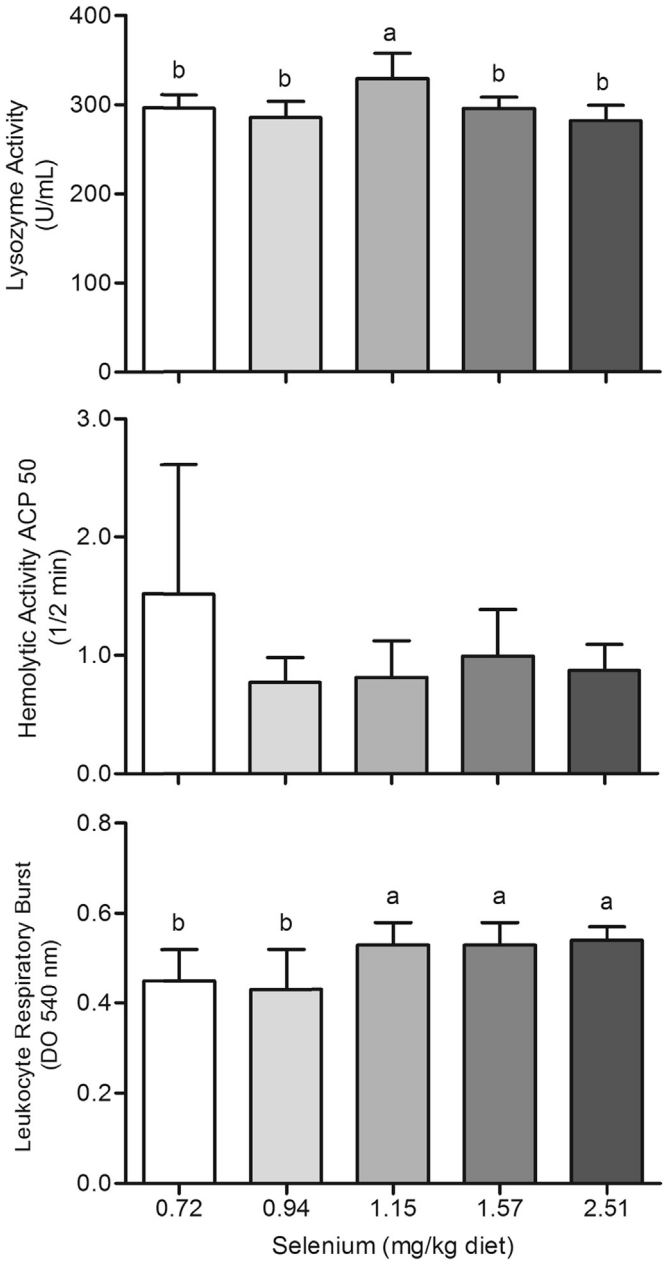

Fig. 1. Lysozyme activity, hemolytic activity of the alternative complement pathway (ACP 50) and leukocyte respiratory burst in juvenile pacu, *Piaractus mesopotamicus*, fed various selenium levels for 65 days. Data are given as means ± sd, n = 16. Within each chart, different letters above bars indicate statistically significant pairwise differences (Tukey's test, $p < 0.05$).

Table 2
Red blood cell (RBC), hematocrit (HCT) and mean corpuscular volume (MCV) of juvenile pacu, *Piaractus mesopotamicus*, fed selenium-supplemented diets for 65 days.

Selenium (mg/kg diet)*	RBC (x 10^6/mm^3)	HCT (%)	MCV (mm^3)
0.72	2.85 ab	36.8 b	130 ab
0.94	2.80 b	36.8 b	132 a
1.15	3.27 a	42.0 a	132 a
1.57	2.93 ab	37.4 ab	128 b
2.51	2.73 b	34.5 b	127 b
SD	0.52	6.04	4.82
SEM	0.07	0.78	0.62
ANOVA P-value	0.0468	0.0354	0.0331

Values are means ± SD. Means in the same column with different superscripts letters are significantly different (Tukey test $P < 0.05$, n = 16).
*Analyzed values.

