Improving Heat Resistance of Nile Tilapia (Oreochromis niloticus) by Dietary Zinc Supplementation

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In this study, we investigated the effects of zinc threonine supplementation on growth, immunity-related genes, antioxidative stress, and resistance to heat stress (HS) in Nile tilapia. A total of 300 Nile tilapia (76.69 ± 0.11 g) were randomly allocated into five zinc treatments, with three replicates of 20 fish in each treatment. Based on the control group with zinc-free feeds, dietary zinc levels of 20 or 40 mg/kg diet were added in the form of zinc sulfate (ZnSO₄) and zinc threonine (Thr-Zn). After 45 days of feeding, part of the fish was exposed to HS. The highest performance of growth and feed intake was found in fish fed with zinc (ZnSO₄ or Thr-Zn) at 40 mg/kg, which also significantly elevated the villus length and absorption area. Additionally, significantly higher values of superoxide dismutase, catalase, and glutathione peroxidase activity but lower value of malondialdehyde both before and after the heat stress were documented in fish fed with zinc at 40 mg/kg in diets. After HS, dietary supplementation with zinc threonine significantly reduced the expression of HSP 70 in the liver but increased the expression of IL-10 and TNF-α. Our study demonstrated that zinc threonine supplementation at 40 mg/kg facilitated the coping of tilapia with HS by improving the growth, intestinal morphology, antioxidative resistance, and immune responses.

1. Introduction

Growing human population has resulted in an increasing demand for food, especially for the animal-based proteins. Aquaculture nowadays provides an important source of animal proteins for human consumption [1, 2]. Quality of aquaculture is dependent on many factors such as environmental and nutritional conditions, disease management, and feeding skills [3]. The rearing water temperature is an important environmental factor affecting the health of cultured fish [4, 5]. Many studies have reported the negative impacts of high water temperature on the health and welfare of farmed fish [6–8]. For example, the reduction of available oxygen in the rearing water due to heat stress may lead to breathing disturbances on fish [9]. Such process can further result in the generation of reactive oxygen radicals causing oxidative damages to proteins, lipids, and DNA and altering the balance between oxidation and antioxidation systems of the body [10–12]. Under heat stress conditions, the fish tend to have a poor feed intake, compromised immune response, and increased susceptibility to diseases, causing large economic losses to the aquaculture industry [13].

The application of functional feed additives is one promising means for the prevention of environmental stress in aquaculture [14], as use of these additives works to relieve growth retardation, inflammatory response, immunosuppression, and oxidative damage in fish [9, 15–17]. Zinc (Zn) is an essential trace element and constituent of a variety of enzymes. It participates in many important physiological functions such as regulation of bone mineralization and enhancing immunity [18–20]. Many studies have revealed the beneficial effects of dietary zinc to alleviate the heat stress
in animals by improving the feed utilization, stimulating the immune system, and enhancing the animal health [21–24]. However, most of these studies were related to livestock production, especially for chickens and pigs [25–27], and there is a paucity of studies on aquatic farming such as fish. Kumar et al. [28] found that dietary supplementation with 10 mg/kg zinc potentially mitigated the stress on fish, and other studies also indicated that Zn supplementation could be used to prevent fish from multiple stresses [29, 30]. However, the efficacy of Zn supplementation differed between fish species, levels, and sources of zinc, as well as environmental factors [31–33]. Traditionally, inorganic Zn (Zn sulfate, Zn oxides) was used in aquaculture system because of their low costs and wide commercial availability. The Zn-amino acid complex as organic Zn source, such as zinc threonine, is more likely to fulfill the Zn requirements of animals with a lower dietary Zn supplementation [34]. Related studies also indicated that organic Zn sources generally displayed better efficacy and bioavailability than the inorganic salts [35–37], but their potential application has seldom been explored in fish farming.

Nile tilapia is one of the important farmed tropical fish species, with a global production of 6.031 million tons in 2020 alone [38, 39]. Although tilapia are well known to tolerate a wide range of temperatures, any water temperature below or above the optimal levels may lead to reduced growth and impaired immune activity [40–42]. Although some studies have demonstrated that the beneficial effects of dietary zinc to alleviate the heat stress [21, 22, 25], to our knowledge, there are no studies that have been done on the use of zinc in Nile tilapia reared under heat stress. Therefore, in the present study, we examined the effects of dietary Zn supplementation on the growth performance, antioxidant capacity, and immunity-related genes of Nile tilapia, with a final goal to test the resistance of tilapia to heat stress under different Zn source and level exposures.

