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RESEARCH ARTICLE

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Effects of the amplitude and frequency of salinity fluctuations on antioxidant responses in juvenile tongue sole, *Cynoglossus semilaevis*

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Abstract

To understand the tolerance of tongue sole, *Cynoglossus semilaevis,* to varying salinities, the effects of the amplitude (2, 4, 6 and 8 g/L) and frequency (2, 4 and 8 days) of salinity fluctuations on the activities of antioxidant responses, including acidic phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT) and superoxide dismutase (SOD) from antioxidant system in liver, muscle, gills and kidney were investigated in this study. The results showed that the antioxidant responses of tongue sole were highly tissue-specific during the varying salinity fluctuations. In all tissues, ACP and AKP activity was found to be highest at moderate salinity fluctuations compared to the control, low and high salinity treatments ($p<0.05$). SOD and CAT activities had significant effect due to salinity fluctuations in all tissues ($p<0.05$), except in hepatic and renal tissues. Variations in branchial SOD activity proved that salinity fluctuations had greater impact on tongue sole at moderate and high fluctuating salinities compared to the control and low fluctuating salinities, whereas the branchial CAT activities showed contrasting trend. Further, cortisol levels were significantly affected in lower and higher salinity fluctuations. However, plasma cortisol levels remained low in moderate salinity fluctuations and control ($p<0.05$). Taken together, the results indicated that salinity fluctuations could effectively stimulate and enhance the antioxidant enzyme activity in the liver, kidney, gills and muscle of the juvenile tongue sole, thus effectively eliminating the excessive reactive oxygen species and minimizing the body damage in tongue sole or could be for any other euryhaline teleosts.

Additional key words: salinity variation; enzyme activity; immunomarker; environmental stress; aquaculture.

Abbreviations used: ACP (acidic phosphatase); AKP (alkaline phosphatase); CAT (catalase); IPNV (infectious pancreatic necrosis virus); RIA (radio immuno assay); ROS (reactive oxygen species); SOD (superoxide dismutase).

Authors' contributions: Conceived and designed the experiments: SOK, XT and SD. Performed the experiments: SOK, ZF and BVS. Analyzed the data: SOK, XT, BVS and HAS. Contributed reagents/materials/analysis tools: XT and SD. Wrote the paper: SOK, XT, SD and HAS.

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Introduction

In marine aquaculture, salinity is one of the most important factors influencing the fish growth and survival (Moser & Gerry, 1989; Sampaio & Bianchini, 2002; Lin *et al.,* 2005; Shi *et al.,* 2008; Hu *et al.,* 2011; Khairnar *et al.,* 2014; Martinez-Cardenas *et al.,* 2014), as its variation may cause a variety of physiological stress responses (Choi *et al.*, 2008; Baysoy *et al.,* 2012; Ern *et al.,* 2014; Khairnar *et al.,* 2015; Wedderburn *et al.,* 2016), which has been associated with enhanced reactive oxygen species (ROS) generation (Livingstone, 2001). In order to counteract the potential damage resulting from excess reactive oxygen and to maintain its homeostasis, living organisms have evolved a delicate antioxidant mechanism consisting mainly of two systems: the enzyme system and the non-enzyme system. In biological research,

antioxidant enzymes are often regarded as important indicators of environment quality (Zaccaron *et al.,* 2005), thus the study on their mechanism under different salinity fluctuations might shed light on clarifying the physiological and biochemical responses resulting from oxidative stress. Cortisol is an important glucocorticoid that functions in the osmotic acclimation and stress response of teleosts (Mommsen *et al.,* 1999). Most of the earlier researches concentrated on the effect of salinity which mainly looked at changes in osmoregulatory organs and their hormonal control, plasma parameters, energy metabolism, growth, etc. (Maetz, 1974; McCormick, 2001). However, not much information is available about the changes in fish antioxidant responses due to the salinity purturbance (fluctuations and amplitude) despite the fact that it is one of the most important environmental factors in the aquatic medium. Perhaps, earlier reports have demonstrated that teleost exposed to either hypo or hyper-osmotic shock increases their susceptibility to infectious pancreatic necrosis virus (IPNV) (Chou *et al.,* 1999; Zhang *et al.,* 2011).

Tongue sole (*Cynoglossus semilaevis* Güther, 1873), Actinopterygii, Cynoglossidae, is an important rare native commercial fishery species in the Bohai Sea, the Yellow Sea, and the East China Sea, China, which inhabits coastal waters and estuaries (Masuda *et al.,* 1984; Ma *et al.,* 2005, 2007). Being one of the most important edible fish species in China, tongue sole enjoys increasing market demand due to its tender meat and high nutrition content. Tongue sole is euryhaline in nature and it inhabits coastal ponds, where they can be exposed to a wide salinity variation. However, due to overfishing and consequent decline in the wild population, there arises the urgent need for commercial production of tongue sole. Therefore, to improve the tongue sole culture, there is a need to understand their different mechanisms in controlled environment to attain high production. Hence, there is a need to study the antioxidant responses of juvenile tongue sole in controlled aquaculture conditions in relation to the salinity frequencies.

