Aquaculture 527 (2020) 735451

Contents lists available at ScienceDirect

Aquaculture

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# *myo*-inositol facilitates salinity tolerance by modulating multiple physiological functions in the turbot *Scophthalmus maximus*

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ARTICLE INFO

*Keywords:*
*Scophthalmus maximus*
*myo*-inositol concentration
Survival time
Physiological function
Transcriptome analysis
Steroids
Salinity stress

ABSTRACT

As a B-complex vitamin that is a compatible organic osmolyte and a component of phosphatidylinositol in biological cell membranes, *myo*-inositol plays vital roles in responses to salinity stress. A significant increase in the *myo*-inositol content of the gill of turbot exposed to salinity stress was detected using the enzyme-linked immunosorbent assay. *myo*-inositol supplementation provided by immersion or inclusion in the diet significantly extended survival under salinity stress, with the exception of immersion at salinity 0. Transcriptome data indicated that *myo*-inositol increased physiological capacities related to the steroid biosynthetic process, steroid metabolic process, circadian rhythm, tryptophan metabolism, metabolism of xenobiotics by cytochrome P450, oxidoreductase activity, iron ion binding, and heme binding in turbot. Furthermore, the qPCR results showed that *myo*-inositol strengthened osmotic regulation, as represented by the expression of ion-channel genes of turbot under salinity stress by stimulating the activities of steroid reductase and antioxidase, modulating immune function, and inhibiting the cell cycle and energy metabolism. GO and KEGG enrichment analyses of differentially expressed genes, PPI analysis, and qPCR data showed that signalling pathways associated with steroids including the steroid biosynthetic process, steroid metabolic process, and steroid hormone biosynthesis, mediated by *myo*-inositol occupy a central place in osmoregulation in turbot.

## 1. Introduction

Euryhalinity in fish is modulated by diverse compatible organic osmolytes, such as *myo*-inositol. *myo*-inositol is synthesized by various animal tissues and commonly exists as a component of phosphatidylinositol in biological cell membranes of animals, where it acts on the reconciling of cellular responses to external stimuli (Mai et al., 2001). Therefore, *myo*-inositol is not only a growth factor in animals and microorganisms (Majumder and Biswas, 2006) but also mediates many physiological functions involving the regulation of hormonal signalling, protein stabilization, osmoregulation, and nerve transmission (Garcia-Perez and Burg, 1991; Di Paolo and De Camilli, 2006; Falkenburger et al., 2010; Goncalves et al., 2012). The effects of *myo*-inositol on cell proliferation, differentiation, oxidative status, antioxidant capacity, immunity, growth performance and structural integrity in teleost fish have been studied in detail (Shiau and Su, 2005; Lee et al., 2008; Jiang et al., 2011, 2013, 2015; Li et al., 2017).

*Myo*-inositol is a major intracellular osmolyte that can be accumulated to protect cells from a variety of stresses, including fluctuations in the osmolality of the survival environment (Michell, 2008). When osmotic stress persists for a long period of time, osmolytes that are more compatible than amino acids are required. During prolonged exposure of cells to hypertonicity, cell survival depends on the synthesis either of a compatible osmolyte or of intracellular accumulation of betaine, taurine and *myo*-inositol (Maurice et al., 1997). Kalujnaia et al. (2010) showed that in freshwater acclimated fish, *myo*-inositol levels were highest in osmoregulatory organs (gill and kidney), with very low levels in other fish tissues. The increases in *myo*-inositol concentrations resulting from increases in the intracellular production of the osmolyte could be responsible for the osmotic protection. Sakaguchi et al. (1993) reported that the chondrocytes of eel gill cartilage may function to produce and release inositol into the bronchial circulation, allowing the epithelial cells to take up the osmolyte via active membrane transport systems such as the sodium-dependent *myo*-inositol transporter. Although the effects of *myo*-inositol and its functions and production in salinity-stressed fish have been investigated, previous studies have

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https://doi.org/10.1016/j.aquaculture.2020.735451
Received 15 April 2019; Received in revised form 2 April 2020; Accepted 6 May 2020
Available online 16 May 2020
0044-8486/ 

focused on physiology, biochemistry, and individual gene action (Majumder and Biswas, 2006; Kalujnaia et al., 2010; Jiang et al., 2011). Information about the molecular regulatory mechanism of *myo*-inositol at the transcriptome level is lacking.

As a euryhaline flounder, turbot *Scophthalmus maximus* has the relatively remarkable ability to adapt to opposing osmotic challenges, which implies the regulation of a wide variety of biological functions (Fouchs et al., 2009). Several observations and studies have been conducted to explore the osmoregulatory mechanism of this species. The effects of salinity changes on seawater-adapted juvenile turbot were studied by examining their plasma osmolarity and ion concentrations, oxygen consumption, gill $Na^+$-$K^+$-ATPase activity, and growth parameters (Gaumet et al., 1995; Imsland et al., 2003). A genome scan for candidate genes induced by an abrupt change in salinity conditions was performed to identify potential adaptive variation in turbot (Vilas et al., 2015). Recently, a transcriptomic analysis was conducted to uncover putative osmoregulatory mechanisms in the kidney of *S. maximus* exposed to hyposaline seawater (Cui et al., 2019). Although many studies have examined osmotic regulation in turbot, few have focused on its regulation by anabolites, especially *myo*-inositol.

The goals of this study were to determine the distribution patterns of *myo*-inositol in turbot *S. maximus* under salinity stress from the physiological viewpoint, analyse the influence of *myo*-inositol on turbot survival time, and explore the effect of *myo*-inositol on the transcriptome. The accuracy of the conclusions was verified by qPCR analysis of functional genes related to steroid reductase and antioxidase, modulation of immune function, inhibition of the cell cycle, energy metabolism, and ion transport, which have been determined to affect the fish via *myo*-inositol. The results of this study also provide theoretical support for studies of the osmoregulatory mechanism in turbot and will be used to expand the ecological niche of this species and provide practical and technical support for the promotion of turbot culture in abnormal salinity areas.

## 2. Materials and methods

### 2.1. Ethics statement

All experimental treatments for artificially cultivated fish were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (Bethesda, MD, USA). The study protocol follows the recommendations of the Experimental Animal Ethics Committee, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, China.

### 2.2. Salinity stress experiment

Healthy juvenile turbot (total length 19.94 ± 1.27 cm) were obtained from Yantai Tianyuan Aquaculture Co., Ltd. (Shandong, China). In this experiment, the fish were exposed to five salinity treatments (5‰, 10‰, 30‰, 40‰, 50‰) with three replicates for each treatment (Zhang et al., 2011). The fish treated with 30‰ were used as the control. The 300 fish were stocked in two 250 L circular fiberglass tanks supplied with seawater for 2 weeks to adapt them to the experimental facilities and conditions (photoperiod, 14 h light: 10 h dark; temperature, 14.0 ± 1.0 °C, pH 8.1). At the end of the acclimation period, 300 healthy fish were abruptly transferred to 15 circular fiberglass tanks (250 L) at a density of 20 fish per tank. The number of fish used per tank was determined according to the experimental needs. The salinity grades of 5‰ and 10‰ were prepared by mixing aerated freshwater with seawater, and hypersaline seawater (40‰, 50‰) was prepared by melting seawater crystals (Blue Treasure, Qingdao Sea-Salt Aquarium Technology Co., Ltd., Shandong, China) in the seawater. Water was aerated using an air pump to ensure full oxygenation and exchanged every 12 h. The turbot used for analysis were anaesthetized with MS222 (Maya Reagent, Zhejiang, China) at a concentration of 100 ppm. Kidney and gill tissues and arterial blood ($n$ = 3) were collected from turbot that had been acclimated in different seawater for 5 min, 1 h, 12 h, and 24 h (9 individuals per group at each time point). The fresh tissues were frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

The concentration of *myo*-inositol in the kidney, gill, and plasma of experimental fish, was measured using the Fish IS ELISA Kit following the manufacturer's protocol (JL46316-96 T, Jianglaibio Co. Ltd., Shanghai, China) (Ma et al., 2020). The reliability and stability of the kit were validated using a serial dilution curve (Fig. S1A) and high-temperature (37 °C) destructive experiment (Fig. S1B), respectively. The supernatant (plasma) was obtained by centrifuging the blood at 2–8 °C for 15 min (1000 × g) within 30 min after collection; EDTA was used as the anticoagulant.

### 2.4. Experimental diets and challenge trial

In this part of the study, we created experimental diets and conducted a challenge trial, for which healthy juvenile turbot (total length 19.94 ± 1.27 cm) were obtained from Yantai Tianyuan Aquaculture Co., Ltd. Individuals from the same batch of fish in Section 2.2 were maintained for 2 weeks in 250-L tanks of seawater before the experiment to acclimate them to the experimental facilities and conditions (photoperiod, 14 h light: 10 h dark; temperature, 14.0 ± 1.0 °C, pH 8.1). Water was flowing and aerated using a pipette to ensure full oxygenation.