Table 3
Lymphocyte, monocyte and eosinophil of juvenile pacu, *Piaractus mesopotamicus*, fed selenium-supplemented diets for 65 days.

Selenium (mg/kg diet)*	Lymphocyte (x 10^3/mm^3)	Monocyte (x 10^3/mm^3)	Eosinophil (mm^3)
0.72	38.0	0.64	650
0.94	24.1	0.31	690
1.15	30.7	0.66	877
1.57	42.5	1.07	870
2.51	39.8	0.49	784
SD	16.4	0.82	733
SEM	2.72	0.14	124
ANOVA P-value	0.2031	0.5349	0.9731

Values are means ± SD. Different letters indicate significant differences by Tukey test ($p < 0.05$, n = 16).
*Analyzed values.

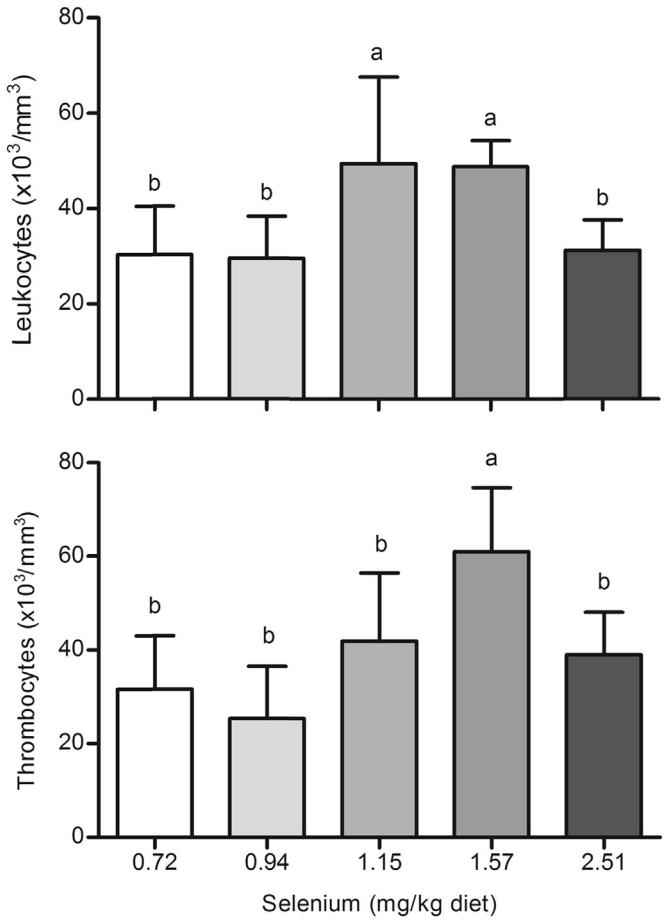

Fig. 2. Leukocyte and thrombocyte in juvenile pacu, *Piaractus mesopotamicus*, fed various selenium levels for 65 days. Data are given as means ± sd, n = 16. Within each chart, different letters above bars indicate statistically significant pairwise differences (Tukey's test, $p < 0.05$).

substrate for both GST and GPx enzymes [4]. Similar results were also reported by Abdel-Tawwab et al. [39] and Hao et al. [40], who found the highest GPx levels in catfish (*Clarias gariepinus*) and loach (*Paramisgurnus dabryanus*) fed diets supplemented with 0.5 mg Se-yeast/kg.

Elevated GPx activity was also found by Ashouri et al. [12] in common carp fed 0.5 and 2 mg nano-Se/kg; however, the latter Se concentration resulted in increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which may be a sign of toxicity. Thus, the authors recommended adding 1 mg nano-Se/kg to carp diets to improve fish growth. In our study, unsupplemented (control) pacu exhibited lower GST and GPx activity and also impaired immunity, highlighting the need for dietary Se supplementation to meet requirements of pacu.

