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ORIGINAL PAPER

Osmoregulatory adaptations of freshwater air-breathing snakehead fish (*Channa striata*) after exposure to brackish water

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Abstract NaCl-rich rock salt dissolved in natural water source leads to salinity fluctuation that profoundly affects freshwater ecosystem and aquatic fauna. The snakehead (*Channa striata*) can live in saline water, but the osmoregulatory mechanisms underlying this ability remain unclear. Herein, we found that exposure to salinities ≥10 ‰ NaCl markedly elevated plasma cortisol and glucose levels, and caused muscle dehydration. In a study of time-dependent response after being transferred from fresh water (0 ‰ NaCl, FW) to salt-dissolved brackish water (10 ‰ NaCl, SW), FW–SW, cortisol increased rapidly along with elevations of plasma glucose and lactate. Interestingly, plasma cortisol returned to baseline after prolonged exposure, followed by a second peak that probably enhanced the branchial Na^+/K^+-ATPase activity. Under SW–FW condition, Na^+/K^+-ATPase activity was not altered as compared to SW-adapted fish. In conclusion, salinity change, especially FW–SW, induced a stress response and hence cortisol release in *C. striata*, which might increase plasma glucose and lactate to energize the branchial Na^+/K^+-ATPase.

Keywords Cortisol · Na^+/K^+-ATPase activity · Osmoregulation · Salinity fluctuation · Snakehead

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Introduction

Salinity is an important environmental factor, which influences the distribution of fish. Salinity effects have been studied in several freshwater fish (Altinok and Grizzle 2001; Luz et al. 2008), but while most studies have focused on the effects of seawater, little is known about the consequence of rock salt contamination (Bringolf et al. 2005). Approximately, 85 and 96 % of mineral contents is NaCl in seawater and rock salt-contaminated water, respectively. Seawater also contains sulfate, magnesium, potassium, and calcium (Bringolf et al. 2005); therefore, the effects of two water sources on aquatic fauna are different. The natural water sources in northeastern Thailand are contaminated by NaCl-rich rock salt (also known as halite) from extensive outcrop of Mesozoic rock as well as the underground NaCl-rich rock salt (El Tabakh et al. 1999). In the dry season, the salinity can be as high as 32 ‰ and is similar to seawater (Suwannatrai et al. 2011). This condition thus inevitably has a widespread ecological impact on the local water dwelling flora and fauna.

Environmental salinity fluctuations induce stress response in freshwater fish (Mommsen et al. 1999; Barton 2002; Kammerer et al. 2010), as indicated by the elevated plasma cortisol level (Wendelaar Bonga 1997). The rise of cortisol is important for survival as it increases blood glucose via glycogenolysis (rapid response) and gluconeogenesis (long-term response) to provide energy and enhance the ability of teleosts to adapt to environmental osmotic changes (Morgan and Iwama 1996; Vijayan et al. 1996; Mommsen et al. 1999). In euryhaline teleosts, cortisol has a role in seawater adaptation by stimulating differentiation of the gill chloride cells (mitochondria-rich cells) and branchial Na^+/K^+-ATPase activity, thus enhancing seawater tolerance in euryhaline fish (Madsen and Bern 1993;

Table 1 Osmolality and ion concentrations of freshwater and saltwater samples collected from freshwater and NaCl-dissolved saltwater (SW) tanks

Ion concentrations/osmolality	Salinity (‰)						
	0	2	5	10	15	20	35
Na^+ (mmol L^{-1})	2	30	80	150	226	315	559
Cl^- (mmol L^{-1})	2	30	79	149	227	314	560
K^+ (mmol L^{-1})	0	0	0.05	0.1	0	0.1	0.3
Osmolality (mOsm kg^{-1} H_2O)	9	70	166	310	460	640	1126

Madsen et al. 1995; McCormick 1996). However, the exact role of cortisol and branchial Na^+/K^+-ATPase activity in stenohaline freshwater teleosts in their osmoregulatory response to saltwater is not known.

Previous reports revealed that air-breathing fish, namely *Monopterus albus* (Pedersen et al. 2014), *Anabas testudineus* (Chang et al. 2007), and *Clarias batrachus* (Sarma et al. 2013), could survive in brackish water. Specifically, *M. albus* thrived in 10 ‰ salinity, while *A. tesudineus* tolerated up to 30 ‰. For *C. batrachus*, although they could endure in as high as 8 ‰ salinity, growth and survival rates were low when compared to fish living in fresh water. However, little is known regarding the salinity tolerance of air-breathing fish in the Channidae family. The snakehead (*Channa striata*), a freshwater teleost of Family Channidae and Order Perciformes, is economically important and is distributed throughout southern and southeastern Asia (Vidthayanon 2002). This species normally inhabits ponds, rice field and rivers, preferring stagnant and muddy water of plains. They can survive in dry season by burrowing in bottom mud as long as skin and breathing apparatus remain moist (Musikasinthorn 2003). The natural habitat of snakehead ranges from fresh water to brackish water. Although they live in brackish environments especially in the summer, the snakehead is normally cultured in freshwater in Asia, except in northeastern Thailand where they are cultured in brackish water (De Silva 2008). Since snakehead is a freshwater fish species that live in areas with NaCl-rich rock salt contamination, it is important to understand their salinity tolerance and osmoregulatory response, especially how an increase in salinity affects the physiochemical blood parameters and branchial Na^+/K^+-ATPase activity.

In the present study, we aimed to investigate (1) the acute effect (1 h) of different level of salinity on blood chemistry (glucose, lactate, Na^+, Cl^-, and K^+) of *C. striata*, and (2) the long-term effects (15 days) of osmotic changes caused by transferring fish from fresh water to 10 ‰ NaCl, and vice versa. We also determined time-dependent changes in plasma cortisol levels, and the correlation between the increased cortisol levels and plasma osmolality or branchial Na^+/K^+-ATPase activity.

