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Analyses of the molecular mechanisms associated with salinity adaption of *Trachidermus fasciatus* through combined iTRAQ-based proteomics and RNA Sequencing-based transcriptomics

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Abstract

Osmoregulation mechanism underlying acclimation and adaptation of migratory fish to different salinities has been studied for decades. Recently developed transcriptomic and proteomic techniques would make it possible to provide more reliable data to decipher the mechanism study at a molecular level. Here in this study, an integrative analysis of the kidney-specific transcriptome and proteome was performed to identify important regulators and pathways involved in salinity adaption of roughskin sculpin (*Trachidermus fasciatus*). Fish were subjected to seawater-to-freshwater transfer that was achieved in 24 hours, samples were collected at 12, 24 and 48 h after the start of the experiment. Time course profiling of gene and protein expressions were examined using RNA-seq and iTRAQ methods. A total of 1504 differentially expressed genes (DEGs) and 378 differentially expressed proteins (DEPs) were identified at the three time points. 66 proteins were filtered by correlation of transcriptome and proteome results. When comparing the data obtained from the three time points, 12 h exhibited the largest number of DEGs and DEPs, suggesting the time course experiment is critical to clarify the framework of regulatory genes/proteins in response to salinity change. This study provides the first time-course, kidney-specific, combined transcriptomic and proteomic profiling associated with salinity adaption of *Trachidermus fasciatus*. The results revealed the possibility of *T.*
fasciatus as an experimental animal for osmoregulation studies, showed
the powerfulness of combining transcriptomic and proteomic approaches
to provide molecular insights of osmoregulation mechanisms in migratory
fish.

**Keywords:** Proteomics, Transcriptomics, Kidney, *Trachidermus fasciatus*,
RNA-sequencing, iTRAQ

1. Introduction

Vertebrates possess a suite of adaptive behavioral and physiological
strategies to cope with destabilizing challenges (or stressors), enabling the
animal to maintain homeostasis and overcome the threat (Flik et al.,
2006). Accordingly, adaptation can be defined as the process of change in
animals to conform better with variable environmental conditions, i.e.,
the organism acquires characteristic changes (morphology, physiology or
behavior changes) to improve their survival and reproductive success in
the particular environment (Bijlsma and Loeschcke, 2005). In comparison
with other vertebrates, fish are subjected to a larger variety of stressors
because the high variability of water environments. Environmental
changes in salinity, temperature and dissolved oxygen are the most
common causes of stress, and will affect physical and biological system
in fish (Harper and Wolf, 2009). These environmental challenges promote
the variability of acclimatization in fish to different abiotic factors,
ultimately lead to adaptive evolution.

For aquatic organisms, the maintenance of body fluid composition in dilute or concentrated salinity environments are achieved by activating osmoregulation (Marshall and Grosell, 2005). Normally, freshwater species inhabit a hypotonic environment where they tend to gain water and lose salts via exposed membranes; marine species live in a hypertonic environment in which salts are gained and water is lost to the external environment (Hasan et al., 2017). Yet, a minority of species, i.e., the euryhaline fish, have a broad capacity to better adapt to salinity changes. The underlying physiological mechanisms comprise a complex physiological process involving structural and functional modifications in the osmoregulatory organs (gill, kidney and intestine) (Eddy and Handy, 2012; Gonzalez, 2012; Marshall and Grosell, 2005).

In seawater, physiological regulation of these organs includes a combination of branchial and renal excretion of salts, and oral ingestion and intestinal uptake of water; in weakly brackish or freshwater, salts are reabsorbed across the gill and intestine whereas excess water is filtered by the kidney (Evans and Somero, 2008; Marshall and Grosell, 2005). To date, specific mechanisms have been well studied in the gill (Evans et al., 2005; Hwang et al., 2011) and intestine (Grosell, 2006) of fish in response to salinity transfer. The kidney has been studied to understand the mechanisms of ion transport in marine and freshwater species.
(Yancheva et al., 2016), however, the specific role of kidney in euryhaline species has received less attention in comparison with those of gill and intestine.

The kidney functions in integrating ion and water transport in maintaining body fluid concentrations (Varsamos et al., 2005). Hence, studying aspects of renal function in osmoregulation can provide insights to their role in salinity adaptation of euryhaline fish. Although relative studies on cellular morphology (Hasan et al., 2017; Jarial and Wilkins, 2010) and transcriptional changes (Mu et al., 2015; Wang et al., 2014) of kidney have been reported in euryhaline species, the scope of molecular studies is still limited, and the data are not sufficient in revealing the molecular targets to explain the underlying adaptive mechanism.

Recently, evaluation of potential candidate genes involved in salinity tolerance of striped catfish (Pangasianodon hypophthalmus, S.) has been reported in three tissues (gill, kidney and intestine) using the RNA-Seq approach (Nguyena et al., 2016). In addition, proteomic studies have focused on the comparison of fish under salt stress treatments and normal environment to survey differentially expressed proteins (DEPs). Recently developed proteomic techniques, such as isobaric tag for relative and absolute quantitation (iTRAQ), allows identification of more proteins, provides more reliable quantitative measurements and large-scale comparisons than traditional two dimensional electrophoresis (2DE)
analysis (Karp et al., 2010). It is also possible to use iTRAQ for pathway and protein-protein interaction analyses. The proteomic analysis by iTRAQ technique was carried out in gill of marbled eel (*Anguilla marmorata*), DEPs were identified under brackish water/freshwater and seawater/freshwater conditions (Jia et al., 2016).