The current study was undertaken to explore the effects of salinity fluctuations on antioxidants responses of the tongue sole. Therefore, an experiment was conducted by placing juveniles in different ranges of frequency and amplitude of salinity. Activities of acidic phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT) and superoxide dismutase (SOD) in hepatic (liver), muscular (muscle), branchial (gills) and renal (kidney) tissues and plasma cortisol levels were monitored as biomarkers of salinity stress. This research was intended to identify the key ecological factors whose variations might threaten the growth and survival of the tongue sole juveniles, thus providing reliable information on the regulation of certain environmental factors for large-scale aquaculture.

Material and methods

Experimental fish

Juvenile tongue soles were provided by Mingbao Aquatic Product Co., Ltd. (Yantai, China) and transferred to the laboratories at the Aoshanwei Research Centre of Ocean University of China. They were acclimated to seawater (30 g/L) in a 1000 L fiberglass tank for at least one week before the experiment started. For the experiment, fish of same age and similar sizes were selected and during the experiment they were fed commercial pellets (Guangzhou Yuequn Technology Co., Ltd, China) to satiation twice daily at 07:00 and 18:00 h. Animal handling procedures were followed according to international guidelines and approved by the Animal Ethics Committee, Ocean University of China, Qingdao, Shandong Province, China.

Experimental design

The effects of three fluctuating frequencies (every 2, 4, or 8 days) and four fluctuation amplitudes (2, 4, 6, or 8 g/L) were compared to an unfluctuating control. The salinity of control was constant *i.e.* 30 g/L, while treatments S30 ± 2, S30 ± 4, S30 ± 6 and S30 ± 8 (S stands for salinity and values are in g/L) were subjected to different salinity fluctuations. In each amplitude treatment, there were three frequency treatments including D2, D4 and D8 (D stands for days) (Fig. 1). Experimental design was conducted according to Khairnar *et al.* (2014). Briefly, there were five replicates for

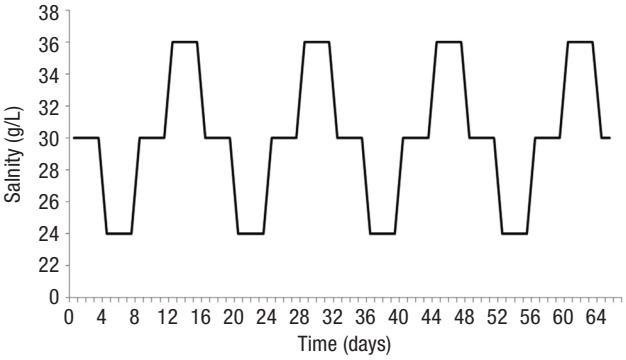

Figure 1. Example of salinity fluctuation treatment at the amplitude of ± 6 g/L and frequency of 4 days (D4S30 ± 6). The same fluctuation pattern was maintained at different amplitudes (± 2, ± 4, ± 6, and ± 8 g/L) and frequencies (2, 4, and 8 days).

each treatment, and each replicate had six juveniles. During the experiment, water quality parameters including temperature, pH and ammonia were monitored daily. Throughout the experiment, photoperiod was set at 14 h light: 10 h dark.

Sampling procedure

Fish were anaesthetized using 60 mg/L of MS-222 (Sigma Aldrich), and then they individually weighted. Blood was collected from the caudal peduncle using 1 mL syringes that were rinsed with a solution containing 25,000 units of ammonium heparin in 3 mL of 0.6% NaCl. Plasma was separated from cells by centrifuging whole blood (3 min, 10000 g, 4 °C). Then from each fish, the second gill arch on the left side was excised and dried with absorbent paper. Further, other key tissues including kidney, liver and muscles were dissected and frozen in liquid nitrogen and stored at –80°C for further analyses. Frozen samples were thawed at 4°C before analyzed. Samples of 0.2 g of gill, kidney, liver and muscle tissue were homogenized with 1.8 mL of cold sterile normal saline solution (NaCl 0.85%, w/v, pH 7.5) using hand homogenizer. All samples were then centrifuged at 3000 g for 10 min, and supernatants were collected and stored at 4 °C prior to analysis. All enzymatic assays were conducted within 24 h upon extraction.

Protein content of the homogenates was measured according to the method of Spector (1978), using bovine serum albumin as a standard. All assays for enzyme activities were carried out in duplicate and measured using UV 2102PC spectrophotometer (Unico, Shanghai, China).

Antioxidant parameters

Acid and alkaline phosphatase. ACP and AKP activity assays were carried out according to Barrett's (1972) method using a commercial kit (Nanjing Jiancheng Biotech Company, China). Concentration of phenol was measured spectrophotometrically at 520 nm after incubation at 37 °C for 30 min (for ACP), or 15 min (for AKP). ACP and AKP activity was defined as the amount of phenol (mol) produced per milligram of protein.