2. Materials and Methods

2.1. Synthesis of Thr-Zn Chelate. Thr-Zn complex was prepared by adding 4.8 g of ZnSO₄ to 7.1 g of threonine and stirring at 90°C until it was completely dissolved. The solution pH was then adjusted to 6.2 with aqueous solution of NaOH (2.5 M) and stirred again for 2.5 h. After standing at room temperature for 12 h, a large number of colorless crystalline solids was obtained, and the solution was filtered under reduced pressure and washed with double-distilled water. The structure of Thr-Zn (Figure 1) was determined by single crystal X-ray diffraction (SMART APEX II, Bruker, Germany) and identified direct methods using SHELXS and refined against F² using SHELXL. The chemical formula of Thr-Zn was identified as C₆H₁₅N₃O₇Zn·2H₂O, and the Zn content was 19.04% as determined by the inductively coupled plasma-optical emission spectroscopy (ICP-OES, iCAP 6300, Thermo Scientific, USA). This value was similar to that reported in previous study [27].

2.2. Experimental Design and Fish Culture. The experimental protocol was approved by the Committee on the Ethics and Welfare of Animal Experiments of Zhongkai University of Agriculture and Engineering. A total of 300 Nile tilapia (average weight 76.69 ± 0.11 g), originally obtained from a farm in Guangzhou, Guangdong Province, China, were randomly allocated into five zinc treatments, with three replicates of 20 fish in each treatment. The fish in the control group received the basal diet without zinc supplement, whereas those in the tested group were fed with the basic diet supplemented with 20 or 40 mg/kg of inorganic Zn (ZnSO₄·7H₂O 24%; Sinopharm Chemical Reagent Co., Ltd. Shanghai, China) or organic Zn (Thr-Zn). The feed ingredients were finely ground through a 60-mesh sieve, mixed, and then prepared into pellets of 2.0 mm size. The pellets were air-dried and placed in sealed plastic bags, marked, and stored at 4°C until use. The composition and nutrient content of the basal diets (dry matter) are shown in Table 1. Total Zn concentrations in the experimental diets, as measured by ICP-OES, were 91.0, 112.0, 90.8, and 111.2 mg/kg diet, respectively, for the treatment of 20 inorganic, 40 inorganic, 20 organic, and 40 organic, respectively.

The tilapia were placed into the plastic tanks (400 L) and provided with the commercial feed for 2 weeks for acclimation prior to the exposure experiments. After acclimation,
Table 1: Basal diet and chemical composition (dry matter).

| Ingredients          | (%) | Chemical composition (%) |
|----------------------|-----|--------------------------|
| Soybean meal         | 23.0| Dry matter 95.0 ± 0.37    |
| Fish meal            | 20.0| Crude protein 37.0 ± 0.35 |
| Rapeseed meal        | 20.5| Crude fiber 4.2 ± 0.10    |
| Wheat flour          | 10.0| Ash 11.6 ± 0.08           |
| Yellow corn          | 1.5 | Gross energy 18.6 ± 0.30  |
| Zinc-free premix     | 2.0 | Total Zn (mg/kg) 71.2 ± 3.67 |

1Zinc-free premix (vitamin and mineral mixture, mg/kg premix): vitamin A (3000 IU), vitamin D3 (1000 IU), vitamin E (2350 mg), vitamin K3 (2800 mg), vitamin B1 (300 mg), vitamin B2 (600 mg), vitamin B6 (400 mg), vitamin B12 (20 mg), D-biotin (40 mg), folic acid (2500 mg), nicotinic acid (2000 mg), inositol (570 mg), D-pantothenic acid (1200 mg), FeSO4·H2O (1500 mg), MgSO4·7H2O (850 mg), MnSO4·H2O (800 mg), CuSO4·5H2O (200 mg), KIO3·H2O (5 mg), CoCl2·6H2O (850 mg), and Na2SeO3 (0.05 mg). 2NFE (nitrogen – free extract) = 100 – (crude protein % + crude lipid% + ash % + crude fiber%). 3Gross energy was calculated by calorific values for protein, lipid, and carbohydrate as 23.6, 39.5, and 17.2 kcal/g, respectively.