The *myo*-inositol concentration of the basal diet used in this study was 617 mg MI $kg^{-1}$ diet, which was the optimum level of dietary *myo*-inositol for juvenile olive flounder (Lee et al., 2008). The other *myo*-inositol concentrations were prepared as described by Waagbø and Sandnes (1998). *myo*-inositol (Solarbio, Beijing, China) was added to the basal diet to provide graded concentrations of 600, 900, and 1200 mg MI $kg^{-1}$. A diet containing no *myo*-inositol was used as the control. The experimental diets and preparation and storage of diets were the same as described in a previous study (Shiau and Su, 2005). The experiment was conducted using six replicates (30 fish per replicate) for each dietary treatment. The fish were fed a commercial diet containing *myo*-inositol twice a day (8:00 and 18:00 h) for 2 weeks at a feeding rate of 1% body weight during the feeding trial, after which each dietary treatment was acutely transferred to lethal salinity of 70‰ (three replicates) and 0‰ (three replicates), respectively. The survival time of the fish was recorded for each treatment. The lethal salinities were established based on a pre-test.

We conducted an immersion experiment in which *myo*-inositol was dispersed to a final concentration of 10 ppm and 65 ppm both in hypersalinity seawater (70‰) and freshwater (0‰). Hypersalinity seawater and freshwater containing no *myo*-inositol were used as the controls, respectively. The fish were randomly divided into18 tanks (triplicate groups per treatment) at a density of 30 fish per tank. The survival time of the fish was recorded for each treatment.

### 2.5. Illumina deep sequencing

Transcriptome profiling of turbot fed with different concentrations of *myo*-inositol in seawater was performed to decipher the mechanisms by which *myo*-inositol modulates physiological functions. We sampled gill tissue from turbot that were fed the commercial diet containing different concentrations of *myo*-inositol (600, 1200 mg MI $kg^{-1}$) and the control group. Total RNA was extracted from gill tissue ($n$ = 3 per treatment) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA), and its integrity was confirmed by 1% agarose gel electrophoresis. RNA from individual fish in each

group was used to construct the cDNA library. The nine cDNA libraries were constructed and subjected to paired-end sequencing on an Illumina HiSeq 4000 platform at Majorbio Medicine Technology Co., Ltd. (Shanghai, China). Reference genomes were downloaded directly from the turbot genome database (http://denovo.cnag.cat/genomes/turbot/). Differential expression analysis of the three conditions/groups (three biological replicates per condition) was performed using the DESeq2 package. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was implemented in the GOseq R package, in which gene length bias was corrected. We used KOBAS software to test the statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Protein-protein interaction (PPI) networks were constructed by extracting the target gene list from the STRING database using Cytoscape software.

### 2.6. Fluorescence quantitative PCR and statistical analysis

We performed gene expression analysis using qPCR to evaluate crucial genes associated with biological processes involved in immunity (*HSP70*, *HSP90*)(Xu et al., 2014), ion channels (*SLC40A1*, *AE2*, *AQP1*, *CFTR*, *NHE*, *NCC*, *AQP11*) (Marshall and Grosell, 2006; Hwang et al., 2011; Hiroi and McCormick, 2012), cell cycle (*VEGF-A*, *CIART*, *hPER1*, *GADD45γ*)(Bjarnason et al., 2001; Benjamin et al., 2007; Kammerer et al., 2009; Nikkola et al., 2018), sterol reductase (*Delta-24-SR*, *HMG-CoA reductase*)(Roberts et al., 2004), antioxidase (*Mn-SOD*, *GSTA*, *GPx1*) (Jiang et al., 2015; Li et al., 2017), and energy (*MDH1*) (Lo et al., 2005) in the kidney and gill of turbot fed with different concentrations of *myo*-inositol. The results of the qPCR analysis not only verified the accuracy of the transcriptome data but also further detected the effect of *myo*-inositol consumption on crucial physiological functions (except steroid-related process).

Gill and kidney tissues were sampled from turbot that were fed the commercial diet containing different contents (0, 600, 900, 1200 mg MI $kg^{-1}$) of *myo*-inositol. Total RNA was extracted from these tissues using an RNA prep pure Tissue Kit (Tiangen, Beijing, China). The total RNA quality and concentration were checked (Table S1). First-strand cDNA was synthesized using reverse transcriptase with *TranScript* First-Stand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co. Ltd., Beijing, China). With the exception of the *AQP1*, *CFTR*, *NHE*, *NCC*, and *AQP11* genes, which were from Ma et al. (2020), the sequence corresponding to a gene with reads mapping to the reference genome was used to design the primers with Primer 6.0 software. The specificity and efficiency of all primers (Table 1) for qPCR were validated by sequencing and performing serial dilutions and generating a calibration curve, respectively. The 18S housekeeping gene was used to adjust the gene expression data to maintain the correct rate of pre-analysis gene expression data (Zhang et al., 2019). qPCR was implemented on a 185–5096 CFX96 Real-Time PCR Detection System (Beijing yue da Biotechnology Co. Ltd., Beijing, China) following the protocol for the *TransStart* Top Green qPCR SuperMix Kit (Beijing TransGen Biotech Co. Ltd.). The $2^{-\Delta\Delta}$Ct method was used to analyse the relative gene expression (Schmittgen & Zakrajsek, 2000).

### 2.7. Statistical analysis

All statistical analyses were performed using SPSS19.0 for Windows. Results are expressed as mean values with the standard error mean (SEM). Normality and homoscedasticity assumptions were tested prior to analysis. One-way analysis of variance (ANOVA) was employed to distinguish significant differences between different treatment and control groups. Tukey HSD tests were then used to find significant groupings within the data set. In all analyses, the significance level was set at $p = .05$.

## 3. Results

### 3.1. Characteristics of myo-inositol content under salinity stress

In seawater (salinity 30), the *myo*-inositol content was higher in the kidney (7.53 ± 0.11 ng $g^{-1}$) than the gill (6.89 ± 0.29 ng $g^{-1}$) of turbot, but this difference was not statistically significant. However, the *myo*-inositol concentration was significantly lower in plasma (4.34 ± 0.32 ng $ml^{-1}$) than kidney and gill ($p < .05$). In all treatments, the content of *myo*-inositol was highest in the gill 5 min after salinity stress, reached the lowest level at 1 h and then increased over time (Fig. 1A). The time-course data showed no effect of salinity on *myo*-inositol abundance in the kidney (Fig. 1B). The level of *myo*-inositol in the plasma was significantly higher in the seawater group (salinity 30) than the other salinities tested based on the time-point data ($p < .05$). The concentration of *myo*-inositol showed a similar varying tendency at the same time in plasma, in which the content of *myo*-inositol in all treatments tended to be inhibited compared with the seawater group (Fig. 1C).

**Table 1**
Gene primers used for qPCR.

| Name | Forward primer | Reverse primer | Accession numbers/Origin |
|---|---|---|---|
| *HSP70* | AAGCCGAGGACGACCTTCAGAG | GTTCTCCAGCCAAGCGATGATCTC | SMAX5B020032 |
| *HSP90* | TGAGGAGAAGAAGAAGCAGGAGGAG | GCCGTAGGTGCTCGTGACAATG | SMAX5B008133 |
| *CIART* | GTTATGCCACCGTACTCCGATCTG | CGCCGACGATGAGCCTAATGTG | SMAX5B015827 |
| *VEGF-A* | TGGTGGATGTGGAGCAGGAGTTC | CAGCAACCAGAGCATCGCCATAG | SMAX5B008295 |
| *HMG-CoA* | GACTGCCCCAAGACAGAAGA | GACCAGCGATGCCTAAAATA | SMAX5B005723 |
| *hPER1* | CACCCTCAAAAACACTGACACG | GCTCCGAAAACATAATCCCC | SMAX5B001490 |
| *AE2* | CCTTGCTGCCTCATCACTACATGG | TCTGTGGATGTGGTGAGAGGAGTG | SMAX5B010728 |
| *SLC40A1* | ACCATCGCCAACATCGCCAAC | TCCTGACCTGCCACGACCAC | SMAX5B021652 |
| *Delta-24-SR* | GAAGCATCGCAGAACAAGCAGAAC | TCAGGCTCCGCATGGTCAGTC | SMAX5B017665 |
| *Mn-SOD* | CCGAACTGACCTACGACTATGG | TGTGGCGTGGTGTTTGCTGT | SMAX5B019682 |
| *GSTA* | GCTTTGTCCTGAACGAGTCCT | TCCGCCATTTTCTGGGTGAG | SMAX5B013227 |
| *GPx1* | TCCGTCCAGGGAATGGCTTT | GGCAGGCTGTTTTTCAGGTAG | SMAX5B002217 |
| *GADD45 γ* | GAGGAAAGCGGCGGAGATAA | AGCAGACACAGCACCACATTG | SMAX5B007882 |
| *MDH1* | CGGCGAACACCAACTGTCTGATC | CCAGGCGAGTCAGGCAGGAG | SMAX5B009203 |
| *AQP1* | GTCGCAGCAGCTCTCGTCTAC | CCGCTGACCAGGACCTTCATG | Ma et al., 2020 |
| *CFTR* | ATTTACGCCCGCCGCTTATC | CCAGCAACCTCAATCACGAA | Ma et al., 2020 |
| *NHE* | GCCAACATCTCCCACAAGTCC | GACGAAGGTCCAGTTCCAGGC | Ma et al., 2020 |
| *NCC* | GCAACAAAGGACCCGTAAAGT | GACCAAACACCCAGGACAGAC | Ma et al., 2020 |
| *AQP11* | GCAGGTGGCAGTATGACAGGAG | AGACCAGACAGTACTCCAGGAAGG | Ma et al., 2020 |
| *18S* | GTGGAGCGATTTGTCTGGTT | CTCAATCTCGTGTGGCTGAA | Zhang et al., 2019 |

