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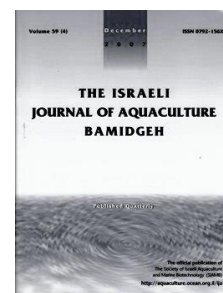
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## Physical Responses of *Lates calcarifer* to Acute Nitrite Stress

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**Keywords:** *Lates calcarifer*; nitrite; antioxidant enzyme; digestive enzyme; cortisol concentration

### Abstract

The acute toxicity of nitrite (133.33, 266.66, 399.99mg/L) to 15 days post-hatched *Lates calcarifer* (barramundi) was studied by semi-static bioassay method; 0 mg/L nitrite was used as control. Samples were obtained at 0, 6, 12, 24, 36, 48, 72 and 96 h respectively to measure and analyze the activity of antioxidant, digestive enzymes, and concentration of serum cortisol. Nitrite concentration and exposure time significantly affected the antioxidant and digestive enzymes activities. By the end of the experiment, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in the experimental group were significantly higher than in the control group, while peroxidase (POD) and catalase activities were significantly lower than in the control group except for in higher concentrations. Differing from POD and catalase, the acid phosphatase (ACP) activity of sub-concentration treatment was significantly higher than in the control group. Amylase activity in the nitrite stressed group was significantly lower than the control group, while the results of protease and lipase activity were reversed. Cortisol activity was delayed because of acute nitrite stress in the barramundi. *Lates calcarifer* showed certain short-term tolerance to nitrite stress. Results from the present study indicate that nitrite stress stimulates the responses of antioxidant and digestive enzyme activity and cortisol in fish, and these parameters can be used as physical indicators of stress.

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## Introduction

The constantly growing human population is increasing the demand for fish food. This has raised worldwide intensification of the aquaculture industry (Roques et al., 2015;). In the past few decades, global farmed fish production has more than doubled in intensive production (Naylor et al., 2000). Some side effects of this intensive production are increase in waste products and higher risk of the spread of pathogens (Naylor et al., 2000). Fish from aquaculture are often subject to high temperature, poor water quality, and crowded environments (Allais et al., 2018).

Nitrite is not an important toxicant in open aquaculture systems; however, it can be of great concern in closed or semi open systems when it exceeds 50 mg/L (Van Rijn et al., 2006; Parra & Yúfera, 1999; Handy & Poxton, 1993). Three nitrogen conversion pathways are naturally present to remove nitrogenous elements in aquaculture systems, including photoautotrophic removal by algae, autotrophic bacterial conversion of ammonia to nitrate, and heterotrophic bacterial conversion of ammonia directly to microbial biomass (Ebeling et al., 2006). The process of nitrification has been found to be affected by a range of parameters (e.g. dissolved oxygen concentration, pH, organic matter, temperature, salinity etc.). In addition, nitrifying bacteria and highly sensitive organisms, are extremely susceptible to a broad variety of inhibitors such as high concentrations of ammonia and nitrous acid, low dissolved oxygen levels and pH outside the optimal range. With those changes in environmental conditions, the metabolic ability of nitrifying bacteria to transform nitrite is not sufficient. In aquaculture systems, changes in the nitrification process due to crowded environment, food excess in the tanks, or disturbance of the nitrifying bacteria often result in a drastic increase in nitrite concentrations (Svobodova et al., 2005). Fish are then subject to accumulation of nitrogenous elements in blood plasma affecting physiological metabolism, immune performance, red blood cell and ion regulation, and also damage to the liver, kidney, skin and gills (Lin et al., 2018). Hence, inefficient processes of bacterial nitrification can occur and may be especially problematic for aquaculture practices (Roques et al., 2015; Ebeling et al., 2006; Jensen et al., 1987). Nitrogenous concentration has been recorded up to 800mg/L in semi closed systems (Van Rijn & Rivera, 1990). Under nitrite poisoning, nitrite oxidizes hemoglobin to methemoglobin which reduces the total oxygen-carrying capacity of the blood because of its inability to bind with oxygen (Lin et al., 2018; Ciji et al., 2012). Entering intracellular tissue compartments, nitrite can also cause hypoxia in vital organs such as the liver (Jensen et al., 1987).

Barramundi *Lates calcarifer*, commonly known as Asian sea bass, is a valued catadromous fish species in aquaculture because of its high fecundity, fast growth, and wide environmental tolerance (fresh to saline waters; Ma et al., 2018). This species has been a well-established industry in fresh or saline water ponds, tanks or cage aquaculture systems for around thirty years in Indo-West Pacific regions, and generated annual production of almost 66,000 tons in 2010 worldwide (Ma et al., 2018; Domingos et al., 2013). Barramundi are carnivorous fish, requiring a protein-rich diet of approximately 400–600 g/kg of crude protein (Glencross, 2006). Protein-rich diets leading to high rates of ammonia excretion (Carter et al., 1998), often result in water with strong nitrite concentrations in intensive barramundi culture systems. Previous studies have shown that high nitrite concentrations influence fish physiology by impacting on metabolism, immune performance, erythrocyte function, ion regulation, and gill tissue structure (Kroupova et al., 2005). Nitrite toxicity involves over production of reactive oxygen species and leads to immune system reaction (Xian et al. 2011). With exposure to contaminants, aquatic organisms can stimulate the use of endogenous antioxidant enzyme and hormonal regulation systems to eliminate excessive reactive oxygen species (ROS) that cause oxidative stress and then protect cells against oxidative damage (Qiu et al., 2011; Lesser, 2006; Barata et al., 2005). The change in antioxidant enzymes activity has been hypothesized as a suitable biomarker of nitrite stress, as well as determining nitrite toxic effect (Ciji et al., 2012).