Fish were fed with respective diets twice a day for 45 days. Within the first week, the fish were daily fed at 3% of average body weight and then increased by 1% every two weeks to a maximal 6% feeding rate. During the experimental periods, the water quality was checked and water was exchanged once daily with 30% of the tank volume. Throughout the experiments, the water temperature was 25 ± 2°C, dissolved oxygen was 6.5 ± 0.5 mg/L, the pH was 7.3 ± 0.2, and the ammonia nitrogen was less than 0.25 mg/L. After the experiment, all fish from each tank were counted and the body weight and length were measured after 24 h of starvation. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (CF), and survival were calculated as follows:

\[ WG (g) = \text{final body weight (FBW)} - \text{initial body weight (IBW)} \]
\[ SGR (%) = 100 \times \left( \frac{\ln \text{FBW} - \ln \text{IBW}}{\text{experiment period}} \right) \]
\[ FCR = \frac{\text{feed intake}}{\text{weight gain}} \]
\[ PER = \frac{\text{weight gain}}{\text{protein intake}} \]
\[ CF = 100 \times \left( \frac{\text{body weight}}{\text{body length}^3} \right) \]

2.3. Sampling. Fish were anesthetized with MS-222 (25 mg/L) in order to minimize suffering and distress. Subsequently, the whole blood samples (3 fish/replicate) were collected from the caudal vessels at the caudal peduncle region and divided into two aliquots (without anticoagulant). One sample was clotted for 30 min at 4°C and centrifuged at 4000 × g for 10 min to obtain the serum and then kept at -80°C until use for evaluation of oxidative status. Another sample was kept in trace element-free tubes and stored at 4°C prior to quantify the trace element content. After blood collection, the tissues including intestinal, liver, and muscle were carefully removed and washed with ice-cold saline. Immediately after dissection, anterior intestine was prefixed in 4% paraformaldehyde for 24 h at room temperature for histomorphology analysis, and the remaining tissues were stored at -80°C for subsequent analyses.

2.4. Heat Stress Challenge. After sampling, a total of 24 fish were selected randomly from each group (8 fish/replicate) for high-temperature stress test based on the previous studies [9, 40, 43]. Briefly, the initial water temperature was 27°C, which was then gradually increased every 2 h by 2°C until it reached 32°C and maintained for 48 h using aquarium heaters. The other water quality parameters remained unchanged throughout this period. Subsequently, the number of dead fish was recorded every 3 h. Fish were sampled when 50% of mortality was reached for each treatment. The liver and blood samples were collected from the remaining fish after anesthesia, and specific operation was performed as described previously.

2.5. Sample Processing. Antioxidant indices such as malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in serum (before and after heat challenges) were quantified by the commercial kits based on the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, China). Metal (Fe, Zn, Mn, and Cu) contents in serum, muscles, and liver were determined using ICP-OES following tissue dissection. Standard reference materials were concurrently subjected to digestion, and the recovery was within 90-110%. After fixation, the intestinal tissues were dehydrated by gradient alcohol, cleared by xylene, and embedded in paraffin. They were then sectioned at 4 μm and stained with hematoxylin and eosin (H&E). Subsequently, the stained sections were imaged using photomicrography (Nikon Eclipse Ci-L, Japan) under a microscope through 40x magnification. The villus height and width were measured by Image-Pro Plus 6.0 software (Media Cybernetics, USA) using an average of five measurements per picture. The absorption surface area (ASA) was calculated as ASA (mm²) = villus height × villus width.

2.6. Gene Expression. Approximately 100 mg of frozen liver tissue (before and after heat challenges) was pulverized by mortar and pestle in liquid nitrogen, and then, the total RNA was extracted using Trizol reagent (Thermo Fisher Scientific) based on the manufacturer’s protocol. RNA concentration and quality ratios were quantified at 260/280 nm by a DeNovix DS-11 Spectrophotometer (DeNovix Inc., USA). Afterwards, complementary DNA (cDNA) was synthesized by cDNA synthesis kit (Thermo Scientific, USA) and stored at -80°C for further analysis.

The primers (Table 2) used to amplify the selected genes were designed by NCBI Primer-BLAST and then
were expressed as mean ± standard error.

