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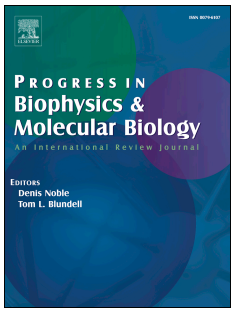


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# Analyses of the molecular mechanisms associated with salinity adaption of *Trachidermus fasciatus* through combined iTRAQ-based proteomics and RNA Sequencing-based transcriptomics

Qian Ma[1,2], Xinfu Liu[1], Wenrong Feng[1], Shufang Liu[1,2], Zhimeng Zhuang[1,3*]

1. Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China
2. Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, China
3. Function Laboratory for Marine Biology and Biotechnolgy, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, China



*Correspondence to: Zhimeng Zhuang, Yellow Sea Fisheries Research Institute, 106 Nanjing Road, Qingdao 266071, P.R. China. Email: zhuangzm@ysfri.ac.cn; Fax: 0086-532-85811514; Tel: 0086-532-85836344

## 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 osomoregulation (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)

111 analysis (Karp et al., 2010). It is also possible to use iTRAQ for pathway
112 and protein-protein interaction analyses. The proteomic analysis by
113 iTRAQ technique was carried out in gill of marbled eel (*Anguilla*
114 *marmorata*), DEPs were identified under brackish water/freshwater and
115 seawater/freshwater conditions (Jia et al., 2016).
116 Moreover, the transcriptomic profile has been combined with proteomic
117 analysis (iTRAQ), as it has been reported that transcriptome deduced
118 proteins could cover almost all of the proteins found in iTRAQ (Tse et al.,
119 2013; Tse et al., 2014). As reported, combination of RNA-seq and iTRAQ
120 was used to reveal the physiological and molecular responses to osmotic
121 stress. Hence, this combination provides us the omics approach to fill the
122 knowledge gap of the genetic basis of salinity adaptation of euryhaline
123 fish, especially for non-model organisms and those lacking reference
124 genomes. In addition, the samples in previously reports were normally
125 collected at only one time point post treatment, and the set time varied
126 depending on different researches. Still, a combined proteomic and
127 transcriptomic analysis regarding the time-course governing of
128 osmoregulation in response to salinity stress is limited.
129 In the past decade, some euryhaline species such as salmons and eels
130 have been widely used for studying the mechanism of osmoregulation,
131 because of their wide distribution in different geographical locations and
132 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 be 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

155 al., 2009).

156 In this study, transcriptome sequencing and quantitative shotgun
157 liquid-chromatography mass spectrometry (LC-MS) were conducted to
158 identify salinity adaptation related proteins in *T. fasciatus*. This study will
159 be helpful developing a comprehensive understanding of how the
160 proteome and transcriptome change in association with salinity changes
161 in the kidney, provide more genomic and proteomic background data for
162 the candidacy of *T. fasciatus* to serve as an experimental animal, and gain
163 insight into the osmoregulation net work of euryhaline teleosts.

164 **2. Materials and Methods**

165 **2.1 Animal collection, maintenance and salinity control**

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

175 At the start of the experiment (time 0 h), samples were collected as
176 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℃ 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 $\leqslant$ 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 $1 \times 10^{-5}$), 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 $\geqslant$ 2 and a false discovery rate (FDR)

221 < 0.05 in a comparison as significant DEGs at each time point (12, 24 and
222 48 h) in comparison with 0 h, and numbers of DEGs at all the three time
223 points were summed to obtain the overall DEGs number. The DEGs were
224 used for gene ontology (GO) and Kyoto Encyclopedia of Genes and
225 Genomes database (KEGG, http://www.genome.jp/kegg/pathway.html)
226 enrichment analyses (Minoru Kanehisa et al., 2007; Zhang et al., 2013).
227 Both GO terms and KEGG pathways with a Q-value ≤ 0.05 are
228 significantly enriched in DEGs. All expression data statistic and
229 visualization was conduction with R package (http://www.r-project.org/).
230 Short Time-series Expression Miner (STEM, version 1.2.2b) software
231 was used to perform trend analysis and clustering of gene expression. The
232 software uses a specialized calculation method to cluster genes according
233 to their changing trend of expression level, and each gene cluster has a
234 similar gene expression curve. The clustered profiles with p-value ≤
235 0.05 were considered as significant profiles.
236 The RNA-Seq data have been submitted to the NCBI Short Read Archive
237 (SRA) with an accession number SRA552729 (http://
238 www.ncbi.nlm.nih.gov/sra).

239 **2.3 Protein extraction and iTRAQ analysis**

240 Total proteins were extracted from the same samples as those for
241 RNA-Seq using the cold acetone method (Wu et al., 2014). Protein
242 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℃ 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 $\leqslant$ 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

309 out by analyzing co-expression status of each pair of selected proteins
310 (between the 19 selected ion transport and metal binding proteins and 378
311 DEPs identified by iTRAQ, listed in Table S1 and S6). The Pearson
312 correlation was calculated, and paired proteins of ion transport and metal
313 binding protein interactors with a significant correlation based on Pearson
314 correlation coefficient > 0.90 were selected for further analysis.
315 Cytoscape 3.2.1 software was applied to integrate the co-expression
316 relationship of ion transport and metal binding protein interactors with
317 their associated pathways (Kong et al., 2017).

318 **2.5 The cDNA synthesis and quantitative real time PCR**

319 The first-strand cDNA was synthesized from total RNA using
320 PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio., China)
321 following the manufacturer's instructions.

322 Transcriptomic data were validated by performing qRT-PCR on a total of
323 11 genes to detect their mRNA levels at all the four time points (0, 12, 24
324 and 48 h). Primer sequences are tabulated in Table S2. The qRT-PCR was
325 conducted using SYBR® *Premix Ex Taq*™ (Takara Bio., China) by a 7500
326 ABI Real time PCR system (Applied Biosystems, USA). A dissociation
327 protocol was always performed after thermocycling to determine target
328 specificity. Control amplifications were always included. PCR
329 amplifications were performed in triplicate. Expression of 18s was used
330 as the internal control. The ratio changes in the target genes relative to the

control gene were determined by the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001) and the transcript level was described in terms of its relative concentration ($RC_{target}$ / $RC_{control}$).

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*

375 *rubripes*, and 2,076 to the sequence of *Oryzias latipes* (Fig.S1).

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

383 **3.2 Identification of DEGs at the mRNA level**

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

394 GO assignments of the 1504 DEGs showed that these unigenes were
395 categorized into 44 functional groups, which could be classified into three
396 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,

441 metabolic process, and single-organism processes in BPs; cell, as well as
442 cell part in CCs; and binding in MFs (Fig.6).
443 A total of the 125 pathways were identified under KEGG analysis (Table
444 S7), 14 of which were significantly enriched ($P < 0.05$). The high
445 representation of 'Focal adhesion', 'Phagosome', 'Ribosome',
446 'ECM-receptor interaction' and 'Spliceosome' pathways were shown in
447 Fig.7.