Materials and methods

Animals

Snakehead (*C. striata*) weighing 120 ± 1.24 g were obtained from natural water sources in Khon Kaen Province in the northeast of Thailand, and maintained in four aerated fiberglass tanks (160 × 110 × 55 cm^3). Each tank held 150 L of dechlorinated tap water equipped with charcoal filters and air stones. Water temperature was 22–26 °C. Fifty percent of water was changed daily. They were fed brine shrimp (*Artemia salina*) twice a day (~2 % of body weight per day) and acclimated for more than a week. Since *A. salina* was cultured in seawater, they were washed several times with fresh water before use. Once fish looked healthy and ate well, they were divided into two groups. The first group was transferred to salt water, which was prepared by dissolving NaCl (Anivar, Australia) in dechlorinated tap water, and salinity was determined by a light refractometer. Salinity in the tank was increased at a rate of 2 ‰ NaCl per day and fish were allowed to acclimate to new salinity for 24 h until the endpoint salinity of 10 ‰ NaCl. A half of water in each tank was replaced with 10 ‰ NaCl every 4 days. The second group was acclimated in dechlorinated tap water (fresh water). Both groups were kept under these conditions for at least 4 weeks prior to the experiment. Fish were maintained under natural photoperiod. Stressors (e.g., noise, crowding, and vibration) were kept to minimum to avoid variation of cortisol levels. This study has been approved by the Animal Ethics Committee of Khon Kaen University, Thailand (no. 0514.1.12.2/26). All animals were cared for in accordance with the Ethics of Animal Experimentation of National Council of Thailand.

Experimental design

Experiment 1: acute effect of the various salinities on blood chemistry of C. striata

Chemical and ion compositions of fresh water and salt water are shown in Table 1. Animals were fasted for 24 h prior to the experiment. The freshwater-acclimated fish were randomly placed into each concentration of salt water

for 1 h. The control group [0 ‰ NaCl, fresh water] was exposed to dechlorinated tap water. Finally, all fish were subjected to determinations of plasma osmolality, muscle water content, and plasma concentrations of cortisol, glucose, Na^+, Cl^-, and K^+. The muscle water content after an acute salinity exposure reflected the ability of fish to restrict water loss.

Experiment 2: time-dependent adaptation to salt-dissolved brackish water (SW)

The experimental group (FW–SW) was fish exposed to abrupt salinity change from fresh water, FW, (0 ‰ NaCl) to salt-dissolved brackish water (10 ‰ NaCl), whereas the control group (FW–FW) was fish transferred from fresh water to fresh water. To avoid handling stress, the old water in the tanks was rapidly pumped out and concurrently replaced with the experimental medium. Plasma osmolality, blood chemistry (plasma concentrations of cortisol, glucose, lactate, Na^+, and Cl^-) and branchial Na^+/K^+-ATPase activity were measured at 0 h (fresh water), and then at 1, 3, 4, 6, 12, 24, 72, 168, 240, and 360 h after exposure ($n = 8$ animals per each time point).

Experiment 3: response of SW-acclimated fish to fresh water

In the experimental group (SW–FW), fish acclimated in SW (10 ‰ NaCl) for 4 weeks were transferred to fresh water (0 ‰ NaCl), whereas the control group (SW–SW) was transferred from brackish to brackish water. Plasma osmolality, blood chemistry (plasma concentrations of cortisol, glucose, lactate, Na^+, and Cl^-) and branchial Na^+/K^+-ATPase activity were measured at 0 h (salt water), and then at 1, 3, 4, 6, 12, 24, 72, 168, 240, and 360 h after exposure ($n = 8$ animals per each time point).

Sample collections

Prior to blood collection by cardiac puncture, fish were anesthetized with 0.5 % tricaine methanesulfonate (catalog no. MS222; Sigma, St. Louis, MO, USA). The water used for anesthesia had the same temperature and electrolyte composition as that previously used in the experiment. It took about 5 min for the fish to become unconscious. Under anesthesia, the heart was exposed immediately, and blood sample was drawn from the bulbus arteriosus using a syringe coated with 200 U mL^{-1} ammonium heparin (Sigma). Plasma was stored at −80 °C until analysis. The muscle tissue samples (~0.5 g) were collected from epaxial side in duplicate and weighed immediately to obtain wet weight. Thereafter, they were dried at 60 °C for 48 h and then weighed (dry weight). The percent muscle water content was calculated as: (wet weight − dry weight)/wet weight × 100.

Blood and water analyses

Plasma and water osmolality was determined by a freezing point-based osmometer (Advanced Instruments, Norwood, MA, USA). Concentrations of Na^+, Cl^-, and K^+ in plasma and water were determined by ion-selective electrode system of Cobas 6000 analyzer module c501 (Roche Diagnostics, Rotkreuz, Switzerland). Concentrations of glucose and lactate were also measured by Cobas 6000 analyzer. Plasma samples were analyzed in triplicate or duplicate, depending on available plasma volume. Hematocrit measurement was performed in duplicate using microhematocrit capillary tubes centrifuged at 11,000 rpm for 5 min in a microhematocrit centrifuge.

Measurement of cortisol level

Plasma cortisol concentrations were determined by radioimmunoassay using a commercial kit (ImmuChemCoated Tube Cortisol ^{125}I RIA kit, MP Biomedicals, NY, USA). Plasma samples and standards were pipetted into anti-cortisol-coated tubes. Cortisol-3-carboxymethyloxime-BSA was used as an immunogen to produce anti-cortisol antibody in rabbits. The anti-serum was coated and bound to the inner surface of a polypropylene tube. Cortisol ^{125}I was added to all tubes followed by 45-min incubation. The coated tubes were then analyzed in a gamma counter to determine the amount of antibody-bound ^{125}I-cortisol. Levels of cortisol in the plasma samples were obtained from the standard curve. The anti-serum (primary antibody) reacted 100 % with cortisol, 12.3 % with 11-desoxycortisol, 5.5 % with corticosterone, 2.1 % with cortisone, 0.25 % with progesterone, and <0.1 % with testosterone.

