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Short communication

Metabolite and gene expression responses in juvenile flounder *Paralichthys olivaceus* exposed to reduced salinities

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A B S T R A C T

Seawater salinity is one of the most important changeable environmental factors influencing the behavior, survival, growth and production of marine organisms. In this work, metabolite and gene expression profiles were used to elucidate the biological effects of reduced salinities in juvenile flounder *Paralichthys olivaceus*. Metabolic profiling indicated that both reduced salinities (23.3‰ and 15.6‰) enhanced proteolysis and disturbed osmotic regulation and energy metabolism in juvenile flounder *P. olivaceus*. Furthermore, the low salinity (15.6‰) enhanced anaerobic metabolism indicated by the elevated lactate in flounder tissue extracts. Gene expression profiles exhibited that reduced salinities could induce immune stress and oxidative stress and disturb energy metabolism in juvenile flounder *P. olivaceus*. In addition, reduced salinities might promote the growth and gonadal differentiation in juvenile flounder *P. olivaceus*.

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1. Introduction

The marine environmental factors, such as salinity, temperature, hypoxia, photoperiod and so on, may impact on the physiological and biological status of marine animals in a wide variety of ways [1]. Natural changes in seawater salinity occur frequently due to rainwater diluting seawater or mixing of estuarine waters. Evidences have indicated that salinity changes can effectively affect different processes in marine animals [1]. Li et al. reported the significant up-regulation of heat shock proteins induced by the low salinity, which suggested that low salinity influenced the innate immunity in pearl oysters *Pinctada martensii* [2]. In marine fishes, most studies focused on the effects in osmoregulation, immune system and energy metabolism induced by salinity changes [1,3,4]. For example, Cuesta et al. have found that salinity influences the humoral immune parameters of gilthead seabream (*Sparus aurata* L.) [5].

Olive flounder *Paralichthys olivaceus* is an economically important fish species in marine aquaculture industry in China. It widely distributes in open seas with high salinities and estuaries with low salinities, suffering dramatic salinity alterations [6]. The early life stages of *P. olivaceus*, larva and juvenile, can survive in a salinity with a range from 5‰ to 45‰ [6]. Previously, researchers mainly tested the physiological parameters to characterize the effects of salinity in *P. olivaceus* at early life stages. Tang et al. studied the effects of low salinity stress on the growth of juvenile fish *P. olivaceus* [7]. They demonstrated that the low salinity (5‰) could significantly decrease the growth rate of juvenile *P. olivaceus* stressed by this salinity for 1 week [7]. However, a medium salinity (16‰) may increase the growth rate and decrease the albinism rate of both larval and juvenile fish *P. olivaceus* [6]. Recently, the biochemical indices have been measured to interpret salinity-induced effects and mechanisms in juvenile *P. olivaceus* [8]. Basically, low salinity (10‰) may increase the activities of superoxide dismutase (SOD) and catalase (CAT) in juvenile *P. olivaceus*, which suggested that low salinity could induce oxidative stress in juvenile *P. olivaceus* [8]. In addition, low salinity (5‰) can influence the plasma osmolality in gills of juvenile *P. olivaceus* by decreasing the activity of Na^{+}-K^{+}-ATPase [8]. However, there is a lack of knowledge related to salinity-induced effects in *P. olivaceus* at molecular levels.

With the development of -omic techniques, including genomics,

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transcriptomics, proteomics and metabolomics, these top-down approaches can be used to explore the global profiles related to the biological alterations induced by environmental stressors in organisms [9,10]. Researchers can comprehensively profile one type of molecules such as genes, proteins and metabolites and their alterations to characterize the biological effects with high-throughput analysis. Basically, metabolomics focuses on the low molecular weight (<1000 Da) metabolites that are the end products of metabolism in biological samples [11]. This newly established -omic approach can directly detect the alterations in multiple metabolic pathways. Therefore, it may present a broader view into the stressor-induced effects in marine animals [10,12]. In this study, the biological responses induced by two reduced salinities (75% and 50% normal salinity) were investigated using metabolic and gene expression profiling to elucidate the biological effects of reduced salinities in juvenile *P. olivaceus* after exposure for 48 h.