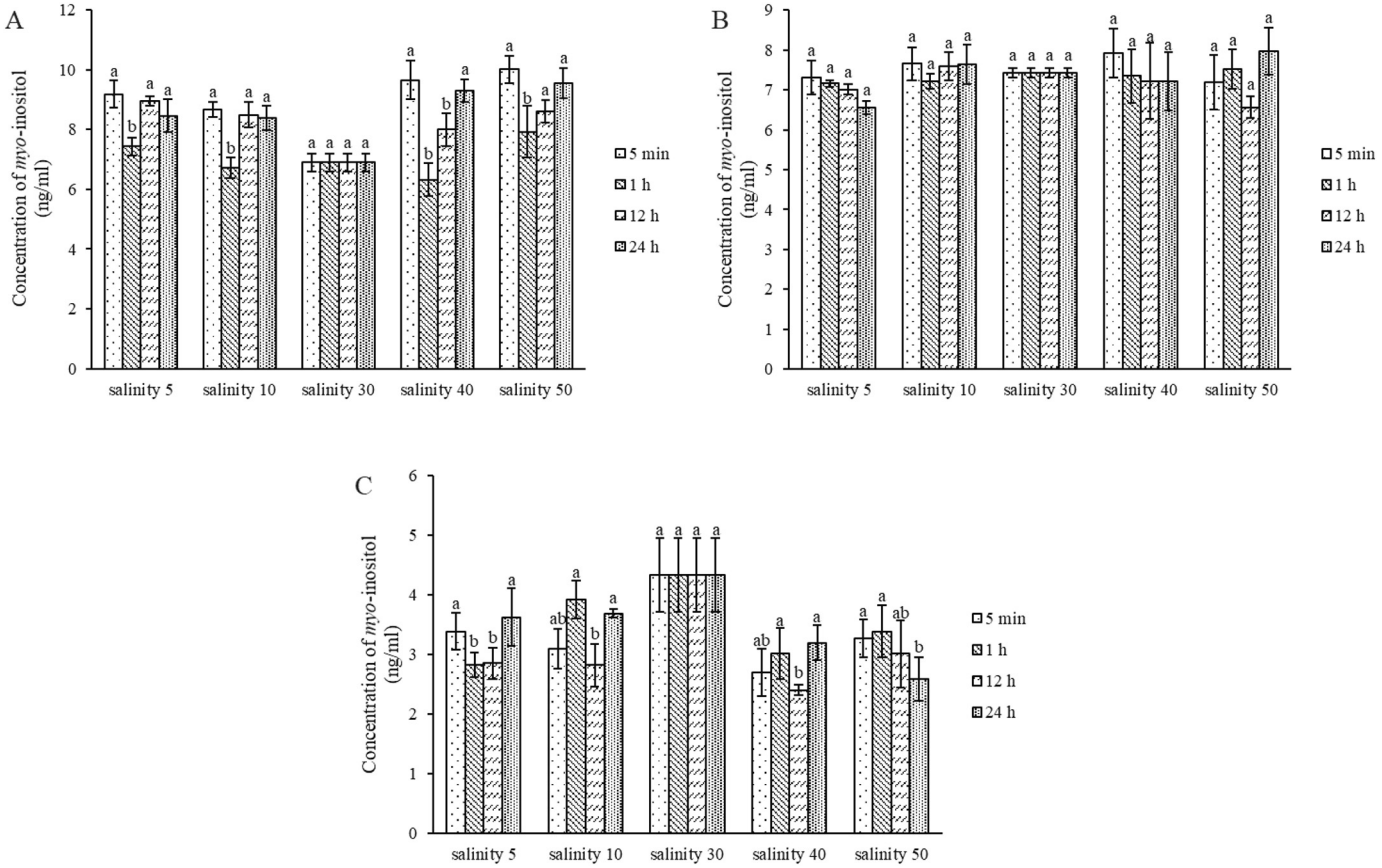


**Fig. 1.** *myo*-inositol (MI) concentration in gill (A), kidney (B), and plasma (C) tissues in different salinities. Different letters represent significant differences ($p < .05$) under the same salinity.

### 3.2. Effects of myo-inositol on the survival of turbot under salinity stress

With or without *myo*-inositol immersion, the survival time of turbot was close to 13 h in the freshwater environment. The survival time of turbot was not significantly affected by immersion in *myo*-inositol under low salinity (salinity 0) (Fig. 2A). Turbot only survived for approximately 5 h under salinity 70, but the survival time after *myo*-inositol immersion in 10 ppm and 65 ppm reached nearly 10 h and 58 h, respectively. The results of the immersion test demonstrated that *myo*-inositol immersion significantly increased the survival time of turbot in the high-salinity treatment (salinity 70) ($p < .05$). The survival time of turbot soaked in 65 ppm *myo*-inositol was 11 times longer than in the no *myo*-inositol treatment at salinity 70 (Fig. 2B).

The survival time increased by 8 h, 9 h, and 11 h after consumption of different contents of *myo*-inositol (600, 900, 1200 mg $kg^{-1}$) compared with the survival time of turbot in freshwater, which was approximately 13 h (Fig. 2C). In the high-salinity treatment, the turbot only survived for approximately 5 h, but the survival time reached nearly 6 h, 7 h, and 11 h, respectively, after consumption of different concentrations of *myo*-inositol (Fig. 2D). Therefore, the turbot fed diets containing *myo*-inositol had significantly longer survival time than fish without *myo*-inositol in the diet at both salinity 70 and 0 ($p < .05$) (Fig. 2C, D). The survival time increased with the increase in *myo*-inositol concentration, and the survival time of turbot fed 1200 mg/kg of *myo*-inositol was twice as long as the group without *myo*-inositol at salinity 70.

### 3.3. Transcriptome sequencing analysis results

After checking the purity, concentration (Table S2), and integrity (Fig. S2), the high quality RNAs from individual fish in each group were used to construct cDNA libraries and conduct high-throughput sequencing. The gill transcriptomes of turbot fed different concentrations of *myo*-inositol were generated by Illumina deep sequencing. The turbot fed the commercial diet containing *myo*-inositol concentrations of 600 and 1200 mg MI $kg^{-1}$ and the control group were named the G600, G1200, and CK group, respectively. The $R^2$ value of the Pearson' correction coefficient between samples was > 0.8 within the group, which indicated reliable repeatability of the transcriptome data in the biological repeats (Fig. S3). We identified 143 DEGs in the G600 group compared with the control group, of which 79 were up-regulated and 64 were down-regulated (Fig. S4A). We found 220 DEGs in the G1200 group compared with the control group, of which 114 were up-regulated and 106 were down-regulated (Fig. S4B). GO annotation analysis showed that the number of DEGs was prominently higher in the G1200 group than the G600 group in all the same functional categories. The functional categories that contained the majority of enriched genes in the G600 group were different from those in the G1200 group. They were single-organism process, metabolic process, cellular process, membrane, and binding in the G600 group, whereas in the G1200 group they were metabolic process, single-organism, binding, catalytic activity, and membrane (Fig. 3).

CK represents the control group, G600 represents turbot fed the commercial diet containing 600 MI $kg^{-1}$of *myo*-inositol, and G1200 represents turbot fed the commercial diet containing 1200 MI $kg^{-1}$ of *myo*-inositol.

KEGG enrichment analysis indicated that all significant DEGs in both groups were significantly enriched in steroid biosynthesis, steroid hormone biosynthesis, circadian rhythm-fly, circadian rhythm, terpenoid backbone biosynthesis, glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate, biosynthesis of unsaturated fatty acids, fatty acid elongation, monobactam biosynthesis, butanoate metabolism, tryptophan metabolism, protein processing in endoplasmic

