Cortisol regulation of Na\(^+\), K\(^+\)-ATPase β1 subunit transcription via the pre-receptor 11β-hydroxysteroid dehydrogenase 1-like (11β-Hsd1L) in gills of hypothermal freshwater milkfish, *Chanos chanos*

Yau-Chung Hu\(^{a,c}\), Keng-Fu Chu\(^{a}\), Lie-Yueh Hwang\(^{b}\), Tsung-Han Lee\(^{a,c,*}\)

\(^{a}\) Department of Life Sciences, National Chung Hsing University, Taichung, 402, Taiwan
\(^{b}\) Taishi Station, Mariculture Research Center, Fisheries Research Institute, Council of Agriculture, Yulin, 636, Taiwan
\(^{c}\) The iEGG and Animal Biotechnology Center, National Chung Hsing University, Taichung, 402, Taiwan

**ARTICLE INFO**

**Keywords:**
Milkfish
Cortisol
11β-Hsd
Na\(^-\), K\(^+\)-ATPase β1 subunit
Hypothermal stress
Euryhaline fish

**ABSTRACT**

Hypothalamic stress changes the balance of osmoregulation by affecting Na\(^+\), K\(^+\)-ATPase (Na-K-ATPase) activity or inducing modulation to epithelium permeability in fish. Meanwhile, cellular concentrations of cortisol can be modulated by the pre-receptor enzymes 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-Hsd1 and 2). In fish, increasing levels of exogenous cortisol stimulate Na\(^+\) uptake via specific interaction with cortisol. This study investigated cortisol effects on expression of Na-K-ATPase subunit proteins and activity in gills of milkfish under hypothermal stress and revealed that the plasma cortisol contents as well as gill 11β-hsd11 and na-k-atpase β1 mRNA abundance were decreased in fresh water (FW) milkfish. Meanwhile, in the seawater (SW) milkfish, the plasma cortisol contents and gill 11β-hsd11 and na-k-atpase β1 mRNA abundance was increased under hypothermal stress. On the other hand, the abundance of 11β-hsd2 mRNA increased in both FW and SW. In addition, 11β-hsd11 expression increased in FW milkfish but decreased in SW milkfish after cortisol injection. Accordingly, the results that gill Na-K-ATPase activity of FW milkfish was affected by environmental temperatures as well as cortisol-dependent Na-K-ATPase β1-subunit levels might be due to increased expression of 11β-hsd11 that elevated intracellular cortisol contents. In hypothermal SW milkfish, decreasing abundance of Na-K-ATPase β1 protein due to reduced expression of 11β-hsd11 was found after cortisol injection. Thus, under hypothermal stress, 11β-HSD1L in FW milkfish gills was used to modulate cortisol and the following effects on increasing the transcription of Na-K-ATPase β1 protein.

1. Introduction

Hypothalamic stress leads to an imbalance in fish osmolarity. Metabolism-dependent ion transport is more sensitive to temperature decreases as compared to passive ion diffusion [1]. To compensate for ion loss, either ion uptake can be increased through an increase in Na\(^+\), K\(^+\)-ATPase (Na-K-ATPase) activity or epithelium permeability can be decreased, resulting in slower down-gradient diffusion [1–3]. Low temperature-exposed of fishes found shifts in plasma osmolesytes in Mozambique tilapia (*Oreochromis mossambicus*) [4], alewife (*Alosa pseudoharengus*) [5], and milkfish (*Chanos chanos*) [6]. Gill Na-K-ATPase activity has also been found to change, with a general trend toward reduced enzyme activity at lower temperatures [7,8]. Na-K-ATPase is involved in ion secretion of gill ionocytes by providing an electrochemical gradient for Na\(^+\) and Cl\(^-\) movement between blood and water [3]. Regardless of the increased and decreased magnitude of temperature change, initial decreases in plasma Na\(^+\) and Cl\(^-\) levels occur, followed by a decline in tissue water content and rise in extracellular fluid [9–12], suggesting that the strength of physiological responses is correlated with the magnitude of difference from the original regulatory set point. Hu et al. [13] further illustrated that protein levels of both α1- and β1-subunits are correlated to mRNA levels as well as to Na-K-ATPase activity in gills of milkfish and exhibit co-localization of α1- and β1-subunits in epithelial ionocytes. Co-immunoprecipitation of α1- and β1-subunits indicates the formation of an α1-β1 complex in milkfish gills [13].

