Effects of Vitamin C on Ammonia Nitrogen Stress Resistance in Juvenile Starry Flounder (Verasper variegatus) Postprint

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Abstract

Ammonia nitrogen pollution represents a significant contaminant in aquaculture; consequently, enhancing the resistance of fish to pollution stress is of paramount importance. This study investigated the effects of dietary vitamin C levels on the capacity of juvenile starry flounder (Verasper variegatus) to resist ammonia nitrogen stress. The experiment was conducted at a water temperature of $(12.5\$\pm1.5)°C, employinghealthyjuvenilestarryflounderwithabodyweightof(38.0\pm\$0.8)$ g, which were randomly allocated into seven groups (three replicates per group, 30 fish per replicate) and fed experimental diets containing vitamin C at concentrations of 10.2 (control), 249.1, 402.8, 616.2, 769.5, 909.4, and 1,177.8 mg/kg for eight weeks. Upon completion of the feeding trial, 10 fish from each replicate were exposed to ammonia nitrogen stress at a concentration of 20 mg/L for 24 h. The results indicated that both before and after ammonia nitrogen stress, vitamin C accumulation in liver and muscle tissues reached saturation when dietary vitamin C content attained 769.5 mg/kg; further elevation of dietary vitamin C levels did not elicit significant increases in hepatic or muscular vitamin C concentrations ($P>0.05$). Except for the 1,177.8 mg/kg vitamin C group, serum catalase (CAT) and superoxide dismutase (SOD) activities in all vitamin C-supplemented groups were significantly higher than those of the control group both before and after ammonia nitrogen stress ($P<0.05$). Ammonia nitrogen stress significantly decreased serum CAT and SOD activities in all groups ($P<0.05$), with the exception that the 616.2 mg/kg vitamin C group exhibited no significant alteration in serum CAT activity ($P>0.05$); however, vitamin C supplementation attenuated the magnitude of this reduction. Both before and after ammonia nitrogen stress, gill filament Na+/K+-ATPase activity in all vitamin C-supplemented groups was significantly higher than that of the control group ($P<0.05$). Ammonia nitrogen stress significantly reduced gill filament Na+/K+-ATPase activity

in the control group and the 909.4 and 1,177.8 mg/kg vitamin C groups (P<0.05), whereas no significant changes were observed in the remaining groups (P>0.05). Ammonia nitrogen stress significantly elevated serum glucose and lactate concentrations in all groups (P<0.05), and significantly increased serum cortisol concentration in the control group (P<0.05). Additionally, ammonia nitrogen stress significantly decreased total iron binding capacity in all groups (P<0.05). Based on comprehensive evaluation of all measured parameters, dietary vitamin C levels of 402.8–616.2 mg/kg were effective in enhancing the capacity of juvenile starry flounder to resist ammonia nitrogen stress.

Full Text

Effect of Dietary Vitamin C Content on Anti-Ammonia-Nitrite Stress Ability of Juvenile Spotted Halibut (Verasper variegatus)

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Abstract: Ammonia-nitrogen pollution is a critical pollutant in aquaculture, making it essential to improve fish resistance to pollution stress. This study investigated the effects of dietary vitamin C content on the anti-ammonia-nitrite stress ability of juvenile spotted halibut. The experiment was conducted at a water temperature of (12.5$±1.5)°C. Healthy juvenile spotted halibut with a body weight of (38.0±$0.8) g were randomly divided into 7 groups (3 replicates per group, 30 fish per replicate) and fed experimental diets containing 10.2 (control), 249.1, 402.8, 616.2, 769.5, 909.4, and 1,177.8 mg/kg vitamin C for 8 weeks.

After the feeding trial, 10 fish from each replicate were exposed to 20 mg/L ammonia-nitrite for 24 h. The results showed that both before and after ammonia-nitrite stress, vitamin C accumulation in liver and muscle reached saturation when dietary vitamin C content reached 769.5 mg/kg; further increases in dietary vitamin C did not significantly elevate vitamin C levels in these tissues (P>0.05). Except for the 1,177.8 mg/kg vitamin C group, serum catalase (CAT) and superoxide dismutase (SOD) activities in all vitamin C-supplemented groups were significantly higher than those in the control group both before and after stress (P<0.05). Ammonia-nitrite stress significantly decreased serum CAT and SOD activities in all groups except the 616.2

mg/kg group for CAT (P>0.05), though vitamin C supplementation reduced the magnitude of this decline. Gill Na+/K+-ATPase activity in all vitamin C-supplemented groups was significantly higher than in the control group both before and after stress (P<0.05). Ammonia-nitrite stress significantly reduced gill Na+/K+-ATPase activity in the control group and the 909.4 and 1,177.8 mg/kg vitamin C groups (P<0.05), but caused no significant changes in other groups (P>0.05). The stress significantly increased serum glucose and lactate levels in all groups (P<0.05) and elevated cortisol levels in the control group (P<0.05), while decreasing serum total iron binding capacity (TIBC) in all groups (P<0.05). Based on comprehensive analysis of all measured indices, dietary vitamin C content of 402.8–616.2 mg/kg effectively improved the anti-ammonia-nitrite stress capacity of juvenile spotted halibut.