Superoxide dismutase. SOD activity was determined according to Ji (1991) with an assay kit (Nanjing Jiancheng Biotech Company, China). Assay conditions were 65 μmol phosphate buffer, pH 7.8, 1 μmol hydrochloric hydroxylamine, 0.75 μmol xanthine and $2.3 \cdot 10^{-3}$ IU xanthine dismutase. The supernatant (50 μL) had no blank reaction and was incubated in the system for 40 min at 37 °C, and terminated with 2 mL of 3.3 g/L p-aminobenzene sulfonic acid and 10 g/L of naphthylamine. An SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation (monitored at 550 nm) by 50%.

Catalase. CAT activity was measured according to Goth (1991) with an assay kit (Nanjing Jiancheng Biotech Company, China). The base system including 4.60 μmol phosphate buffers (pH 7.4), $6.5 \cdot 10^{-3}$ μmol H2O2 and incubated for 1 min at 37°C. The reaction was terminated immediately using 32.4 μmol ammonium molybdate. A CAT unit is defined as catalyzing the use of 1 μmol H_2O_2 per second.

Plasma cortisol. Plasma cortisol was measured by Radio Immuno Assay (RIA) technique according to manufacturer's instructions (Beijing Beifang Biotech Research Institute, China) and the method used was that of Pickering & Pottinger (1995) modified by Liu *et al.* (2012).

Statistical analysis

Statistical analysis of the data was performed with a statistical package (SPSS 16.0, SPSS Inc., Richmond, CA, USA). Values were presented as means ± standard error of the mean. Data for the immune parameters were tested for homogeneity of variances, and then possible differences were tested using one-way ANOVA for immune responses and followed by a Duncan's multiple comparison to find out the difference between treatments.

Results

Water quality

During the course of the experiment, the temperature ranged from 18 to 22 °C, pH was around 8.0 and ammonia nitrogen was less than 0.2 mg/L. No mortality, health disturbances or any alterations in behavior were observed in any treatments during the experiment ($p>0.05$).

Growth

There were no siginificant differences in the initial body weights among all treatments ($p>0.05$). The ANOVA analysis showed that both the frequency and the amplitude of salinity fluctuation had significant effects on the final weights of tongue sole juveniles ($p<0.01$) (Fig. 2). Further, there was a significant interaction between frequency and amplitude of salinity

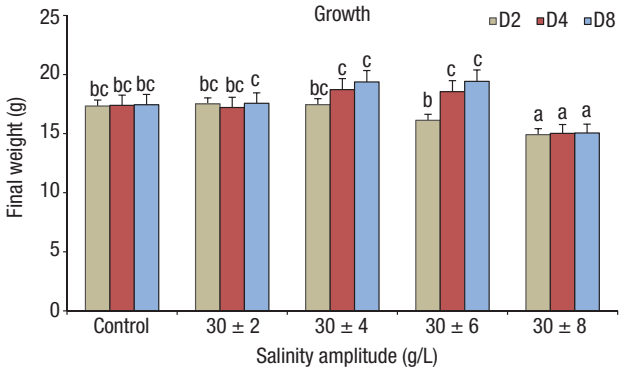

Figure 2. The effect of salinity fluctuations on final weight of juvenile tongue sole. Means with different letters indicate significance difference among the groups ($p<0.05$). Error bars represent the standard error and D2, D4 and D8 are salinity frequencies (2, 4 and 8 days).

fluctuations ($p<0.01$), and the tongue sole exhibited higher specific growth rate at the amplitude of 4–6 g/L at the frequencies of 4 and 8 days than other treatments and the control ($p<0.05$).

Acidic phosphatase activities

ACP activities in different tissues of a tongue sole in the control was highly tissue-specific having 66.47 ± 0.18, 17.32 ± 0.08, 112.20 ± 0.07 and 199.73 ± 4.62 U/g protein in hepatic, muscular, branchial and renal tissues respectively (Fig. 3). ACP activity was significantly influenced in all the tissues of tongue sole by the amplitude and frequency of salinity ($p<0.05$). The hepatic ACP activity ranged from 60.29-77.90 U/g protein and it was significantly higher at D4S30 ± 6 and D8S30 ± 6 than other treatments ($p<0.05$) (Fig. 3A). The muscular ACP activity showed less variation as compared to other tissues and had increasing trend with increasing frequency and amplitude levels compared to control and D8 treatments ($p<0.05$) (Fig. 3B). The branchial ACP activity at D8S30 ± 4, D4S30 ± 6 and D8S30 ± 6 were significantly higher compared to control and other treatments ($p<0.05$) (Fig. 3C). The renal ACP activity in juvenile tongue sole were significantly higher at salinity amplitude of 4–6 g/L with the frequencies of 2–8 days compared to control ($p<0.05$) (Fig. 3D).