The stress response in teleosts is characterized by the release of corticosteroids/cortisol [14,15]. This endocrine response promotes physiological adjustments that support the increased energetic demands associated with stressful events [16–18]. Circulating levels of cortisol change with several factors, including the type and severity of stressor and the rate of hormone synthesis, release, and degradation

---

*Corresponding author at: Department of Life Sciences, National Chung Hsing University, 145 Xingda Road, Taichung, 402, Taiwan.
E-mail address: thlee@email.nchu.edu.tw (T.-H. Lee).*
In fish, plasma cortisol concentrations generally vary from 2 to 42 ng mL\(^{-1}\) (5.51–115.88 nM) in resting fish to 20–500 ng mL\(^{-1}\) (55.18–1379.50 nM) post-stress \cite{18,19}. In zebrafish larvae, increasing levels of exogenous cortisol stimulate Na\(^+\) uptake via the specific interaction of cortisol with the glucocorticoid receptor \cite{20}. The roles of cortisol in osmoregulatory mechanisms of FW fish are well known. In SW fish, however, the signaling pathways through which these effects are exerted remain controversial. Cortisol level increased in the plasma with higher Na-K-ATPase expression when salinity changes in black porgy (\textit{Acanthopagrus schlegelii}) \cite{21}, rainbow trout (\textit{Salmo gairdneri}) and Atlantic salmon (\textit{Salmo salar}) \cite{22}. In addition, cortisol injection increases Na-K-ATPase activity in sea bream (\textit{Sparus aurata}) \cite{23} and mRNA/protein expressions in Atlantic salmon \cite{24,26}. Thus, changes in plasma cortisol concentration may alter Na-K-ATPase activity by modulating expression of Na-K-ATPase subunits.

The main corticosteroids isolated from fish blood are cortisol and cortisone, which vary with species, sex, and reproductive status \cite{27}. In the target tissues, 11β-hydroxysteroid dehydrogenases (11β-Hsds) are the key enzymes regulating the cortisol signaling pathway, catalyzes the conversion of cortisol and controls the cortisone switch \cite{28}. To our knowledge, there is no experimental evidence of 11β-Hsds activities in milkfish. Functional data of 11β-Hsds from other fishes are also rare. Most mammalian cells express 11β-Hsd1 to produce cortisol from the inactive cortisone \cite{29}. Baker \cite{30}, on the other hand, reported that the zebrafish (\textit{Danio rerio}) genome contains no homologue of 11β-Hsd1. Instead, zebrafish expresses 11β-HSDD1 which lacks the function of converting cortisone to cortisol \cite{31}. In most fishes, the analogue of zebrafish 11β-hsdl1 is 11β-hydroxysteroid dehydrogenase 1-like, abbreviated as 11β-hsdl1 \cite{31}. 11β-Hsd2, however functions in the reverse direction to inactivate cortisol \cite{28}. Studies on the activity and function of 11β-Hsd2 have revealed its dual roles in fish in the inactivation of glucocorticoids and activation of androgens \cite{32,33}. Accordingly, we hypothesized that in milkfish, pre-receptor enzymes such as 11β-Hsds regulate cortisol levels in tissues.

In this study, differential responses of cortisol signaling between SW- and FW-acclimated milkfish under hypothermal stress were investigated. In addition, the presence of pre-receptor metabolism of cortisol in gills was hypothesized and tested by determining the expression of 11β-hsds identified in the milkfish transcriptome database \cite{36}. Positive correlations were found between temperature-enhanced changes and cortisol treatment in Na-K-ATPase activity and 11β-hsdl1 expression, as well as Na-K-ATPase β-subunit protein levels. Low-temperature effects and cortisol injection increased the abundance of 11β-hsdl2 mRNA in both FW and SW. Meanwhile, under hypothermal stress, 11β-hsdl1 and Na-K-ATPase β1 protein increased in FW milkfish but decreased in SW milkfish after cortisol injection. In hypothermal SW milkfish, less abundance of Na-K-ATPase β1 protein was found after cortisol injection, due to the expression of 11β-hsdl1.

2. Materials and methods

2.1. Experimental fish

Juvenile milkfish were obtained from a local fish farm. Average standard length and body weight were 10.2 ± 0.2 cm and 13.7 ± 1.3 g, respectively. After rearing for one month in BW (15%) prepared from local fresh tap water with the appropriate amount of Blue Treasure Tropical Fish Sea Salt (Qingdao Sea-Salt Aquarium Technology, Qingdao, China), milkfish were transferred to either SW (35%) or FW (0%) at 28 ± 1 °C for at least 3 weeks before sampling. Water was continuously circulated through fabric-floss filters and partially replaced every 2 weeks. The photoperiod was 12 h light: 12 h dark. Fish were fed daily with commercial pellets. Before sampling, the fish were anesthetized with 500 mL/L 2-phenoxyethanol. After anesthesia, fish were sacrificed by cutting their spinal cord over crushed ice. The gills were dissected, rapidly frozen in liquid nitrogen, and stored immediately at −80 °C for the following analyses. The protocol used for the experimental milkfish was reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 102-114 to T.H. Lee).

