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ORIGINAL ARTICLE

Physiological, biochemical and molecular responses of *Sepia pharaonis* juveniles to low salinity

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Abstract

Sepia pharaonis (cuttlefish) has a narrow salinity adaptation range, therefore, it is highly sensitive to changes in salinity. This limits breeding practices for *Sepia pharaonis*. The aim of this study was to explore effects of low salinity concentrations on gills of cuttlefish. Larvae were randomly categorized into two groups and acclimatized to 2 different salinity conditions (22 psu and 29 psu). Salinity was adjusted by diluting salinity water (29 psu) with fresh water then aeration was carried out overnight. Observation of gills under light and electron microscopy showed that cuttlefish survived up to 22 psu salinity levels. Further, light and transmission electron microscopic examination showed that gills exposed to low salinity stress (22 psu) showed significant pathological changes compared with the control group (29 psu). Biochemical and molecular analyses showed that Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase, TIP4-1 and Zfand4 were involved in regulation of osmotic pressure in *S. pharaonis*.

KEYWORDS
biochemical response, gill, low salinity, molecular response, *Sepia pharaonis*

1 | INTRODUCTION

Sepia pharaonis mainly inhabits the Red Sea, the Persian Gulf from Japan to the Gulf of Thailand and northern Australia, Indian Ocean and Andaman Sea (Nair et al., 2011). This species is characterized by rapid growth, short life span, tolerance to crowding and human handling, resistance to diseases and feeding habits, therefore, it preferred for commercial breeding (Anderson et al., 2011; Minton et al., 2001; Samuel & Pattersonl, 2011; Sareban et al., 2014; Tuanapaya & Nabhitabhata, 2017). Salinity is an important parameter in artificial propagation of *S. pharaonis*. Optimum salinity range for *S. pharaonis* is approximately 24–35 psu implying that *S. pharaonis* is highly sensitive to low salinity levels (Le et al., 2014; Wen et al., 2011). *S. pharaonis* are affected by salinity fluctuations during artificial culturing due to heavy rainfall. These fluctuations significantly affect mortality and commercial production of *S. pharaonis*. Therefore, fish farms using seawater with varying salinity levels should acclimatize juvenile *S. pharaonis* to adapt to the local seawater salinity, especially for seawater with low salinity. Notably, *S. pharaonis* responds to changes in salinity through osmoregulation.

Seawater salinity alters locomotor activity and food intake, thus affecting growth of aquatic animals (Navarro & Gonzalez, 1998; Phuc et al., 2014). In addition, salinity levels directly affect serum osmotic pressure, ion concentration, gill Na^+/K^+-ATPase (NKA) activity and free amino acids. Several studies have explored the effects of salinity on aquatic animals (Charmantier & Wolcott, 2001; De Vos et al., 2019; Esparza-Leal et al., 2019; Hines et al., 2019; Jaffer et al., 2020; Long et al., 2019; Lu et al., 2019; May et al., 2017; Schrandt et al., 2018; Taheri & Ghelichpour, 2019; Wang et al., 2018, 2019; Zou et al., 2019). However, the mechanism of osmotic pressure in regulation of salinity levels in cephalopods has not been fully explored.

The aim of this study was to explore the effect of varying salinity levels on gills of *S. pharaonis*. Histological and scanning electron microscopy analyses of *S. pharaonis* gills were conducted to determine

effects of low salinity on *S. pharaonis* cell structure. Furthermore, regulatory mechanism employed by cuttlefish to adapt to low-salt habitats were analysed by determining expression levels of osmotic regulation-related enzymes, and penetration-related genes including Na^+/K^+-ATPase α subunit gene, aquaporin TIP4-1 and Zfand4. The findings of this study provide information on osmotic pressure adjustment mechanism of *S. pharaonis*, which are important in promoting large-scale breeding.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Experimental juveniles of cuttlefish (*S. pharaonis*) were obtained from an artificial breeding nursery based in Ningbo City, China (N29°35′, E121°59′). Larvae (mantle length 4.17 ± 0.46 cm, body weight 13.85 ± 3.42 g) with uniform size and good vitality were selected and subjected to 3 days temporary cultivation in a different pond. Larvae were temporarily maintained in 6 blue plastic barrels (40 individuals/barrel; barrel dimensions: diameter 1.0 m; height, 0.6 m) for 3 days before the experiment. They were fed on small dried shrimps (after washing) twice a day. After 30 min of feeding, their faeces and food remains were drained. Experiments were conducted on water at a temperature of 24.2 ± 1.5℃ and salinity levels at 29.1 ± 0.2 psu. Approximately 80% of the water volume was replaced daily.