Na^+/K^+-ATPase activity assay

The measurement of Na^+/K^+-ATPase activity was modified from the method of McCormick (1993). Gill tissues taken from the fish were homogenized in 200 µL of SEID buffer (150 mM sucrose, 10 mM Na_2EDTA, 10 mM imidazole, 0.1 % deoxycholic acid), and then centrifuged at 5000*g* for 1 min. Homogenate samples (10 µL) were added into 200 µL of solution A [4 U mL^{-1} lactate dehydrogenase, 5 U mL^{-1} pyruvate kinase, 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole, pH 7.5] or solution B [solution A plus 0.5 mM ouabain (Na^+/K^+-ATPase inhibitor)] in 96-well microplates at 25 °C, followed by reading at 340 nm for 10 min by a microplate reader (Bio-Rad, QC, Canada) to determine a decrease in NADH level, which was used to

Table 2 Blood chemistry and percent muscle water content in the snakehead (*C. striata*) after 1 h exposure to water with various salinities

	Salinity (‰)					
	0	2	5	10	15	20
Muscle water content (%)	79.72 ± 1.82	85.69 ± 2.64*	78.16 ± 1.51	74.79 ± 1.48*	74.05 ± 2.01*	75.10 ± 1.47*
Hematocrit (%)	39.40 ± 13.22	38.33 ± 7.58	38.33 ± 8.88	35.17 ± 6.05	40.83 ± 2.14	44.5 ± 3.62*
Glucose (mmol L^{-1})	6.25 ± 0.87	7.66 ± 1.49*	8.06 ± 0.91*	10.00 ± 1.13*	7.31 ± 0.69*	9.50 ± 1.37*
K^+ (mmol L^{-1})	8.42 ± 1.14	9.17 ± 1.80	9.33 ± 6.54	9.93 ± 0.81	8.23 ± 2.04	6.28 ± 1.97

Values are mean ± SE ($n = 8$/group)

* $P < 0.05$ vs. 0 ‰ salinity (one-way ANOVA)

calculate ATP hydrolysis (McCormick 1993). Each pair of wells (with and without ouabain) was determined for Na^+/K^+-ATPase activity (μmol ADP mg protein^{-1} h^{-1}), which was calculated from the difference of ATP hydrolysis in the absence and presence of ouabain. The total protein concentrations were determined by bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA).

Statistical analysis

All data were analyzed by GraphPad Prism 6.0 (San Diego, CA, USA). Results are expressed as mean ± SE. Differences between groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post test. Comparison between the two factors, i.e., salinity and time, were performed by two-way ANOVA. The interaction between salinity and time was also analyzed. The level of significance for all statistical analyses was $P < 0.05$.

Results

Experiment 1: acute effect of the various salinities on the blood chemistry of *C. striata*

In natural water sources, there is a periodic change in salinity, and fish might be exposed to a brief salinity fluctuation. Therefore, the present experiment was performed to demonstrate an abrupt change in blood chemistry after 1-h exposure to NaCl-contaminated water, which mimicked a sudden exposure to natural salinity fluctuation. Snakeheads could survive after 1-h exposure to 2, 5, 10, 15, and 20 ‰ NaCl, but none survived in 35 ‰ NaCl. Fish exposed to 15 and 20 ‰ NaCl showed stress behaviors, such decreased movement and increased mucus secretion, and eventually died within 6 h. Their blood chemistry and percent muscle water content are shown in Table 2. Percent muscle water content increased significantly at 2 ‰ NaCl and decreased at salinities >5 ‰ NaCl, while hematocrit was significantly increased at salinity 20 ‰ NaCl. Plasma glucose was elevated in all salinities and reached the highest level at 10 ‰ NaCl. Plasma levels of K^+ were not altered after exposure to salt water.

The osmolality and ion concentrations (Na^+, Cl^-, and K^+) of FW and SW (2, 5, 10, 15, 20, and 35 ‰ NaCl) were determined (Table 1). The osmolality values of media with 0, 2, 5, 10, 15, 20, and 35 ‰ NaCl were 9, 70, 166, 310, 460, 640, and 1126 mOsm kg^{-1} H_2O, respectively. Since plasma osmolality of the fish exposed to fresh water (9 mOsm kg^{-1} H_2O) was about 281.8 ± 16.0 mOsm kg^{-1} H_2O, plasma cortisol levels remained unchanged after 1-h exposure to hypoosmotic media (70 and 166 mOsm kg^{-1} H_2O), but were significantly increased in hyperosmotic solution (310, 460, and 640 mOsm kg^{-1} H_2O) (Fig. 1a). Plasma concentrations of Na^+ and Cl^- as well as the plasma osmolality were elevated significantly with increasing salinities, starting from 310 mOsm kg^{-1} H_2O (Fig. 1b, c).

Experiment 2: time-dependent adaptation to SW

No evidence of mortality in FW–SW and FW–FW groups was found, but there were conspicuous changes in blood ion levels, plasma osmolality, and cortisol levels in the FW–SW group as compared to FW–FW group. Changes in the plasma cortisol levels were complex. Specifically, they were transiently increased during the first hour after being transferred from FW to SW, but returned to the control level at 4 h, increased again at 72–240 h, and then returned to the baseline value at 360 h (day 15). There was no change in cortisol level in the FW–FW group (Fig. 2a).

Plasma osmolality of FW-adapted fish was elevated at 1 h after being transferred to SW as compared to the value at 0 h (Fig. 2b). After exposure to water salinity 10 ‰ (310 mOsm kg^{-1} H_2O) for 168 h, the plasma osmolality could increase from ~280 mOsm kg^{-1} H_2O (at 0 h) to 390 mOsm kg^{-1} H_2O, which was greater than that of the plasma baseline and medium by ~40 and ~25 %, respectively. The plasma levels of Na^+ and Cl^- significantly increased at 3 and 1 h, respectively, after being transferred to SW (Fig. 2c). Ion concentrations remained higher than that of the control levels even after 360 h.

