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Effects of osmotic stress on the expression profiling of aquaporin genes in the roughskin sculpin (*Trachidermus fasciatus*)

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Abstract

Aquaporins (AQPs) are a family of integral membrane proteins that have been shown to be important for osmoregulation in many vertebrates. To identify potential stress resistance-related *aqp* genes in salinity adaptation of the roughskin sculpin *Trachidermus fasciatus*, we investigated the time-course expression dynamics of seven aquaporin genes (*aqp1*, *4*, *7*, *8*, *10*, *11* and *12*) in three osmoregulatory tissues (kidney, gill and intestine) and one metabolic tissue (liver). The fish were subjected to two different acute osmotic treatments (seawater-to-freshwater transfer respectively achieved in 1 h and 24 h, namely, E-acute and acute group). The expression profiling of the seven *aqp* genes were performed using quantitative real-time PCR (qRT-PCR). At the time of all sampling time points (0 h, 12 h, 24 h and 48 h), no expression of *aqp4* was found in the gill, liver and intestine; no expression of *aqp7* was found in the gill and liver. Significant differences of *aqp* expression were determined in the four target tissues, and the mRNA levels were largely variable among gene members and tissues. Similar patterns of the time-course expression were detected in most of the *aqp* genes in *T. fasciatus* between the two acute groups, except that only one gene (*aqp12*) in the kidney and three genes (*aqp7*, *aqp8* and *aqp10*) in the intestine revealed different expression patterns. These results suggest that the expression response of *aqp* genes was similar under osmotic changes with different rates.

Key words: *Trachidermus fasciatus*, aquaporin, salinity changes, stress response, gene expression

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1 Introduction

The ability of teleosts to cope with environmental salinity changes depends on their capacity in the maintenance of water homeostasis (Harper and Wolf, 2009). Membrane intrinsic proteins (MIP) such as aquaporins (AQPs) are a family of integral membrane proteins that transport water and small molecular weight solutes across biological membranes (Agre et al., 2002; King et al., 2004). The first report on fish *aqp* appeared in year 2000 (Cutler and Cramb, 2000). As reported, the role of AQPs is suspected to be related to conservation of water in seawater (SW) and possibly excretion of water in freshwater (FW) (Cerdà and Finn, 2010). To date, a total of 13 different subfamilies have been described in the AQP superfamily of fish, which differ in tissue expression, regulation and selectivity (King et al., 2004; Takata et al., 2004). These subfamilies include classical AQPs (AQP-0, -1, -2, -4, -5 and -6), aquaglyceroporins (AQP-3, -7, -9 and -10), aquaporin-8 (AQP-8), and unorthodox aquaporins (AQP-11 and -12) (Finn and Cerdà, 2011).

In mammals, AQP1 and AQP2 are essential for water resorption in the kidney (Nielsen et al., 2002), AQP4 is involved in cerebral water balance, astrocyte migration and neural signal transduction (Verkman et al., 2006), while AQP3 and AQP7 seem to play important roles during skin hydration and metabolism of adipocytes (Hara-Chikuma and Verkman, 2006). In teleosts, water transport performances at the molecular level seem to be uncertain across different organs, species and their respective ecophases (Aoki et al., 2003; Martinez et al., 2005). The physiological role of teleost AQPs is particularly important in osmoregulatory organs (kidney, gill and intestine), where certain AQPs would be significantly modulated during osmotic challenges (Cutler and Cramb, 2002; Watanabe et al., 2005; Kim et al., 2010; Giffard-Mena et al., 2011; Choi et al., 2013; Lema et al., 2018; Cao and Shi, 2019). As for kidney, 11 orthologs of AQPs (AQP-1aa, -1ab, -3a, -3b, -7, -8aa, -8ab, -9a, -10a, -10b and -12) have been reported in various teleost species (Cerdà and Finn, 2010). Research on the role of these *aqp* genes in fish has so far focused on *aqp1*, *aqp3* and *aqp4*, which are mainly expressed in osmoregulatory tissues (the gill, the gastro-intestinal tract and the kidney) (Engelund and Madsen, 2011, 2015).