2.2. Low temperature-exposure experiments

For the low temperature-exposure experiments, the FW- or SW-acclimated milkfish were transferred to other tanks with cooling systems (PF-225 M, PRINCE, Tainan, Taiwan). The temperature of the water was cooled at a constant rate (2 °C/h) from 28 °C to 18 °C. Because a preliminary study revealed that mRNA and protein expression of Na-K-ATPase α1- and β1-subunits were significantly changed after the second day (48 h) of 18 °C exposure, the plasma cortisol levels of FW and SW milkfish were determined after 2 d for the groups exposed to 28 °C (the control group) and 18 °C (the low-temperature group). The number of animals per group is 6 in each time point and group.

2.3. Cortisol injection experiments

Cortisol doses were chosen based on the results of related studies in tilapia \cite{23,26,37–39}. Hydrocortisone (Cortisol, Sigma-Aldrich, St Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, Sigma) was used for the intraperitoneal injections \cite{20,40}. The injections were given between 9 and 10 a.m. \cite{41}. Then, the milkfish were anesthetized with 500 μL/L 2-phenoxyethanol before being subjected to one of the following four treatments: (1) 100 μL DMSO injection (the control group), (2) 4 μg cortisol/g body weight injection, (3) 50 μg cortisol/g body weight injection, or (4) non-injection (the mock group) \cite{42}. The preliminary data revealed significant changes in expression of gill Na-K-ATPase β1 protein after transfer of milkfish to 18 °C for 48 h. Thus, the milkfish were injected with cortisol before cold exposure and sampled for analyses of plasma cortisol contents as well as total RNA and protein of gills after exposure to 18 °C for 48 h. The number of animals per group is 6 in each time point and group.

2.4. Analysis of plasma cortisol contents

Blood was collected from the dorsal veins of anesthetized milkfish using heparinized 1 mL syringes. The plasma was stored at 4 °C for experiments after centrifugation at 1000 × g at 4 °C for 15 min. Cortisol levels were analyzed using an fish ELISA kit (Cusabio Biotech, Wuhan, China), according to the manufacturer’s instructions. The manufacturer declared no significant cross-reactivity or interference between fish cortisol and analogues. The optical density of the solution in each well was determined within 10 min using a microplate reader (VERSAMax, Molecular Devices) set to 450 nm. The number of animals per group is 6 in each time point and group.

2.5. Total RNA extraction and real-time PCR

Total RNA samples from gills of experimental fish were extracted using the TriPure Isolation Reagent (Roche, Mannheim, Germany) after freezing in liquid nitrogen. The RNA pellet was treated according to the RNA clean-up protocol of the RNAspin Mini RNA Isolation Kit (GE Health Care, Piscataway, NJ, USA). Integrity of the RNA was verified on a 1% agarose gel. The concentration and quality of the RNA were determined using a NanoDrop 2000 (Thermo, Wilmington, DE, USA). First-strand cDNA was synthesized from 2 μg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. For the optimal primer annealing test, PCR amplification was performed using EX Taq polymerase (Takara, Otsu, Shiga, Japan) and 0.25 μM of each primer. The PCR products were verified on 1% agarose gels. Expression of milkfish \textit{na-k-aptase a1, na-k-aptase β1, 11β-hsdl1} and \textit{11β-hsdl2} was quantified using the SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a MiniOpticon Real-Time
presents upregulation of over 5-fold in the ratio of FW milk to SW milk.

Abbreviations of Fig. 1

KCNJ1: potassium voltage-gated channel subfamily J member 1
IRS1: insulin receptor substrate 1

2.6. Preparation of the membrane vesicle fraction

Protein samples from the gills of experimental fish were frozen with liquid nitrogen and steeped in a mixture of homogenization buffer.

PCR System (Bio-Rad). Elongation factor 1α (ef1α) was used as an internal control for the target gene. All samples were normalized to the control, a pool of cDNA from many organs including the heart, brain, kidney, gill, esophagus, intestine and liver [13], the corresponding control, a pool of cDNA from many organs including the heart, brain, kidney, gill, esophagus, intestine and liver [13], the corresponding control, a pool of cDNA from many organs including the heart, brain, kidney, gill, esophagus, intestine and liver [13]. The letter “n” indicates each cDNA sample used in these experiments, and “c” indicates the control. Accession numbers and primer sequences are listed in Table 1.