The purpose of the present study was to examine the physiological response of *L. calcarifer* to acute changes in nitrite concentration, particularly regarding antioxidant enzymes, digestive enzymes, and cortisol in barramundi. The study aims to improve the rearing efficiency of this species and provide scientific basis for the management of the aquaculture water environment.

## Materials and methods

### Experimental fish

Fertilized eggs of the same batch of barramundi were produced by broodstocks held in the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Lingshui Town. All eggs were stocked into 500 L fiberglass incubators for hatching and holding. On 3 days post hatching (DPH), fish larvae were transferred to 1000 L seawater fiberglass tanks. The rearing temperature was maintained at  $29.0 \pm 1.0^\circ\text{C}$ , pH was 8.0-8.2, and salinity was  $33 \pm 0.8\text{‰}$  throughout the experiment. The light regime was set to 14hr light/ 10hr dark, and light intensity was maintained at 2000 lux. In each tank, two air stones were used to maintain dissolved oxygen  $> 6.5$  mg/L. The nitrite content was lower than 0.03 mg/L. Water was changed 2-times per day with a daily exchange rate of 20% during the experiments.

Rotifers *Brachionus roundiformis*, at a density of 10-20 ind/mL were used to feed the larvae from 3 DPH to 9 DPH ( $4.50 \pm 0.10$  mm). *Artemia* nauplii were first introduced at 20-30 nauplii/mL on 7 DPH until 15 DPH. *Artemia* nauplii and rotifers were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following manufacturer's instruction before they were added into the larval rearing tanks. Instant microalgal paste, *Nannochloropsis* sp., (Qingdao Hongbang biotechnology co. LTD) was also added into larval fish tanks to create a green-water background.

### Experimental design

Initial range-exploring tests were conducted before the formal test to determine the nitrite test concentrations. Sodium nitrite ( $\text{NaNO}_2$ ) crystal was used to prepare solutions with different concentrations. Behavior and survival conditions of barramundi were recorded. Half of the individuals died at 96 h ( $\text{LC}_{50}$ , 96 h) under 399.99 mg/L, so it was chosen to be the highest test concentration.

Acute effects of nitrite on barramundi ( $8.2 \pm 3.93$  mm, total length) were investigated in a semi-static (International Organization for Standardization, 1997) 96 h experiment that involved a strict pH 8.1 and temperature  $29^\circ\text{C}$  regime. To eliminate the differences between different batches, test fish were chosen from the same batch and not reused. During the experimental period, no food was supplied to the test animals to reduce the buildup of metabolic ammonia. The experiment consisted of four different target test concentrations of nitrite at 0, 133.33, 266.66, and 399.99 mg/L. The experiment was conducted in 50 L aerated glass tanks. Each treatment had three replicates with 120 barramundi and a total of 360 fish per treatment. Five samples were collected from every tank at 0, 6, 12, 24, 36, 48, 72 and 96 h. Water samples were collected from each tank every 3h for nitrite concentration determination to adjust to the required concentrations using the diazo-azo colorimetric method (Chen et al., 2016).

During the experimental period, symptoms of poisoning were recorded continuously, and survival rate and physiological activity such as unusual swimming behaviors, hyperactive respiration of the barramundi were recorded in each test group. The criterion for the death was defined as more than 15 seconds immobilization of the gill cover flapping. Fish samples were anesthetized with an overdose of tricaine methane sulfonate (MS-222, Sigma, USA), and were thoroughly rinsed with distilled water to remove external salt. They were then immediately stored in liquid nitrogen. The whole frozen fish were thawed, weighed, and homogenized for enzymatic assays, using a glass homogenizer on ice in 0.2 M NaCl (w/v) (Gawlicka et al., 2000). The homogenate was centrifuged at 13000 g for 10 min at  $2^\circ\text{C}$ . Then, the aqueous supernatant was collected and incubated in the enzyme substrate at 25 or  $37^\circ\text{C}$  and read on a spectrophotometer

(UV-1800BPC, LiuYi Biotechnology co., Ltd, China) at a specific wavelength. Enzyme activities were tested in triplicate.

#### *Sample processing procedure*

After sampling, the specimens were thoroughly rinsed in distilled water to remove external salt and then immediately stored in liquid nitrogen. The whole frozen fish were thawed, weighed, and homogenized for enzymatic assays, using a glass homogenizer on ice in 0.2 M NaCl (w/v) (Gawlicka et al., 2000) according to the specified ratio. The homogenates were centrifuged at 2°C and 15000 r/min for 10 min. Then, the aqueous supernatant was collected and incubated in the enzyme substrate under 25 or 37°C and read on a spectrophotometer (UV-1800BPC, LiuYi Biotechnology co., Ltd, China) at the target wavelength. Enzyme activities and cortisol concentration were carried out in triplicate.

The activity of antioxidant enzymes including acid phosphatase (ACP, E.C. 3.1.3.2), catalase (CAT, E.C. 1.15.1.1), glutathione peroxidase (GSH, E.C.1.11.1.9), peroxidase (POD, E.C.1.11.1.7), superoxide dismutase (SOD, E.C.1.15.1.1), and digestive enzyme, including lipase (LPS, E.C.3.1.1.3), amylase (AMS, E.C.3.2.1.1), pepsin (PES, E.C.3.4.23.1), and the cortisol concentration were assayed by corresponding assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The operation process and units of specific activity followed the instructions. Soluble protein of crude enzyme extracts was quantified by Bicinchoninic Acid method (Walker, 2002) using the bicinchoninic acid protein assay kit (Catalog No. A045-4; Jiancheng Bioengineering Institute, Nanjing, China).