### 3. Results

#### 3.1. Growth Performance

The growth performance parameters of Nile tilapia feeding on diets supplemented with different Zn sources and levels for 45 days are summarized in Table 3. Zn supplementation levels at 40 mg/kg significantly increased the FBW, WG, and SGR compared with the control group (p < 0.05), but its sources did not significantly affect these parameters (p > 0.05). Similarly, FCR was significantly lower in the high-Zn (40 mg/kg) group than the control group, while no difference was found between the two Zn sources (ZnSO₄ and Thr-Zn, p > 0.05). There were no significant differences in PER, CF, body length, and survival among all treatments (p > 0.05).

### Table 3. Effect of different levels (20 or 40 mg Zn/kg feed) and sources of dietary zinc on growth performance and nutrient utilization of Nile tilapia for 45 days.

| Item      | CT       | 20 ZnSO₄ | 40 ZnSO₄ | 20 Thr-Zn | 40 Thr-Zn |
|-----------|----------|----------|----------|-----------|-----------|
| IBW (g)   | 76.7 ± 1.51 | 76.7 ± 1.54 | 76.8 ± 1.55 | 76.7 ± 1.01 | 76.6 ± 1.51 |
| FBW (g)   | 201.9 ± 3.22b | 210.0 ± 4.44ab | 214.4 ± 2.17a | 209.8 ± 1.57ab | 213.2 ± 2.82a |
| WG (g)    | 125.2 ± 3.20b | 133.3 ± 4.40ab | 137.7 ± 2.08a | 133.1 ± 1.59ab | 136.6 ± 2.77a |
| SGR (%/day) | 2.15 ± 0.04b | 2.24 ± 0.05ab | 2.28 ± 0.02a | 2.24 ± 0.02ab | 2.27 ± 0.03a |
| FCR       | 1.36 ± 0.04a | 1.28 ± 0.04ab | 1.24 ± 0.02b | 1.28 ± 0.02ab | 1.25 ± 0.03b |
| PER       | 2.02 ± 0.05 | 2.12 ± 0.07 | 2.18 ± 0.03 | 2.08 ± 0.03 | 2.16 ± 0.04 |
| Body length (cm) | 18.2 ± 0.26 | 18.20 ± 0.11 | 18.24 ± 0.05 | 18.34 ± 0.20 | 18.57 ± 0.17 |
| CF        | 3.31 ± 0.15 | 3.48 ± 0.08 | 3.51 ± 0.02 | 3.38 ± 0.08 | 3.31 ± 0.05 |
| Survival (%) | 88.3 ± 11.7 | 88.3 ± 11.7 | 98.3 ± 1.7 | 100.0 ± 0.0 | 100.0 ± 0.0 |

IBW: initial body weight; FBW: final body weight; WG: weight gain; SGR: specific growth rate; FCR: feed conversion ratio; PER: protein efficiency ratio; CF: condition factor. Different superscript letters indicate significantly different (p < 0.05).

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2.7. Statistical Analysis. Prior to running a one-way ANOVA method (SPSS v24.0), the normality and homoscedasticity of the data were checked, followed by Duncan’s multiple range test for differences among groups before or after the heat stress. After that, paired t-tests were used to determine if there was a significant difference in the same zinc treatment before and after HS. Significant level was at p < 0.05. All data were expressed as mean ± standard error (SE).

Table 2: Sequences of primers for qRT-PCR analysis.

| Gene      | qPCR primers, forward/reverse (5’-3’) | GenBank number |
|-----------|---------------------------------------|----------------|
| β-Actin   | F: GGAAATCGTGCGTGACATCA R: TACCAAGGGAAGGCTGGG | XM_003443127   |
| HSP70     | F: GCACAAGAAGGACTCACCC R: ATCTGCCGCCCTGTCCAGCTTT | NM_001279671.2 |
| IL-10     | F: CTCGGCTTCTTCTTCAGCTTC R: GCAAAGGGCTCCTTTAAAGGA | KP645180.1     |
| TNF-α     | F: GGCACTCAATATCTGCAGCACC R: AACACGCCAACAGGATCC | AY428948.1     |
| INF-γ     | F: AAAGATGGAGCTCTCGACCAT R: GTGTGATTGCGTGTGCTTC | XM_005448319.1 |

β-Actin: internal reference gene (housekeeping gene); HSP70: heat shock protein 70; IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; INF-γ: interferon gamma.
3.3. Mineral Deposition. The concentrations of 4 metals accumulated in fish serum and muscle/liver are shown in Table 5. In serum, the contents of Fe, Cu, and Zn at 40 mg/kg Thr-Zn was significantly higher than those in the control group (p < 0.05), but no significant difference in Mn content was documented (p > 0.05). The other Zn treatments also did not show significant difference from the control treatment. Similarly, Cu concentrations in the muscle and liver were significantly higher in fish fed with high level (40 mg/kg) of Thr-Zn diet compared with the control diet (p < 0.05). Bioaccumulation of Zn in the muscle and liver did not exhibit significant difference among treatments, although Zn concentrations were relatively higher at 40 mg/kg Thr-Zn.