2.7. Antibodies

The primary antibodies used in this study included the (1) mouse monoclonal antibody (α6F, DHSB; 1:5000) raised against the α1-subunit of avian Na-K-ATPase with predicted size of 100 kDa; and (2) goat polyclonal antibody (β1, AVIVA, San Diego, CA, USA; 1: 500) raised against the β1-subunit of human Na-K-ATPase with predicted size of 40 kDa. The secondary antibody for immunoblots was horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:20,000) and rabbit anti-goat IgG (1:5000) (Pierce).

2.8. Immunoblotting

The sample mixture of protein buffer dye and 20 μg of total membrane fraction of gill proteins (v/v = 1/5) was heated to 95 °C and held at that temperature for 5 min to denature the proteins. The pre-stained protein molecular weight marker was purchased from Fermentas (SM0671, Hanover, MD, USA). All samples were separated using Fig. 1. The suggested pathway of cortisol action modified from the pathway of aldosterone-regulated sodium reabsorption in the KEGG database in FW milkfish as compared to SW milkfish. (http://www.genome.jp/kegg-bin/show_pathway?map=hsa04960&show_description=show). Metabolites or compounds are represented in rectangles with black frames. Rectangles with blue frames are mRNA expression of enzymes that participate in the mineralocorticoid pathway. Up- and down-regulation of genes is represented by the FPKM ratio of FW milkfish to SW milkfish. The green color indicates a decrease in expression of more than 0.5-fold (downregulation). The red color indicates an increase of over 2-fold (upregulation). The less intense shades of green and red indicate changes in expression levels between 0.5- and 2-fold. The purple color re-
electrophoresis on a 10% SDS-polyacrylamide gel (20 μg of protein/lane) on a Mini-protein II electrophoresis cell (Bio-Rad). The separated proteins were then transferred to 0.45 mm PVDF (polyvinyl difluoride) membranes (Millipore, Bedford, MA, USA). The blots were preincubated to minimize nonspecific binding for 1 h at room temperature in phosphate buffer saline with Tween 20 buffer (PBST; final concentration: NaCl, 137 mM; KCl, 3 mM; Na2HPO4, 10 mM; KH2PO4, 2 mM; Tween 20, 0.2% (v/v)) containing 5% (w/v) nonfat dried milk. The blots were then incubated first with the primary antibody and then with the secondary antibody. Images were developed with Immobilon™ Western HRP Substrate (Millipore) under a cooling CCD (charge-couple device) camera (Chemidoc XRS+®, Bio-Rad) with associated software (Quantity One version 4.6.8, Bio-Rad). The bands of blots were converted to numerical values using ImageLab 3.0 (Bio-Rad) to quantify and compare the relative protein abundance in the immunoreactive bands. The immunoblotting procedures were carried out according to Tang et al. [44] and Hu et al. [13].

2.9. Determination of Na-K-ATPase activity

Na-K-ATPase activity was assayed by observing the inorganic phosphate generated using a colorimetric assay following Tang et al. [45]. A 340 μL reaction medium buffer (final concentration: imidazole, 100 mM; NaCl, 125 mM; KCl, 75 mM; MgCl2, 7.5 mM; pH 7.6) was prepared for the reaction mixture, which was mixed with a 10 μL protein sample, 100 μL 10 mM Na2ATP, and 50 μL 10 mM ouabain (specific inhibitor of Na-K-ATPase, minimum activity group) or deionized water (maximum activity group). To test the potential of Na-K-ATPase activity, both minimal and maximal activity groups were incubated in parallel at 28 °C for 10 min and terminated using an immediate ice bath for 10 min to stop the reaction [4]. The colorimetric reagent (final concentration: ammonium molybdate, 0.75%; H2SO4, 0.9 M; Tween-20, 1%) was mixed in equal volumes of the reacted samples, and inorganic phosphate was measured at 405 nm with a microplate reader (VERI-SAmax, Molecular Devices, Sunnyvale, CA, USA) to calculate Na-K-ATPase activity. The absorbance of each sample was determined in triplicate.

2.10. Statistics

Results were compared using Minitab software (Minitab® 16.1.0, Minitab Inc., State College, PA, USA). Results of the cortisol injection experiments were analyzed using one-way ANOVA followed by Tukey’s test for mRNA and protein expressions among different groups. Cortisol concentrations, gill 11β-hsds mRNA expression, and Na-K-ATPase activity in SW and FW milkfish were compared at 28 °C and 18 °C using a two-way ANOVA followed by Tukey’s tests. P < 0.05 was set as the significance level. Values were expressed as means ± SEM (standard error of the mean).