The roughskin sculpin *Trachidermus fasciatus* exhibits a catadromous lifestyle and migrates between freshwater and seawater during its life cycle (Goto, 1990). Previously, the possibility of selecting *T. fasciatus* as an experimental animal for osmoregulation studies was proposed; a kidney-specific transcriptome was performed to identify important regulators and pathways involved in salinity adaption of this euryhaline species, and a total of seven *aqp*s were identified (Ma et al., 2018). As for certain *aqp*s, tissue-specific or ubiquitous expression patterns at mRNA

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levels would be identified depending on isoforms (Cerdà and Finn, 2010). Hence, the aim of this study was to investigate the expression pattern of each *aqp* in different tissues of *T. fasciatus* to better understand the role of AQPs and regulatory mechanism of euryhaline fish in response to salinity stress.

A complex physiological process, involving structural and functional modifications in the osmoregulatory organs (gill, kidney or intestine), was involved in salinity adaptation in teleosts (Marshall and Grosell, 2006; Gonzalez, 2012). Additionally, the liver plays a primary role in maintaining metabolic homeostasis; and it is also an organ essential for stress response in fish. Since the responses to salinity challenges are likely to vary among tissues, all the four aforementioned tissues were selected. In the present study, fish were subjected to two salinity treatments (one extreme acute and the other relatively chronic seawater-to-freshwater transfer), mRNA levels of the seven *aqp*s were determined in the aforementioned four tissues at different time points along with the salinity treatments. Comparison of the tissue expression pattern of *aqp*s was performed to better understand the role of the seven genes as well as the molecular mechanism involved in osmoregulation of *T. fasciatus*.

2 Materials and methods

2.1 *Animal collection, maintenance and salinity control*

Trachidermus fasciatus were collected at the Yuhai Hatchery Station (Shandong, China) in December 2014, and then transported to the Tongyong Hatchery Station (Qingdao, China) where the experiments were carried out. A total of 216 fish (standard length (12.22±0.91) cm, body mass (19.54±5.17) g) were equally separated and every 18 fish was acclimated in a flat bottom FRP (Fiber Reinforced Plastic) tank with an effective volume of 100 L, the fish reared in 12 tanks were under a 12 h light:12 h dark photoperiod for two weeks prior to the beginning of the experiments. Over 600 L sand-filtered natural sea water (salinity of 30, temperature of 10–12°C) was supplied to each tank per day.

At the start of the experiments (time 0 h), three individuals from each tank were collected as controls while fish remained at seawater (30). Salinity change commenced thereafter by varying the inflowing seawater to freshwater (from 30 to 3) to each tank. Two different experiments were set up as followed: (1) salinity was sharply reduced and the water change took about 1 h (extreme acute group, E-acute, salinity changing rate of 27 h^{-1}); (2) salinity was gradually reduced and the water change took about 24 h (acute group, relatively chronic comparing to the E-acute group, salinity changing rate of 1.1 h^{-1}). Each experiment was performed with six replicates. Three individuals were randomly collected from each group at the time of 12 h, 24 h and 48 h, tissues (gill, intestine, kidney and liver) from each fish were firstly dissected and frozen in liquid nitrogen, and then samples from the same time point of the same treatment were respectively pooled to form sample pools. These sample pools were used to get tissue homogenates of total RNA. 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 *Total RNA extraction and the first-strand cDNA synthesis*

Total RNA was extracted from frozen tissues using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The isolated RNA samples were suspended in DEPC-treated water, quantified using NanoVue™ (GE Healthcare) at $A_{260\ nm}$ and $A_{280\ nm}$, and then analyzed for integrity on agarose gel. 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. The cDNA synthesis included a genomic DNA elimination reaction and the RT Primer Mix contained both Oligo dT Primer and Random 6 mers.