2.2 | Experimental protocol

2.2.1 | Experiment 1: Tolerance of cuttlefish juveniles to varying salinity levels

Tolerance of cuttlefish to different salinity levels was determined by gradually decreasing the salinity levels of seawater using 3 schemes. In Plan A, salinity was reduced by 4 psu every 24 h; in Plan B, salinity was reduced by 3 psu every 24 h; in Plan C, salinity was reduced by 2 psu every 24 h; whereas in the control group salinity was maintained at 29 psu. Survival rate was determined at 24, 48, 72 and 96 h. Dead juveniles were removed immediately after detection.

2.2.2 | Experiment 2: Long-term exposure to low salinity

Juveniles were randomly categorized into 2 groups and acclimatized to 2 different salinity challenges (22 psu and 29 psu). Five cuttlefish were randomly sampled from the control group and low-salt group at 6 time points (0 day, 0.5 day, 1 day, 2 days, 7 days and 14 days). Cuttlefish were then treated with 5% alcohol (diluted with seawater). Samples were dissected, gills were removed and immediately placed in liquid nitrogen. All experiments were conducted following guidelines by the Animal Care and Use Committee of Ningbo University. This study was approved by the Animal Care and Use Committee of Ningbo University.

2.3 | Haematoxylin and Eosin (H-E) staining

Isolated gills were preserved in 10% formalin solution and embedded in paraffin. Tissues were sectioned into 4 μm sections, dewaxed and rehydrated. Tissues were then subjected to H-E staining and visualized under a standard light microscope.

2.4 | Transmission electron microscopy (TEM)

Tissues were isolated and cut into several sections of 1 mm^3. The tissue blocks were fixed in a fixative solution overnight at 4°C. After several washes in phosphate-buffered salinity, tissue blocks were postfixed in 1% (w/v) osmium tetroxide, then dehydrated severally using different concentration of ethanol as follows: 50% (v/v) ethanol for 15 min, 75% (v/v) ethanol for 15 min, 95% (v/v) of ethanol for 15 min (twice) and absolute ethanol for 30 min (twice). After washing with alcohol, tissue blocks were dehydrated with absolute acetone for 10 min (twice). Tissues sections were then embedded with Spurr's resin and cured in an oven at 60°C for 48 h. Ultrathin sections (90 nm thickness) of the tissue blocks were obtained using Leica EMUC7 ultrathin slicer. Sections were placed on a 150-mesh copper grid, and double-stained with uranyl acetate and lead citrate. The ultrathin sections were visualized under H-7650 Transmission Electron Microscope (Hitachi—Science & Technology, Japan).

2.5 | Determination of biochemical indicators

Gills were removed and washed in normal salinity and 10% homogenate prepared in 1.15% w/v of potassium chloride. The homogenate was centrifuged at 7,000 × *g* at 4°C for 10 min. The supernatant was then used to determine oxidative stress by estimating activity of antioxidant enzymes. Na^+/K^+-ATPase activity and Ca^{2+}/Mg^{2+}-ATPase activity were determined using analysis kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6 | Determination of osmotic pressure regulatory genes