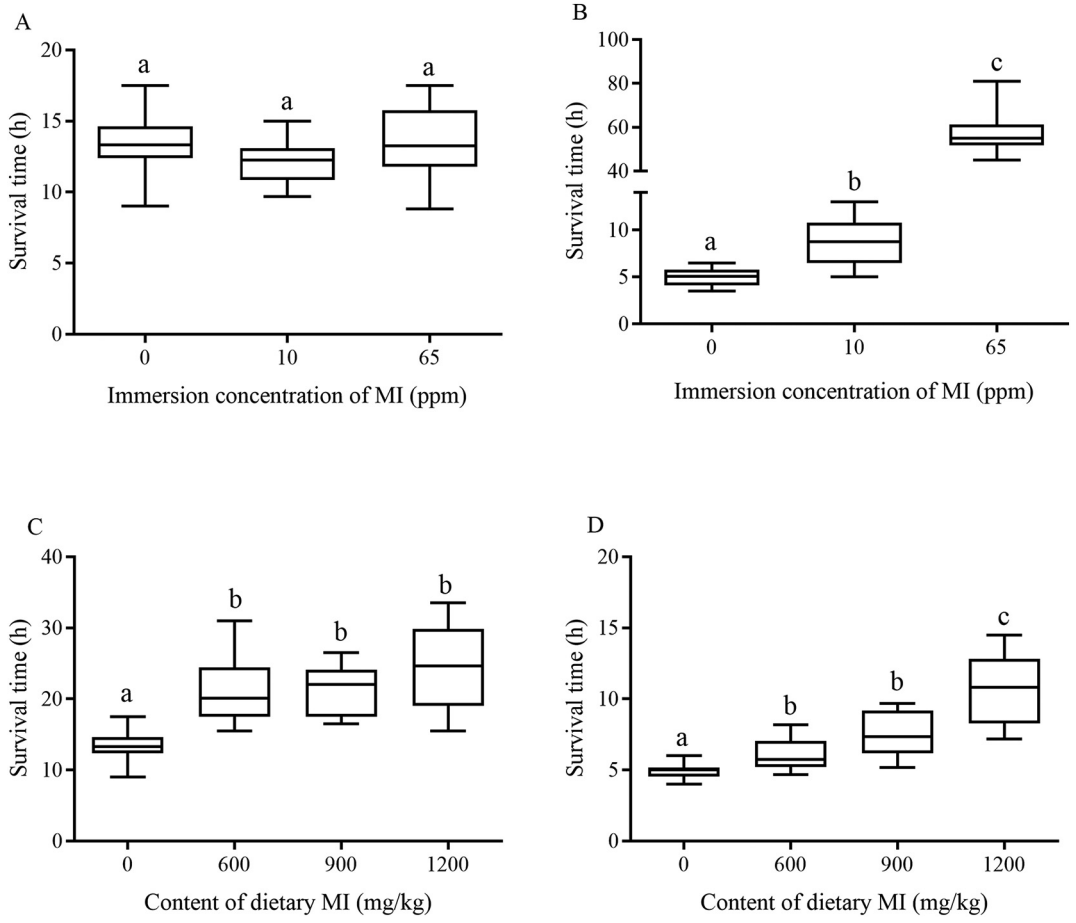


**Fig. 2.** Survival time of turbot treated with *myo*-inositol (MI) under salinity stress. Survival time in freshwater (A) and salinity 70 (B) by immersion in *myo*-inositol; survival time in freshwater (C) and salinity 70 (D) of turbot fed diets containing *myo*-inositol. Different letters represent significant differences ($p < .05$).

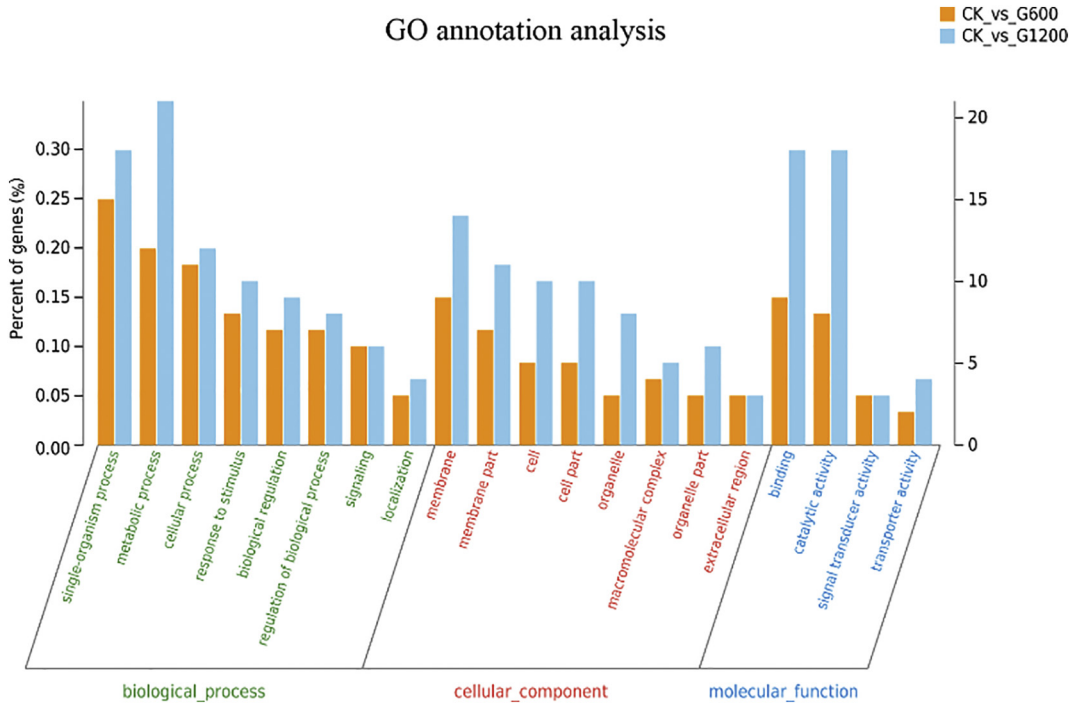


**Fig. 3.** Results of the GO annotation analysis of differentially expressed genes.

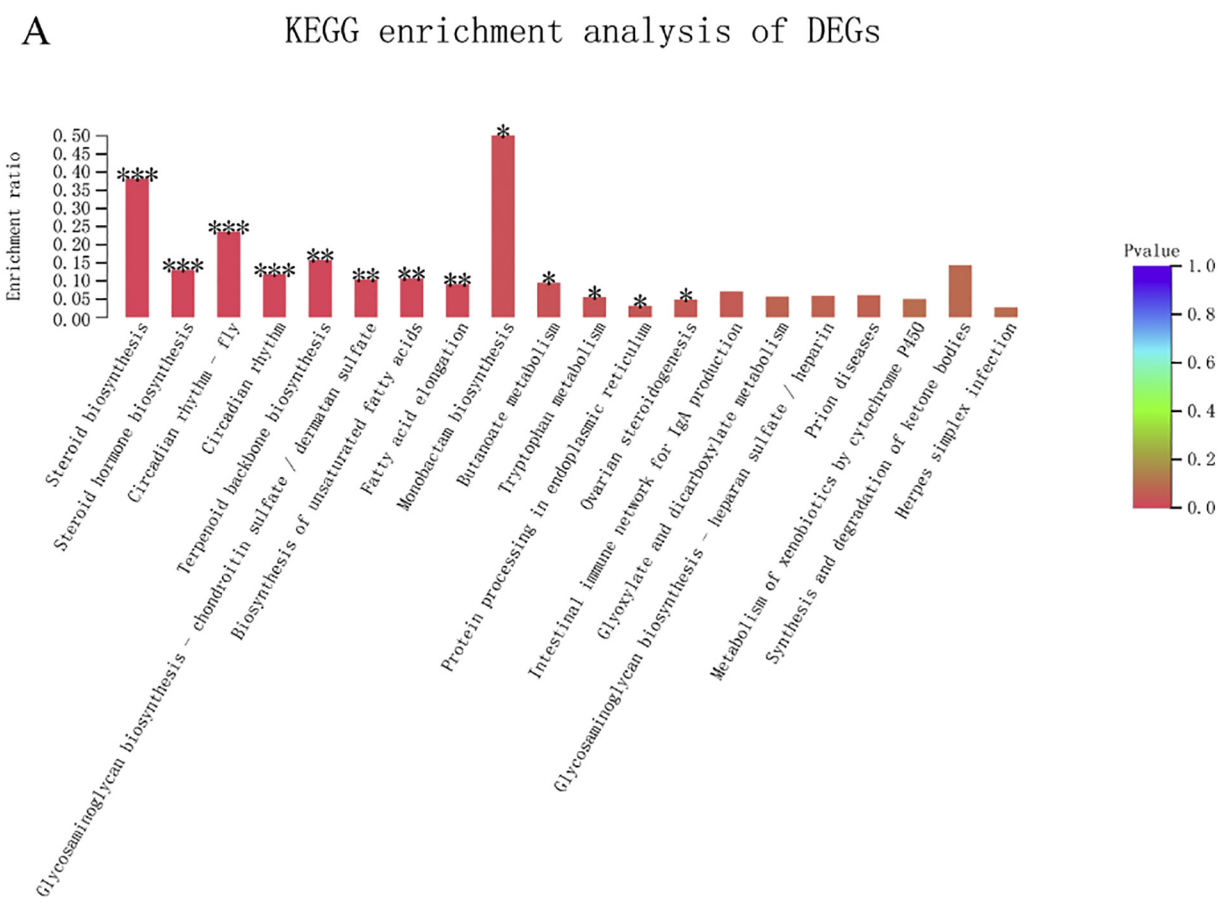


**Fig. 4.** KEGG (A) and GO (B) enrichment analysis of DEGs.

reticulum, and ovarian steroidogenesis ($p < .05$) (Fig. 4A). The GO enrichment analysis data for all significant DEGs in both groups suggested that the DEGs were significantly enriched in steroid biosynthetic process and steroid metabolic process ($p < .01$) (Fig. 4B).

Red indicates significant enrichment, and other colours indicate non-significant enrichment. Three asterisks (***) indicate $p < .001$, two asterisks (**) indicate $p < .01$, and one asterisk (*) indicates $p < .05$.

The PPI network (Fig. 5) was constructed with Cytoscape software using all significant DEGs in the transcriptome ($p < .05$). According to the PPIs and protein degrees in the network, the genes encoding the highest-degree proteins were involved in cytochrome P450 1A1, lathosterol oxidase, lanosterol 14-alpha demethylase, cryptochrome-1, acetoacetyl-CoA synthetase, cytochrome P450 1B1, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, 78 kDa glucose-regulated protein, and heat shock protein (HSP) 90-alpha 1. Apart from the genes for cytochrome P450 1A1 and cytochrome P450 1B1, which were down-regulated, all the other genes were significantly up-regulated ($p < .05$) (Table 2).

Annotation analysis of the genes encoding the highest-degree proteins in the KEGG and GO databases was conducted to evaluate pathway enrichment. KEGG enrichment analysis indicated that these genes were significantly enriched ($p < .05$) in steroid biosynthesis, steroid hormone biosynthesis, tryptophan metabolism, and metabolism of xenobiotics by cytochrome P450 (Fig. 6A). The functional classification results in the GO database indicated that these genes were significantly enriched ($p < .01$) in oxidoreductase activity, iron ion binding, and heme binding (Fig. 6B).