2.3 *Quantitative real time PCR*

Primers for the quantitative real time PCR (qRT-PCR) were designed according to the sequences of *T. fasciatus aqp* genes based on the RNA-Seq data (NCBI accession number SRP103494) obtained in our lab. The primer sequences were listed in Table 1. The qRT-PCR was conducted by a 7500 ABI real time PCR system (Applied Biosystems, USA). Amplifications were performed in a 20 μL final volume containing 1 μL cDNA sample, 10 μL SYBR® *Premix Ex Taq™* (Takara Bio., China), 0.4 μL ROXII, 0.4 μL of each primer and 7.8 μL ddH_2O. Control amplifications were always included. PCR amplifications were performed in triplicate, using the following conditions: initial denaturing at 95°C for 10 s, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. A dissociation protocol was always performed after thermocycling to determine target specificity. Expression of 18s was used as the in-

Table 1. Oligonucleotide primers used in this study

Name	Primer sequences (5′ to 3′)	Amplification target
AQP1-RT-F	TGACACCGTTGAGAGAGTTGAG	Expression of *aqp1*
AQP1-RT-R	CTTGTTCAAGGCCGTCATGTAC	
AQP4-RT-F	CCAATTGAGAGGCTGGCAGA	Expression of *aqp4*
AQP4-RT-R	GCTGCTGTCAGAGGGTCATT	
AQP7-RT-F	TGATGGCTTTGTCGGATCAGAA	Expression of *aqp7*
AQP7-RT-R	TGCTGCCCAGAGAAATACCAAT	
AQP8-RT-F	AAACAGGCTGGTCCCAAACA	Expression of *aqp8*
AQP8-RT-R	CAGCTGAGAGAGGCAACACA	
AQP10-RT-F	AGAGCCGCATCCAAACAGAA	Expression of *aqp10*
AQP10-RT-R	CTCACCTGCATGCAGAGGAA	
AQP11-RT-F	AAACTCCCACCTGGAATACTGC	Expression of *aqp11*
AQP11-RT-R	CTCTTCTTGGTCTCCTGGAGGA	
AQP12-RT-F	CTGGAGGTGCAGACCATCG	Expression of *aqp12*
AQP12-RT-R	CTCCAGCTGCAGGAACCTC	
18S-F	TTTCGAGGCCCTGTAATTGGAA	Expression of 18s
18S-R	CCGAGATCCAACTACGAGCTTT	

ternal 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 ($RC_{target}/RC_{control}$).

2.4 *Statistical analysis*

The heatmap analysis of aquaporin genes (scale=“row”, cluster rows TRUE) was performed using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools). All data were expressed as mean±standard deviation (SD) and analyzed by one-way ANOVA (analysis of variance) to determine significant differences between the treatments and control using the Statistical Package for the Social Sciences, SPSS (version 16.0). Values were considered statistically significant when $P<0.05$.

3 Results

As shown in Fig. 1, no expression of *aqp4* and *aqp7* could be detected in the gill. The expression of *aqp1*, *aqp10*, *aqp11* and *aqp12* were up-regulated at 12 h, and then followed by a down-regulation in both E-acute and acute group in response to salinity changes. The increment of *aqp8* transcripts in the acute group was detected at 24 h, and then followed by a decrement at 48 h; while in the E-acute group, *aqp8* expression kept increasing from 12 h to 48 h, and reached a two-fold higher level. As for the patterns of the five *aqp* genes expressed in the gill, *aqp10* and *aqp11* clustered in samples from both the two acute groups (Fig. 1), but a cluster of *aqp1* and *aqp12* was only found in the E-acute group