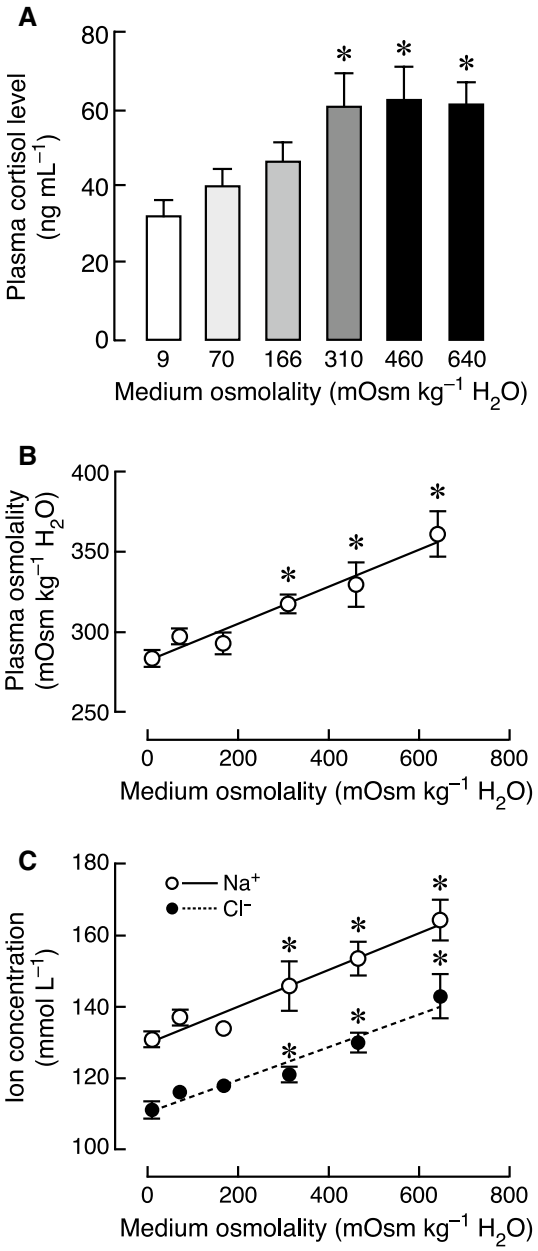

Fig. 1 Acute effect of different medium concentrations on **a** plasma cortisol, **b** plasma osmolality, and **c** plasma Na^+ and Cl^- levels in *C. striata*. Media (70, 166, 310, 460 and 640 mOsm kg^{-1} H_2O) were prepared by dissolving NaCl in dechlorinated tap water. The osmolality of dechlorinated tap water (control) was 9 mOsm kg^{-1} H_2O. Fish were exposed to dechlorinated tap water or different media for 1 h. Each value is mean ± SE ($n = 8$ independent samples per group for each sampling time). *$P < 0.05$ compared with the corresponding control group (one-way ANOVA)

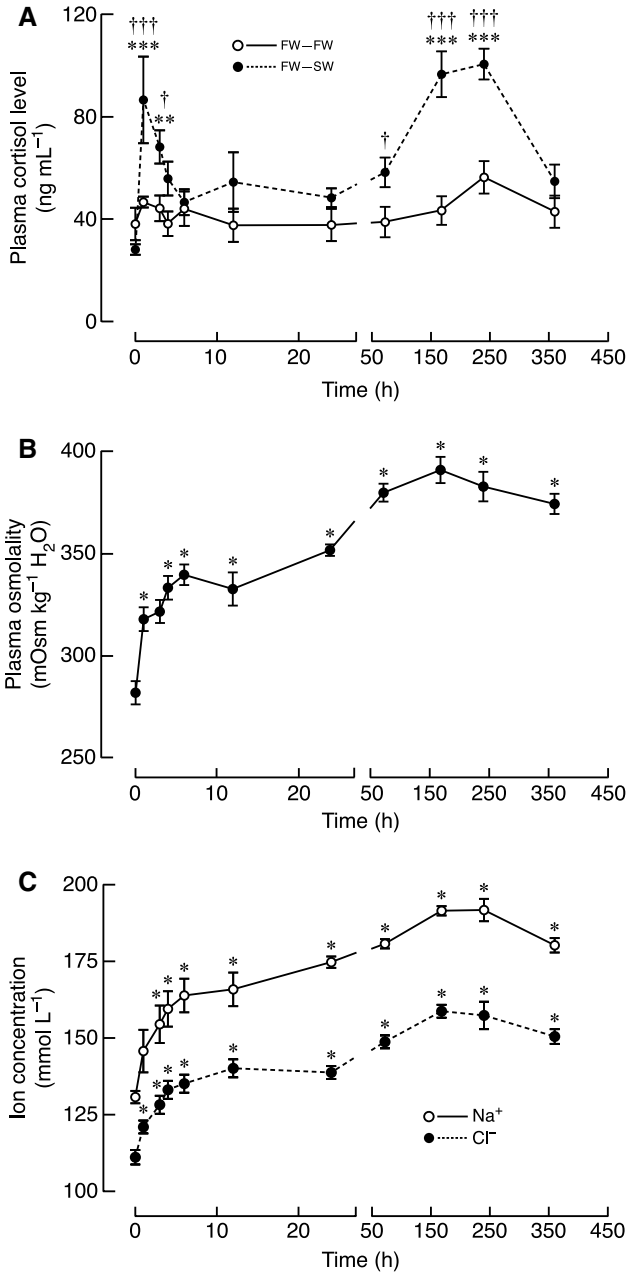

Fig. 2 **a** Changes in the plasma cortisol levels in *C. striata* after an abrupt transfer from fresh water to salt-dissolved brackish water (FW–SW), or from fresh water to fresh water (FW–FW). FW (0 ‰) was dechlorinated tap water. Salt-dissolved brackish water (SW) was 10 ‰ NaCl dissolved in dechlorinated tap water. **$P < 0.01$, ***$P < 0.001$ compared with the value at 0 h; $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the FW–FW group (two-way ANOVA). **b** Changes in plasma osmolality and **c** plasma Na^+ and Cl^- levels in *C. striata* after an abrupt transfer from fresh water to salt-dissolved brackish water. *$P < 0.05$ compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE ($n = 8$ per group for each sampling time)