Illumina Hiseq2500 tool was used to map the gill transcriptome of *S. pharaonis*. Genes associated with salinity stress were identified (PRJNA430775) as described previously (Ren et al., 2020). Further, relative expression of osmoregulatory-related genes (Na^+/K^+-ATPase α subunit (Atpalpha), water channel protein (TIP4-1), and AN1 type zinc finger protein 4 (Zfand4) were determined. Total RNA of the gill was extracted using Trizol method. RNA quality and quantity

were analysed using a Bio Analyzer 2100 (Agilent Technology, Santa Clara, CA) and Nano Drop 2000 spectrophotometer (Infinigen Biotechnology Inc., City of Industry, CA), respectively. First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase at 37°C for 1 h with oligo dT primers following the manufacturer's protocol. RT-PCR was conducted in a 20 μl reaction mixture system containing 10 μl of SYBR reagent, 5 μl of template cDNA, 3 μl of water and 2 μl of each primer. The reaction procedure was as follows: Initial denaturation at 95°C for 5 min and 45 cycles at 95°C for 10 s; 60°C for 20 s; and 72°C for 30 s. The melting reaction procedure was as follows: 95°C for 5 s; 65°C for 1 min; and 97°C for 15 s. RPL18 was used as an internal reference gene (Table 1).

TABLE 1 Primer sequence

Name	Sequences (5′–3′)	Length (bp)
RPL18-F	AAGAGTGGAGGCGAGATTC	157
RPL18-R	CGTAGGGTTTGGTATGACTG	
Atpalpha-F	CAGGGCTTCTTGGTGGTT	182
Atpalpha-R	CCCGCATTCTGGTTCTTT	
TIP4-1-F	TGAACCGCAGAACATAAAGA	97
TIP4-1-R	CTACAACAATGATGGCGACTA	
Zfand4-F	GGATTGCCTCCCTTGTTCAT	183
Zfand4-R	CAGCACCCAAGCTACCAAAA	

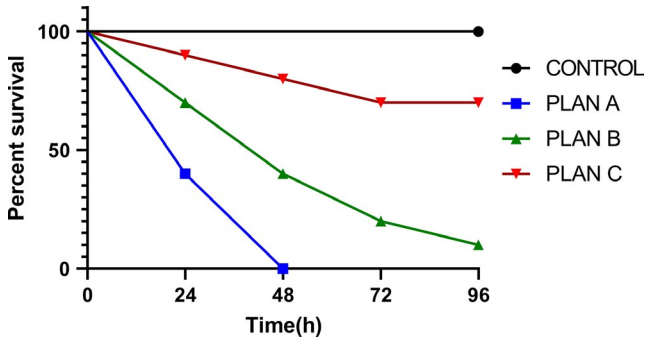

FIGURE 1 Survival rate of different salinity gradient schemes of *S. pharaonis* larvae

2.7 | Statistical analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 19.0, was used for all statistical analyses. Comparisons between groups at different time points were performed using Student's *t* tests, and results were expressed as mean ± SD. Differences were considered statistically significant at $p < 0.05$. $p < 0.05$ was considered statistically significant, whereas $p < 0.01$ was considered highly significant. Prism 5 software (version 5.01, GraphPad software Inc.) was used for generation of charts.

3 | RESULTS

3.1 | Effects of different salinity gradient schemes on survival rate of cuttlefish larvae

A sharp change in salinity causes death of *S. pharaonis*. A reduction in 4 psu salinity every 24 h resulted in death of all cuttlefish within 48 h (Figure 1). A reduction in 3 psu salinity every 24 h showed a 48-h survival rate at 50%. In addition, the 96-h cumulative survival rate was 10%. Reduced of salinity by 2 psu every 24 h showed a survival rate of 75% at 96 h. These findings show that Plan C was a more effective scheme for a gradual decrease in salinity. Salinity was then maintained at 22 psu for long-term culture.

3.2 | Histological analysis and scanning electron microscopic evaluation of gills

Histological changes of gills of cuttlefish after exposure to different salinity levels are illustrated in Figure 2. Gill filaments comprise single columnar cells and single-layer flat cells and are basic functional units. Epithelial tissue of gills is composed of columnar cells and flat cells. In the control group, the edge of gill epithelial tissue was clear with a complete structure (Figure 2a). In the low-salt group, the edge of gill epithelial tissue was incomplete and cells were exfoliated (Figure 2b).