448 **3.5 Association analysis of transcriptome and proteome data**

449 As the transcriptomic and proteomic data were obtained from exactly the
450 same samples at the same time point, we determined the number of
451 identified proteins for which corresponding transcripts were represented
452 in the RNA-seq data to examine their congruence. The distribution of the
453 corresponding mRNA : protein ratios was shown by a scatter plot analysis
454 of the $\log_2$-transformed ratios (Fig.8). Analyses of data from different
455 experimental groups (12, 24 and 48 h) revealed a total of 102 concordant
456 dots, representing a correspondence of protein abundance with transcript
457 accumulation (red dots) were identified. Up-regulated trend of 22, 9 and
458 26 genes respectively at the time points of 12, 24 and 48 h was
459 significantly associated between DEGs and DEPs. Down-regulated trend
460 of 14, 7 and 17 genes were also revealed. In addition, 216 green dots
461 (transcripts only) and 901 blue dots (proteins only) were identified,
462 indicating differential expression was only found on the transcript or the

463 protein levels.

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

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

482 **3.6 Validation of selected genes by quantitative real-time polymerase**
483 **chain reaction**

484 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 osomoregulation-related genes (Cldn4 and Aqp1) were used for validation of osomoregulation 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 (osomoregulation-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 (*sirt1*, *hsf1* and *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 *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,

573 some of the DEGs and DEPs would be related to stress resistance. As
574 shown in Table S8, a total of 8 of the 18 up-regulated proteins detected at
575 12 h had roles in immune and stress response. These proteins included
576 MHC class I alpha antigen, fibrinogen beta chain, G-type lysozyme B,
577 GTPase IMAP family member 4-like, etc. Immune factors, such as MHC
578 class I antigen etc., have been reported to be linked to osmotic stress, the
579 decreased expression levels of immune-related proteins in the seawater
580 might indicate that their functional roles are more important in the
581 freshwater environment (Narnaware et al., 1998; Tse et al., 2013). Under
582 this circumstance, the up- and down- regulation of aforementioned
583 stress-related proteins identified in this study might also be related to
584 freshwater adaptation.
585 In this study, the protein G-type lysozyme B was not only identified at 12
586 h, but also found to be the only up-regulated immune-related protein
587 identified at 24 h (Table S8). Lysozymes were shown to be involved in
588 innate immunity and physiological activities in fish (Gao et al., 2016;
589 Wang et al., 2016). It has been reported by Schmitz et al. (2016) that
590 salinity significantly enhanced plasma lysozyme activity in striped catfish
591 (*Pangasianodon hypophthalmus, S.*). Higher lysozyme activities during
592 both acute and chronic hyperosmotic stress have also been described in
593 euryhaline species (Dominguez et al., 2005; Jiang et al., 2008; Yada et al.,
594 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

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

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

633 Previous studies have demonstrated that calcium in oestrogen-treated
634 freshwater trout was bound to Vitellogenin, i.e., the circulating plasma
635 levels of Vitellogenin was induced by oestrogen-treatment, which resulted
636 in increased circulating levels of Vitellogenin and Vitellogenin-bound
637 calcium (Persson et al., 1994). Similarly, circulating plasma levels of
638 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-methyltansferase 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., Cldn4 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,

683 Wong et al. found that the Aqp1 expression as a control for the salinity
684 acclimation was puzzling since the expression trend could differ from
685 different species (Wong et al., 2014). For instance, the decreased Aqp1
686 expression in medaka and seabream intestine was detected after seawater
687 transfer, while seawater acclimation up-regulated Aqp1 expression in the
688 eel intestine (An et al., 2008; Aoki et al., 2003; Wong et al., 2014). Even
689 though the species-specific difference in Aqp1 expression might indicate
690 various ion transporting mechanisms existing in different teleost lineages
691 (Wong et al., 2014), the Aqp1 expression trend in *T. fasciatus* kidney
692 could also suggest the activation of cell water volume regulation. In
693 addition, mammalian Aqp1 antibody specifically detected a separate
694 protein in the kidney of *T. fasciatus*, which also resulted in a similar
695 decrease trend.
696 Calmodulin, a multifunctional calcium sensor protein that participates in
697 various cellular processes under normal, stress and pathological
698 conditions (Li et al., 2014), was also found to be significantly regulated
699 under freshwater transfer. In comparison with the RNA-seq and
700 Western-blot results, the qRT-PCR revealed dramatically variation of
701 Calm1 mRNA levels. Still, high correlation was identified among the
702 aforementioned three data. Moreover, CaM binding protein
703 (plasmalemma vesicle-associated protein) was found to be inhibited at 12
704 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.

## Reference

**An, K.W., Kim, N.N. and Choi, C.Y., 2008. Cloning and expression of aquaporin 1 and arginine vasotocin receptor mRNA from the black porgy, *Acanthopagrus schlegeli* : effect of freshwater acclimation, Fish physiology and biochemistry. 34, 185-194.**

**Aoki, M., Kaneko, T., Katoh, F., Hasegawa, S., Tsutsui, N. and Aida, K., 2003. Intestinal water absorption through aquaporin 1 expressed in the apical membrane of mucosal epithelial cells in seawater-adapted Japanese eel, Journal of Experimental Biology. 206, 3495-3505.**

**Bijlsma, R. and Loeschcke, V., 2005. Environmental stress, adaptation and evolution: an overview, Journal of evolutionary biology. 18, 744-749.**

**Cao, L., Wang, W., Yang, C. and Wang, Y., 2010. Threatened fishes of the world: *Trachidermus fasciatus* Heckel, 1837 (Cottidae), Chinese Fishes. Springer, pp. 63-64.**

**Ciano, C.D., Nie, Z., Szászi, K., Lewis, A., Uruno, T., Zhan, X., Rotstein, O.D., Mak, A. and Kapus, A., 2002. Osmotic stress-induced remodeling of the cortical cytoskeleton, American Journal of Physiology-Cell Physiology. 283, C850-C865.**

**Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M. and Robles, M., 2005. Blast2GO: a**

universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics. 21, 3674-3676.

Dominguez, M., Takemura, A. and Tsuchiya, M., 2005. Effects of changes in environmental factors on the non-specific immune response of Nile tilapia,*Oreochromis niloticus L*, Aquaculture Research. 36, 391-397.

Eddy, F.B. and Handy, R.D., 2012. Ecological and Environmental Physiology of Fishes, Oxford University Press, Oxford, UK.

Evans, D.H., Piermarini, P.M. and Choe, K.P., 2005. The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste, Physiological Reviews. 85, 97-177.