Figure 3a and b show the plasma glucose and lactate levels, respectively. The control and treatment fish were obtained from FW–FW and FW–SW groups, respectively. In the FW–SW group, marked increases in both plasma glucose and lactate levels were observed within the first hour post-transfer as compared to the corresponding FW–FW group, before returning to the control levels at 4 h. The control group exhibited a relatively constant value

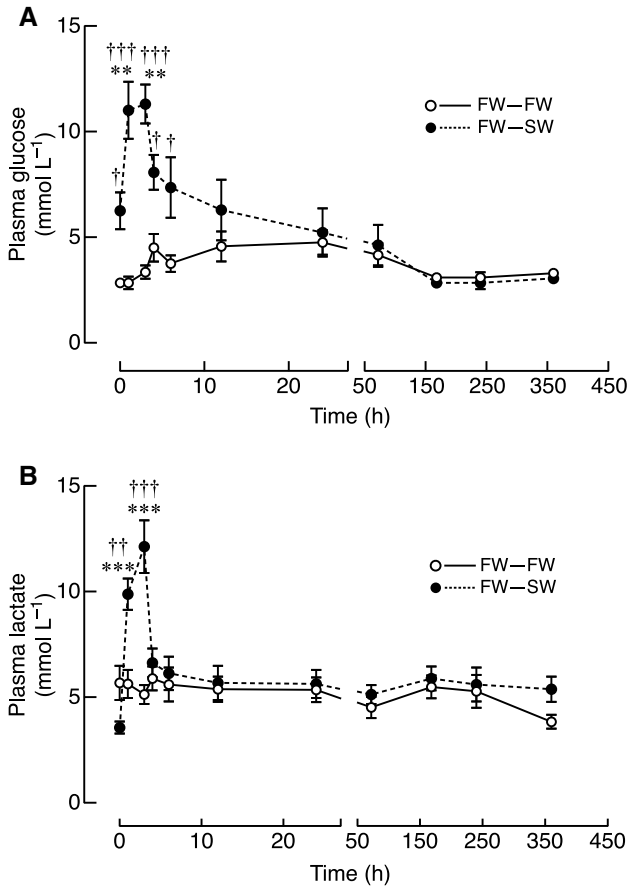

Fig. 3 **a** Changes in plasma glucose and **b** plasma lactate in *C. striata* after an abrupt transfer from fresh water to salt-dissolved brackish water (FW–SW), or from fresh water to fresh water (FW–FW). Each value is mean ± SE ($n = 8$ per group for each sampling time). $^{**}P < 0.05$ and $^{***}P < 0.001$ compared with the value at 0 h; $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the FW–FW group (two-way ANOVA)

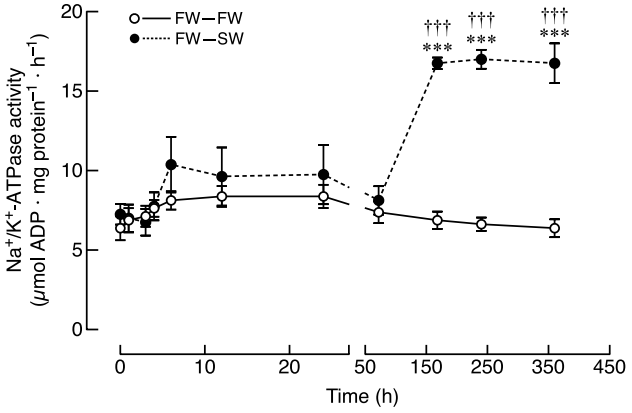

Fig. 4 Changes in the branchial Na^+/K^+-ATPase activity in *C. striata* after an abrupt transfer from fresh water to salt-dissolved brackish water (FW–SW), or from fresh water to fresh water (FW–FW). Each value is mean ± SE ($n = 8$ per group for each sampling time). $^{***}P < 0.001$ compared with the value at 0 h; $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the FW–FW group (two-way ANOVA)

throughout the experimental period. Moreover, FW–SW group showed a two-fold increase in the branchial Na^+/K^+-ATPase activity on day 7 (168 h) compared to the baseline level (0 h), which remained at this high level until the end of the experiment (Fig. 4). The branchial Na^+/K^+-ATPase activity of FW–FW group was not altered.

Experiment 3: response of SW-acclimated fish to fresh water

In this series of experiments, the SW-acclimated fish were transferred to either FW (experimental group; SW–FW) or 10 ‰ NaCl (control group; SW–SW). Plasma cortisol levels in both control and experimental groups appeared higher at the first hour post-transfer as compared to the baseline level at 0 h (Fig. 5a), before returning to the baseline level thereafter. Plasma osmolality and concentrations of Na^+ and Cl^- rapidly decreased during the first hour post-transfer, and remained relatively constant until the end of experiment (Fig. 5b, c). Plasma glucose peaked at 3 h and then decreased to the baseline level, while that of the control fish (SW–SW) remained unaltered throughout the experiment (Fig. 6a). Both SW–FW and SW–SW groups had similar levels of plasma lactate (Fig. 6b) and branchial Na^+/K^+-ATPase activities (Fig. 7).