#### *Statistical analysis*

All data were expressed as mean  $\pm$  SD and were analyzed with SPSS19.0 using ANOVA to determine whether there was any significant difference among the concentrations. When ANOVA was significant, the Duncan test was performed to determine where the differences lay.

## **Results**

### *Acute nitrite stress effects to behavior and survival*

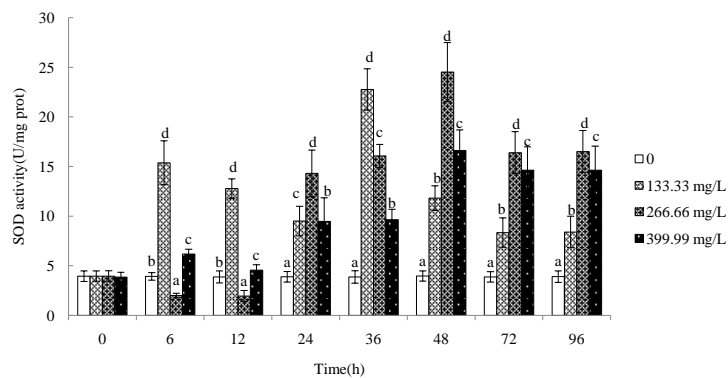
After 96 hours, fish survival rate was not significantly affected by acute nitrite for all the treatments except the one with a nitrite concentration of 399.99 mg/L where it dropped to 50%.

During the experimental period, with the increase of nitrite content, the fish showed different degrees of stress behavior with different general responses of individuals such as stiffening, bending, breathing difficulty, a diminution in the ability to evade, and sudden death. The behaviors observed were physiological, congestive body, and acceleration of gill cover flapping. Mucus production on the surface of the body returned to normal after 24 hours of adaptation. Some individuals subjected to the highest concentrations of nitrite (i.e. 399.99 mg/L) faced bleaching, and a small number of individuals died after 48 hours.

### *Acute nitrite stress effects to antioxidant enzymes*

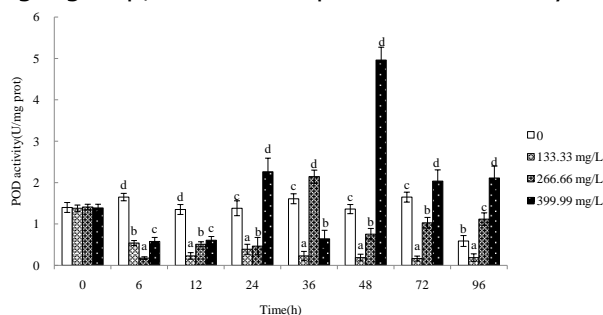
When subjected to acute nitrite stress, all fish in our experiment showed a significant change ( $P < 0.01$ ) in antioxidant enzyme activity compared to fish in the control where antioxidant enzyme activity remained constant during the 96 hours of the experiment.

At the lowest concentration of nitrite (133.33 mg/L), SOD activity significantly tripled ( $P < 0.01$ , Fig. 1) after 6 hours compared to control conditions and reached a peak five times higher than in the control after 36 hours. SOD activity increased with an increase in nitrite concentration to 266.66 mg/L and 399.99 mg/L. When nitrite concentration was 266.66 mg/L, a peak was recorded after 48 hours. This was five times higher than in the control. Maximum SOD activity was recorded at a concentration of nitrite of 399.99 mg/L, (Fig. 1). In general, the SOD activity significantly increased ( $P < 0.01$ ) in relation to time.



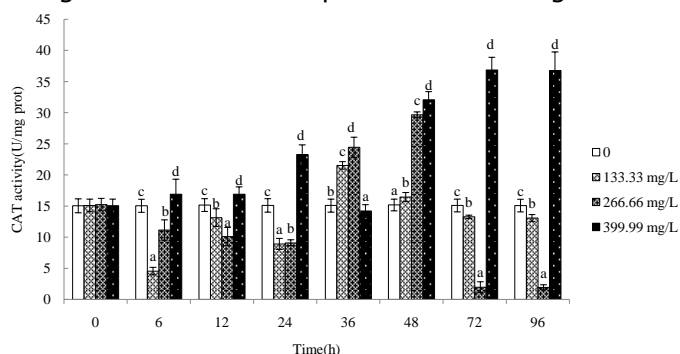
**Fig.1** Activity response of superoxide dismutase (SOD) to nitrite stress in *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

In general, peroxidase activity was significantly ( $P < 0.01$ ) impacted by nitrite (Fig 2). After 6 hours of exposure, peroxidase activity in all the stress groups was significantly lower ( $P < 0.05$ ) than in the control group. After 24 hours of exposure, the peroxidase activity was still low for 133.33 mg/L and 266.66 mg/L treatment but increased in the 399.99 mg/L group. A similar trend was also observed after 48 hours, and the peroxidase activity reached the highest level, five times the control level, with 399.99mg/L of nitrite. At the end of this experiment, the highest peroxidase activity was observed in 399.99 mg/L group, and lowest peroxidase activity was observed in 133.33 mg/L group.