Evans, T.G. and Somero, G.N., 2008. A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors, Journal of Experimental Biology. 211, 3636-3649.

Fiol, D.F. and Kültz, D., 2007. Minireview: Osmotic stress sensing and signaling in fishes, The FEBS Journal. 274, 5790-5798.

Flik, G., Klaren, P.H., Van den Burg, E.H., Metz, J.R. and Huising, M.O., 2006. CRF and stress in fish, General and comparative endocrinology. 146, 36-44.

Gao, C., Fu, Q., Zhou, S., Song, L., Ren, Y., Dong, X., Su, B. and Li, C., 2016. The mucosal expression signatures of g-type lysozyme in turbot (*Scophthalmus maximus*) following bacterial challenge, Fish Shellfish Immunol. 54, 612-619.

Gao, T.X., Bi, X.X., Zhao, L.L. and Li, C.J., 2013. Population genetic structure of roughskin sculpin *Trachidermus fasciatus* based on the mitochondrial Cytb sequence, Acta Hydrobiologica Sinica. 37, 199-207.

Giffard-Mena, I., Boulo, V., Aujoulat, F., Fowden, H., Castille, R., Charmantier, G. and Cramb, G., 2007. Aquaporin molecular characterization in the sea-bass (*Dicentrarchus labrax*): The effect of salinity on AQP1 and AQP3 expression, Comparative Biochemistry and Physiology. Part A: Molecular and Integrative Physiology. 148, 430-444.

Gonzalez, R.J., 2012. The physiology of hyper-salinity tolerance in teleost fish: a review, Journal of Comparative Physiology B. 182, 321-329.

Goto, A., 1990. Alternative life-history styles of Japanese freshwater sculpins revisited, Environmental Biology of Fishes. 28, 101-112.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., Palma, F. di, Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N. and Regev A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nature Biotechnology. 29, 644–652.

Grosell, M., 2006. Intestinal anion exchange in marine fish osmoregulation, Journal of Experimental Biology. 209, 2813-2827.

Guerreiro, P.M., Fuentes, J., Canario, A.V.M. and Power, D.M., 2002. Calcium balance in sea bream (*Sparus aurata*): the effect of oestradiol-17beta, Journal of Endocrinology. 173, 377-385.

Guhab, Y.-J. and Hwangb, P.-P., 2016. Insights into molecular and cellular mechanisms of hormonal actions on fish ion regulation derived from the zebrafish model, Gen Comp Endocrinol.

Harper, C. and Wolf, J.C., 2009. Morphologic effects of the stress response in fish, Ilar Journal. 50, 387-396.

**Hasan, M.M., DeFaveri, J., Kuure, S., Dash, S.N., Lehtonen, S., Merilä, J. and McCairns, R.J.S., 2017 Sticklebacks adapted to divergent osmotic environments show differences in plasticity for kidney morphology and candidate gene expression, Journal of Experimental Biology. 220, 2175-2186.**

**Hwang, P.-P., Lee, T.-H. and Lin, L.-Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms, American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 301, R28-R47.**

**Imsland, A.K., Gunnarsson, S., Foss, A. and Stefansson, S.O., 2003. Gill $Na^+$, $K^+$-ATPase activity, plasma chloride and osmolality in juvenile turbot (*Scophthalmus maximus*) reared at different temperatures and salinities, AQUACULTURE. 218, 671-683.**

**Jarial, M.S. and Wilkins, J.H., 2010. Structure of the kidney in the coelacanth *Latimeria chalumnae* with reference to osmoregulation, Journal of Fish Biology. 76, 655-668.**

**Jia, Y., Yin, S., Li, L., Li, P., Liang, F., Wang, X., Wang, X., Wang, L. and Su, X., 2016. iTRAQ proteomic analysis of salinity acclimation proteins in the gill of tropical marbled eel (*Anguilla marmorata*), Fish physiology and biochemistry. 42, 935-946.**

**Jiang, I.-F., Bharath Kumar, V., Lee, D.-N. and Weng, C.-F., 2008. Acute osmotic stress affects Tilapia (*Oreochromis mossambicus*) innate immune responses, Fish and shellfish immunology. 25, 841-846.**

**Karp, N.A., Huber, W., Sadowski, P.G., Charles, P.D., Hester, S.V. and Lilley, K.S., 2010. Addressing Accuracy and Precision Issues in iTRAQ Quantitation, Molecular and Cellular Proteomics 9, 1885-1897.**

**Kong, F.-Y., Zhu, T., Li, N., Cai, Y.-F., Zhou, K., Wei, X., Kou, Y.-B., You, H.-J., Zheng, K.-Y., Tang, R.-X., 2017. Bioinformatics analysis of the proteins interacting with LASP-1 and their association with HBV-related hepatocellular carcinoma, Scientific Reports. 7, 44017.**

**Li, S.-F., Zhang, G.-J., Zhang, X.-J., Yuan, J.-H., Deng, C.-L. and Gao, W.-J., 2017. Comparative transcriptome analysis reveals differentially expressed genes associated with sex expression in garden asparagus (*Asparagus officinalis*), BMC Plant Biology. 17, 143.**

**Li, S., Jia, Z., Li, X., Geng, X. and Sun, J., 2014. Calmodulin is a stress and immune response gene in Chinese mitten crab *Eriocheir sinensis*, Fish Shellfish Immunol. 40, 120-128.**

**Liping Ma, Laijin Su, Hui Liu, Feng Zhao, Deqing Zhou and Duan, D., 2017. Norovirus contamination and the glycosphingolipid biosynthesis pathway in Pacific oyster: A transcriptomics study, Fish Shellfish Immunol. 66, 26-34.**

**Liu, H.L., Zhang, Q., Tang, Y.L., YU, F.Y. and Zhou, J.Y., 2010. Structure and genetic diversity of mtDNA D-Loop sequences among Trachidermus fasciatus stocks in Yellow Sea and Bohai Sea of China, Marine Science Bulletin. 3, 010.**

**Liu, Y., Yu, S., Chai, Y., Zhang, Q., Yang, H. and Zhu, Q., 2012. Lipopolysaccharide-induced gene expression of interleukin-1 receptor-associated kinase 4 and interleukin-1β in roughskin sculpin (*Trachidermus fasciatus*), Fish & shellfish immunology. 33, 690-698.**

**Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods. 25, 402-408.**

**Loretz, C.A., Pollina, C., Herberger, A.L., Hyodo, S. and Takei, Y., 2012. Skeletal tissues in Mozambique tilapia (*Oreochromis mossambicus*) express the extracellular calcium-sensing receptor, Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology. 163, 311-318.**

**Loretz, C.A., Pollina, C., Hyodo, S. and Takei, Y., 2009. Extracellular calcium-sensing receptor distribution in osmoregulatory and endocrine tissues of the tilapia, General and Comparative Endocrinology. 161, 216-228.**