3. Results

3.1. Prediction of cortisol action on the mineralocorticoid pathway according to milkfish transcriptome sequencing

On the basis of the milkfish transcriptome database, the prediction of mineralocorticoid pathway was made in the reference of the KEGG database. The expressions of genes encoding proteins for the mammalian mineralocorticoid pathway were upregulated in the FW group, except for SGK1, SLC9A3R2, and IGF1 (Fig. 1). Higher expression of all downstream genes of the mineralocorticoid pathway was made in the reference of the KEGG database. The expressions of genes encoding proteins for the mammalian mineralocorticoid pathway were upregulated in the FW group, except for SGK1, SLC9A3R2, and IGF1 (Fig. 1). Higher expression of all downstream genes of the mineralocorticoid pathway, such as the Na-K-ATPase α, β, and γ subunits, were found in FW milkfish rather than SW ones.

3.2. Low-temperature effects on plasma cortisol concentration and gill 11β-hsds expression

Plasma cortisol levels as well as gill 11β-hsds mRNA abundance were determined on the second day after exposure to 18 °C. Significant increases in plasma cortisol levels were found in SW milkfish when exposed to 18 °C for 2 d (9.66 ± 0.82 nM vs. 16.28 ± 1.11 nM). In contrast, the plasma cortisol concentration of FW milkfish decreased significantly after two-day exposure to 18 °C (23.73 ± 0.82 nM vs.4.69 ± 1.1 nM) (Fig. 2A). 11β-hsd1l expression was significantly higher in SW milkfish after exposure to 18 °C for 2 d, whereas it decreased significantly after exposure to 18 °C for 2 d in FW milkfish.

Fig. 2. Effects of hypothermal stress (18 °C) on (A) plasma cortisol concentrations and (B, C) gill 11β-hsd1l and 2 mRNA expression in milkfish. The milkfish were sampled 2 days after transfer from 28 °C to 18 °C. Different letters indicate significant differences among groups in SW (lower case) or FW (upper case). Different letters (a vs. b and A vs. B) indicate significant differences between the 28 °C and 18 °C groups at the same salinity using a two-way ANOVA followed by Tukey’s post hoc method (n = 6, P < 0.05). No interaction was found between salinity and temperature in (C). Values are means ± SEM.
There was a significant interaction between the effects of salinity and temperature on cortisol concentration and $11\beta$-hsd1l expression (Table 2A, B). The low temperature thus increased the cortisol concentration and $11\beta$-hsd1l expression of the SW group, but decreased those of the FW group. $11\beta$-hsd2l expression increased under cold exposure in both the FW and SW groups, and it was significantly higher in FW milkfish than in SW individuals (Fig. 2C). No interaction between the effects of salinity and temperature was found (Table 2C).

### 3.3. Effects of cortisol injection on gill $11\beta$-hsd2 expression

In SW milkfish, the mRNA abundance of $11\beta$-hsd1l was significantly decreased in gills of the cortisol-injection group as compared to the cold-exposed group (Fig. 3A). In contrast, the $11\beta$-hsd1l mRNA abundance was significantly increased in the cortisol-injection group as compared to the 18 °C group in FW milkfish (Fig. 3A). There was a significant interaction between the effects of salinity and temperature on the expression of $11\beta$-hsd1l, revealing that cortisol recovered $11\beta$-hsd1l expression to the level of the 28 °C group in SW, but not in FW (Table 3A). On the other hand, gills of both hypothermal SW and FW milkfish significantly expressed higher levels of $11\beta$-hsd2l in the cortisol injection groups (Fig. 3B). No interaction, however, was found between the effects of salinity and temperature (Table 3B).

### 3.4. Na-K-ATPase expression and activity in gills of milkfish after cortisol injection

In low temperature-exposed FW milkfish, the expression of Na-K-ATPase $\alpha_1$ mRNA was not significantly different among the various injection groups (Fig. 4A). Compared to the normal temperature (28 °C) group, however, the mRNA levels of Na-K-ATPase $\beta_1$ decreased significantly in the low-temperature (18 °C) group while increasing significantly after injection with 50 μg/g cortisol (Fig. 4B). Similarly, relative intensities of Na-K-ATPase $\alpha_1$ immunoreactive bands among various injection groups of low temperature-exposed FW milkfish were not significantly different (Fig. 5A). In addition, relative protein abundance of Na-K-ATPase $\beta_1$ in the 18 °C group decreased significantly and was induced significantly by the 50 μg/g cortisol injection (Fig. 5B). Conversely, in low temperature-exposed SW milkfish, expression of na-k-atpase $\alpha_1$ was not significantly altered (Fig. 4C), while expression of na-k-atpase $\beta_1$ increased significantly (Fig. 4D). After injection of the hypothermal SW milkfish with 4 μg/g cortisol or 50 μg/g cortisol, as well as DMSO, the solvent of cortisol, na-k-atpase $\beta_1$ abundance decreased significantly, but was still higher than that of the 28 °C group (Fig. 4D). Among various groups of SW milkfish, there was no significant difference in relative intensities of immunoreactive bands of gill Na-K-ATPase $\alpha_1$ (Fig. 5C). Meanwhile, the abundance of Na-K-ATPase $\beta_1$-subunits in the 28 °C group was significantly lower than that of the other groups (Fig. 5D). In low temperature-exposed FW milkfish, Na-K-ATPase activity was significantly decreased as compared to the normal temperature group and significantly induced in the 50 μg/g cortisol injection group (Fig. 6A). On the other hand, no significant difference in Na-K-ATPase activity was found among different injection groups of low temperature-exposed SW milkfish gills (Fig. 6B).