Discussion

The snakehead fish occasionally encounter fluctuations in environmental salinity. The present study revealed that the abrupt transfer of the snakehead fish to media of various salinities could induce increases in plasma cortisol, plasma osmolality and ions (Na^+ and Cl^-). Abrupt changes in cortisol and glucose levels that occurred shortly after salinity change likely provided extra energy for coping with unfavorable conditions. The fish endured salt-dissolved brackish water (10 ‰) for a long duration, and interestingly exhibited a second peak of plasma cortisol. This response may in fact account for the enhancement of branchial Na^+/K^+-ATPase activity that allowed the fish to maintain ionic homeostasis. Under SW–FW condition, rapid loss of Na^+ and Cl^- and a brief stress were observed, and the considerably higher activity of Na^+/K^+-ATPase of SW–SW and SW–FW fish, as compared to FW-adapted fish, remained unchanged. Hence, the snakehead was capable of adapting to salt-dissolved brackish water, similar to other freshwater teleosts, e.g., climbing perch (*A. testudineus*) (Chang et al. 2007), Asian swamp eel (*M. albus*) (Pedersen et al. 2014), white sucker (*Catostomus commersoni*) (Wilkes and McMahon

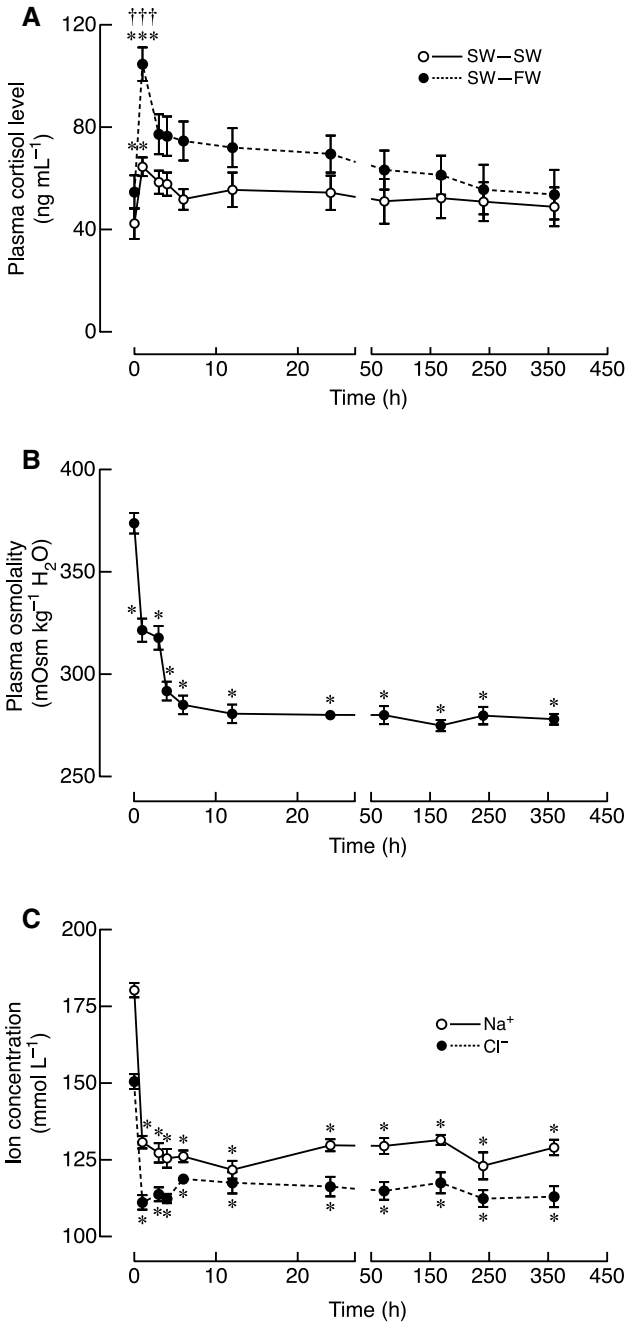

Fig. 5 **a** Changes in the plasma cortisol in *C. striata* after being transferred from salt-dissolved brackish water to fresh water (SW–FW), or from salt-dissolved brackish water to salt-dissolved brackish water (SW–SW). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the value at 0 h; $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the SW–SW group (two-way ANOVA). **b** Changes in plasma osmolality, and (**c**) plasma Na^+ and Cl^- levels in *C. striata* after being transferred from salt-dissolved brackish water to fresh water. $^{*}P < 0.05$ compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE ($n = 8$ per group for each sampling time)

1986), common carp (*Cyprinus carpio*) (Wang et al. 1997), flathead catfish (*Pylodictis olivaris*) (Bringolf et al. 2005), and goldfish (*Carassius auratus*) (Luz et al. 2008).

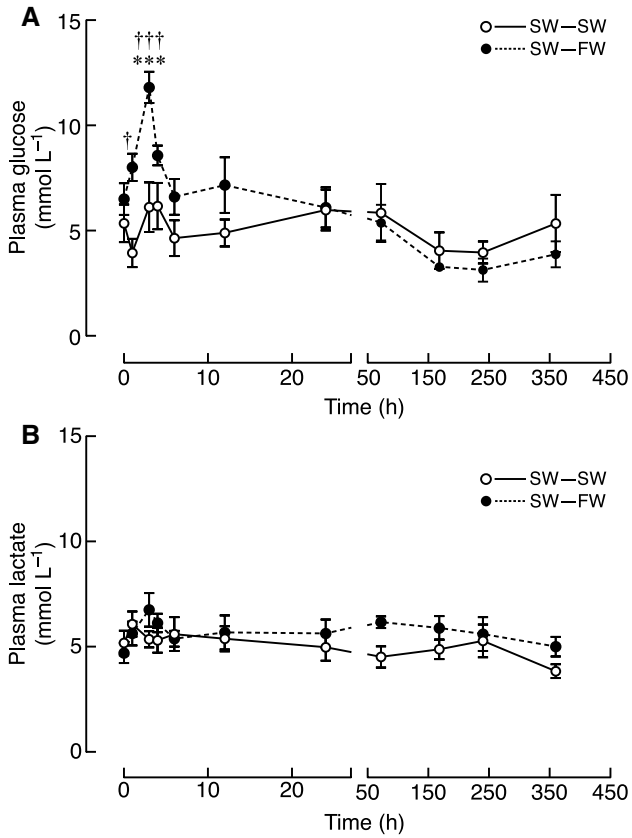

Fig. 6 **a** Changes in the plasma glucose and **b** plasma lactate in *C. striata* after being transferred from salt-dissolved brackish water to fresh water (SW—FW), or from salt-dissolved brackish water to salt-dissolved brackish water (SW–SW). Each value is mean ± SE ($n = 8$ per group for each sampling time). $^{***}P < 0.001$ compared with the value at 0 h; $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the SW–SW group (two-way ANOVA)