**Marshall, W. and Grosell, M., 2005. Ion transport, osmoregulation, and acid-base balance, The physiology of fishes. 3, 177-230.**

**Martínez-Álvarez, R.M., Sanz, A., García-Gallego, M., Domezain, A., Domezain, J., Carmona, R., del Valle Ostos-Garrido, M. and Morales, A.E., 2005. Adaptive branchial mechanisms in the sturgeon *Acipenser naccarii* during acclimation to saltwater, Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 141, 183-190.**

**Minoru Kanehisa, Michihiro Araki, Susumu Goto, Masahiro Hattori, Mika Hirakawa, Masumi Itoh, Toshiaki Katayama, Shuichi Kawashima, Shujiro Okuda, Toshiaki Tokimatsu and Yamanishi, Y., 2007. KEGG for linking genomes to life and the environment, Nucleic Acids Research. 36, D480–D484.**

**Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq, Nature methods. 5, 621-628.**

**Mu, X., Su, M., Gui, L., Liang, X., Zhang, P., Hu, P., Liu, Z. and Zhang, J., 2015. Comparative renal gene expression in response to abrupt hypoosmotic shock in spotted scat (*Scatophagus argus*), General and Comparative Endocrinology. 215, 25–35.**

**Narnaware, Y.K., Kelly, S.P. and Woo, N.Y.S., 1998. Stimulation of macrophage phagocytosis and lymphocyte count by exogenous prolactin administration in silver sea bream (*Sparus sarba*) adapted to hyper- and hypo-osmotic salinities, Veterinary Immunology and Immunopathology. 387-391.**

**Nguyena, T.V., Jungb, H., Nguyenc, T.M., Hurwooda, D. and Mathera, P., 2016. Evaluation of potential candidate genes involved in salinity tolerance in striped catfish (*Pangasianodon hypophthalmus*) using an RNA-Seq approach, Marine Genomics. 25.**

**Olsen, H.S., Cepeda, M.A., Zhang, Q.Q., Rosen, C.A. and B.L.Vozzolo, 1996. Human stanniocalcin: a possible hormonal regulator of mineral metabolism, Proceedings of the National Academy of Science of the United States of America. 93, 1792-1796.**

**Persson, P., Sundell, K. and Björnsson, B.T., 1994. Estradiol-17β-induced calcium uptake and resorption in juvenile rainbow trout, *Oncorhynchus mykiss*, Fish physiology and biochemistry. 13, 379-386.**

**Robinson, M.D., McCarthy, D.J. and Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics. 26, 139-140.**

**Sardella, B.A., Kültz, D., 2009. Osmo- and ionoregulatory responses of green sturgeon (*Acipenser medirostris*) to salinity acclimation, Journal of Comparative Physiology B. 179, 383-390.**

**Schmitz, M., Douxfils, J., Mandiki, S.N.M., Morana, C., Baekelandt, S. and Kestemont, P., 2016. Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection, Fish Shellfish Immunol. 55, 550-558.**

**Song, H., Wang, H.-Y. and Zhang, T., 2016. Comprehensive and Quantitative Proteomic Analysis of Metamorphosis-Related Proteins in the Veined Rapa Whelk, *Rapana venosa*, International Journal of Molecular Sciences 17, 924.**

**Takeshita, N., Onikura, N., Matsui, S. and Kimura, S., 1997. Embryonic, larval and juvenile development of the roughskin sculpin, *Trachidermus fasciatus* (Scorpaeniformes: Cottidae),**

910 **Ichthyological Research. 44, 257-266.**
911 **Takeshita, N., Onikura, N., Matsui, S. and Kimura, S., 2004. Comparison of early life-history in two**
912 **catadromous sculpin, *Trachidermus fasciatus* and *Cottus kazika*, Journal of the Shimonoseki**
913 **University of Fisheries (Japan).**
914 **Tse, W.K.F., Sun, J., Zhang, H., Law, A.Y.S., Yeung, B.H.Y., Chow, S.C., Qiu, J.-W. and Wong, C.K.C., 2013.**
915 **Transcriptomic and iTRAQ proteomic approaches reveal novel short-term hyperosmotic**
916 **stress responsive proteins in the gill of the Japanese eel (*Anguilla japonica*), Journal of**
917 **Proteomics. 89, 81-94.**
918 **Tse, W.K.F., Suna, J., Zhang, H., Law, A.Y.S., Yeung, B.H.Y., Chow, S.C., Qiu, J.-W. and Wong, C.K.C.,**
919 **2014. iTRAQ-based quantitative proteomic analysis reveals acute hypo-osmotic responsive**
920 **proteins in the gills of the Japanese eel (*Anguilla japonica*), Journal of Proteomics. 105,**
921 **133-143.**
922 **Tseng, D.Y., Chou, M.Y., Tseng, Y.C., Hsiao, C.D., Huang, C.J., Kaneko, T. and Hwang, P.P., 2009. Effects**
923 **of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo, American Journal of**
924 **Physiology: Regulatory, Integrative and Comparative Physiology. 296, 549-557.**
925 **Varsamos, S., Nebel, C. and Charmantier, G., 2005. Ontogeny of osmoregulation in postembryonic**
926 **fish: a review, Comparative Biochemistry and Physiology. Part A: Molecular and Integrative**
927 **Physiology. 401-429.**
928 **Wang, G., Yang, E., Smith, K.J., Zeng, Y., Ji, G., Connon, R., Fangue, N.A. and Cai, J.J., 2014. Gene**
929 **expression responses of threespine stickleback to salinity: implications for salt-sensitive**
930 **hypertension, Frontiers In Genetics. 5, 312.**
931 **Wang, J., Cheng, G. and Tang, Z., 2000. The distribution of roughskin sculpin (*Trachidermus fasciatus***
932 ***Heckel*) in Yalu river basin, China, Journal of Fudan University. Natural science. 40, 471-476.**
933 **Wang, M., Zhao, X., Kong, X., Wang, L., Jiao, D. and Zhang, H., 2016. Molecular characterization and**
934 **expressing analysis of the c-type and g-type lysozymes in Qihe crucian carp *Carassius***
935 ***auratus*, Fish Shellfish Immunol. 52, 210-220.**
936 **Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E. and Kinne, R.K.H., 2003. Cell volume regulation:**
937 **osmolytes, osmolyte transport, and signal transduction, Reviews of Physiology,**
938 **Biochemistry and Pharmacology. 148, 1-80.**
939 **Wong, M.K.-S., Ozaki, H., Suzuki, Y., Iwasaki, W. and Takei, Y., 2014. Discovery of osmotic sensitive**
940 **transcription factors in fish intestine via a transcriptomic approach, BMC Genomics. 15,**
941 **1134.**
942 **Wu, X., Xiong, E., Wang, W., Scali, M. and Cresti, M., 2014. Universal sample preparation method**
943 **integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop**
944 **proteomic analysis, NATURE PROTOCOLS. 9, 362-374.**
945 **Xu, D., Sun, L., Liu, S., Zhang, L. and Yang, H., 2016. Understanding the Heat Shock Response in the**
946 **Sea Cucumber *Apostichopus japonicus*, Using iTRAQ-Based Proteomics, International**
947 **Journal of Molecular Sciences 17, 150.**
948 **Xu, J.R., Han, X.L., Yu, J.F., Bao, F. and Xu, P., 2009. Analysis of genetic diversity of *Trachidermus***
949 ***fasciatus* by ISSR, Freshwater Fisheries. 1, 003.**
950 **Yada, T., Azuma, T. and Takagi, Y., 2001. Stimulation of non-specific immune functions in**
951 **seawater-acclimated rainbow trout, Oncorhynchus mykiss, with reference to the role of**
952 **growth hormone, Comparative Biochemistry and Physiology. Part B: Biochemistry and**
953 **Molecular Biology. 129, 695-701.**