3.4. Antioxidant Activity. Antioxidant activity in the serum of fish was quantified before and after heat stress following 45 days of feeding with Zn supplementation (Table 6). Before the heat challenge and right after the 45 days of feeding, the activities of SOD, CAT, and GPx increased significantly in the ZnSO₄ (40 mg/kg) and Thr-Zn (20 or 40 mg/kg) treatment groups compared with the control group (p < 0.05), while the MDA activity was significantly decreased (p < 0.05). After the heat challenge, the SOD, CAT, and MDA activities in the ZnSO₄ (40 mg/kg) and Thr-Zn (20 or 40 mg/kg) treatment groups were all enhanced (p < 0.05) and showed almost similar results as the preheat challenge. Again, the 40 mg/kg Thr-Zn group showed the highest CAT, SOD, and GPx activities and the lowest MDA level after heat stress.

3.5. Gene Expressions. As shown in Figures 3(a)–3(d), there was a significantly higher (p < 0.05) expression of IL-10 in the 40 mg/kg ZnSO₄ group relative to all other groups before the heat stress. Concurrently, the expression level of TNF-α increased significantly in 20 mg/kg Thr-Zn, 40 mg/kg ZnSO₄ and 40 mg/kg Thr-Zn groups compared to the control group (p < 0.05), while the INF-γ values decreased in the 20 mg/kg Zn-treated group. After heat stress, although treatment groups showed no significant change compared to the control group in the expression of INF-γ (p > 0.05), treatment groups showed higher IL-10 levels (p < 0.05) than the control group. On the other hand, there was no significant difference in the relative expression of HSP 70 in the liver of tilapia before HS, while the HSP 70 content in the zinc threonine group was significantly reduced after HS. After the heat challenge, the expression levels of IL-10 in the ZnSO₄ (20 or 40 mg/kg) and Thr-Zn (40 mg/kg) treatment groups were all enhanced (p < 0.05) as the preheat challenge; the expression level of TNF-α in the ZnSO₄ (20 or 40 mg/kg) treatment and INF-γ in 20 mg/kg ZnSO₄ showed similar results (p < 0.05).

4. Discussion

Zn is an essential micronutrient for fish with absorption mainly from feeding. Zn levels in vegetable-based diets to
Table 5: The content of copper, iron, manganese, and zinc in the serum, liver, and muscle of Nile tilapia fed on diets with different concentrations (20 or 40 mg zinc/kg feed) and sources of dietary zinc for 45 days.