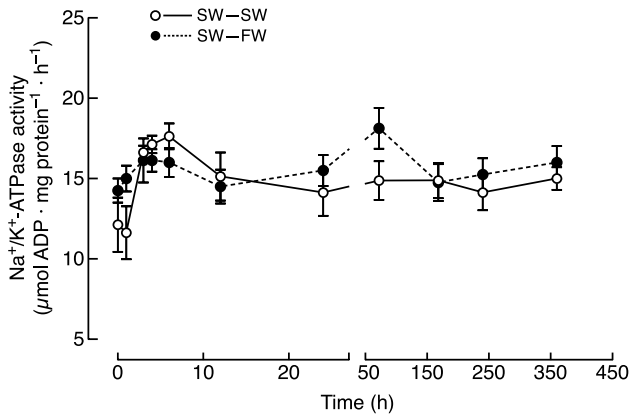

Fig. 7 Changes in the branchial Na^+/K^+-ATPase activity in *C. striata* after being transferred from salt-dissolved brackish water to fresh water (SW–FW), or from salt-dissolved brackish water to salt-dissolved brackish water (SW–SW). Each value is mean ± SE ($n = 8$ per group for each sampling time)

The common responses of stenohaline freshwater fish to salinity changes are the increased plasma osmolality and ion concentrations (Eckert et al. 2001; Tam et al. 2003; Luz et al. 2008), both of which were observed in snakehead. Since Na^+ and Cl^- are the major electrolytes in body fluid, regulation of both Na^+ and Cl^- is critical for osmoregulation (Evans 2008; Kaneko et al. 2008). When fish are exposed to hyperosmotic medium relative to plasma osmolality, water tended to be lost from the body with salt influx across the gill epithelium. The plasma hyperosmolality-induced tissue water loss—as indicated by muscle dehydration—was not only found in snakehead but also reported in common carp (Van der Linden et al. 1999) and goldfish (Luz et al. 2008). In addition, a decrease in plasma free water also raised the hematocrit in snakehead, similar to that observed in white sucker (*C. commersonii*) (Wilkes and McMahon 1986). In Asian swamp eel, hematocrit decreased after a long exposure to saline water (Pedersen et al. 2014). However, hematocrit was not altered in some other species, such as goldfish (*C. auratus*), tilapia (*O. mossambicus*), and gray snapper (*Lutjanus griseus*) after a direct salinity exposure (Luz et al. 2008; Kammerer et al. 2010; Serrano et al. 2011). The upper limit of salinity tolerance in stenohaline freshwater fish appeared to be determined by their ability to deal with soft tissue dehydration and to restrict an increase in plasma osmolality. If freshwater fish are exposed to a relatively high salinity, they may eventually die of continuous water loss and blood electrolyte imbalance (Maceina and Shireman 1979; Wang et al. 1997; Luz et al. 2008). As shown here, exposure of the snakehead to >10 ‰ salinity levels resulted in 100 % mortality within 24 h, indicating that salinities around 10 ‰ were the upper limit of tolerance for this species.

Plasma cortisol was increased as early as 1 h following exposure to 10 ‰ NaCl (Fig. 2a), and subsequently decreased after 3 h of exposure. The increases in plasma osmolality, Na^+, and Cl^- at 1, 3, and 1 h, respectively (Fig. 2b, c) together with the initial rise in cortisol level after SW exposure indicate a state of stress. Increased plasma cortisol following seawater transfer has previously been observed in killifish (*F. heteroclitus*) (Jacob and Taylor 1983; Marshall et al. 1999) and tilapia (*O. mossambicus*) (Kammerer et al. 2010). Since acute stress is an energy-consuming process as evidenced by the stress-induced increases in metabolic rate and oxygen uptake in fish (Barton 2002), the first peak of cortisol in response to acute stress from salinity change might play an important role in a rapid production of glucose, which could be used as an energy source for cell metabolism (Soivio et al. 1981; Mommsen 1984). The rapid elevation of plasma glucose level after hyperosmotic exposure, suggests that glycogenolysis—known to be enhanced by cortisol (Vijayan et al. 1997; Mommsen et al. 1999)—contributed to the rapid provision of glucose. Such cortisol action appeared to be non-genomic, and probably involved alteration in the phosphorylation-dephosphorylation status of glycogen phosphorylase (Gomez-Munoz et al. 1989). However, since this hyperglycemia was transient, gluconeogenesis that took longer time was not involved in providing glucose during saltwater exposure (van der Boon et al. 1991; Wendelaar Bonga 1997). Meanwhile, during exposure to environmental salinities, an increase in oxygen consumption for branchial ATP production often occurred to stimulate pumps and ion transporters (Chang et al. 2007; Tseng et al. 2007), consistent with the present finding that Na^+/K^+-ATPase was activated. The enhanced ATP production through glycolysis might eventually lead to excessive lactate production.