2. Materials and methods

2.1. Experimental animals and conditions

After breeding for forty days, the juvenile flounder *P. olivaceus* were purchased from a fish farm in Yantai, China. These fish were acclimatized in aerated normal seawater with a salinity of 31.3‰ for 1 week before reduced salinity exposure. After acclimatization, the healthy fish with similar sizes (length 4.30 ± 0.17 cm, body weight 0.62 ± 0.06 g) were randomly divided into six tanks each containing 10 individual fish in 20 L of seawater. In the reduced-salinity exposure experiment, there were three groups, including control (normal salinity, 31.3‰), medium salinity (23.5‰, prepared from 75% normal seawater and 25% de-ionized water) and low salinity (15.6‰, prepared from 50% normal seawater and 50% de-ionized water) treatments. There were two replicate tank containers for each of the treatments. The seawater in tank containers was exchanged daily. During the acclimatization and exposure periods, fish were kept at 25 °C under a photoperiod of L12:D12 and the mortality was 0. Ten individual fish in each group were randomly sampled after exposure to reduced salinities for 48 h. These fish samples were flash frozen in liquid nitrogen and ground into powder. Then the powder of each sample was divided into two tubes and stored at −80 °C before metabolite and RNA extractions.

2.2. Metabolite extraction

Polar metabolites in ground tissues of juvenile *P. olivaceus* ($n = 10$ for each treatment) were extracted by a modified extraction protocol as described previously [12]. Briefly, the tissue (ca. 100 mg wet weight) was homogenized and extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts were then re-suspended in 600 μL phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4, including 0.5 mM TSP, pH 7.0) in D_2O. The mixture was vortexed and then centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.3. ^1H NMR spectroscopy

Metabolite extracts of juvenile *P. olivaceus* were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C), as described previously [12]. All ^1H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

2.4. Spectral pre-processing and multivariate data analysis

All one dimensional ^1H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (V7.0, the Mathworks Inc., Natwick, USA) [12]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with a transformation parameter $\lambda = 2.0 \times 10^{-7}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [13]. The NMR spectral data were mean-centered before principal components analysis (PCA).

The supervised multivariate data analysis methods, partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (OPLS-DA), were then sequentially used to uncover and extract the statistically significant

Table 1
The primers of selected genes for quantification of expressions by qRT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
Immunity:			
HSP 70	GGACACGATTACAGCAAGGAACTC	ACTGGTGCTCATACTCGTCCTTCTCT	DQ662230
IFN γ	TCGCACCAGGCAGGACTTTG	AGACACAGGGACAGACAGACCACT	AB435093.1
IL 6	AACCCCAGCACTTTCCACAGG	GTTTGGGCATCTCTCTTTTACGAAT	DQ267937
Anti-oxidant:			
SOD	GACCTCACTTCAATCCCCACG	TTTCCAGCAGTCACATTCCCC	EF681883.1
GST	TCCTGGCATCCTCTCAGACTTTC	AAACTTGCTGAGGGCGGGG	EU182592.1
CAT	CGTGGGCAAAATGGTTCTCAA	ATCTTGTCGGGGCTCGCTTC	GQ229479.1
Growth and Development:			
IGFBP 4	GCATCAAATACCCGCACACAT	TTCTGATCCACACACCAGCACT	KF917548.1
IGF 1	CGCATCTCATCCTCTTTCTCCC	TGAGAGGGTGTGGCTACAGGAGA	AF016922.1
SOX 9	CCTTTCTACCAGGTCAATCCCA	GCTGGAGGGGAGGTCTGTCTT	FJ231468.1
Energy metabolism:			
NADH dehydrogenase	CTACCTACGCCCTTGCTGACATT	CTAACTGCCTATTCAGCCCACTCT	GQ229479.1
LDH	CTCCAAGGTGGTAATGGTGACTG	ACTTGACAATGTTGGGGATGATG	CX725614.1
ATP synthase	GCAGAGAATGCCTCCCTCCTTG	CTCCAGCACCGATGAACTTAGC	AY316304.1
Reference:			
β-actin	GGAAATCGTGCGTGACATTAAG	CCTCTGGACAACGGAACCTCT	ADP88939.1

Abbreviations: CAT, catalase; GST, glutathione S-transferase; HSP 70, heat shock protein 70; IFN γ, interferon γ; IGF 1, insulin-like growth factor 1; IGFBP 4, insulin-like growth factor binding protein 4; IL 6, interleukin 6; LDH, lactate dehydrogenase; SOD, superoxide dismutase and SOX 9, Sry-related high mobility group box 9.