### 4. Discussion

The characteristics and central roles of gill Na-K-ATPase in maintaining ion homeostasis are the most extensively studied osmoregulatory components in both FW and SW euryhaline teleosts [46–53]. Kang et al. [6] reported different Na-K-ATPase responses to hypothermal stress in SW- and FW-acclimated milkfish. Na-K-ATPase
activity is significantly downregulated in gills of low-temperature-exposed FW milkfish without alteration of Na-K-ATPase α-subunit protein expression. Kang et al. [6] also suggested that Na-K-ATPase α-subunits are retained and protected by HSP70 under low-temperature stress. Moreover, the Na-K-ATPase α1-subunit was found to be associated with β1-subunits in milkfish gills [13]. In accordance with the findings of Kang et al. [6] that gill Na-K-ATPase activity of FW milkfish is lower at 18 °C compared to that at 28 °C, the present study revealed downregulation of FW gill Na-K-ATPase β1-subunit at 18 °C. Conversely, the gill Na-K-ATPase β1-subunit in SW milkfish at 18 °C was upregulated without simultaneous increases in the α1-subunit, which also indicated that the Na-K-ATPase α1-β1 complex was disrupted under hypothermal stress. Acclimation of fish to low temperatures involves a compensatory increase in Na-K-ATPase functional capacities, either by changing the protein expression or by enhancing the capacity of the enzyme [54–58]. In the case of acclimation to warmer temperatures, lower [59] or unchanged Na-K-ATPase capacities were found in the common carp, Cyprinus carpio [54]. In the goldfish (Carassius auratus), increases in Na-K-ATPase activity and larger Na-K-ATPase-immunoreactive ionocytes were reported in gills of 15 °C-acclimated individuals as compared to 29 °C-acclimated individuals [54]. However, in cold-adapted stenothermal Antarctic fish, lower [60,61] or higher [58] Na-K-ATPase capacities of cold-compensated fish were determined at the same temperatures by in vitro assay of Na-K-ATPase. The Antarctic fish T. bernacchii modulates the numbers of α1-expressing cells to increase Na-K-ATPase activity when exposed to warmer environments [62]. Such differences in Na-K-ATPase capacity found in cold-adapted stenothermal Antarctic fish might be associated with different levels of stenothermy of the investigated fish species [58]. However, few studies have focused on differential expression of Na-K-ATPase subunits in response to hypothermal challenge.

### Table 3


<table>
<thead>
<tr>
<th></th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) 11β-HSD1L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salinity</td>
<td>1</td>
<td>0.003</td>
<td>0.003</td>
<td>16.404</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Temperature</td>
<td>2</td>
<td>0.045</td>
<td>0.023</td>
<td>108.133</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>2</td>
<td>0.002</td>
<td>&lt; 0.001</td>
<td>0.586</td>
<td>0.561</td>
</tr>
<tr>
<td>Error</td>
<td>30</td>
<td>0.042</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| (B) 11β-HSD2         |                   |                |             |       |        |
| Salinity             | 1                 | 0.046          | 0.046       | 32.758| < 0.001|
| Temperature          | 2                 | 0.108          | 0.054       | 38.288| < 0.001|
| Interaction          | 2                 | 0.002          | < 0.001     | 0.591 | 0.561  |
| Error                | 30                | 0.042          | 0.001       |       |        |