Moreover, the epithelium of the gill from the control group comprised micro crest and complete structure. More secretory

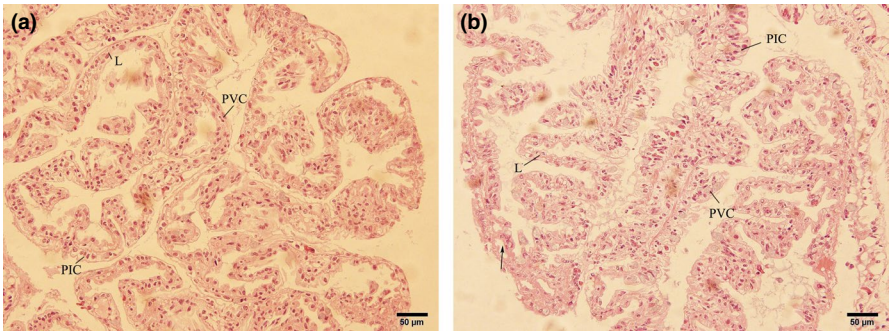

FIGURE 2 Observation of *S. pharaonis* gills under a light microscope. (a) gills from the control group. (b) gills from the treatment group. The arrow shows the epithelial cells that came off. PIC, pillar cells; PVC, pavement cells; L, gill lamella

granules were observed in flat cells, whereas abundant black vesicles were observed in the cytoplasm between nucleus and micro crest (Figure 3a). Chromatin of columnar cells was loose, and cytoplasm contents were abundant (Figure 3c). A uniform cell density was observed in mucus cells, with a regular shape (Figure 3e). In the low-salt group, gill epithelial micro crest was not visible, the structure was incomplete, and secretory granules and black vesicles were few in the flat cells (Figure 3b). Besides, chromatin of columnar cells shrank, the number of mitochondria increased and were swollen, cristae increased; high levels of rough endoplasmic reticulum were observed and the cytoplasm was not complete (Figure 3d). Additionally, electron density of the mucus cells was uneven, and the boundary between some particles was fuzzy (Figure 3f). These observations indicate varying degrees of adaptive changes in flat cells, columnar cells and mucous cells.

3.3 | Osmotic pressure regulation response to salinity stress

Levels of the main biochemical parameters of Na^+/K^+-ATPase and activity of Ca^{2+}/Mg^{2+}-ATPase were used to explore how *S. pharaonis* responds to osmotic pressure regulation under salinity stress (Figure 4). Analysis of activity of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase first exhibited an increasing trend, then later showed a decreasing trend, with a maximum level observed on day 2. Biochemical parameters of low salinity group were significantly lower compared with levels of these parameters in the control group at day 0 and day 14 ($p < 0.01$). Activity of Na^+/K^+-ATPase in low salinity group was significantly higher compared with that of the control group at 0, 12 h, day 2 and day 7 ($p < 0.01$). Activity of Ca^{2+}/Mg^{2+}-ATPase was significantly higher compared with that of the control group at day 2 and day 7 ($p < 0.05$).