Keywords: juvenile spotted halibut (Verasper variegatus); vitamin C; stress response; anti-ammonia-nitrite stress

Introduction

With the intensification of industrial aquaculture in recent years, uneaten feed and excretory products from aquatic animals generate substantial ammonia-nitrogen, which represents a major factor inducing disease outbreaks in farmed species. For teleost fish, ammonia-nitrogen is the primary product of nitrogen metabolism, and most species are highly sensitive to its toxic effects. High concentrations of ammonia-nitrogen in culture water can damage the antioxidant system, reducing antioxidant enzyme activities and increasing free radical content, which subsequently impairs non-specific immunity, disrupts metabolic processes, and increases susceptibility to bacterial and viral pathogens, thereby elevating the risk of disease epidemics.

Vitamin C (L-ascorbic acid) is an essential micronutrient for animal growth, production, and normal physiological function, playing important roles in lipid metabolism regulation and maintenance of cardiac, central nervous, and hematopoietic functions, as well as synthesis of various hormones. Vitamin C promotes fish growth, alleviates stress responses to environmental challenges, and enhances immunity. Since most fish lack L-gulonolactone oxidase necessary for vitamin C synthesis, they must obtain it from dietary sources. Previous studies have demonstrated that ammonia-nitrite stress decreases gill Na+/K+-ATPase activity and causes severe damage to gill and liver tissues and antioxidant systems in black carp. Spotted halibut is an important commercial species that experiences water quality deterioration (sudden ammonia-nitrogen spikes) and disease problems under intensive culture conditions. This study investigated the effects of dietary vitamin C supplementation on the anti-ammonia-nitrite stress capacity of juvenile spotted halibut to provide scientific guidance for using vitamin C to enhance environmental stress resistance.

Materials and Methods

Experimental Materials

Experimental Fish Juvenile spotted halibut used in this study were obtained from Yantai Tianyuan Aquatic Products Co., Ltd., with an average body weight of (38.0$±$0.8) g.

Experimental Diets Based on reference [10], a basal diet containing 50.2% crude protein and 8.6% crude lipid was formulated . Vitamin C polyphosphate (35% active vitamin C, purchased from Qingdao Jinhaili Aquatic Technology Co., Ltd.) was used as the vitamin C source, added to the basal diet at levels of 0 (control), 1,500, 3,000, 4,500, 6,000, 7,500, and 9,000 mg/kg to produce seven experimental diets with measured vitamin C concentrations of 10.2, 249.1, 402.8, 616.2, 769.5, 909.4, and 1,177.8 mg/kg, respectively. Feed ingredients were ground through a 60-mesh sieve, mixed thoroughly, and pelleted into 5 mm diameter pellets, which were dried at 65 °C and stored at -20 °C until use.

Experimental Methods

Feeding Method Fish were randomly allocated into 7 groups with 3 replicates each (30 fish per replicate) and acclimated in cylindrical tanks (53 cm diameter × 60 cm height) for one week before the 8-week feeding trial. Fish were fed to satiation once daily at 14:00. Continuous aeration maintained adequate dissolved oxygen, and the environment was kept quiet. Water was exchanged daily at 08:00 and 15:00. During the trial, water temperature was $(12.5\$\pm1.5)°C, pHwas7.8\pm\$0.2$, dissolved oxygen was >6.0 mg/L, and total ammonia-nitrogen concentration was monitored and maintained at $ $0.1 mg/L.

Ammonia-Nitrite Stress After the feeding trial, fish in each tank were counted and weighed, and 10 fish per tank were exposed to 20 mg/L total ammonia-nitrite for 24 h following the method of Hu et al. [9]. During stress, continuous aeration maintained dissolved oxygen $ $5.0 mg/L and pH at 7.31, resulting in a non-ionized ammonia concentration of 0.272 mg/L. Non-ionized ammonia concentration was calculated as follows [11]:

$C_1 = 1.216 \times f \times C_2 / 100$
$f = 100 / (10\hat{}(pKa-pH) + 1)$
$pKa = 0.09018 + 2729.92 / T$
$T = 273.15 + t$

Where: C_1 = non-ionized ammonia concentration (mg/L); f = molar percentage of non-ionized ammonia in aqueous solution (%); C_2 = ammonia-nitrogen concentration (mg/L); T = absolute temperature (K); t = temperature (°C).