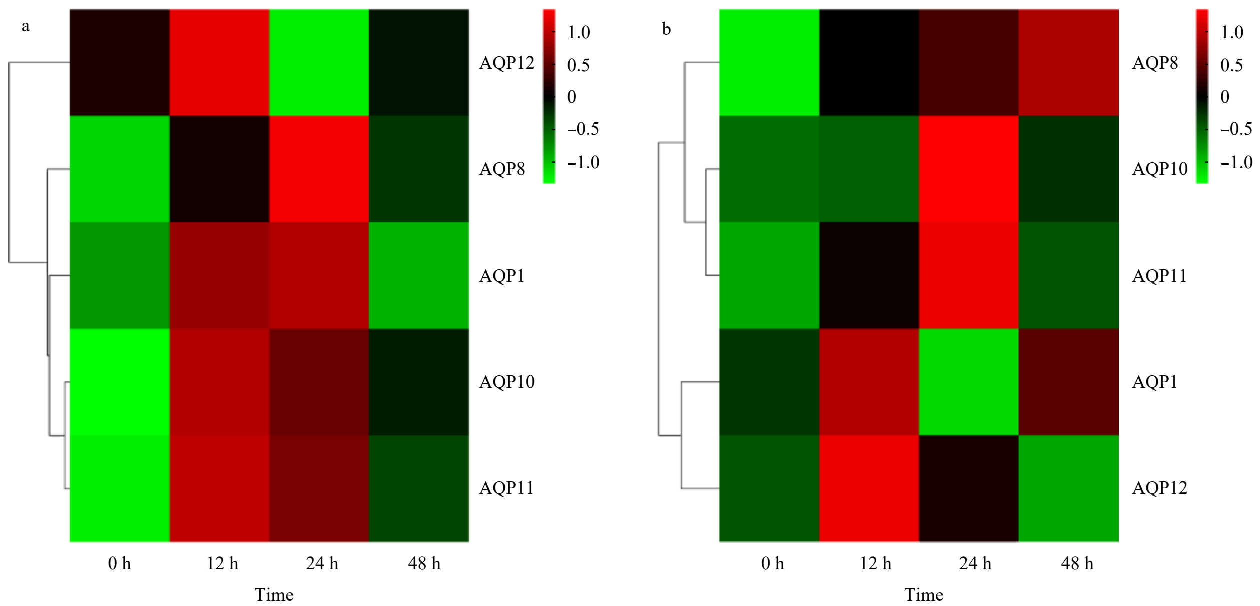

Fig. 1. Clustered heatmap of aquaporin genes expressed in the gill of *Trachidermus fasciatus* in response to seawater-to-freshwater transfer. Genes were clustered according to their expression pattern. a. Acute group and b. extreme acute group.

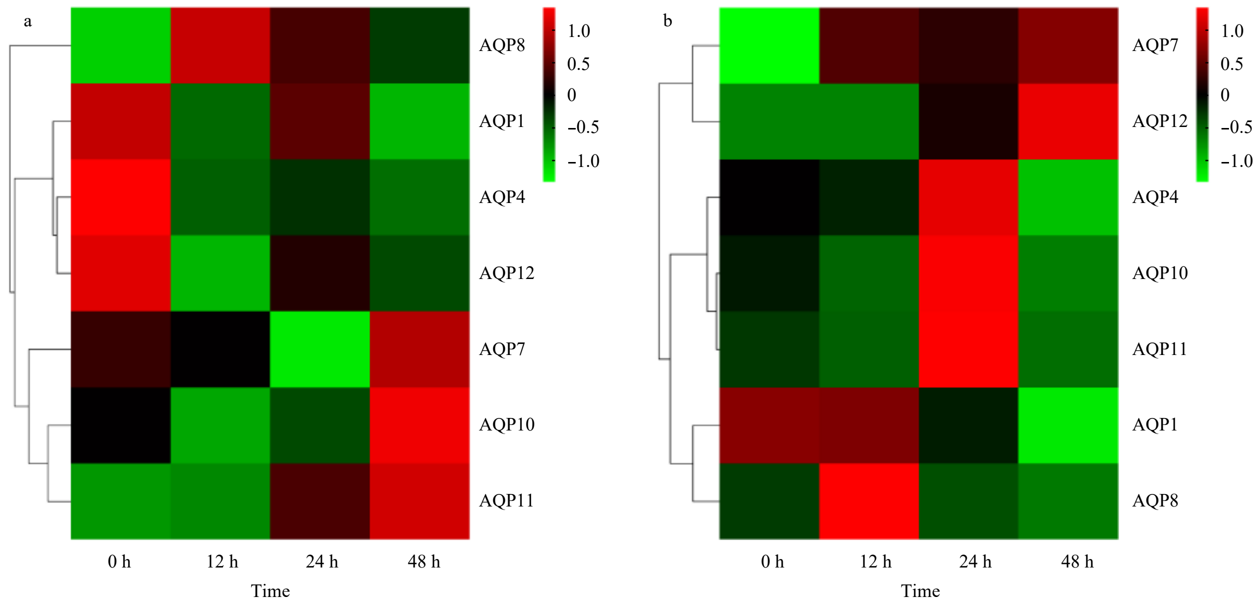

Fig. 2. Clustered heatmap of aquaporin genes expressed in the kidney of *Trachidermus fasciatus* in response to seawater-to-freshwater transfer. Genes were clustered according to their expression pattern. a. Acute group and b. extreme acute group.