Alkaline phosphatase activities

AKP activities in hepatic, muscular, branchial and renal tissues for the control were 5.71 ± 0.14, 5.19 ± 0.02, 12.33 ± 0.10 and 41.12 ± ± 0.52 U/g protein respectively (Fig. 4). The hepatic AKP activity at D8S30 ± 4, D4S30 ± 6 and D8S30 ± 6 were significantly higher compared to control and other treatments ($p<0.05$) (Fig. 4A). The renal AKP activity was significantly higher among the four different tissues and the AKP

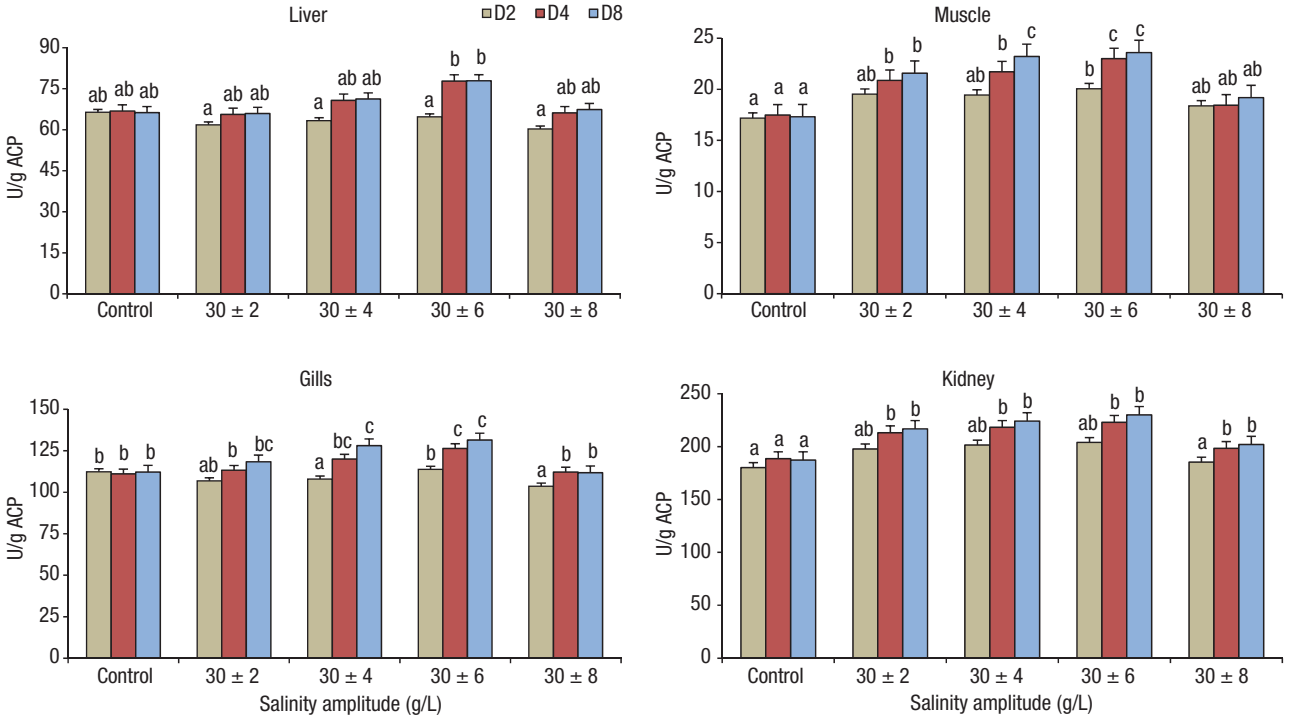

Figure 3. The effect of salinity fluctuations on ACP activities in different tissues of juvenile tongue sole. Different letters above the histogram bars indicate significant differences between groups ($p<0.05$). Error bars represent the standard error and D2, D4 and D8 are salinity frequencies.