Moreover, the transcriptomic profile has been combined with proteomic analysis (iTRAQ), as it has been reported that transcriptome deduced proteins could cover almost all of the proteins found in iTRAQ (Tse et al., 2013; Tse et al., 2014). As reported, combination of RNA-seq and iTRAQ was used to reveal the physiological and molecular responses to osmotic stress. Hence, this combination provides us the omics approach to fill the knowledge gap of the genetic basis of salinity adaptation of euryhaline fish, especially for non-model organisms and those lacking reference genomes. In addition, the samples in previously reports were normally collected at only one time point post treatment, and the set time varied depending on different researches. Still, a combined proteomic and transcriptomic analysis regarding the time-course governing of osmoregulation in response to salinity stress is limited.

In the past decade, some euryhaline species such as salmons and eels have been widely used for studying the mechanism of osmoregulation, because of their wide distribution in different geographical locations and spawning migration between freshwater (FW) and seawater (SW) habitats.
Even though much has been achieved in these species, we are attempted to select another experiment animal with smaller size and shorter life cycle, which would be more suited for relative small culture systems. The roughskin sculpin (*Trachidermus fasciatus*) used to distribute widely along the eastern coasts of China, however, wild populations of this species have seriously declined since 1970s due to overfishing and destruction of natural habitats (Cao et al., 2010). *T. fasciatus* exhibits a catadromous lifestyle, i.e., the adults migrate from FW to SW for spawning and the fingerlings migrate from SW to FW (Goto, 1990). This fish has been previously listed as a critically endangered species in China Red Data book of Endangered Animals-Pisces (Yue and Chen, 1998). However, some populations have recently reappeared in traditional habitats, successful farming of these species has been conducted in many fish farms and hatchery stations. Regarding the current facilitation for sampling, here we propose the potential of this species as an experimental animal in biological and ecological studies, for its short life cycle (one year life-span), as well as the high tolerance to different salinity and temperature. So far, the life history and spawning habitats of *T. fasciatus* have been reported (Takeshita et al., 1997; Takeshita et al., 2004; Wang et al., 2000). Recent publications are focusing on molecular mechanism of immune responses (Liu et al., 2012; Yu et al., 2013), genetic diversity and population genetic structure (Gao et al., 2013; Liu et al., 2010; Xu et
In this study, transcriptome sequencing and quantitative shotgun liquid-chromatography mass spectrometry (LC-MS) were conducted to identify salinity adaptation related proteins in *T. fasciatus*. This study will be helpful developing a comprehensive understanding of how the proteome and transcriptome change in association with salinity changes in the kidney, provide more genomic and proteomic background data for the candidacy of *T. fasciatus* to serve as an experimental animal, and gain insight into the osmoregulation net work of euryhaline teleosts.

2. Materials and Methods

2.1 Animal collection, maintenance and salinity control

Adults of *T. fasciatus* (one year-old) were collected at Yuhai Hatchery station (Shandong, China) in December 2014, and then transported to Tongyong Hatchery station (Qingdao, China) where the experiment was carried out. A total of 90 fish were equally separated into three groups, each group was domesticated in a flat bottom FRP tank with an effective volume of 100 L under a 12 h light : 12 h dark photoperiod for two weeks prior to the beginning of the experiment. Over 600 L sand-filtered natural sea water with a salinity of 30 ppt and temperature of 10-12°C was supplied to each tank per day.

At the start of the experiment (time 0 h), samples were collected as control group while fish remained at seawater (30 ppt). Salinity change
commenced thereafter by adding freshwater (3 ppt) into the inflowing seawater to each tank, salinity was gradually reduced at a rate of 1.1 ppt/h over a 24-hour period, then the fish were maintained in freshwater afterwards. Samples were collected at the time points of 12 h (during the salinity treatment), 24 h (immediately after salinity change was achieved) and 48 h (24 hours after the salinity change was achieved). All the fish were collected under dark conditions. The kidney was collected from three individuals of each tank at different time point of 0, 12, 24 and 48 h, tissues from three tanks at each time point were pooled to generate sufficient amounts of sample for both RNA-seq and iTRAQ experiments. Two independent biological replicates for each time point were conducted, and a total of eight samples were collected. The samples were frozen in liquid nitrogen for RNA isolation and protein extraction. All the experimental animal procedures involved in this study were approved by the Yellow Sea Fisheries Research Institute’s animal care and use committee.

2.2 RNA isolation, Illumina sequencing and raw data processing

Total RNA was extracted from mixed kidney tissue of nine fish (three individuals per tank, three tanks) at each time point using the Trizol Kit (Promega, USA) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Takara Bio, Japan) for 30 min at 37°C to remove residual DNA. RNA quality and quantity were
assessed by RNase free agarose gel-electrophoresis and determined using Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA), respectively. Afterwards, RNA samples were used for cDNA library construction following NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, USA). RNA-Seq was performed on the Illumina sequencing platform (Illumina HiSeq™ 2000) using the paired-end technology by Gene Denovo Biotechnology Co. (Guangzhou, China). RNA-sequencing data were filtered by a Perl program to remove low quality reads containing more than 50% of low quality (Q-value ≤ 10) bases, reads with more than 5% of unknown nucleotides (N), and reads containing adapters. Downstream analyses were based on high-quality clean data. The high-quality clean reads were assembled using trinity software as described for de novo transcriptome assembly without a reference genome (Grabherr et al., 2011). Functional annotations and classifications were performed by using Blast2GO (Conesa et al., 2005) and WEGO (Ye et al., 2006) (E value threshold 1 × 10⁻⁵), respectively. The gene expression level is calculated by using RPKM method (Reads Per kb per Million reads) (Mortazavi et al., 2008). To identify differentially expressed genes (DEGs) across samples, the edgeR package (http://www.r-project.org/) was used (Robinson et al., 2010). We identified genes with a fold change ≥ 2 and a false discovery rate (FDR)
< 0.05 in a comparison as significant DEGs at each time point (12, 24 and 48 h) in comparison with 0 h, and numbers of DEGs at all the three time points were summed to obtain the overall DEGs number. The DEGs were used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes database (KEGG, http://www.genome.jp/kegg/pathway.html) enrichment analyses (Minoru Kanehisa et al., 2007; Zhang et al., 2013). Both GO terms and KEGG pathways with a Q-value ≤ 0.05 are significantly enriched in DEGs. All expression data statistic and visualization was conduction with R package (http://www.r-project.org/). Short Time-series Expression Miner (STEM, version 1.2.2b) software was used to perform trend analysis and clustering of gene expression. The software uses a specialized calculation method to cluster genes according to their changing trend of expression level, and each gene cluster has a similar gene expression curve. The clustered profiles with p-value ≤ 0.05 were considered as significant profiles.