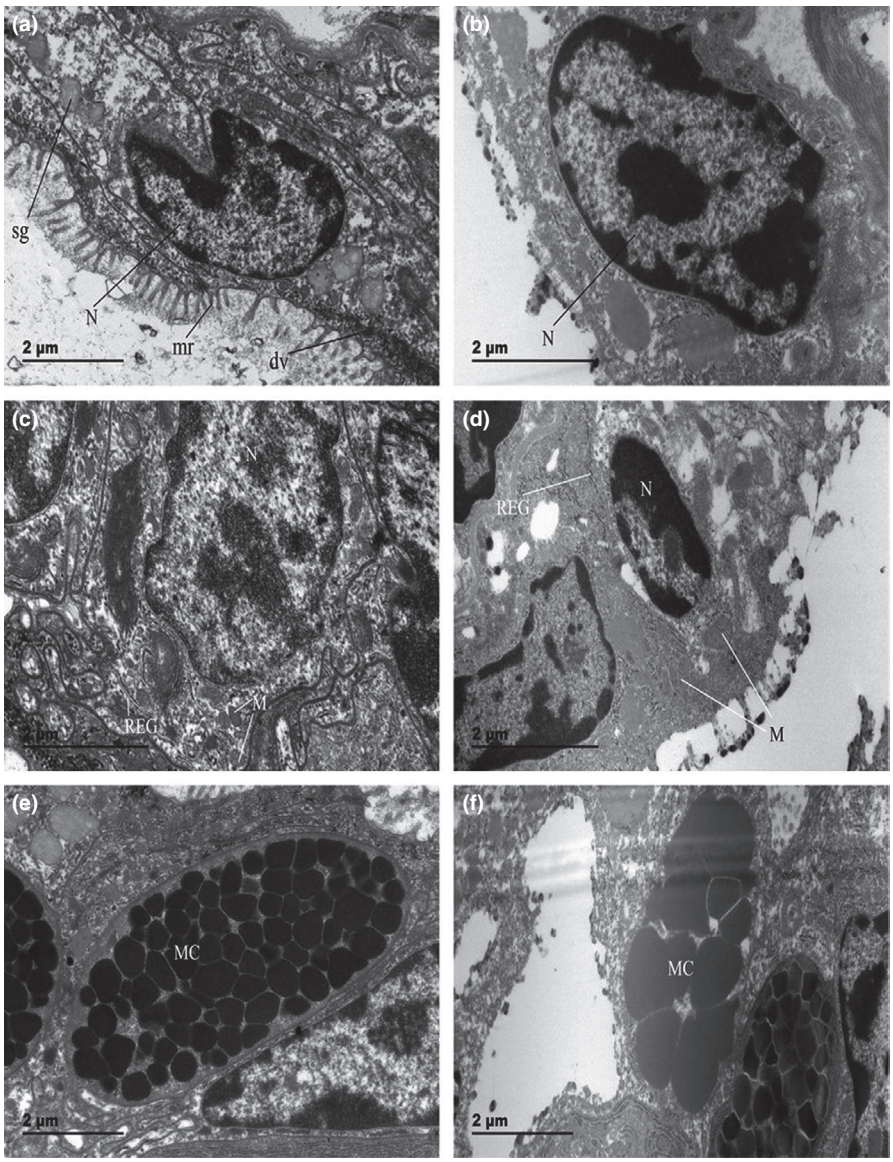

FIGURE 3 Observation of *S. pharaonis* gills under TEM. (a) pavement cell (PVC) of the control group, ×25,000; (b) pavement cell (PVC) of the treatment group, ×25,000; (c) pillar cell (PIC) of the control group, ×25,000; (d) pillar cell (PIC) of treatment group, ×25,000; (e) mucous cell (MC) of the control group, ×20,000; (f) mucous cell (MC) of treatment group, ×20,000. M, mitochondria; N, nucleus; PVC, pavement cell; PIC, pillar cell; MC, mucous cell; REG, rough endoplasmic reticulum; dv, black vesicle; mr, microridge; sg, secretory granule