Sample Collection and Analysis Fish were sampled before and after ammonia-nitrite stress. Five fish per tank were randomly selected, and

approximately 2 mL blood was collected from the caudal vein using 1% heparin sodium as anticoagulant. After 4 h at low temperature, blood was centrifuged at 4,000 r/min for 10 min, and serum was stored at -20 °C for biochemical analysis. After blood collection, fish were dissected to isolate gill, liver, and muscle tissues for determination of gill Na+/K+-ATPase activity and vitamin C content in liver and muscle. Na+/K+-ATPase activity was measured using assay kits from Nanjing Jiancheng Bioengineering Institute, and vitamin C content was determined by the 2,4-dinitrophenylhydrazine method.

Statistical Analysis

Results are expressed as mean ± standard deviation. One-way ANOVA was performed using SPSS 17.0, with $P<0.05$ considered significant. Duncan' s multiple range test was used for post-hoc comparisons. Paired t-tests were used to compare data before and after stress.

Results

Effect of Dietary Vitamin C on Tissue Vitamin C Content Before and After Stress

As shown in , before stress, serum vitamin C content increased significantly with dietary vitamin C level ($P<0.05$). Liver and muscle vitamin C content increased significantly with dietary vitamin C up to 769.5 mg/kg, after which accumulation reached saturation and further dietary increases did not significantly elevate tissue levels ($P>0.05$). After stress, serum vitamin C content was highest in the 769.5 and 1,177.8 mg/kg groups, significantly higher than other groups ($P<0.05$). Muscle and liver vitamin C content were highest in the 769.5 mg/kg group ($P<0.05$). Compared with pre-stress values, serum vitamin C decreased significantly in the 616.2 mg/kg group but increased significantly in the 769.5 and 1,177.8 mg/kg groups after stress ($P<0.05$). Muscle vitamin C decreased significantly after stress in the control and 769.5, 909.4, and 1,177.8 mg/kg groups ($P<0.05$), while liver vitamin C decreased significantly in the control and 616.2, 909.4, and 1,177.8 mg/kg groups ($P<0.05$).

Effect of Dietary Vitamin C on Serum Antioxidant Enzyme Activities Before and After Stress

As shown in , except for the 1,177.8 mg/kg group, serum CAT and SOD activities in all vitamin C-supplemented groups were significantly higher than the control group both before and after stress ($P<0.05$). Ammonia-nitrite stress decreased serum CAT and SOD activities, but vitamin C supplementation reduced the magnitude of this decline. Except for CAT activity in the 616.2 mg/kg group, which showed no significant change ($P>0.05$), all other groups exhibited significant differences in CAT and SOD activities before and after stress ($P<0.05$).

Effect of Dietary Vitamin C on Gill Na+/K+-ATPase Activity Before and After Stress

As shown in , gill Na+/K+-ATPase activity in all vitamin C-supplemented groups was significantly higher than the control group both before and after stress ($P<0.05$), with no significant differences among supplemented groups ($P>0.05$). Ammonia-nitrite stress decreased gill Na+/K+-ATPase activity, but significant reductions were only observed in the control group and the 909.4 and 1,177.8 mg/kg groups ($P<0.05$).

Effect of Dietary Vitamin C on Serum Stress Indices Before and After Stress

As shown in , before stress, dietary vitamin C had no significant effect on serum cortisol or lactate ($P>0.05$) but significantly increased glucose and TIBC ($P<0.05$). After stress, vitamin C supplementation significantly increased TIBC while decreasing glucose, cortisol, and lactate levels ($P<0.05$). Ammonia-nitrite stress elevated serum glucose, cortisol, and lactate, with significant differences before and after stress for glucose and lactate in all groups ($P<0.05$) and for cortisol only in the control group ($P<0.05$). Additionally, stress decreased serum TIBC, with significant differences observed in all groups ($P<0.05$).