metabolite variations between control and reduced salinity-treated samples. The results were visualized in terms of scores plots to show the classifications and corresponding loadings plots to show the NMR spectral variables contributing to the classifications. The model coefficients were calculated from the coefficients incorporating the weight of the variables in order to enhance interpretability of the model. Then metabolic differences responsible for the classifications between control and reduced salinity-treated groups could be detected in the coefficient-coded loadings plots. The coefficient plots were generated by using MATLAB with an in-house developed program and were color-coded with absolute value of coefficients (r). A hot color (i.e., red) corresponds to the metabolites with highly positive/negative significances in discriminating between groups, while a cool color (i. e. blue) corresponds to no significance. The correlation coefficient was determined according to the test for the significance of the Pearson's product-moment correlation coefficient. The validation of each model was conducted using 10-fold cross validation and the cross-validation parameter Q^2 was calculated, and an additional validation method, permutation test (permutation number = 200), was also conducted in order to evaluate the validity of the PLS-DA models. The R^2 in the permutated plot described how well the data fit the derived model, whereas Q^2 describes the predictive ability of the derived model and provides a measure of the model quality. If the maximum value of Q^2 max from the permutation test was smaller than or equal to the Q^2 of the real model, the model was regarded as a predictable model. Similarly, the R^2 value and difference between the R^2 and Q^2 were used to evaluate the possibility of over-fitted models [14]. Metabolites were assigned following the tabulated chemical shifts and by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada).

2.5. RNA extraction and quantitation of gene expressions

In this study, twelve genes related to diverse functions were selected for the quantification of mRNA expression in juvenile flounder *P. olivaceus* samples (Table 1). These genes were basically divided into four classes, including immunity, anti-oxidant, growth and development, and energy metabolism. As a member of heat shock protein (HSP) family, HSP 70 plays an important role in the immune response by functioning as a chaperone in immune system [15]. Both interferon γ (IFN γ) and interleukin 6 (IL 6) are involved in immune and inflammatory responses in fishes [16]. In fishes, the insulin-like growth factor (IGF) system plays a crucial role in stimulating cell growth, differentiation, metabolism and reproduction, while insulin-like growth factor binding proteins (IGFBPs) are present in various biological fluids, regulating the availability of IGFs to the IGF receptors [17]. Sry-related high mobility group box 9 (SOX 9) is one of the most important transcription factors in gonadal differentiation in vertebrates [18]. As known anti-oxidative enzymes, superoxide dismutase (SOD, EC 1.15.1.1), glutathione S-transferases (GST, EC 2.5.1.18) and catalase (CAT, EC 1.11.1.6) are usually used as scavengers for reactive oxidative species (ROS) to reduce oxidative stress in organisms [19]. NADH dehydrogenase, lactate dehydrogenase (LDH) and ATP synthase are three known enzymes involved in energy metabolism. In this work, the expression levels of these representative genes were measured by qRT-PCR to test the effects of reduced salinities in juvenile flounder *P. olivaceus*.

Total RNA from juvenile *P. olivaceus* was extracted following the manufacturer's directions (Invitrogen, LifeTechnologies, Carlsbad, CA, USA), and the first-strand cDNA was synthesized according to M-MLV RT Usage information (Promega, Madison, WI, USA). Gene-specific primers employed for quantification of selected mRNA expression were listed in Table 1. The fluorescent real-time quantitative PCR amplifications were carried out in triplicate in a total volume of 50 μL containing 25 μL of 2 × SYBR Premix Ex Taq™ (TaKaRa), 1.0 μL of 50 × ROX Reference DYE II, 12.0 μL of DEPC-treated H_2O, 1.0 μL of each primer, 10.0 μL of 1:20 diluted cDNA. The fluorescent real-time quantitative PCR program was as following: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 45 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the ABI