| Parameters | CT | 20 ZnSO₄ | 40 ZnSO₄ | 20 Thr-Zn | 40 Thr-Zn |
|------------|----|-----------|-----------|-----------|-----------|
| Serum      |    |           |           |           |           |
| Copper     | 0.26 ± 0.07ᵇ | 0.39 ± 0.07ᵇ | 0.39 ± 0.04ᵇ | 0.43 ± 0.03ᵃ | 0.58 ± 0.01ᵃ |
| Iron       | 18.5 ± 0.76ᵇ | 22.7 ± 1.01ᵇ | 23.2 ± 2.66ᵃ | 21.5 ± 0.91ᵇ | 24.4 ± 0.97ᵃ |
| Manganese  | 6.88 ± 0.14 | 7.95 ± 0.52 | 7.41 ± 0.64 | 7.97 ± 0.47 | 7.93 ± 0.30 |
| Zinc       | 29.3 ± 0.23ᵇ | 30.3 ± 2.19ᵇ | 31.4 ± 1.26ᵃ | 31.0 ± 0.91ᵃ | 34.4 ± 0.97ᵃ |
| Muscle     |    |           |           |           |           |
| Copper     | 4.70 ± 0.86ᵇ | 3.79 ± 0.48ᵇ | 4.86 ± 0.76ᵇ | 5.07 ± 0.22ᵇ | 10.4 ± 0.57ᵃ |
| Iron       | 59.3 ± 4.51 | 52.0 ± 2.77 | 50.5 ± 4.54 | 50.8 ± 4.26 | 49.7 ± 2.56 |
| Manganese  | 14.6 ± 1.17 | 13.1 ± 0.71 | 12.5 ± 0.51 | 14.3 ± 1.43 | 13.0 ± 0.87 |
| Zinc       | 30.3 ± 2.85 | 30.8 ± 2.16 | 31.8 ± 2.72 | 28.7 ± 2.24 | 36.7 ± 2.31 |
| Liver      |    |           |           |           |           |
| Copper     | 310.1 ± 25.1ᵇ | 302.8 ± 33.2ᵇ | 341.2 ± 12.9ᵇ | 328.5 ± 27.4ᵃ | 389.4 ± 15.4ᵃ |
| Iron       | 214.6 ± 19.3 | 193.3 ± 31.8 | 234.3 ± 3.81 | 222.1 ± 9.20 | 250.3 ± 26.8 |
| Manganese  | 26.2 ± 2.63 | 27.9 ± 3.61 | 31.0 ± 2.25 | 23.9 ± 1.68 | 26.8 ± 2.81 |
| Zinc       | 71.3 ± 2.60 | 76.4 ± 10.3 | 79.5 ± 8.90 | 76.6 ± 4.04 | 85.9 ± 4.76 |

Different superscript letters indicate significant difference (p < 0.05).

Table 6: Changes in antioxidant biomarkers of Nile tilapia fed different concentrations (20 or 40 mg zinc/kg feed) and sources of dietary zinc for 45 days and postchallenge.

| Heat stress (HS) | Groups | SOD (U/mL) | CAT (U/mL) | GPx (U/mL) | MDA (nmol/mL) |
|-----------------|--------|------------|------------|------------|---------------|
| Before HS       | CT     | 25.42 ± 0.47ᶜ | 4.16 ± 0.11ᶜ | 50.15 ± 1.11ᶜ | 6.26 ± 0.27ᵃ |
|                 | 20 ZnSO₄ | 26.18 ± 0.53ᶜ | 4.16 ± 0.09ᶜ | 51.69 ± 1.65ᵇᶜ | 4.19 ± 0.14ᵇ |
|                 | 40 ZnSO₄ | 31.55 ± 0.61ᵇ | 5.13 ± 0.06ᵇ | 55.08 ± 1.61ᵇ | 4.29 ± 0.24ᵇ |
|                 | 20 Thr-Zn | 32.77 ± 0.40ᵇ | 4.90 ± 0.18ᵇ | 54.77 ± 1.70ᵇᶜ | 4.04 ± 0.20ᵇ |
|                 | 40 Thr-Zn | 37.01 ± 0.69ᵃ | 5.30 ± 0.09ᵃ | 58.15 ± 1.56ᶜ | 2.83 ± 0.17ᶜ |
| After HS        | CT     | 40.21 ± 0.68ᵃ | 4.29 ± 0.19ᵇ | 49.85 ± 1.58ᵇ | 6.92 ± 0.18ᵃ |
|                 | 20 ZnSO₄ | 43.03 ± 1.17ᵃ | 4.70 ± 0.20ᵇ | 51.38 ± 1.30ᵇ | 5.15 ± 0.22ᵇ |
|                 | 40 ZnSO₄ | 49.81 ± 1.12ᵇᵃ | 7.02 ± 0.63ᵃ | 60.92 ± 1.17ᵇ | 5.40 ± 0.26ᵇ |
|                 | 20 Thr-Zn | 46.99 ± 1.20ᵇᵃ | 7.29 ± 0.13ᵃ | 59.08 ± 1.72ᵃ | 4.80 ± 0.18ᵇ |
|                 | 40 Thr-Zn | 52.83 ± 0.65ᵃ | 7.29 ± 0.15ᵃ | 61.54 ± 0.78ᵃ | 3.73 ± 0.13ᵃ |

Small superscript letters refer to differences for fish fed different levels and sources of zinc before or after HS in Duncan’s multiple range test, while asterisks indicate significant differences between values obtained before and after HS for each zinc treatment in t-tests (p < 0.05).