954 **Yancheva, V., Velcheva, I., Stoyanova, S. and Georgieva, E., 2016. Histological biomarkers in fish as a**
955 **tool in ecological risk assessment and monitoring programs: A review, Applied Ecology and**
956 **Environmental Research. 14, 47-75.**
957 **Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., Bolund, L. and Wang, J.,**
958 **2006. Wego: A Web Tool for Plotting Go Annotations, Nucleic Acids Research. 34,**
959 **W293-W297.**
960 **Yeung, B.H.Y., Law, A.Y.S. and Wong, C.K.C., 2012. Evolution and roles of stanniocalcin, Molecular**
961 **and Cellular Endocrinology. 349, 272-280.**
962 **Yu, S., Yang, H., Chai, Y., Liu, Y., Zhang, Q., Ding, X. and Zhu, Q., 2013. Molecular cloning and**
963 **characterization of a C-type lectin in roughskin sculpin (*Trachidermus fasciatus*), Fish &**
964 **shellfish immunology. 34, 582-592.**
965 **Yue, P.Q. and Chen, Y.Y., 1998. China red data book of endangered animals—Pisces, Science Press,**
966 **Beijing.**
967 **Zhang, J., Wu, K., Zeng, S., Silva, J.A.T.d., Zhao, X., Tian, C.-E., Xia, H. and Duan, J., 2013.**
968 **Transcriptome analysis of Cymbidium sinense and its application to the identification of**
969 **genes associated with floral development, BMC Genomics. 14, 279.**
970 **Zhu, X., Liang, J., Shrubsole, M.J., Ness, R.M., Cai, Q., Long, J., Chen, Z., Li, G., Wiese, D., Zhang, B.,**
971 **Smalley, W.E., Edwards, T.L., Giovannucci, E., Zheng, W. and Dai, Q., 2014. Calcium Intake**
972 **and Ion Transporter Genetic Polymorphisms Interact in Human Colorectal Neoplasia Risk in**
973 **a 2-Phase Study, The Journal of Nutrition. 144, 1734-1741.**
974

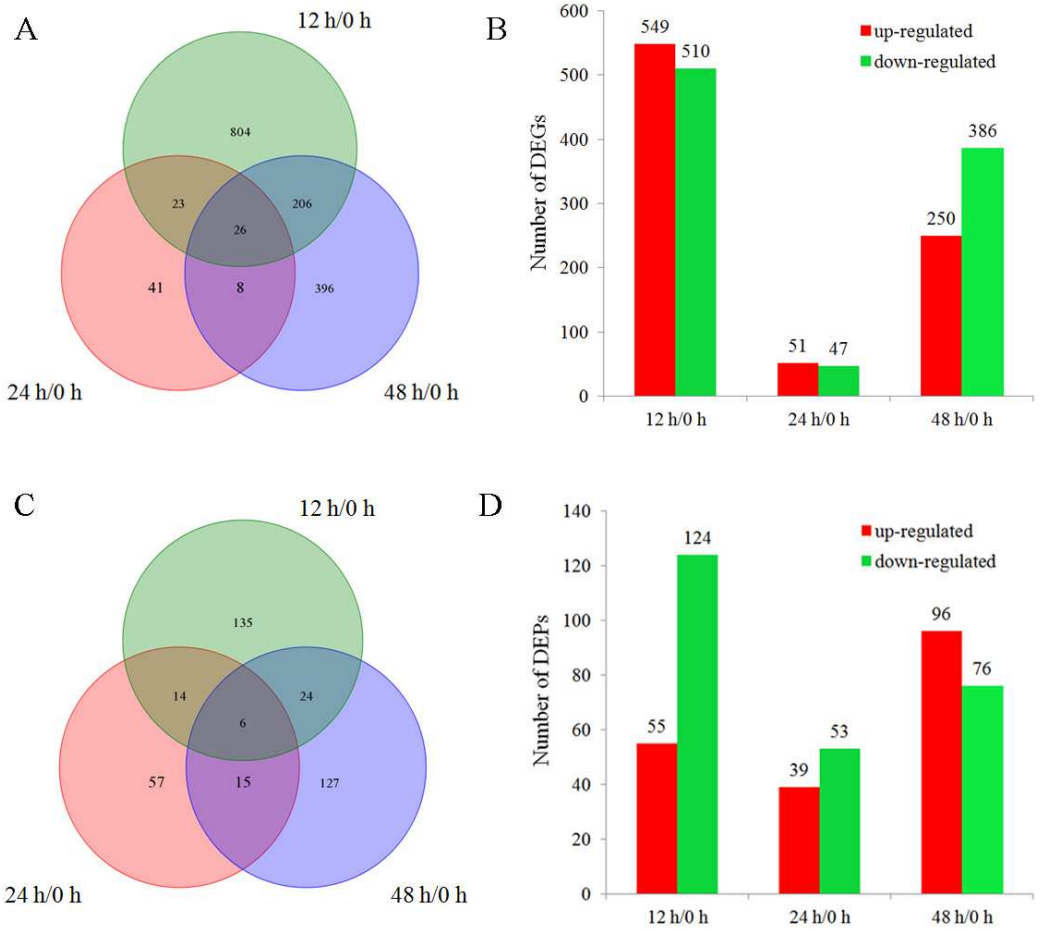


Fig.1 Differentially expressed genes or proteins at different time points (12, 24 and 48 h respectively compared to 0 h) in *Trachidermus fasciatus*. (A) Venn diagram showing the number of genes differentially expressed. (B) Clusters of differently expressed genes (up-regulated and down-regulated). (C)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