**Fig.2** Activity response of peroxidase (POD) to nitrite stress in *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

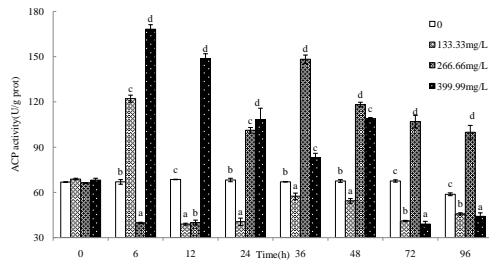
The catalase activity was significantly affected by increasing nitrite concentration ( $P < 0.01$ , Fig. 3). Catalase at 133.33 mg/L and 266.66 mg/L treatments showed similar activities. Their activities started with a decrease until 36 hours when they rose slightly above the control treatment. Then catalase activity from 133.33 mg/L treatment went back to original activity and the 266.66 mg/L group drastically decreased, ten times more than its peak. Regarding the 399.99mg/L group, catalase activity increased constantly during the 96hours of experiment reaching more than twice its initial activity level.



**Fig.3** Activity response of catalase (CAT) to nitrite stress in time of *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

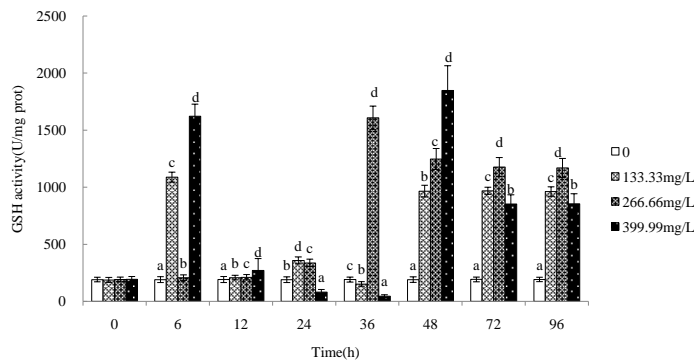
While subjected to nitrite stress, acid phosphatase (ACP) activity of *L. calcarifer* was significantly ( $P < 0.05$ ) affected (Fig. 4). At 133.33 mg/L concentration of nitrite, ACP activity doubled after 6 hours of exposure and then dropped and stayed low until the end of the experiment. In 399.99 mg/L nitrite concentration group, activity was similar with a

peak after 6 hours tripling ACP activity. It was followed by a drop after 72 hours of treatment. Treatment at 266.66 mg/L of nitrite showed a slower rise to a peak of 2.5 times the original after 36 hours of treatment and then a slight decrease.



**Fig.4** Activity response of acid phosphatase (ACP) to nitrite stress in time of *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

Fish from both the 133.33 mg/L and 399.99mg/L nitrite treatments showed a peak of glutathione peroxidase (GSH-Px) activity ( $P < 0.01$ , Fig. 5) with a highest peak at the stronger concentration (7 time more than for controlled conditions). Those peaks were followed by clear drop in activity. For both, GSH-Px activity reached a peak again after 48 hours of the experiment and stayed high until the end of the experiment with a slight decrease in the 399.99mg/L group. Regarding treatment with 266.66 mg/L of nitrite, GSH-Px activity remained low until 36 hours when the activity rose six times than before. It slightly diminished and stayed the same until the end of the experiment.

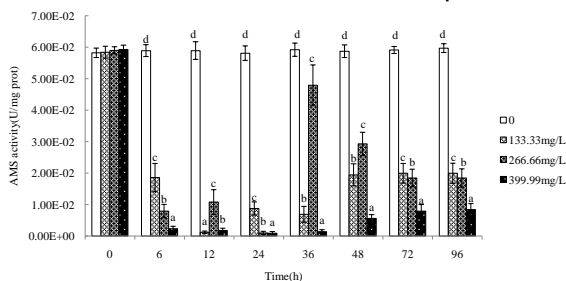


**Fig.5** Activity response of glutathione peroxidase (GSH-Px) to nitrite stress in time of *Lates calcarifer*. Different letters represent significantly different ( $P < 0.05$ ).

#### Acute nitrite stress effects on digestive enzymes

Digestive enzymes activity was analyzed and quantified during our experiment. For all enzymes, digestive enzymes activity during nitrite stress was significantly different ( $P < 0.01$ ) from the controlled conditions.

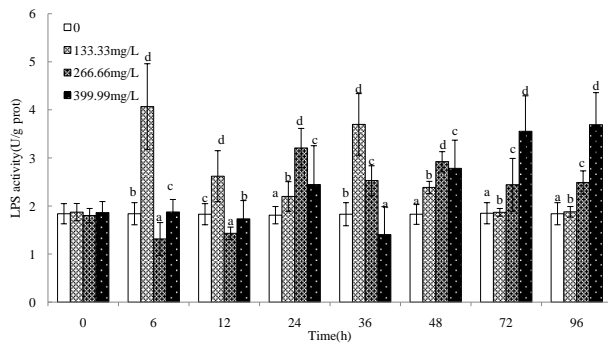
The amylase activity of fish from the nitrite stress group significantly decreased ( $P < 0.05$ ) through the experiment time (Fig.6). While amylase activity stayed low at 133.33 mg/L and 399.99mg/L of nitrite, amylase activity reached a peak after 36 hours for 266.66 mg/L of nitrite group. This peak was still lower than controlled activity and diminished until the end of the experiment.