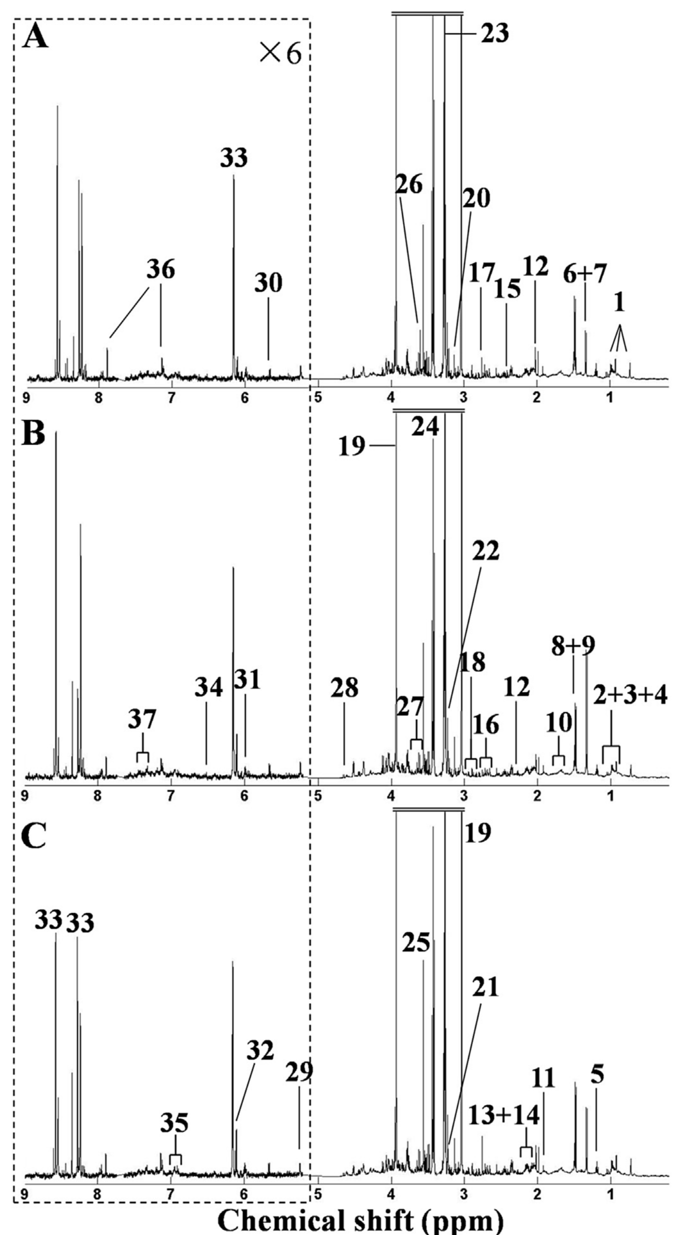

Fig. 1. Typical ^{1}H NMR spectra of tissue extracts of juvenile flounder *P. olivaceus* from the control (normal salinity, A), 75% normal salinity (23.5‰, B) and 50% normal salinity treatments (15.6‰, C). **Keys**: (1) Cholate, (2) Isoleucine, (3) Leucine, (4) Valine, (5) Ethanol, (6) Lactate, (7) Threonine, (8) Alanine, (9) Unknown 1 (1.48 ppm), (10). Arginine, (11) Acetate, (12) *N*-Acetylglutamate, (13) Glutamate, (14) Glutamine, (15) Succinate, (16) Aspartate, (17) Dimethylamine, (18) Asparagine, (19) Creatine phosphate, (20) Malonate, (21) Choline, (22) Phosphocholine, (23) Trimethylamine *N*-oxide, (24) Taurine, (25) Glycine, (26) Sarcosine, (27) Myo-inositol, (28) β-Glucose, (29) α-Glucose, (30) Unknown 2 (5.65 ppm), (31) Unknown 2 (5.98 ppm) (32) AMP, (33) ATP, (34) Fumarate, (35) Tyrosine, (36) Histidine and (37) Phenylalanine.

7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative expression level of the genes [20]. One way analysis of variance (ANOVA) with Tukey's test was performed on gene expression levels between control and reduced salinity-treated groups. A P value less than 0.05 was considered statistically significant. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.