### 3.4. Fluorescence quantitative PCR

#### 3.4.1. Gene expression in the gill after consumption of myo-inositol

The relative expression of genes in the gill determined by qPCR was in accordance with the results obtained by high-throughput sequencing (Fig. 7A, B), which verified the accuracy of the transcriptome data. In the gill of turbot, expression of the *HSP70* and *HSP90* genes associated with immunity was up-regulated, while that of the *SLC40A1* and *AE2* genes was down-regulated as the concentration of *myo*-inositol in the diet was increased (Fig. 7A). The expression levels of a cluster of genes related to the cell cycle, including *CIART*, *Hper1*, and *VEGF-A*, were down-regulated whereas the *GADD45*γ gene was up-regulated in the gill of turbot with increasing levels of *myo*-inositol in the diet (Fig. 7A). As the concentration of *myo*-inositol was increased, the expression of genes encoding delta (24)-sterol reductase and HMG-CoA reductase, which are related to sterol reductase, first increased and then decreased (Fig. 7A). For antioxidant-related genes, the mRNA of the *GSTA* gene increased with an increasing *myo*-inositol concentration, whereas that of the *GPx1* and *Mn-SOD* genes remained relatively stable (Fig. 7C). The expression of *MDH1* was suppressed, but this effect did not increase with the increasing *myo*-inositol concentration (Fig. 7D).

#### 3.4.2. Gene expression in the kidney after consumption of myo-inositol

In the kidney of turbot, the expression of *HSP70* showed zigzag-like changes. The expression of *HSP90* was significantly up-regulated only with 1200 mg $kg^{-1}$ of *myo*-inositol ($p < .05$) (Fig. 7E). As the concentration of *myo*-inositol in the feed was increased, the expression of *SLC40A1*, which is related to ion transport, was up-regulated, whereas that of *AE2* was significantly inhibited ($p < .05$) (Fig. 7F). The expression of genes related to the cell cycle (*CIART*, *Hper1*, and *VEGF-A*) was down-regulated, but *GADD45*γ was significantly up-regulated as

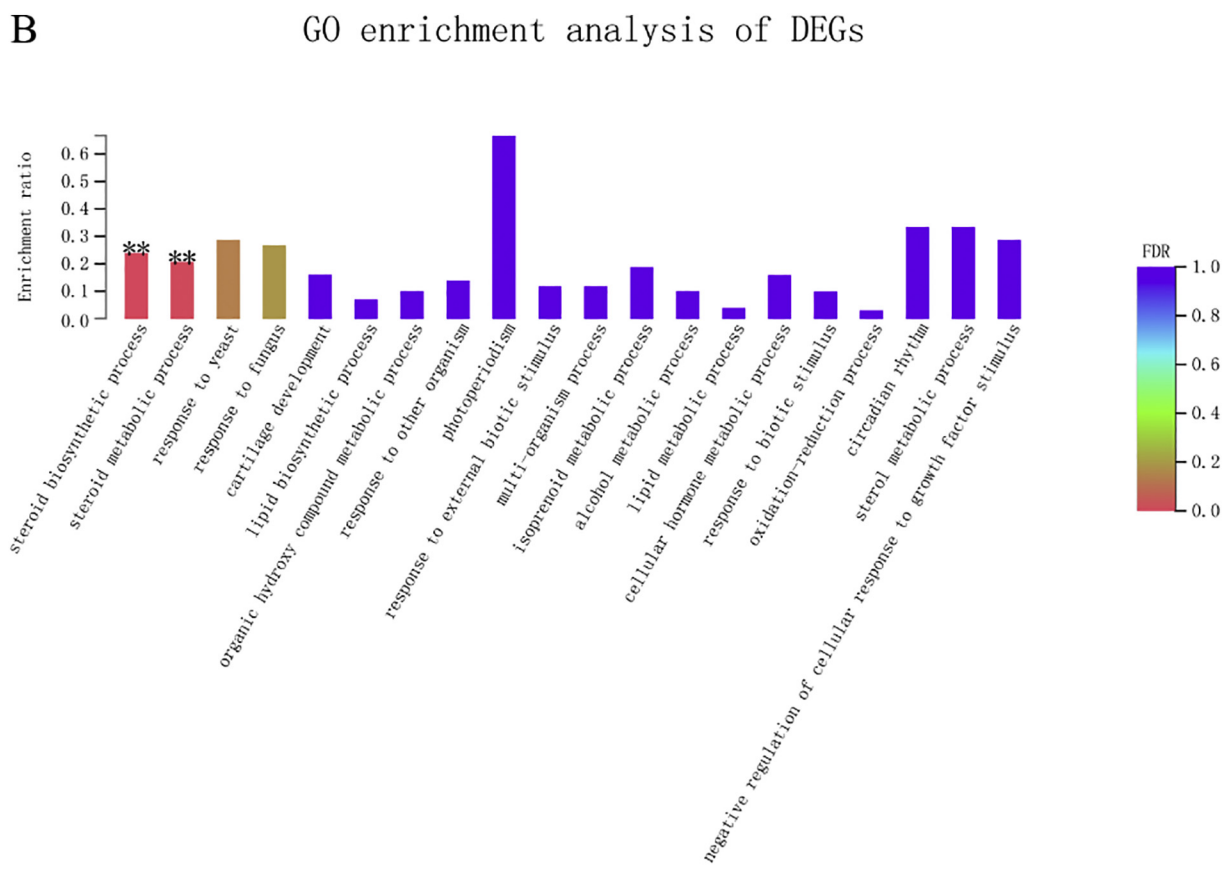


**Fig. 4.** (*continued*)

the concentration of *myo*-inositol in the feed was increased ($p < .05$) (Fig. 7G). With the exception of the 1200 mg $kg^{-1}$*myo*-inositol treatment, the mRNA of *delta (24)-sterol reductase* slightly increased, as did *HMG-CoA* except with the 600 mg $kg^{-1}$ treatment (Fig. 7H). The expression of the cluster of antioxidant-associated genes, including *GPx1*, and *GSTA*, was significantly promoted by increasing *myo*-inositol concentrations ($p < .05$) (Fig. 7I). Expression of the *MDH1* gene was diminished, yet this effect decreased with the increase in *myo*-inositol concentration in the kidney (Fig. 7J).

### 3.5. Expression analysis of ion-channel genes

Expression analysis of ion-channel genes after feeding *myo*-inositol was performed by qPCR to determine the effects of changes in *myo*-inositol-mediated physiological activities on osmotic regulation. The expression levels of *AQP1* and *NCC* were significantly up-regulated in the gill of turbot by increasing concentrations of *myo*-inositol ($p < .05$), while those of the genes *CFTR* and *NHE* were down-regulated (Fig. 8). The gene expression trend in the kidney was opposite to that in the gill. The expression of *AQP1* was significantly inhibited at dietary concentrations of *myo*-inositol of 900 and 1200 mg $kg^{-1}$ ($p < .05$). Although the trend was not linear, expression of the *NHE* gene was remarkably promoted in the kidney after *myo*-inositol feeding, whereas that of *NCC* and *AQP11* was suppressed (Fig. 8). The *CFTR* gene was only expressed in the gill, while the *AQP11* gene was only expressed in the kidney.

## 4. Discussion

### 4.1. Response of myo-inositol content to salinity transfer

Inositol, in particular *myo*-inositol, is an essential vitamin-like nutrient for most aquatic animals (Michael and Koshio, 2008). Many species of fish and their intestinal microbial flora can synthesize *myo*-inositol de novo in an amount sufficient to support normal physiological functions, such as tilapia (*Oreochromis mossambicus*) (Sacchi et al., 2014), channel catfish (*Ictalurus punctatus*) (Burtle and Lovell, 1989), and sunshine bass (*Morone chrysops* ♀ × *Morone saxatilis* ♂) (Deng et al., 2002). The results of the present study showed that *myo*-inositol could be accumulated in all the crucial osmoregulatory organs of turbot to protect cells from salinity stress (Yancey, 2005; Burg and Ferraris, 2008). However, all the salinity treatments significantly increased *myo*-inositol contents in the gill, but not in the kidney ($p < .05$), which suggests that *myo*-inositol is more important in the gill than the kidney during osmotic regulation in turbot (Kalujnaia et al., 2013; Sacchi et al., 2014). Seawater transfer was also shown to induce a 1.8-fold increase in *myo*-inositol content within the gill in the euryhaline eel (*Anguilla anguilla*) (Kalujnaia et al., 2010), whereas an up to 2-fold increase in the content of *myo*-inositol in the kidney was associated with the transfer of the fish to seawater (Fiess et al., 2007). These findings suggest that the magnitude of importance of *myo*-inositol in osmotic regulation may depend on the fish species (Shirmohammad et al., 2016). A decrease in the *myo*-inositol concentration may be the result of antagonism by other compatible organic osmolytes involving triglycerides in the plasma (Wen et al., 2007).