**Fig.6** Activity response of amylase (AMS) to nitrite stress in time of *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

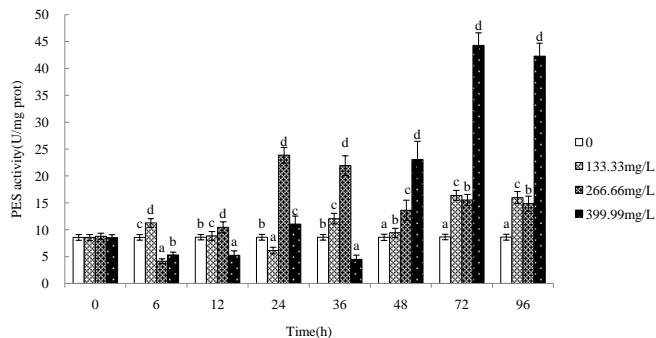
Lipase activity significantly increased ( $P < 0.01$ ) when subjected to nitrite stress (Fig.7). After 6 hours of exposure, lipase activity almost doubled in the group exposed to 133.33 mg/L of nitrite and decrease back to initial value after 48 hours of exposure. With

exposure to 266.66 mg/L of nitrite, lipase activity practically doubled after 24 hours. It was only after 48 hours that lipase activity slowly increased in 399.99mg/L group.



**Fig.7** Activity response of lipase (LPS) to nitrite stress in time of *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

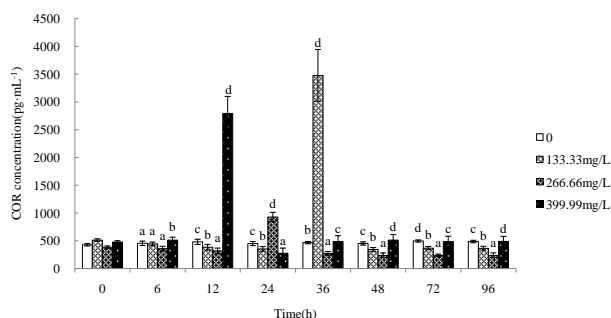
Pepsin activity increased significantly ( $P < 0.01$ ) in time compared to control conditions. (Fig. 8) After 6 hours of treatment with 133.33 mg/L of nitrite, pepsin activity started a slow rise till the end of the experiment, reaching 1.5 time the level of control conditions. With treatment at 266.66 mg/L of nitrite, pepsin activity peaked after 24 hours with 2.5 times the level of control. It diminished back to 1.5 times the level of control conditions after 96 hours of experiment. For 399.99mg/L group, pepsin activity raised after 48 hours and stayed around 5 times higher than control until the end of the experiment.



**Fig.8** Activity response of pepsin (PES) to nitrite stress in time of *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).

#### Acute nitrite stress effects to cortisol concentration

During the experiment, nitrite stress conditions significantly ( $P < 0.01$ ) affected cortisol activity in barramundi (Fig.9). The higher the concentration, the earlier the peak of activity occurred. With a concentration of nitrite of 399.99 mg/L, 266.66 mg/L and, 133.33 mg/L, cortisol activity rose drastically after 12 hours, 24 hours, and 36 hours, respectively. Cortisol activity was five, two, and seven times higher than the control conditions at 399.99 mg/L, 266.66 mg/L, and, 133.33 mg/L, respectively. After 48 hours, the cortisol activity of fish remained at low level until completion of the experiment. At the end of this experiment, the lowest cortisol concentration was observed in 266.66 mg/L group, and the highest concentration was in 399.99mg/L group.



**Fig.9** Activity response of cortisol (COR) to nitrite stress in *Lates calcarifer*. Different lowercase letters indicate statistically significant differences ( $P < 0.05$ ).



## Discussion

### *Effect of acute nitrite stress on behavior and survival*

In our experiment, barramundi affected by nitrite stress showed physiological responses concordant with previously studies on crustaceans, demonstrating that high concentrations of nitrite can strongly affect fish skin and sputum, causing the fish to struggle vigorously and suffocate (Romano & Zeng, 2013). In our case, barramundi exposed to high concentration of 399.99 mg/L also experienced color fading/whitening of the fish skin. This whitening is consistent with the poisoning phenomenon described by (Tomasso, 1986) and similar to the physical affliction reported on *Takifugu rubripe* (Wang et al., 2018) and *Pelteobagrus fulvidraco* (Li et al., 2016) when exposed to nitrite stress. Our results suggest that the phenomenon of whitening of the barramundi skin is caused by nitrite stress, especially which is accompanied by erratic behaviors such as congestion or difficulty in breathing.

Previous studies on fish have shown different tolerance levels to acute nitrite stress especially regarding survival rates. *Epinephelus coioides* fry, under 4.5-5.5 mg/L of dissolved oxygen, had an LC50 value from 208.4-354.8 mg/L (Leyun, 2012). Under 7.0 mg/L of dissolved oxygen, *Takifugu rubripe* had an LC50 value from 159.25-201.88 mg/L (Wang et al., 2018). With dissolved oxygen kept at >6.5 mg/L, barramundi survival rate was 50% when subjected to nitrite concentration of 399.99 mg/L and 100% for the other treatment groups. It appeared that barramundi showed stronger resistance from both in their tolerance to nitrite and survival rate.