Fig. 4. Effects of cortisol injection on mRNA expression of Na-K-ATPase α1 (A, C) and β1 (B, D) subunits in gills of hypothermal seawater (SW) and freshwater (FW) milkfish. The milkfish were sampled at 2 days after injection. Groups are listed as 28 °C, 18 °C, 18 °C + 4 μg/g cortisol, 18 °C + 50 μg/g cortisol, and 18 °C + DMSO injection. Different letters indicate significant differences among groups by one-way ANOVA and Tukey’s test (P < 0.05). Values are means ± SEM (n = 6).
In mammals, both mineralocorticoids and glucocorticoids have been found to increase mRNA expression of \textit{na-k-atpase} $\alpha$- and $\beta$-subunit genes [63]. Mineralocorticoids also increase expression of \textit{na-k-atpase} through mineralocorticoid receptors in the toad bladder [64], mammalian kidney [65], and hippocampus [66]. Meanwhile, glucocorticoid binding to glucocorticoid receptors has similar effects in the rat colon [67,68] and cultured liver cells from rat [69]. High doses of glucocorticoids have also been found to upregulate Na-K-ATPase concentration in the skeletal muscle [70]. Because of the lack of mineralocorticoids in fish [71], the corticosteroid biosynthesis pathways differ between fish and mammals [72]. Previous studies illustrated that, in teleosts, the corticosteroid response is followed by osmoregulatory changes under cold stress [73,74]. In low temperature-exposed FW milkfish, decreases in Na-K-ATPase activity might be caused by downregulation of mRNA and protein levels of the Na-K-ATPase $\beta_1$-subunit by lower cortisol content, since the plasma cortisol concentration of hypothermal FW milkfish decreased after 2 days of 18 °C exposure in the present study. Plasma cortisol concentration and gill Na-K-ATPase $\beta_1$-subunit expression were both significantly increased in SW milkfish after 2 days of 18 °C exposure. Moreover, comparison of cortisol levels and the predicted pathway of cortisol action between FW and SW milkfish at 28 °C indicated that cortisol levels are highly correlated with the expression of Na-K-ATPase subunits. In previous studies, when tilapia were transferred from FW to SW, gill \textit{na-k-atpase} $\alpha_1$ mRNA abundance increased significantly at 24 h, whereas plasma cortisol levels increased rapidly after 3 h [75,76]. In FW-acclimated black sea bream (\textit{Acanthopagrus schlegelii}), gill Na-K-ATPase activity as well as plasma cortisol contents are higher than those of SW-acclimated fish [21,77]. McCormick et al. [26] reported that cortisol induces \textit{na-k-atpase} $\alpha_1$ mRNA expression and Na-K-ATPase activity via the glucocorticoid receptor in gills of the Atlantic salmon (\textit{S. salar}) during smoltification. Furthermore, Tipsmark and Madsen [39] reported that cortisol increases \textit{na-k-atpase} $\beta_1$ and \textit{na-k-atpase} $\alpha_1$ mRNA abundance in FW-acclimated Atlantic salmon. Both \textit{na-k-atpase} $\beta_1$ and \textit{na-k-atpase} $\alpha_1$ mRNA abundance of FW salmon are induced by cortisol treatment. Conversely, an in vitro study revealed that cortisol only increases \textit{na-k-atpase} $\alpha_1$ abundance in gills of the silver sea bream (\textit{Sparus sarba}) [78]. We inferred that differential effects of cortisol on Na-K-ATPase expression might be dependent on whether the promoter of the Na-K-ATPase subunits contains specific response elements in various teleosts.

Cortisol is known to play multiple roles in the regulation of a wide range of physiological processes, including metabolism, immune responses, growth, reproduction, and osmoregulation [79]. Hanke et al. [80] analyzed the plasma cortisol concentration of milkfish acclimated...
to 5% brackish water with a gradual temperature increase and found significantly higher cortisol concentrations in milkfish exposed constantly to 33°C rather than 26°C. Cortisol treatment has been reported to stimulate Na\(^+\), Cl\(^-\), and Ca\(^{2+}\) uptake [81-83] and increase the functional surface area of ionocytes [84,85]. Cortisol treatment also increases development of epidermal ionocytes through the glucocorticoid receptor to enhance fox3 transcription factor expression [86,87]. Thus, cortisol promotes Na\(^+\) absorption by stimulating sodium chloride cotransporter (NCC) expression and the differentiation of NCC-expressing ionocytes in zebrafish [88]. Na-K-ATPase activity is also induced after cortisol injection in the rainbow trout, gilthead sea bream, and Atlantic salmon [26,48,89]. Hunter [90] stated in a review article that the glucocorticoid receptor is widely expressed in mammalian kidneys, with mRNA detected in most renal epithelial cells. In mammals, the pre-receptor metabolism of cortisol by 11β-HSD1 converts cortisone into active cortisol, whereas 11β-HSD2 catalyzes the reverse reaction [60]. However, 11β-HSD1 is located in the S3 proximal tubule, whereas the mineralocorticoid receptor and 11β-HSD2 have a more restricted distribution in the connecting tubule and collecting duct in mammalian kidneys [90]. Baker [30], on the other hand, reported that the zebrafish (Danio rerio) genome contains no homologue of 11β-Hsd1. Instead, 11β-Hsd1L [91-93], a gene phylogenetically close to 11β-Hsd1, is found in the zebrafish [30,94]. Human 11β-HSD1L converts cortisone to cortisone, but does not cause the reverse reaction [92]. In the zebrafish, 11β-HSD1La and 11β-HSD1Lb lack the function of converting cortisone to cortisol [31]. The phylogenetic tree of milkfish 11β-Hsd1 with zebrafish 11β-HSD1L and human 11β-HSD1/L1 were constructed (Suppl. Fig. 1). Accordingly, 11β-HSD1L of milkfish is more similar to human 11β-HSD1 than 11β-HSD1L. However, the construction of alignment and phylogenetic tree to human 11β-HSD1, 11β-HSD1L, zebrafish 11β-HSD1La, 11β-HSD1Lb and milkfish 11β-HSD1L revealed that milkfish 11β-HSD1L was more similar to zebrafish 11β-HSD1La/b. So far, the function of fish 11β-HSD1L was only discussed in zebrafish [31]. 11β-HSD1L was able to reduce cortisone levels in the whole body, liver, brain and testis of zebrafish. However, expressions of 11β-HSD1L and 11β-hsd2 were nearly equal in the milkfish gills. In the reports comparing 11β-HSD1L and 11β-HSD2 abundances in various organs of human [95], 11β-HSD1/2/L1 revealed similar expressions in the small intestine with few amounts of mRNA expression. To date, there is no reference studying the co-factor of fish 11β-HSD1L. Mammalian 11β-HSD1L was found to have the activities of high-affinity NADP\(^+\)-dependent cortisol oxidation in different tissues or cells, e.g., the rat Leydig cells [96], pig testis [93] and sheep kidney [91]. However, human and zebrafish recombinant 11β-HSD1L did not exhibit activity even in the presence of excessive substrate and cofactor [31].