### 4.2. myo-inositol extends survival under salinity stress

Vargas-Chacoff et al. (2015) reported that dietary *myo*-inositol improves growth, osmoregulation, energy metabolism, and digestive capacity in juveniles of the Notothenioid fish *Eleginops maclovinus* acclimated at different salinities. Moderate effects of *myo*-inositol supplementation on growth were found only during the first 4 weeks of the feeding process of Atlantic salmon (*Salmo salar* L., fry) (Waagbø &

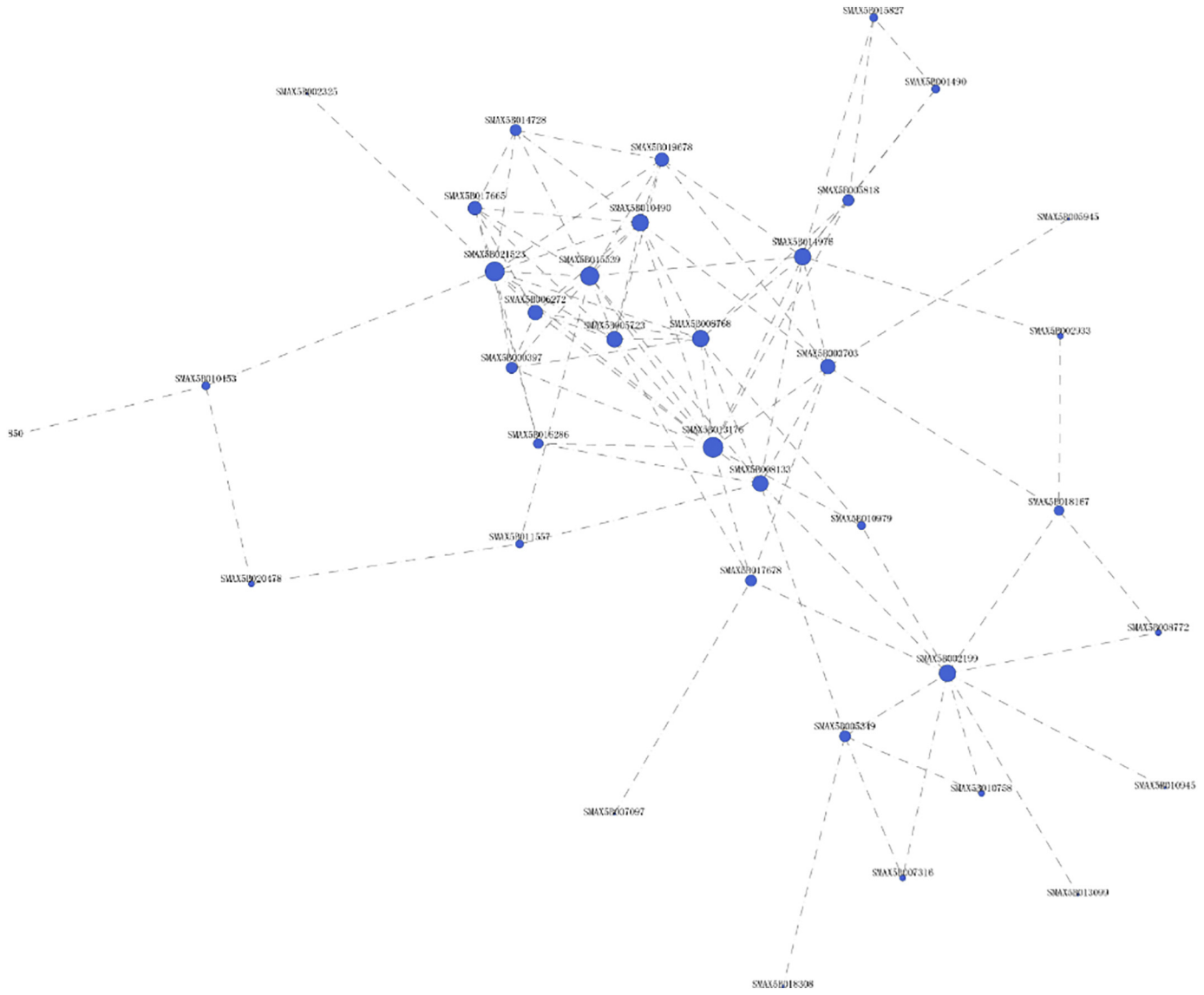


**Fig. 5.** Protein-protein interaction networks.

**Table 2**
Genes encoding the highest-degree proteins in the protein-protein interaction networks.

| Gene ID | Gene name | *p* value | Regulate |
|---|---|---|---|
| SMAX5B013176 | Cytochrome P450 1A1 | 2.34E-16 | down |
| SMAX5B021523 | Lathosterol oxidase | 0.001683 | up |
| SMAX5B015539 | Lanosterol 14-alpha demethylase | 1.13E-07 | up |
| SMAX5B014976 | Cryptochrome-1 | 8.08E-11 | up |
| SMAX5B010490 | Acetoacetyl-CoA synthetase | 1.02E-06 | up |
| SMAX5B008768 | Cytochrome P450 1B1 | 2.99E-08 | down |
| SMAX5B005723 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | 2.52E-05 | up |
| SMAX5B003703 | 78 kDa glucose-regulated protein | 0.004407 | up |
| SMAX5B008133 | Heat shock protein HSP 90-alpha 1 | 0.002369 | up |

Sandnes, 1998). Cell viability, as measured by proliferation and differentiation, was increased by *myo*-inositol supplementation in carp (*C. carpio* var. Jian) enterocytes in primary culture (Jiang et al., 2013). Our results showing that *myo*-inositol supplementation provided by immersion or dietary supplementation significantly increased survival time under salinity stress are consistent with the findings for *P. olivaceus*, which showed that sufficient *myo*-inositol supplementation significantly increased survival ($p < .05$) (Lee et al., 2008). In contrast, the survival of parrot fish (*Oplegnathus fasciatus*) (Khosravi et al., 2015) and juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) (Shiau and Su, 2005) fed different dietary *myo*-inositol levels was not affected. Furthermore, no significant variations in mortality related to dietary *myo*-inositol supplementation were detected in *S. salar* L., fry during a long-term feeding experiment (WaagbØ et al., 1998). These findings may be explained as follows. (1) Distinct species of fish can produce and demand different amounts of *myo*-inositol (Shirmohammad et al., 2016), and (2) the functions of *myo*-inositol on osmoregulation in fish differ during diverse treatments (Gardell, 2013) (for example, salinity stress was applied following feeding in the current study, whereas other studies implemented long-term feeding). In the present study, the survival time of turbot dipped in different concentrations of *myo*-inositol was not significantly affected at low salinity (salinity 0). An explanation for this finding may be that fish inhabiting a freshwater environment were actively minimizing absorption of *myo*-inositol dissolved in the water and thus were not significantly affected by low contents of *myo*-inositol; in contrast, the opposite phenomenon has been reported for seawater teleost fish (Lam et al., 2014).