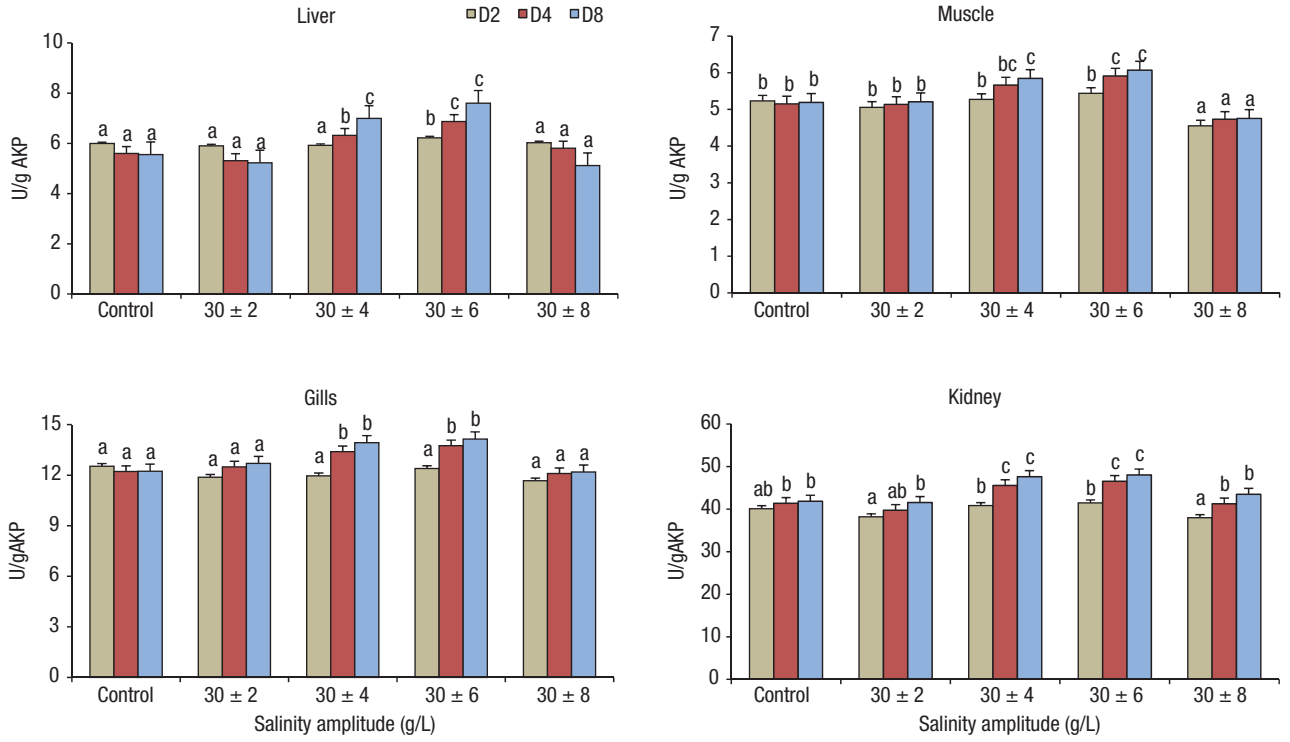

Figure 4. The effect of salinity fluctuations on AKP activities in different tissues of juvenile tongue sole. Different letters above the histogram bars indicate significant differences between groups (p<0.05). Error bars represent the standard error and D2, D4 and D6 are salinity frequencies.

activity in the branchial tissues were significantly higher than muscular tissues, which were influenced by the amplitude and frequency of salinities (p<0.05) (Fig. 4B). The muscular AKP activity was significantly enhanced at D4S30 ± 4, D4S30 ± 6, D8S30 ± 4 and D8S30 ± 6 as compared with control and other treatments (p<0.05) (Fig. 4C). The renal AKP activities were found significantly higher in D8S30 ± 4 and D8S30 ± 6 than control and other treatments (p<0.05) (Fig. 4D). The increasing trend in AKP activity was observed in renal and branchial tissues with decrease in frequency and amplitude of salinity (up to ± 6 g/L fluctuation levels), while it dropped at higher amplitude (± 8 g/L). However, there were no significant differences between branchial and renal AKP activity at ± 4 and ± 6 g/L amplitude at D4 and D8 frequencies (p>0.05).

Superoxide dismutase activities

SOD activity was significantly affected in muscular, renal and branchial tissues of tongue sole (p<0.05) (Fig. 5), whereas the hepatic SOD activities were not found significantly influenced by the fluctuating frequency and the amplitude of salinity compared to the constant salinity (Fig. 5A, p>0.05). The renal SOD activity was significantly higher among the four different tissues, and muscular tissue had the lowest SOD activity (p<0.05) (Fig. 5D). The muscular SOD activity was significantly higher at salinity amplitude of 4–6 g/L at the frequencies of 2–8 days compared to control (p<0.05) (Fig. 5B). The renal SOD activity followed the same trend as in muscular tissues, and was significantly higher at D4S30 ± 6 and D8S30 ± 6 in all the treatments. The branchial SOD activity was significantly influenced by treatments D4S30 ± 4, D4S30 ± 6, D8S30 ± 4 and D8S30 ± 6 as compared to control and other treatments (p<0.05) (Fig. 5C) and showed a tendency to increase with the increase of amplitude from ± 2 to ± 6 g/L. However at ± 8 g/L amplitude, fluctuation was lowest (p<0.05).