The RNA-Seq data have been submitted to the NCBI Short Read Archive (SRA) with an accession number SRA552729 (http://www.ncbi.nlm.nih.gov/sra).

2.3 Protein extraction and iTRAQ analysis

Total proteins were extracted from the same samples as those for RNA-Seq using the cold acetone method (Wu et al., 2014). Protein concentration and quality were determined using the Pierce™ BCA
Protein Assay Kit (Thermo Scientific, USA) and confirmed by SDS-PAGE. 100 µg protein of total protein from each sample was used for protein digestion, the protein was adjusted to a final volume of 100 µL with 8 M Urea. 11 µL of 1 M DTT (DL-Dithiothreitol) was added and samples were incubated at 37 °C for 1 hour. Then 120 µL of the 55 mM iodoacetamide was added to the sample and incubated for 20 min protected from light at room temperature.

For each sample, proteins were precipitated with ice-cold acetone, then re-dissolved in 100 mM TEAB (triethylammonium bicarbonate). Proteins were then tryptic digested with sequence-grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. The resultant peptide mixture was labeled with iTRAQ tags 113-119 and 121, respectively. The labeled samples were combined and dried in vacuum. Protein samples were subject to iTRAQ labeling, strong cation exchange (SCX) fractionation and reverse-phase nanoliquid chromatography/tandemMS (LC-MS/MS) analysis.

All of the mass spectrometry data were collected using Triple TOF™ 5600 LC/MS/MS and analyzed using the Data Analysis Software. The mass spectrometry data were transformed into MGF files with Proteome Discovery 1.2 (Thermo, Pittsburgh, PA, USA) and analyzed using Mascot search engine (Matrix Science, London, UK; version 2.3.2). Mascot database was set up for protein identification using Trachidermus.
*fasciatus* reference transcriptome. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and peptide Mass Tolerance of 20.0 ppm. The Mascot search results were averaged using medians and quantified. Proteins with fold change in a comparison > 1.2 or < 0.83 and unadjusted significance level $P < 0.05$ were considered differentially expressed. DEPs number at each time point (12, 24 and 48 h) in comparison with 0 h was calculated, and numbers of DEPs at all the three time points were summed to obtain the overall DEPs number.

The search results were passed through additional filters before exporting the data. For protein identification, the filters were set as follows: significance threshold $P < 0.05$ (with 95% confidence) and an ion score or expected cut-off of less than 0.05 (with 95% confidence). For protein quantitation, the filters were set as follows: ‘median’ was chosen for the protein ratio type (http://www.matrixscience.com/help/quant_config_help.html); the minimum precursor charge was set to 2+, and the minimum peptide was set to 2 (Xu et al., 2016); only unique peptides were used to quantify the proteins. The median intensities were set to normalization, and outliers were removed automatically. The peptide threshold was set as above for identity.

To predict the functions of the differentially expressed proteins (DEPs), we analyzed the proteins with regard to three aspects. Proteins were
annotated using blastp against GO, KEGG and COG/KOG database to obtain their functions. Significant GO functions and pathways were examined within differentially expressed proteins with $P$ value $\leq 0.05$.

The category gene enrichment test of all proteins was performed using Blast2GO to determine whether the DEPs were significantly enriched in any functional subcategories (Conesa et al., 2005). An FDR significance threshold of 0.05 was selected. Lastly, we allocated the DEPs to biological pathways using the KEGG resource (www.genome.jp/kegg/). A 1.2-fold cutoff value was used to identify up-regulated and down-regulated proteins with a $P$-value of less than 0.05 (Song et al., 2016). The iTRAQ was conducted by the Guangzhou Gene denovo Biotechnology Co., Ltd.

2.4 Association analysis and co-expression analysis

To investigate the concordance between transcriptome and proteome results in this study, we calculated the Pearson’s correlation for these data and created scatter plots with the expression ratios of each time point during or post salinity change (12, 24 and 48 h) versus the control group (0 h). Values were considered significantly positively correlated when $R > 0.80$, while moderate positive correlation was determined when $0.50 < R < 0.80$.

To illustrate the molecular mechanisms associated with salinity adaptation, the protein/protein regulatory network analysis was carried
out by analyzing co-expression status of each pair of selected proteins (between the 19 selected ion transport and metal binding proteins and 378 DEPs identified by iTRAQ, listed in Table S1 and S6). The Pearson correlation was calculated, and paired proteins of ion transport and metal binding protein interactors with a significant correlation based on Pearson correlation coefficient > 0.90 were selected for further analysis. Cytoscape 3.2.1 software was applied to integrate the co-expression relationship of ion transport and metal binding protein interactors with their associated pathways (Kong et al., 2017).