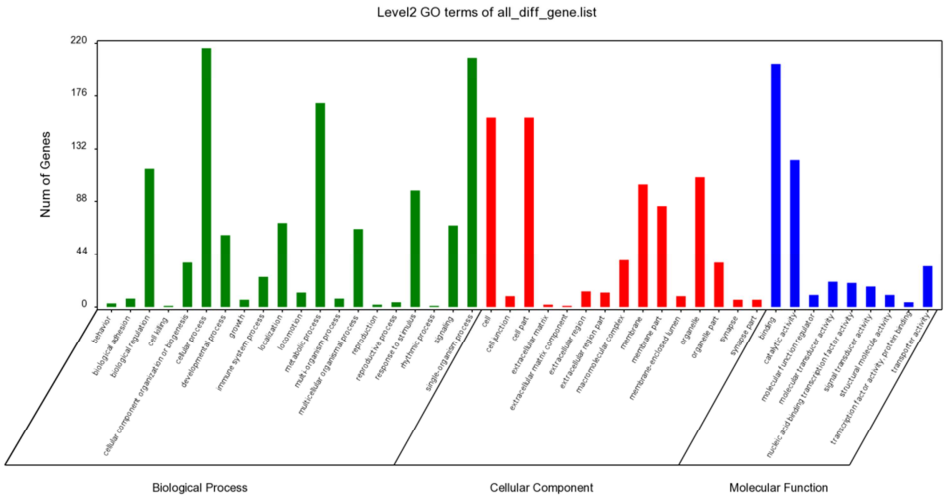


Fig.2 GO assignments of the 1504 differentially expressed genes (DEGs).

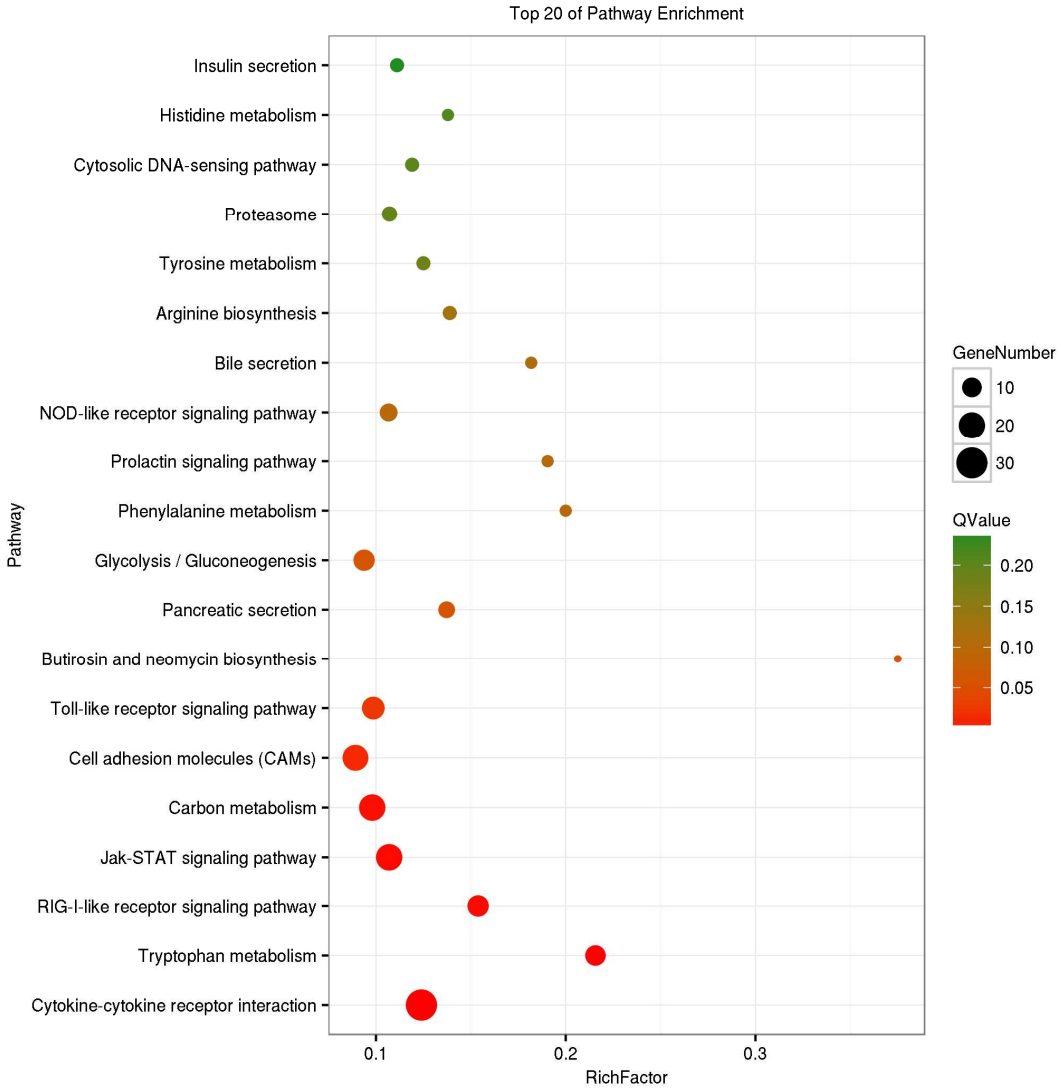

Fig.3 Top 20 of pathway enrichment of the 1504 differentially expressed genes by KEGG.