3. Results and discussion

Fig. 1 presents the typical ^{1}H NMR spectra of tissue extracts of juvenile flounder *P. olivaceus* from the control (normal salinity, 31.3‰), medium (75% normal salinity, 23.5‰) and low (50% normal salinity, 15.6‰). Several metabolite classes were identified, including amino acids (branched chain amino acids: valine, leucine and isoleucine, threonine, alanine, aspartate, glutamate, glutamine, phenylalanine, etc.), energy storage compounds (AMP, ATP and glucose), intermediates in Krebs cycle (succinate and fumarate), organic acids (cholate and molonate) and osmolytes (dimethylamine, taurine and trimethylamine *N*-oxide). Visibly, the NMR spectra (Fig. 1) are dominated by an amine oxide, trimethylamine *N*-oxide (3.27 ppm), which is a common metabolite used as an osmolyte in marine fishes [21]. Principal components analysis (PCA) was conducted on the NMR spectral data from control, medium salinity (23.5‰) and low salinity (15.6‰) treatments. PCA resulted in significant ($P < 0.05$) separations between control and reduced salinity-treated groups (data not shown), which demonstrated the significant metabolic responses induced by reduced salinities in juvenile flounder *P. olivaceus*. However, no significant ($P > 0.05$) separation between the medium salinity- and low salinity-treated groups was found, suggesting that these two reduced salinities induced similar biological effects in juvenile flounder *P. olivaceus*.

Further OPLS-DA was performed on the NMR spectral data from control and each reduced salinity-treated group (Fig. 2). Obviously, OPLS-DA resulted in clear classifications between control and reduced salinity-treated groups (Fig. 2A and D), respectively, with reliable Q^2 values (>0.5). From the loading plot (Fig. 2B), the medium induced significant ($P < 0.05$) increases in arginine, glutamate, dimethylamine, glycine, ATP and an unknown metabolite (3.48 ppm), and decreases in phosphocholine, taurine and sarcosine. In the low salinity-treated flounder samples, these metabolites, including arginine, glutamate, dimethylamine, glycine, ATP, phosphocholine, taurine and sarcosine were similarly altered (Fig. 2D), which confirmed these two reduced salinities induced similar biological effects in juvenile flounder *P. olivaceus*. However, two differentially altered metabolites were observed in the low salinity-treated flounder samples. In particular, lactate was