2.5 The cDNA synthesis and quantitative real time PCR

The first-strand cDNA was synthesized from total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio., China) following the manufacturer’s instructions. Transcriptomic data were validated by performing qRT-PCR on a total of 11 genes to detect their mRNA levels at all the four time points (0, 12, 24 and 48 h). Primer sequences are tabulated in Table S2. The qRT-PCR was conducted using SYBR® Premix Ex Taq™ (Takara Bio., China) by a 7500 ABI Real-time PCR system (Applied Biosystems, USA). A dissociation protocol was always performed after thermocycling to determine target specificity. Control amplifications were always included. PCR amplifications were performed in triplicate. Expression of 18s was used as the internal control. The ratio changes in the target genes relative to the
control gene were determined by the $2^{\Delta \Delta CT}$ method (Livak and Schmittgen, 2001) and the transcript level was described in terms of its relative concentration ($RC_{\text{target}} / RC_{\text{control}}$).

All data were expressed as mean ± standard deviation (S.D.) and analyzed by one-way ANOVA (analysis of variance) to determine significant differences between means using the Statistical Package for the Social Sciences, SPSS (version 16.0). Values were considered statistically significant when $P < 0.05$. The strength of association between qRT-PCR and RNA-seq results was evaluated by calculating the Pearson product-moment correlation coefficient (R).

2.6 SDS-PAGE and Western blotting assay

Protein concentrations were determined using the BCA method. Samples (40 µg per lane) were separated on a 12% polyacrylamide gel under reducing conditions and then blotted onto a PVDF membrane (Millipore, USA). The membranes were blocked in blocking buffer (TBST, 5% skimmed milk in TBS containing 0.05% Tween-20) for 2 h at room temperature, and incubated overnight at 4 °C with primary antibodies in TBST containing 1% skimmed milk. The incubation were respectively conducted using rabbit antibodies to Aquaporin 1 (Aqp1, 1:300; Boster, China), Solute carrier family 12 member 3 (Slc12a3, 1:200; Boster, China), Calmodulin (Calm1, 1:800; Cusabio, China) or β-actin (1:200; Boster, China), followed by incubation with goat anti-rabbit horseradish
peroxidase (HRP)-conjugated IgG (1:50000; Boster, China) for 2 h at 37 °C. The reactive protein bands on the membrane were visualized using ECL (Tiangen, China) and exposed in the darkroom. The expression intensities of gene-specific bands were normalized against the β-actin bands.

3. Results

3.1 Data obtained from the T. fasciatus kidney transcriptome analysis

To identify DEGs that respond to salinity change, gene expression profiles in kidney at different time points (0, 12, 24 and 48 h) were investigated using the RNA-Seq technique. The transcriptome sequencing of eight samples resulted in a total of 387,485,698 reads. These reads were then de novo assembled into 85,994 transcripts. Using the BLASTx algorithm (E-value < 10^-5), all assembled unigenes was searched against the databases of NCBI Nr, Swiss-Prot, COG, and KEGG (Li et al., 2017; Liping Ma et al., 2017). Of all the 28,010 unigenes that could be annotated by all four databases, 27,912 and 24,276 had homologous sequences in the Nr and Swiss-Prot protein databases, while 8,074 and 14,188 unigenes could be classified by COG and KEGG databases, respectively.

A BLASTx top-hit species distribution showed that 10,092 unigenes exhibited similarity to the sequences of Maylandia zebra, 6,931 to the sequences of Oreochromis niloticus, 2,856 to the sequence of Takifugu
rubripes, and 2,076 to the sequence of *Oryzias latipes* (Fig.S1).

For functional prediction and classifications, all unigenes were aligned to the COG database and grouped into 25 COG classifications. Gene Ontology analysis of our dataset showed that 9,114 genes were grouped into cellular component, 10,211 were grouped into molecular function, and 10,683 were grouped into biological process (Fig.S2). For further identification of the biological pathways in *T. fasciatus*, we mapped the assembled sequences to 240 different KEGG pathways.

### 3.2 Identification of DEGs at the mRNA level

The number of DEGs exhibiting significant (FDR < 0.001) and differential (ratio value > 2 or < 0.5) expression pattern at 12, 24 and 48 h time points was respectively shown in Fig.1B. Compared with 0 h, 549 up- and 510 down-regulated genes at 12 h, 51 up- and 47 down-regulated genes at 24 h, 250 up- and 386 down-regulated genes at 48 h were respectively detected. Overall, a total of 1504 DEGs were detected at all the three time points in comparison with 0 h. The list of DEGs was presented in Table S3. Obviously, salinity change led to dramatic alteration of gene expression at early time point when the fish were exposed to the ambient stress.

GO assignments of the 1504 DEGs showed that these unigenes were categorized into 44 functional groups, which could be classified into three main categories (biological process, cellular component and molecular
function), which respectively contains 20, 15 and 9 functional subcategories (Fig. 2). The assembled unigenes were annotated against the KEGG database and assigned to the 175 KEGG pathways (Table S4). The majority of these pathways were shown in Fig. 3, with the top three of pathway enrichment as ‘Cytokine-cytokine receptor interaction’ (30 genes), ‘Jak-STAT signaling pathway’ (20 genes) and ‘Carbon metabolism’ (20 genes).