Promote the growth of juvenile Nile tilapia were least 44.5 mg/kg [45]. The increase of growth performance could be explained by a potential role of Zn in fasten glycolysis and nitrogen metabolism activity [46, 47]. Additionally, the growth rates of fish were independent of their origin, similar to earlier studies which showed no significant difference of weight gain, body composition, and survival of Nile tilapia, common carp (Cyprinus carpio), and rainbow trout (Oncorhynchus mykiss) fed with different forms of Zn [37, 46, 48, 49]. Other studies showed that organic Zn or nano-ZnO was better than the mineral Zn for the growth performance of Nile tilapia [32, 50]. The inclusion of zinc threonine may improve the absorption and utilization of nutrients and enhance the growth of Nile tilapia. In our study, we also demonstrated that Thr-Zn significantly improved FBW, WG, and SGR of Nile tilapia and decreased FCR.

Villus width and length are the common indicators for intestinal function and absorption of nutrients [51]. In our results, dietary Zn supplementation at 40 mg/kg significantly increased the villus length and absorption area in the fish intestine, and their highest values were found in fish fed with zinc threonine-containing diet. Such improvement may be due to the role of Zn in intestinal cell proliferation [52]. Besides, the higher bioavailability of zinc threonine ensured the continual release of Zn into the intestinal tract with better absorption of nutrients [34]. In a similar study, Hu et al. [50] reported that the intestinal villus length of Nile tilapia increased significantly at a Zn level of 30 mg/kg added in the basic feeds.

The mineral contents in the whole body, serum, liver, and muscle are generally used to assess the status of mineral utilization in aquatic animals [53, 54]. In our study, Zn

Aquaculture Nutrition
Before heat stress

Relative expression of HSP-70 mRNA

CT
20 ZnSO₄
40 ZnSO₄
20 Thr-Zn
40 Thr-Zn

(a)

After heat stress

Before heat stress

After heat stress

Figure 3: Continued.
concentrations in the serum of tilapia gradually enhanced with the amount of Zn added, particularly in the zinc threonine group, but such effect was not observed in liver and muscle. Previously, Do Carmo e Sá et al. [45] and Huang et al. [53] reported that the increase of Zn concentration in bone resulted from dietary Zn supplement in tilapia. Increased Zn accumulation in tissues with organic Zn supplementation was also reported in other animals, including yellow catfish (Pelteobagrus fulvidraco) [54] and juvenile grouper (Epinephelus coioides) [55]. One of the interesting results from our study was that the Zn supplementation facilitated the accumulation of other metals such as Cu and Fe, even though their concentrations in the diets were the same. In contrast, Sahin et al. [56] found no significant effect on Mn and Cu concentrations in the whole body of rainbow trout (Oncorhynchus mykiss) with dietary Zn addition, and Luo et al. [54] indicated that supplemented Zn did not significantly affect the Mn concentration in catfish but reduced the Fe and Cu concentration in the whole body. In Nile tilapia, excessive Zn supplementation reduced the Fe, Mn, Mg, and Ca contents in the liver or muscle [53, 55]. In our study, we found no observable change of Fe and Mn in the liver...
and muscle, but a significant increase in Cu in the liver, muscle, and serum after supplementation with 40 mg/kg of zinc threonine. Absorption and metabolism of trace elements are very complicated processes and influenced by factors such as animals, diet types, or chelated zinc sources [46]. Organic zinc may be absorbed differently from inorganic Zn and reduce interaction with other elements in the gastrointestinal tract [37, 57]. Another possibility was the improved gut absorption ability for Cu which displayed chemical similarity with Zn. Consequently, supplementing the appropriate amount of Zn in tilapia diet helped the deposition of Zn in the tissues, and zinc threonine may relieve the antagonistic effect with other trace elements, but the specific absorption mechanism remains to be further studied.