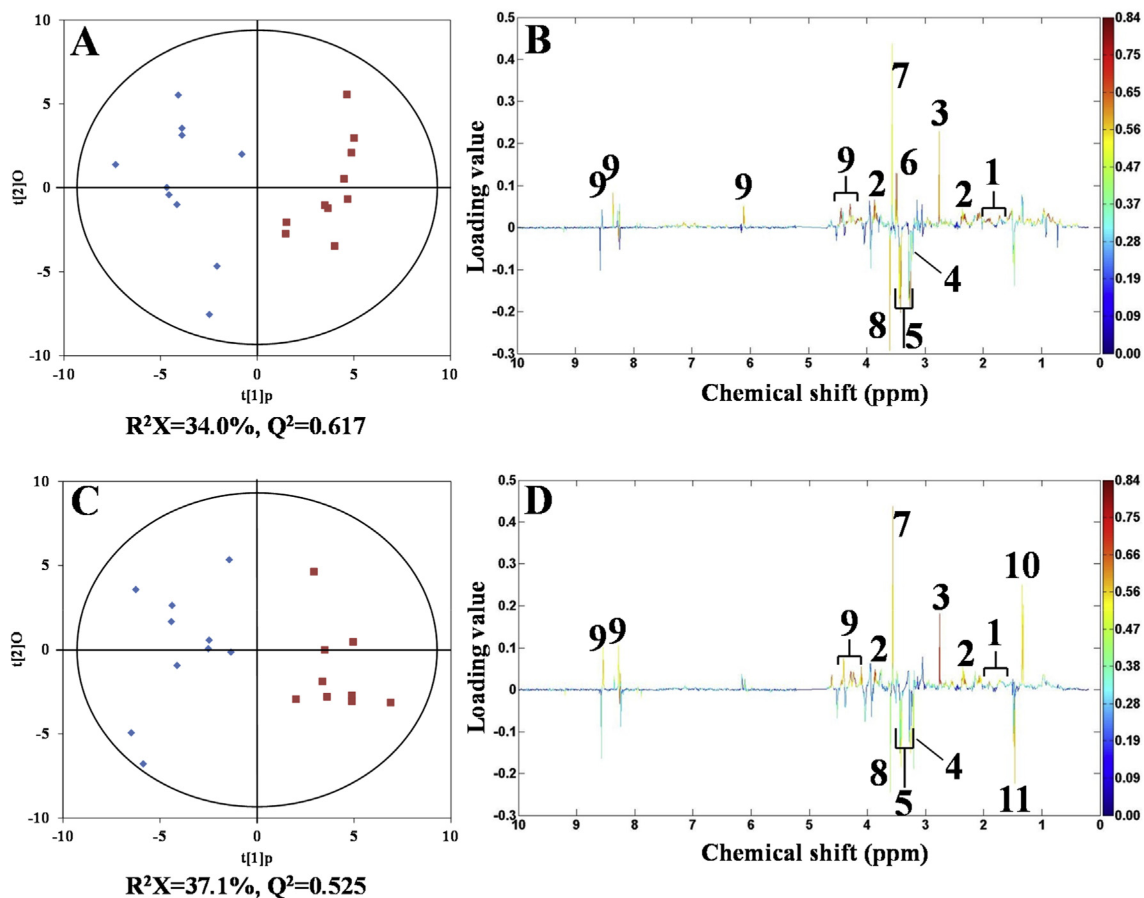

Fig. 2. OPLS-DA scores derived from ^{1}H NMR spectra of the tissue extracts from juvenile flounder *P. olivaceus* from control (normal salinity, 31.3‰) (◆) and reduced salinity treatments (■), (A) control Vs. 75% normal salinity (23.5‰) treatment and (C) control Vs. 50% normal salinity (15.6‰) treatment and corresponding coefficient plots (B) and (D). The color map shows the significance of metabolite variations between the two classes (control and reduced salinity treatments). Peaks in the positive direction indicate metabolites that are more abundant in the reduced salinity treatments. Consequently, metabolites that are more abundant in control group are presented as peaks in the negative direction. **Keys:** (1) Arginine, (2) Glutamate, (3) Dimethylamine, (4) Phosphocholine, (5) Taurine, (6) Unknown 1 (3.48 ppm), (7) Glycine, (8) Sarcosine, (9) ATP, (10) Lactate and (11) Unknown 2 (1.48 ppm).

elevated, whereas an unknown metabolite (1.48 ppm) was depleted.

In both reduced salinity treatments, several amino acids including arginine, glutamate and glycine were significantly elevated, which indicated the disruption of amino acid metabolism induced by reduced salinities (23.5‰ and 15.6‰). This is consistent with the enhanced proteolysis that is a known adverse effect of some toxicants, such as diazinon, in marine fishes [22]. Sarcosine is an intermediate and byproduct in glycine synthesis and degradation. As an amino acid derivative, it can be metabolized to glycine by the enzyme, sarcosine dehydrogenase, and is naturally found in muscles. Interestingly, the elevated glycine and depleted sarcosine were consistently observed in both reduced salinity treatments. It seemed that reduced seawater salinities might promote the conversion of sarcosine to glycine in juvenile flounder *P. olivaceus*. Both dimethylamine and taurine are known organic osmolytes in marine organisms, regulating their intracellular osmolarity with their environment [9,12]. The reduced salinities could induce hypo-osmotic stress and therefore disturbed the osmolarity between juvenile flounder *P. olivaceus* and the seawater, resulting in decreased taurine. The increased concentration of dimethylamine might be used to compensate the decreased osmolyte, taurine. In