3.3 Trend analysis and clustering of gene expression

In this study, we tracked the mRNA level changes of DEGs during the salinity-treatment time course (from 12 h to 48 h) by using k-mean clustering. As a result, 26 clusters were retrieved, of which seven were evaluated as statistically significant \( (P < 0.05) \) (Fig. 4). Of the seven significant trends, profile 18, 19 and 21 represented genes whose expression were significantly increased at 12 h; in profile 18 and 19, a total of 161 and 123 genes showed similar expression pattern with the mRNA levels decreasing at 24 h but then followed by opposite trend; profile 21 consisted of the maximum numbers of genes \( (N = 188) \), whose expression increased at 12 h and then stayed constant afterwards. The other four profiles (3, 4, 6 and 7) represented genes whose expression initially decreased at 12 h and then followed by different variation trend in mRNA expression. GO analyses of genes from these seven clusters indicated that the most number of genes enriched in cellular process and
metabolic process classes in BPs, as well as binding in MFs (Table S5).

### 3.4 Identification of DEPs through iTRAQ

Four time points (0, 12, 24, and 48 h) were selected for profiling the osmoregulatory responsive proteome changes. By using the iTRAQ labeling and LC–MS/MS analysis, a total of 4997 proteins in *T. fasciatus* kidney from all the time points were identified at a 95% confidence level. The identified proteins cover a wide range of cellular components (1722, 34%), molecular functions (1908, 38%), and biological processes (1929, 39%).

Proteomic changes were first examined between the three time points (12, 24 and 48 h) and 0-h control. Fish at the 12 h time point showed the most changes, with 179 (55 up- and 124 down-regulated) proteins differentially expressed under salinity treatment. When compared with 0 h, 39 up- and 53 down-regulated proteins were identified at 24 h, 96 up- and 76 down-regulated proteins at 48 h were detected (Fig. 1D). Overall, a total of 378 DEPs were identified (Table S6). Hierarchical clustering results revealed that salinity stress response varied dramatically at the three time points (Fig. 5).

Under GO analysis of the 378 identified DEPs, significant enrichment (*P* < 0.05) was respectively found for 18, 17, and 10 categories in the biological process (BP), cellular component (CC), and molecular function (MF) domains. The most enriched GO terms were cellular process,
metabolic process, and single-organism processes in BPs; cell, as well as cell part in CCs; and binding in MFs (Fig.6).

A total of the 125 pathways were identified under KEGG analysis (Table S7), 14 of which were significantly enriched ($P < 0.05$). The high representation of ‘Focal adhesion’, ‘Phagosome’, ‘Ribosome’, ‘ECM-receptor interaction’ and ‘Spliceosome’ pathways were shown in Fig.7.

3.5 Association analysis of transcriptome and proteome data

As the transcriptomic and proteomic data were obtained from exactly the same samples at the same time point, we determined the number of identified proteins for which corresponding transcripts were represented in the RNA-seq data to examine their congruence. The distribution of the corresponding mRNA : protein ratios was shown by a scatter plot analysis of the log$_2$-transformed ratios (Fig.8). Analyses of data from different experimental groups (12, 24 and 48 h) revealed a total of 102 concordant dots, representing a correspondence of protein abundance with transcript accumulation (red dots) were identified. Up-regulated trend of 22, 9 and 26 genes respectively at the time points of 12, 24 and 48 h was significantly associated between DEGs and DEPs. Down-regulated trend of 14, 7 and 17 genes were also revealed. In addition, 216 green dots (transcripts only) and 901 blue dots (proteins only) were identified, indicating differential expression was only found on the transcript or the
protein levels.

When analyzing the 102 concordant dots, the proteins that overlapped at different time points as well as those shared the same annotation were excluded. As a result, the expression of 66 proteins were altered significantly at three time points post seawater to freshwater transfer in this study, 38 proteins were up-regulated and 28 were down-regulated (Table S8). Among the 66 proteins, 19 were identified as ion transport and metal binding proteins, 9 were immune- and stress-related proteins, etc.

We further investigated the co-expression status of ion transport and metal binding protein interactors in the 378 DEPs identified by iTRAQ, and 169 co-expression proteins with the 19 selected ion transport and metal binding proteins were identified (Table S1). Next, the networks of the co-expression of the aforementioned interactors with their associated pathways were integrated through Cytoscape 3.2.1 software. Our data showed that a complex co-expression network was formed (Fig.S3). In addition, through pathway analysis, we found that 75 of the 169 co-expression proteins were annotated with significantly enriched KEGG pathways.

3.6 Validation of selected genes by quantitative real-time polymerase chain reaction

Concordance between qPCR and RNA-seq based mRNA expression for a
total of 11 genes was examined in this study (Fig. 9). Of the 11 genes selected for validation of transcriptome data, nine were chosen from the concordant red dots in the aforementioned association analysis whose protein abundance were shown to be correspondence with mRNA transcription levels. These genes were shown to be involved in response to stimulus (F13a1, Vrk2, Msrb2 and Cmpk2), ion transport (Pvalb, Calm1 and Slc12a3), fatty acid metabolism (ES1) and immunity (MHC class I). Moreover, two osmoregulation-related genes (Cldn4 and Aqp1) were used for validation of osmoregulation expression pattern in our results. A linear regression analysis was performed to confirm the reliability of RNA-seq data, as shown in Fig. 9, a high correlation of gene expression level was detected between RNA-Seq and qRT-PCR in all the 11 genes.

In accordance with the association analysis, data indicated that mRNA expression of these genes, even with the genes of similar functions, exhibited different expression patterns after salinity change. For instance, among the four genes involving in response to stimulus, the expression of Cmpk2 and Vrk2 were up-regulated and then followed by down-regulated; while the expression of F13a1 and Msrb2 showed decreasing trend. Pvalb expression was significantly down-regulated after salinity change, while the other two genes related to ion transport showed different patterns. Moreover, Aqp1 (osmoregulation-related gene) mRNA level decreased
sharply after salinity change, while Cldn4 expression was slightly down-regulated and then followed by an increment at 48 h.