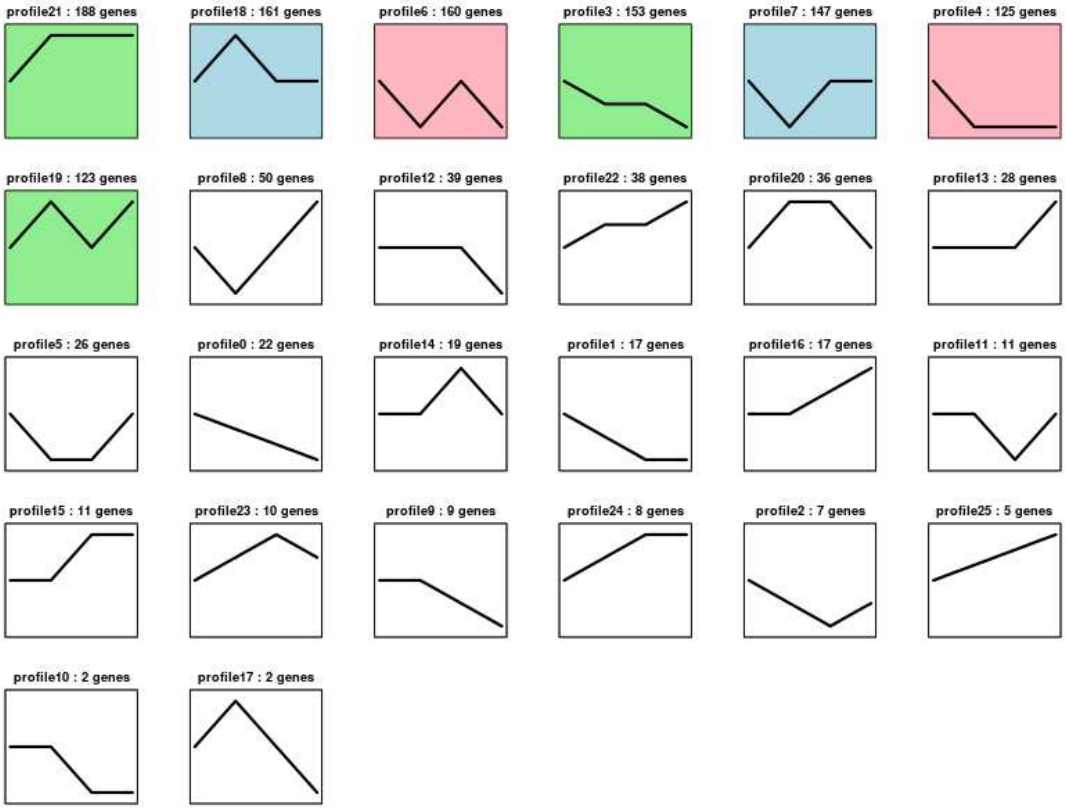


Fig.4 Sketch map of the cluster analysis of differentially expressed genes. Filled color clusters show clusters with significant trends (*P* value < 0.05). The number at the lower left corner of each cluster represents the number of genes in the cluster.

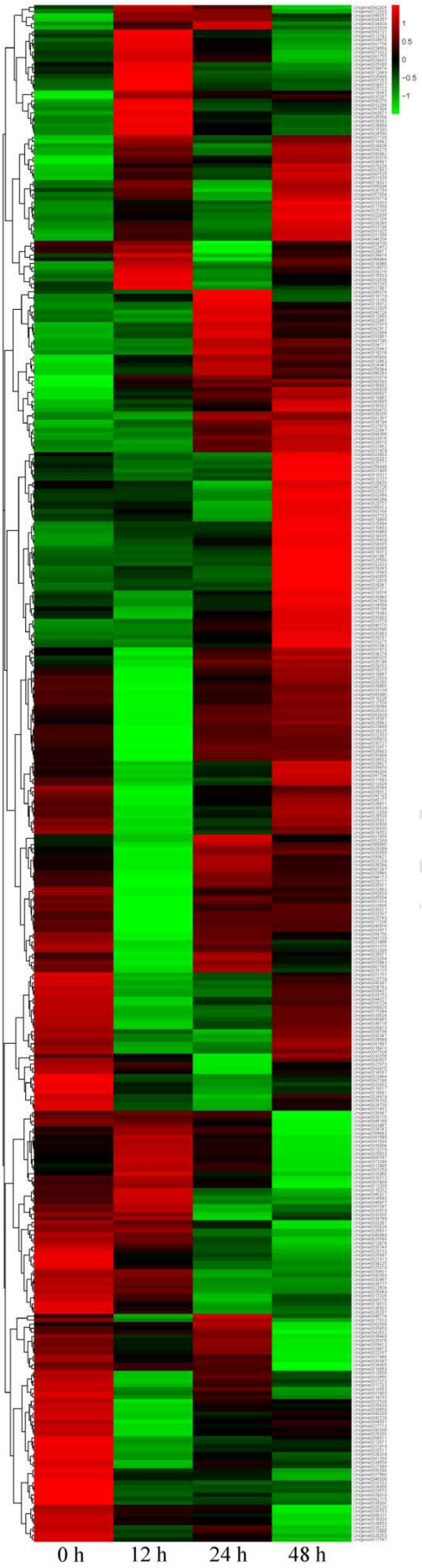

Fig.5 Hierarchical clustering of the 378 differentially expressed proteins. Left, protein tree. The color scale bar in the right, bottom corner indicates increased (red) and decreased (green) levels, and no significant changes (black) of proteins in response to salinity change.

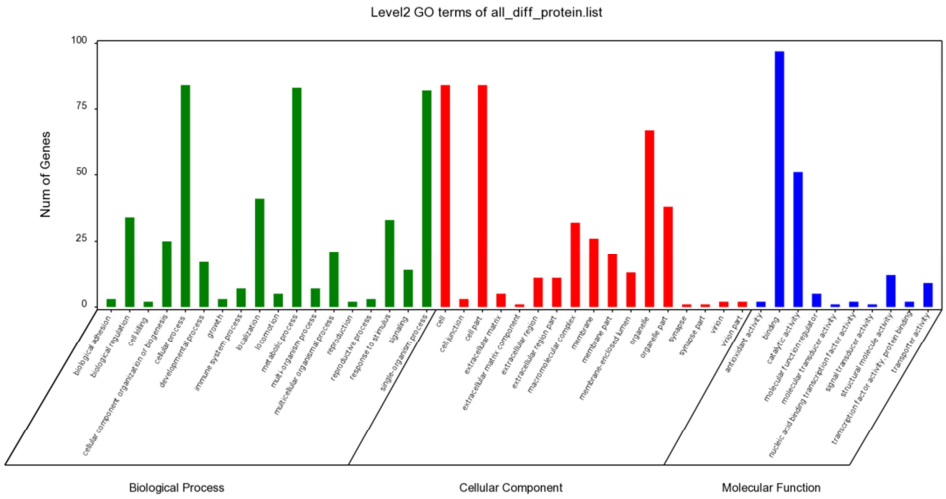


Fig.6 Gene ontology (GO) analysis of the 378 differentially expressed proteins (DEPs) responded to salinity change.