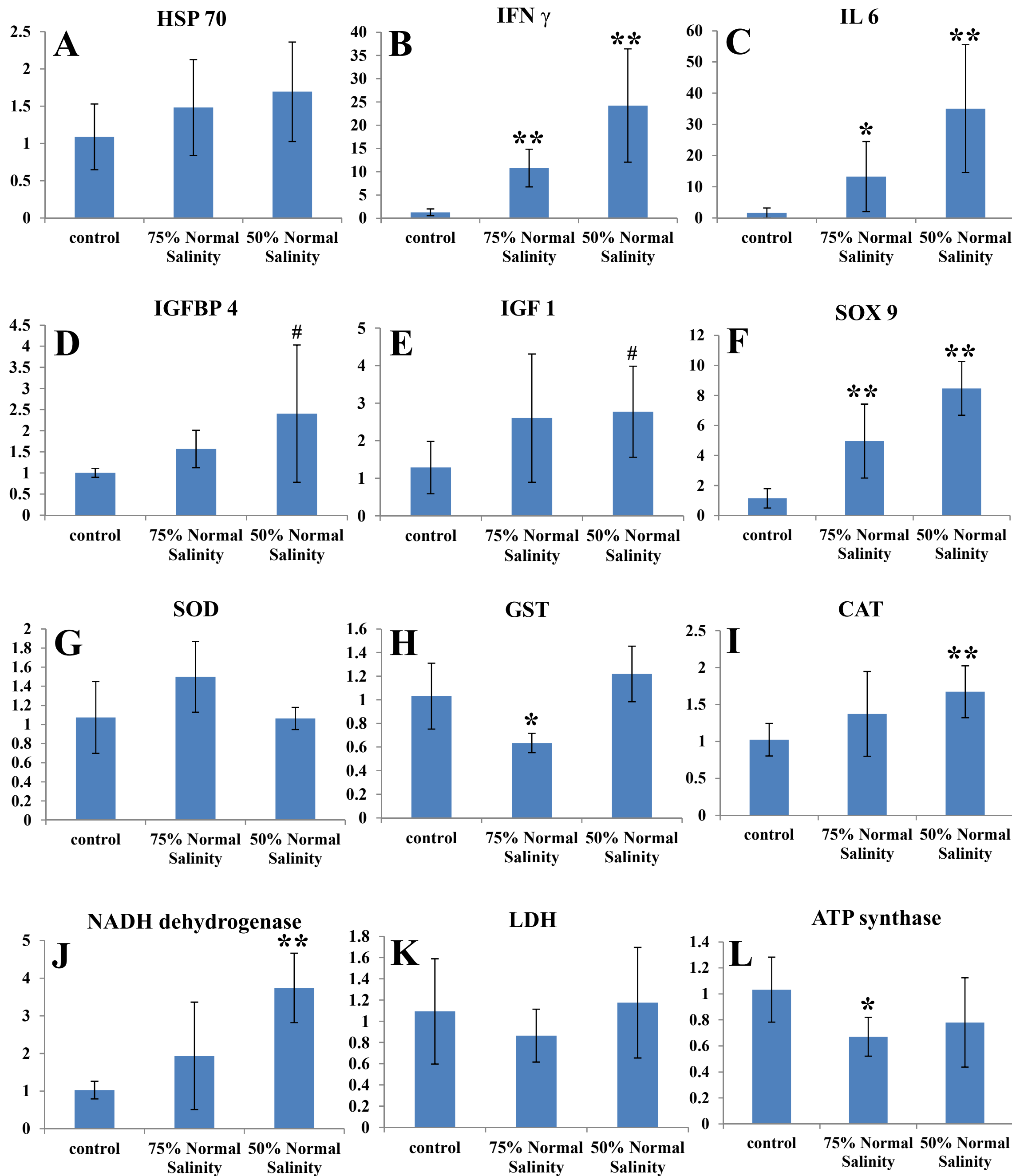

Fig. 3. Expression levels of HSP 70, IFN γ, IL 6, IGFBP 4, IGF 1, SOX 9, SOD, GST, CAT, NADH dehydrogenase, LDH and ATP synthase mRNAs relative to β-actin by qPCR in whole soft tissues of *P. olivaceus* after exposure to reduced salinities for 48 h. Statistical significances ($P < 0.05$, * and $P < 0.01$, **) between control and reduced salinity treatments were determined by one-way ANOVA. The symbol '#' means that the P value determined by one-way ANOVA approaches 0.05. **Abbreviations:** CAT, catalase; GST, glutathione S-transferase; HSP 70, heat shock protein 70; IFN γ, interferon γ; IGF 1, insulin-like growth factor 1; IGFBP 4, insulin-like growth factor binding protein 4; IL 6, interleukin 6; LDH, lactate dehydrogenase; SOD, superoxide dismutase and SOX 9, Sry-related high mobility group box 9.

one known metabolic pathway involved in energy metabolism, phosphocholine is synthesized in the reaction catalyzed by choline kinase, converting ATP and choline into phosphocholine and ADP. In the reduced salinity-treated juvenile flounder *P. olivaceus* samples, phosphocholine and ATP were significantly decreased and increased, respectively, which implied the inhibited conversion of ATP and choline to phosphocholine and ADP. This probably meant that the juvenile flounder *P. olivaceus* reserve a high concentration of ATP to cope with the hypoosmotic stress induced by these two reduced salinities. In the low salinity-treated juvenile flounder *P. olivaceus* samples, the elevated lactate indicated the enhanced anaerobic metabolism caused by the low salinity.