(Fig. 1b).

The seven candidate genes revealed different expression patterns in the kidney (Fig. 2). Significant decrease of *aqp1* and *aqp4* expression was detected at the 48 h time point, no significant difference of the gene expression pattern was found between the two treatments. Significant increase of *aqp7* could be identified at 12 h in the E-acute group, and the average increment is about four times as much as that at 0 h; *aqp7* was not significantly altered in the acute group. As for *aqp8*, the mRNA level firstly increased and then followed by a decrement in both group. The *aqp10* had an opposite trend that the expression firstly decreased and then increased. No significant change of *aqp11* expression was found in the kidney. When comparing the mRNA level of *aqp12* under the two treatments, an opposite trend was observed that the *aqp12* mRNA level was significantly decreased in the acute group but increased in the E-acute group. As shown in Fig. 2, similar pattern of *aqp10* and *aqp11* expression were identified in both E-acute and an acute group, other than that, expression pattern of the other five genes did not reveal any similarities in the clustering results between the two groups.

As for the intestine, no expression of *aqp4* could be detected in the two groups (Fig. 3). Similar expression pattern of *aqp1* was found in E-acute and acute group; the mRNA level increased at 12 h, then decreased at 24 h and followed by a increment at 48 h. The expression of *aqp11* and *aqp12* were increased following the salinity treatment in both groups. However, the expression of *aqp8* and *aqp10* in the two groups showed different patterns; the mRNA level in the acute group was slightly increased at 48 h following the significant decrease at 12 h, but in the E-acute group the expression at 48 h was significantly higher comparing to that at 0 h. The *aqp7* gene also showed different expression pattern under different treatments. According to the heatmap, two major clusters in the acute group were shown in Fig. 3a; one was composed of *aqp11* and *aqp12*, the other was composed of *aqp1*, *aqp7*, *aqp8* and *aqp10*. In the E-acute group, two different major clusters was identified, one was composed of *aqp1* and *aqp11*, one with the other four genes.

The *aqp* genes showed different expression patterns in the liver in response to the two salinity treatments (Fig. 4). No expression of *aqp4*, *aqp7* could be detected. No expression of *aqp8* could be found before the treatments; but the *aqp8* expression was respectively activated at 12 h in the acute group and 48 h in the E-acute group. The expression trend of *aqp1*, *aqp11* and *aqp12* in the acute group was in accordance with that in the E-acute group; an up-regulation of *aqp1* as well as a down-regulation of *aqp10*, *aqp11* and *aqp12* was found in the two groups. In the acute group, expression of *aqp10* at 12 h was over 8-fold higher than that at 0 h. The heatmap showed very similar clustering patterns between the two groups, one major cluster consisted of *aqp1*, *aqp8* and *aqp10*, and the other consisted of *aqp11* and *aqp12*.

4 Discussion

4.1 *The aquaporin genes identified in T. fasciatus*

The putative aquaporin genes (*aqp1*, *4*, *7*, *8*, *10*, *11* and *12*) were identified based on sequencing data of a kidney-specific transcriptome of *T. fasciatus* (Ma et al., 2018). As for other teleosts, the first aquaporin reported in kidney was an *aqp10*-like paralog in gilthead seabream (Santos et al., 2004). The *aqp3* paralogs have also been investigated in the kidney of a few fish species (Engelund and Madsen, 2011; Cutler et al., 2007), however, no expression of *aqp3* was found in the kidney of *T. fasciatus*. In the gill, several *aqp* paralogs have been detected, and these genes are believed to be more important in cell volume regulatory response than in transepithelial water exchange (Madsen et al., 2015). In the intestine, the *aqp* expression pattern often varies within different intestinal segments (Kim et al., 2010).