Zn is important in anti-inflammatory, immunomodulatory, and antioxidant activity and affects the phagocytic capacity of macrophages and neutrophils as well as differentiation of immune system cells [58–61]. Direct antioxidant activity of Zn is a result of its association with thiol groups [62–64]. Oxidative stress is caused by ROS and impairment of antioxidant defense, and CAT, SOD, and GPx activities as well as MDA levels are generally used as early warning indicators of oxidative stress [65, 66]. In our study, the serum SOD, CAT, and GPx activities increased significantly in tilapia fed on zinc threonine (40 mg/kg), whereas MDA content decreased as compared to other treatments. These results suggested the increasing antioxidant capacity of fish with Thr-Zn supplementation. Correspondingly, Huang et al. [53] observed a significant increase in serum SOD and decrease in MDA levels with increasing dietary Zn levels in tilapia. Other studies also indicated that Zn supplementation contributed to the increase of the antioxidant capacity in juvenile carp (Cyprinus carpio) [67], turbot (Scophthalmus maximus) [68], and juvenile yellow catfish (Pelteobagrus fulvidraco) [54]. In addition, Zn homeostasis is essential in maintaining proper immune function [69]. Under Zn deficiency conditions, two genes IFN-γ and IL-2 of Th1 cell products which are known to promote inflammation and antitumor effects, were downregulated, whereas IL-4, IL 6, and IL-10 (products of Th2 cells) which promote humoral immune responses against extracellular pathogens and inhibit Th1 cytokines remained unchanged [70]. These immune-related cytokines such as TNF-α, IL-10, and INF-γ are generally the biomarkers for immune responses in animals [40]. In our study, TNF-α and IL-10 were upregulated in tilapia supplemented with Zn at 40 mg/kg, but INF-γ was downregulated at 20 mg/kg. In our study, the basic diet may already meet the daily Zn requirements of tilapia, and the immune regulation may be greatly affected by the Zn level. Awad et al. [71] similarly suggested that Zn supplementation with nano-Zn oxide or traditional Zn oxide in diets improved the immune function in tilapia.

The optimal growth temperature of tilapia is between 25 and 28°C [41], beyond which the fish displayed immune disorders and oxidative stress due to imbalance between ROS production and antioxidant defenses [10, 11]. HSP 70 played an essential molecular chaperone function in the folding and assembly of synthesized proteins as well as in protecting the cells from heat stress. Thus, HSP 70 can be used as an indicator of stress levels [9, 72, 73]. In this study, the expression of HSP70 in the liver of tilapia was not significantly different among the groups before the heat stress but decreased significantly in the Thr-Zn group after heat stress. Similarly, HSP70 was significantly downregulated under multiple conditions of stress (lead, temperature, and pathogenic infection) in the liver of Pangasius hypophthalmus following 10 mg/kg and 20 mg/kg Zn supplements [28, 74].

An important finding in our study was that Zn supplementation as feed additives reduced the oxidative stress and improved the immune function after heat stress, especially for the Thr-Zn group. Mohamed et al. [29] found that zinc sulfate has a positive role in protecting against inflammation and suppressing both oxidative stress and lipid peroxidation. Similarly, Kumar et al. [28] demonstrated that supplementation of zinc in the diet has a positive beneficial role in the mitigation of multiple stresses in Pangasius hypophthalmus. Furthermore, Zhu et al. [27] suggested that the antidiabetic effect of zinc threoninate might be due to its antioxidative stress ability in diabetic rats. Apparently, Zn played an important role as signaling molecule in the immune function as well as antioxidative stress under heat stress. Zn can activate the antioxidant proteins and depress the oxidant promoting enzymes, thereby weakening the cellular site-specific oxidative injury. Moreover, Zn also reduced the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) caused by temperature or other stresses and increases the expression of zinc finger proteins with specific anti-inflammatory properties, such as A20 and PPAR-α [18, 69, 75]. Our study demonstrated that heat stress could cause oxidative stress and inflammation, whereas dietary Zn supplementation significantly improved the defense against heat stress and this may be linked to the beneficial effects of zinc on enhancing growth performances, intestinal morphology, antioxidant capacity, and immune functions of tilapia.

5. Conclusion

Our study showed that dietary supplementation of zinc (ZnSO₄ or Thr-Zn) at 40 mg/kg potentially boosted the growth, intestinal morphology, antioxidative defense, and immunity of Nile tilapia. It also offers an applicable method for improving the antioxidative function and immune status of Nile tilapia during the heat stress conditions without paradoxical side effects. Additionally, zinc threonine might have alleviated the antagonistic effect between Zn and other trace elements and increased the accumulation of Cu in the serum, liver, and muscle. In conclusion, the results from this study demonstrated that improving heat resistance of Nile tilapia by dietary zinc supplementation is feasible and the effect of organic Zn is superior to inorganic Zn.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Conflicts of Interest

The authors declare that they have no conflict of interest.

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