In this study, GSH levels were not affected by Se supplementation. In contrast, Ashouri et al. [12] found higher GSH in common carp fed diets supplemented with 2 mg nano-Se/kg. Interestingly, the GSH levels reported in that study were lower than the ones observed in this study. Nevertheless, supplementation with organic selenium has been shown to overcome the trade-off between the immune and antioxidant systems [6].

Even though feed intake was higher in fish fed Se-supplemented diets, Se supplementation had no significant effect on weight gain, feed efficiency and feed conversion. In addition, fish of this study reached the final weigh similarly at commercial or others experimental condition [13,41,42]. However some negative effects of Se supplementation including reduced growth, poor feed efficiency, reduced GPx activity, and high mortality have been shown in juvenile Atlantic salmon (*Salmo salar*) [43], rainbow trout [8], fingerling channel catfish [9], and juvenile crucian carp (*Carassius carassius*) [44], but it was not observed in this study.

Practical diets deprived of a specific source of Se may meet Se requirements for some fish species, especially considering that the main animal protein source of diets is fishmeal, a key source of Se in practical diets [9,45]. As observed in our study, the final Se concentrations in the experimental diets was much higher than the amount supplemented, indicating that the final value reflects the sum of Se present in the practical diet and the one supplemented with Se. Practical diets have met the Se requirements in juvenile Sacramento splittail [46], juvenile grouper [10], juvenile black seabream [47], and juvenile hybrid striped bass (*M. chrysopx* x *M. saxatilis*) [45,48]. Moreover, direct uptake of Se and other essential minerals from the water across the gills, digestive tract, and skin has also been shown in fish [38,49]. However, the Se requirement greatly varies according to ROS production, besides the fact that stress condition increase Se utilization in fish [29]. Nevertheless, fish fed the Se-free diet exhibited suboptimal antioxidative status, even though increased immune response and hematological parameters after supplementation. This suboptimal antioxidative status may impair the immune system response and represent a trade-off between Se availability and production of immune factors.

In our study, no signs of toxicity such as reduced growth, poor feed efficiency, deformities, tissue damage, and increased mortality were observed in pacu fed diets supplemented with up to 2.51 mg Se-yeast/kg, information that could be confirmed through Se