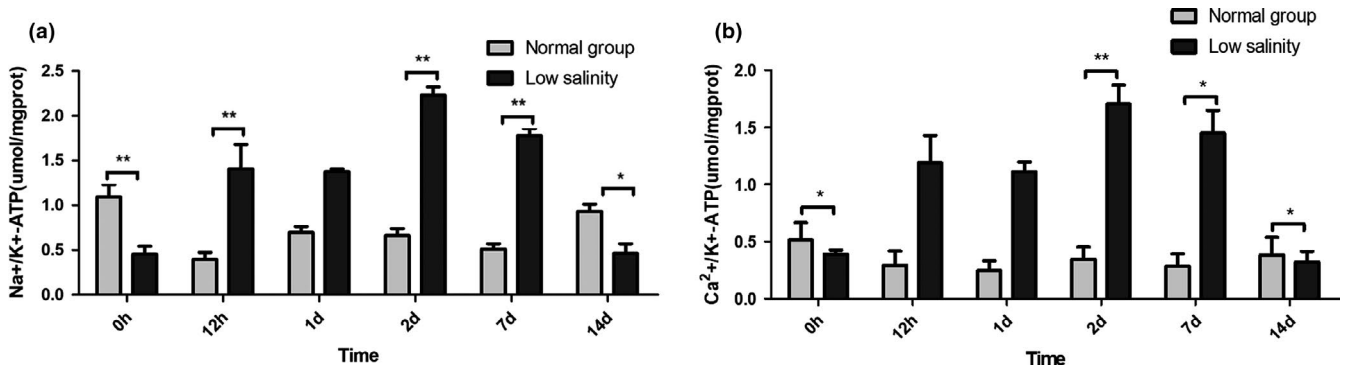

FIGURE 4 Effect of low salinity on biochemical indicators in gills of *S. pharaoni*. (Mean ± SD, $N = 5$) (a) Na^+/K^+-ATPase activity and (b) Ca^{2+}/Mg^{2+}-ATPase activity

These findings imply that ATPase is involved in osmotic pressure regulation in *S. pharaonis*. Na^+/K^+-ATPase showed a synergistic activity with Ca^{2+}/Mg^{2+}-ATPase. Notably, Na^+/K^+-ATPase was more sensitive to salinity stress compared with Ca^{2+}/Mg^{2+}-ATPase.

3.4 | Effects of osmotic pressure on regulatory-related genes activity under different salinity levels

Relative expression of Na^+/K^+-ATPase α subunit in gill significantly increased and reached a maximum at day 14 d ($p < 0.01$). The expression level at day14 was significantly different compared with the level on day 1 ($p < 0.01$) (Figure 5a). Relative expression of aquaporin TIP4-1 in the gill first increased then decreased, with a maximum level observed at day 0.5 ($p < 0.01$) (Figure 5b). Relative expression of Zfand4 in gill decreased over the study period with maximum level observed on day 0 (Figure 5c).

4 | DISCUSSION

Growth and development of aquatic animals are dependent on salinity levels of their habitats, as they directly affect their survival, feeding and growth (Phuc et al., 2014). The optimum salinity range for *S. pharaonis* is approximately 24–33 psu in mainland China. In this study, a gradual decrease in salinity from 29 psu showed that the cuttlefish only survived up to a salinity of 22 psu. Low salinity levels affect cell osmotic adjustment of aquatic animals. Animals must adapt to changes in the environment through various regulatory mechanisms to maintain normal body functions (Schipp et al., 1979). Similar results were observed in this study.

The gill is an important organ for osmotic pressure regulation. Gill filaments comprise flat cells, column cells, mucus cells, mesenchymal cells and chlorine cells. Flat cells have micro cristae on their surfaces, which increase the contact area between gills and water, facilitating gas and substance exchange. Damage to micro cristae on the surface of the flat cells in the low-salt group implies that some cells could not adapt efficiently to the low-salt environment. Analysis of the low-salt column cells showed that the chromatin was shrivelled, the number of mitochondria was high and the structure was expanded, high levels of rough endoplasmic reticulum were observed and the cytoplasm was not enriched. Notably, chromatin shrinkage is a major morphological feature of apoptosis. Changes in these organelles imply that low-salt column cells avoid stress through energy and material metabolism. However, the cell structure was severely damaged; therefore, osmotic pressure was not effectively regulated. Mucus cells are located in the gill epithelium of aquatic animals. Previous studies report that the mucus on the body surface of shellfish contains large amounts of antibacterial and bacteriolytic substances, implicated in their non-specific immunity (Ming et al., 2001; Wang & Fang, 2009; Wang et al., 2003). In addition, mucus cells in the gill tissue of *S. pharaonis* may function by secreting mucus. Electron density of mucus cells in the control group was uniform with regular shape. On the other hand, the electron density of mucus cells in the low-salt group was not uniform, and the boundary between some mucus cells was fuzzy. These changes imply that non-specific immunity of the gill tissue of the cuttlefish was affected. Notably, mesenchymal cells and chlorine cells were not identified; therefore, further studies should be performed to explore the effect of low salinity levels on these cells.