Catalase activities

CAT activity in different tissues of tongue sole in control was highly tissue-specific having 24.95 ± 0.18, 8.29 ± 0.02 and 16.08 ± 0.53 U/g protein in hepatic, branchial and renal tissues respectively (Fig. 6). However, renal CAT activity showed no significant effect on frequency and amplitude of salinity fluctuations (p>0.05) (Fig. 6C). Muscular CAT activity was not recorded, as in muscle CAT

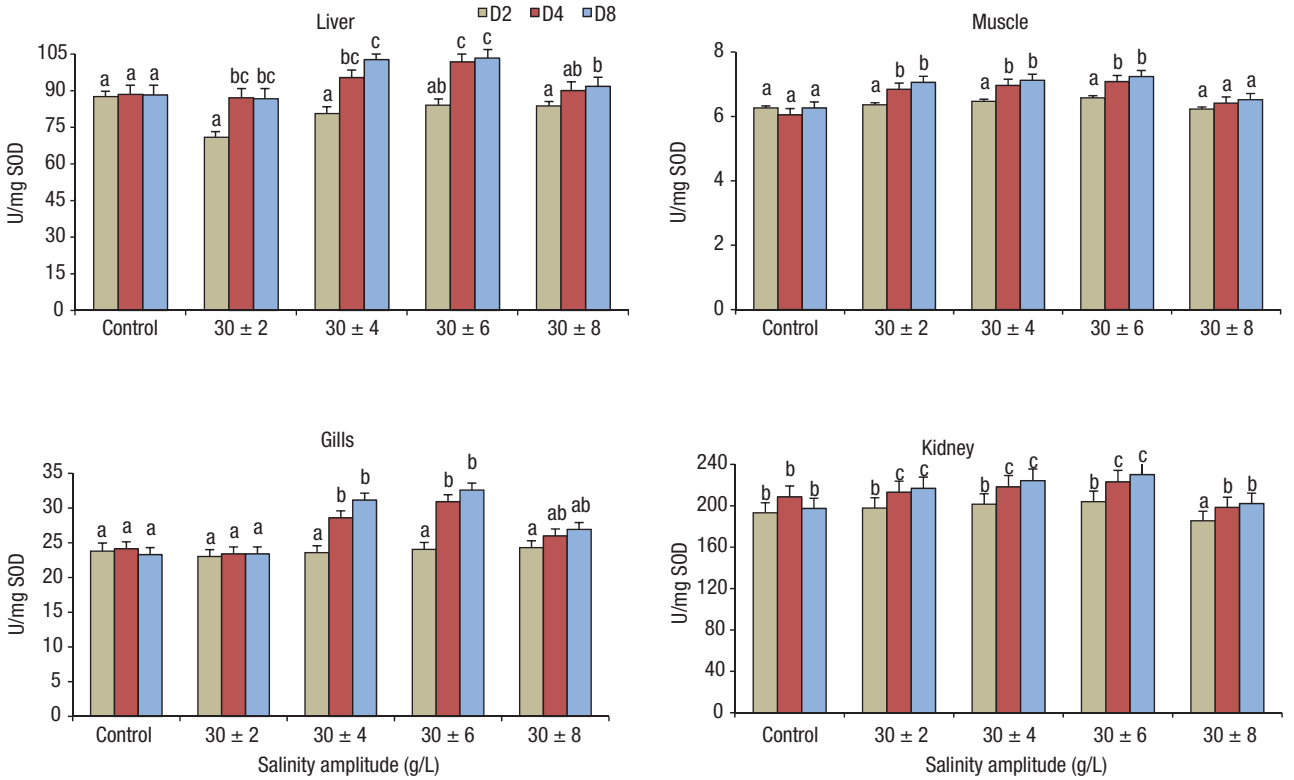

Figure 5. The effect of salinity fluctuations on SOD activities in different tissues of juvenile tongue sole. Different letters above the histogram bars indicate significant differences between groups (p<0.05). Error bars represent the standard error and D2, D4 and D6 are salinity frequencies.