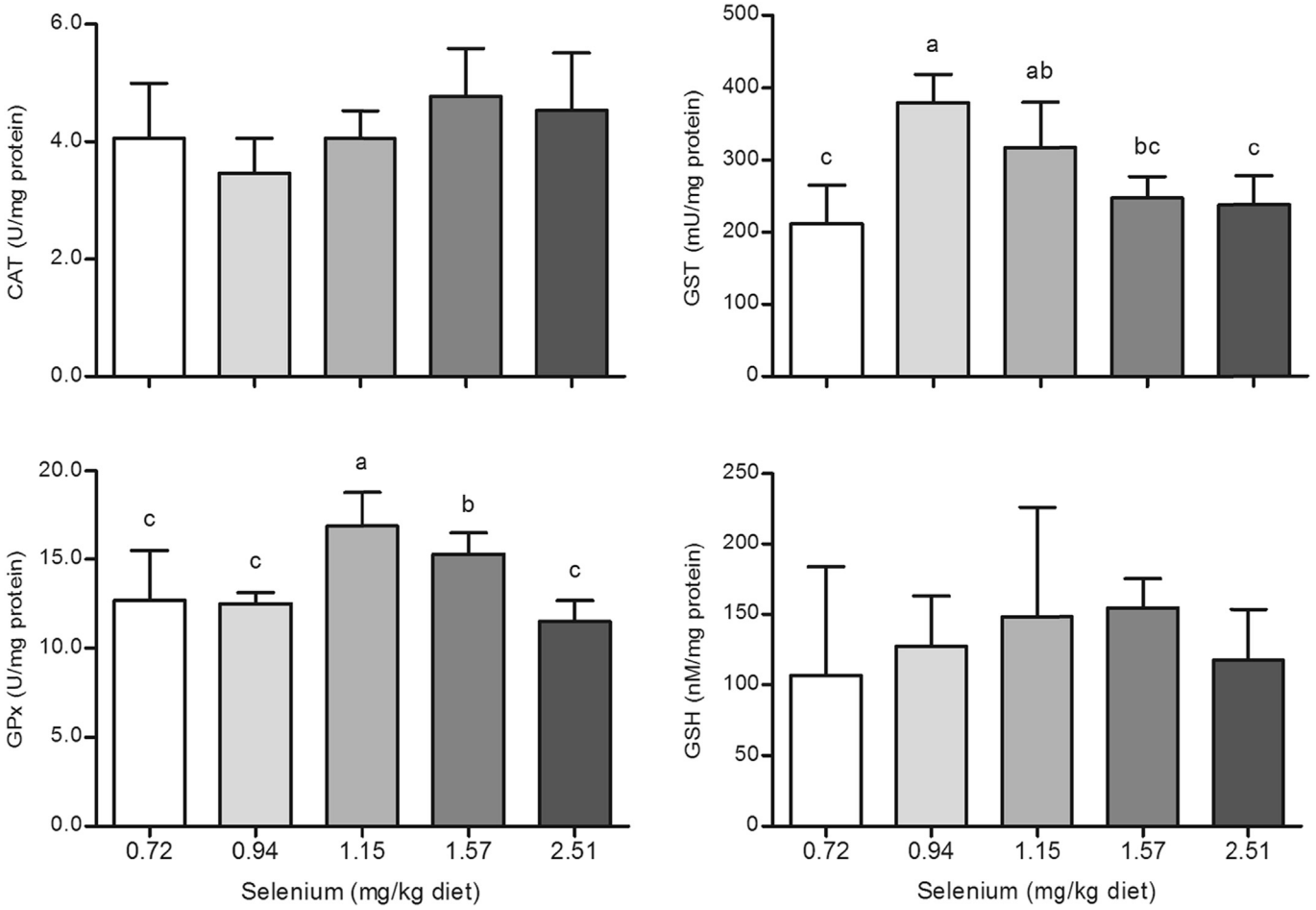

Fig. 3. Enzymatic activity of liver catalase (CAT), glutathione-S-transferase (GST), and glutathione peroxidase (GPx), and hepatic levels of reduced glutathione (GSH) in juvenile pacu, *Piaractus mesopotamicus*, fed various levels of selenium for 65 days. Data are given as means ± sd, n = 16. Within each chart, different letters indicate statistically significant pairwise differences (Tukey's test, $p < 0.05$).

Table 4
Feed intake, weight gain and feed conversion ratio of juvenile pacu, *Piaractus mesopotamicus*, fed selenium-supplemented diets for 65 days.

Selenium (mg/kg diet)*	Feed intake (g)	Weight gain (g)	Feed conversion ratio
0.72	118 ab	88.5	1.4
0.94	118 ab	99.7	1.2
1.15	122 a	85.8	1.4
1.57	115 b	91.0	1.3
2.51	118 ab	88.9	1.3
SD	3.99	10.2	0.17
SEM	0.89	2.28	0.04
ANOVA P-value	0.0479	0.3848	0.3369

Values are means ± SD. Different letters indicate significant differences by Tukey test ($p < 0.05$, n = 16).
*Analyzed values.

determination in several tissues and blood in futures studies. Feed intake was lowest in fish fed diets supplemented with 1.57 mg Se-yeast/kg, but it was not significantly different from that of fish fed 2.51 mg Se-yeast/kg or sufficient to worsen the growth performance. However, Se toxicity depends on several factors, including fish species, age, and nutritional condition; Se chemical form and nature; and exposure conditions, transport, and bioaccumulation [50]. In this study, we used Se-yeast, which is a highly bioavailable form of Se [29].

In conclusion, the dietary selenium requirement of pacu was estimated to be 1.56 mg/kg of diet and the long-term dietary supplementation with organic Se at 1.15 mg Se-yeast/kg prevented the immunosuppressive action of oxidative stress and improved immune response and antioxidant status in pacu. However, long-term Se supplementation did not affect growth performance, indicating that Se is not a limiting factor to weight gain.

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