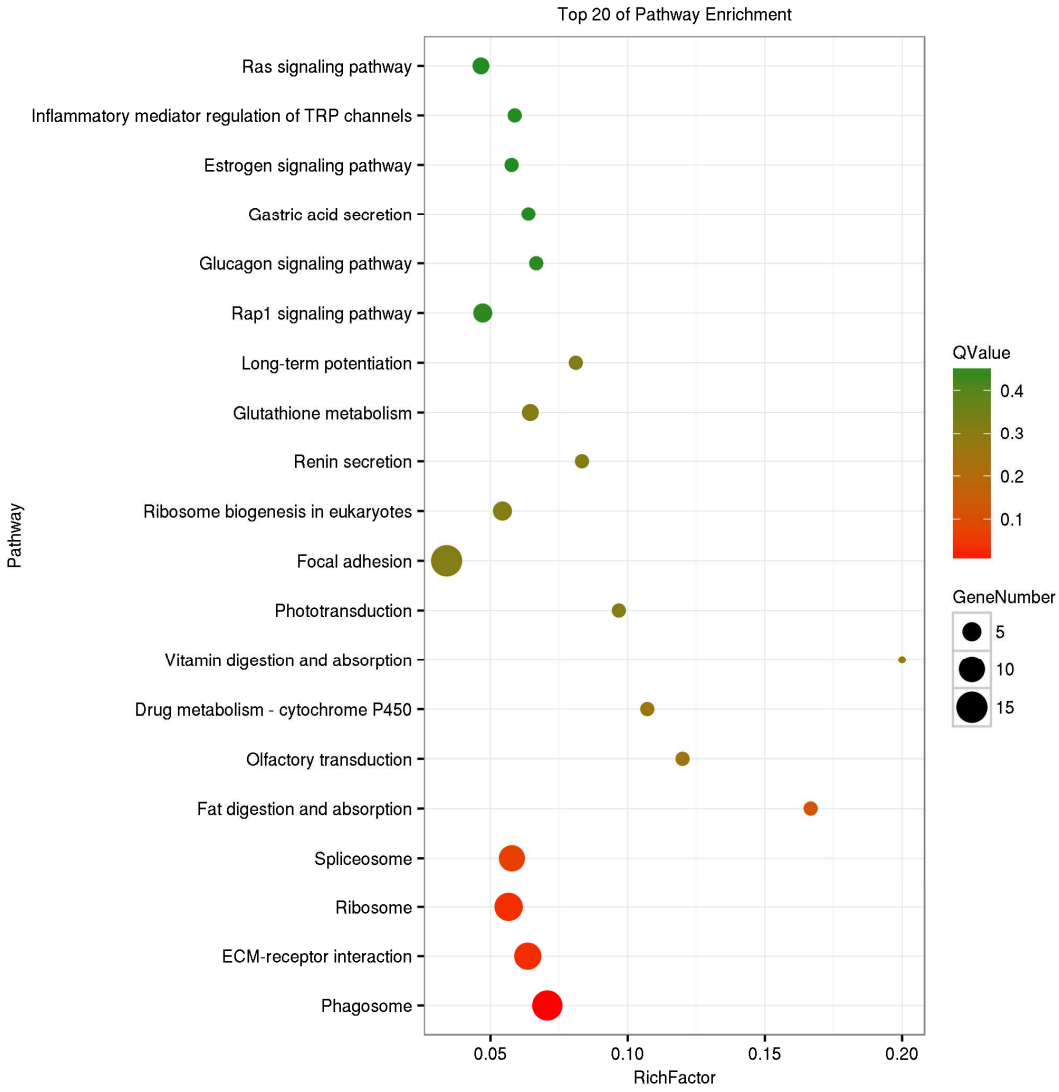


Fig.7 Top 20 of pathway enrichment of the 378 differentially expressed proteins (DEPs) by KEGG.

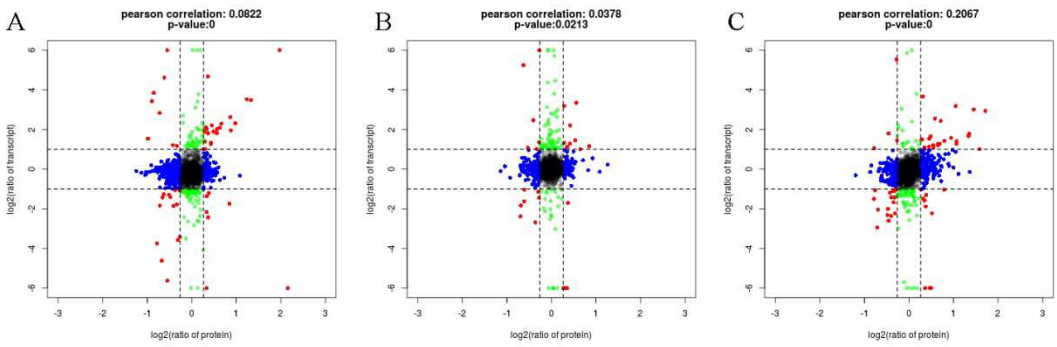


Fig.8 Comparison of expression ratios from transcriptomic (y-axis) and

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.

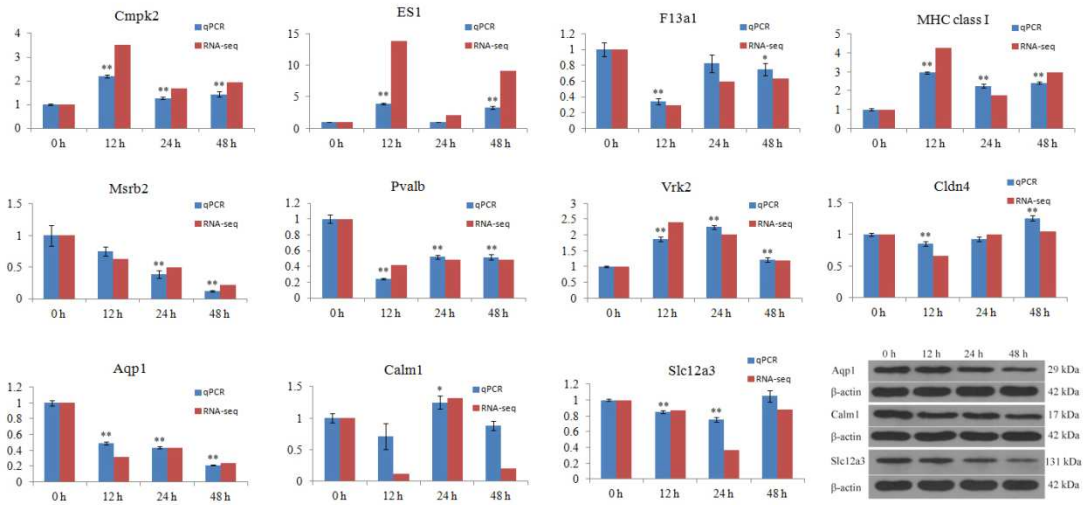


Fig.9 Extension and validation of the RNA-Seq and iTRAQ results. A-K: Relative mRNA expression levels at different time points by qPCR-PCR. I: The altered expression of the three identified proteins by western blot, controls of equal protein loading were confirmed by β-actin expression. Cmpk2: *UMP-CMP kinase 2*; ES1: *ES1 protein homolog*; F13a1: *coagulation factor XIII A chain*; MHC class I: *MHC class I alpha antigen*; Msrb2: *Methionine-R-sulfoxide reductase B2*; Pvalb: *Parvalbumin beta*; Vrk2: *serine/threonine-protein kinase VRK2*; Cldn4: *claudin 4*; Aqp1: *aquaporin 1*; Calm1: *Calmodulin 1*; Slc12a3: *solute carrier family 12 member 3*. Statistical significances are indicated with an asterisk at $P < 0.05$, and two asterisks at $P < 0.01$.