After exposure to reduced salinities for 48 h, both *IFN* γ and *IL 6* were significantly ($P < 0.05$) up-regulated (Fig. 3B and C). In a previous work, the *Edwardsiella tarda* infection similarly up-regulated the mRNA expression levels of *IFN* γ and *IL 1*β in flounder *P. olivaceus*. Therefore, the up-regulation of *IFN* γ and *IL 6* suggested that reduced salinities might induce immune stress in reduced salinity-treated juvenile flounder *P. olivaceus* samples. This meant that the low salinity induced more severe immune stress in juvenile *P. olivaceus*. In the low salinity (15.6‰) treatment, both *IGF 1* and *IGFBP 4* were up-regulated with *P* values approaching 0.05 (Fig. 3D and E), which implied that the low salinity (15.6‰) could stimulate the growth of juvenile flounder *P. olivaceus*. Zhang et al. reported the highest growth rate of juvenile flounder *P. olivaceus* at a seawater salinity ~19‰ that was close to the low salinity (15.6‰) used in this study [23]. In another work, Wang found that the salinity at 16‰ increased the growth rate of juvenile fish *P. olivaceus* as well [6]. The significant ($P < 0.01$) up-regulation of *SOX 9* (Fig. 3F) exhibited the promoted gonadal differentiation in juvenile flounder *P. olivaceus* exposed to reduced salinities. Among the genes of anti-oxidant enzymes, *GST* was significantly down-regulated in the medium salinity (23.3‰) treatment (Fig. 3H). In a previous study, the GST activity was also decreased in clam *Ruditapes philippinarum* under this hyposaline condition that could induce oxidative stress in clams and resulted in redox balance alterations [19]. In the low salinity (15.6‰) treatment, *CAT* was significantly ($P < 0.05$) up-regulated (Fig. 3I), which confirmed the oxidative stress caused by the low salinity in juvenile flounder *P. olivaceus*. This finding was consistent with the tested CAT activity in juvenile flounder *P. olivaceus* under a similar hyposaline condition reported by Guo et al. [8]. As a flavoprotein, NADH dehydrogenase contains iron-sulfur centers and is involved in both energy metabolism and production of reactive oxygen species in mitochondria [24]. The low salinity (15.6‰) induced significant up-regulation of *NADH dehydrogenase* (Fig. 3J), which indicated the disturbed energy metabolism and oxidative stress in juvenile flounder *P. olivaceus* induced by the low salinity. The expression levels of *ATP synthase* were down-regulated in both reduced salinity-treated groups, with or without statistical significance (Fig. 3L). Interestingly, the amounts of ATP in juvenile flounder *P. olivaceus* were increased in both reduced salinity treatments. The disparity between ATP synthase mRNA and ATP, was not surprising, since mRNA expression means the tendency of the corresponding encoded protein which does not always happen due to the posttranscriptional and post-translational modifications [25]. ATP synthase is an important enzyme involved in energy metabolism through the synthesis of ATP. As the end product of ATP synthase, therefore, ATP concentrations did not correlate well with the expression levels of *ATP synthase*.

In summary, the biological effects of reduced salinities were characterized by metabolite and gene expression responses in juvenile flounder *Paralichthys olivaceus*. Basically, metabolic responses indicated that both reduced salinities (23.3‰ and 15.6‰) enhanced proteolysis and disturbed osmotic regulation and energy metabolism in juvenile flounder *P. olivaceus*. Especially, the low salinity (15.6‰) enhanced anaerobic metabolism as well, marked by the elevated lactate in juvenile flounder *P. olivaceus*. Based on the expression profiles of selected genes, reduced salinities could induce immune stress and oxidative stress and disturb energy metabolism in juvenile flounder *P. olivaceus*. In addition, reduced salinities could promote the growth and gonadal differentiation in juvenile flounder *P. olivaceus*. Overall, this work demonstrates that metabolite and gene expression profiles can be used to elucidate the adverse effects of reduced salinities in juvenile flounder *P. olivaceus*.

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