Unlike 11β-Hsd1 which is capable of converting inert cortisone to cortisol, 11β-Hsd2 functions in the reverse direction to inactivate cortisol [28]. 11β-Hsd2 has been identified in gills of several teleosts, including the rainbow trout (Oncorhynchus mykiss) [32], Japanese eel (Anguilla japonica), and Nile tilapia (Oreochromis niloticus) [33]. Studies on the activity and function of 11β-Hsd2 have revealed its dual roles in fish in the inactivation of glucocorticoids and activation of androgens [32-35]. In cortisol-injected milkfish, higher plasma cortisol levels led to elevated expression of 11β-hsd2 to transform cortisone from excessive cortisol in gills. Decreased na-k-atapase β1 expression in low temperature-exposed SW milkfish after cortisol injection might result from higher 11β-hsd2 and lower 11β-hsd1l expression. Meanwhile, increased Na-K-ATPase activity of FW milkfish gills after cortisol injection at low temperatures might be induced by higher expression of 11β-hsd1l in cells converting the inactive form of cortisol into an active form. Due to their higher plasma cortisol content, expression of 11β-hsd1l and 11β-hsd2 was modulated to maintain the balance of cortisol in the gills after injection. Although the expressions of 11β-hsds affected by (i) environmental temperatures as well as (ii) cortisol injection partly explain the increased abundance of Na-K-ATPase β1, the detailed mechanism of Na-K-ATPase β1 modulation is not yet clear in low temperature-exposed milkfish. Overall, based on the Na-K-ATPase activity results, this study demonstrated that cortisol plays an important role in the osmoregulatory capability of FW milkfish when exposed to hypothermal stress.

In conclusion, changes in branchial Na-K-ATPase activity of FW milkfish were found affected by environmental temperatures as well as cortisol-dependent Na-K-ATPase β1-subunit levels due to elevated expression of 11β-hsd1l. Accordingly, 11β-HSD1L was thought to play the role of a pre-receptor in gills of hypothermal milkfish. In contrast, in hypothermal SW milkfish, the protein amounts of Na-K-ATPase β1-subunit were not significantly different after cortisol injection, because 11β-hsd1l also decreased after cortisol injection. Therefore, SW milkfish may have different mechanisms to modulate expression of the Na-K-ATPase-β1 subunit as compared to FW milkfish under hypothermal stress.
Acknowledgements

This study was partly funded by a grant from the Ministry of Science and Technology (MOST), Taiwan [MOST-106-2313-B-005-038-MY3] to T.H.L. This work was also in part funded by the iEGG and Animal Biotechnology Center from The Feature Area Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE), Taiwan [MOE-107-S-0023-F] to T.H.L.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2019.105381.

References


S.A. Cruz, C.H. Lin, P.L. Chao, P.P. Hwang, Glucocorticoid receptor, but not mineralocorticoid receptor, mediates cortisol regulation of epidermal ionocyte development and ion transport in zebrafish (Danio rerio), Fluoi 8 One B (2013) e77997.