3.7 Validation of selected proteins by Western blot

Validation of the iTRAQ results was limited by the availability of antibodies for the fish species. We used mammalian antibodies and some of them resulted in specific bindings. As shown in Fig.9, the expression of three proteins (Aqp1, Calm1 and Slc12a3) showed a gradually decreasing trend. Further comparison between the Western blot results and time-course qPCR data revealed a mainly positive correlation, two inconsistent data were detected, i.e., the expression of Calm1 at 24 h and that of Slc12a3 at 48 h.

4. Discussion

The fish were subjected to a salinity transfer achieved in 24 hours in this study, our previous research on gill physiological responses (including the branchial Na⁺/K⁺-ATPase relating to osmoregulation and caspase 3/7 relating to apoptosis) of exactly the same fish here revealed no significant difference in the enzyme activities at all the three time points (Manuscript under review). Since fish gills are directly exposed to water environments, the branchial enzyme activities were shown to be indicative of salinity acclimation in fish (Imsland et al., 2003; Martínez-Álvarez et al., 2005). Our data might have reflected the stable physiological levels of T. fasciatus during, and even post the salinity treatment.
In addition to the physiological data, we did observe significant variations in the expression of three stress-related genes (\textit{sirt1}, \textit{hsf1} and \textit{hsp70}) at the corresponding time points (Manuscript under review). These results suggested that the expression of stress-related genes were significantly altered, even though the relatively sensitive physiological indicators were not affected. Regarding these previous results, questions have been raised such as what genes were contributing to sustain the stable physiological level, and how their expression patterns would be along with the salinity change.

In this study we monitored the variation of DEGs and DEPs for three time points within the 48 h period after the onset of salinity change, to pursue a scoping on the trends in multi omics of \textit{T. fasciatus}. Firstly, we found the largest number of DEGs and DEPs at 12 h time point, which was in the middle of the 24-h salinity transfer. One possible reason for this is that fish normally suffer the most severe challenges during the environment change, expression changes of many genes would be identified at this time. Although some of the genes identified at this time might not be classified as closely related gene (such as salt-response genes in this study), they should not be overlooked, since further studies would clarify their function even though they were identified as nonresponsive at first (like the role of NFAT5 listed by Wang et al., 2014).
Wang et al. (2014) also suggested that changes in the transcription rates of genes could be missed due to the experimental design such as single time-point sampling. Multiple time points spanning pre- and post- salinity change were performed in our research. Variations in DEGs and DEPs identified at different time points supported Wang’s point of view, showing that the alteration in proteins and genes, as well as activation of relative pathways, were time-dependent under salinity treatments.

4.1 Proteomic responses during and post the seawater-to-freshwater transfer

Of the 378 identified DEPs in this study, 66 quantifiable proteins were significantly changed at both the transcript and protein levels, with 38 displaying increased abundance levels and 28 displaying decreased abundance levels. Based on the annotation of these DEPs, most of the immune- and stress- related proteins were identified to be up-regulated, while other proteins related to ion transport, metal binding, cytoskeleton, etc. were found in both up- and down- regulated proteins. Moreover, the altered immune-related proteins overlapped at different time points, but most of the proteins related to ion transport and other functions rarely exist simultaneously at different sampling time.

Immune- and stress- related proteins were up-regulated

In this study, the salinity change was achieved in a relative short time (24 h), which might lead to an acute stress response. Under this circumstance,
some of the DEGs and DEPs would be related to stress resistance. As shown in Table S8, a total of 8 of the 18 up-regulated proteins detected at 12 h had roles in immune and stress response. These proteins included MHC class I alpha antigen, fibrinogen beta chain, G-type lysozyme B, GTPase IMAP family member 4-like, etc. Immune factors, such as MHC class I antigen etc., have been reported to be linked to osmotic stress, the decreased expression levels of immune-related proteins in the seawater might indicate that their functional roles are more important in the freshwater environment (Narnaware et al., 1998; Tse et al., 2013). Under this circumstance, the up- and down-regulation of aforementioned stress-related proteins identified in this study might also be related to freshwater adaptation.

In this study, the protein G-type lysozyme B was not only identified at 12 h, but also found to be the only up-regulated immune-related protein identified at 24 h (Table S8). Lysozymes were shown to be involved in innate immunity and physiological activities in fish (Gao et al., 2016; Wang et al., 2016). It has been reported by Schmitz et al. (2016) that salinity significantly enhanced plasma lysozyme activity in striped catfish (*Pangasianodon hypophthalmus*, S.). Higher lysozyme activities during both acute and chronic hyperosmotic stress have also been described in euryhaline species (Dominguez et al., 2005; Jiang et al., 2008; Yada et al., 2001). Here we reported the potential role of G-type lysozyme B in
freshwater acclimation of *T. fasciatus*. Similar to G-type lysozyme B, five stress-regulated proteins were found to be induced at both 12 and 48 h time points (Table S8). The up-regulation of these proteins at different time points revealed that the activation of the stress-related proteins could last for 24 more hours after the treatment.

Among the 28 down-regulated proteins observed at all the three time points, only one (heat shock protein 90 alpha) was shown to be related to stress-response. However, nearly half of the up-regulated proteins were immune- or stress- related factors. The observation of up-regulated expression of immune factors in the freshwater environment suggested that the salinity change might activate the stress response process, the association between the role of these proteins and osmoregulation still need further investigation.