ATPase enzyme is an important protease in ion regulation. In the normal physiological state, the internal environment of the body of the fish is stable, whereas the balance is broken when the environmental salinity changes. Na^+/K^+-ATPase activity and Ca^{2+}/Mg^{2+}-ATPase activity are then activated to establish a new balance (Lin et al., 2004). Na^+/K^+-ATPase is a transmembrane protein, also known as sodium pump or sodium-potassium pump, which is ubiquitous in eukaryotic cell membrane. It is expressed at significantly high levels in salt transporting tissues such as gills (Zhang et al., 2017). In this study, activity of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in gills of cuttlefish in the low salinity group initially increased, then normalized. Similar results were reported in a previous study on *Penaeus vannamei* (Jaffer et al., 2020). This finding implies that change in Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activity was due to 'stress response' by *S. Pharaonis* in adapting to the salinity changes. The trend and time point of the activity of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in gills were similar, indicating that the two enzymes may have a synergistic effect in osmotic regulation. In addition, a

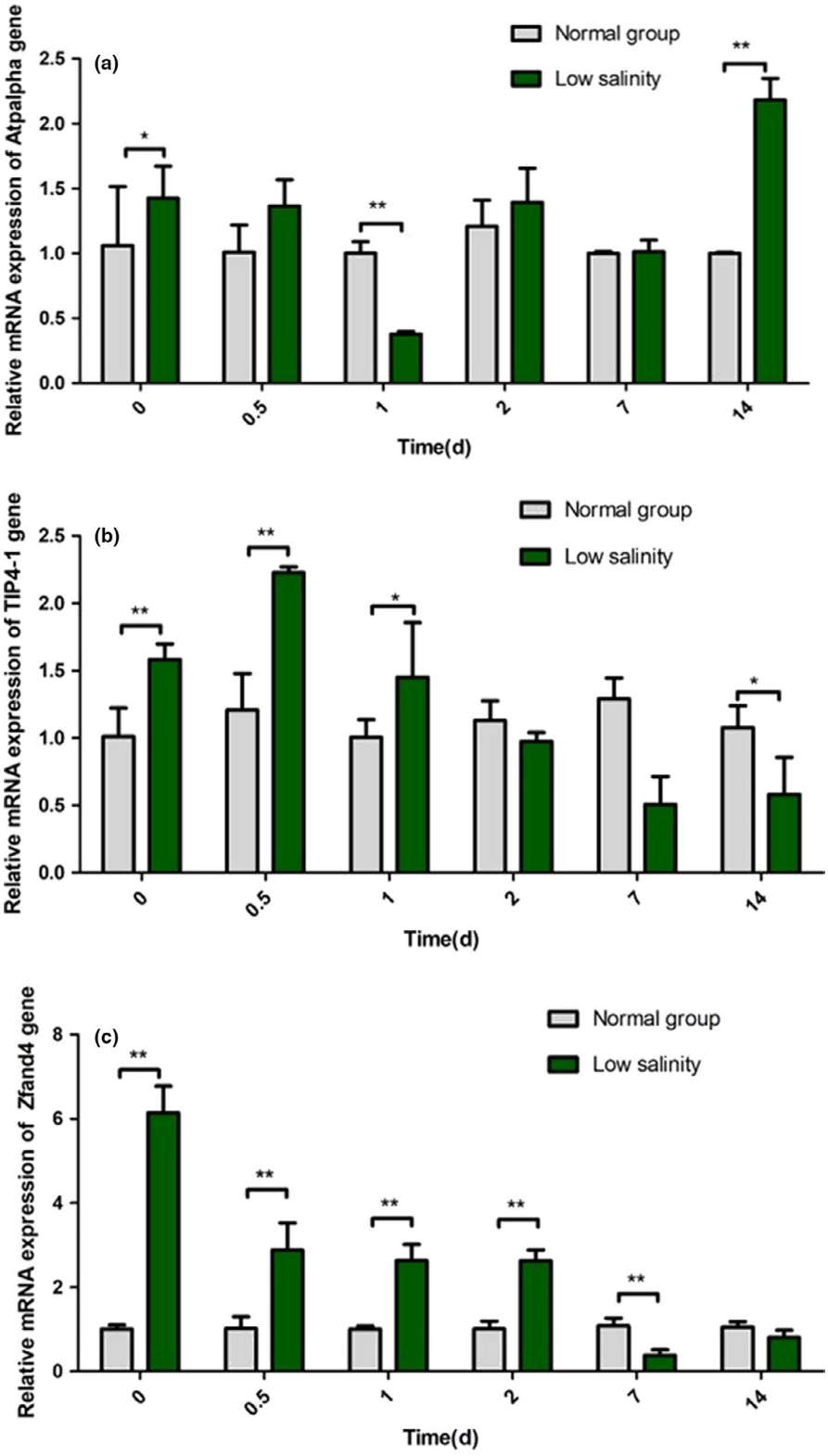

FIGURE 5 Expression of osmotic pressure regulatory genes in gills of *S. pharaonis* larvae at different durations (Mean ± SD, *N* = 5). (a) Atpalpha gene; (b) TIP4-1 gene; (c) Zfand4 gene

significant change in Na^+/K^+-ATPase activity (compared with the control group) occurred earlier, indicating that Na^+/K^+-ATPase in gill was more sensitive to salinity stress compared with Ca^{2+}/Mg^{2+}-ATPase.

Osmotic adjustment-related genes play an important regulatory role in response to salt stress. In this study, we evaluated the expression levels of 3 osmoregulatory-related genes, including Na^+/K^+-ATPase α subunit gene, aquaporin TIP4-1 and Zfand4 gene.

The α subunit of Na^+/K^+-ATPase contains nucleotide and cation binding sites, catalytic sites, chemical modification regulatory sites and ligand binding sites (Imsland et al., 2003). These sites