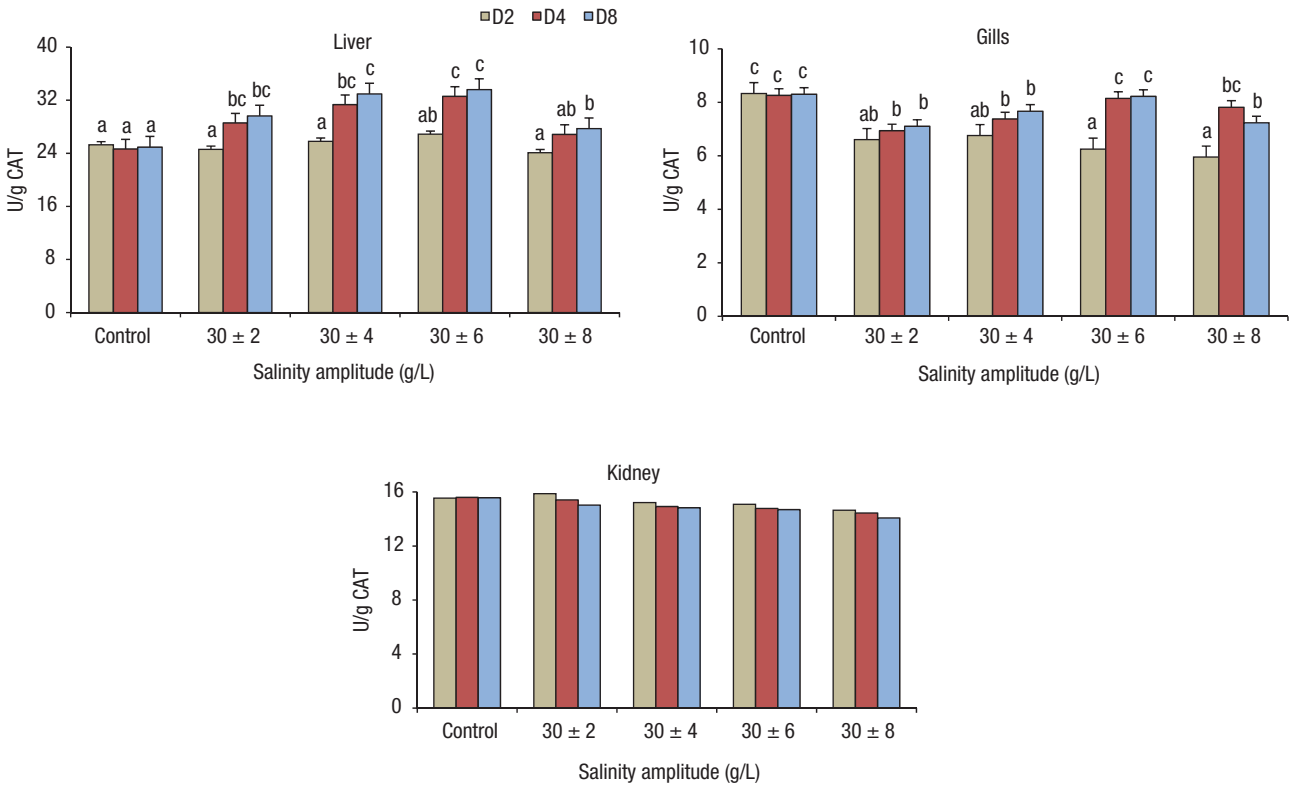

Figure 6. The effect of salinity fluctuations on CAT activities in different tissues of juvenile tongue sole. Different letters above the histogram bars indicate significant differences between groups (p<0.05). Error bars represent the standard error and D2, D4 and D6 are salinity frequencies.

activity is low or even absent. The hepatic CAT activity was significantly higher than in branchial and renal tissues, and the branchial tissue had the lowest CAT activity (p<0.05). The hepatic CAT activity tended to increase with increase in amplitude fluctuations except ± 8 g/L (Fig. 6A). The branchial CAT activity was significantly influenced in control (S30 ± 0), D4S30 ± 6 and D8S30 ± 6 compared to other treatments (p<0.05) (Fig. 6B).

Plasma cortisol

The plasma cortisol levels observed in all treatments ranged between 30.49 and 52.29 nmol/L. Cortisol levels were significantly affected in lower (S30 ± 2) and higher (S30 ± 8) salinity fluctuations. However, plasma cortisol levels remained low in moderate salinity fluctuations (S30 ± 4 and S30 ± 6) and control (30 gL) (p<0.05) (Fig. 7).

Discussion

The fluctuation of salinity often brings about many physiological stress responses, which disturbs the balance of serum hormone, energy metabolism and electrolyte in aquatic animals (Choi *et al.,* 2008). However, the health of animals could be evaluated by using sensitive immunomarkers. The ideal immunomarker indicates not only the health of the animals but also the degree of environmental stress on immune systems. Susceptibility to disease may increase if immunomarkers are below the normal standard (Wang & Chen, 2005). To evaluate the immunity status with respect to salinity fluctuation, the antioxidant responses were measured in the current study using key immune responsive enzyme activities

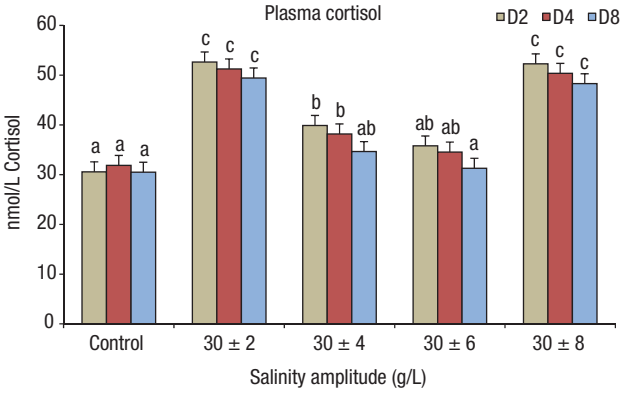

Figure 7. The effect of salinity fluctuations on plasma cortisol levels in different tissues of juvenile tongue sole. Different letters above the histogram bars indicate significant differences between groups (p<0.05). Error bars represent the standard error and D2, D4 and D6 are salinity frequencies.

including ACP, AKP from the hydrolytic system and SOD and CAT from the antioxidant system.