### *Effect of acute nitrite stress on antioxidant enzymes*

The overproduction of ROS led to potential oxidant damage on cellular macromolecules such as DNA, protein, and lipids (Sies, 1997). The non-specific immune system plays a dominant role in the response of fish to environmental stress and is considered the first line of defense against ROS. The antioxidant enzymes that were measured during our experiments (e.g. superoxide dismutase, peroxidase, catalase, acid phosphatase, and glutathione peroxidase) account for a significant portion of the immune regulatory process. Antioxidant enzymes can up-regulate antioxidant defense against excessive ROS production and oxidant damage (Qiu et al., 2011; Lesser, 2006; Barata et al., 2005). Lipid peroxidation leads to impaired cellular function and alterations in physicochemical properties of cell membranes (Barata et al., 2005). Previous studies have shown that antioxidant enzyme activity increases when organisms are exposed to a certain nitrite dose and is inhibited with increase in nitrite concentration and exposure duration (e.g. *Oreochromis niloticus*, (Qiang et al., 2015); *Cyprinus carpio*, (Han et al., 2007); *Takifugu rubripe*, (Wang et al., 2018). Catalase and SOD enzymes can be coupled to form a system providing the first immune defense against oxygen toxicity. First, SOD catalyzes the dismutation of the superoxide anion radical to H<sub>2</sub>O and H<sub>2</sub>O<sub>2</sub>. Then, H<sub>2</sub>O and H<sub>2</sub>O<sub>2</sub> are detoxified by both catalase and GSH-Px activity. GSH-Px protects cell membranes against oxidative damage by eliminating strong prooxidants such as hydroperoxides (Ilham et al., 2018). Due to the inhibitory effects on oxyradical formation, the SOD-CAT system provides the first defense line against oxygen toxicity. In our experiments, the SOD and catalase activity of SOD rose initially and later decreased, matching other findings (Xu et al. 1995) which indicated that under mild stress, fish SOD and catalase activity is induced, and when under severe stress conditions, their activity is inhibited. Nevertheless, catalase activity in higher nitrite concentrations (399.99 mg/L) was not inhibited. The rise of catalase and SOD activities in our study confirmed the presence of excessive ROS and the attempt to neutralize ROS impact (Monteiro et al., 2006). The results from our current research together with previous published data demonstrate that specific POD activity increased to a high level after acute nitrite stress. The results suggest that POD enzyme activity expands over time with a high level of exposure.

ACP activity can be now used as early warning of fish stress (Das et al., 2004). ACP in fish responded to low nitrite concentrations (Bai (2009). The occurrence of the above phenomenon may result from changes in cell metabolism rather than oxidant stress (Wang et al., 2018; Bai, 2009; Martinez-Alvarez et al., 2002). With exposure to long-

term and high concentrations of nitrite, the antioxidant system cannot remove ROS in time, and the oxidation and antioxidant proportions are imbalanced causing free radicals to accumulate, thus causing oxidant damage to the cells. At the same time, the anti-oxidation system of the fish is also damaged, manifested as a continuous decrease in enzyme activity (Wang et al., 2018; Lv et al., 2010; Bai, 2009). Therefore, to a certain extent, the time that lapses until the antioxidant enzyme activity returns to a normal level reflects the body's ability to adapt to the environment.

The lowest value of ACP activity in the 399.99 mg/L group was significantly lower than in the control group, which may be related to the above conclusions about low-level stress promoting antioxidant enzyme activity and high-concentration stress inhibiting antioxidant enzyme activity. This may be related to the physiological characteristics such as wide salinity, high resistance to stress, or different developmental stages of the barramundi. It may also be that the stress of nitrite caused a certain disorder effect on its antioxidant system, but the measurement of stress treatment is still within its scope of adaptation. At the same time, many studies also showed that the experimental subjects have different specifications, culture densities, and environmental factors.

#### *Acute nitrite stress on digestive enzymes activities*

Research on the tissue, organs, blood parameters, and immune function of the cultured individuals has been conducted (Sun et al., 2014; Ciji et al., 2012). Nitrite can be absorbed and accumulated in the intestine and gills of fish, that affect the digestive performance of the individual by affecting the functional structure of the tissue or organ (Gao et al. 2008). When analyzing barramundi early stages, it was found that under normal conditions, protease activity increased significantly, while the lipase and amylase activity fluctuated (Srichanun et al. 2013). This may suggest that reduced amylase activity in fish in the present study may be due to barramundi selective digestion that absorbs proteins to produce more energy to counter environmental stress.

#### *Acute nitrite stress on cortisol concentration*

Stress responses in fish are first manifested as changes in hormone content in the blood with elevation in blood cortisol, recognized as an indicator of fish stress (Hontela et al., 1992). In the course of environmental stress, the hypothalamus-pituitary-interstitial axis (HPI) predominates, and cortisol and other hormones respond, induced by the synthesis and release, and then affect the ion permeability and metabolism to maintain the physiological balance of the body (Bernier & Peter, 2001). Here, the cortisol content of each stress group at the end of the experiment was similar or lower than that of the control, except for the peaks after 12, 24, and 36 hours for the 133.33, 266.66 and 399.99 mg/L group respectively. Presumably, under different nitrite concentrations toxicity accumulated reaching the tolerance limit of the fish, who reacted with a peak of activity of physical tolerance. It has been previously suggested that elevation of cortisol observed in fish exposed to nitrite was the consequence of an activation of the hypothalamic-pituitary-interrenal (HPI) axis (Ciji et al. 2012).

Concerning nitrite stress and toxicological experiments on fish, our research studied changes of antioxidative and digestive enzyme activity and serum cortisol concentration in fish. The consequence of stress events to the organisms depends on the ability of an individual to quickly raise antioxidant potential in response to oxidative stress. Increase in nitrite concentration is more likely to disrupt the action of antioxidant and digestive enzymes and to slow down their defense actions against stress. This study enriched the basic data of nitrite on the digestive performance of fish and provided the data basis and scientific basis for other fish nitrite stress experiments.