are implicated in activation and/or inhibition of the activity of the enzyme (He et al., 2001). Transition from high-salt to low-salt water increases the expression levels of Na^+/K^+-ATPase gene in the gills of *Eriocheir sinensis* larvae (Sun et al., 2013) and *Salmo salar* (Bystriansky & Schulte, 2011). A similar phenomenon was reported with Na^+/K^+-ATPase α subunit of the gill in this study. Notably, expression levels of Na^+/K^+-ATPase α subunit gene in gills is different from that of Na^+/K^+-ATPase, which can be attributed to the fact that the enzyme and gene expression does not occur at the same level.

Aquaporin is a transmembrane protein that transports water and plays an important role in maintaining osmotic pressure balance (Ge & Zhao, 2016). TIP4-1 expression level in the gill initially increased then it decreased. This indicates that cuttlefish maintained osmotic pressure balance in the body by regulating expression of TIP4-1, thus maintaining homeostasis of cells. Similar findings have also been reported in aquaporin levels in *Portunus trituberculatus* (Wang et al., 2014) and *Seabream gilthead* (Deane & Woo, 2006).

Moreover, A20/AN1 zinc-finger domain-containing proteins play an important role in immune response regulation. For instance, A20/AN1 zinc-finger proteins are associated with stress response in plants (Jin et al., 2007; Vij & Tyagi, 2008). Similarly, A20/AN1 zinc-finger proteins represent common elements of stress response in plants and animals (Vij & Tyagi, 2006). In the present work, Zfand4 expression level in gill decreased implying that low salt levels potentially inhibits its expression.

In summary, biochemical and molecular analysis shows that Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase, TIP4-1 and Zfand4 are involved in regulation of osmotic pressure in *S. pharaonis*. Histological analysis of hepatocytes showed significant damage at the cellular level due to low salinity, implying that *S. pharaonis* does not fully adapt to extremely low salinity levels (from 29 psu to 22 psu in 48 h). These findings show that gradual decrease in salinity levels can effectively lead to acclimatization of *S. pharaonis*.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTION

WWS, KLW and CLW conceived and designed the experiments. KLW, YMY and HWX performed the experiments. WWS, KLW, HWX, CLW, YZ, RHL and CKM analysed the data. WWS, KLW, YZ, YMY, HWX, CLW and CKM contributed reagents/materials/analysis tools. KLW, WWS and HWX wrote the paper. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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