The second phase of cortisol release after being exposed to SW could be directly associated with the long-lasting osmoregulatory mechanism. We observed that after the snakehead were exposed to SW, the plasma osmolality continued to increase during the initial phase (1–72 h) and then remained high (~380 mOsm kg^{-1} H_2O) thereafter until the end of the experiment (Fig. 2b) coincidently with the second cortisol surge (Fig. 2a). In addition, the plasma osmolality of the 4-week SW-acclimated fish was ~380 mOsm kg^{-1} H_2O (Fig. 5b, at 0 h). These results indicated that fish could adjust the osmoregulatory variables (i.e., plasma osmolality and ion concentrations) to a new homeostatic level. Since increase in plasma osmolality paralleled increases in the plasma Na^+ and Cl^- levels, the hyperosmotic plasma was likely to result from influx of Na^+ and Cl^-. An increase in plasma osmolality was also evident in other stenohaline species (De Boeck et al. 2000; Eckert et al. 2001; Tam et al. 2003). For example, in goldfish exposed to 10 ‰ salinity (~284 mOsm kg^{-1} H_2O), plasma osmolality can reach up to ~350 mOsm kg^{-1} H_2O (Luz et al. 2008).

Cortisol has been reported to directly activate Na^+/K^+-ATPase in several teleost species, such as *Oncorhynchus kisutch* (Björnsson et al. 1987), *Anguilla rostrata* (Butler and Carmichael 1972), *O. mossambicus* (Dange 1986), and *F. heteroclitus* (Pickford et al. 1970). In addition, a decrease in the gill Na^+/K^+-ATPase activity in hypophysectomized killifish (*F. heteroclitus)* could be restored by cortisol supplementation (Pickford et al. 1970). In vitro treatment of primary gill tissue culture of coho salmon with cortisol (*O. kisutch*) was also found to increase Na^+/K^+-ATPase activity in a dose-dependent manner (McCormick and Bern 1989). Taken together, the osmoregulatory adaptation in SW-acclimated snakehead, which occurred about ~3 days after high salinity exposure, triggered the release of cortisol and activated Na^+/K^+-ATPase activity for body adjustment to the new homeostasis.

When SW-acclimated snakehead fish were transferred to fresh water, they exhibited a rapid ion loss, leading to

acute stress, as indicated by elevated plasma cortisol and glucose. The rapid loss of ions was a necessary osmoregulatory mechanism to maintain plasma ion concentrations. The energy required to drive Na^+ uptake in fresh water can be generated by both V-type H^+-ATPase and Na^+/K^+-ATPase. If external Na^+ concentration is in the 1 mmol L^{-1} range, the energy requirement can be solely generated by Na^+/K^+-ATPase (Kirschner 2004). However, the contributions of these pumps to generate electrochemical gradient vary with tissues, cell types, and expression patterns of the two transporters. For example, in frog skin, Na^+/K^+-ATPase in the granular cells is the main transporters to generate the gradient for Na^+ uptake, while V-type H^+-ATPase in the chloride cells works in concert with Na^+/K^+-ATPase (Ehrenfeld and Klein 1997). In the present study, the snakehead fish showed high Na^+/K^+-ATPase activity throughout the 15 days (360 h) post-transfer. Glucose could be used to energize the branchial Na^+/K^+-ATPase in snakehead.

Several investigations suggested that cortisol played a role in ion uptake in freshwater- and brackish-water adapted fish by increasing Na^+/K^+-ATPase density in the gill chloride cells (Laurent and Perry 1990; Dang et al. 2000; Eckert et al. 2001). A number of studies have also shown upregulation of Na^+/K^+-ATPase activity in freshwater-acclimated fish (Ciccotti et al. 1994; Woo and Chung 1995; Kelly et al. 1999; Lin et al. 2003). Therefore, it was not surprising to observe high Na^+/K^+-ATPase activity in both FW–SW and SW–FW adaptations in the snakeheads. However, the transient elevation of cortisol observed in the SW–SW group (1 h after exposure) could have resulted from handling stress during water exchange, and the elevated level was much smaller than that in the SW–FW group. Similar findings regarding handling stress were also reported in killifish (Scott et al. 2004), tilapia and jundiá (Barcellos et al. 2011). Indeed, stressors of various causes, such as sudden water temperature change, pollutants, and handling, have been reported to increase cortisol release, passive ion influx, and water loss in many fish species (Wendelaar Bonga and Lock 1992).

Meanwhile, consistent with findings in *O. mossambicus* (Morgan et al. 1997), plasma K^+ level did not change significantly after exposure to different salinity levels. This was probably due to the lower gradient of K^+ concentration between the blood (8.42 ± 1.14 mmol L^{-1}) in freshwater-acclimated fish and salt water (ranging 0–0.3 mmol L^{-1}) compared to those of Na^+ and Cl^-. Exchange of K^+ between plasma and the intracellular compartment could also prevent fluctuation in the plasma K^+ level (Eddy 1985).

In conclusion, we have provided evidence to explain how snakehead survives in natural water sources with salinity fluctuation. Specifically, the snakehead was capable of living in brackish water (<10 ‰ NaCl) for an extended period of time (i.e., up to 4 weeks, duration for acclimated fish in SW). This stressful condition induced cortisol secretion from the interrenal cells, which, in turn, enhanced the production of glucose, presumably through glycogenolysis and glycolysis, respectively. Glucose further energized Na^+/K^+-ATPase, that was the important transporter for adaptation to the new condition during FW–SW acclimation. In addition, glucose could generate a driving force for sodium uptake during SW–FW acclimation. Cortisol itself also has a direct stimulatory effect on the Na^+/K^+-ATPase activity, which, in turn, maintained osmoregulation and ionoregulation in the new level of homeostasis (Björnsson et al. 1987). When SW-acclimated snakehead was placed in fresh water, the plasma osmolality and ion concentrations were restored to normal level, but acute stress was still observed as indicated by transient increases in the plasma cortisol and glucose levels. Thus, the present findings underline the reason why salinity must be monitored and tightly controlled in commercial fish farming even when using natural water sources.

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