4.2 *Tissue distribution of different aqp genes and their role in salinity-acclimation of T. fasciatus and other fish*

The wide distribution of *aqp1* transcripts has been detected in endothelial barriers of almost all tissues in mammals (Mobasheri and Marples, 2004; Tingaud-Sequeira et al., 2008, 2010). The *aqp1* mRNA could be found in most tissues of fish but its expression in the intestine and kidney predominated. The transcript

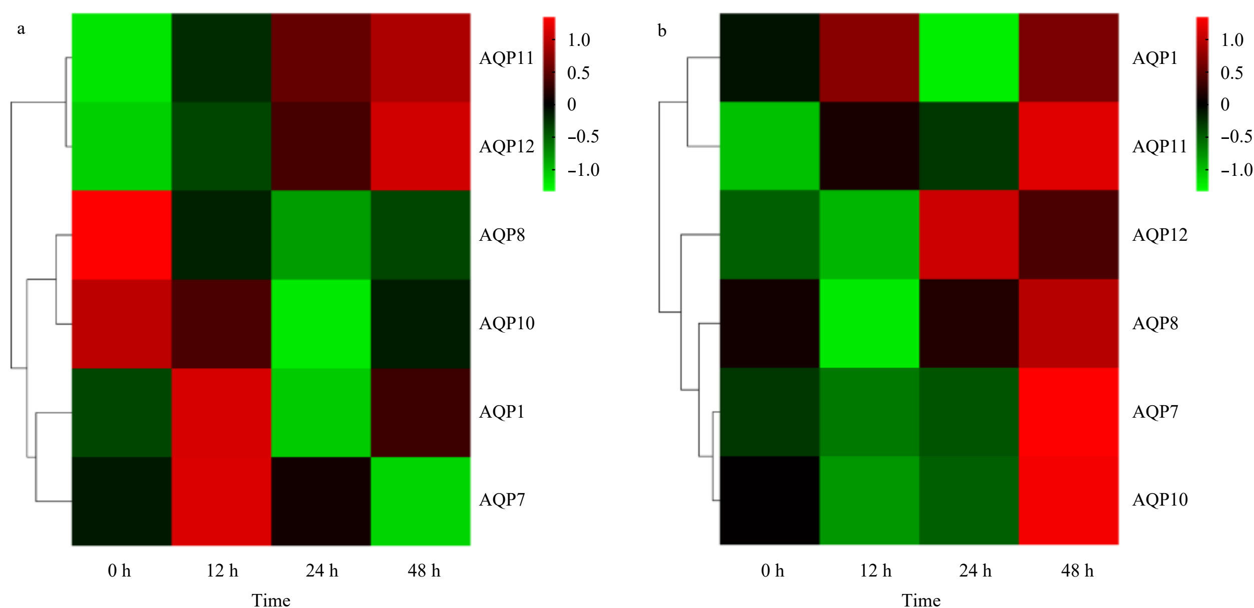

Fig. 3. Clustered heatmap of aquaporin genes expressed in the intestine of *Trachidermus fasciatus* in response to seawater-to-freshwater transfer. Genes were clustered according to their expression pattern. a. Acute group and b. extreme acute group.