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## References

- Allais, L., Zhao, C., Fu, M., Hu, J., Qin, J. G., Qiu, L., Ma, Z.** (2018). Nutrition and water temperature regulate the expression of heat-shock proteins in golden pompano larvae (*Trachinotus ovata*, Linnaeus 1758). *Biochemistry*, 1-13.
- Bai X. J.** (2009). Influence of Nitrite to the Growth, Phosphatase and Phenoloxidase of *Branchiostoma belcheri* Gray. *Shandong fisheries*, 8, 008.
- Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., & Porte, C.** (2005). Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basin (NE Spain). *Aquatic toxicology*, 74(1), 3-19.
- Bernier, N. J., & Peter, R. E.** (2001). The hypothalamic-pituitary-interrenal axis and the control of food intake in teleost fish. *Comparative biochemistry physiology Part B: Biochemistry molecular biology*, 129(2-3), 639-644.
- Carter, C., Houlihan, D., & Owen, S.** (1998). Protein synthesis, nitrogen excretion and long-term growth of juvenile *Pleuronectes flesus*. *Journal of fish biology*, 53(2), 272-284.
- Chen, X., Wang, K., Guo, A., Dong, Z., Zhao, Q., Qian, J., & Zhang, D.** (2016). Excess phosphate loading shifts bacterioplankton community composition in oligotrophic coastal water microcosms over time. *Journal of experimental marine biology ecology*, 483, 139-146.
- Ciji, A., Sahu, N., Pal, A., Dasgupta, S., & Akhtar, M.** (2012). Alterations in serum electrolytes, antioxidative enzymes and haematological parameters of *Labeo rohita* on short-term exposure to sublethal dose of nitrite. *Fish physiology biochemistry*, 38(5), 1355-1365.
- Das, P., Ayyappan, S., Jena, J., & Das, B.** (2004). Nitrite toxicity in *Cirrhinus mrigala* (Ham.): acute toxicity and sub-lethal effect on selected haematological parameters. *Aquaculture*, 235(1-4), 633-644.
- Domingos, J. A., Smith-Keune, C., Robinson, N., Loughnan, S., Harrison, P., & Jerry, D. R.** (2013). Heritability of harvest growth traits and genotype-environment interactions in barramundi, *Lates calcarifer* (Bloch). *Aquaculture*, 402, 66-75.
- Ebeling, J. M., Timmons, M. B., & Bisogni, J.** (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. *Aquaculture*, 257(1-4), 346-358.
- Gao, M., Ma, L., Ge, L., Mei, C., Xu, H., & Chen, W.** (2008). Nitrite uptake mechanism and the influencing factors of accumulation in aquatic animals. *South china fisheries science*, 4(4), 73-79.
- Gawlicka, A., Parent, B., Horn, M. H., Ross, N., Opstad, I., & Torrissen, O.** (2000). Activity of digestive enzymes in yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*): indication of readiness for first feeding. *Aquaculture*, 184(3-4), 303-314.
- Glencross, B.** (2006). The nutritional management of barramundi, *Lates calcarifer*—review. *Aquaculture Nutrition*, 12(4), 291-309.
- Han, Y., Zahng, H., & Wang, K.** (2007). Activity of SOD and GSH-Px in the Blood of Fingerling *Cyprinus carpio* under Different Nitrite Concentration. *Freshwater fisheries*, 1, 013.
- Handy, R., & Poxton, M.** (1993). Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. *Reviews in fish biology fisheries*, 3(3), 205-241.
- Hontela, A., Rasmussen, J. B., Audet, C., & Chevalier, G.** (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Archives of environmental contamination toxicology*, 22(3), 278-283.
- Ilham, I., Hapsari, F., & Fotedar, R.** (2018). Growth, enzymatic glutathione peroxidase activity and biochemical status of juvenile barramundi (*Lates calcarifer*) fed dietary fermented lupin meal supplemented with organic selenium. *Aquaculture research*, 49(1), 151-164.