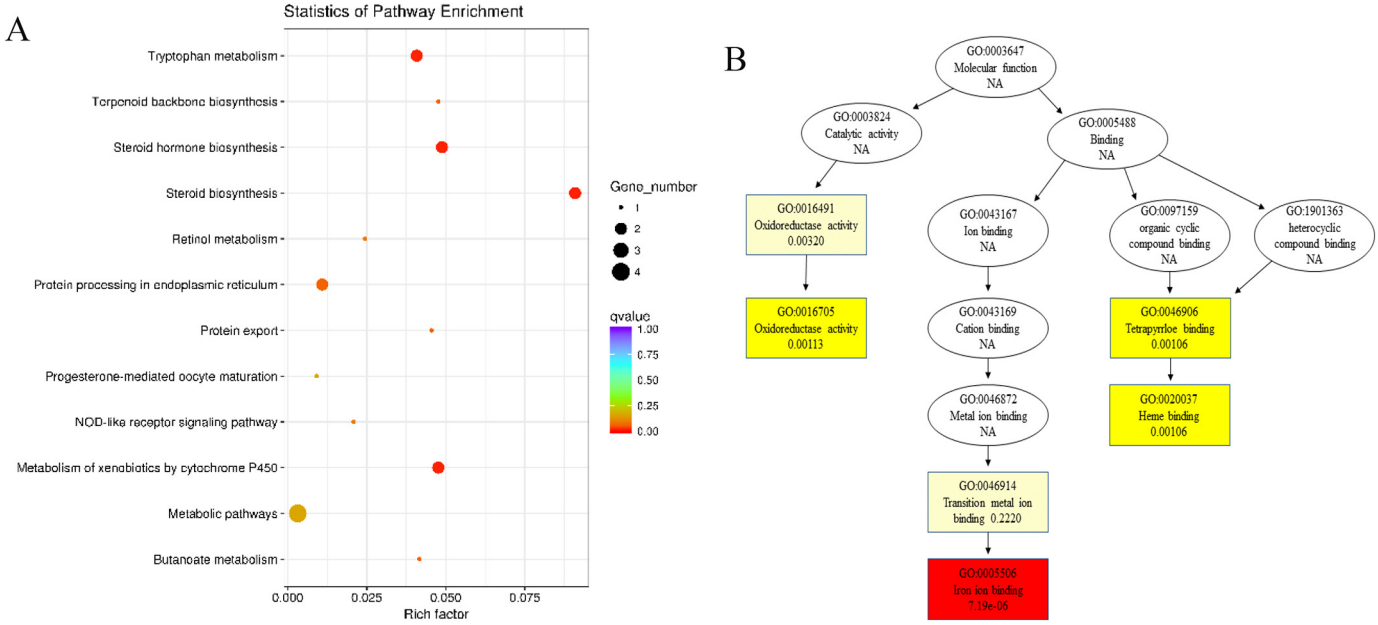


**Fig. 6.** Results of the KEGG (A) and GO (B) annotation analysis of genes encoding the highest- degree proteins.

### 4.3. *myo*-inositol improves physiological functions

As a part of B-complex vitamin, a component of phosphatidylinositol, and a growth factor in animals, *myo*-inositol mediates multiple physiological functions (Shirmohammad et al., 2016). In the present study, transcriptome profiling of turbot fed with different *myo*-inositol concentrations was performed to decipher the mechanisms by which *myo*-inositol modulates physiological functions. The number of DEGs in the G1200 group was much higher than in the G600 group in all the same functional categories, which indicated that *myo*-inositol probably mediated gene expression to promote physiological activities of turbot (Jiang et al., 2015; Li et al., 2018). The GO and KEGG enrichment analyses suggested that *myo*-inositol likely enhanced salinity tolerance by mediating the processes associated with steroid biosynthesis and metabolism. These results reinforce those of Mancera and Mccormick (1999), who reported that steroids related to cortisol improved the ability of euryhaline fish to maintain plasma osmolality, increase gill $Na^+$-$K^+$-ATPase activity, and increase osmoregulatory ability during seawater acclimation. Lock et al. (2010) reported that the steroid (vitamin D) is intimately involved in the smoltification process and participates in the preparatory physiological adaptations that occur in freshwater. Pathway enrichment analysis conducted in the present study and accumulating evidence indicate that *myo*-inositol can affect the circadian rhythm of animals (Kazuki et al., 2015). The great magnitude of the effect of the circadian rhythm on physiological activities involved in light adaption, oxygen consumption, and regulation of ion channels, as well as how it impacts environmental adaptation in many species of marine animals, have been researched previously (Kim et al., 1997, Ren and Li, 2004, Ko et al., 2009).

A PPI network was constructed to explore the major effects of *myo*-inositol on physiological activities, and to screen genes encoding the highest-degree proteins. The up-regulated expression of the majority of the highest-degree genes indicated that *myo*-inositol probably promoted physiological activities by increasing the expression of crucial genes (Jiang et al., 2015; Li et al., 2018). The results of the pathway annotation analysis of these genes in the PPI network showed that the pathways involved in steroid biosynthesis, steroid hormone biosynthesis, tryptophan metabolism, and metabolism of xenobiotics by cytochrome P450 played important roles in improving the physiological activities of turbot in response to *myo*-inositol, as previously documented in other fish (Mancera and Mccormick, 1999; Zhao et al., 2014; Hoseini et al., 2017; Zhao et al., 2017). Furthermore, annotation analysis based on the GO database revealed that the functions of oxidoreductase activity, iron ion binding, and heme binding, which have been identified in other fish (Waagbø et al., 1998; Jiang et al., 2013), were affected by dietary *myo*-inositol in turbot.

Taken together, the transcriptome analysis results revealed that *myo*-inositol enhanced salinity tolerance likely by improving multiple physiological functions. Moreover, the signalling pathways associated with steroids, including the steroid biosynthetic process, steroid metabolic process, and steroid hormone biosynthesis, mediated by *myo*-inositol played a major role in osmoregulation in turbot.

### 4.4. Effect of *myo*-inositol on the physiological function of the gill and kidney

Many studies have evaluated the effects of dietary *myo*-inositol on multiple physiological activities, including immune response, antioxidant capacity, circadian rhythm, hormone profile, and metabolism in fish (Jiang et al., 2013, 2015; Li et al., 2018). To decipher the mechanisms by which *myo*-inositol acts in turbot, the expression of genes in different functional categories were evaluated at the molecular level. The results revealed that *myo*-inositol modulated immune function in the gill by promoting the expression of immune-related genes and in the kidney by supplying optimum dietary *myo*-inositol levels, as described by Jiang et al. (2015). In agreement with the findings of previous studies (Jiang et al., 2013; Jiang et al., 2015), the analysis demonstrated that *myo*-inositol stimulated the activities of steroid reductase and antioxidase, especially steroid reductase in the gill and antioxidase in the kidney. Although there is no evidence that *myo*-inositol directly acts on ion transport, it has been demonstrated that *myo*-inositol phosphate affects intracellular ion regulation (Putney, 1987; Putney Jr, 1990). Consistent with those findings, in the current study, the ion transport functions related to iron and anions were selectively suppressed by *myo*-inositol in the osmoregulatory organs (gill and kidney) of turbot. Moreover, these findings corroborated the results of Kazuki et al. (2015), who reported that *myo*-inositol stalls the cell cycle both in the gill and kidney by inhibiting the expression of genes involved in cell growth and by up-regulating growth arrest and DNA-damage-inducible genes. These findings also showed that the regulation of *myo*-inositol in

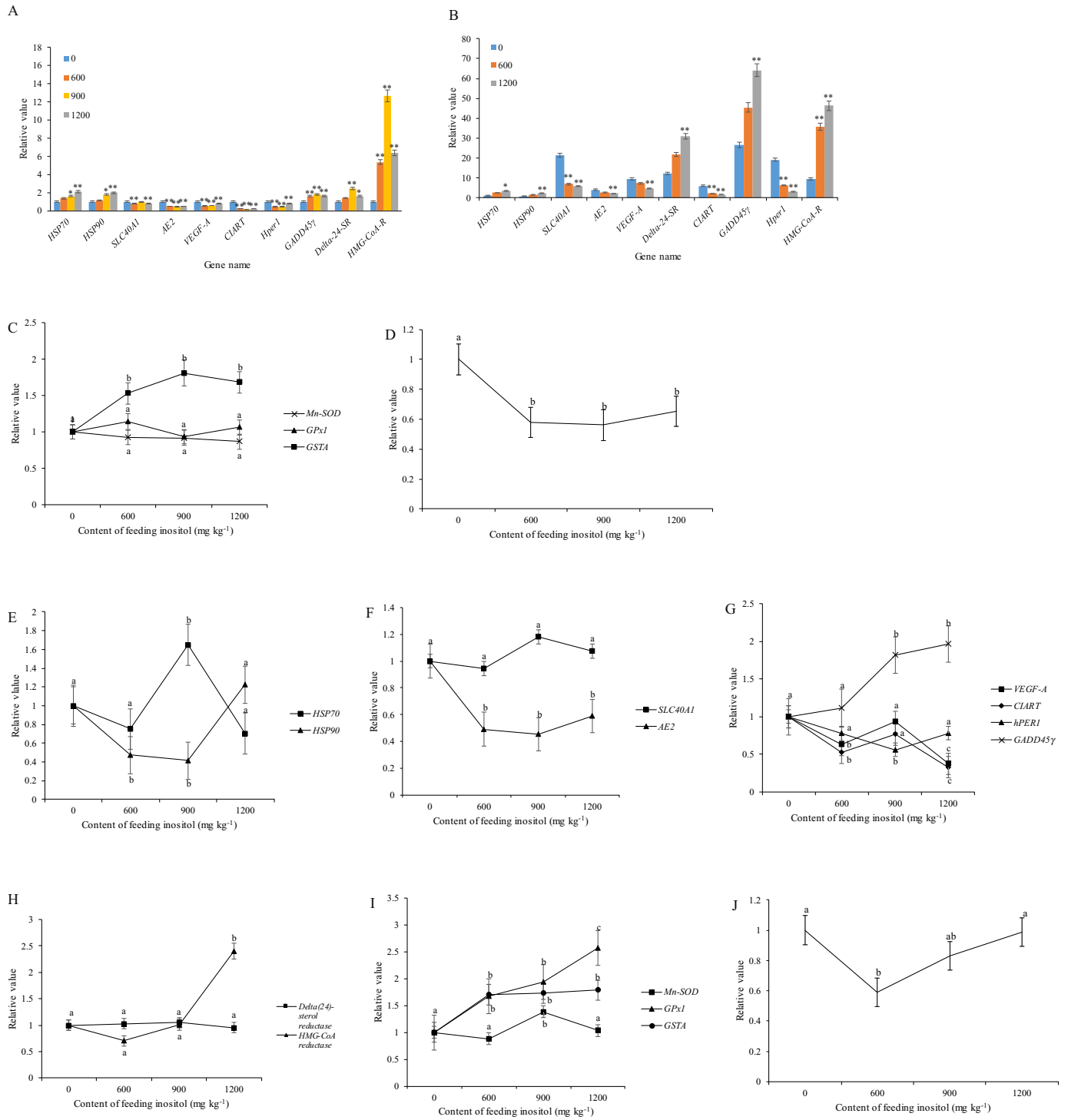


**Fig. 7.** Gene expression after feeding turbot different concentrations of *myo*-inositol. Relative expression of genes in the gill by qPCR (A) and in the transcriptome (B). Relative expression of genes related to antioxidase (C) and the *MDH1* gene (D) in the gill. Relative expression of genes related to immunity (E), ion channels (F), cell cycle (G), sterol reductase (H), antioxidase (I), and the *MDH1* gene (J) in the kidney. *HSP70*, heat shock 70 kDa protein; *HSP90*, heat shock 90 kDa protein; *SLC40A1*, solute carrier family 40 member 1; *AE2*, anion exchange protein 2; *VEGF-A*, vascular endothelial growth factor A; *CIART*, circadian-associated transcriptional repressor; *hPER1*, period circadian protein homolog 1; *GADD45*γ, growth arrest and DNA damage-inducible protein GADD45 gamma; *delta (24)-sterol reductase*; *HMG-CoA reductase*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *Mn-SOD*, manganese superoxide dismutase; *GPx1*, glutathione peroxidase 1; *GSTA*, glutathione S-transferase A; *MDH1*, malate dehydrogenase 1. One asterisk (*) indicates $p < .05$, and two asterisks (**) indicate $p < .01$. Different letters represent significant differences ($p < .05$) between columns.