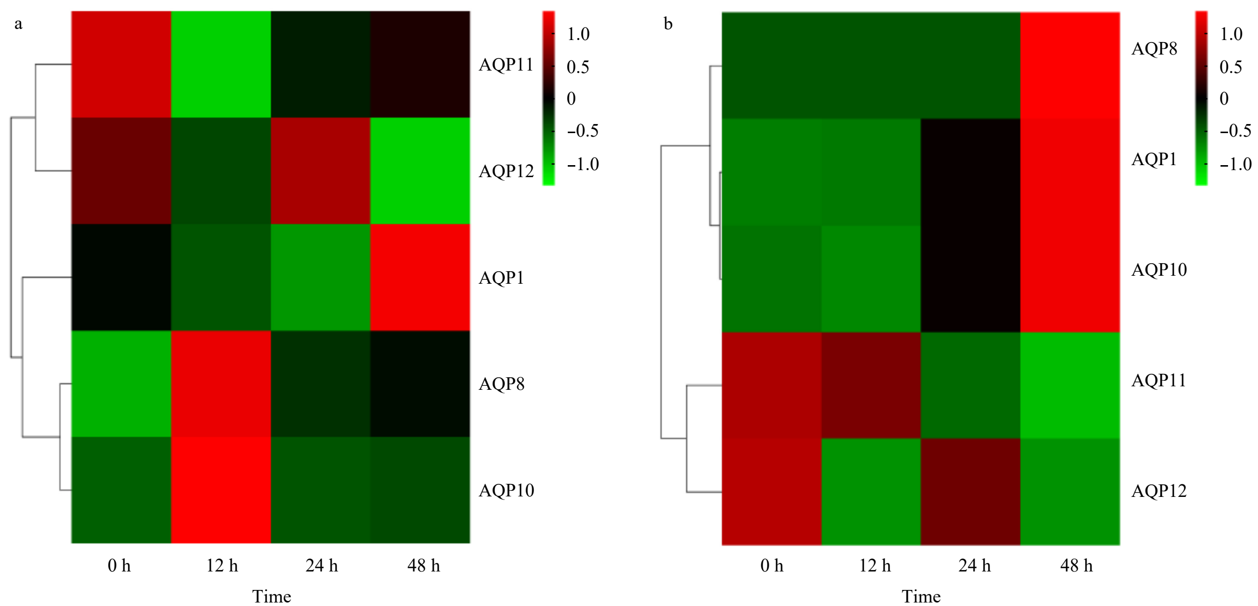

Fig. 4. Clustered heatmap of aquaporin genes expressed in the liver of *Trachidermus fasciatus* in response to seawater-to-freshwater transfer. Genes were clustered according to their expression pattern. a. Acute group and b. extreme acute group.

level of *aqp1* was present in the kidney of various fish, and the role of *aqp1* in osmoregulation has been widely studied (Kim et al., 2014; Ip et al., 2013; An et al., 2008; Engelund and Madsen, 2015). Here in this study, the *aqp1* was expressed in all the four target tissues (the gill, kidney, intestine and liver). The *aqp1* mRNA level in the kidney of fish from the two groups both decreased following the salinity decline, which is as what would be expected. It has been previously reported that the *aqp1* mRNA in the kidney of seabass *Dicentrarchus labrax* was four to five times higher in SW- than in FW-acclimated fish (Giffard-Mena et al., 2008). Tipsmark et al. (2010) reported an increasing mRNA level of *aqp1aa* in the Atlantic salmon *Salmo salar* kidney during SW-acclimation.

In marine medaka, SW-acclimated fish exhibited higher levels of *aqp1* transcripts in the kidney, whereas lower levels in gill, muscle and ovary (Kim et al., 2014). Moreover, other teleosts such as black porgy *Acanthopagrus schlegelii* (An et al., 2008), river pufferfish *Takifugu obscurus* (Jeong et al., 2014) exhibited higher gill *aqp1* expression in FW than in SW. In the gill of FW-acclimated roughskin sculpin, *aqp1* was also found to be up-regulated. Similarly, an increment of *aqp1* expression was found in the intestine and liver along with the decreasing salinity. The results was not consistent with previous findings reporting more stimulated expression of *aqp1* in the intestines of SW-acclimated fish than in those of FW-acclimated fish (Giffard-Mena et al., 2007; Raldúa et al., 2008).

In fish, the information on the expression and function of *aqp8* has been mostly focused on intestinal regulation (Cerdà and Finn, 2010; Choi et al., 2013; Cutler et al., 2009; Kim et al., 2010). The *Oryzias dancena aqp8* was dominantly expressed in the intestine and spleen, moderately expressed in the kidney, while barely expressed in the liver (Kim et al., 2014). The presence of *aqp8* in the gill, kidney and intestine of *T. fasciatus* has been confirmed in this study; no expression of *aqp8* was detected in the liver before the treatments, however, the *aqp8* expression was shown to be significantly activated by the osmotic treatments.