**Ion transport and metal binding proteins were significantly altered**

A total of five of the 38 up-regulated proteins, as well as 14 of the 28 down-regulated proteins were shown to be related to ion transport and metal binding. As described by Tse et al. (2014), the ion transporters were considered as effectors to induce different mechanisms to compensate for the osmotic challenge. As the end point effectors for the latter phase of the osmotic response, their fast response were triggered by ambient salinity change.

Among the aforementioned 17 altered proteins, eight were involved in
calcium binding and transport. Ca$^{2+}$ is an important and universal second messenger participating in different signaling cascades, especially the osmosensing pathways (Fiol and Kültz, 2007). Proteins related to Ca$^{2+}$ homeostasis, such as calcium-sensing receptor, were shown to be salinity dependent in some osmoregulatory tissues (Loretz et al., 2012; Loretz et al., 2009). In this study, we found that eight proteins (Stanniocalcin, Vitellogenin B, Calmodulin, etc.) were down-regulated in kidney of freshwater-acclimating fish.

In fish, Stanniocalcin is a key endocrine factor that acts on gill, intestine and kidney to regulate serum calcium and phosphate homeostasis (Yeung et al., 2012). The primary function of stanniocalcin is the inhibitory effects on Ca$^{2+}$ uptake in response to excess serum calcium in fish (Olsen et al., 1996; Tseng et al., 2009). It is also involved in negative regulation of Cl$^{-}$ uptake (Guhab and Hwangb, 2016). Yet the down-regulation of Stanniocalcin at 12 h in this study was induced by the seawater to freshwater transfer, which might result in the fall in Ca$^{2+}$ and Cl$^{-}$ levels.

Previous studies have demonstrated that calcium in oestrogen-treated freshwater trout was bound to Vitellogenin, i.e., the circulating plasma levels of Vitellogenin was induced by oestrogen-treatment, which resulted in increased circulating levels of Vitellogenin and Vitellogenin-bound calcium (Persson et al., 1994). Similarly, circulating plasma levels of Vitellogenin and total calcium were also found to be simultaneously
induced by oestradiol-17 beta treatment in sea bream (Guerreiro et al., 2002). These reports suggested the association between Vitellogenin and calcium levels in response to exogenous induction. In addition to the calcium binding and transport proteins, an ion channel impairing toxin (Stonustoxin) and a calcium channel impairing toxin (Stonustoxin), were found to be significantly up-regulated at 12 h in T. fasciatus kidney, indicating the negative effect of salinity transfer on permeability of calcium ions. Although our current understanding of calcium signaling in fish osmotic responses is limited, the identification of these proteins may help in clarifying their roles in mechanisms of ionic regulation.

Other ion transport proteins, such as solute carrier family 22 member 6, coagulation factor XIII A chain, betaine homocysteine s-methyltransferase and Methionine-R-sulfoxide reductase B2, were identified as the significantly down-regulated concordant dots. On the other hand, proteins like trypsin, 60S ribosomal protein L27a and epidermis-type lipoxygenase 3-like, were induced by the environmental salinity decrement. To our knowledge, the regulation of these factors at both RNA and protein levels induced by salinity variation was first reported in our study, and the mechanism underlying this phenomenon needs further investigation.

Other proteins related to salinity change
Achieving ion homeostasis during osmotic stress is contingent upon the cell’s ability to recognize and quantify environmental osmolality and arrange an appropriate response (Evans and Somero, 2008). Upon osmotic challenge, effective regulation of cytoskeleton dynamics is essential for cell–cell adhesion and cell volume regulation (Ciano et al., 2002; Wehner et al., 2003). Therefore, work must be aimed at exploring the proteins and their roles in cellular component. Our study showed that short-term seawater to freshwater transfer induced expression of several cytoskeleton proteins. Cellular component (complement component 7, protamine-like protein), cytoskeleton (formin-like protein 1-like, tubulin alpha), cell adhesion (ribosomal protein L34), etc., were significantly altered, these findings highlighted the role of the cytoskeleton in response to osmotic stress.

4.2 Further association analysis and verification of RNA-seq data

As shown in Fig.9, high correlations were detected between qPCR- and RNA-seq based expression in most of the detected genes, confirming the reliability of RNA-seq data we obtained. Two osomoregulation-related genes, i.e., Clcn4 and Aqp1, were used as control for the seawater/freshwater transfer effects. Aqp1 showed a gradual decrease in gene expression along with the freshwater transfer in this study. Similar results were also reported by Giffard-Mena et al. (2007) that the freshwater-acclimated fish exhibited lower mRNA levels. However,
Wong et al. found that the Aqp1 expression as a control for the salinity acclimation was puzzling since the expression trend could differ from different species (Wong et al., 2014). For instance, the decreased Aqp1 expression in medaka and seabream intestine was detected after seawater transfer, while seawater acclimation up-regulated Aqp1 expression in the eel intestine (An et al., 2008; Aoki et al., 2003; Wong et al., 2014). Even though the species-specific difference in Aqp1 expression might indicate various ion transporting mechanisms existing in different teleost lineages (Wong et al., 2014), the Aqp1 expression trend in *T. fasciatus* kidney could also suggest the activation of cell water volume regulation. In addition, mammalian Aqp1 antibody specifically detected a separate protein in the kidney of *T. fasciatus*, which also resulted in a similar decrease trend.

Calmodulin, a multifunctional calcium sensor protein that participates in various cellular processes under normal, stress and pathological conditions (Li et al., 2014), was also found to be significantly regulated under freshwater transfer. In comparison with the RNA-seq and Western-blot results, the qRT-PCR revealed dramatically variation of Calm1 mRNA levels. Still, high correlation was identified among the aforementioned three data. Moreover, CaM binding protein (plasmalemma vesicle-associated protein) was found to be inhibited at 12 h in *T. fasciatus* kidney, which happened 12-h prior to the
down-regulation of CaM, suggesting the action of CaM and its related proteins might be time-dependent in response to salinity change.