ACP is a phosphatase, a type of enzyme used to free the attached phosphate groups from other molecules during digestion. It is stored in lysosomes and functions when these molecules fuse with endosomes (Cajaraville *et al.,* 2000; Rajalakshmi & Mohandas, 2005). Meanwhile, AKP is a metalloenzyme, which catalyzes the non-specific hydrolysis of phosphate monoesters (Zhang *et al.,* 2004; Zang *et al.,* 2012). When exposed to a variety of environmental stressors, lysosomal enzymes ACP and AKP will participate in degradation of foreign proteins, carbohydrates and lipids (Ottaviani, 1984; Pipe *et al.,* 1993; Xue & Renault, 2000). In the present study, salinity fluctuations significantly affected the activities of hydrolases in tongue sole except renal and hepatic activities of ACP and AKP. In all tissues, ACP and AKP activities were found higher at moderate salinity fluctuations than the control and other fluctuating treatments, and renal ACP as well as AKP activities were found higher compared to other tissues. These mutual increase in both ACP and AKP activities at moderate fluctuating salinities not only suggest enhancement of the capacity of degradation and defense to foreign materials, but also improvement of metabolic intensity to provide more energy to maintain its homeostasis in tongue sole.

SOD as well as CAT plays rather important role in scavenging free radicals, particularly in their involvement against oxidization and phagocytosis resulting from body cell damage. It is believed that SOD takes initiative in the scavenging of reactive oxygen species, catalyzing the dismutation of O^{-2} into H_2O and H_2O_2, with the latter being further reduced into H_2O and O_2 through catalysis of CAT for detoxification (Zhang KF *et al.,* 2007; Zhang Z *et al.,* 2011). SOD plays an essential role to minimize the oxidative damage to host cells during immune defense, while CAT is considered as important and sensitive biomarker of oxidative stress as SOD, revealing biological effects on the redox status of the marine organisms (Regoli *et al.,* 2002a,b). In the present study, SOD activities had a significant effect due to salinity fluctuations in all tissues, except for hepatic tissue. The variation in branchial SOD activities proved that the salinity fluctuations had great impact on tongue sole at moderate as well as high fluctuating salinities compared to control and low fluctuating salinities, whereas the branchial CAT activities showed a contrasting trend. Further, the renal CAT activities showed no significant differences and the hepatic CAT activity showed a similar trend to SOD. This explains that the salinity fluctuations had a prominent role in the changes of SOD and CAT activities, in view of cellular antioxidants. Generally, a high antioxidant enzyme activity indicates that there is a large amount

of free radicals awaiting elimination (Andersen *et al.,* 1998; Ross *et al.,* 2001), so the rising activities of SOD and CAT in this study fully shows that the accumulation of free radicals has reached a remarkable level needing to be lowered, otherwise it will cause severe oxidant damage to the body cells (Winston & Di Giulio, 1991). Thus, the living organisms have evolved effective anti-oxidant self-defense mechanism in order to maintain their homeostasis and act against oxidative stress, so the rise in SOD and CAT activities could effectively minimize the damage, otherwise the body would suffer loss of immunity and eventually of its survival. Similarly, the study on amur sturgeon, *Acipenser schrenckii* showed that salinity could have affected the activity of SOD and CAT to some extent, but their activity recovered more or less with the elongation of domestication time, which might have some close relation with the adaptation to osmotic pressure in *A. schrenckii* (Zhao *et al.,* 2008). According to the present study, it is considered that fluctuations in salinity could activate SOD and CAT to defend the body against the damage caused by excessive amount of oxygen free radicals but their activity might be inhibited as the salinity drops below their tolerance range, which partly explains the fatality occurring in juvenile fish (Yin *et al.,* 2010).

In teleost fish, plasma cortisol level is normal when the range observed is around 20-102 nmol/L. In the present study, plasma cortisol levels were relatively low in all treatments except in those in intermediate salinity fluctuations (S30 ± 4 and S30 ± 6) and in control. In lower (S30 ± 2) and higher (S30 ± 8) salinity fluctuations, the higher production of plasma cortisol level is caused by an osmotic imbalance in the fish. The results of the present study shows that levels of plasma cortisol were high due to the fish being exposed to hypertonic and hypotonic environments, which is in agreement with the study carried out by Pickering & Pottinger (1995).

In summary, collectively, the immune responses significantly affected the enzyme activity of tongue sole. Effects of salinity fluctuations were tissue-specific and changed either with increase or decrease in amplitude and frequency of salinity. However, further investigation is required at farm conditions for better understanding of the physiological responses of the tongue sole or it could be for any other euryhaline teleosts.

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