the organism is carried out in existing cells (Jiang et al., 2013). Furthermore, by reducing the expression of energy-related genes such as *MDH1*, energy metabolism was likely reduced by the *myo*-inositol-shortened circadian rhythms (Kazuki et al., 2015). Although some genes have similar expression trends, the effects of *myo*-inositol on the gill are generally different from those on the kidney (Kalujnaia et al., 2013). It is noteworthy that all the various physiological activities (i.e., those involved in immunity, steroid reductase, antioxidase, ion

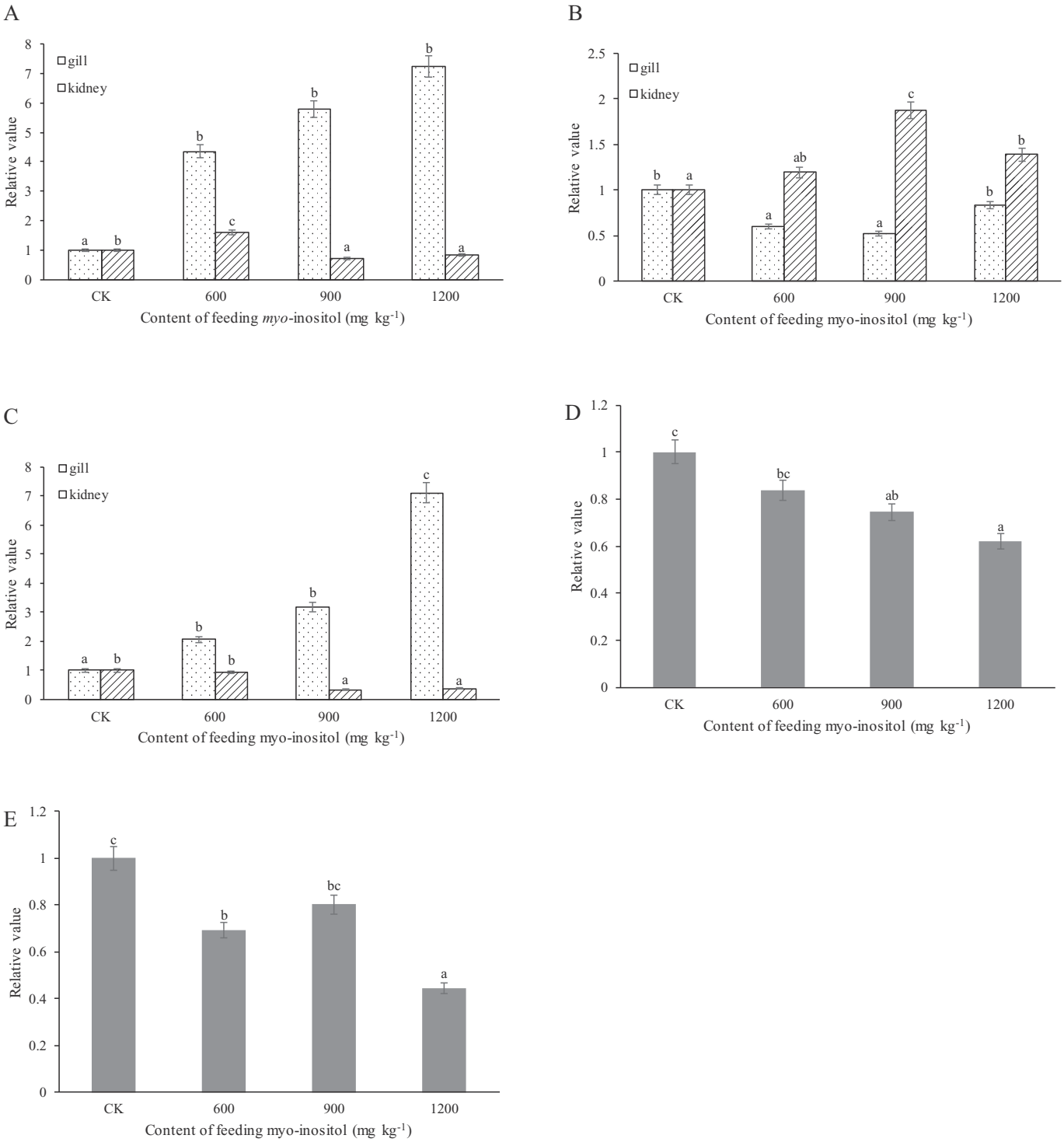


**Fig. 8.** Gene expression of ion-channel genes *AQP1*(A), *NHE3* (B), *NCC* (C), *CFTR* (D), and *AQP11*(E). *AQP1*, aquaporin 1; *CFTR*, cystic fibrosis transmembrane regulator; *NHE1*, $Na^+/H^+$ exchanger; *NCC*, $Na^+/Cl^-$ cotransporter; *AQP11*, aquaporin 11. CK, control. Different letters represent significant differences ($p < .05$) between columns.

regulation and cell cycle) that were found to be affected by *myo*-inositol in turbot play crucial roles in osmotic regulation (Kalujnaia et al., 2007; Lam et al., 2014; Brennan et al., 2015).

### 4.5. Effect of myo-inositol on ion channels

To determine the effects of changes in *myo*-inositol-mediated physiological activities on osmotic regulation, expression analysis of ion-channel genes was performed. The results showed that *myo*-inositol facilitated the transport of water and small neutral solutes across cell membranes of the gill but inhibited it in the kidney. This finding is consistent with the results of a previous study (Cutler et al., 2007), in which the seawater-acclimating corticosteroid hormone cortisol was shown to regulate the transport of water mediated by aquaporins in eel osmoregulatory tissues during acclimation to salinity changes. The present results also showed that *myo*-inositol promoted the cotransport

of sodium and chloride ions but depressed chloride ion transport by CFTR in the gill. These findings corroborate those of Bodinier et al. (2009), who reported that CFTR contributes to ion regulation and thus to adaptation of the salinity transitions coupled to other transport. NHE3 is a critical component of one of the currently proposed ion-uptake models in fish ionocytes (Dymowska et al., 2012). In turbot, the function of NHE3 was inhibited in the gill but promoted in the kidney, which reinforces the role of NHEs in the $Na^+$ uptake mechanism in specific types of ionocytes in fish (Hwang et al., 2011). Conversely, evidence of changes in osmotic regulation was also found in the transcriptome data. These results demonstrated that *myo*-inositol changed the osmotic regulatory capacity of the fish (Shirmohammad et al., 2016). Furthermore, the turbot fed diets containing different contents of *myo*-inositol had significantly longer survival than those without *myo*-inositol at both salinity 70 and 0. The results of the immersion test also demonstrated that *myo*-inositol immersion significantly increased the survival time of turbot in the high-salinity treatment. Altogether, these results indicated that *myo*-inositol enhanced the osmotic regulation ability of turbot, as observed in tilapia (*O. mossambicus*) (Gardell et al., 2013). Additionally, a previous study has suggested that ion-channel genes play important roles in hormonal regulation in osmoregulatory organs of the black porgy *Acanthopagrus schlegeli*, thereby improving its hyperosmoregulatory ability in hypoosmotic environments (An et al., 2008). In summary, *myo*-inositol strengthened osmotic regulation, as represented by the expression of ion-channel genes, by stimulating the activities of steroid reductase and antioxidase, modulating immune function, and inhibiting the cell cycle and energy metabolism.

## 5. Conclusion

In the present study, a significant increase in *myo*-inositol content was detected in the turbot gill during salinity stress. Additionally, *myo*-inositol supplementation via immersion or in the diet significantly increased the survival time of turbot under salinity stress. Transcriptome and qPCR data indicated that *myo*-inositol strengthened osmotic regulation, as represented by the expression of ion-channel genes, by stimulating the activities of steroid reductase and antioxidase, modulating immune function, and inhibiting the cell cycle and energy metabolism. GO and KEGG enrichment analyses of DEGs, PPI analysis, and qPCR data showed that signalling pathways associated with steroids, including the steroid biosynthetic process, steroid metabolic process, and steroid hormone biosynthesis, mediated by *myo*-inositol played a major role in osmoregulation in turbot.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735451.

## Availability of supporting data

The raw reads for the next generation sequencing data have been submitted to the NCBI Sequence Read Archive (SRA) under accession number PRJNA527368.

## Submission declaration

The authors declare that the work described herein has not been published previously elsewhere in the same form, in English or in any other language.

## Author contributions

Wenxiao Cui participated in the experimental design, investigations, data analyses and interpretation, and drafted the manuscript. Aijun Ma and Zhihui Huang participated in the experimental design and coordinated the study. Zhifeng Liu, Kai Yang and Wei Zhang helped to perform the fish exposure experiments and carried out sample preparation.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

## Acknowledgments

This work was supported by the Earmarked Fund for Modern Agro-Industry Technology Research System [grant numbers CARS-47-G01]; and the AoShan Talents Cultivation Program supported by Qingdao National Laboratory for Marine Science and Technology [grant numbers 2017ASTCP-OS04]; the National Natural Science Foundation of China [grant numbers 41706168]; the Agricultural Fine Breed Project of Shandong [grant numbers 2016LZGC031]; the Key Research and Development Plan of Nation [grant numbers 2018YFD0900301-12]; and the Central Public-interest Scientific Institution Basal Research Fund, YSFRI, CAFS [grant numbers 20603022019019].

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