In addition, higher *aqp8* mRNA level was found in SW reared *T. fasciatus*, and the *aqp8* mRNA level in the intestine decreased along with the decreasing salinity. Similarly, *aqp8* in *Anguilla japonica* tended to be expressed to a higher level in the intestinal segments of SW-acclimated eel than in those of FW-acclimated eel (Kim et al., 2010). The intestinal *aqp8* in *Oncorhynchus nerka* also increased after SW acclimation (Choi et al., 2013). Conversely, the intestinal *aqp8* mRNA level in the marine medaka is higher in FW than in SW (Kim et al., 2014). Accordingly, significant difference was identified among species in the expression pattern of *aqp8* when the fish were facing with salinity changes or osmotic stress.

The distribution pattern of *aqp10* was similar among different teleosts, in the sense of its abundant expression levels in the gonad, gill, intestine and kidney (Kim et al., 2014; Hamdi et al., 2009; Tingaud-Sequeira et al., 2010). However, this tissue distribution pattern could be greatly altered by salinity. For instance, the *aqp10* level was exclusively higher in the gill, intestine, liver and spleen in the SW-acclimated than that in the FW-acclimated Japanese eel *A. japonica*. In this study, the *aqp10* expression in the afore-mentioned tissues of *T. fasciatus* was found to be differently altered by the two different salinity treatments, but mostly the *aqp10* mRNA level in the SW-acclimated fish was lower than that in the FW-acclimated fish.

Expression profiles of *aqp11* and *aqp12* in different tissues of *T. fasciatus* were investigated under two acute salinity treatments (from SW to FW). As a result, expression of *aqp11* and *aqp12* in the osmoregulatory tissues were both increased along with the FW-acclimation, but their expression in the liver were down-regulated. In the gill and kidney of *O. dancena*, the *aqp12* mRNA could be detected, but not in the same tissues of zebrafish (Kim et al., 2014; Gorelick et al., 2006). As two unorthodox aquaporins, *aqp11* and *aqp12* did not significantly induce the water permeability in oocytes expressing, but might be related to their intracellular localization in vivo (Gorelick et al., 2006; Itoh et al., 2005).

In this study, variation of *aqp11* and *aqp12* expression in os-

moregulatory tissues of *T. fasciatus* revealed the effect of salinity on mRNA level of these two genes, indicating that they might have participated in the molecular response to osmotic stress. Moreover, the heatmap indicated a similar trend of *aqp11* and *aqp12* expression in the liver and intestine, revealing a consistency between mRNA levels and orthologs.

4.3 *Comparison of expression pattern of different aqps among different tissues and treatments*

In this study, two salinity treatments including one extreme acute and the other relatively chronic treatment was performed. In all the four tissues, the seven *aqp* genes exhibited a similar expression pattern between the two different treatments in most cases, suggesting that the *aqp* expression pattern may not be affected by the treatment strength. In addition, expression pattern of each *aqp* gene was tissue-specific, i.e., each tissue possessed its own expression patterns of *aqp* genes in response to the same salinity stress.

5 Conclusions

In this study, we investigated the effect of two different acute salinity treatments on various tissue distribution and expression pattern of *aqp1*, *4*, *7*, *8*, *10*, *11* and *12* in *T. fasciatus*. The qRT-PCR analyses showed that *aqp* transcripts are expressed in not only osmoregulatory tissues but also nonosmoregulatory tissues like liver. As for certain *aqp* gene such as *aqp 4* and *aqp7*, their presence is only localized at specific tissues such as kidney. Although the overall tissue distribution pattern of *aqp*s was not significantly different between FW- and SW-acclimated fish, the mRNA levels were largely variable among genes and tissues. Moreover, the salinity-dependent patterns of different *aqp* genes were also different among fish species, suggesting that the changes in transcription levels of *aqp*s might be species- or lineage- specific. Further studies on the distribution of *aqp*s in different cell types by performing salinity challenges are needed in order to specify the function of each *aqp*. Data from this study could serve fundamental basis to design gene expression assays with *T. fasciatus aqp* genes for a comprehensive understanding on the coordinated role of multi-genes in *aqp* superfamily in salinity adaptation of this euryhaline species.

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