Another ion transporter, Slc12a3, was also used for qRT-PCR and Western-blot to examine whether the gene/protein was affected by freshwater transfer. High correlation was determined between the qPCR- and RNA-seq result, and the protein levels also showed a similar trend excepted for the 48 h time point. Basically, these results clarified the concordance of regulated gene and protein expression by salinity treatment. So far, our researches first reported the potential role of Slc12a3 in salinity adaptation. However, SLC12A1 (Solute Carrier Family 12: Member 1) was shown to be critical for calcium (re)absorption and homeostasis in the kidney (Zhu et al., 2014). Previous studies indicated that SLC12A1 showed a gradual increase in gene expression under seawater transfer (Wong et al., 2014). These findings provides us a new direction to take the Solute Carrier Family as a candidate gene family for further functional studies.

5. Conclusions

The present study showed the successful acclimation of *T. fasciatus* to a transfer from seawater to freshwater achieved within 24 hours, suggesting the excellence of this species as an experimental animal revealing the regulatory mechanism of salinity adaption in euryhaline fish. To better understand the time course gene and protein expression mechanisms
underlying the relative physiological functions, we presented the kidney
specific analysis using a combined method of RNA-seq and iTRAQ
technologies. A total of 66 differentially expressed proteins were filtered
by correlation of transcriptome and proteome results. Among these
proteins, the class of ion transport and metal binding proteins, especially
those related to calcium binding and transport, showed the most
prominent differences between the experimental and control groups.
From these differentially expressed proteins, candidate genes (Calm1,
SLC12A1, Pvalb, Aqp1, etc) as well as their gene families for further
studies were revealed. In addition, strong variation of immune-/stress-
related and cytoskeleton proteins in the experimental group suggested an
important role of these proteins in the process of salinity stress response.
Overall, the integrative transcriptomic and proteomic data can provide a
large number of valuable dataset to reveal the physiological process of
osmoregulation in _T. fasciatus_, and a significant step forward towards a
elucidation of the mechanism of salinity response and adaptation
underlying fish migration.

**Declarations**

**Ethics approval and consent to participate**

All the experimental animal procedures involved in this study were
approved by the Yellow Sea Fisheries Research Institute’s Animal Care
and Use Committee.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

The study was conceived by Q M, X-F L and Z-M Z. Sample collection and preparation was done by Q M, X-F L, W-R F and S-F L. The qRT-PCR and Western blot assay was performed and analyzed by Q M and W-R F. Q M wrote the paper and all authors provided valuable feedback on the manuscript and approved the paper for submission.

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Fig. 1 Differentially expressed genes or proteins at different time points (12, 24 and 48 h respectively compared to 0 h) in *Trachidermus fasciatus*. (A) Venn diagram showing the number of genes differentially expressed. (B) Clusters of differently expressed genes (up-regulated and down-regulated). (C) Venn diagram showing the number of proteins differentially expressed. (D) Clusters of differently expressed proteins (up-regulated and down-regulated).

Numbers on the top of the columns represent the gene or protein numbers. Red indicates up-regulated and green indicates down-regulated.
Fig. 2 GO assignments of the 1504 differentially expressed genes (DEGs).
Fig. 3 Top 20 of pathway enrichment of the 1504 differentially expressed genes by KEGG.

Fig. 4 Sketch map of the cluster analysis of differentially expressed genes. Filled color clusters show clusters with significant trends ($P$ value < 0.05). The number at the lower left corner of each cluster represents the number of genes in the cluster.
Fig. 5 Hierarchical clustering of the 378 differentially expressed proteins. Left, protein tree. The color scale bar in the right, bottom corner indicates increased (red) and decreased (green) levels, and no significant changes (black) of proteins in response to salinity change.

Fig. 6 Gene ontology (GO) analysis of the 378 differentially expressed proteins (DEPs) responded to salinity change.
Fig. 7 Top 20 of pathway enrichment of the 378 differentially expressed proteins (DEPs) by KEGG.

Fig. 8 Comparison of expression ratios from transcriptomic (y-axis) and
proteomic (x-axis) profiling. (A) 12 h/0 h; (B) 24 h/0 h; (C) 48 h/0 h.

Log2 expression ratios were calculated from time points post salinity change (12, 24, 48 h) versus 0 h. Significant changes in expression are color-coded: blue, proteins only; green, transcripts only; red, both.

Fig.9 Extension and validation of the RNA-Seq and iTRAQ results. A-K: Relative mRNA expression levels at different time points by qPCR-PCR. I: The altered expression of the three identified proteins by western blot, controls of equal protein loading were confirmed by β-actin expression.

Cmpk2: \textit{UMP-CMP kinase 2}; ES1: \textit{ESI protein homolog}; F13a1: \textit{coagulation factor XIII A chain}; MHC class I: \textit{MHC class I alpha antigen}; Msrb2: \textit{Methionine-R-sulfoxide reductase B2}; Pvalb: \textit{Parvalbumin beta}; Vrk2: \textit{serine/threonine-protein kinase VRK2}; Cldn4: \textit{claudin 4}; Aqp1: \textit{aquaporin 1}; Calm1: \textit{Calmodulin 1}; Slc12a3: \textit{solute carrier family 12 member 3}. Statistical significances are indicated with an asterisk at \( P < 0.05 \), and two asterisks at \( P < 0.01 \).