Discussion

Dietary vitamin C promotes fish growth and physiological metabolism, but excessive intake can inhibit metabolic processes. Previous studies have shown that tissue vitamin C content correlates positively with dietary vitamin C level until reaching a saturation plateau. In this study, vitamin C accumulation in muscle, liver, and serum increased with dietary level, with liver and muscle saturation occurring at 769.5 mg/kg dietary vitamin C. The liver serves as the primary storage site for vitamin C, containing substantially higher levels than muscle and serving as the main site of vitamin C metabolism. Ammonia-nitrite stress did not significantly affect muscle vitamin C in the 249.1, 402.8, and 616.2 mg/kg groups or liver vitamin C in the 249.1, 402.8, and 769.5 mg/kg groups. As an important physiological regulator with antioxidant and immunoenhancing properties, vitamin C may be mobilized from hepatic stores during stress to scavenge oxidative free radicals and mitigate oxidative damage. The control diet contained insufficient vitamin C to counteract stress-induced radicals. The minimal decrease or even increase in serum vitamin C after stress may reflect hepatic mobilization into circulation. These findings align with studies on grouper, rainbow trout, and parrotfish.

Animal antioxidant defense systems comprise enzymatic and non-enzymatic components, with SOD and CAT constituting key enzymatic antioxidants that scavenge free radicals to maintain redox balance and prevent peroxidative damage. Environmental stress generates oxidative stress through excessive free radical production, reducing antioxidant enzyme activities and compromising

defense capacity. This study demonstrates that dietary vitamin C effectively elevated serum SOD and CAT activities, enhancing antioxidant capacity. The reduced decline in antioxidant enzyme activities following ammonia-nitrite stress in vitamin C-supplemented groups indicates that vitamin C mitigates damage to the antioxidant system. Previous research has linked vitamin C' s antioxidant capacity to improved resistance against acid, salinity, and ammonia stress in aquatic animals, consistent with our results.

Gill tissue performs respiratory and osmoregulatory functions in fish, with Na+/K+-ATPase being a key enzyme in chloride cells involved in osmoregulation. Environmental stress can reduce gill Na+/K+-ATPase activity. This study showed that dietary vitamin C increased gill Na+/K+-ATPase activity, and while stress decreased activity in all groups, the reduction was less severe in vitamin C-supplemented groups. This suggests vitamin C alleviates the negative impacts of ammonia-nitrite stress on gill tissue and protects osmoregulatory function. These results agree with findings in black carp but differ from studies on freshwater prawn, possibly reflecting species-specific vitamin C requirements.

Cortisol is a stress hormone secreted via the hypothalamic-pituitary-interrenal axis in response to environmental stressors, with blood cortisol serving as a sensitive stress indicator. Elevated cortisol increases blood glucose by activating gluconeogenic enzymes such as glucose-6-phosphatase to meet energy demands during stress. In this study, serum cortisol increased significantly after stress only in the control group, while the 616.2 mg/kg group showed no significant change, indicating that this vitamin C level enhances stress resistance. Vitamin C modulates cortisol synthesis by preventing unsaturated fatty acid oxidation and reducing steroid production while also regulating neurotransmitter synthesis in the brain to enhance stress tolerance. The significant post-stress increase in serum glucose in all groups, particularly in the control and 1,177.8 mg/kg groups, reflects rapid mobilization of carbohydrates for energy during stress, consistent with studies on rockfish and sea bass.

Lactate, a product of anaerobic metabolism, reflects respiratory patterns. Ammonia-nitrite stress damages gill tissue, impeding gas exchange and causing hypoxia that shifts metabolism toward anaerobic pathways, increasing blood lactate. The significant post-stress lactate increase in all groups, with the greatest elevation in the control group and smallest in the 402.8 mg/kg group, demonstrates that 402.8 mg/kg vitamin C effectively mitigates ammonia-induced impairment of aerobic metabolism. Combined with cortisol and glucose data, dietary vitamin C at 402.8–616.2 mg/kg effectively reduces stress damage.

Iron is essential for microbial proliferation, and transferrin's high iron-binding capacity exerts bacteriostatic effects. All vitamin C-supplemented groups showed significantly higher TIBC than the control, with optimal levels at 249.1–616.2 mg/kg, indicating enhanced bactericidal capacity. Although stress decreased TIBC in all groups, the decline was less pronounced in vitamin C-supplemented

groups, suggesting vitamin C enhances resistance to environmental stress and pathogen infection. While vitamin C' s effect on TIBC in aquatic animals has not been previously reported, our results align with studies on pantothenic acid and vitamin B6 in juvenile Jian carp.

Based on comprehensive evaluation of all measured parameters, dietary vitamin C content of 402.8–616.2 mg/kg effectively improves the anti-ammonia-nitrite stress capacity of juvenile spotted halibut.

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Note: Figure translations are in progress. See original paper for figures.