- International Organization for Standardization.** (1997). ISO 7346-2:1996. Water quality. Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)]. Part 2: Semi-static method. Geneva. Switzerland: *International Organization for Standardization*, 1-11.
- Jensen, F. B., Andersen, N. A., & Heisler, N.** (1987). Effects of nitrite exposure on blood respiratory properties, acid-base and electrolyte regulation in the carp (*Cyprinus carpio*). *Journal of comparative physiology B*, 157(5), 533-541.
- Kroupova, H., Machova, J., & Svobodova, Z.** (2005). Nitrite influence on fish: a review. *Veterinarni medicina (praha)*, 50(11), 461.
- Lesser, M. P.** (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. *Annual review of physiology*, 68, 253-278.
- Leyun, Z.** (2012). Acute toxic effects of ammonia and nitrite on *Epinephelus coioides* fry. *Marine sciences*, 36(5), 81-86.
- Li, M., Gong, S., Li, Q., Yuan, L., Meng, F., & Wang, R.** (2016). Ammonia toxicity induces glutamine accumulation, oxidative stress and immunosuppression in juvenile yellow catfish *Pelteobagrus fulvidraco*. *Comparative biochemistry physiology part C: Toxicology pharmacology*, 183, 1-6.
- Lin, Y., Miao, L. H., Pan, W. J., Huang, X., Deng, J. M., Zhang, W. X., Ge, X. P., Liu, B., Ren, M. C., Zhou, Q. L., Xie, J., Pan, L. K.** (2018). Effect of nitrite exposure on the antioxidant enzymes and glutathione system in the liver of bighead carp, *Aristichthys nobilis*. *Fish shellfish immunology*, 76, 126-132.
- Lv, X., Li, J., Fang, Y., Guo, Z., Zhao, Y., & Li, K.** (2010). Nitrite stress on immune-related enzymes and the ultrastructure in different tissue of redclaw crayfish (*Cherax quadricarinatus*). *Journal of fisheries of China*, 12, 005.
- Ma, Z., Hassan, M. M., Allais, L., He, T., Leterme, S., Ellis, A. V., MacGraw, B., Qin, J. G.** (2018). Replacement of fishmeal with commercial soybean meal and EnzoMeal in juvenile barramundi *Lates calcarifer*. *Aquaculture research*, 49(10), 3258-3269.
- Martinez-Alvarez, R., Hidalgo, M., Domezain, A., Morales, A., García-Gallego, M., & Sanz, A.** (2002). Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity. *Journal of experimental biology*, 205(23), 3699-3706.
- Monteiro, D. A., De Almeida, J. A., Rantin, F. T., & Kalinin, A. L.** (2006). Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). *Comparative biochemistry physiology part C: Toxicology pharmacology*, 143(2), 141-149.
- Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., Folkek, C., Lubchencos, J., Mooney, H., Troell, M. J. N.** (2000). Effect of aquaculture on world fish supplies. *Nature*, 405(6790), 1017-1024.
- Parra, G., & Yúfera, M.** (1999). Tolerance response to ammonia and nitrite exposure in larvae of two marine fish species (gilthead seabream *Sparus aurata* L. and Senegal sole *Solea senegalensis* Kaup). *Aquaculture research*, 30(11-12), 857-863.
- Qiang, J., Yang, H., Ma, X., Wang, H., Xu, P., He, J., & Zhu, Z.** (2015). Using response surface methodology to determine effects of chronic nitrite exposure and stocking density on growth of GIFT tilapia *Oreochromis niloticus* juveniles. *Oceanologia et limnologia sinica*, 46, 1166-1174.
- Qiu, J., Wang, W.-N., Wang, L. J., Liu, Y. F., & Wang, A. L.** (2011). Oxidative stress, DNA damage and osmolality in the Pacific white shrimp, *Litopenaeus vannamei* exposed to acute low temperature stress. *Comparative biochemistry physiology Part C: Toxicology pharmacology*, 154(1), 36-41.
- Romano, N., & Zeng, C.** (2013). Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: a review on factors influencing their toxicity, physiological consequences, and coping mechanisms. *Reviews in fisheries science*, 21(1), 1-21.
- Roques, J. A. C., Schram, E., Spanings, T., van Schaik, T., Abbink, W., Boerrigter, J., de Vries P., van de Vis H., & Flik, G.** (2015). The impact of elevated water nitrite concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822). *Aquaculture research*, 46(6), 1384-1395.

- Sies, H.** (1997). Oxidative stress: oxidants and antioxidants. *Experimental physiology: Translation integration*, 82(2), 291-295.
- Srichanun, M., Tantikitti, C., Utarabhand, P., & Kortner, T. M.** (2013). Gene expression and activity of digestive enzymes during the larval development of Asian seabass (*Lates calcarifer*). *Comparative biochemistry physiology Part B: Biochemistry molecular biology*, 165(1), 1-9.
- Sun, S., Ge, X., Zhu, J., Xuan, F., & Jiang, X.** (2014). Identification and mRNA expression of antioxidant enzyme genes associated with the oxidative stress response in the Wuchang bream (*Megalobrama amblycephala* Yih) in response to acute nitrite exposure. *Comparative biochemistry physiology Part C: Toxicology pharmacology*, 159, 69-77.
- Svobodova, Z., Machova, J., Poleszczuk, G., Hůda, J., Hamáčková, J., & Kroupova, H. J. A. V. B.** (2005). Nitrite poisoning of fish in aquaculture facilities with water recirculating systems. *Acta veterinaria brno*, 74(1), 129-137.
- Tomasso, J.** (1986). Comparative toxicity of nitrite to freshwater fishes. *Aquatic toxicology*, 8(2), 129-137.
- Van Rijn, J., & Rivera, G.** (1990). Aerobic and anaerobic biofiltration in an aquaculture unit—nitrite accumulation as a result of nitrification and denitrification. *Aquacultural engineering*, 9(4), 217-234.
- Van Rijn, J., Tal, Y., & Schreier, H.** (2006). Denitrification in recirculating systems: theory and applications. *Aquacultural engineering*, 34(3), 364-376.
- Wang, J., Tang, H., Zhang, X., Xue, X., Zhu, X., Chen, Y., & Yang, Z.** (2018). Mitigation of nitrite toxicity by increased salinity is associated with multiple physiological responses: A case study using an economically important model species, the juvenile obscure puffer (*Takifugu obscurus*). *Environmental pollution*, 232, 137-145.
- Xian, J. A., Wang, A. L., Chen, X. D., Gou, N.-N., Miao, Y. T., Liao, S. A., & Ye, C. X.** (2011). Cytotoxicity of nitrite on haemocytes of the tiger shrimp, *Penaeus monodon*, using flow cytometric analysis. *Aquaculture*, 317(1-4), 240-244.
- Xu, L., Zhang, Y., & Chen, Y.** (1995). The advances of molecular ecotoxicology and its significance in water environment protection. *Acta hydrobiologica sinica*, 19(2), 171-185.